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Editors

Liver Immunology

Principles and Practice

Third Edition

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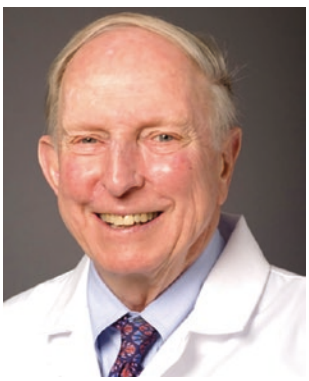
The Editors and Authors dedicate with gratitude and respect this third edition of Liver Immunology: Principles and Practice to the memory and legacy of our dear friend and colleague, Edward L. Krawitt, M.D. Ed was internationally recognized for his contributions to the scientific understanding of autoimmune liver diseases, especially autoimmune hepatitis. His patients admired and esteemed him as a consummate clinician, who always sought better diagnostic and therapeutic options for their care. His professionalism, scientific rigor, depth of knowledge, clinical acumen, and humble style contributed to his success as an educator, mentor, and administrator. All of us who were privileged to know and work with him cherished his warmth, friendship, and enthusiasm for life.



Preface

The Babylonians appreciated the liver as a vital organ in the nineteenth century B.C., but today, some 4000 years later, few educated adults know that the liver is a vital organ on par with the brain, heart, lungs, and kidneys. People worldwide, including healthcare providers, know too little about the complex, essential functions of the liver. Outbreaks of icteric hepatitis during World War II, now known to be caused by hepatotrophic viruses, triggered our modern appreciation of the role of the liver in health and disease. Remarkable progress during the last half of the twentieth century and continuing today has led to recognition of liver diseases caused by viral infections, steatohepatitis (due to alcohol or non-alcoholic metabolic disorders), xenobiotics (especially drug-induced liver injury), autoimmunity, and genetic defects altering alpha-1-antitrypsin secretion or metabolism of iron or copper. In the 1980s, liver transplantation provided unexpected evidence that the microenvironment of the liver is naturally immunosuppressive, which explained why it is unnecessary to match donors and recipients for HLA alleles.

We now realize the liver as the second largest immune organ in the human body (after the gut) and that hepatobiliary injury and liver diseases, regardless of etiology, result from inflammation generated by innate and adaptive immune responses. This third edition of *Liver Immunology: Principles and Practice* provides a timely update of important advances in our understanding of the liver's role as an immune organ and the roles of innate and adaptive immunity in the pathogenesis of all liver diseases. We welcome Atsushi Tanaka, M.D., from Tokyo, Japan, as an Editor and appreciate his dedication to ensuring that this edition represents the views of our global community of scholars and clinician investigators. The editors are grateful to our international team of authors who have donated so much of their time, intellects, expertise, and unique perspectives to create "state-of-the-art" chapters. We hope this third edition inspires clinicians and investigators to fresh approaches in thinking about liver physiology and liver diseases. Such knowledge is a prerequisite for understanding evidence-based care of children and adults afflicted with liver diseases and for acceleration of the pace of investigations required to develop better diagnostics and therapeutics. Appreciation of the liver as an immune organ and the immunologic mechanisms of pathogenesis of all liver diseases is essential for progress in hepatology.

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Core Concepts in Immunology: The Definition of Autoimmunity and Its Unique Application to the Seat of Tolerance, the Liver

1

Ehud Zigmond and Shishir Shetty

Key Points

- The first line of defense against pathogens is the innate immune system, which is activated following the detection of danger molecules known as PAMPs/DAMPs by highly conserved receptors.
- Activation of the innate immune system results in inflammatory response leading to targeted attack by phagocytosis or the release of cytotoxic agents.
- Adaptive immunity is the second line of defense and displays extreme diversity in antigen recognition, providing the immune system with an enormous anticipatory repertoire of antigen-specific effector cells and antibodies.
- Full activation of a naive T cell requires the presentation of antigen by antigen-presenting cells (APCs) and the engagement of a series of accessory molecules on the T cell with corresponding co-stimulatory molecules on the APC.
- Liver-derived products initiate, mediate, regulate, and resolve systemic inflammation.
- The liver is an immune organ, with unique anatomy enabling the generation of distinct immune responses.
- The liver is constantly exposed to enormous antigen load from the gut; however, generally the immune responses elicited in the liver result in tolerance.
- An important mechanism leading to liver tolerance is antigen presentation by nonprofessional and/or immature hepatic APCs.
- A combination of genetic and environmental factors plays a role in the pathogenesis of autoimmune diseases.
- Liver autoimmunity is a great paradox for an organ with unique tolerizing properties.

Danger Signal Recognition by Innate Immune Cells

The immune system is an evolutionary network responsible for the activation of specific cellular changes and events in response to stimuli. The innate immune system designates a conserved set of responses to danger signals in which the nature of the response is similar each time. In contrast, the adaptive immune arm that is found only in vertebrates provides a specific response to each threat and induces immunological memory. Effective function of adaptive response requires recognition of threat by the innate immune system. Inappropriate activation of the immune system, however, is often associated with the development chronic inflammatory disorders. The immune system comprises of a wide-ranging repertoire of cell types, physical and physiological processes, and functional effector molecules.

The innate immune system is activated following detection of molecules expressed by microbes or released during cell death or tissue damage [1]. These highly conserved moieties are known as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and include lipopolysaccharides, lipoproteins, glycolipids, flagellin, viral RNA, and bacterial DNA, as well as endogenous ligands such as heat-shock proteins released by damaged or necrotic host cells. Recognition of these danger

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signals is mediated by highly conserved receptors including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors [2, 3] (Table 1.1). On binding of their ligands, these receptors signal through pathways of conserved components to initiate expression of a large number of genes that code for proteins with effector, messenger, and regulatory functions, such as antimicrobial peptides (AMPs), cytokines, and chemokines (Fig. 1.1a). The result is initiation and amplification of the inflammatory response leading to targeted destruction of the activating organism, infected cell, or tumor cell by phagocytosis or the release of cytotoxic agents. Innate effector mechanisms activated by the above recognition systems during inflammation cause the target to be dispatched and include natural killer cell cytotoxicity, complement activation, opsonization, phagocytosis, respiratory burst, and AMP activity and are carried out by macrophages, neutrophils, as well as other innate cells such as basophils, mast cells, and eosinophils.

The innate immune system is equipped with a second type of detection system, used by innate lymphoid cells,

Table 1.1 Exogenous pathogen recognition receptors and their ligands

Receptor	Bacterial ligand	Fungal ligand	Parasite ligand	Viral ligand
TLR1	Triacyl lipopeptides			
TLR2	Peptidoglycan, porins	Phospholipo-mannan	tGPI-mutin	Hemagglutinin protein
TLR4	LPS	Mannan	Glycoinositol-phospholipids	Envelope proteins
TLR5	Flagellin			
TLR6	Lipoteichoic acid	Zymosan		
TLR11			Profilin-like molecule	
TLR3				dsRNA
TLR7				ssRNA
TLR8				ssRNA
TLR9	CpG-Island		Hemozoin	DNA
NOD1	Meso-diaminopimelic-acid			
NOD2	Muramyl-dipeptide			
NLCR4	Flagellin			
NLRP3	DNA and RNA			
RIG-1				ss/dsRNA
MDA5				ss/dsRNA
LPA2				ss/dsRNA

Adapted from [1, 3]

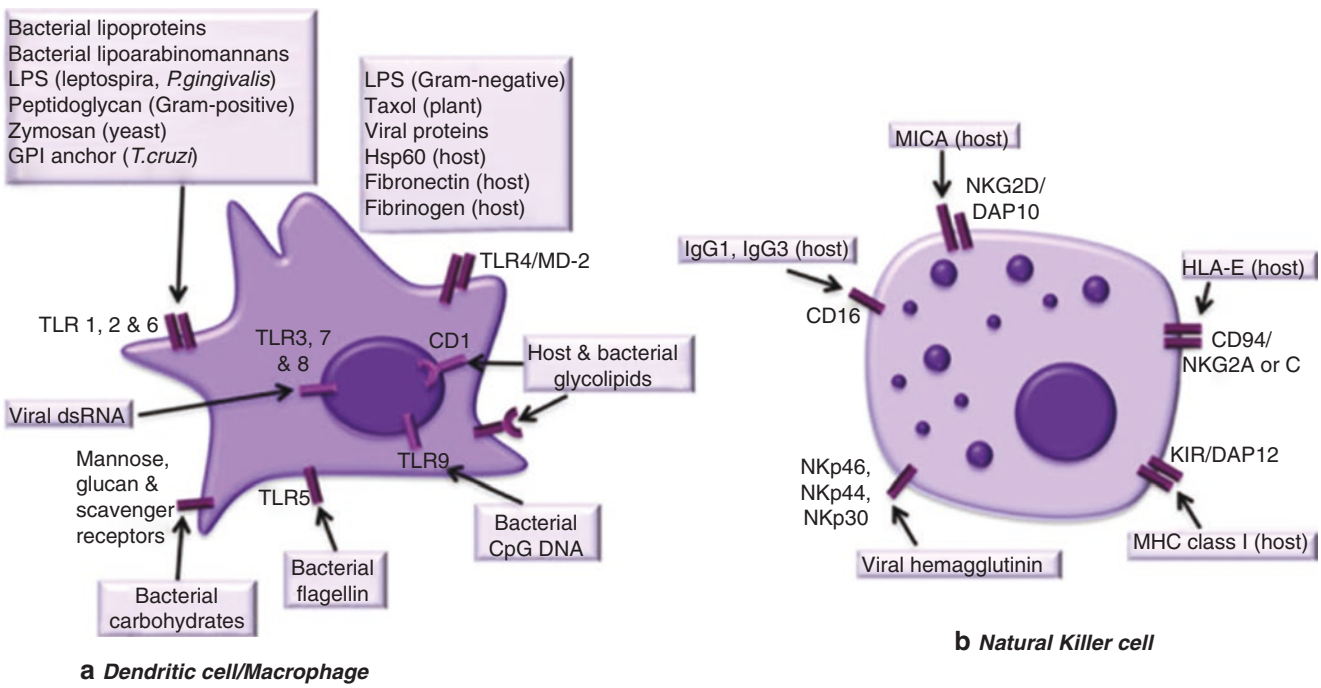

Fig. 1.1 Key phenotypic features of dendritic cell (**a**) and natural killer cell (**b**), important innate immune cells of myeloid (DC) and lymphoid (NK) lineages

especially natural killer cells (Fig. 1.1b), which identify changes to host cells that signify danger such as infection or tumor transformation [4, 5]. This detection system uses "natural cytotoxicity receptors" including NKG2D, which recognizes the stress-inducible molecule MICA (upregulated on tumor and virus-infected cells), and NKp46, which recognizes influenza hemagglutinin. Ligation of these receptors results in immediate killing of the infected or tumor cell by the NK cell. NK cells also express stimulatory and inhibitory receptors (killer immunoglobulin-like receptors [KIRs] that detect changes in the levels of major histocompatibility complex (MHC) class I molecules, which occur during times of abnormal protein synthesis such as tumor transformation or viral infection).

Adaptive Immunity

If a microorganism or tumor evades or overcomes innate defense mechanisms and inflammation is not resolved, an adaptive immune response is initiated. The first and crucial step is the activation of T lymphocytes. Naive, antigen-inexperienced T cells circulate between the blood and peripheral lymphoid tissues as small inactive cells with condensed chromatin, few organelles, and minimal metabolic and transcriptional activity. They remain in this inactive state until they encounter an infectious agent or danger signal, which usually occurs in lymph nodes. Recognition of an antigen or danger signal results in their proliferation and differentiation into effector lymphocytes capable of responding to the infection or danger by cytokine production or cytotoxicity.

Antigen Recognition by T-Cell Receptors

Naive T cells are activated by "professional" antigen-presenting cells (APCs), which are myeloid cells, capable of capturing, processing, and displaying antigen on their cell surface [6, 7]. These functions are performed by macrophages, B cells, and, particularly, dendritic cells (DCs) which have the additional ability to transport antigens from the site of activation to lymphocyte-rich lymph nodes (Fig. 1.2). APCs digest protein antigens into short peptides and present them on their cell surface where they are displayed complexed with MHC molecules. MHC molecules are highly polymorphic and can thus present a diverse range of different peptides. T cells recognize peptide/MHC complexes by highly specific clonotypic T-cell receptors (TCRs). During T-cell development, a great diversity of TCR specificities is generated by the rearrangement of multiple germline gene segments that code for different regions (variable, diversity, joining, and constant) of the molecules. This is followed by the variable addition of nucleotides and hypermutation of antigen receptor genes at positions that generate further diversity in the antigen recognition sites of these molecules. Thus, T cells display extreme diversity in antigen recognition, with up to 10^{16} possible specificities of TCRs, providing the immune system with an enormous anticipatory repertoire of antigen-specific effector cells [8, 9]. However, this number is greatly reduced by the removal of T cells whose TCRs are potentially autoreactive (negative selection). Only T cells whose TCRs are able to recognize self-MHC molecules are allowed to survive (positive selection). These processes occur during T-cell maturation in the thymus.

Distinct classes of T cells recognize intracellular and extracellular antigens presented by class I and class II major

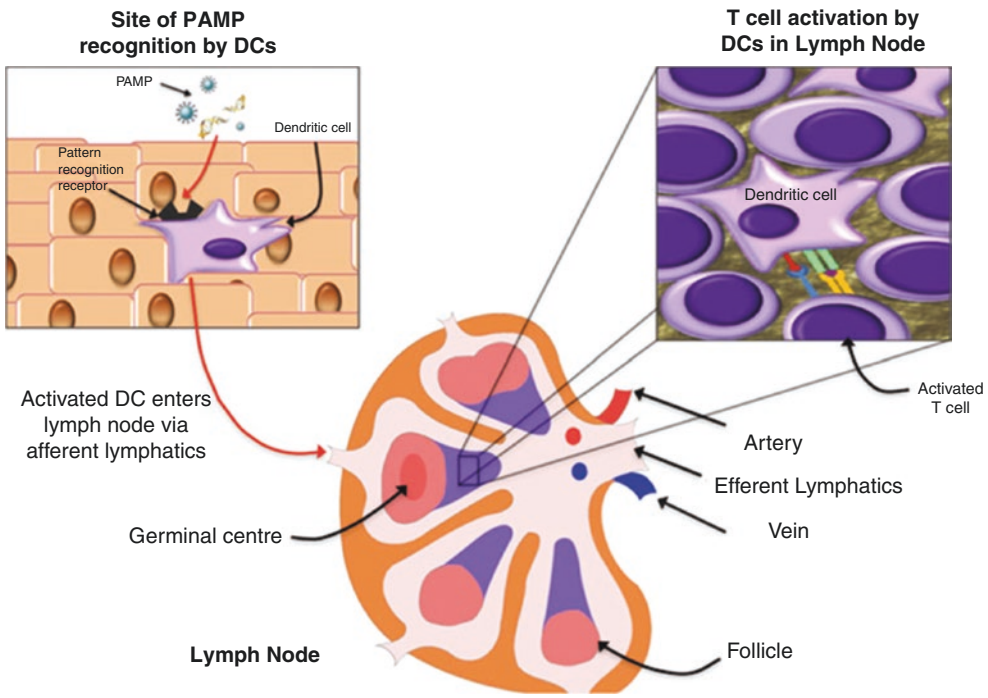

Fig. 1.2 Dendritic cells are activated on recognition of pathogen-associated molecular patterns (PAMPs) by specialized receptors such as TLRs (Toll-like receptors). They phagocytose and undergo phenotypic changes before trafficking to lymph nodes and present antigen to naive T cells

histocompatibility molecules on APCs. Peptides derived from endogenously synthesized antigens, such as self-peptides or viral peptides (in infected cells), are loaded onto MHC class I molecules in the endoplasmic reticulum and presented on the cell surface to CD8+ T cells, which typically kill the infected or tumor cell by Fas- or granzyme-mediated induction of apoptosis and the release of interferon gamma (IFN-γ), which disrupts viral replication [10, 11]. Peptides derived from extracellular antigens, which are internalized by APCs, are loaded onto MHC class II molecules for presentation to CD4+ T cells, which, in turn, activate other cells of the adaptive immune response [12]. Importantly, specific APCs are equipped with the ability to present exogenous antigens on MHC class I molecules, known as cross-presentation, a process essential for the initiation of $CD8^+$ T-cell responses [13].

T-Cell Activation

Engagement of the TCR by peptide/MHC complexes, in the absence of additional signals, is insufficient for the activation of naive T cells. Instead, it induces T-cell inactivation, a process known as anergy, which protects against unwanted immune responses against harmless or self-antigens. Full activation of a naive T cell requires the simultaneous engagement of a series of accessory molecules on the T cell with corresponding co-stimulatory molecules on the APC that are induced by danger signals from the innate immune system [14]. The B7 family of molecules, CD80, CD86, and B7-homolog expressed by an APC, transduce co-stimulatory signals to T cells through CD28 and inducible co-stimulatory receptors (ICOS). Additionally, CD40 on the APC interacts with its T-cell ligand, CD154, upregulating B7 expression. Further nonspecific interactions between adhesion molecules on the APC and the T cell strengthen the physical association between the two cells (Fig. 1.3).

If the interaction between the TCR and the peptide/MHC is maintained over a threshold amount of time, the naive T cell is activated, and it undergoes clonal proliferation and differentiation into effector T cells. Full activation of naive T cells takes 4–5 days and requires a third signal provided by cytokine binding to receptors expressed by the responding T cell. These cytokines are provided by the APCs, reflect prior pattern

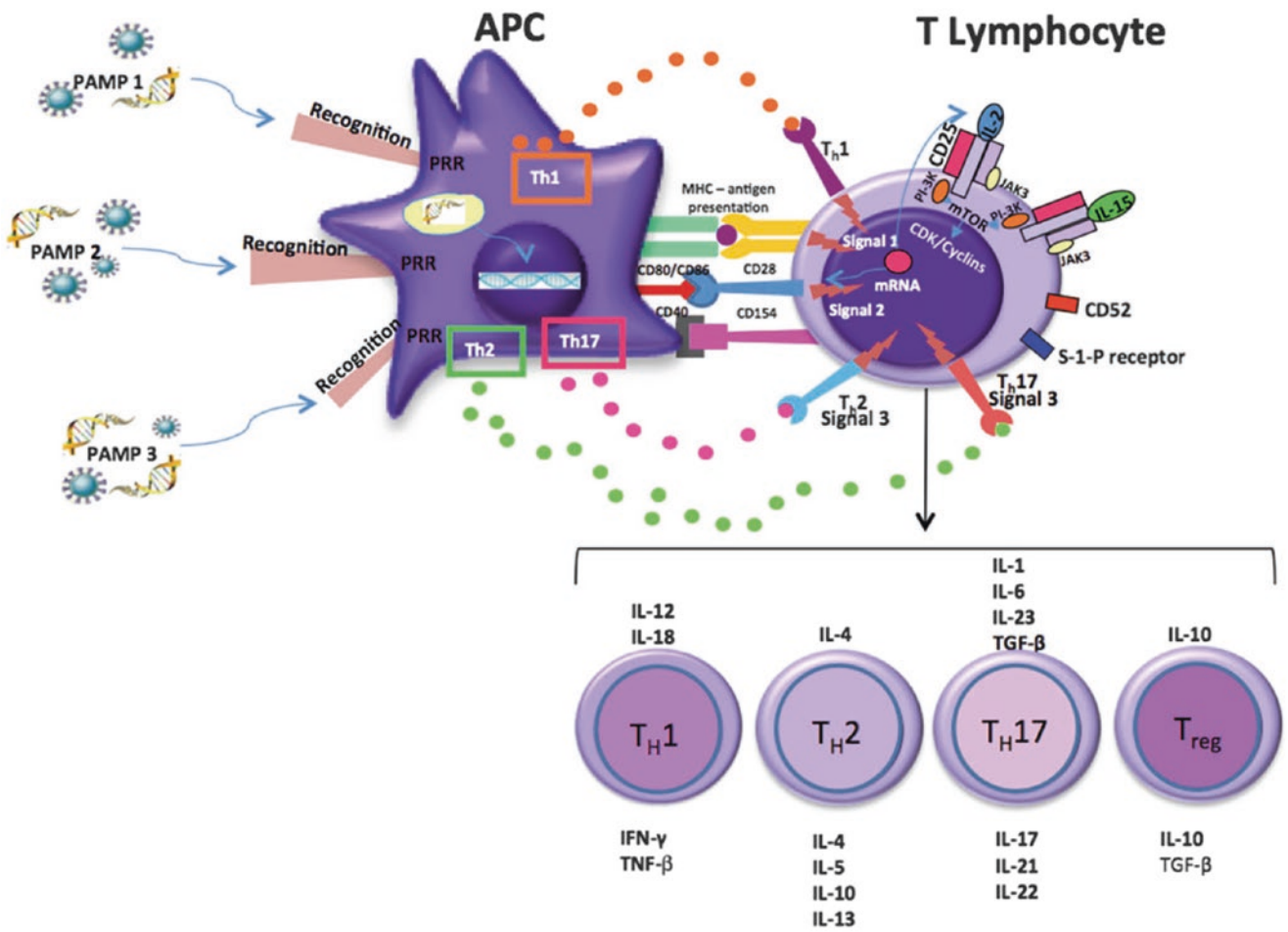

Fig. 1.3 T-cell activation. An activated dendritic cell presents antigen to T cells in the context of major histocompatibility complex class II molecules. A second signal is provided through engagement of CD80 and CD86. Effective T-cell activation and proliferation will only occur in the appropriate cytokine environment

recognition receptor (PRR) engagement, and ultimately induce different subpopulations of cytokine-secreting T cells including TH1, TH2, T regulatory cells, and TH17 cell populations. T-cell activation is also accompanied by changes in cell-surface adhesion molecules that direct effector T cells from the lymphoid tissues to the sites of infection or danger in the periphery.

Effector Functions of the Adaptive Immune System and Their Regulation

The differentiation of naive T cells into functional effector cells is controlled by signals from the innate immune system [7, 11, 14]. Release of IL-12 and IL-18 by macrophages and DCs and IFN-γ by NK cells promotes the development of CD8+ cytotoxic T cells and CD4+ T-helper 1 (Th1) cells. Release of IL-4 and IL-6 promotes the development of CD4+ Th2 cells. Th1 cells are generally induced by viruses and intracellular bacteria, whereas Th2 cells are induced by allergens and helminth pathogens. Th1 cells secrete IFN-γ and TNF-β and activate macrophages but also provide helper function for B-cell production of complement-fixing and virus-neutralizing antibodies. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and are considered to be the true helper cells, activating differentiation and class switching of B cells to secrete IgE, IgA, and IgG1 [7, 11, 14]. Other populations of CD4+ T cells with regulatory function, termed T regulatory 1 cells, produce IL-10 and transforming growth factor-β (TGF-β). They suppress Th1 responses, have important roles in the maintenance of immunological tolerance at mucosal surfaces, and initiate tissue repair [15–17].

B-Cell Antigen Receptors (Antibodies)

An additional arm of the adaptive immune system is B cells which are the cellular source of antibody secretion. Antibodies, like TCRs, are coded for by sets of rearranging gene segments (Fig. 1.4) and thus possess as much diversity and specificity for antigen as the TCR [18]. Antibodies

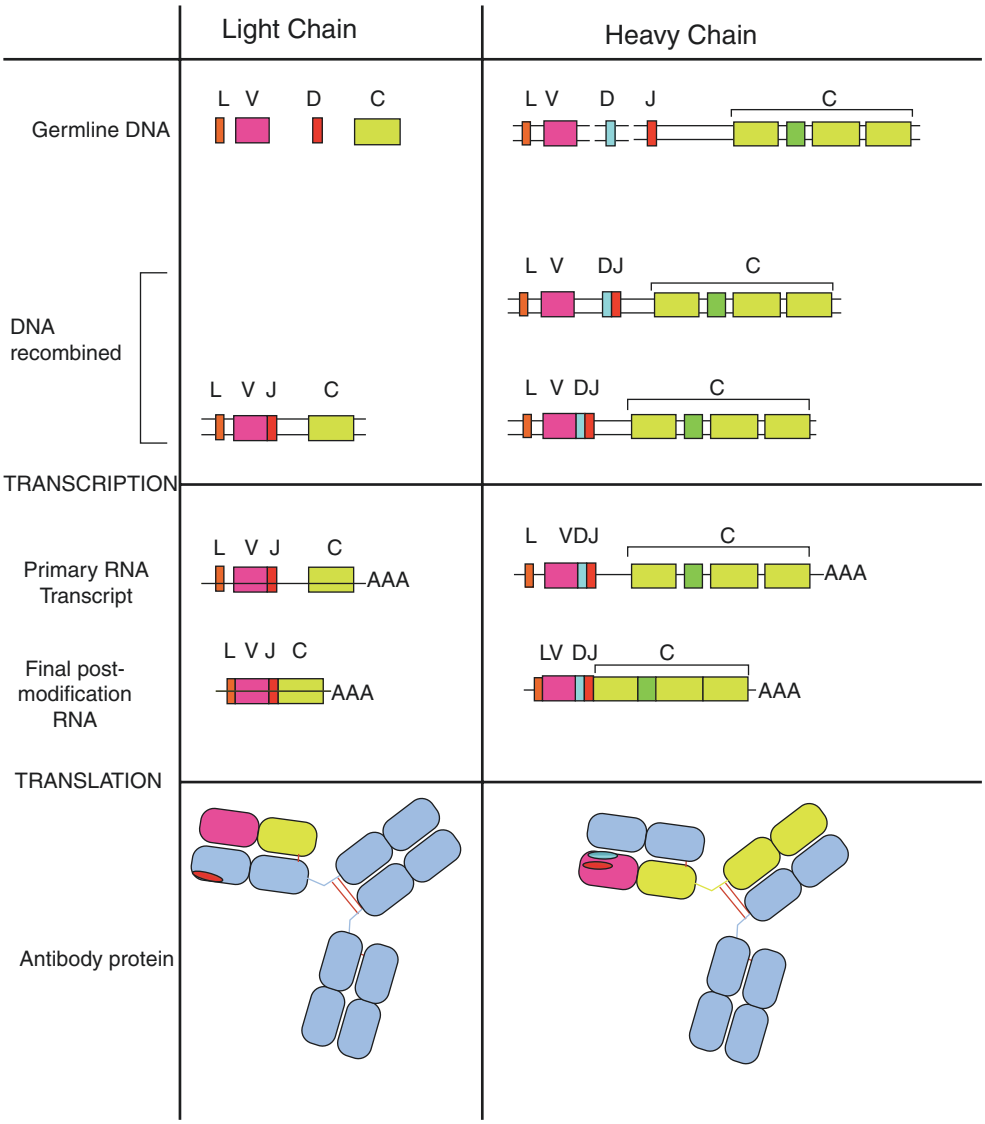

Fig. 1.4 Gene rearrangement required for the generation of antibodies (and T-cell receptors). During B-cell development, families of immunoglobulin gene segments undergo rearrangement to generate a unique DNA sequence for each B-cell antigen receptor. On differentiation to a plasma cell, additional posttranslational modification results in the generation of secreted forms of the molecule (antibodies)

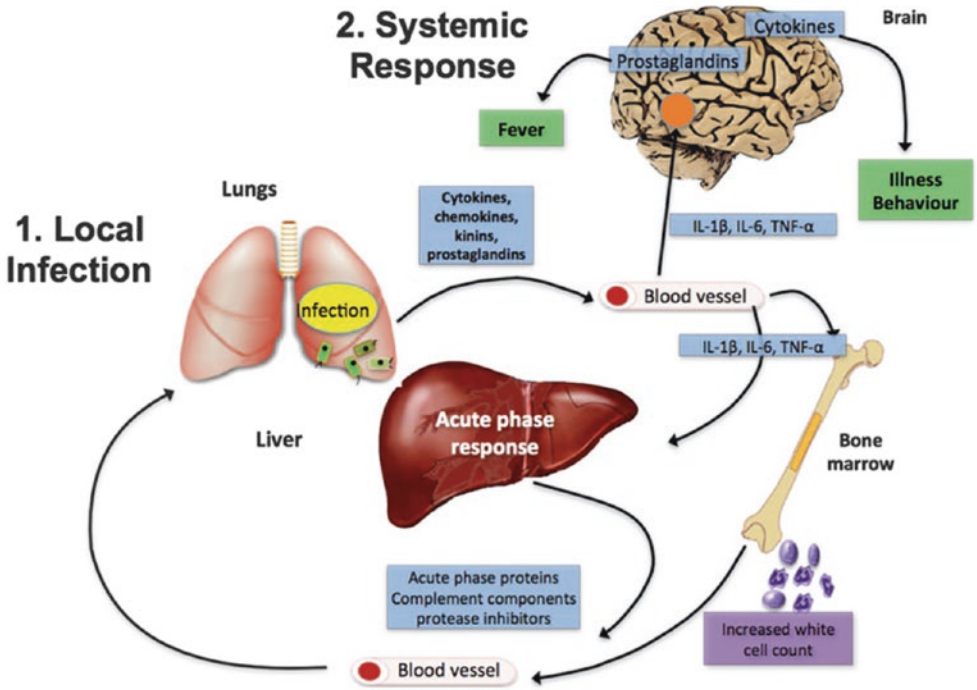

Fig. 1.5 Systemic inflammation. The liver has a key role in detecting circulating inflammatory cytokines, producing acute-phase proteins, and alerting the body to inflammation. Induction of the acute-phase response has significant metabolic implications

released in soluble form can neutralize toxins and viruses and also opsonize pathogens for phagocytosis by macrophages, cytotoxicity by NK cells, and directed histamine release by mast cells and basophils. Antibodies can also activate complement leading to the lysis of bacteria [19]. B lymphocytes also function as APCs as they express class II MHC molecules and their membrane-bound antibodies can specifically bind antigens, leading to their internalization and presentation to T cells. Generation of antigen-specific responses by B lymphocytes (and also T cells) is associated with the generation of specific memory cells, which can be rapidly reactivated by the same antigens.

Local and Systemic Inflammation

Inflammation is a general term given to the mobilization and effector activities of the immune system that are activated by responses to signals of danger. Chemical messengers from activated cells of the innate immune system and from pathogen-infected cells and are responsible for mediating inflammation. These chemical messengers include chemokines, cytokines, and growth factors that recruit additional inflammatory cells [20, 21]. Inflammatory cytokines, carried to the liver from sites of inflammation or damage, are detected by hepatocytes, which are activated to synthesize complement components as well as acute-phase proteins including serum amyloid A, fibrinogen, mannose-binding lectin, and C-reactive protein. Acute-phase proteins and complement components bind to microorganisms, targeting them for destruction and phagocytosis [19, 22]. They also alert the whole body to danger, mobilizing immune cells, inducing proliferation and additional synthesis of cellular and molecular immune components. Thus, liver-derived products initiate, mediate, regulate, and resolve systemic inflammation, emphasizing a major role for the liver in innate immunity [23] (Fig. 1.5).

Regulation of Inflammation

Innate immune strategies are activated within seconds of detection of danger, damage, or abnormal growth. They are regular events in the healthy individual, occurring throughout the body, perhaps more frequently at sites of high cell turnover (where there is likely to be a higher incidence of mutation) and increased exposure to foreign antigens (such as the gastrointestinal tract, liver, lungs, and uterus). Inflammatory effector functions continue to be activated until the stimulating structure is destroyed or removed, at which time anti-inflammatory cytokines, such as IL-10 and TGF-β, and other regulatory mechanisms induce resolution of innate immune responses [24, 25]. MicroRNAs are major regulators of the inflammatory response [26], while autophagy also has a role through its effect on endogenous inflammasome activators and inflammasome components which modulate IL-1β and IL-18, as well as IL-1α, release [12]. Resolution of inflammation is accompanied by activation of extensive tissue repair and remodeling mechanisms; e.g., the IL-10 cytokine family is known to have major effects

on epithelial cell biology [25, 27]. In some situations, activator and effector functions fail to be regulated, leading to chronic inflammation which results in permanent scarring, tissue damage, or fibrosis, such as fibrosis and cirrhosis in chronic hepatitis.

Liver Anatomy and Microanatomy

In order to understand the special challenges and processes of liver immunology, its unique anatomy must be first approached. It receives a dual blood supply arising from the hepatic artery and from the portal vein. The arterial supply provides oxygenated blood with a variable vasculature, but the commonest anatomical pattern involves the common hepatic artery arising from the coeliac axis along with the left gastric and splenic arteries [28]. It is the portal vein that provides the main nutritional supply of blood draining the gut and splanchnic organs. The hepatic artery and portal vein go on to form branches that drain into the hepatic sinusoidal channels, and the blood flows from these portal areas into the hepatic venules, which are at the center of the hepatic lobule. The venules merge to form the hepatic vein which then drains into the inferior vena cava [29].

Apart from the unique dual blood supply, there is also a striking heterogeneity of the endothelial cell populations which line the hepatic vasculature [30]. The hepatic arterioles and portal venules are lined by endothelium that is similar to conventional endothelium. The hepatic sinusoids form a vascular bed that is lined by liver sinusoidal endothelial cells (LSEC). Compared to conventional endothelium, they have a unique structure and phenotype which provide a multifaceted ability to mediate several vital functions ranging from filtration, scavenging, and regulating immune responses to both harmless gut-derived products and pathogenic organisms [31]. The sinusoidal endothelium is discontinuous containing fenestrae, which are open pores 100–220 nm in size and lack a classical basement membrane [32]. The channels are characterized by a low flow sinusoidal environment and allow the sinusoidal endothelium to function as a sieve and the fenestrae acting as dynamic filters for solutes and particles.

This vasculature perfuses through the liver, which is composed of epithelial and mesenchymal populations that are arranged in repetitive microscopic structures. The structural units are often characterized as either a lobule or acinus [33]. The lobule is comprised of a central structure which is the central venule and surrounded by the peripheral structures of the portal tract (Fig. 1.6). The portal tract contains several structures including the hepatic arteriole, portal venule, lymphatic vessels, and bile duct with ductules. In contrast, the acinus is taken as a structure with the portal tract at the center and the hepatic venules at the periphery and is divided into zones with a decreasing oxygen and nutrient content across the lobule with zone 1 surrounding the portal tract and zone 3 surrounding the hepatic venule. Within these functional units, the parenchyma is made up of hepatocytes which are organized in cords, one or two cells thick separated by the sinusoidal channels. These polygonal-shaped cells have a basolateral surface facing the sinusoidal channel and a canalicular surface, which forms a structure, termed the canaliculus with the adjacent hepatocyte. These canaliculi drain bile produced by hepatocytes into bile ducts which are found at the portal tract and are lined by a specialized cuboidal/columnar epithelium termed the cholangiocyte [34]. On the basolateral surface of the hepatocyte, between the hepatocyte and sinusoidal channel is the space of Disse; this compartment contains the hepatic stellate cell, a member

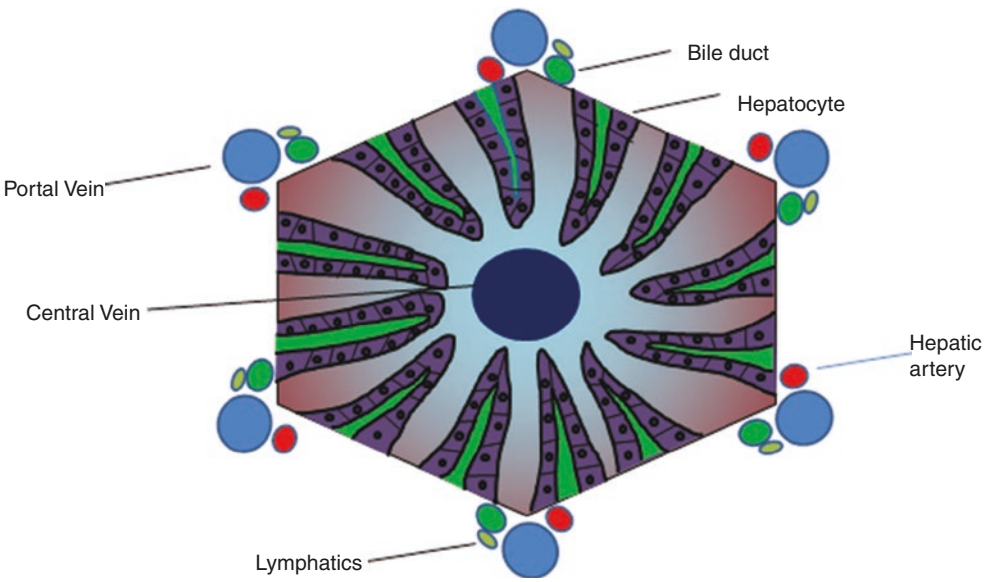

Fig. 1.6 Liver lobule. Schematic of the structural organization of the liver (hepatic) lobule, demonstrating the inflow of blood from branches of both the hepatic artery and hepatic portal vein and outflow through central venules. Bile is produced within lobules by hepatocytes and is drained in the opposite direction to blood flow into branches of the bile ducts located in the portal areas

of the myofibroblast family [35]. Part of the lymph formed in the liver passes through the space of Disse and then drains into lymphatic vessels found in the portal tract. The structure of the liver is therefore adapted to the continual recirculation of blood immune cells captured from peripheral blood flow, passing through the liver parenchyma and then migrating into the lymphatic drainage.

Liver Immune Tolerance

The liver is continuously exposed to food and microbial antigens from the intestine and displays barrier functions toward environmental antigens. Additionally, the liver as a metabolic organ produces a variety of neo-antigens. Hence, the risk of immune activation in the liver seems higher than in other organs. In order to avoid immune activation to this enormous load of antigens, it appears that the liver has in turn acquired specialized mechanisms of immune tolerance.

The comprehension that immune responses in the liver are biased toward tolerance originates from a 1969 classic experiment revealing that allogeneic liver transplants between unrelated pigs were generally tolerated, while transplantation of other organs resulted in rejection [36]. Moreover, the tolerance induced by the transplanted liver was not simply a result of a lack of relevant antigens, because the transplanted liver induced tolerance to other transplanted organs from the same donor [37, 38]. It is well known today that combined transplantation of the human liver together with the kidney or lung from the same donor protects the non-liver graft from rejection and improves allograft survival [39], proving that the transplanted liver induces systemic immune tolerance.

The immune tolerogenic properties of the liver are further demonstrated by its roles in oral tolerance and portal venous tolerance. Thus, administration of antigens or donor cells by the oral route or directly via the portal vein (passing the gut) induces both local and systemic tolerance to the antigen, resulting in donor antigen-specific anergy or hyporesponsiveness [40]. Of note, induction of oral tolerance is abolished by a portocaval shunt to bypass the liver, confirming the role of the liver in oral tolerance induction [41].

This tolerogenic microenvironment leads to liver T-cell dysfunction, including clonal deletion, anergy, senescence, deviation, and exhaustion. Clonal deletion is a process whereby T and B cells expressing antigen-specific receptors with self-reactive specificities are deleted during their development. Clonal anergy denotes to a state of inactivation of lymphocytes that cannot induce strong immunity. Clonal deviation is the process whereby naive CD4+ T cells differently accept the Th2 but not the Th1 or the Th17 phenotype. T-cell exhaustion is another form of T-cell dysfunction often associated with chronic infection and tumorigenesis [42]. An exhausted T cell is characterized by impaired effector functions and proliferative capacity, as well as altered transcriptional, epigenetic, and metabolic signatures, including the overexpression of inhibitory receptors such as PD-1, CTLA-4, LAG-3, and TIM-3 and a dysregulated cytokine production [43, 44]. Of note, all of these states of T-cell dysfunction leading to tolerance instead of immunity were shown to be the results of T-cell priming in the liver [45]. The causes for this phenomenon are probably multifactorial and include the type and specific function of the APC involved, the site of immune priming and the cytokine milieu, and the unique composition and function of the hepatic immune cellular compartment as will be discussed below.

Immunotolerance state ensures that the liver does not mount a robust immune response against gastrointestinal tract-derived molecules and pathogens. However, this hepatic immune tolerogenic environment is also exploited by hepatitis viruses, parasites, and tumors and can lead to persistent infection and rapid cancer progression in the liver.

The Unique Characteristics of Hepatic Immune Cells and Their Role in Supporting Immune Tolerance

Within the microanatomical structures of the liver reside a variety of immune cell populations strategically positioned to deal with the significant antigen load that perfuse through the organ. The unique characteristics of these hepatic cellular immune components and their position within the liver are critical for the performance of this task – eliminating pathogens yet avoiding over activation of the immune system that would potentially lead to unwanted harmful inflammation.

A key process of the immune system is the presentation of antigen that either enters the liver through the circulation or is cell derived from dying parenchymal cells that have been infected by pathogens. Antigen is presented to T cells in order to induce T-cell-mediated immune responses. Naive CD8+ T cells and CD4+ T cells in secondary lymph nodes are activated by two independent signals: the presentation of antigen by MHC class I and II molecules, respectively, triggering the TCR receptor and a second co-stimulatory signal which is required for full activation. For CD8+ to provide full effector function and develop memory, they also require help from CD4+ T cells. This is facilitated by a process termed licensing where antigen is presented to both antigen-specific CD4+ and CD8+ and requires the APC to express both MHC class I and class II [46, 47]. Within the liver, the cells that express both MHC class I and II are the Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic dendritic cells (DCs). These cells have been shown to present antigen to T cells, but a large body of evidence suggests that within the liver this process is skewed toward immunosuppression and tolerance.

Kupffer cells (*KCs*) comprise the largest population of tissue-resident macrophages in the body [48]. Innovative studies exploring the cellular origin of macrophages demonstrate that KCs originate from erythromyeloid progenitors in the yolk sac rather than being bone marrow derived and have been shown to maintain by self-renewal [49, 50]. These cells are positioned within the sinusoidal space on the luminal surface predominantly near the portal tract, directly exposed to the circulation. They phagocytose debris and invading pathogens and appear to be fixed to their position and produce long cytoplasmic extensions which allow them to cover large areas [51]. KCs can directly interact with hepatocytes and phagocytose apoptotic hepatocytes [52]. KC-derived cytokines have a significant role in modulating the differentiation and proliferation of other cells and make a major contribution to maintaining the balance between tolerance and the ability of the host to mount an immune response to pathogens [53, 54]. Alongside a range of cytokines, they also release prostanoids, reactive oxygen species, and nitric oxide which have been shown to inhibit T-cell activation [55]. The positioning of KCs in the liver sinusoids is ideal for interactions with circulating lymphocytes. They present antigen to lymphocytes but also secrete immunosuppressive factors such as IL-10 and prostaglandin E2 [53, 56]. The depletion of these cells in murine models leads to the loss of oral tolerance and liver transplant tolerance [57, 58]. Together, these observations indicate that KCs seem to represent a tolerogenic cell population within the liver contributing to the tolerogenic properties of this organ, thereby avoiding detrimental inflammatory and immune reactions toward gut-derived antigens.

Liver sinusoidal endothelial cells (*LSECs*) constitutively express MHC class I and II as well as co-stimulatory molecules. They can take up antigen and present it to CD4+ T cells and have the capability of cross-presenting antigen to CD8+ T cells by taking up antigens by scavenger receptors and transferring to MHC class I.

However, the presentation of antigen by LSEC has been shown to drive tolerogenesis in CD8+ T cells [59, 60]. Naive CD8+ T cells primed by LSECs are first activated to proliferate, secrete cytokines, and express CD69 and CD25 but finally exhibit low IL-2 and IFN-γ production and low cytotoxicity [25]. This tolerance was shown to depend on PD-L1, since LSECs from PD-L1-deficient mice failed to induce CD8+ T-cell tolerance [27]. Recent data suggests that this LSEC-driven tolerogenesis can be overcome by the concentration of antigen, where high concentrations of antigen lead to a shift from tolerogenic to effector T-cell differentiation [61]. IL-6 trans-signaling also drives LSEC to trigger rapid effector cell differentiation and sustained CD8+ responses [62]. The bias toward tolerance is also seen in CD4+T-cell interactions with LSEC. The expression of MHC class II on LSEC enables them to present antigen to CD4+ T cells, but the low level of co-stimulatory molecules leads to the induction of immunosuppressive regulatory T cells (Tregs) rather than T-helper cells [63, 64].

The liver also contains a population of hepatic resident *dendritic cells* (*DCs*) comprised of myeloid as well as plasmacytoid DCs. DCs are known to be the most powerful antigen-presenting cell in the body and play a key role in mediating immune responses which are triggered in the secondary lymph nodes. Within the normal liver, the DCs are situated around the portal tract and have the capability to take up and process antigen like other DC populations. But like the other APCs in the liver, their activation of T cells is skewed toward immunotolerance. This appears to be due to the fact that hepatic DCs are in an immature state within the liver compared to other organs, and while they express MHC molecules, they have low expression of pattern recognition receptors such as TLR-4 as well as low expression of co-stimulatory molecules required for T-cell activation.

An important innate population is *natural killer* (*NK*) cells, which are enriched in the liver and can make up to 50% of the intrahepatic lymphocyte population. These cells are characterized by their ability to rapidly clear virally infected cells and cells undergoing malignant transformation and detect cells undergoing stress responses [65]. These recognition mechanisms are independent of antigen specificity. The functional activity of NK cells is balanced by the activity of activating and inhibitory receptors on the cell surface [66]. The expression on the cell surface of alleles of major histocompatibility complex class I (MHC-I) binds to inhibiting receptors on NK cells and promotes tolerance of NK cells to normal cells of the body [67]. Target cells which have lower expression or absence of MHC-I lead to the triggering of activating receptors on the NK cell surface which bind ligands on the cell surface and lead to targeted killing [68]. NK cell effector function is mainly mediated via cytotoxicity and release of IFN-γ [66]. The NK cell compartment within the liver can be divided into transient conventional NK cells and liver-resident NK cells which have distinct phenotypes, and there is gathering evidence that they develop from separate innate lineages [69]. The distribution of NK subset cells in human tissues such as the liver is very different from the peripheral blood, and it is likely that the hepatic microenvironment and the chronic exposure to foreign antigens play an important role in regulating this balance. These subsets seem to have distinct functional capabilities, for example, the liver-resident NK cells have higher granzyme and perforin levels and higher surface expression of TRAIL and FasL compared to the circulating subsets suggesting that they mediate cellular elimination by apoptotic methods [70–72]. Liver-resident NK cells directly suppress T-cell responses through the programmed cell death-1 ligand-receptor (PDL1-PD1) axis. Impaired NK cell function is associated with declining cytotoxic CD8+ T-cell activity in persistent viral infections like chronic hepatitis B [73, 74].

Hepatic unconventional T cells can be broadly divided into two major populations, the first of which expresses NK cell markers (known as NKT cells) and the second which does not express these markers. NKT cells express TCR-αβ chains and typical NK cell markers and are a bridge between innate and adaptive immunity [75]. They are characterized by their ability to recognize lipid antigens through the expression of MHC-like molecule CD1d and can themselves be divided into type I, or invariant subset, and type II or diverse NKT cells and nonclassical subsets [76, 77]. NKT cells are most abundant in the liver compared to other organs [78]. The type I subset is more abundant in mice, while the type II subset is abundant in humans [79]. Type I NKT cells express a semi-variant αβTCR that is encoded by a Vα chain (Vα24 in humans and Vα14 in mice) and Jα18 gene segments which are paired with more diverse non-germline Vβ chains [79]. The type I NKT cells have been shown to be capable of releasing TH1-, TH2-, and TH17-type cytokines, and so their cytokine profile response is dictated by the microenvironment, type of antigen-presenting cell, and lipid antigen. Studies have shown that type I NKT cells drive pro-inflammatory pathways and can stimulate conventional T cells and NK cells to mediate liver damage [80]. In contrast type II NKT cells have a more immunoregulatory role and can play a counterbalance to the responses driven by type I NKT cells [81, 82]. Imaging studies have demonstrated that NKT cells perform intravascular effector functions in the eradication of pathogens, and they perform a surveillance role by crawling along the hepatic sinusoidal channels [83, 84]. By bridging the innate and adaptive responses, NKT cells act as immunoregulators during immunological liver disease. Activated NKT cells contribute to the recruitment of Tregs via the CXCR3-CXCL10 pathway [85] and were also shown to promote the priming of IL-10-producing CD8+ T cells by hepatocytes in order to limit liver injury [86].

Another major population of hepatic unconventional T cells is γδT cells, which have a γδTCR rather than an αβTCR [87, 88]. As seen with NKT cells, the liver is an organ that is enriched for this population of T cells, where 15–25% of intrahepatic T cells have been shown to be made up of γδT cells. γδT cells have been shown to leave the thymus as fully mature T cells and therefore already have a defined functional status [89]. While they do recognize antigen presented by MHC molecules, they also recognize ligands independent of TCR engagement. This property allows them to respond to cytokine stimulation in a more rapid manner than conventional T cells, and they can release a range of cytokines including IFN-γ, IL-4, IL-10, and TGFβ in large amounts [90, 91]. Apart from cytokine release, they also have cytolytic capability by releasing cytolytic granules and killing via death receptor-mediated apoptosis [92, 93]. With this broad repertoire of functions, they have been shown to be able to drive inflammatory processes in certain situations, while being protective in other models [94, 95]. In terms of localization, lymphocytes are enriched around portal areas, but a significant proportion of lymphocytes within the parenchyma have been shown to be γδT cells [96]. More recently another unconventional T-cell subset has been described, termed the mucosal-associated invariant T (MAIT) cell, which has been found to make up a significant proportion of the innate-like T-cell compartment of the liver (up to 20–50% of T cells) [97]. MAIT cells express a semi-variant TCR that recognizes a MHC-like protein (MR-1). MR-1 presents vitamin B metabolites derived from commensal and pathogenic bacteria, and through this pathway, MAIT cells are activated by a variety of bacterial strains [98]. Originally, high levels of these cells were found in human gut biopsies with accumulation in the lamina propria which led to them being named MAIT cells [99]. Their significant contribution to the immune population within the normal liver has led investigators to speculate that MAIT cells play a part in the ability of the liver to act as a firewall between the host and gut-derived bacteria [100]. They are predominantly found in the portal tract specifically localized around the peribiliary regions [101].

Hepatic stellate cells (*HSCs*) are perivascular cells possessing multiple diverse functions. They store vitamin A in cytoplasmic lipid droplets, regulate the flow of blood through the sinusoids, and undergo transdifferentiation into myofibroblasts contributing to liver fibrosis. Immunologically, they have two well-documented roles. The first is secretion of various chemokines that may be involved in recruitment of inflammatory cells to the liver. In addition, they can present antigen and activate T cells, particularly NKT cells [102]. Of note, the fate of classical T cells activated by HSCs may be intricate and in most cases supports immunosuppressive behaviors. Thus, it has been demonstrated that human suppressed T-cell activation through PD-L1 [103], and HSCs following exposure to IFN-γ can activate and expand Treg cells in an IL-2-dependent manner and independent of PD-L1 [104]. Moreover, mouse HSCs co-transplanted with allogeneic pancreatic islets promoted graft acceptance, mediated by PD-L1 [105].

Of note, the *hepatocytes* also seem to participate in immunoregulation by their ability to function as APCs. In order to achieve this purpose, direct interactions between lymphocytes and hepatocytes are essential. Electron microscopy has shown that direct contacts occur through cytoplasmic extensions penetrating the liver endothelial fenestrations [106]. Besides their constitutive MHC class I expression, hepatocytes express MHC class II under inflammatory conditions, e.g., in viral or autoimmune hepatitis (AIH), which seems to be unique in case of parenchymal cells [107]. MHC class II expressing hepatocytes can present antigen and activate CD4+ T cells; however, this was not sufficient to cause hepatitis in a transgenic mouse model [107]. Rather, in mice

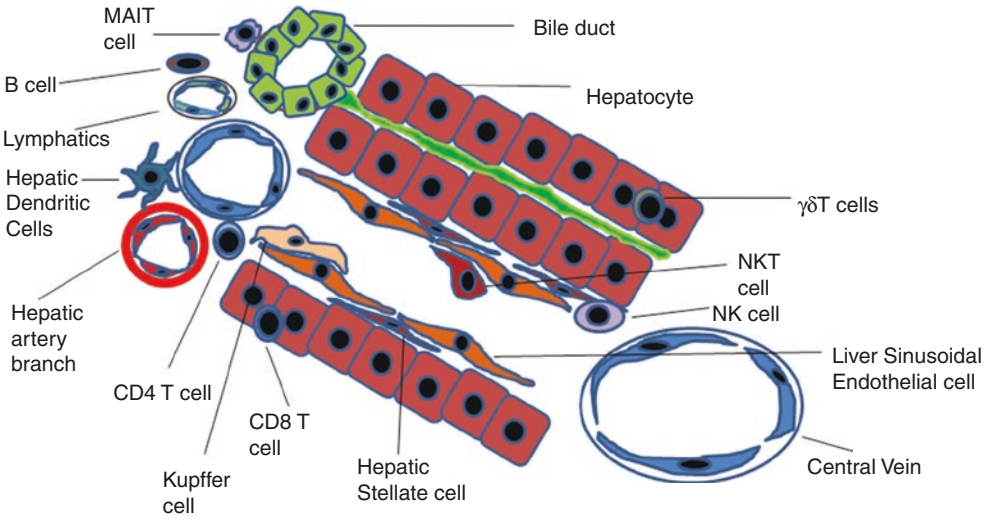

Fig. 1.7 Immune cell populations within the hepatic sinusoids. Schematic overview of the subpopulations of resident hepatic immune cells and their localization in relation to the key cellular populations of the hepatic sinusoids

constitutively expressing MHC class II in hepatocytes, the ability of lymphocytic choriomeningitis virus-specific CD4+ and CD8+ T cells to produce IFN-γ was abrogated, and viral persistence was prolonged [108]. Thus, it is suggested that MHC class II expression by hepatocytes in response to inflammation contributes to the liver tolerogenic effect and thereby to chronicity of viral hepatitis infection.

PD-L1 is induced in hepatocytes by viral infection as well as by type I and type II interferons [109]. Given that PD-L1 is also inducible by IL-10 [110], a central cytokine in the liver produced by resident DCs, KCs, and LSECs, it appears that PD-L1 induction in hepatocytes in response to inflammation contributes to the tolerogenic effect mediated by these cells. Figure 1.7 outlines the location of these hepatic immune cellular subpopulations within the liver sinusoids.

The Influence of Hepatic Exposure to Gut-Derived Products on Liver Tolerance

The mechanisms underlying the bias of hepatic APC function toward tolerance have been postulated to be related to the hepatic microenvironment. The liver is continually exposed to bacterial products from the intestinal system. Low levels of endotoxin from gram-negative bacteria such as lipopolysaccharide (LPS) are found in the normal liver circulation. Experimental data have shown that these continual low levels of LPS induce LPS tolerance, for example, the exposure of LPS to Kupffer cells leads to release of immunoregulatory cytokines such as IL-10. The high proportion of innate immune cell populations may also have a role in this process. NK cells and NKT cells can produce large amounts of interferon family cytokines. These cytokines have a key role in promoting immune cell activation and function, but their continual secretion has also been shown to have a negative feedback effect. This has been shown to downregulate effector functions of both CD8+ and CD4+ T-cell function in response to certain stimuli and also promote the generation of regulatory T cells. Interestingly, in contrast to TLR4 activation, stimulation of TLRs relevant for viral infection, i.e., activation of TLR3 by polyI:C or activation of TLR9 by CpG oligonucleotides, has been shown to induce CD8+ T-cell-mediated hepatitis in murine models and has been suggested to induce autoimmunity by breaking immune tolerance in the liver [111, 112]. Also aggravation of CD4+ T-cell-mediated liver disease by TLR9 activation has been described [113]. It seems noteworthy that TLR9 signaling in CD4+CD25+ regulatory T cells alleviates their regulatory function [114].

The Definition of Autoimmunity and Loss of Immune Tolerance

Autoimmune disease occurs when a specific adaptive immune response is mounted against self-antigens. When an adaptive immune response develops against self-antigens, it is usually impossible for immune effector mechanisms to eliminate the antigen completely, and so a sustained response occurs. The consequence is that the effector pathways of immunity cause chronic inflammatory injury to tissues, which may prove lethal. The mechanisms of tissue damage in these disorders are essentially the same as those that operate in protective immunity. Thus, it is expected to find autoreactive B lymphocytes (autoantibodies) and autoreactive T lymphocytes targeted against autoantigen(s). The autoreactive lymphocytes expand polyclonally because the mechanisms that normally keep them at bay fail. In other words, autoimmune diseases can be considered a manifestation of immune dysregulation.

Autoimmune diseases represent a major health problem because of their chronic nature, the associated healthcare cost, and their prevalence in young populations. Because

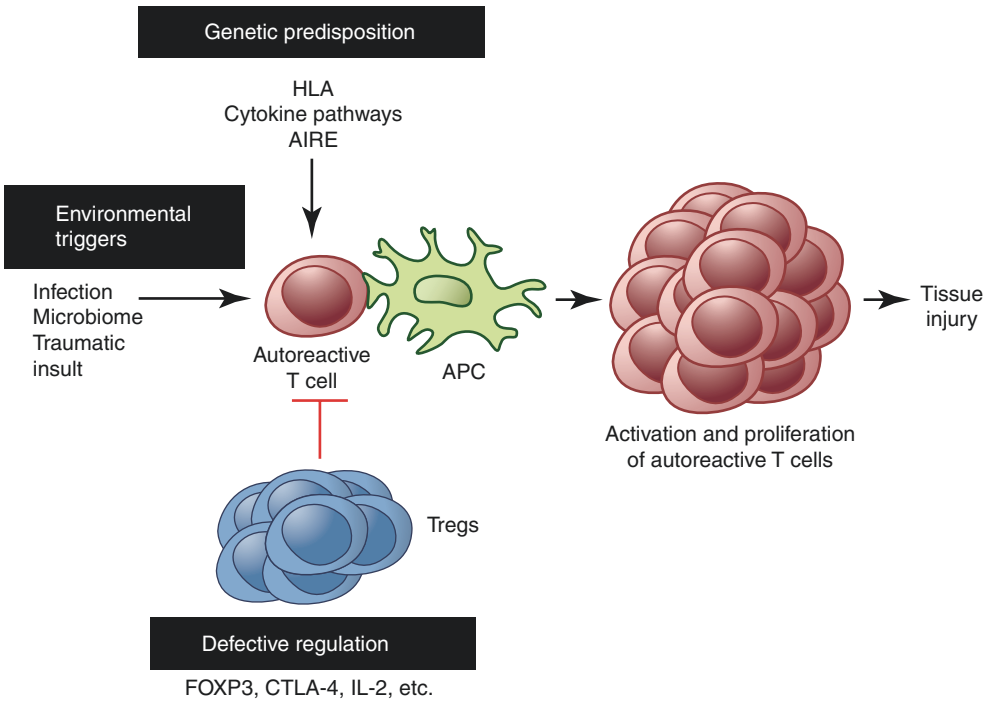

Fig. 1.8 Genetic susceptibility, environmental stimuli, and defective regulation are responsible for initiating autoimmunity. Genetic polymorphisms in immune-related genes (including HLA, cytokines/receptors, and those involved in central tolerance) may lower the threshold for the activation of autoreactive T cells. Environmental triggers such as infection, the microbiome, and tissue injury generate a pro-inflammatory environment that supports the activation of autoreactive lymphocytes. Tregs normally function to suppress autoreactive T cells, but defects in development, stability, or function may render these cells dysfunctional and unable to control autoreactive T-cell responses. Alone or in combination, these factors can contribute to the escape, activation, and proliferation of autoreactive lymphocytes that result in tissue injury and clinical disease. (Reproduced from Rosenblum et al. [125]. Open Access)

most patients with autoimmune disease develop symptoms long after the abnormal immune reactions begin, it is regularly hard to identify the factors responsible for the initiation of disease. It is believed that a combination of genetic and environmental factors plays a role in the pathogenesis of these disorders. Thus a simple theory would be that polymorphisms in various genes result in defective regulation/reduced threshold for lymphocyte activation and environmental factors initiate/augment activation of self-reactive lymphocytes that have escaped regulation. Genome-wide association studies have suggested a role for plentiful genetic polymorphisms in different autoimmune diseases. The contribution of each gene seems to be small, and it is expected that multiple polymorphisms contribute to disease development [115, 116]. The strongest associations are with HLA alleles, yet it is not known how different HLA alleles contribute to disease development [117].

Assuming loss of self-tolerance is the fundamental abnormality in autoimmune diseases, it is worthwhile to investigate which mechanisms of tolerance collapse and lead to the initiation of the disease. Imbalance between effector T cells and functional Treg cells is supported by animal models of autoimmunity [118]. Decreases in the number of functional Tregs, or resistance of effector T cells to regulation, have shown to play a role in several human autoimmune disorders.

In systemic lupus erythematosus (SLE) patients, it has been demonstrated that mature naive B cells can produce autoantibodies before encounter with antigen, suggesting that defects in early B-cell tolerance checkpoints may contribute to disease development [119]. In addition, an inappropriate exaggerated innate immune response can be a trigger for autoimmunity [120]. An example for such a scenario is demonstrated in mice that lack the ubiquitin-modifying enzyme A20 and develop lethal autoimmunity due to unregulated TLR signals [121]. The mechanisms leading to the initiation of autoimmunity are summarized in Fig. 1.8.

Autoimmunity in a Tolerogenic Organ, the Liver

Autoimmune liver diseases (ALD) can be categorized according to the target of the autoimmune response, i.e., immune attack against hepatocyte or cholangiocyte, and as a consequence, the location of inflammation within the liver [122]. The clinical presentation and the immunological characteristics of these disorders differ considerably. The autoimmune

cholangiopathies comprise of a heterogeneous group of disorders including primary biliary cholangitis (PBC) involving the small intrahepatic bile ducts and two different conditions that can affect both intra- and extrahepatic bile ducts named primary sclerosing cholangitis (PSC) and immunoglobulin G4-associated cholangitis (IgG4-AC).

There is no uncertainty regarding the autoimmune nature of AIH and PBC; however, PSC should be probably considered an immune-related disorder rather than a classical autoimmune disorder since there is lack of imperative criteria necessary to define it as autoimmune, i.e., the lack of specific serum autoantibodies [123]. IgG4-related disease is a systemic disease potentially involving many organs that in some of the cases affect the liver as well. The findings imply that its autoimmune character includes the IgG4+ clones that dominate the B-cell receptor repertoire and the robust responsiveness to steroids in this disorder [124]. Occasionally, the simultaneous autoaggression against hepatocytes and cholangiocytes results in "overlap syndromes" between PBC and AIH or PSC and AIH.

AIH and PBC have a strong female preponderance, while PSC is more frequent in male. AIH and PSC affect all ages and races, while PBC is rarely seen in children. Immunosuppression is an effective treatment for AIH, while in PBC and PSC, these drugs generally lack significant efficacy.

In recent years the microbiota has gained great attention for its role in the development of various disease conditions, including liver disorders and autoimmune diseases. The exposure of the liver to gut-derived products via the portal blood supply and the potential influence of the bile on the intestinal microbiota is of particular interest for the pathogenesis of liver disorders. The human intestinal microbiota is believed to be especially important in the pathogenesis of PSC, because up to 80% of patients with PSC have concomitant inflammatory bowel diseases.

The development of autoimmunity specifically in the liver, a tolerogenic organ, is indeed a great paradox. The reasons for that are largely obscured currently and hopefully will be uncovered utilizing novel scientific methods in the near future.

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The Liver as a Lymphoid Organ

2

Zhe-Xiong Lian and Liang Li

Abbreviations

APCs	Antigen-presenting cells
BECs	Biliary epithelial cells
cNK	Conventional NK
ConA	Concanavalin A
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
ECM	Extracellular matrix
ECM1	Extracellular matrix protein 1
FXR	Farnesoid X receptor
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF-1α	Hypoxia-inducible factor 1-alpha
HLA	Human leukocyte antigen
Hobit	Homolog of Blimp-1 in T cells
HSCs	Hepatic stellate cells
HSPCs	Hematopoietic stem and progenitor cells
ICAM-1	Intercellular adhesion molecule 1
IgA	Immunoglobulin A
ILCs	Innate lymphoid cells
IRAK-M	Interleukin-1 receptor-associated kinase M
KCs	Kupffer cells
KLF2	Kruppel-like factor 2
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
Lr-NK	Liver-resident NK
LSECs	Liver sinusoidal endothelial cells
MAIT	Mucosal-associated invariant T
MHC	Major histocompatibility complex
MR-1	MHC class 1-related protein
NAFLD	Nonalcoholic fatty liver disease
NK cell	Natural killer cell
NKT cell	Natural killer T cell
PAMP	Pathogen-associated molecular pattern
PBC	Primary biliary cholangitis
PGLYRP2	Peptidoglycan recognition protein 2
PPARγ	Peroxisome proliferator-activated receptor gamma
PSC	Primary sclerosing cholangitis
S1PR1	Sphingosine-1-phosphate receptor 1
Th cell	T helper cell
TLR	Toll-like receptor
TRAIL	TNF-related apoptosis-inducing ligand
Treg cell	Regulatory T cell
Trm cell	Tissue-resident memory T cell

Key Points

- The liver is the largest solid organ in the body. It not only has metabolic and detoxifying functions but also serves as a unique lymphoid organ.
- The liver continuously receives antigens from blood circulation, including diet and commensal microbiota antigens, as well as toxic chemicals and apoptotic cells. The specific microanatomy and cell composition of the liver determine its function to maintain immune homeostasis.
- The liver contains innate immune cells which provide the first line of defense. These cells are important in modulating liver injury and maintaining an anti-inflammatory or tolerant environment.
- There are several liver-specific or liver-resident cell subsets that contribute to the uniqueness of liver immunology, i.e., liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, natural killer T cells, liver-resident nature killer cells, and tissue-resident memory T cells.
- The liver is biased to induce tolerance through negative regulation of T cells: absence of co-stimula-

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tory molecules, secretion of IL-10, activation of $CD8^+$ T cells without the assistance of $CD4^+$ T cells, sequestration of activated T cells, and expression of ligands of inhibitory molecules. These effects are mediated by antigen-presenting cells, as well as other lymphoid populations that have suppressive function.
- The liver microenvironment regulates composition, location, and function of immune cells through various ways, such as modulating the extracellular matrix, chemokines, adhesion molecules, bile acids, oxygen, and dynamics of metabolic changes.
- The liver contains hematopoietic stem and progenitor cells and a unique niche for their differentiation. However, whether the differentiation of such cells can sustain liver tolerance remains unknown.

Introduction

The liver is a unique organ. Fetal liver functions as the major site of definitive hematopoiesis [1], while postneonatal hematopoiesis is switched to the bone marrow. Thereafter the liver primarily functions as an organ for protein synthesis and metabolism. The liver filters over 2000 liters of blood every day, with two-thirds of its blood from the portal vein and others from the hepatic artery. In addition to nutrition and oxygen exchange, continuous stimulation by foreign antigens and toxins from the portal venous blood renders the liver to have exclusive features of innate immune response. The liver can regulate systemic immune homeostasis by producing many circulating factors, such as acute-phase proteins, lipoproteins, clotting factors, and complement proteins. In addition, it contains many resident cells and is biased toward immune tolerance, with strong innate immunity and poor adaptive immune response. Hence the liver has been proposed to be an immune organ, an innate immune organ, and/or a lymphoid organ [2–4]. Functional diversity of the liver and heterogeneity of liver immune cells have been incredibly attracting interests. Knowledge of liver immunology and the differences between the liver and other immune organs will advance our understanding of the mechanisms of liver tolerance.

Liver Anatomy and Cell Composition

The liver exerts its function via microscopically divided units called hepatic lobules (Fig. 2.1). The hepatic lobules are hexagon-structured with a central vein in the center, where the blood converges from the portal tracts. Blood from hepatic artery and portal vein enters the liver and circulates slowly within the lobules through microvessels into centrilobular vein and then drains via the hepatic vein. Hepatocyte-produced bile flows in the opposite direction into the bile duct within the portal tract. The microvessels in hepatic lobules are lined by specific endothelial cells of the liver, called liver sinusoidal endothelial cells (LSECs). However, they are loosely arranged and lack a basement membrane. “Millions” of large fenestrae are found along the liver sinusoid surface, making it one of the most permeable microvessels in the body. Between hepatocytes and LSECs, the space of Dissé is formed; this permeable barrier facilitates the exchange, active uptake, and degradation of circulating molecules [3, 5, 6].

The liver cellular environment contains 80% hepatocytes, which are critical in detoxification, metabolism, and protein synthesis (Fig. 2.2) [7]. Among the non-hepatocytes, about 50% of the cell population are LSECs, which form the walls of microvessels and clear infectious microorganism through endocytosis. Biliary epithelial cells (BECs) make up about 5% of the non-hepatocytes and form bile ducts for bile transport. Hepatic stellate cells (HSCs) reside in the space of Dissé and contribute to extracellular matrix production. HSCs comprise about 1% of non-hepatocytes in a healthy liver but may increase during fibrosis conditions. Hepatocytes, LSECs, BECs, and HSCs are important players in the liver innate immune response.

The liver also contains many other innate immune cells (see Fig. 2.2), including Kupffer cells (KCs), which compose about 20% of non-hepatocytes. There are also macrophages derived from bone marrow myeloid progenitors, which encompass a small part of the liver macrophage population under steady state. Dendritic cells (DCs) are professional antigen-presenting cells (APCs), but their percentage in the healthy liver remains unclear. Although about 25% of non-hepatocytes are lymphocytes, many of them have innate immune function. For example, 30% of liver lymphocytes are natural killer (NK) cells. Other innate lymphocytes include innate lymphoid cells (ILCs), natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ T cells. Furthermore, there are many resident subsets of these cells in the liver, which is different from other lymphoid organs, indicating first-line response against pathogens.

This unique microanatomy also creates a specific microenvironment in the liver, within which extracellular matrix, bile acids, metabolic products, and heterogeneous oxygen concentration deposition can affect the composition and function of liver immune cells. The liver does not have secondary or tertiary lymphoid organ-like microanatomy and functions as a predominance of innate immunity.

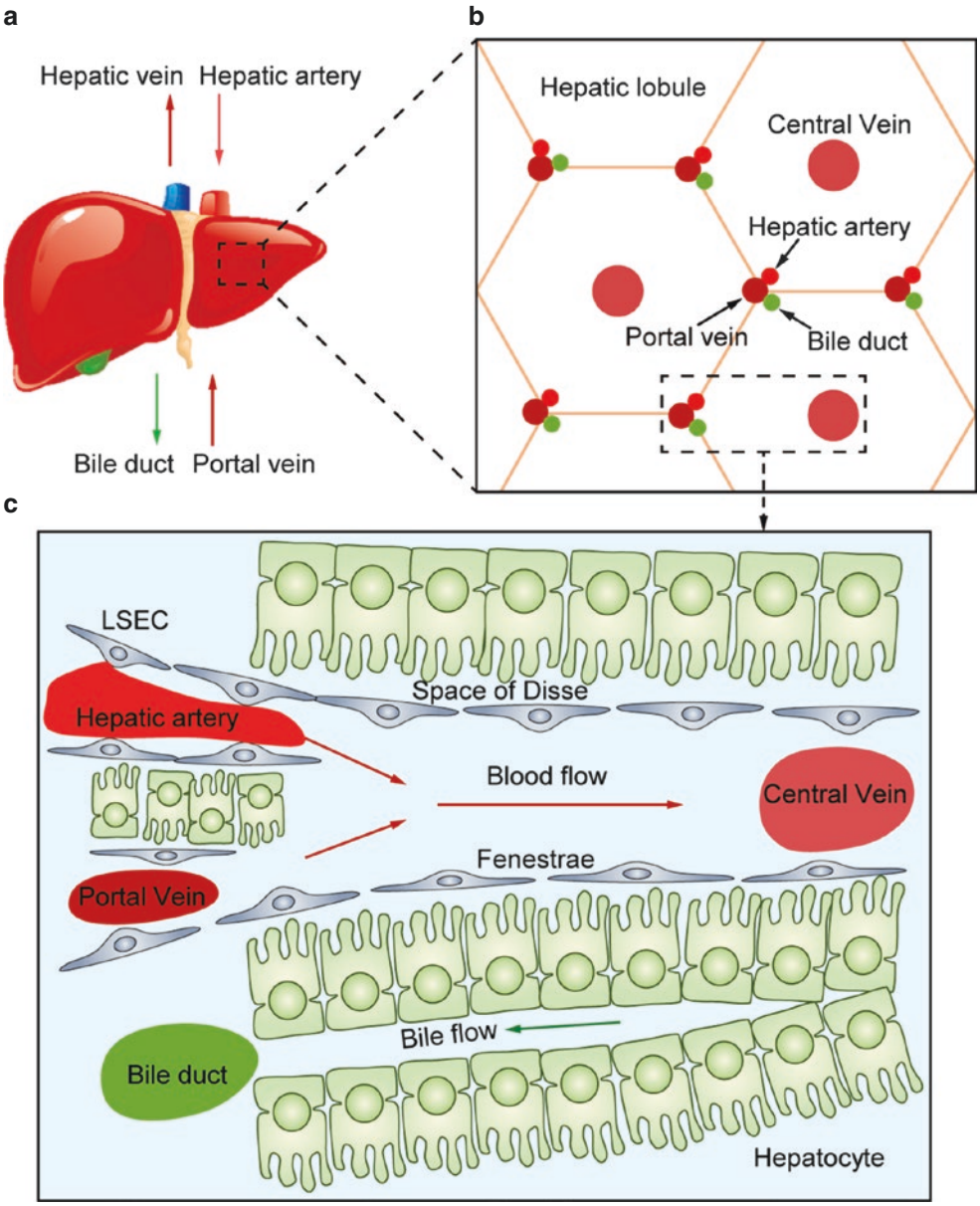

Fig. 2.1 Liver microanatomy. (**a**) Liver receives blood from both the hepatic artery and the portal vein; filtered blood goes out through the hepatic vein. Bile flows out of the liver through the bile duct. (**b**) The liver can be microanatomically divided into functional hepatic lobules. (**c**) Blood flows from the hepatic artery and portal vein in the portal tract through liver sinusoids to the central vein in the center of each hepatic lobule. Bile flows in the opposite direction to the bile duct in the portal tract

Predominance of Innate Immunity in Liver

Antigen-Presenting Cells

Hepatocytes

Hepatocytes are key cells in innate immunity and participate in systemic and local immunity (Fig. 2.3). They generate over 80% of the proteins involved in innate immunity, including acute-phase proteins, complement proteins, clotting factors, cytokines, and chemokines. First, hepatocytes produce most of the proteins in the complement system. The complement system is important for directly killing bacteria by cell lysis and also promotes clearance of bacteria, apoptotic cells, and immune complexes by phagocytes. Second, hepatocytes produce acute-phase proteins such as C-reactive protein, serum amyloid a proteins and serum amyloid p components. The serum level of acute-phase proteins can increase more than 1000-fold after infection or injury. These proteins act as opsonins to facilitate phagocytosis and further pro-inflammatory cytokine production, as well as complement activation. Third, hepatocytes produce many proteins that participate in lipopolysaccharide (LPS) recognition and activation of Toll-like receptor 4 (TLR4) signaling, such as LPS-binding protein, soluble CD14, and soluble MD-2; such production also is induced by inflammatory cytokine stimulation. Fourth, hepatocytes produce several iron metabolism-related proteins, including transferrin, lipocalin-2, hemopexin, and hepcidin. These proteins promote iron uptake by the host, directly bind

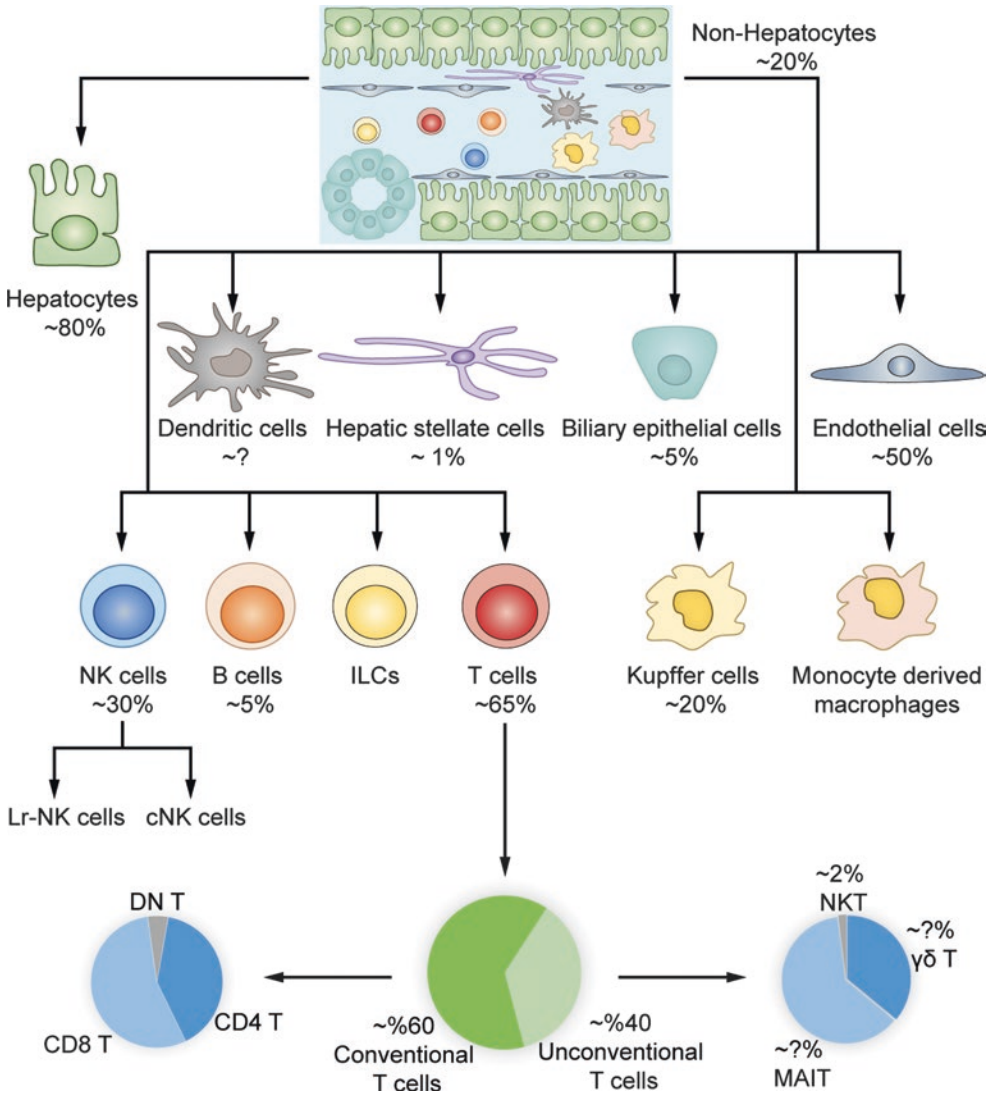

Fig. 2.2 Cell composition of healthy human liver. Hepatocytes compose about 80% of liver cells. Among the non-hepatocytes, about 50% are LSECs. Kupffer cells are the major myeloid part which makes up 20% of non-hepatocytes. There are also bone marrow-derived macrophages and DCs in the liver. About 5% of non-hepatocytes are BECs. HSCs compose only about 1% of non-hepatocytes but are critical for ECM production and liver fibrosis. Among the lymphocytes, NK cells (~30%) and T cells (~65%) are the two major populations. Liver also contains some B cells (~5%) and ILCs. Liver T cells are heterogeneous, including about 40% conventional T cells and 60% unconventional T cells. Among conventional T cells, $CD8^+$ T cells outnumber $CD4^+$ T cells, with a small proportion of DN T cells. Among unconventional T cells, there are MAIT cells (62%), γδ T cells (36%), and NKT cells (2%)

to iron uptake proteins on bacteria, retain heme from bacteria by direct binding, and inhibit the excretion of host cells. These mechanisms all limit the access of bacteria to iron, thus suppressing their survival and proliferation. Fifth, hepatocytes produce fibrinogen. Fibrinogen is a key factor in coagulation, but its active fragment fibrin has antibacterial activity. Fibrinogen can also exert antibacterial function through activation of the complement system and mediate adhesion of monocytes and neutrophils. Sixth, proteinase inhibitors secreted by hepatocytes can inactivate proteases released by pathogens and dead or dying cells, thus activating the innate immune system. They can also express peptidoglycan recognition protein 2 (PGLYRP2), which is an immunomodulatory protein that hydrolyzes the peptidoglycan of bacteria to avoid their recognition by immune cells [7].

There are other ways that hepatocytes participate in innate and adaptive immunity. Hepatocytes express TLR4 for the recognition and clearance of bacteria-derived LPS [8]. Upon infection and liver damage, hepatocytes produce several chemokines such as CCL2 and CXCL1, to attract other immune cells that participate in the protection or aggravation of inflammation [7]. Moreover, hepatocytes can directly interact with T cells through fenestrae [9]. They have polarized expression of major histocompatibility complex (MHC) class I and intercellular adhesion molecule I (ICAM-1) molecules on the perisinusoidal cell membrane, rendering them capable of priming $CD8^+$ T cells. However, due to the lack of co-stimulatory signaling, hepatocyte-primed cells undergo apoptosis [10, 11].

LSECs

LSECs are liver-specific endothelial cells, which represent a permeable barrier between blood cells and hepatocytes. LSECs account for about 50% of the non-parenchymal cells in the liver (see Fig. 2.2) [3]. The hepatic sinusoid is the first site of exposure to these antigens, and LSECs are important for taking up and eliminating soluble antigens that entered through the portal vein [12].

As an important part of innate immunity of the liver, LSECs have a high endocytic capacity based on the level

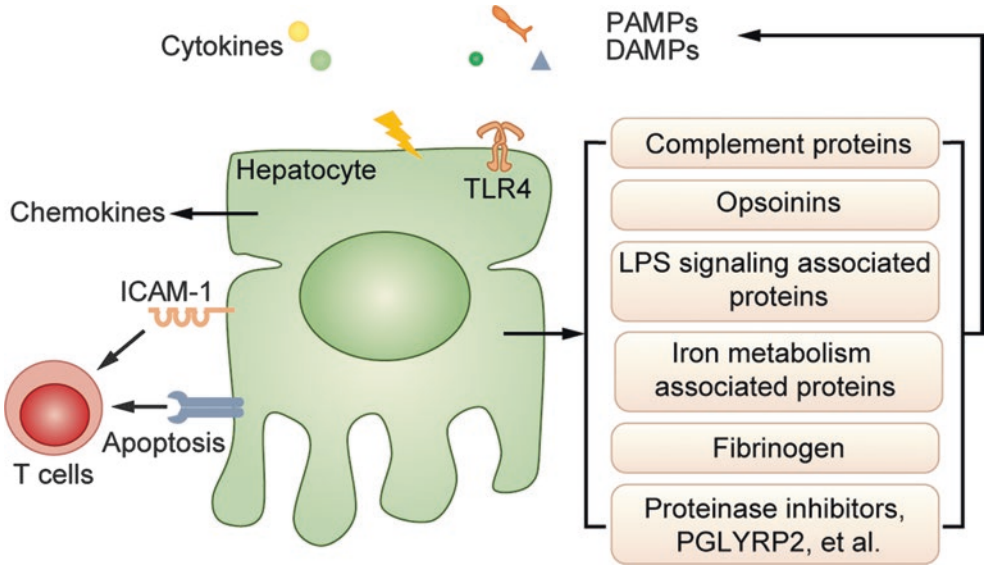

Fig. 2.3 Immunology of hepatocytes. Hepatocytes are critical participants in both liver and systemic immunity. They continuously produce large amounts of circulating proteins that can modulate systemic immune responses. The production of these proteins can be augmented when they are stimulated locally by pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and cytokines. Hepatocytes also express chemokines that can recruit immune cells into the liver and migrate through LSECs for direct contact. They express integrin ligands such as ICAM-1 which can mediate T cell retention. They express MHC molecule but no co-stimulatory molecules, which can lead to T cell apoptosis

of scavenger receptor expression that can recognize antigens flowing through liver sinusoids. For example, nearly all virus can be detected in LSECs within a minute after intravenous infusion in mice [13], demonstrating the role of LSECs in the clearance of blood-borne viruses. LSECs also express TLRs, which are critical in innate immunity. However chronic exposure to LPS leads to IL-10 production and resistance of TLR signaling in LSECs, which may prevent an inappropriate response to gut microbiota products [14]. In contrast, the special location and the expression of many adhesion molecules by LSECs allow them to interact with other liver cells. For example, LSECs participate in imprinting a Kupffer cell identity on monocytes through Notch-induced liver X receptor-α signaling [15, 16] and maintaining quiescence of HSCs [17]. Transmigration of monocytes via LSECs can also lead to the generation of myeloid-derived suppressor cells [18].

LSECs not only regulate innate immune responses but also directly regulate adaptive immune responses through antigen presentation to T cells. LSECs have the potential to prime immunity in the liver, but they are more likely to maintain tolerance. LSECs express MHC-II molecules [19]. However, antigen presentation by LSECs fails to induce $CD4^+$ T cell differentiation toward T helper 1 (Th1) cells [20]. They exert other tolerogenic properties, including inhibiting the expansion of mature Th1 cells [21], promoting the generation of regulatory T (Treg) cells [22], and suppressing the secretion of inflammatory cytokines IFN-γ and IL-17 [23]. LSECs also have the capacity to cross-present exogenous antigens on MHC class I molecules to $CD8^+$ T cells. However, activation of $CD8^+$ T cells induces upregulation of PD-L1 on LSECs which suppresses their development into cytotoxic effector T cells [24, 25].

In summary, LSECs maintain the balance of liver immune activation and leukocyte recruitment, which determine liver immunity and stand between hyperactivation and dysfunction (see Fig. 2.6).

BECs

The biliary tree is composed of biliary ducts that are lined with biliary epithelial cells. The biliary tree spreads over the liver and participates in a first line of defense against microbial components, chemical xenobiotics, and foreign antigens. BECs contribute to maintaining immune tolerance in the liver (Fig. 2.4). BECs from a healthy human liver express low levels of human leukocyte antigen (HLA) class I and no HLA class II molecules. However, during pathological conditions such as virus infection and primary biliary cholangitis (PBC), BECs can be stimulated to upregulate MHC molecules, but whether they express co-stimulatory molecules is not clear [26, 27]. Moreover, neither normal nor cytokine-stimulated BECs can directly induce T cell activation [28]. BECs can also express PD-1 ligands and TNF-related apoptosis-inducing ligand (TRAIL) in PBC and primary sclerosing cholangitis (PSC), which can induce apoptosis of leukocytes to limit the immune response [29, 30].

Aside from antigen presentation, BECs maintain liver tolerance in other ways. They express TLRs that sense microbial and viral components [31]. However, they also express interleukin-1 receptor-associated kinase (IRAK)-M, which

negatively regulates TLR signaling and maintains tolerance against endotoxins [32]. BECs can also secrete defensins and transport immunoglobulin A (IgA) to the bile duct lumen to eliminate pathogens that induce an excessive immune response [33, 34]. Besides, BECs constitutively express peroxisome proliferator-activated receptor gamma (PPARγ) which is a negative regulator of NF-kB signaling that attenuates inflammatory signals [35]. In conclusion, BECs utilize several mechanisms to maintain immune tolerance within the hepatic microenvironment.

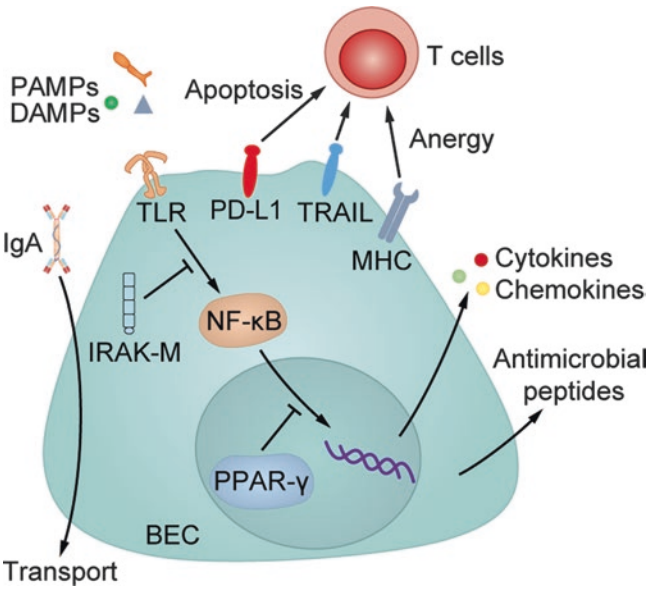

Fig. 2.4 Immunology of BECs. BECs are important players in maintaining liver tolerance. Although they express TLRs that can recognize PAMPs and DAMPs, their response and cytokine/chemokine expression are negatively regulated by IRAK-M and PPAR-γ. They can also transfer IgA to fight against pathogens. They can express MHC molecules with low co-stimulatory molecules and can induce T cell anergy. They also express inhibitory molecules such as PD-L1 and Fas to induce apoptosis of activated T cells

KCs and Bone Marrow-Derived Macrophages

KCs compose over 80% of tissue-resident macrophages and are critical for the elimination of insoluble waste by phagocytosis. KCs reside within the sinusoidal vascular space and account for about 20% of the non-parenchymal cells in the liver (see Fig. 2.2) [3]. Owing to their special localization in the liver sinusoid, KCs perform initial immune surveillance against pathogens from the portal vein via pattern recognition receptors (Fig. 2.5). They also emit extensions into the space of Dissé and function as a bridge between the blood and liver parenchyma. KCs are also heterogeneous, with differential expression of CD11c and MHC molecules, phagocytic capacity, and metabolic functions [36, 37].

KCs play a major role in maintaining immune tolerance and providing an anti-inflammatory micromilieu during homeostasis (Fig. 2.6) [38]. They can produce TNF-α upon stimulation and can also act as "incompetent" APC, expressing low levels of MHC-I/MHC-II and co-stimulatory molecules at a steady state, which leads to the induction of anergy in T cells [39]. Moreover, KCs can suppress the antigen-presenting function of DCs and LSECs to induce immune tolerance [40]. Apart from affecting antigen presentation, several mechanisms contribute to the tolerogenic features of KCs: (1) production of immune regulatory molecules (IL-10, nitric oxide, TGF-β) [41], (2) expression of co-inhibitory molecule PD-L1 [42], (3) induction of Treg cells and stimulation of their suppressive activity [43], and

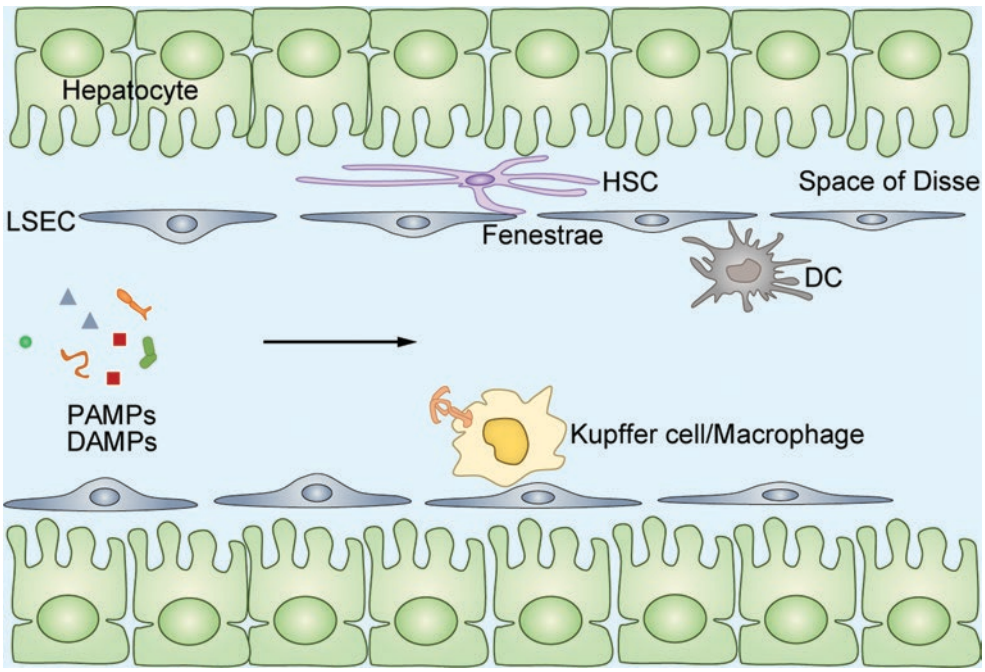

Fig. 2.5 Antigen-presenting cells in the liver. Liver is a lymphoid organ dominated by innate immunity. It is enriched in cells that play a role in innate immune response, including hepatocytes, LSECs, Kupffer cells and monocyte-derived macrophages, HSCs, and DCs. They form the frontline of firewall against PAMPs and DAMPs

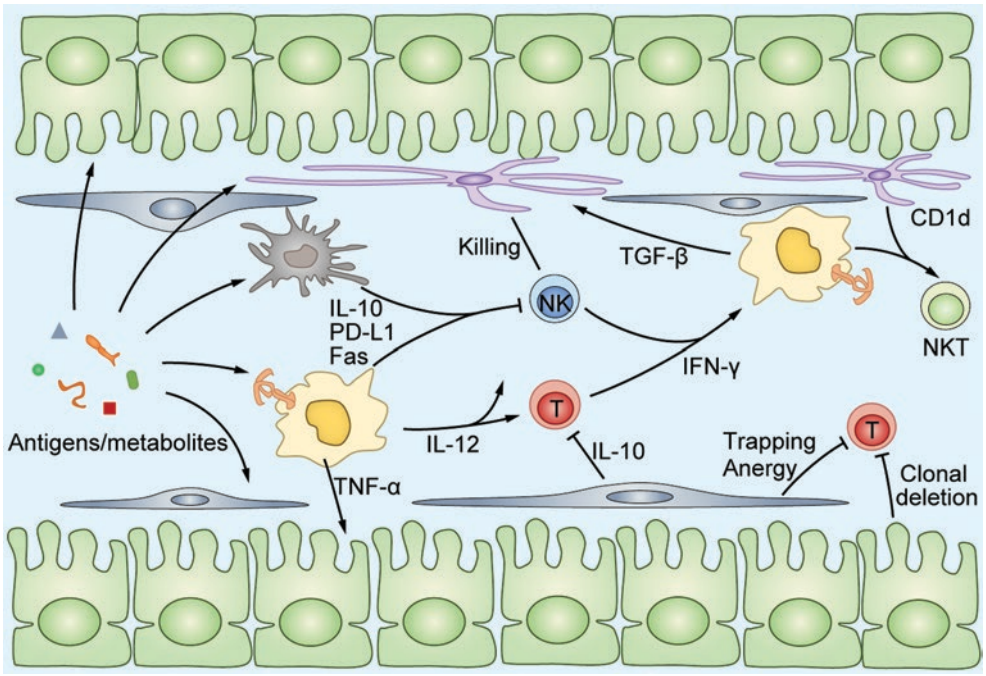

Fig. 2.6 Immunobiology of antigen-presenting cells. PAMPs and DAMPs can stimulate antigen-presenting cells in the liver, including hepatocytes, LSECs, Kupffer cells, DCs, and HSCs. Kupffer cell-derived TNF-α can mediate liver injury. Kupffer cells and DCs can stimulate the activation of T cells and NK cells through IL-12 production. Under some conditions, they can also express PD-L1 and produce IL-10 to suppress T cell and NK cell activation. LSECs can inhibit T cell responses by secreting IL-10 and sequestering activated T cells. Hepatocytes can also mediate clonal deletion of T cells. Kupffer cells and HSCs can present lipid antigens to NKT cells through CD1d. Kupffer cells can also activate HSC through producing TGF-β, while NK cells can suppress HSC activation by direct killing. NK and T cell-derived IFN-γ can stimulate Kupffer cells and induce their activation

(4) expression of FasL to induce T cell apoptosis [44]. The tolerogenic function of KCs may promote the progression of hepatitis virus infection and hepatocellular carcinoma (HCC), rendering them a potential therapeutic target.

Although KCs are poor activators of T cell activation under physiological conditions, stimulation by TLR ligands or cytokines can restore their antigen-presenting function [45]. They can promote antimicrobial immunity in an infectious microenvironment by presenting antigens to NKT cells and cross-presenting antigens to $CD8^+$ T cells [46, 47]. Additionally, KCs can indirectly modulate the immune response during infection by releasing cytokines and chemokines. They can recruit dendritic cells and other leukocytes to the liver and activate infiltrated leukocytes [48]. Thus, via a delicate mechanism, KCs can maintain tolerance in the presence of antigen and alarm the immune system when appropriate. Moreover, KCs, which originate from the yolk sac in the liver, contain bone marrow-derived macrophages, especially during infection and liver injury. Monocytes can also differentiate into resident macrophages that mimic the phenotype and function of KCs. This process is regulated by HSCs, LSECs, and hepatocytes [15, 16]. Studies using single-cell technologies have also revealed new macrophage subsets in the human liver [49, 50], indicating that the heterogeneity of macrophages in the liver needs more investigation.

DCs

DCs are the most important APCs to initiate the adaptive immune response, but classically, they function in the spleen and lymph nodes or other tertiary lymphoid tissues. DCs are also present in the liver and contribute to maintaining a tolerogenic condition under steady state (see Fig. 2.5). Immature DCs or DC progenitors enter the liver via the portal vein and then move through the liver microvessels to the central vein or transmigrate through LSECs into the space of Dissé [45]. During this time, they interact with other immune cells and uptake antigens within the liver. The liver microenvironment, including stromal cells and epithelial cells, can induce the generation of tolerogenic DCs [41]. Liver DCs produce substantial amounts of IL-10 upon TLR4 stimulation and can promote immunologic hyporesponsiveness [51].

Liver DCs are subdivided into two major populations, $CD11b^+$ myeloid DCs and $CD123^+$ plasmacytoid DCs. While myeloid DCs in the liver produce substantial amounts of IL-10 and indoleamine 2,3-dioxygenase that can induce Treg cells, plasmacytoid DCs participate mainly in type I IFN production upon stimulation. Both of the two DC subsets have reduced activity to stimulate T cell activation. Liver plasmacytoid DCs also express PD-L1 to limit T cell activation [6]. There are also $CD8^+$ DCs in the liver, which are important for T cell activation and Th1 responses. Thus, heterogeneity of liver DC subsets may regulate the balance between tolerance and immunity.

HSCs

HSCs are located in the space of Dissé and are a minority subset in the liver, accounting for 5% to 8% of total liver cells (see Fig. 2.2) [52]. HSCs have been identified as the main effector cells in liver fibrosis [53]. They can transdifferentiate into collagen-producing myofibroblasts. Apoptosis of activated HSCs is the best characterized mechanism of fibrosis resolution [54]. HSCs can participate in liver innate immune response and induction of tolerance (see Fig. 2.6). HSCs are reservoirs of vitamin A in the healthy liver; quiescent HSCs store 80% of total liver retinols in their lipid droplets. Activated HSCs lose their lipid droplets and become myofibroblasts. Retinoid acid released by HSCs may modulate the function of other cells such as hepatocytes, LSECs, and KCs [55]. The metabolite of vitamin A, all-*trans* retinoic acid, is also important for the generation of Treg cells [56]. HSCs are also identified as liver-resident APCs and express MHC-I and MHC-II molecules, as well as CD1 and co-stimulatory molecules and can prime T cells efficiently [57].

HSCs also express various kinds of TLRs, which can be activated by hepatotropic viruses and lead to antivirus responses. However, the activation of TLR signals can result in liver fibrosis, inflammation, and injury. The mechanism of how HSCs exert immune function and the possible functional consequences are not well understood [58–60]. HSCs also participate in immune suppression by inducing regulatory T cells [61], inhibiting T cells by PD-L1 or TGF-β [62], and inducing myeloid suppressor cells [63].

Innate Lymphocytes

NKT Cells

NKT cells are a family of innate-like T cells. They express T cell receptors that recognize lipid antigens, as well as surface markers of NK cells. NKT cells are enriched in microvascular compartments of the liver and compose about 2% of unconventional T cells in the human liver (see Fig. 2.2) and up to 50% of hepatic lymphocytes in mice.

NKT cells can be activated by lipid antigens presented by CD1d or by TLR signaling and cytokine stimulation. Thus, they can be a bridge between the innate and adaptive immune response (Fig. 2.7) [64]. NKT cells also act as a double-edged sword in liver immunity: either promoting

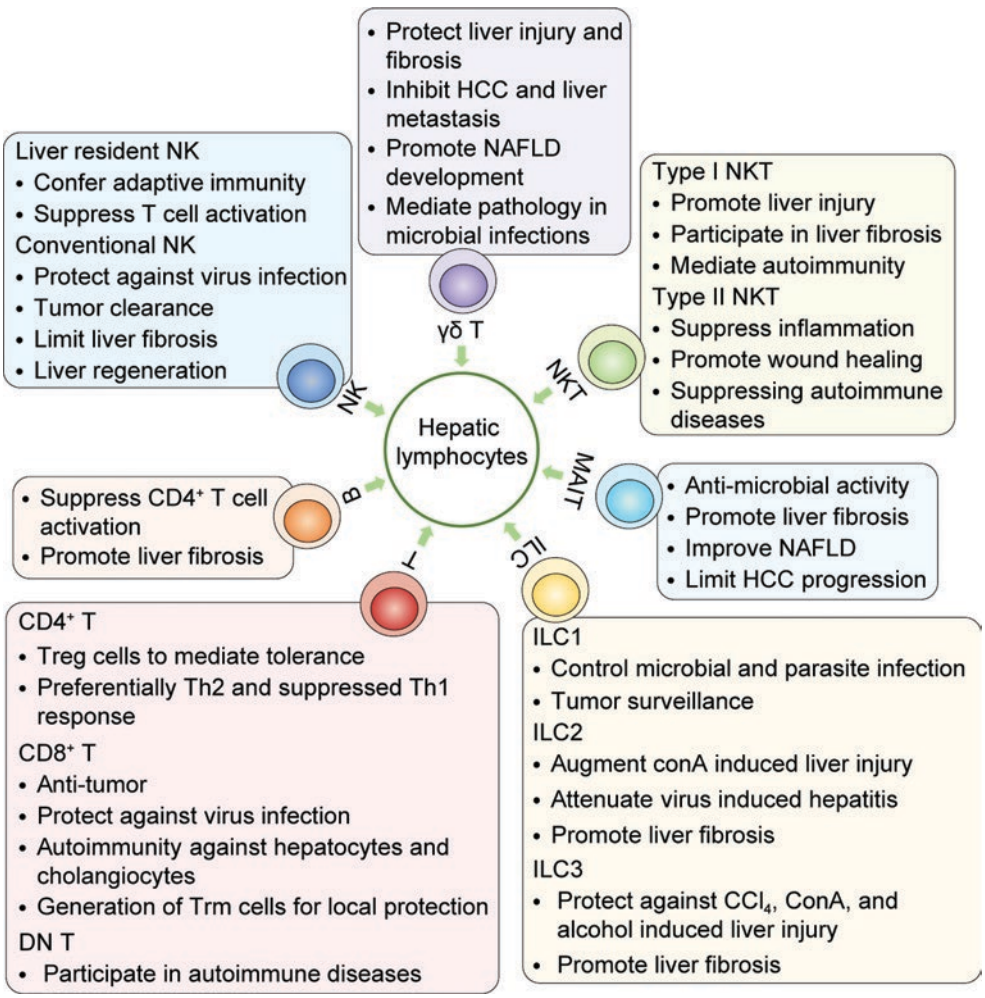

Fig. 2.7 Innate and adaptive immune cells in the liver. Liver lymphocytes are composed of cell populations that have either innate or adaptive function. The innate lymphocytes include NK cells, ILCs, γδ T cells, MAIT cells, and NKT cells. The adaptive lymphocytes include T cells and B cells. Although a lymphoid organ, B cells primarily participate in local liver immunology through the innate function. Innate and adaptive lymphocytes play important roles in the pathogenesis and protection of many liver diseases

or inhibiting inflammation. This is mainly because hepatic NKT cells can be categorized into two subsets—type I or invariant NKTs and type II NKTs. These two subsets have opposing roles in liver inflammation. Type I NKT are pro-inflammatory, whereas type II NKT cells inhibit type I NKT-mediated liver injury [65].

After activation, type I NKT cells can produce pro-inflammatory cytokines, such as IL-4, IL-5, IL-13, IL-17, INF-γ, and TNF-α. They can also express cytotoxic mediators, such as perforin, FasL, and TRAIL, to mediate liver injury, recruit, and activate other leukocytes [64, 66]. Type I NKT cells can promote fibrogenesis through the Hedgehog pathway and secrete osteopontin to stimulate HSC activation [67, 68]. However, they can also exert anti-fibrotic activity by inhibiting HSC proliferation or by killing HSC directly [69]. It has also been reported that type I NKT cells are linked to immune tolerance breakdown in PBC when stimulated by mucosal commensals, which provide a clue that NKT cells have a role in the development of autoimmunity [70]. Furthermore, type I NKT cells also participate in the protection against HBV infection and inhibition of tumor progression.

In contrast to the promoter role of type I NKT cells in liver damage, type II NKT cells act to protect against liver damage. They express more diverse TCRs and are mainly activated by self-antigens, such as glycolipid sulfatide. Interactions among type II NKT cells and hepatic DCs result in the regulation of type I NKT cell activity [71]. Activation of invariant NKT cells by self-antigens induces IL-4, but not IFN-γ production, which promotes hepatocyte proliferation, monocyte transition, and improved wound healing [72]. Type II NKT cells have been demonstrated to play a role in the protection against type I diabetes, experimental autoimmune encephalomyelitis, and ischemia reperfusion injury [64].

MAIT Cells

MAIT cells are characterized by the expression of CD3, TCRVα7.2, and CD161, which comprise about 60% of unconventional T cells in the liver (see Fig. 2.2). They are innate-like T cells and play an important role in the first-line defense of the liver (see Fig. 2.7). They are restricted by MHC class 1-related protein (MR-1) [73], which presents microbial-derived vitamin B metabolites and mediates their differentiation and expansion in the periphery [74]. They can also be activated by IL-12 and IL-18. Upon activation, MAIT cells can secret large amounts of inflammatory cytokines and mediate cytolysis by releasing granzyme B and perforin [75].

Both blood and liver MAIT cells exhibit an activated phenotype with high CD69, HLA-DR, and CD38 expression [76]. They express CXCR6 and CCR6, and the liver constitutively expresses their ligands CXCL16 and CCL20. They predominantly reside around bile ducts within the portal tracts in both healthy and diseased livers [76]. BECs exposed to bacteria are able to activate MAIT cells in an MR1-dependent, cytokine-independent manner, suggesting that MAIT cells can defend the biliary mucosa against infection from the gut. In the inflamed liver, MAIT cells may be further recruited to the sinusoids through CXCR3, lymphocyte function-associated antigen-1 (LFA-1), and integrin α4β1.

MAIT cells participate in many liver diseases related to infection or chronic inflammation and may be a promising therapeutic target. MAIT cells may participate in the development of liver fibrosis through enhancing the pro-inflammatory properties of monocyte-derived macrophages and promoting fibrogenic function of myofibroblasts [77, 78]. They may also improve nonalcoholic fatty liver disease (NAFLD) through promoting M2 macrophage polarization [79]. In PBC, cholic acid stimulation of hepatocytes induces IL-7 production, which can stimulate MAIT activation [80]. The antibacterial potency of MAIT cells in alcoholic liver disease is compromised, which increases their susceptibility to infection [81]. MAIT cells are decreased in HCC and are reprogrammed to a tumor-promoting direction, with down-regulated expression of cytokine secreting and cytolysis effector function genes [82, 83].

NK Cells/Liver-Resident NK Cells

NK cells were first described in the liver as pit cells as they contain cytoplasmic granules. Inside these granules are cytotoxic molecules, such as perforin and granzyme B. NK cells rapidly recognize and clear virus-infected and tumor cells without antigen specificity. They also participate in shaping the adaptive immune response [84]. Various activating and inhibitory NK cell receptors expressed on NK cells control their recognition, priming, and activation. For example, in the "missing-self hypothesis," MHC-I molecules expressed on tissue cells are recognized by inhibitory killer Ig-like receptors, and absence of MHC-I triggers NK cell-mediated killing.

Although NK cells develop from the bone marrow, they are enriched in the liver and compose about 30% of the total lymphocytes (see Fig. 2.2). Within the liver, NK cells are found to be attached to endothelial cells and KCs, but not in the space of Dissé [85]. Hepatic NK cells are phenotypically and functionally different from circulating NK cells and participate in various liver diseases and liver regeneration [86]. NK cells are important in controlling hepatic viral infection, including hepatitis B virus (HBV) and hepatitis C virus (HCV), but they can also directly kill hepatocytes and cholangiocytes and promote the development of autoimmune liver diseases. NK cells can promote liver regeneration through cytotoxicity or negatively regulate liver regeneration through IFN-γ production under different circumstances [85, 87]. In liver fibrosis, NK cell-mediated killing of activated HSCs through RAE1-NKG2D interaction is important for fibrosis resolution [54]. Additionally, a higher number of

tumor-infiltrating NK cells slow the progression and predict a better outcome of HCC patients [86].

Hepatic NK cells are heterogeneous, including conventional NK cells (cNK) and liver-resident NK cells (Lr-NK cells) (see Fig. 2.7). Lr-NK cells were first identified in mice as $CD49a^+DX5^-$ NK cells and $CD49a^+$ NK cells in human [88, 89]. Lr-NK cells develop from different lineages from cNK cells. cNK cells require Eomesodermin for development, while Lr-NK cells require T-bet for development and aryl hydrocarbon receptor for maintenance [90]. Although the roles of cNK cells have been widely reported, functions of Lr-NK cells still need to be investigated. Aside from their ability to confer adaptive immunity in skin-contact inflammation, Lr-NK cells are reported to have suppressive functions. They can suppress hepatic T cell antiviral activity through PD-L1 [91]. They have also been shown to suppress $CD4^+$ T cell proliferation in an animal model of autoimmune cholangitis [92].

Innate Lymphoid Cells (ILCs)

ILCs are a family of lymphocytes that do not express antigen-specific receptors like T and B cells [93]. They can be divided into NK cells, ILC1s, ILC2s, ILC3s, and Treg cells, which mirror the classification of T helper cell subsets and participate in maintaining tissue homeostasis. They are reported to contribute to controlling commensals and pathogens at barrier tissues, promoting adaptive immunity and regulating tissue inflammation [93]. The liver is enriched with ILC1s, but it has very few ILC2s and ILC3s. These ILC2s and ILC3s, however, show liver residency signatures, indicating they also play an important role in local liver immunity (see Fig. 2.7).

Aside from NK cells, the heterogeneity and roles of ILCs in liver homeostasis and diseases have recently gained increasing attention. ILC1s play an important role in the control of microbial and parasite infections and tumor surveillance [94]. Liver ILC2s express ST2 and play either a pro- or anti-inflammatory role in response to liver injury induced by IL-33. They can augment concanavalin A (ConA)-induced liver inflammation, while attenuating adenovirus-induced hepatitis [95]. ILC3s in the liver can secrete IL-22 and protect against carbon tetrachloride-, ConA-, and alcohol-induced liver injury [95]. Both ILC2s and ILC3s can promote liver fibrosis. ILC2s can trigger HSC activation through IL-13-STAT6 signaling pathway, and ILC3s can directly activate HSC through IL-17 and IL-22, as well as suppress IFN-γ production [95].

γδ T Cells

γδ T cells are a subset of T cells that expresses γδ T cell receptors and functions as a bridge between innate and adaptive immunity. The liver is one of the richest sources of γδ T cells, which comprise about 15% of liver T cells, but there is less than 5% of these cells in the blood [96]. γδ T cells are heterogeneous and can recognize self- or nonself-proteins, lipids, and phosphorylated isoprenoids. The ligands of many γδ TCRs have not been identified. A proportion of the known ligands for γδ TCRs are lipid antigens, which are utilized by the gut microbiota to sustain IL-17-producing γδ T cell homeostasis, including activation, survival, and proliferation in the liver [97].

γδ T cells are rapid responders and exert cytotoxic function or secrete large amounts of cytokines that participate in regulating liver homeostasis. They play protective roles in ConA-induced liver injury; hepatitis virus, adenovirus, and cytomegalovirus infection; CCl_4 and methionine choline-deficient diet-induced liver fibrosis; liver cancer; and liver metastasis of colon cancer. They have also been reported to mediate pathology in *Schistosoma japonicum*, mouse hepatitis virus, HCV, and adenovirus infections and promote NAFLD development [98, 99].

The human liver contains clonal expanded circulating and resident γδ T cells [100]. One subset of liver-resident $V\delta1^+$ γδ T cells expresses low level of CD27 and CD45RA but high IFN-γ and TNF-α and participates in chronic virus infection [100]. In conclusion, the heterogeneity of γδ T cells results in the potential for distinct functions under different liver pathophysiology conditions.

Adaptive Immune Cells

T/B Cells

The healthy liver also contains conventional T cells and B cells (see Fig. 2.2), but liver adaptive immune cells have specific properties (see Fig. 2.7). Although $CD8^+$ T cells and activated T cells are predominant liver T cell counterparts, many of them undergo apoptosis and deletion within the liver [5]. $CD8^+$ T cells usually outnumber $CD4^+$ T cells in the liver and may play a pathogenic role in many autoimmune liver diseases, such as PBC [101]. $CD4^+$ T cells can be polarized into different helper T cell subsets. However, liver APCs preferentially polarize naive $CD4^+$ T cells into Th2 cells and Treg cells and induce apoptosis of polarized Th1 cells [102]. The liver also contains $CD4^-CD8^-$ T cells, which expand during autoimmune diseases and may play a role in disease progression [103].

Within $CD4^+$ T cells, Treg cells participate in the generation of liver tolerance. During the neonatal period, a rapid increase in hepatic Treg cells is critical for self-tolerance and liver maturation [104]. LSECs, but not KCs, are important in inducing Treg cells in the liver, and delivery of autoantigen peptides to LSECs can induce generation of autoantigen-specific Treg cells to suppress established autoimmune diseases [105, 106]. Liver DCs are also important in the generation of Treg cells [41]. KCs can also induce the genera-

tion of IL-10-producing CD4+ T cells, which contribute to tolerance of HBV [107]. Additionally, the hepatic environment is deficient in IL-2 and enriched in pro-inflammatory cytokines, which can impair Treg cell function [108].

B cells participate in adaptive immune responses via antigen presentation, cytokine secretion, and antibody production. B cells comprise only about 5% of hepatic lymphocytes, and little is known about the population and functional diversity of B cells in the liver, especially their function in situ. Hepatic B cells are activated by TLR signaling in the liver and may participate in promoting liver fibrosis and forming intraportal lymphoid follicles during chronic HCV infection [109, 110]. In the tolerogenic liver environment, IL-10 induces CD11b expression on B cells and their production of IL-10, which in turn suppresses CD4+ T cells in autoimmune hepatitis [111].

Tissue-Resident Memory T (Trm) Cells

Trm cells were recently characterized as a subset of memory T cells that resides in barrier tissues, such as the skin, lung, gut, and liver [112]. They respond more rapidly before the recruitment of other memory T cells and provide localized protective immunity and immunosurveillance through direct cytolysis or pro-inflammatory cytokine secretion. The liver also contains Trm cells. Liver Trm cells reside in the liver sinusoids and express several signature genes of tissue residency, such as high levels of homolog of Blimp-1 in T cells (Hobit), CD69, and CXCR6, and low levels of CCR7, CD62L, Kruppel-like factor 2 (KLF2), and sphingosine-1-phosphate receptor 1 (S1PR1) [95]. They may also require IL-2 for antigen-specific proliferation. Liver Trm cells are important in the protection against malaria, HBV, and lymphocytic choriomeningitis mammarenavirus infection, but their roles remain to be elucidated in other pathological conditions, such as liver cancer and autoimmunity (see Fig. 2.7) [112].

Liver as a Tolerogenic Organ: Mechanisms

Liver tolerance was first observed in the 1960s, when liver allografts transplanted between pigs were shown to survive without immunosuppressive treatments [102]. Furthermore, it was observed that liver transplantation can increase the acceptance of other grafts that are co-transplanted, indicating an induction of systemic tolerance in mice, rats, and human. Additionally, direct transplantation into the portal vein leads to increased survival of donor cells. Many other findings also support the conclusion that the liver is tolerogenic, such as the persistence of some liver pathogens (HBV, HCV, and malaria) and the induction of oral tolerance to food antigens.

The mechanism of liver tolerance relies on the specific composition and function of hepatic immune cells (Fig. 2.8). The liver is an important place for T cell priming; however, it maintains systemic tolerance through negative regulation of T cells [113]. The liver contains many cells that express MHC molecules without co-stimulatory molecules, which can induce T cell anergy and apoptosis. TGF-β and IL-10 produced by KCs, LSECs, and Treg cells can also induce T cell malfunction. Naive T cells can interact with hepatocytes, LSECs, and BECs, which express MHC-I but not MHC-II. This results in CD8+ T cell priming without the help of polarized helper T cells, thus resulting in defects in

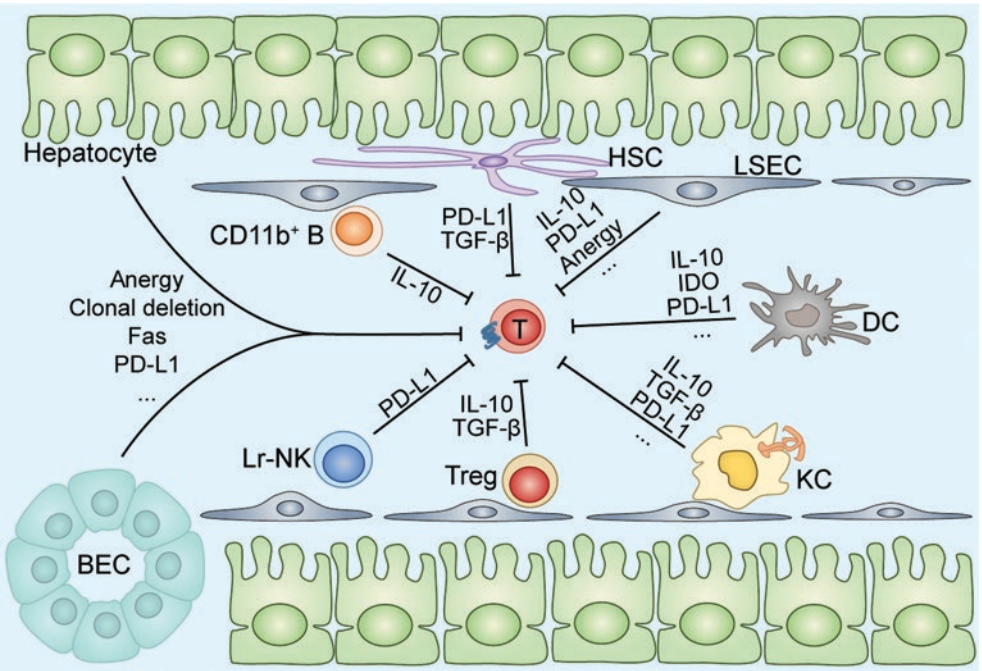

Fig. 2.8 Mechanisms of liver tolerance. T cells in liver microenvironment are suppressed in many ways. Hepatocytes and BECs can mediate T cell anergy or clonal deletion. Suppressive milieu in the liver is composed of immunosuppressive cytokines such as IL-10 and TGF-β1 and inhibitory ligands such as PD-L1. This suppressive milieu is contributed by hepatocytes, LSECs, BECs, Kupffer cells, HSCs, tolerogenic DCs, Treg cells, Lr-NK cells, CD11b+ B cells, and possibly other cell types

$CD8^+$ T cell function and memory formation. Activated T cells can be retained in the liver by LSECs, which facilitate the suppression of their function by other hepatic immune cells and induction of systemic tolerance. Moreover, cells in the liver such as $CD11b^+$ B cells and Lr-NK can suppress T cell activation and contribute to liver tolerance.

Reverse tolerance, or a break in liver tolerance, is important for the treatment of chronic infections and the development of HCC. Blocking inhibitory receptors, such as PD-1, have been shown to be effective in HBV infection and are currently being investigated as a treatment for HCC [114, 115]. However, hepatotoxicity is one of the risks of blocking inhibitory molecules in cancer [116]. Inducing hepatic tolerance against certain antigens, however, may be effective for the treatment of autoimmune diseases. Oral administration or ectopic expression of myelin basic protein in the liver can protect mice from neuroinflammation through induction of antigen-specific Treg cells in the liver [117, 118].

Regulation of the Immune Response by the Liver Microenvironment

Extracellular Matrix (ECM) Regulation of Immune Cell Response in the Liver

ECMs are non-cell components that form an intricate network that provides a physical scaffold, structural support, tensile, compressive strength, and elasticity in tissues and organs. The interaction with ECM components is important for cell fate, activation, and migration. Thus, quantitative and qualitative modification of the liver extracellular matrix will affect its structure and function, including tissue homeostasis, differentiation, and diseases (Fig. 2.9).

Liver ECM components can directly regulate hepatic immune cell activation and function. ECM components change during the development of fibrosis and are different between periportal and pericentral areas. Different components can differentially regulate the proliferation of hepatocytes and biliary cells in vitro [119]. Moreover, one ECM protein, endogenous extracellular matrix protein 1 (ECM1), can maintain architectural and functional homeostasis of the liver by interacting with αv integrin to suppress the activation of TGF-β and HSCs [120].

However, the liver ECM can also provide mechanical signals for hepatic immune cell function. During inflammation or infection, tissue injury is induced and disrupts mechanical homeostasis. Integrin-mediated adhesion exerts forces on cells, which induce cells to sense the physical properties of the surrounding microenvironment [121]. Several mechanotransducers have been identified on endothelial and epithelial cells, including cell adhesion protein complexes, primary cilia, and mechanically gated ion channels [122]. Extracellular deposition increases tissue stiffness, which activates fibroblasts and suppresses COX2 and PGE2 expression to promote fibrosis progression [122–124].

Bile Acid Regulation of Immune Response in the Liver

Bile acids are synthesized by hepatocytes from cholesterol and are important for nutrition absorption. However, bile acids are also regarded as tissue damaging and pro-inflammatory molecules [125]. They are recognized by several receptors such as G protein-coupled bile acid receptor 1 (also known as TGR5) and farnesoid X receptor (FXR). These receptors are expressed by many innate immune cells

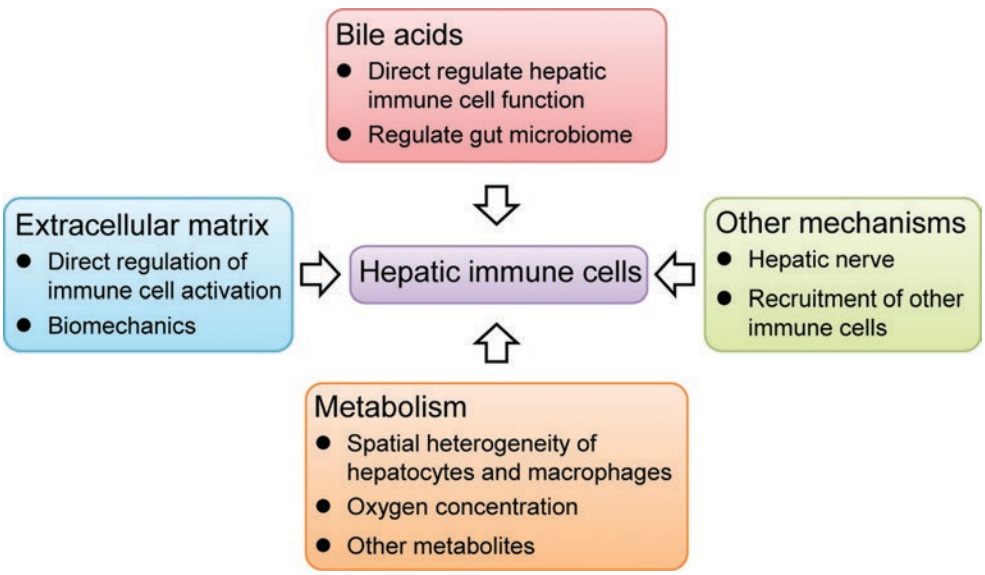

Fig. 2.9 Regulation of hepatic immune cells by the liver microenvironment. The liver immune response can be regulated by its specific microenvironment. Extracellular matrix, bile acids, metabolism, and other factors regulate liver immune cells

including macrophages, DCs, and NKT cells, at the interface of the host immune system, and may participate in the generation of tolerance (see Fig. 2.9) [126]. For example, activation of TGR5 and FXR in macrophages is linked with anti-inflammatory responses, including reduced NF-kB activation, reduced inflammasome activation and cytokine production, and stabilization of the M2 macrophage phenotype [127, 128].

Bile acids can also regulate liver immunity through other cell types. Conversion of primary bile acids to secondary bile acids by the gut microbiota controls the expression of CXCL16 by LSECs, which is critical for $CXCR6^+$ NKT cell accumulation and liver-selected tumor inhibition [129]. FXR activation in HSCs can decrease their sensitivity to TGF-β and reduce liver fibrosis [130]. Viruses can induce the expression of bile acid transporters and biosynthesis enzymes, stimulating the intracellular accumulation of bile acids in various cell types. Accumulated bile acids activate the TGR5-β-arrestin-SRC axis for efficient innate antiviral immune response [131]. A low dose of bile salts can stimulate hepatocyte proliferation and facilitate liver regeneration [132]. However, bile salts are biological detergents. Overload of bile salts in cholestasis conditions such as PBC and PSC can induce the apoptosis and necrosis of hepatocytes and endothelial cells, which can activate TLR signaling and inflammatory cytokine production [133]. They can also induce the expression of ICAM-1 and CXCL1 in hepatocytes, which attracts neutrophils to aggravate liver injury [134]. Treatment of PBC and PSC with ursodeoxycholic acid and obeticholic acid can compete with toxic bile salts and activate FXR signaling, which can protect hepatocytes and BECs from apoptosis, as well as modulate the inflammatory response [135].

In conclusion, bile acids can modulate liver immune responses. However, they can also be metabolized by the gut microbiota and modulate the immune response in the intestine. Changes in bile acids in circulation can also induce a systemic immune response. Thus, they may be a therapeutic target for many inflammatory liver disorders.

Metabolic Regulation of Liver Immune Response

Metabolic activity determines immune cell fate and function. For example, both glucose metabolism and aerobic glycolysis are known to drive classical macrophage activation, while oxidative metabolism, such as fatty acid oxidation, mediates alternative macrophage activation [136]. Amino acid metabolism, glutamine metabolism, glycolysis, and fatty acid oxidation have differing roles in naive T cell activation, effector T cell differentiation, and memory T cell maintenance [136]. Elements, such as oxygen level, glucose level, fatty acids, cytokines, growth factors, and amino acids, guide immune responses within the microenvironment (see Fig. 2.9).

The liver receives two-thirds of its blood from the portal vein, in which oxygen is depleted. Thus, the liver forms an oxygen gradient from the portal area to the central vein. This results in the heterogeneity of hepatocyte metabolism in various locations [137]. However, not much is known regarding immune cells and the liver oxygen conditions. We propose that different oxygen conditions not only affect hepatocytes but directly or indirectly modulate the function of immune cells and, to some extent, maintain a suppressive milieu when cells enter the liver. Some results support this speculation. Periportal hepatocytes express much higher E-cadherin [138], which not only functions as an epithelial "glue" but also suppresses DC maturation [139] and γδ T cell activation [140]. Hypoxia-inducible factor 1-alpha (HIF-1α) can suppress IL-17-producing γδ T cell accumulation in acetaminophen-induced liver injury [141]. Moreover, chronic hypoxia has been shown to reduce the glucose utilization of neutrophils and decrease neutrophil-related pathology [142]. In many liver disease conditions, increased hypoxia is observed. HIF-1α can promote profibrotic and proangiogenic gene expression of KCs to mediate the resolution of inflammation and tissue repair [143]. Also, most of the KCs reside in the sinusoidal zone close to portal spaces, with smaller numbers in the centrilobular zones [36]. Moreover, KCs from the periportal zone have a higher endocytic activity, larger lysosomes, and high lysosomal enzyme activity [37].

Aside from hypoxia, change of metabolism in the liver can also directly or indirectly affect the function of hepatic immune cells [5]. Hepatic blood supply contains a high level of dietary fats and carbohydrates. The metabolites of these nutrients, such as succinate, triglyceride, and cholesterol can act as ligands to induce an inflammatory response within the liver. In NAFLD, saturated fatty acids sensitize hepatocytes to TLR agonists and promote liver inflammation. A metabolic change of HBV-infected hepatocytes facilitates virus replication and resistance to clearance. Additionally, fatty acid oxidation in hepatocytes results in the production of acetoacetate that can shuttle into macrophages and suppress their profibrotic function in liver fibrosis [144]. Ketone body β-hydroxybutyrate, which is produced by hepatocytes, can inhibit NLRP3 inflammasome activation [145]. These data suggest that metabolic signals within the liver can regulate liver immune cell population and function. A deeper understanding of relationship between changes of metabolism and hepatic immune cell functions during pathological conditions will greatly aid in the development of therapeutic targets against liver diseases.

Many other liver properties may also regulate the liver immune response, such as recruitment of other cells for the

assistance of local immunity and hepatic nerve regulation of hepatocytes and other hepatic immune cells. These elements need to be considered in the overall view of liver immunology, where many questions remain.

Hematopoiesis in the Liver

The adult liver contains hematopoietic stem and progenitor cells (HSPCs) that can differentiate into both myeloid cells and lymphocytes. Moreover, extramedullary hematopoiesis often happens under pathological conditions [146, 147]. These data indicate that there is a niche for hematopoiesis in the liver. Actually, KCs can promote extramedullary hematopoiesis in the liver, possibly through the expression of ICAM-1 and LFA-1 [148]. HSPCs, mainly common myeloid progenitors and granulocyte monocyte progenitors, but not megakaryocyte-erythroid progenitors, can suppress T cell activation in vitro through IFN-γ-STAT1-iNOS pathway. However, HSPCs in the liver are primarily megakaryocyte-erythroid progenitors and have minimal suppressive function, indicating a different HSPC composition between the liver and bone marrow [149]. Many questions remain to be answered about hematopoiesis in the adult liver, including (1) whether hepatic HSPCs are prone to differentiate into regulatory cells locally to sustain liver tolerance, (2) how this process is regulated, and (3) whether hematopoietic chimerism contributes to liver transplantation-associated immune tolerance.

Summary

The liver is potentiated for appropriate response, rendering it vulnerable to immune-mediated injury in which hepatocytes are the targets. Liver immunity is a response regulated by balance: a predominance of innate immune cells that can quickly react against pathogens and injuries, along with a tolerance milieu to suppress over-activation. Thus, understanding dynamic regulation of the liver immune balance is very important. We believe that with the increased understanding of basic immunology, along with advances of technologies that facilitate visualizing liver immune response in vivo and describing cell heterogeneity in detail, such as intravital microscopy and single-cell sequencing, the sophistication of the liver immune response will be revealed.

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The Uniqueness of Innate Immunity

3

Gyongyi Szabo and Jaclyn Mallard

Key Points

- The liver has abundant presence of innate immune cells that can rapidly be expanded in response to liver injury and danger signals.
- Predominance of immature dendritic cells and low antigen-presenting capacity of Kupffer cells provides a tolerogenic immune environment in the liver.
- Hepatocytes can mount a robust antiviral defense by producing type I interferons.
- The composition of innate immune cell phenotypes can rapidly change under disease conditions.

Introduction

Owed to its size, the liver can be considered as one of the largest immune organs where immunity in general has some unique features (Box 3.1). Some of this uniqueness is related to the physiological setup of the liver, in that it receives nutrient-rich blood through the portal vein. The portal blood can also deliver gut-derived signals that originate from the gut microbiome in infections and in subclinical manifestation of immune activation related to increased gut permeability. The portal blood as it enters the liver sinusoids, then can directly deliver pathogen-associated molecular patterns (PAMPs) to Kupffer cells (KCs) and other immune cells residing in the liver sinusoids. Another unique feature of the liver is the resident macrophage, KC population that is generally "tolerant" in the normal liver as being constantly exposed to gut-derived PAMPs [1]. However, pattern recognition receptors (PRRs), which sense endogenous and pathogen-derived danger signals, are expressed and functional in the liver not only in immune cells but also in hepatocytes, biliary epithelial cells, hepatic stellate cells, and sinusoidal endothelial cells [2]. While these nonimmune cell types may not all have the intracellular machinery to produce innate immune responses, the contribution of these cells to the liver innate immune environment is substantial and needs to be considered in disease states. Another major function of innate immune cells is induction of adaptive immune responses. The classical antigen-presenting function of dendritic cells (DCs) and monocytes is present in the liver [3]. It should be noted that the DC population in the liver is enriched in plasmacytoid DCs (pDCs) with relative minority of myeloid DCs (mDCs) [4, 5]. Furthermore, these DCs are present in the normal liver in an immature phenotype that is not fully ready for antigen presentation until further maturation signals are provided to them. Furthermore, other cell types in the liver can act as "non-professional" antigen-presenting cells (Fig. 3.1). These include hepatocytes and cholangiocytes to a lesser extent. However, antigen presentation by these nonprofessional antigen-presenting cells is incomplete and results in tolerogenic T-cell phenotype instead of antigen-specific T-cell activation [3]. Thus, the liver immune environment is an immune tolerogenic milieu (see Box 3.1).

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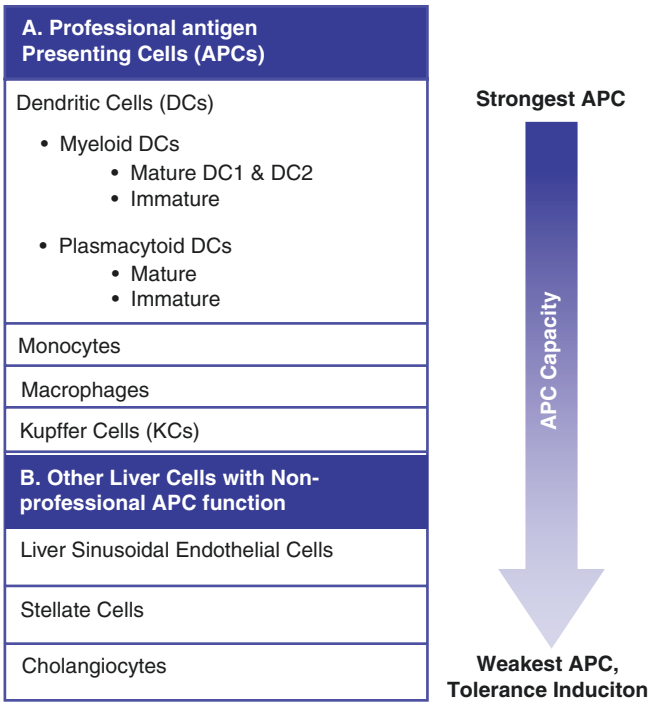

Fig. 3.1 Diversity of antigen-presenting cells in the liver. Antigen-presenting cells (APCs) can be categorized as professional (**a**, top) or nonprofessional (**b**, bottom). (**a**) Professional APCs in the liver include DCs, which have the strongest APC capability, with myeloid DCs (mDCs) being slightly better than plasmacytoid DCs (pDCs), followed by monocytes, macrophages, and liver-resident KCs. (**b**) Nonprofessional APCs include sinusoidal endothelial cells, stellate cells, and cholangiocytes, which are weak APCs, but facilitate tolerance induction in the liver

Box 3.1 Uniqueness of Innate Immunity in the Liver

- Largest of the immune organs:
  - Dendritic cells are predominantly comprised of the immature phenotype in the liver.
  - Enriched population of resident macrophages, called Kupffer cells.
- Unique biologic properties:
  - Organ structure and vasculature that promote close interactions between parenchymal and nonparenchymal cells and circulating immune cells.
  - Sustained exposure to PAMPs and gut-derived antigens from the portal circulation facilitates immediate immune response to pathogens and tolerogenic effect to commensal microbes.
- Cytokine environment promotes immune tolerance to commensal microbes:
  - IL-10, PGE_2, and TGF-ß

PAMPs pathogen-associated molecular patterns, IL-10 interleukin 10, PGE_2 prostaglandin E2, TGF-ß transforming growth factor beta

Overview of Innate Immune Cell Types in the Liver

Pathogenesis of alcoholic and nonalcoholic liver diseases is largely caused by excessive inflammation. Innate immune cells such as monocytes, macrophages, granulocytes, dendritic cells (DC), and natural killer (NK) cells have major roles in the unique tolerogenic milieu of the liver as well as induction and resolution of inflammatory responses that contribute to pathogenesis and progression of liver disease (Fig. 3.2).

Monocytes and Macrophages

Monocytes are derived from hematopoietic stem cells in the bone marrow (BM) and generally comprise three subpopulations in humans: classical monocytes ($CD14^+CD16^-$), proinflammatory monocytes ($CD14^+CD16^+$), and nonclassical monocytes ($CD14^-CD16^+$) [6]. Inflammatory monocytes ($CD14^+CD16^+$) are mobilized from the BM and migrate to tissues in response to infection/injury and in the liver, these cells play a major role in resolving fibrosis [7]. Other monocyte populations conduct routine immune surveillance by patrolling the tissue vasculature for molecular indicators of infection/injury [8]. Once inflammatory monocytes reach the site of injury they differentiate into macrophages ($CD68^+$), which can adopt inflammatory or anti-inflammatory phenotypes to aid in pathogen clearance or tissue repair, respectively [9]. The tissue microenvironment influences whether monocyte-derived macrophages adopt an inflammatory/anti-inflammatory phenotype. Inflammatory macrophages, often defined as $CD68^+CD163^+$ in humans, induce immune responses via pathogen recognition that activates the inflammasome and antigen presentation to T cells. Activated macrophages express tumor necrosis factor alpha (TNF-α) and IL-1β, which can promote necrosis and exacerbate liver injury [10]. Expression of CD44 and detection of extracellular ATP promotes anti-inflammatory macrophages to facilitate tissue repair following sterile liver injury/inflammation [11]. In response to anti-inflammatory cytokines such as transforming growth factor beta (TGF-β) and IL-10, macrophages phagocytose activated neutrophils in the tissues to help turn off the inflammatory response [12]. This leads to decreased expression of proinflammatory cytokines such as IL-23 and IL-17 along with decreased chemokines such as G-CSF that promote neutrophil mobilization from BM. Monocytes also aid in tissue repair by promoting resolution of fibrosis in the liver. In addition to detecting pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) indicative of infection/injury, macrophages also play a crucial role in lipid metabolism in the liver. Insufficient lipid metabolism by macrophages leads to fat accumulation and cholesterol

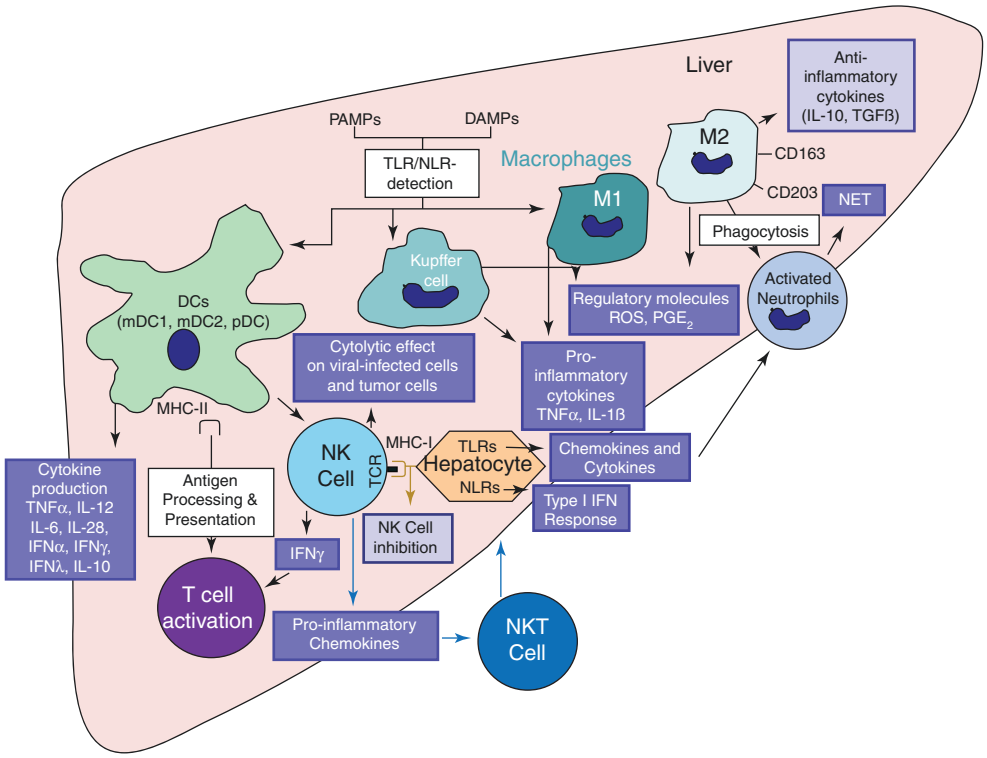

Fig. 3.2 Innate immune responses in the liver. PAMPs and DAMPs are detected by TLRs and intracellular NLRs, which are expressed by DCs, proinflammatory macrophages, and hepatocytes in the liver. This leads to expression of proinflammatory cytokines such as TNFα and IL-1ß from DCs and macrophages. DCs and hepatocytes also induce interferon responses following PAMP/DAMP detection. Hepatocytes release other cytokines and proinflammatory chemokines following TLR-mediated recognition of PAMPs/DAMPs that promote recruitment of proinflammatory monocytes and neutrophils to the liver. Activated neutrophils release NETs, which immobilize pathogens and allow for their degradation by ROS. ROS and PGE_2 are regulatory molecules released by KCs and proinflammatory macrophages and their excessive expression leads to tissue injury and prolonged inflammatory responses. Inflammation is regulated by anti-inflammatory macrophages, which phagocytose activated neutrophils and release regulatory molecules and cytokines that function to suppress the immune response. NK cells are activated by DCs in the liver and release proinflammatory chemokines that recruit NKT cells. In the healthy liver, hepatocyte expression of MHC-I regulates NK cell activation. The strongest APCs in the liver are DCs, which are capable of T-cell activation and prompting T-cell quiescence to maintain immune tolerance in the liver. PAMPs pathogen-associated molecular patterns, DAMPs damaged-associated molecular patterns, TLR toll-like receptor, NLR NOD-like receptor, DC dendritic cell, TNFα tumor necrosis factor alpha, IL-1ß interleukin-1beta, NET neutrophil extracellular trap, ROS reactive oxygen species, PGE_2 prostaglandin E2, KC Kupffer cell, NK cell natural killer cell, MHC-I major histocompatibility complex I, APC antigen-presenting cell

build-up in the liver, leading to increased release of PAMPs and DAMPs from dying hepatocytes, which induces prolonged inflammation characteristic of alcoholic and nonalcoholic fatty liver disease (NAFLD) [13, 14]. While monocyte-derived macrophages accumulate in the tissues following infection/injury, tissue-resident macrophages are a self-renewing population that acts as first responders to PAMPs and DAMPs, which indicate a local infection/injury [15]. Kupffer cells (KCs) constitute the tissue-resident macrophages of the liver [16].

Kupffer Cells

Unlike monocyte-derived macrophages, which are replenished from the bone marrow, Kupffer cells (KCs) are liver-resident macrophages that are derived from the embryonic yolk sac [15, 16]. Previously, KCs were thought to only be self-renewing; however, a recent study has demonstrated that following severe depletion of KCs, blood-derived monocytes are capable of replenishing resident KCs in the liver [17]. In the healthy liver, KCs maintain liver homeostasis by disposing metabolic products [18]. Like monocyte-derived macrophages, KCs also play a major role in excessive inflammation that is associated with NAFLD [19, 20]. Their detection of debris from damaged/dying cells induces expression of the proinflammatory cytokine TNF-α, which prompts hepatocytes to release the chemokine CXCL1 (C-X-C motif chemokine ligand 1) that mobilizes neutrophils to migrate from the BM and clear dead cells from the liver [21]. Under inflammatory conditions, KCs clear fibrotic debris from activated stellate cells and induce immune responses that prompt infiltration of additional innate immune cells into the liver to aid in recovery. During chronic alcohol use KCs become sensitized to toll-like receptor 4 (TLR-4) signaling, which impacts their ability to induce sufficient inflammatory

responses [22]. In mice, KCs are characterized as F4/80^{hi}CD11b^{lo}CD68 $^{+}$ CD11c^{int} in addition to TLR-4 and TLR-9 expressions [22].

Granulocytes: Neutrophils, Basophils, and Eosinophils

Granulocytes are derived from hematopoietic precursors in the BM and can be broken down into three main populations: neutrophils, basophils, and eosinophils. Basophils and eosinophils are involved in inflammatory reactions and are commonly associated with allergic responses. They also function to promote blood flow to tissues. Neutrophils play a more prominent role in liver inflammation and have effector functions such as phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) [23, 24]. These cells are often the first to migrate to tissues in response to infection/injury. Damaged/dying hepatocytes release the chemokine CXCL1, which binds to CXCR2 on neutrophils and is a major chemoattractant involved in their mobilization from the BM [21, 25]. Mature neutrophils that have entered the liver are CD11b^{+} and CD11c^{+} [26]. Inflammatory neutrophils express CD177 and have increased release of effector and regulatory immune modulators such as reactive oxygen species (ROS), NET, and myeloperoxidase (MPO) [26]. Persistent cirrhosis is characterized by malfunctions in neutrophil phagocytosis and increases in inflammatory mediators that prevent these cells from mounting a sufficient effector response [27]. Increasing the number of neutrophils in the liver correlates with survival in patients with alcoholic hepatitis (AH) [28]. However, neutrophils can also induce liver injury as their survival is promoted by hepatic stellate cells (HSC), which in turn are activated by neutrophils and activated HSCs promote liver fibrosis [29].

Dendritic Cells

Depending on their origin, dendritic cells (DCs) can be myeloid DCs or plasmacytoid DCs [3, 30]. Myeloid DCs are derived from myeloid progenitors in the BM and are highly efficient antigen-presenting cells capable of activating T cells to induce an adaptive immune response. Turnover of BM-derived DCs in the liver is approximately 7 days [31]. Generally, DCs are characterized by CD11c^{+}F4/80^{-}csf-1R^{-}FH3^{+} expression, but they can also be further categorized into conventional DCs (cDCs) that prime T cells and plasmacytoid DCs (pDCs) [17, 32, 33]. Three subpopulations of DCs are typically found in the human liver: CD141^{+} cDCs, CD1c^{+} cDCs, and pDCs. CD1c^{+} DCs are associated with inflammatory responses in the liver and express Th1, Th17, and IL-12 to promote T-cell activation [34]. They play a major role in tissue inflammation via inflammasome activation following recognition of PAMPs and/or DAMPs indicative of infection/injury in the tissue. Additionally, they play a major anti-inflammatory role in maintaining tolerance to commensal microbials and antigens in the liver [35, 36]. Hepatic DCs are also unique in that they are characterized by low expression of major histocompatibility complex II (MHC II) expression and poor T-cell stimulation ability [37, 38]. HDCs also induce anti-inflammatory responses by lowering TLR signaling following detection of decreased type I IFN expression or by increasing expression of PD-L1, which decreases T-cell stimulation [39]. The inflammatory/anti-inflammatory phenotype of HDCs can be discerned by their lipid content. High lipid HDCs are associated with increased expression of inflammatory cytokines such as TNF-α, interferon gamma (IFN-γ), IL-12, and IL-6, whereas HDCs with low lipid content promote regulatory T-cell activation and expression of the anti-inflammatory cytokine IL-10 from T cells [40].

NK Cells and NKT Cells

Natural killer (NK) cells and NKT cells are enriched in the liver relative to the blood and function to maintain tissue homeostasis in the healthy liver [41]. Unlike other lymphocyte populations, NK cells and NK T cells do not undergo somatic hypermutation and so do not develop antigen specificity. Instead, these effector cells recognize viral hepatitis and cancer cells in the liver via receptors that detect common molecular patterns.

Liver-resident NK cells are characterized as CD3^{-}CD56^{+} in humans [42]. In the absence of infection/injury, NK cell effector functions are regulated by receptor recognition of MHC-I expressed on healthy hepatocytes. Depletion of healthy hepatocytes prevents NK inhibition and promotes immune activation. Under disease conditions, NK cell function is modulated to increase inflammation and tissue injury [43]. In chronic liver disease, NK cells reduce fibrosis by direct killing of HSCs cells via release of the proinflammatory cytokine IFN-γ [44]. However, chronic hepatitis B (HBV) and C (HCV) infection can decrease NK cell expression of proinflammatory cytokines, thereby impairing their ability to mount an effective immune response to chronic viral infection [45–47]. NK cell effector functions are negatively impacted in cases of hepatocellular carcinoma (HCC), which is associated with impaired cytotoxicity and decreased expression of IFN-γ and thus prevents their ability to reduce fibrosis [48–50]. This dysfunction in HCC is caused by direct inhibition of NK cells by myeloid-derived suppressor cells [51] and release of prostaglandin E2 from fibroblasts [52], which reduces expression of receptors needed for immune activation. Treatment with IL-15 induces NK cell activation and restores cytotoxicity [53] and is currently being evaluated in clinical trials as a potential antitumor therapy [48].

In addition to their primary effector functions, NK cells promote inflammation by recruiting NKT cells to the liver. These are lymphocytes with an invariant T-cell receptor that recognize nonpeptide antigens such as lipids and glycolipids, which are major contributors to liver fibrosis. NKT cells help to remove damaged/dying cells by releasing death-inducing mediators and surface effector molecules such as FAS ligand and CD40. In mice, there are two populations of NKT cells involved in alcoholic liver disease (ALD): type I NKT cells are proinflammatory cells that activate KCs and neutrophils and type II NKT cells promote protection against ALD by inhibiting type I NKT cells [54].

Pattern Recognition Receptors

Unlike adaptive immune cells, innate immune cells are unable to undergo somatic mutation to generate receptors specific for a particular antigen. Instead, monocytes, macrophages and DCs identify sources of infection/injury via recognition of molecular patterns that are commonly found on pathogens and/or are indicators of necrosis/tissue injury [55–57]. Additionally, pattern recognition receptors (PRRs) also facilitate the ability of innate immune cells maintain immune tolerance and regulate the adaptive immune response [57, 58] (Fig. 3.3).

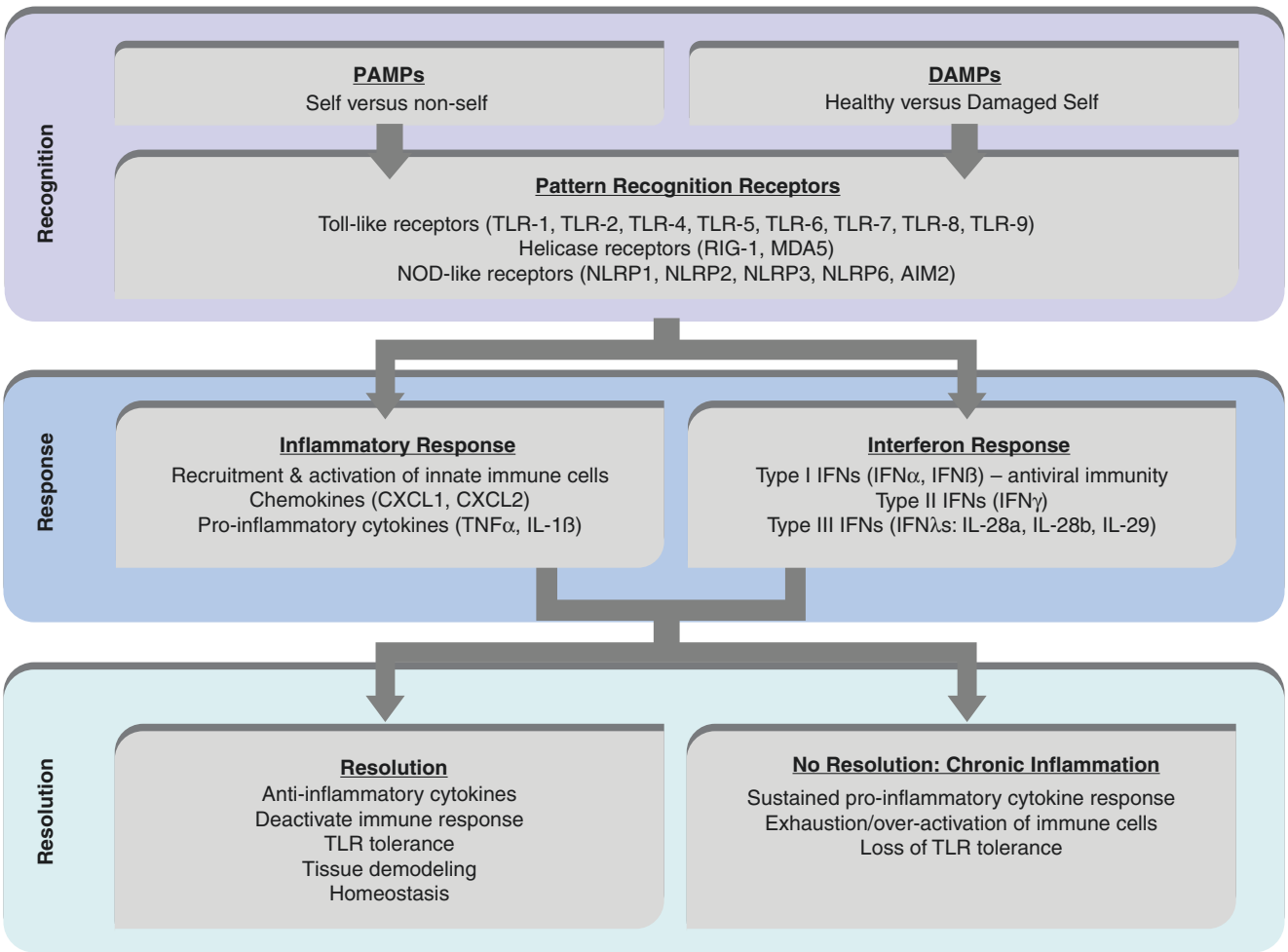

Fig. 3.3 Activation, response, and resolution of inflammation in the liver. Recognition of PAMPs (discriminate between self and nonself for pathogen detection) and DAMPs (discriminate between healthy and unhealthy self for detection of tissue injury) initiates the inflammatory response in the liver. PAMPs/DAMPs are recognized by distinct PPRs, which include TLRs, helicase receptors, and NLRs. TLRs that commonly induce inflammation in the liver are TLR-1, TLR-2, TLR-4, and TLR-5, which are embedded in the plasma membrane and detect extracellular PAMPs/DAMPs and TLR-9, which is located in the endosomal membrane and detects cytoplasmic RNA/DNA indicative of viral infection. PRR detection of PAMPs/DAMPs leads to either an inflammatory response, which prompts recruitment and activation of immune cells via chemokine and cytokine expression and/or an interferon response, which confers antiviral immunity (types I and III) or induces T-cell activation (type II). Inflammatory and interferon responses result in either resolution or no resolution of the infection/injury that prompted the response. Sufficient release of anti-inflammatory cytokines will deactivate the inflammatory/interferon response and promote TLR tolerance, tissue remodeling, and homeostasis. Failure to resolve inflammatory/interferon responses leads to chronic inflammation, which is characterized by sustained proinflammatory cytokine expression, immune cell exhaustion, and loss of TLR tolerance leading to tissue fibrosis and disease progression. PAMPs pathogen-associated molecular patterns, DAMPs damage-associated molecular patterns, PRRs pattern recognition receptors, TLRs toll-like receptors, MDA5 melanoma differentiation-associated protein 5, NLRs NOD-like receptors

PAMPs and DAMPs

Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized by pattern recognition receptors (PRRs) on innate immune cells and stimulate inflammatory responses. PAMPs include molecular patterns common among, but unique to invading species of bacteria/viral pathogens whereas DAMPs are markers of cell death such as high mobility group box 1 protein (HMGB1), mitochondrial DNA, heat shock proteins, and purine metabolites [59, 60]. Excess collagen and lipids in the liver are DAMPs indicative of steatosis/fibrosis. Accumulation of cholesterol crystals is another example of DAMPs found in the liver that induces inflammation associated with nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) pathogenesis [61]. The detection of DAMPs in the liver promotes recruitment of macrophages, which function to repair damaged liver tissue during sterile injury/inflammation [11].

Toll-Like Receptors

TLRs are membrane bound receptors that recognize PAMPs or DAMPs. There are several different TLRs located within the plasma and endosomal membranes of DCs and macrophages, each recognizing a unique type of molecular pattern. TLR signaling pathways that are commonly induced in the liver include TLR-1, TLR-2, TLR-4, TLR-5, and TLR-9, each of which detects distinct microbial patterns [54, 62]. All of these are plasma membrane receptors that detect extracellular PAMPs except for TLR-9, which is expressed on the endosomal membrane and detects viral/bacterial RNA/DNA in the cytoplasm that are indicative of infection. Upon detection of their respective molecular pattern, the TLRs listed above activate the intracellular immune pathway MyD88, which induces an inflammatory response. The MyD88 pathway leads to NF-κB phosphorylation (p65) and nuclear translocation, which prompts inflammatory responses via cytokine/chemokine expression and/or inflammasome activation. Neutrophil expression of TLR-4 has been shown to trigger release of neutrophil extracellular traps (NETs) to combat pathogens [63, 64]. In the liver, TLR-9 detection of DAMPs results in neutrophil mobilization from the BM [65]. TLR signaling can also prompt anti-inflammatory responses. For example, HDCs produce IL-10 in response to TLR-9 signaling to protect the liver from progressive tissue injury [66].

Helicase Receptors

Helicase receptors, commonly referred to as RIG-1-like receptors (RLRs), are cytoplasmic helicases that detect PAMPs indicative of RNA virus infection [67]. RLR detection of RNA viruses induces type I IFN activation that in turn promotes an inflammatory response to the infection [68–71].

NLRs and Inflammasomes

Nod-like receptors (NLRs) are intracellular receptors that detect PAMPs and DAMPs and ultimately form the framework for inflammasome assembly and activation. Like TLRs, several NLRs have been identified that recognize distinct PAMPs/DAMPs. NLR detection of PAMPs/DAMPs prompts inflammasome formation, an oligomeric complex that leads to release of IL-1β and IL-18, proinflammatory cytokines that induce downstream immune responses (Fig. 3.4). While there are many NLRs that recognize distinct PAMPs/DAMPs and activate inflammasome formation, once formed, inflammasomes uniformly function to release IL-1β and IL-18. The NLRs form an oligomeric complex by direct/indirect binding to caspase 3, which in turn cleaves pro-IL-1β and pro-IL-18 into their mature cytokines that are released from the cell and constitute local/peripheral immune responses. NLRP3 recognizes PAMPs/DAMPs indicative of general inflammation/cell damage. Noncanonical activation of the NLRP3 inflammasome is mediated by caspase 11 [72, 73]. Lipopolysaccharide (LPS) is directly detected by caspase 11 [72, 74]. Activated caspase 11 cleaves gasdermin D, which then induces pyroptosis and can also play a role in activation of the NLRP3 inflammasome [75]. NLRP3 is by far the most well-studied of these PRRs and plays a role in pro-IL-1β-mediated inflammatory responses in alcoholic liver disease (ALD) [76]. In the liver IL-1β expression promotes increased expression of promatrix metallopeptidase 9 (MMP9), leading to HSC activation associated with fibrosis [77]. Additionally, NASH pathogenesis in mice is associated with inflammasome activation induced by accumulation of cholesterol crystals in kupffer cells (KCs) [54].

Cytokines and Chemokines

Detection of PAMPs/DAMPs by innate immune cells ultimately leads to release of cytokines and chemokines, which are molecules that induce or regulate inflammatory responses. Cytokines can be pro- or anti-inflammatory and function to activate or inhibit cellular immune responses from both innate and adaptive immune populations. Chemokines are molecules that act as chemoattractant to recruit additional immune cells to sites of infection/injury. Regulatory mediators are molecules that suppress the local immune response during the steady state and/or turn off immune activation once infection/injury is resolved.

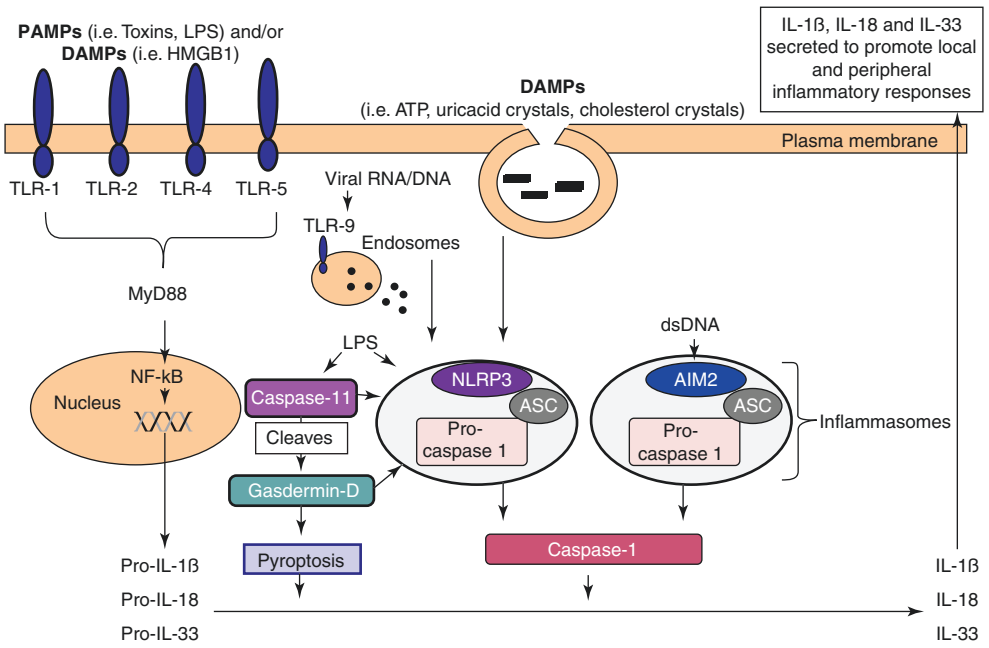

Fig. 3.4 Inflammasome responses in the liver. Extracellular PAMPs and DAMPs detected by TLRs expressed on the cell surface trigger inflammasome activation via the MyD88 pathway and NF-kB. This triggers formation of the inflammasome oligomeric complex that leads to cleavage of procaspase 1 into activated caspase 1, which then cleaves pro-IL-1ß, pro-IL-18, and pro-IL-33 into their active cytokine form so they can be released from the cell and promote additional inflammatory/interferon responses. Extracellular DAMPs such as ATP, uric acid crystals, and cholesterol crystals that are phagocytosed also induce inflammasome assembly and activation once they are detected in the cytoplasm. Cytoplasmic viral RNA/DNA is detected by TLR-9, which is located in the endosomal membrane, and this also induces inflammasome assembly and activation. Additionally, cytoplasmic LPS is directly detected by caspase 11, which then promotes noncanonical inflammasome activation and pyroptosis. Double-stranded DNA in the cytoplasm can directly activate the AIM2 inflammasome. NLRP3 and AIM2 are the most prominent inflammasomes involved in liver disease pathogenesis and although they have different modes of activation, both inflammasomes ultimately result in cytokine expression of IL-1ß, IL-18, and IL-33, which promote downstream inflammatory and interferon responses. PAMPs pathogen-associated molecular patterns, DAMPs damage-associated molecular patterns, TLR toll-like receptor, NF-kB nuclear factor kappa B, IL-1ß interleukin 1beta, ATP adenosine triphosphate, LPS lipopolysaccharide, NLRP3 NOD-like receptor protein 3, dsDNA double-stranded DNA, HMGB1 high mobility group box protein 1

Proinflammatory Cytokines and Chemokines

Proinflammatory cytokines released by innate immune cells include IL-1β, IL-6, IL-18 IFN-γ, and TNF-α. IL-1β and IL-18 are released by DCs and macrophages following inflammasome activation and function to prompt inflammation in the tissues and peripheral blood and TNF-α is a marker of proinflammatory macrophage activation [26]. Neutrophil extracellular traps (NETs) can trigger macrophage activation, leading to expression of TNF-α and IL-1β, which can cause necrosis and increase liver inflammation. NK cells induce apoptosis of hepatic stellate cells (HSCs) by release of IFN-γ. Reduced expression of TNF-α and IFN-γ by NK cells is observed in chronic viral hepatitis, suggesting that chronic viral infection impairs NK cell effector functions. CXCL1, CXCL5, CXCL12, G-CSF, and SDF-1 are chemokines that play prominent roles in liver disease. CXCL1 and G-CSF are largely involved in neutrophil mobilization from the bone marrow (BM) [25, 78]. Neutrophil recruitment is also dependent on TLR-2-mediated signaling of CXCL12 in acute and chronic liver injury in mice [79]. SDF-1 and CXCL12 are also released by activated macrophages. Inflammatory monocytes are recruited to the liver via CD40-CD40L binding [25, 80]. IL-1, IL-8, IL-17, CXCL1, and CXCL5 in particular play a major role in promoting neutrophil recruitment to the liver in patients with severe alcoholic hepatitis [54, 81]. CCL20, another chemokine highly expressed in AH patients, is thought to induce inflammation and fibrosis via activation of HSCs [82].

Anti-inflammatory Cytokines

Anti-inflammatory cytokines function to inhibit/reduce inflammation and induce tissue repair following infection/injury. These include TGF-β and IL-10, both released by anti-inflammatory macrophages in tissues, which in turn prompts these cells to phagocytose-activated neutrophils in order to regulate inflammation. BM-derived macrophages and neutrophils express IL-10 in liver injury to promote tissue repair. Hepatic DCs release anti-inflammatory cytokines such as TGF-β and IL-10 in response to

commensal microbes and antigens thereby preventing an immune response to nonpathogenic bacteria in the liver. The tolerogenic effect of HDCs is attributed to their expression of the anti-inflammatory cytokine IL-10, which is mediated by production of the regulatory molecule prostaglandin [83].

Regulatory Mediators

Regulatory molecules commonly involved in pathogenesis of liver disease include reactive oxygen species (ROS), prostaglandin, and leukotrienes. These molecules function to inhibit inflammatory responses in the steady state [84]. ROS is a known indicator of cell stress and tissue injury and is highly expressed by neutrophils [26]. It directly kills pathogens that have been immobilized by NETs [26]. ROS released from neutrophils in a model of acetaminophen-induced liver injury prompted proinflammatory macrophages to convert to an anti-inflammatory phenotype [85]. Under disease conditions, excess ROS and NETs can cause excessive inflammation and exacerbate tissue injury [86]. NETs also demonstrate regulatory immune functions by degrading cytokines and chemokines to help resolve inflammation [87]. Myeloperoxidase (MPO) is another regulatory mediator that is expressed by recruited macrophages to modulate the immune response during liver injury [88].

Role of Innate Immunity in Liver Diseases

Gut–Liver Axis

In healthy individuals, homeostasis exists between the gut microbiome and the innate immune system aided by intact gut epithelial and mucosal function [89]. Disruption of the gut barrier function and changes in the gut microbiome represent increasingly recognized factors contributing to liver diseases. It should be noted, however, that disruption of gut barrier or changes in the gut microbiome alone are unlikely to cause liver disease without some additional direct liver/hepatocyte insults such as alcohol use or the metabolic syndrome. While changes in different bacterial taxa have been reported in relation to chronic liver diseases, reduction in diversity of the composition of host bacterial taxa appears to be a common abnormality regardless of the type of liver disease [90, 91]. Alteration in epithelial barrier function and mucosal defense mechanisms have been linked to chronic alcohol use and were shown to be directly affected by alcohol or its metabolites [91]. For example, alcohol induces miR-212 that reduces the mRNA levels of the key tight junction protein, ZO1 [92]. Occludin expression is also decreased by alcohol and a recent study showed that changes in the proximal small intestine likely contribute to alcohol-related gut permeability [93]. Chronic alcohol use also results in an increase in the abundance of Paneth cells in the proximal small bowel that are normally rare in this bowel segment [93]. Alcohol feeding in mice resulted in abnormal mucosal barrier composition and increased IL-17 production. This was mediated by the composition of the gut microbiome and can be normalized by antibiotic administration [93, 94].

Alcoholic Liver Disease

Immunity, including innate immunity, is compromised in individuals with chronic excessive alcohol use, and this subclinical immunosuppression is also present in patients with alcoholic liver disease (ALD) [95]. The alcohol-related immune alterations are manifested in impaired host defense mechanisms of circulating monocytes and macrophages [96]. Antimicrobial functions, including phagocytosis and killing of bacteria, are impaired, thereby predisposing patients with ALD to infections. The reduced antimicrobial and antiviral function of the innate immune cells is somewhat paradoxical because in ALD, activation of Kupffer cells and recruitment of inflammatory macrophages have been identified as characteristic and mechanistic features of the disease pathomechanism. Activation of the proinflammatory innate immune cascade is even more pronounced in acute alcoholic hepatitis (AH) where accumulation of neutrophil leukocytes and recruited macrophages is a histological diagnostic marker of the liver disease [97]. Recent studies showed that neutrophil leukocytes are activated in vivo in ALD, resulting in production of neutrophil extracellular trap (NET) formation in the liver [98]. Alcohol-induced NETs are present in animal models of AH as well as in human livers with ALD [98]. However, these in vivo preactivated neutrophils in ALD cannot respond properly to a subsequent infectious stimulus in vitro [98]. While in the healthy liver, NETs are removed by macrophages in the process called “efferocytosis” to prevent prolonged tissue injury, in ALD macrophages have impaired efferocytosis [98].

In addition to the beneficial effects of anti-inflammatory macrophages (M2) in tissue remodeling, the predominant phenotype of macrophages in the liver in ALD is the M1-like proinflammatory phenotype. It has been shown that Kupffer cell and macrophage depletion have protective effects in a mouse model of ALD [99, 100]. A recent study showed that administration of a small molecule inhibitor of CCR2/CCR5 reverses inflammation, liver damage, and steatosis in a mouse model of ALD [101].

The activation of the proinflammatory cascade and elevated levels of a wide range of proinflammatory cytokines have been reported in animal models of ALD as well as in human AH where the magnitude of inflammatory cytokine increase is greater than in ALD [102]. The role of TLR4, a sensor of bacterial LPS, has been extensively studied in ALD

[100, 103]. Increased circulating LPS levels are present after excessive alcohol use in animal models and in humans; however, the lack of TLR4 provides only partial protection from ALD [103]. It has been shown that interferon regulatory factor 3 (IRF3), downstream of TLR4, is an important regulator in ALD as it is activated by ER stress in ALD. IRF3-deficient mice show robust protection from all features of ALD including liver damage, steatosis, and inflammation [104].

In addition to TLRs, the intracellular sensor, NLRP3, was shown to play a critical role in ALD [105–107]. NLRP3 senses increase levels of ATP and uric acid in ALD leading to NLRP3 inflammasome activation, caspase-1 activation, and IL-1ß production [108, 109]. In the intragastric alcohol feeding model in mice, caspase-11 activation was also found and linked to pyroptosis in AH [110]. The importance of inflammasome activation in ALD is underscored by inhibition of all features or ALD in NLRP3 deficient mice or after therapeutic inhibition of IL-1 using the recombinant IL-1 receptor antagonist, anakinra [76, 101]. At the cellular level, inflammasome activation occurs mostly in liver mononuclear cells [macrophages] and not in hepatocytes in ALD [108]. It has been shown that the increased levels of IL-1ß contribute to amplification of the inflammatory pathway, inhibition of hepatocyte regeneration, and promotion of liver fibrosis [76, 111].

NAFLD and NASH

Nonalcoholic fatty liver disease (NAFLD) by definition is fat deposition in hepatocytes without evidence of inflammatory cell infiltration. Nonalcoholic steatohepatitis (NASH) is defined by the presence of inflammatory cells that are mostly activated macrophages [112, 113]. Hepatic recruitment of macrophages was shown to promote steatohepatitis via CCR2 and this could be attenuated by CCR2/5 inhibition [114]. It is important to remember that innate immune activation and macrophage recruitment is a common underlying element in the metabolic syndrome that occurs not only in the liver but also extends to other organs involved in the metabolic syndrome [115].

Kupffer cells and recruited macrophages play key roles in sustaining inflammation in NASH. It has been shown that lipotoxicity from free fatty acids results in release of DAMPs from hepatocytes that directly activate macrophages [116, 117]. In concert with DAMPs, the levels of circulating LPS are also increased in NASH contributing to dual activation of innate immune signaling pathways. Inflammasome activation in NASH was reported in different animal models and in human NASH with NLRP3 and AIM2 inflammasome involvement [106, 118]. Activation of innate immune signaling pathways, particularly inflammasome activation in NASH occurs not only in classical immune cells but also in hepatocytes and sinusoidal endothelial cells that may have a role in sustained inflammation [106, 107]. Finally, inflammatory cell activation and inflammatory cytokines promote hepatic stellate cell activation and drive liver fibrosis.

PBC and PSC

The importance of innate immunity is increasingly recognized in biliary diseases where autoinflammation is a component of damage to the biliary epithelium [119]. In human primary biliary cirrhosis (PBC), immunodominant mitochondrial autoantigen has been identified and innate immunity changes appear critical in initiation and perpetuation of the autoimmune injury [120]. Once the adaptive immune response develops in PBC, the subsequent disease progression is exacerbated by innate immune responses. Indeed, altered monocyte responses were found to TLR ligands in patients with PBC [121]. Elevated circulating $CD14^{low}CD16^{hi}$ monocyte subpopulation in PBC correlated with liver injury and promoted Th1 polarization in blood monocytes [122]. In a mouse model of autoimmune cholangitis, activated invariant NKT cells exacerbated liver injury [123]. In primary sclerosing cholangitis (PSC), involvement of both innate and adaptive immune activation was found [124]. In mouse models of PSC, macrophages were shown to contribute with predominance of M1 polarized inflammatory phenotype [125]. In patient with PSC, significantly higher frequencies of $CD68^{+}CD206^{+}$ macrophages and recruitment of $CD16^{+}$ monocytes were found in the liver compared to other liver disease [126].

Viral Hepatitis

Recognition of invading pathogens, including viruses, is a classical role of innate immunity. Intracellular pattern recognition receptors (TLRs and helicase receptors) sense viral nucleic acids (DNA and RNA) and rapidly induce interferons as first-line antiviral host defense. TLR3, TLR7, TLR9, and helicase receptors [30, 127, 128] are expressed in hepatocytes, the site of hepatitis virus replication. Hepatitis viruses are DNA (HBV, HDV) or RNA (HAV, HCV, HEV) viruses that are recognized by the host innate immune system with varying effectiveness that ultimately contributes to capacity of some of these hepatitis viruses to establish chronic infection.

While the host innate immune system is equipped to recognize viruses and induce antiviral immunity, viruses have unique and effective ways to escape these host recognition systems and/or undermine effective induction of antiviral interferons and/or downstream immune pathways in antiviral host defense.

In addition to recognition of viral nucleic acid sequences as “danger” signals for the host, viral proteins have also been shown to elicit innate immune responses that are mostly pro-inflammatory signals. For example, HCV structural and non-

structural proteins are recognized by TLR2 leading to proinflammatory cytokine production in monocytes and impaired maturation and antigen-presenting function in dendritic cells [129]. These observations indicate that viruses have multiple ways to undermine host defense in favor of establishing chronic infection.

HCC

The role of innate immunity in hepatocellular cancer development is only partially understood. The basic principle of immune escape of cancer cells applies to hepatocellular carcinoma (HCC) and may be linked to the overall depression of immune responses described in liver cirrhosis that could be in addition to the underlying cause for cirrhosis. For example, impaired antigen presentation and antigen-specific T-cell activation has been described in chronic alcohol use, and immune escape of chronic HBV and HCV infections. Furthermore, in most chronic liver diseases prior to progression to cirrhosis a long period of proinflammatory cytokine activation is present. Multiple studies have shown that a proinflammatory cytokine environment may contribute to cancer development [130]. Animal models of HCC support this contention.

Summary

The role of innate immunity and inflammation in liver disease has gained significant attention in the recent decade. Innate immunity and inflammation is a key element to development and progression of most chronic liver diseases, and increasing evidence suggests that interventions that attenuate inflammation have beneficial effect on liver diseases. The fundamental role of innate cells in response to pathogens and the role of innate immunity in initiation of adaptive immune responses remain a major host factor in the liver. Future studies are needed to translate understanding of liver innate immunity and signaling pathways to new therapeutic approaches.

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Adaptive Immunity and the Clinical Definition of Autoantibodies

4

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Key Points

- Detection of autoantibodies in the patient's serum bears witness of occurred activation of the adaptive immune system, namely of antigen-specific B and T cells.
- Autoantibodies are a useful tool in the diagnosis and management of autoimmune liver diseases, provided that the clinician is familiar with the laboratory methods and interprets correctly the results.
- Indirect immunofluorescence on triple rodent tissue should be used at a screening level, since it allows the simultaneous detection of the majority of liver-relevant autoantibodies, that is, anti-nuclear, anti-smooth muscle, anti-liver-kidney, anti-liver cytosol, and anti-mitochondrial antibody.
- Type 1 autoimmune hepatitis is characterized by anti-nuclear and/or anti-smooth muscle antibodies, whereas type 2 autoimmune hepatitis is characterized by anti-liver-kidney microsomal and/or anti-liver cytosolic type 1 antibodies.
- Anti-soluble liver antigen testing by a molecular-based assay should be included in the diagnostic work-up of liver disease of unknown origin, being highly specific for autoimmune hepatitis.
- Anti-mitochondrial antibody is the serological hallmark of primary biliary cholangitis and has high disease specificity. Rim-like and multiple nuclear dots anti-nuclear antibodies are also specific for primary biliary cholangitis and are of particular diagnostic value in anti-mitochondrial antibody negative patients.
- Atypical anti-neutrophil cytoplasmic antibody is detected by indirect immunofluorescence on human neutrophils and is associated with type 1 autoimmune hepatitis, primary/autoimmune sclerosing cholangitis, and inflammatory bowel disease.

Activation of Adaptive Immunity

As mentioned in Chap. 5, detection of antibodies in the patient's serum is key to the diagnosis of liver disease, including viral hepatitis and autoimmune liver diseases. The presence of antibodies, either directed against a foreign or a self-antigen, bears witness to occurred activation of the adaptive immune system, since antibodies are produced by terminally differentiated B cells after antigen recognition. Antibody-mediated immune reaction is referred to as humoral, from the Latin word *humor* meaning liquid, as opposed to cell-mediated immunity, executed by different types of T cells. Adaptive immunity, characterized by specificity, memory, and variable intensity, becomes activated by processes initiated by the innate immune system, which acts as the first-line defense mechanism but lacks specificity and memory. In the following sections, steps leading to the activation of cell-mediated and humoral immunity will be briefly reviewed.

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T-Cell Activation

Dendritic cells (DCs) play a central role in activating cell-mediated adaptive immunity following innate immune reactions elicited by an antigen.

In a first step, DCs themselves become activated after recognition through their pattern recognition receptors (PPRs) of exogenous or endogenous danger molecules, referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The number of PPRs is limited, but their ligands are molecules shared by many different pathogens, such as lipopolysaccharide, common to many Gram-negative bacteria, or single-stranded RNA, common to many viruses, allowing recognition of a wide array of pathogens. DAMPs are host biomolecules that can initiate a non-infectious inflammatory response. PPRs endow DCs to discriminate between normal self-molecules, harmless foreign molecules, and dangerous self- or foreign molecules. The main representatives of the PPRs are the Toll-like receptors and the NOD-like receptors, expressed not only by DC but also by other cell types, including macrophages and natural killer cells [1]. After their activation via interaction of PPRs with PAMPs or DAMPs, DCs undergo profound changes, including the following:

- Upregulation of molecules needed to activate T cells, such as CD80, CD86, and HLA
- Processing of microbial antigens for T-cell presentation
- Massive cytokine secretion, particularly IL-12
- Migration to lymph nodes, where antigen presentation to T cells takes place

When DC become mature, they traffic to the draining lymph nodes by upregulating the chemokine receptor CCR7, which responds to two chemokine ligands, CCL19 and CCL21 [2, 3]. These chemokines are expressed by peripheral lymphatic endothelial cells, as well as lymph node stromal cells and guide DC to home in the lymph nodes. DC present to naïve T cells peptides derived from antigens either loaded on MHC class II molecules, in case of externally acquired antigens, or loaded on MHC class I molecules, in case of endogenously derived antigens, mostly of viral origin. Thus, during viral infections, a process termed "cross presentation" allows priming of cytotoxic CD8 cells through presentation of viral antigens acquired by DC from the outside and loaded on MHC class I molecules. Typically, class II MHC molecules accommodate peptides of 12–25 amino acid residues, while class I MHC molecules accommodate in their groove smaller peptides of 8–10 amino acids. CD4 T cells recognize peptide antigens only in the context of MHC II, while CD8 T cells recognize peptide antigens in the context of MHC I. Activation of naïve T cells via antigen presentation by antigen-presenting cells (APCs), such as DC, requires three different signals (Fig. 4.1). The interaction between the T-cell receptor of a naïve T cell and a fitting peptide–MHC complex on an APC is referred to as signal 1, which is insufficient to activate naïve T cells into effector T cells, either CD4 or CD8. A second signal, named signal 2, is provided by the co-stimulatory molecules CD80 and CD86 located on the DC cell membrane, interacting with CD28 on the T cell surface, leading to expression of transcription factors upregulating the synthesis of a variety of cytokines, especially IL-12, by APC. Interaction between another set of

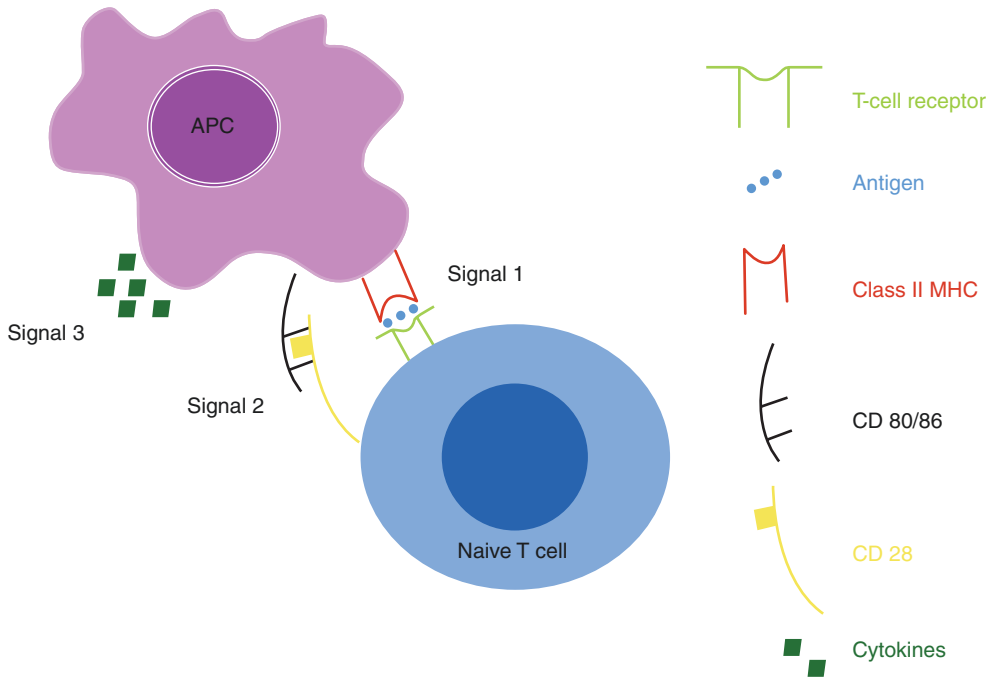

Fig. 4.1 Activation of a naïve T cell requires three signals. Signal 1 is provided by the interaction of the T cell receptor with an MHC class II molecule loaded with a fitting peptide antigen. Signal 2 is given by the interaction of CD28 on the T cell with CD80/86 on the antigen-presenting cell. Finally, cytokines secreted by the antigen-presenting cell provide the signal 3

molecules, that is, CD40 expressed on DC and CD40 ligand, expressed on T lymphocytes further enhances the expression of CD80 and CD86 and IL-12 secretion by DC. However, if CD80/CD86 or structurally related proteins interact with molecules on T cells named cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), the T cell response is terminated through delivery of T cell inhibitory signals and downregulation of CD80/CD86 on APCs. This process prevents immune responses against self-antigens, as demonstrated by autoimmune-like side effects elicited by anti-cancer drugs blocking CTLA-4 or PD- [4]. Cytokine secretion by APC is the third signal required for the full activation of the naïve T cells: the set of secreted cytokines depends on the nature of the antigen and drives the polarization of the T cells in order to achieve an adaptive immune response tailored to the nature of the offending antigen. In presence of high levels of IL-12, a naïve T cell, upon interaction with an APC, differentiate into interferon-gamma (IFN-γ)-producing Th1 cells leading to activation of macrophages, and consequently to killing of intracellular, phagocytized bacteria. The macrophages activated by this pathway are termed M1 macrophages, and the pathway is called classical macrophage activation. If APC secrete predominantly IL-4, a Th2 response is achieved, leading to eosinophils and mast-cells activation, which is particularly effective in counteracting parasitic infections. Th2 cells secrete IL-4, IL-5, and IL-13, inducing the alternative activation of M2 macrophages, which promote collagen synthesis and fibrosis. Finally, in presence of IL-23, IL-6, and transforming growth factor β (TGFβ), a Th17 response emerges through activation of the transcription factors RORγt and STAT-3, equipped for fighting extracellular bacteria and fungi by recruitment of neutrophils and monocytes [5]. Immature DC, that is, DC cells whose PPRs have not interacted with PAMPs, produce IL-10, which in association with TGFβ, leads to polarization of T cells toward regulatory (i.e., immunosuppressive, Treg) functions, which inhibit the activation of T cells, DC, and macrophages [6].

B-Cell Activation

Similarly to T-cell activation, B-cell activation takes place in lymph nodes, more precisely in secondary follicles, composed, beside B cells, of macrophages and follicular dendritic cells (FDCs), which are specialized DC with long cytoplasmic processes. The first signal for B-cell activation is provided by the interaction of an antigen with its specific B cell receptor, represented by membrane-bound immunoglobulins belonging to the M or D class (Fig. 4.2). In contrast to T cells, B cells are able to recognize native antigens directly, without need for processing. However, protein antigens, which are preferentially recognized by B cells located in the follicles of the lymphoid organs, and therefore termed follicular B cells, elicit a stronger antibody response as compared to non-protein antigens, which are recognized by B cells located in the peripheral region of the splenic white pulp and mucosal tissues [7]. T cell-independent B-cell activation is polyclonal and gives rise to an IgM response with weak affinity maturation and memory generation. Membrane-bound antigens are more potent in activating B-cells than soluble antigens, membrane binding being mediated by a wide array of surface receptors, including complement receptors, Fc-receptors, and lectins [8]. FDC play a key role in presenting native antigens to B cells. In order to initiate proliferation and differentiation of the B cell, binding of two or more identical epitopes to adjacent surface immunoglobulins, acting as B cell receptors, is required, bringing them together, a process referred to as cross-linking. Polysaccharides and other microbial non-protein antigens often contain multiple identical epitopes, thus leading to a more effective cross-linking of surface immunoglobulins. By contrast, protein antigens tend to have a less repetitive structure; therefore, in order to achieve an effective B-cell activation, assistance by T-cells is needed, which is easily provided in lymph nodes, since numerous T cells reside in the paracortex, located next to the follicles. Therefore, B-cell activation by non-protein antigens is typically T-cell independent, whereas B-cell acti-

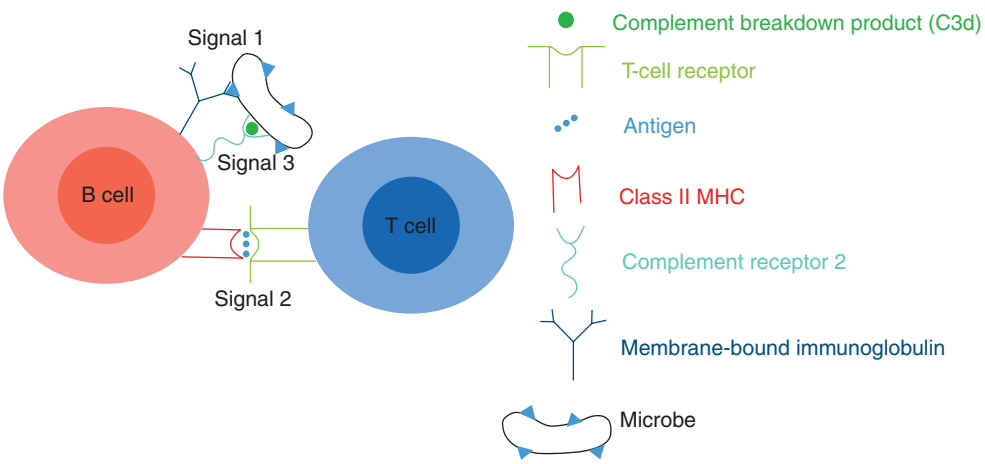

Fig. 4.2 Activation of naïve B cells requires three signals. Signal 1 is provided by the interaction of the surface immunoglobulin on the B cell with an antigen present on a microbe. Signal 2 is provided by the presentation of the processed microbial peptide antigen by B cells to helper T cells. Signal 3 derives from B cell recognition of innate immunity components, such as complement fragment d (C3d)

vation by protein antigens is largely T-cell dependent, making B-cell activation by protein antigens more efficient [8]. When an antigen enters the lymph node via the afferent lymphatic channel or via the artery, virgin B and naïve T cells specific for that antigen recognize it, begin to activate and migrate toward one another, the migration being driven by differential expression of the chemokine receptors CXCR5 and CCR7 by B and T cells.

The second signal for B-cell activation is provided by interaction with T helper cells, whereby protein antigens are endocytosed by B cells and protein-derived peptides are presented to T cells on the B cell membrane within the groove of MHC II molecules. After antigen recognition by T cells presented by B cells, the expression of CD40 on B cells and of CD40 ligand on T cells is induced, as well as cytokine production (mainly IL-4 and IL-21) by T cells, these processes further contributing to B-cell activation and leading to immunoglobulin heavy chain isotype switching and affinity maturation, which are central to achieve an effective humoral immune response.

The third signal for B-cell activation is provided by molecules of the innate immune system either expressed by B cells (e.g., complement receptors recognizing complement breakdown products bound to microbes, and TLR recognizing PAMPs and DAMPs) or secreted by FDC (e.g., B cell activating factor (BAFF) and IL-15). Therefore, similarly to what happens for T-cell activation, the innate immune system plays a central role in initiating the activation of humoral immunity.

The outcome of B-cell activation is clonal expansion leading to generation of antibody-producing plasma cells and memory B cells. Activated B cells undergo two central processes, namely, heavy chain isotype switching and affinity maturation. The first is characterized by production of antibodies with IgG, IgA, and IgE heavy chains, in contrast to surface Ig expressed by virgin B cells which belong to the IgM and IgD classes, as mentioned above. The second leads via somatic hypermutations to IgG, IgE, or IgA of high affinity to the antigenic epitope, whereby only B cells with the highest affinity receive the signals required to continue their expansion.

Autoimmunity and Autoantibodies

The extreme diversity of B- and T-cell antigen receptors, achieved by germ-line gene rearrangement and, in the case of B cells, also by somatic hypermutations, inevitably leads to emergence of receptors recognizing self-antigens. Lymphocytes expressing these receptors represent a danger and need to be regulated: central and peripheral tolerance mechanisms are in place for this purpose in the human immune system. However, they may fail, leading to subclinical autoimmune phenomena and, at times, overt autoimmune diseases. One of the most powerful clinical diagnostic tools in this context is the detection of autoantibodies in the patient's serum, and liver autoimmune diseases are no exception.

Autoimmune hepatitis type 1 (AIH-1) is characterized by the presence of anti-nuclear antibody (ANA) and/or anti-smooth muscle antibody (SMA), whereas autoimmune hepatitis type 2 (AIH-2), which is rarer and affects mostly children, adolescents, and young adults, is diagnosed in presence of anti-liver-kidney microsomal type 1 antibody (anti-LKM1) and/or anti-liver cytosol type 1 antibody (anti-LC1). Anti-soluble liver antigen antibody (anti-SLA) is shared between AIH-1 and AIH-2, being detected in up to 58% of the patients if sensitive assays are used [9]. The serological hallmark of primary biliary cholangitis (PBC) is anti-mitochondrial antibody (AMA), PBC-specific ANA being a powerful diagnostic tool in AMA-negative cases. The most frequently detected autoantibody in primary sclerosing cholangitis (PSC) is anti-neutrophil cytosolic antibody (ANCA), being present in up to 94% of the patients [10]. The pediatric clinical entity referred to as autoimmune sclerosing cholangitis (ASC) is associated with ANA and/or SMA, with or without concomitant anti-SLA (up to 41%) and ANCA (up to 74%). Finally, de novo *AIH,* that is, AIH arising after liver transplantation in a patient transplanted for conditions different from AIH, is associated with ANA and/or SMA or with typical/atypical anti-LKM1.

Laboratory Methods to Detect Autoantibodies

An essential knowledge of the principles the laboratory methods are based on is a prerequisite for the correct clinical usage of liver autoimmune serology. Such knowledge allows also the cross-talk between the laboratory and the clinician, which is crucial for the correct interpretation of the results. The reference method to detect autoantibodies relevant to autoimmune liver diseases is indirect immunofluorescence (IIF) on fresh triple (liver, stomach, and kidney) rodent tissue [11]. The main advantages of this method are the simultaneous detection of all the main liver autoantibodies, that is, ANA, SMA, anti-LKM1, anti-LC1, and AMA, the detection of autoantibodies targeting unknown antigens, and the recognition of disease-characteristic patterns. However, the technique is poorly standardized, observer-dependent and requires trained laboratory personnel, explaining the uneven frequency of autoantibodies reported in the literature [11]. Moreover, the studies reporting the associations of autoantibodies with autoimmune liver diseases date back to the 1970s and 1980s: The fresh rodent tissues used at that time as IIF substrate have been nowadays replaced by commercially available, fixed substrates, whose quality is variable, and comparative studies with fresh substrates are lacking

[12]. In an attempt to overcome these problems, immunochemical techniques are either already available or under development but only for the autoantibodies, whose target antigen has been identified. Since this is not the case for all autoantibodies, IIF remains the gold standard method for autoimmune liver serology.

The Committee for Autoimmune Serology of the International Autoimmune Hepatitis Group (IAIHG) published in 2004 detailed recommendations to guide testing of liver-relevant autoantibodies, in an attempt to ameliorate consistency among laboratories [11]. According to these guidelines, diluted patient's serum is incubated with the tissue substrates, leading to tissue binding of any autoantibody contained in the serum recognizing antigens present on the substrates. Unbound antibodies are removed by washing. A second, fluorochrome-labeled anti-human antibody is added, and after re-washing, the substrates are examined by ultraviolet microscopy (Fig. 4.3). Positive sera give characteristic IIF staining patterns and should be titrated to extinction. Anti-nuclear reactivities should be further characterized on HEp2 cells, a cell line derived from a laryngeal carcinoma which, thanks to their prominent nuclei, allows detection of the nuclear staining patterns, which are of paramount clinical importance in the setting of autoimmune liver diseases (see below). ANCA are detected using human neutrophils as an IIF substrate, being directed against autoantigens located in neutrophils.

The starting serum dilution is by convention 1:10, the positivity cut-off being 1:40 in adults, whereas in children and adolescents titers from 1:20 for ANA and SMA and from 1:10 for anti-LKM1 and for anti-LC1 are considered positive, since positive autoantibodies are infrequently detected in healthy subjects younger than 18 years [11].

The identification of the target antigens of anti-LKM1, anti-LC1, AMA, and to some extent, ANA and SMA, has

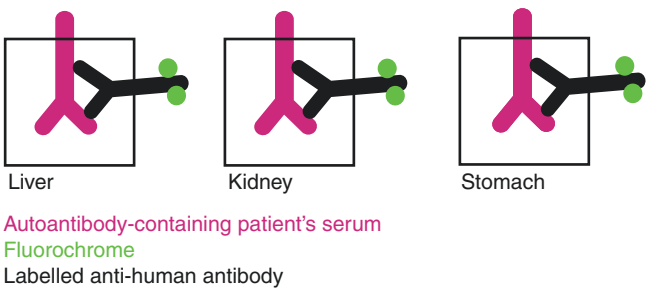

Fig. 4.3 Indirect immunofluorescence. Rodent stomach, liver, and kidney tissue sections are used as a composite substrate. Diluted patient's serum is added, and sections are washed to remove unbound autoantibodies. A second fluorochrome-labeled antibody which targets the human immunoglobulin constant region is added to the preparation, which is washed again and examined under an ultraviolet microscope. The light emitted by fluorochrome-labeled antibodies allows the detection of any tissue-bound autoantibody together with its characteristic immunofluorescence pattern

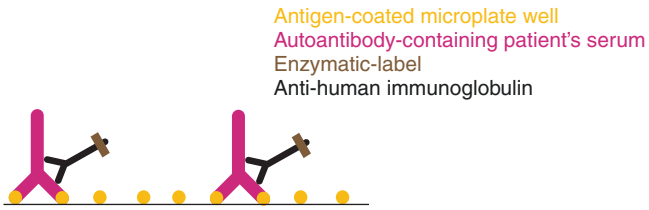

Fig. 4.4 Enzyme-linked immunosorbent assay (ELISA) as an example of a solid-phase assay. In a first step, diluted patient's serum is added to a microplate well coated with purified or recombinant antigen, leading to binding of any circulating antibody to the coated antigen. A second, enzyme-labeled antibody specific for human immunoglobulins is then added to detect antigen-bound autoantibody. In a third step, a chromogenic enzymatic substrate solution is added, promoting a color reaction by the antibody-bound enzyme. The concentration of the autoantibody in the patient's serum is proportional to the intensity of the color

led to the establishment of solid-phase immuno-assays to detect autoantibodies, which are observer-independent. These assays are based on purified or recombinant antigens attached to a solid phase, to which diluted patient's serum is added, leading to antigen binding of the corresponding antibody if present in the test serum. Bound antibodies are detected in a subsequent step by adding labeled anti-human antibodies, whereby labeling can be obtained with a chemiluminescence or fluorescent agent or with an enzyme- or radio-label (Fig. 4.4). The result may be quantitative or semi-quantitative, according to each technique.

Importantly, a solid-phase assay to detect anti-SLA, which is the most disease-specific AIH autoantibody, should be used in the diagnostic work-up of patients with suspected autoimmune liver disease, since this specificity is undetectable by IIF [13].

Anti-Nuclear Antibody

History

ANA was the first autoantibody associated with AIH. It is also the serological hallmark of lupus erythematosus and has been first reported in lupus patients, being responsible for the "lupus erythematosus" cells detected in their blood, which represent neutrophils engulfed with nuclear debris of damaged cells, the phagocytosis being mediated by ANA. The same cells were detected by Ian Mackay in 1956 also in ascites of patients with the so-called chronic hypergammaglobulinemic hepatitis," and for this reason, Mackay named the condition "lupoid hepatitis," the original name of AIH [14]. Later, it became clear that AIH and lupus erythematosus are distinct clinical entities, highlighting that ANA lacks disease-specificity. The technique of IIF was introduced in 1954 by Waller and Coons and replaced the cumbersome lupus erythematosus test in the 1960s.

Methods of Detection, Immunofluorescence Reactivities, and Antigenic Targets

ANA, as the name implies, recognizes antigens located in the cell nucleus; therefore, in IIF, it stains the cell nuclei on all of the three tissue substrates. Nuclei contain a wide variety of potential antigens, explaining the many different staining patterns seen on the nuclei of HEp2 cells (https://anapatterns.org). IIF remains the method of choice to screen for ANA, usually performed on commercially available cell or tissue preparations [15]. In the context of autoimmune liver diseases, ANA should be screened on triple rodent tissue rather than on HEp2 cells, the latter being a very sensitive test, leading to a positive result (titer ≥1/40) in up to 30% of healthy adults [12, 15]. ANA-positive sera on triple rodent tissue should be tested on HEp2 cells in order to characterize the IIF staining pattern, which is easily recognized on the prominent nuclei of this cell line. The homogeneous pattern (AC-1 according to Chan et al. [16]) is the most frequently observed in patients with type 1 AIH, being present in about two-thirds of the ANA-positive patients [17], the remainder displaying a speckled or nucleolar pattern [12] (Fig. 4.5). A wide variety of ANA molecular targets in AIH type 1 have been identified, including double- and single-stranded DNA, histones, centromere, chromatin, small nuclear ribonucleoproteins, and cyclin A [12]. In this context, the clinician should be aware that some ANA antigenic targets considered disease-specific, such as double-stranded DNA for lupus erythematosus and the centromere for systemic sclerosis, may also be encountered in AIH type 1. Molecular-based commercial kits have been established for the majority of the identified ANA antigenic targets, but they should not be used at a screening level in AIH, because as much as one-third of ANA-positive AIH patients do not react with any of the known molecular targets and would be missed if not tested by IIF [12].

Cytoplasmic and mitotic spindle IIF patterns on HEp2 cells are increasingly reported by the laboratories, challenging the term ANA [15]. The frequency and clinical significance of these patterns in autoimmune liver diseases remain to be established. Notably, AMA is detectable at IIF on HEp2 cells giving a reticular cytoplasmic IIF pattern, which should be reported by the laboratories when present.

Two disease-specific IIF ANA patterns are key to the diagnosis of PBC in presence of a cholestatic biochemical profile, especially when AMA is negative, namely the multiple nuclear dots (MNDs) (AC-6 according to Chan et al. [16]) and the rim-like/membranous pattern (AC-12 according to Chan et al. [16]) (Fig. 4.6). Up to half of the PBC patients are ANA-positive, the majority having either of these specific IIF patterns, highlighting the importance for the laboratory to report the IIF pattern on HEp2 cells, besides the titer. Anti-MND stains the nuclear bodies, which are complex structures composed of a number of distinct subunits, the IIF pattern being characterized by 5–30 nuclear dots sized 0.2–1 μm [18] (see Fig. 4.6). Its identified molecular antigenic targets include sp-100, promyelocytic leukemia protein, sp140, and small ubiquitin-related modifiers. The rim-like/membranous pattern is characterized by a bright staining of the nuclear rim, corresponding to the nuclear envelope, which is a complex structure comprising a double bilayer nuclear membrane, the nuclear pore com-

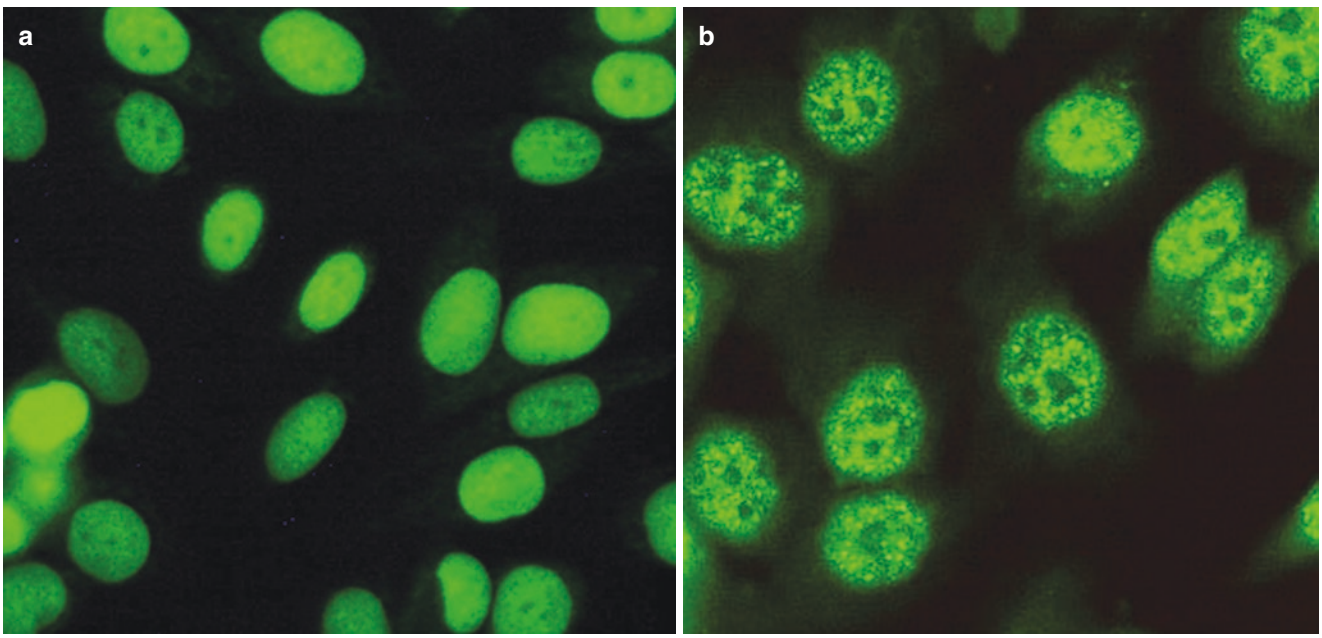

Fig. 4.5 Indirect immunofluorescence on HEp2 cells. Anti-nuclear antibody giving a homogeneous (**a**, characteristic of type 1 AIH) and a speckled (**b**) nuclear staining pattern

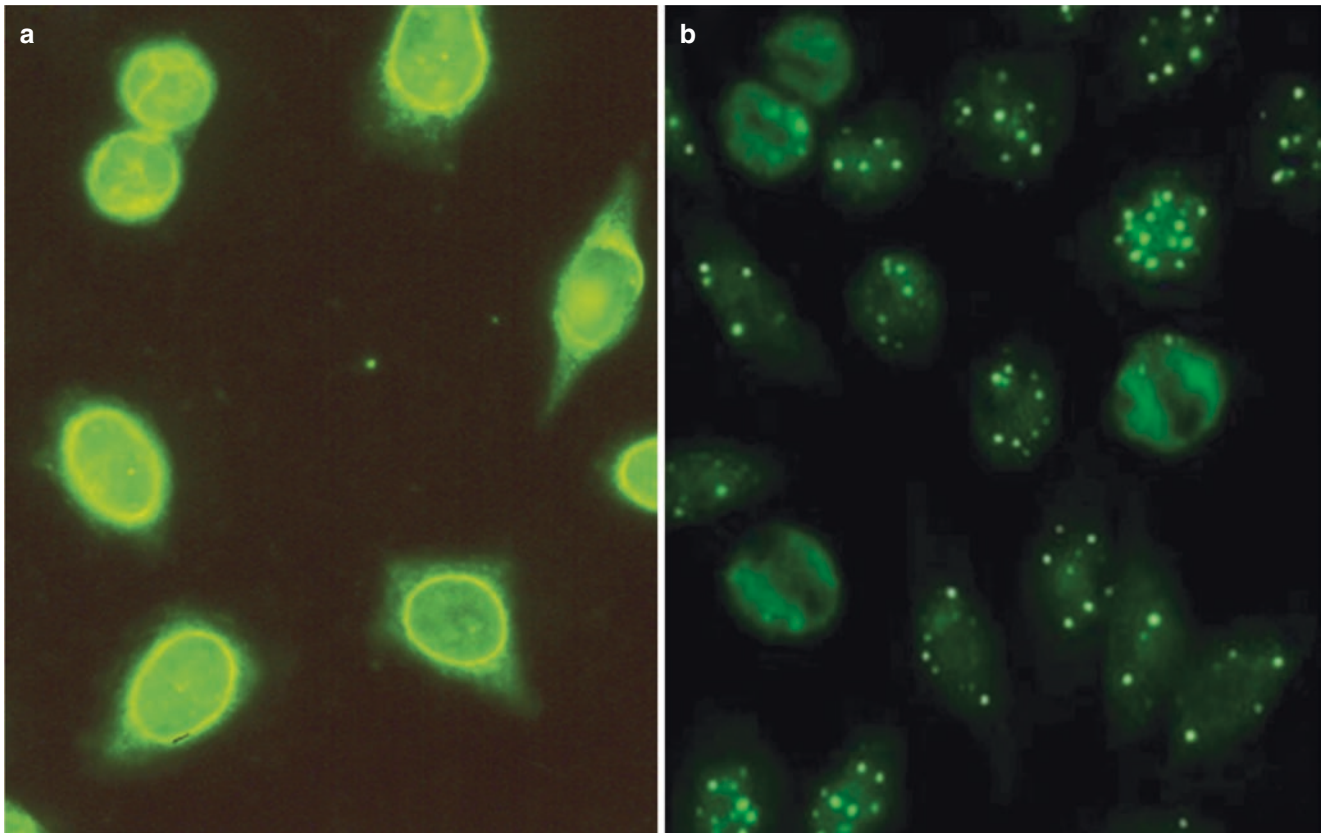

Fig. 4.6 Indirect immunofluorescence on HEp2 cells. Anti-nuclear antibody giving a rim-like/membranous (**a**) and a multiple nuclear dots (**b**) nuclear staining pattern. Both are specific for primary biliary cholangitis

plexes, and the nuclear lamina. Identified molecular targets of rim-like/membranous ANA include the nuclear pore complex components gp210, p62, and Tpr, and less frequently, the nuclear lamina component lamin-B and the nuclear membrane component LBR [18]. ELISA and immunoblotting assays for sp100 and gp210 based on recombinant antigens are commercially available but should be used in complementary manner to IIF, because they do not include all antigenic targets and may lack post-translational modifications and conformational epitopes. In addition to these PBC-specific ANA IIF patterns, the speckled, homogeneous and nucleolar ANA patterns and the pattern given by anti-centromere antibody (ACA) can be present in PBC. ACA stains the kinetochore, a protein structure attaching centromeres to the mitotic spindle fibers during the prophase of mitosis: as a consequence, ACA are best identified on mitotic cells, the Hep-20-10 form of HEp2 cells being the best substrate for its detection due to the high number of mitotic cells contained in this cell line. On interphase cells, ACA appears as multiple discrete dots strewn on the nucleus and corresponding to the centromeres. In contrast to ACA, MND do not stain mitotic cells, allowing its differentiation from ACA. The kinetochore ACA immunodominant epitope is CENP-B. ACA antigens are part of the so-called extractable nuclear antigens, a heterogeneous group of nuclear antigens detected by widely used ELISA or immunodiffusion tests. Of importance, ACA may be the only serological marker in a small subgroup of PBC patients [19].

Clinical Significance in Autoimmune Liver Diseases

Type 1 AIH is characterized by the presence of ANA, being detected in up to 75% of the patients, in association with SMA in half of the cases [20] (Table 4.1). ANA is rare in type 2 AIH [21]. There is no correlation of ANA titers with AIH activity [22, 23]. Besides a reported association between anti-double stranded DNA positivity with AIH/PBC variant syndrome, there is no correlation of specific ANA IIF patterns or molecular targets with AIH clinical features [24, 25].

As mentioned above, ANA lacks disease specificity, being detected in a number of autoimmune systemic and organ-specific extrahepatic diseases, such as lupus erythematosus, Sjögren syndrome, systemic sclerosis, and rheumatoid arthritis. Its presence may predate clinical disease onset by decades [26]. Of note, it is also detected in healthy subjects, with a frequency increasing with age. In addition to autoimmune diseases, it is also found in metabolic, toxic, neoplastic, and infectious conditions.

Table 4.1 Clinical significance of anti-nuclear antibody in autoimmune liver diseases

	Autoimmune hepatitis	Primary biliary cholangitis	Primary sclerosing cholangitis
Frequency if tested on triple rodent tissue	75% in AIH type 1 and ASC	10–65%	8–77%
IIF pattern on HEp2 cells	Homogeneous in ~75% Speckled or nucleolar in ~25%	Nuclear-rim/membranous pattern in 10–40% Multiple nuclear dots in 20–40% Centromere in 9–30%	Homogeneous in 46% Speckled 42–49% Nucleolar 25%
Target antigens	Unknown in 30% Chromatin Histones Centromeres Cyclin A Ribonucleoproteins Double-stranded DNA Single-stranded DNA	Rim-like/membranous pattern: gp210 Nucleoporin p62 Lamin B receptor Multiple nuclear dots pattern: Sp100 Promyelocytic leukemia protein Sp140 Small ubiquitin-related modifiers Centromere pattern: CENP-A CENP-B CENP-C CENP-D CENP-E CENP-F	Unknown in about 20% Double-stranded DNA SSA and SSB Ribonucleoproteins Scl70 Smith Single-stranded DNA
Diagnostic role	Concomitant SMA confers 99% diagnostic specificity	Rim-like/membranous and multiple nuclear dots patterns are virtually diagnostic of PBC Anti-centromere is rarely present in isolation	Lacks disease specificity, may suggests AIH-overlap
Prognostic role	Unknown	Rim-like/membranous and, with less solid evidence, multiple nuclear dots are associated with worse outcomes Anti-centromere is associated with portal hypertensive phenotype	Unknown

Abbreviations: *IIF* indirect immunofluorescence, *AIH* autoimmune hepatitis, *ASC* autoimmune sclerosing cholangitis, *PBC* primary biliary cholangitis, *AMA* anti-mitochondrial antibody, *SS* Sjögren antigen, *ASC* autoimmune sclerosing cholangitis, *SMA* anti-smooth muscle antigen

In the clinical context of acute or chronic liver disease, ANA may be positive in a wide range of clinical entities, including viral hepatitis A, B, C, D, and E, acute liver failure, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), Wilson disease, hepatocellular carcinoma, and alcohol-induced liver disease, though usually at titers lower than in AIH. It may also appear in liver graft recipients who were seronegative before transplantation. The differential diagnosis between drug-induced liver injury (DILI) presenting with a hepatocellular biochemical pattern and AIH is particularly challenging, since ANA are often positive in the so-called AIH-like drug-induced hepatitis, particularly when induced by drugs such as nitrofurantoin, minocycline, statins, and anti-tumor necrosis factor α, among many others. In these cases, only long-term follow-up may lead to the correct diagnosis, since AIH-like drug-induced hepatitis usually does not recur after steroid discontinuation [27].

In contrast to AIH, ANA IIF patterns have a prognostic role in PBC, the MND, and rim-like/membranous patterns being associated with a more severe disease course. Moreover, ACA has been associated with an increased risk of portal hypertension in PBC patients. It remains to be established if PBC-specific ANA in absence of a cholestatic biochemical profile may predict disease onset or may be associated with histological bile duct damage. Recently, a Chinese genome-wide association study identified HLA alleles associated with anti-sp100 but not with anti-gp210 [28].

In PSC patients, ANA are detected in up to three quarters of the patients, without associations with particular IIF patterns or molecular antigenic targets and without established diagnostic or prognostic roles [29, 30].

In conclusion, ANA positivity in the context of liver disease must be evaluated within the clinical context of the individual patient.

Anti-Smooth Muscle and Anti-Actin Antibodies

History

SMA has been first reported in 1965 in the United Kingdom: at that time, rat stomach had replaced human tissue as an IIF substrate [31]. SMA was detected in sera from patients with "lupoid hepatitis" but not in sera from patients with lupus erythematosus, thus representing a fundamental step in recognizing that these are two distinct entities [12]. A decade later, Gianfranco Bottazzo reported that SMA-positive sera display heterogeneous IIF patterns on kidney tissue, some sera staining only small arteries (V), others staining small arteries and glomerular mesangium (VG), and still others,

staining small arteries, glomerular mesangium, and peritubular fibers (VGT) [32]. While sera giving the V pattern were mostly low-titer and from patients with a variety of hepatic and systemic diseases, the VG and VGT patterns were mostly high-titer and restricted to patients with steroid-responsive hepatitis, highlighting for the first time the AIH-specificity of the VG and VGT patterns [32].

Methods of Detection, Immunofluorescence Reactivities, and Antigenic Targets

SMA is detected by IIF on triple rodent tissue: it stains the muscularis mucosae and the lamina propria on gastric substrate, and the smooth muscle of the arterial wall on all of the three substrates [12]. Importantly, as described above, it displays three different patterns on renal tissue, that is, the V, VG, and VGT patterns; the VG and VGT patterns being highly characteristic but not entirely specific for type 1 AIH (Fig. 4.7). If vinblastine-arrested cultured fibroblasts are used as a substrate, the VGT pattern corresponds to the so-called "F-actin" or microfilament pattern, a staining of actin-containing cytoskeleton components. In contrast, the V pattern corresponds to staining of nonactin-containing intermediate filaments [20]. Vascular smooth muscle (VSM) 47 cells from rat embryonic thoracic aorta are an additional IIF substrate used by laboratories to test SMA, on which VGT-positive sera give the "F-actin" pattern (see Fig. 4.7).

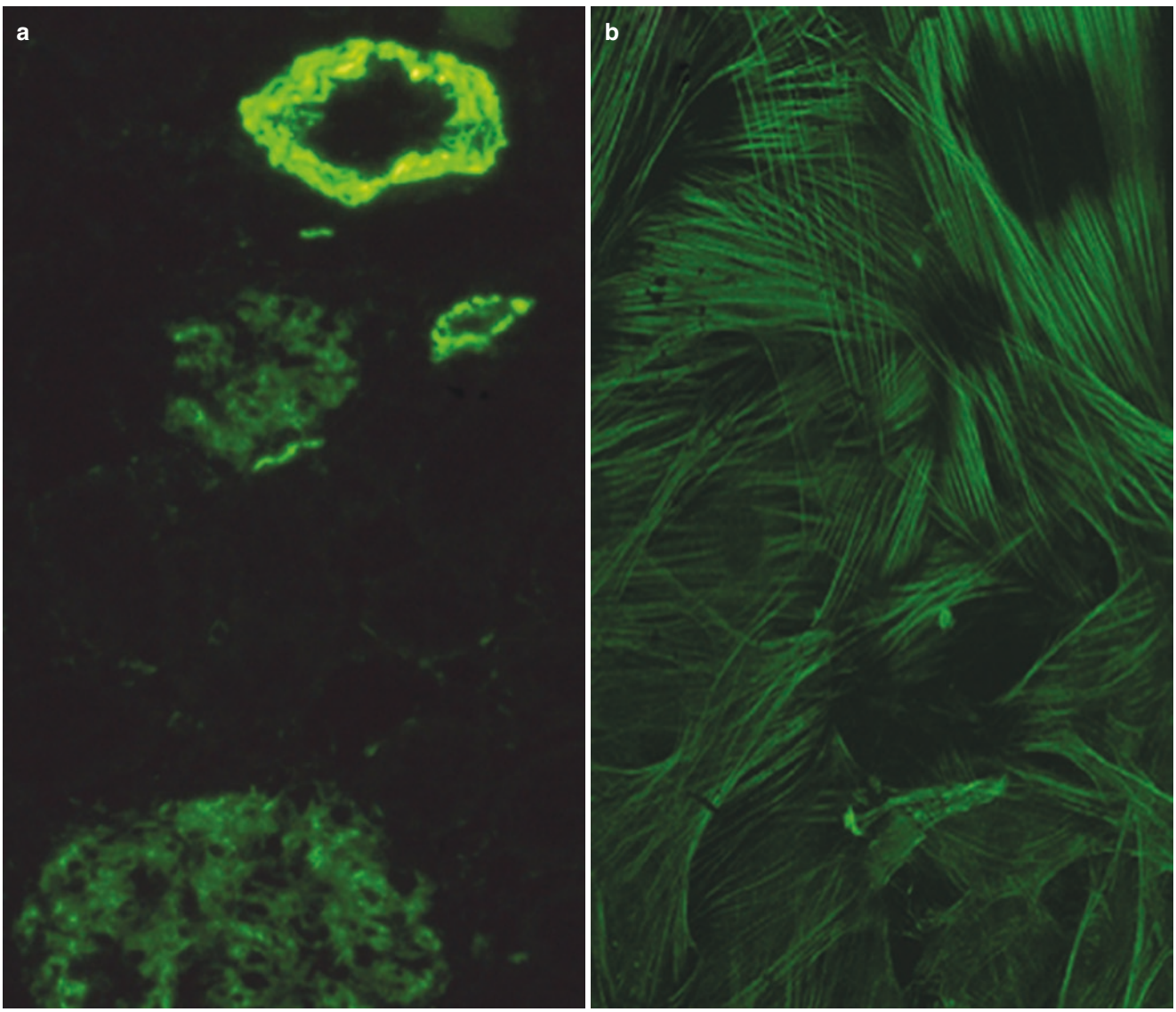

Fig. 4.7 Anti-smooth muscle antibody. (**a**) Indirect immunofluorescence pattern on rodent kidney tissue showing staining of the arterial wall and of the smooth muscle present in the glomerulus, corresponding to the VG pattern, highly specific for type 1 AIH. (**b**) Indirect immunofluorescence pattern on vascular smooth muscle (VSM) 47 cells showing the "F-actin" pattern

Cultured fibroblasts and VSM 47 cells should be used only in a complementary manner to IIF on triple rodent tissue, because some 20% of type 1 AIH patients lack the VGT/F--actin pattern, and therefore would be missed if tested only on the above cell lines. The SMA IIF patterns suggest that its antigenic target is part of filamentous actin, leading to the establishment of assays based on purified filamentous actin to test SMA in a more automated way. However, these assays have proved to be less specific than the VG and VGT IIF pattern on triple rodent tissue, giving positive results in a number of liver diseases distinct from type 1 AIH, including viral hepatitis, NAFLD, type 2 AIH, PSC and PBC [12]. To improve specificity of these assays, higher positivity cut-offs have been proposed, leading however to decreased sensitivity [33]. For this reason, commercially available kits have relatively low cut-off values, and it is the clinician's responsibility to correctly interpret the laboratory results in the clinical context of the individual patient. These assays should not be used alone at a screening level, since about 20% of AIH type 1 patients with the IIF VG or VGT patterns do not react with purified actin and would be missed. Possible causes for this incomplete overlap are loss of epitopes during antigen purification or presence of yet unknown SMA antigenic targets different from actin.

Clinical Significance

SMA characterizes type 1 AIH, where it is found in up to 85% of cases, in conjunction with ANA in at least half of the cases. Concomitant positivity for ANA and SMA has a diagnostic specificity of 99%, a positive predictive value of 97% and a diagnostic accuracy of 74% for AIH type 1 [34]. As alluded to above, the VG and VGT IIF patterns on kidney tissue are more specific for AIH type 1 than the V pattern; therefore, the clinician is significantly helped by the laboratory reporting of the IIF SMA renal pattern. Though SMA lacks disease specificity, in patients who do not have AIH-1, the most frequent finding is the isolated V pattern, and titers are lower as compared to AIH-1 patients [33]. Besides its high diagnostic value, SMA is useful to follow-up AIH patients, since its titer correlates with disease activity in both adults and children [12].

In pediatrics, presence of ANA and/or SMA is found in both AIH-1 and ASC patients [35].

The SMA frequency in PSC patients is as high as 83% but studies investigating the IIF patterns on kidney tissue are lacking. Nevertheless, this high frequency may suggest a link with ASC, but whether PSC represents the adult form of ASC remains to be established [36].

Anti-Liver Kidney Microsomal Antibody

History

Anti-LKM was first reported in 1973 by Mario Rizzetto in Deborah Doniach's London laboratory [12]. He observed an autoantibody giving a bright granular staining pattern of hepatocyte cytoplasm, using liver substrates from humans and from various animal species. This new specificity stained also human and animal kidney tissue. The name anti-LKM stems from the observation that the reactivity was abolished by incubation of the serum with a "microsomal fraction" obtained by ultracentrifugation of a liver homogenate, the fraction containing the endoplasmic reticulum, where the protein antigen is located [12]. The sera used for these early experiments were from only 16 patients, the majority with liver diseases, representing a tiny fraction of the sera tested in Doniach's laboratory. The liver disease associated with anti-LKM was characterized by the group of the pediatric hepatologist Daniel Alagille in Paris in 1987, who reported 65 patients, the majority being younger than 25 years, affected by an aggressive form of chronic hepatitis.

Anti-LKM was renamed anti-LKM1 following the description in the early 1980s of two other autoantibodies giving slightly different IIF patterns on triple rodent tissue, referred to as anti-LKM2 and anti-LKM3, respectively [12]. The former was reported in patients affected by ticrynafen- (also called tielinic acid) induced hepatitis, an anti-hypertensive drug withdrawn from the market for its hepatotoxicity. The latter was reported by Rizzetto's group in 13% of patients affected by chronic hepatitis delta infection, and later by Manns' group in a small minority of AIH-2 patients.

Methods of Detection, Immunofluorescence Reactivities, and Antigenic Targets

On IIF on triple rodent tissue, anti-LKM1 stains brightly the hepatocyte cytoplasm and on kidney tissue, it stains the larger, proximal tubules (Fig. 4.8). Gastric tissue is not stained by anti-LKM1, allowing its easy differentiation from AMA and underlying the importance of using triple tissue as an IIF substrate. Moreover, the renal IIF pattern given by anti-LKM1 is slightly different from the one given by AMA, which stains predominantly the smaller, distal, mitochondria-rich tubules. However, this distinction needs an expert observer. Anti-LKM2 stains predominately the first and second portion of the proximal tubules on kidney tissue, whereas on liver tissue, it shows a more intense staining

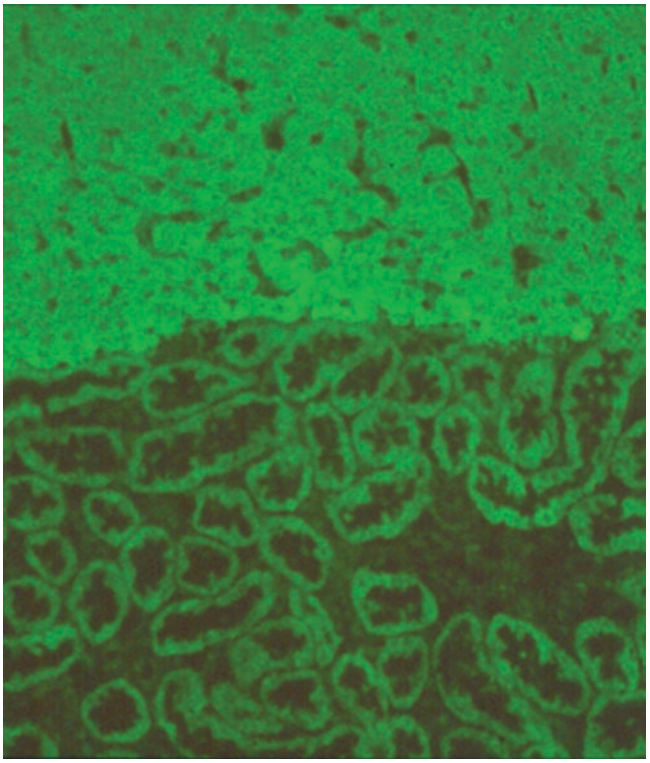

Fig. 4.8 Indirect immunofluorescence pattern on rodent liver (top) and kidney (bottom) tissue of anti-liver-kidney type 1 antibody. On liver substrate, it stains brightly the hepatocyte cytoplasm and on kidney substrate, it stains preferentially the larger proximal tubules

of the centrilobular zone. Anti-LKM3 needs to be tested on human tissues, hampering its use in clinical practice.

The antigenic target of anti-LKM1 was identified between 1986 and 1989 by three independent groups as the cytochrome (CYP) P450 IID6 (CYP2D6) [33]. In contrast, anti-LKM2 and anti-LKM3 were found to target CYP2C9 and uridine diphosphate glucuronosyltransferase (UGT), respectively. A rare inherited condition referred to as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is associated with AIH in about 20% of patients, who are positive for an autoantibody with an IIF pattern indistinguishable from anti-LKM1. This reactivity was found to target CYP1A2, 2A6, and 2D6 [33]. The identification of CYP2D6 allowed the establishment of highly sensitive and specific but unstandardized, molecular-based assays to detect anti-LKM1, whereas this is not the case for anti-LKM2, which is of historical interest, and for anti-LKM3, which is not used in clinical routine. Identified linear immunodominant epitopes within the large protein CYP2D6 are $CYP2D6_{193–212}$, $CYP2D6_{254–271}$, and $CYP2D6_{321–351.}$ It has been reported that CYP2D6 is expressed also on the external aspect of the hepatocyte cell membrane, thus in a location amenable to recognition by anti-LKM1, suggesting a potential pathogenic role of anti-LKM1 in AIH-2 [12].

De-novo AIH is a condition undistinguishable from AIH but affecting liver graft recipients having undergone liver transplantation for diseases distinct from AIH. A subgroup of patients with de novo AIH are positive for anti-LKM1 staining only kidney tissue, referred to as atypical anti-LKM1 [37]. Once again, it is of paramount clinical relevance that laboratories report to the clinician all observed reactivities, even if atypical. The use of IIF on triple rodent tissue is the only method allowing the detection of atypical anti-LKM1.

Clinical Significance

Anti-LKM1 is the serological hallmark of type 2 AIH. In addition to its high diagnostic value, it is useful in clinical practice to monitor patients, since it correlates with disease activity. Considering the antibody half-life of 21–25 days, it is advisable to test anti-LKM1 initially every 3 months and then every 6 months in patients with type 2 AIH. This specificity usually disappears after liver transplantation, and its re-appearance may predict post-transplant AIH-2 recurrence.

Anti-LKM1 lacks disease specificity, being detected in up to 13% of patients with chronic hepatitis C virus (HCV) infection. In clinical practice, it is essential to rule out HCV infection in anti-LKM1 positive patients. The mechanism leading to anti-LKM1 production in HCV is probably molecular mimicry, a process redirecting immune reactivity from external pathogens to structurally similar self-antigens. Indeed, HCV protein sequences shared in common with CYP2D6 have been identified. Anti-LKM1-positive HCV patients were at risk of severe hepatitic flares while treated with interferon; however, this is no longer a clinical issue with the current use of direct acting antiviral agents (DAAs). Recent data indicate that rarely anti-LKM1 may appear after DAA-induced HCV clearance, suggesting that the autoimmune imprint given by HCV persists after viral elimination [38]. The clinical relevance of this phenomenon remains to be clarified.

Anti-Liver Cytosol Type 1 Antibody

History

Anti-LC1 was first reported in 1988 by Martini et al. in the group of Jean Claude Homberg, who detected this reactivity in the serum of 21 young patients affected by type 2 AIH, associated with anti-LKM1 in 14 of them [12]. Anti-LC1 was not detected in a large control group including healthy

controls as well as patients with a variety of systemic and hepatic conditions.

Methods of Detection, Immunofluorescence Reactivities, and Antigenic Targets

At IIF on triple rodent tissue, anti-LC1 selectively stains liver tissue, giving a bright pattern, sparing the centrilobular area (Fig. 4.9). The pattern is obscured by concomitant presence of anti-LKM1, which is often the case. The name is derived from the observation that anti-LC1 reacts only with the cytosolic fraction of liver homogenate when tested by double-dimension immunodiffusion, counterimmunoelectrophoresis, or immunoblotting. Thanks to the identification of the liver-restricted enzyme formiminotransferase cyclodeaminase as the anti-LC1 antigenic target, reliable molecular-based assays for anti-LCM1 have become commercially available, which should be used to investigate anti-LC1 in anti-LKM1-positive patients [33].

Clinical Significance

Anti-LC1 is considered a specific marker of type 2 AIH. It has been rarely reported in patients affected by HCV infection, highlighting the mandatory exclusion of acute and chronic viral hepatitis before making a diagnosis of AIH. About two-thirds of type 2 AIH patients are positive for both anti-LKM1 and anti-LC1, a proportion ranging from 10% to 30% being positive for anti-LC1 in isolation. Positivity for anti-LKM1 and/or anti-LC1 is universal in type 2 AIH. Isolated anti-LKM3 positivity has been anecdotally reported, but this specificity is very difficult to be tested in clinical practice.

Anti-LC1 titers correlate with disease activity, and similarly to anti-LKM1, should be used to monitor patients during follow-up [39].

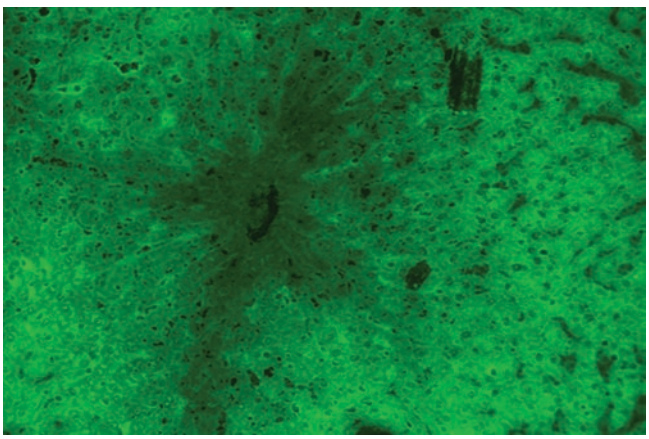

Fig. 4.9 Indirect immunofluorescence pattern on rodent liver tissue of anti-liver-cytosolic type 1 antibody showing bright staining of the hepatocyte cytoplasm with decreased intensity around the central vein

Anti-Soluble Liver Antigen Antibody

History

The German scientist Thomas Berg reported in 1981 a new autoantibody reacting with the supernatant of liver and pancreas rodent homogenates and proposed to name it anti-liver pancreas antibody. Patients with this reactivity were mostly women affected by an aggressive form of chronic hepatitis, responsive to steroids and azathioprine treatment [12]. Importantly, Berg detected anti-liver pancreas antibody by complement fixation test, since this reactivity was undetectable by IIF. Subsequent studies by the same group led to the proposal of a third AIH type, characterized by the presence of anti-liver pancreas antibody. However, due to lack of availability of validated tests to detect this specificity, this proposal was not endorsed by the scientific community. Six years after Berg's report, Michael Manns, in Karl-Hermann Meyer zum Büschenfelde's group, reported an autoantibody reacting with the supernatant of liver homogenate from different species, including guinea pig, rat, rabbit, mouse, and humans, which he named anti-soluble liver antigen (SLA) and which, similarly to the anti-liver pancreas antibody, was not detectable by IIF. Patients with this specificity were similar to the ones in Berg's report, being mostly young women with chronic, hypergammaglobulinemic, steroids-responsive hepatitis. One additional similarity with Berg's report was the AIH-specificity of anti-SLA, being extremely rare in healthy controls, in patients with non-AIH liver diseases or extrahepatic conditions. Manns also suggested a third type of AIH, characterized by isolated anti-SLA positivity. This proposal was again not embraced by the international community, after the IAIHG consensus statement on AIH autoantibody testing recommended a cut-off for ANA positivity on triple rodent tissue of $\geq$1:40. Using this cut-off, most patients with isolated anti-SLA positivity are ANA-positive, and therefore should be classified as AIH type 1.

Subsequently, it became clear that anti-liver pancreas antibody and anti-SLA are the same autoantibody, targeting a liver-specific protein identified as O-Phosphoseryl-tRNA (Sec) selenium transferase, a selenocysteine synthase (SEPSECS) [40]. The nomenclature has been currently simplified to anti-SLA.

Methods of Detection and Antigenic Targets

As mentioned above, anti-SLA cannot be detected by immunofluorescence. For this reason, international guidelines recommend to include anti-SLA testing by ELISA or immunoblot in the initial diagnostic work-up of patients with suspected AIH, in addition to IIF on triple rodent tissue [13].

The sensitivity of commercially available assays is limited, since they use linear epitopes of recombinant, procaryotically expressed antigen. In contrast, conformational epitopes are preserved by radioligand assays, which unfortunately are too cumbersome for clinical laboratories but have been shown to have a much higher sensitivity, leading to anti-SLA detection in up to 58% of AIH patients [9].

SEPSECS is a cytoplasmic 422-amino acid protein expressed in liver, lung, kidney, pancreas, and in activated lymphocytes. It catalyzes the final step of selenocysteine synthesis, the only amino acid synthetized on its cognate tRNA. SEPSECS has been mapped to chromosome 4p15.2; homozygous or compound heterozygous mutations in this locus are associated with pontocerebellar hypoplasia type 2D (OMIM 613811), demonstrating that selenoproteins are crucial for brain development and function. The immunodominant epitope of anti-SLA has been reported to be SEPSECS $_{395–414}$ [41].

Preliminary data show that SEPSECS epitopes are targeted also by autoreactive T-cells in anti-SLA positive AIH patients, but these T cells have not been fully characterized [42].

Clinical Significance

Anti-SLA is the most specific autoantibody for AIH, with a specificity as high as 98.8% [34]. However, its sensitivity is limited, anti-SLA being detected by commercially available assays in only about one-third of AIH patients. Interestingly, it is shared by both types of AIH. Using highly sensitive radioligand assays, its frequency increases to up to 58% of type 1 and type 2 AIH patients, and to 41% of ASC patients [12]. In a very small proportion of AIH patients, it is the only autoantibody reactivity detected: These patients are currently classified and managed as type 1 AIH. In addition to its high diagnostic value, anti-SLA has a prognostic value, positive patients having a more aggressive disease, with higher relapsing rates and decreased transplant-free survival [9, 43].

It is of clinical importance that anti-SLA is strongly associated with anti-Ro52, a subtype of the anti-Sjögren syndrome antigen A. Anti-Ro52 can cross the placenta barrier leading to fetal disease, including potentially fatal high-grade heart block. Therefore, it is advisable to look for this specificity in anti-SLA positive young women and to carefully monitor the fetal heart rhythm in anti-Ro52 positive mothers. A higher risk of fetal loss has been reported in anti-SLA positive mothers. Anti-Ro52-associated neonatal disease often includes a skin rash and is referred to as neonatal systemic lupus erythematosus, which is a confusing nomenclature, being a clinical entity distinct from pediatric and adult systemic lupus erythematosus.

Anti-Neutrophil Cytoplasmic Antibody

History

ANCA has been first reported in the 1980s in patients with systemic vasculitis and glomerulonephritis [33]. The association with inflammatory bowel disease and with PSC has been recognized in the early 1990s, soon followed by the association with AIH.

Methods of Detection and Antigenic Targets

While triple rodent tissue is used as an IIF substrate to detect ANA, SMA, anti-LKM1, and AMA, ANCA is detected by IIF using human ethanol- and formaldehyde-fixed neutrophils as an antigenic source, since, as the name implies, it is directed against antigens found in neutrophils. ANCA is a heterogeneous family of autoantibodies, giving different staining patterns. The classical perinuclear staining pattern (pANCA) (Fig. 4.10) is the result of an artifact due to the ethanol fixation of neutrophil, leading to migration of positively charged cytoplasmic antigens to the negatively charged nuclear membrane. As a consequence, this pattern is not recognized on formaldehyde-fixed neutrophils, where pANCA gives a cytoplasmic staining. If the IIF staining pattern is unaffected by ethanol fixation, the reactivity is referred to

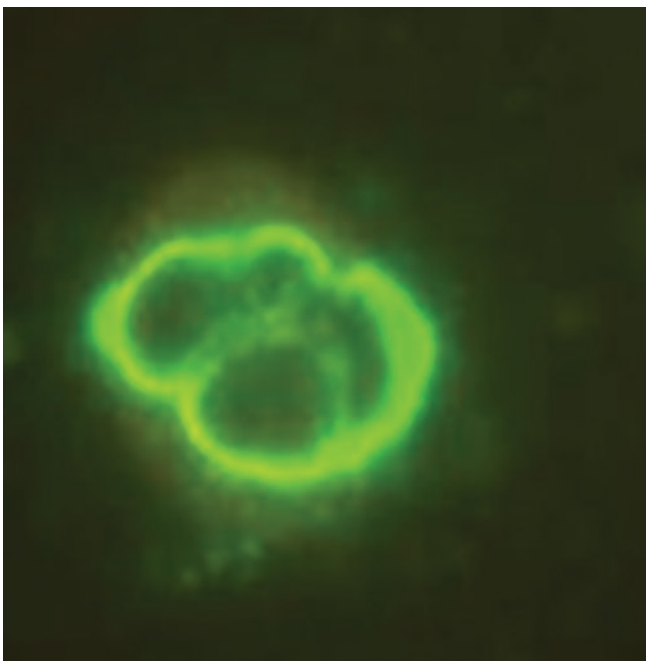

Fig. 4.10 Indirect immunofluorescence pattern of atypical antiperinuclear neutrophil cytoplasmic antibody (p-ANNA). This antibody is found in a variable proportion of patients with autoimmune hepatitis type 1, primary sclerosing cholangitis, autoimmune sclerosing cholangitis and inflammatory bowel disease

as atypical pANCA, also going under the name of perinuclear anti-neutrophil nuclear antibody (pANNA) or nuclear anti-neutrophil antibody (NANA), owing to the fact that the antigenic target is located in the nuclear envelope. cANCA gives a cytoplasmic staining pattern on both ethanol- and formaldehyde-fixed neutrophils. While pANCA is typically detected in patients affected by microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg–Strauss syndrome), cANCA characterizes granulomatosis with polyangiitis (GPA, formerly Wegener granulomatosis); their main antigenic targets are the neutrophil cytoplasmic protein myeloperoxidase, and the cytoplasmic protein leukocyte proteinase 3, respectively. Molecular-based assays detecting anti-myeloperoxidase and anti-leucocyte proteinase 3 are commercially available, which should be used in a complementary manner to IIF, because cANCA and pANCA targeting a variety of additional antigenic targets have been reported and would be missed by the molecular-based assays. Most importantly, pANNA is not detected by these assays, being directed against nuclear antigens, including the nuclear cytoskeleton component beta-tubulin isotype 5, the high-mobility group non-histone chromosomal proteins HMG1 and HMG2, histones and probably other, yet unidentified constituents of the nuclear envelope. Beta tubulin has been described as the molecular target of pANNA in PSC, but the frequency of anti-tubulin antibodies in this condition and the overlap with ANCA-positivity have been questioned. While pANCAs are the most common antibodies in PSC, whether these antibodies target the same antigen in all patients remains to be established. Cytoplasmic ANCA antigenic targets reported in PSC include H1, enolase, bactericidal/permeability increasing protein, lactoferrin, elastase, cathepsin G, catalase, and human lysosomal-associated membrane protein 2.

The clinician should be aware that coexisting ANA may mask a perinuclear staining pattern on neutrophils, since ANA stains the neutrophils nuclei. Substrate containing both HEp2 cells and neutrophils may be helpful in detecting both specificities if present in the same serum.

Clinical Significance

pANNA is associated with PSC, ASC, inflammatory bowel disease and AIH type 1, being the most frequently detected specificity on a neutrophil substrate in these conditions. However, detection of other ANCA subtypes is also possible [44]. Of note, ANCA is virtually absent in type 2 AIH [21]. The reported ANCA frequency in type 1 AIH ranges from 20% to 96% [12]. In rare cases, it can be the only positive specificity, leading to the recommendation to look for ANCA in patients with suspected AIH, but negative IIF on triple rodent tissue and negative anti-SLA [13]. ANCA is very frequent in PSC patients, being present in up to 94% of the cases. Its presence has been associated with less severe disease. There are no data on ANCA frequency in small duct PSC, since patients with this condition have been excluded from the studies.

In pediatrics, ANCA frequency in ASC ranges from 48% to 74%, being higher than in type 1 AIH, where it is detected in 20% to 36% of the patients and being associated with inflammatory bowel disease. In clinical practice, pANNA-positive AIH patients should undergo endoscopic investigations to exclude intestinal pathology, even if asymptomatic [12].

Anti-Mitochondrial Antibody

History

AMA is the serological hallmark of PBC: it was first reported by Ian Mackay in 1958 when, in an attempt to shed some light on the enigmatic pathogenesis of PBC, he thoroughly investigated a 38-year-old female patient in whose serum he detected by complement fixation test an autoantibody reacting with human liver and kidney homogenates [45]. This preliminary report was followed 7 years later by a landmark paper by Sheila Sherlock's group reporting on a unique IIF reactivity on human tissue sections detected in the serum of 32 PBC patients but not in the serum of patients with non-PBC cholestasis. This finding represented a major breakthrough both for patient's care and for the laboratory, as up to that time, investigations of cholestatic conditions included surgical exploration of the biliary tree, and serology testing of the antibody described by Mackay was based on cumbersome techniques like the complement fixation test. The IIF used to detect AMA in this early paper still represents the reference technique to investigate the presence of AMA, with the only difference that rodent substrates are used nowadays instead of human substrates [11]. The first suggestion that this PBC-associated serological specificity was directed against mitochondrial antigens dates back to 1967 when Thomas Berg's group reported reactivity to mitochondrial fractions of liver homogenates in the serum of patients with PBC. The same group extensively worked on AMA, demonstrating that the antigenic target was a 74 kDa enzyme located on the inner mitochondrial membrane, which they named M2-antigen, as opposed to M1 targeted by anti-cardiolipin antibody in syphilis [12]. In addition to the 74 kDa protein, AMA recognized, although less frequently, mitochondrial proteins of 56, 41, 48, and 36 kDa. Subsequently, Berg proposed a nomenclature spanning from M2 to M9 based on the different anti-mitochondrial patterns and reactivities. This nomenclature has been abandoned after cloning of the 74 kDa AMA target molecule by Eric Gershwin in 1987, who identified it as the

E2 subunit of the pyruvate dehydrogenase complex (PDC), allowing the establishment of reliable molecular-based assays using recombinant antigen. Additional AMA targets are other components of the 2-oxo acid dehydrogenase multienzyme complexes, namely 2-oxoglutarate dehydrogenase complex (OGDC), branched-chain 2-oxo acid dehydrogenase complex (BCOADC) and the E3 binding protein of PDC [46].

Methods of Detection and Antigenic Targets

As inferred from the above described historical aspects, AMA can be detected both by IIF and by molecular-based assays, mainly ELISA or dot-blot. At IIF, AMA stains all mitochondria-rich tissues: on kidney sections, it stains preferentially the mitochondria-rich distal, smaller tubules; on gastric sections, it gives a bright granular pattern of gastric parietal cells; and on liver sections, it stains faintly the hepatocyte cytoplasm (Fig. 4.11). This IIF pattern on triple rodent tissue is pathognomonic, anti-LKM1 being similar on kidney substrate but easily distinguished by its lack of gastric staining. In addition, AMA stains the cytoplasm of HEp2 cell giving a granular diffuse pattern, which, however, is not unique to AMA, making the use of HEp2 cells as an IIF substrate for AMA detection inadequate.

The AMA target proteins are all components of the 2-oxo acid dehydrogenase multienzyme complexes, which are key players in the mitochondrial respiratory chain. They are high molecular weight multimers composed of multiple copies of three enzymes, that is, E1, E2, and E3, E2 providing the structural core to which multiple copies of E1 and E3 are non-covalently bound. The E2 enzymes contain the lysine-bound inner lipoyl domain, which has a central role in the catalytic cycle and which is the immunodominant AMA epitope.

Immunoblotting, also named Western blot, can be used to detect AMA using mitochondrial preparations from diverse tissues and species, or more recently, recombinant PDC-E2, as an antigen source; however, this highly sensitive and specific technique is very demanding for the laboratory. Nowadays, the most widely used molecular-based AMA

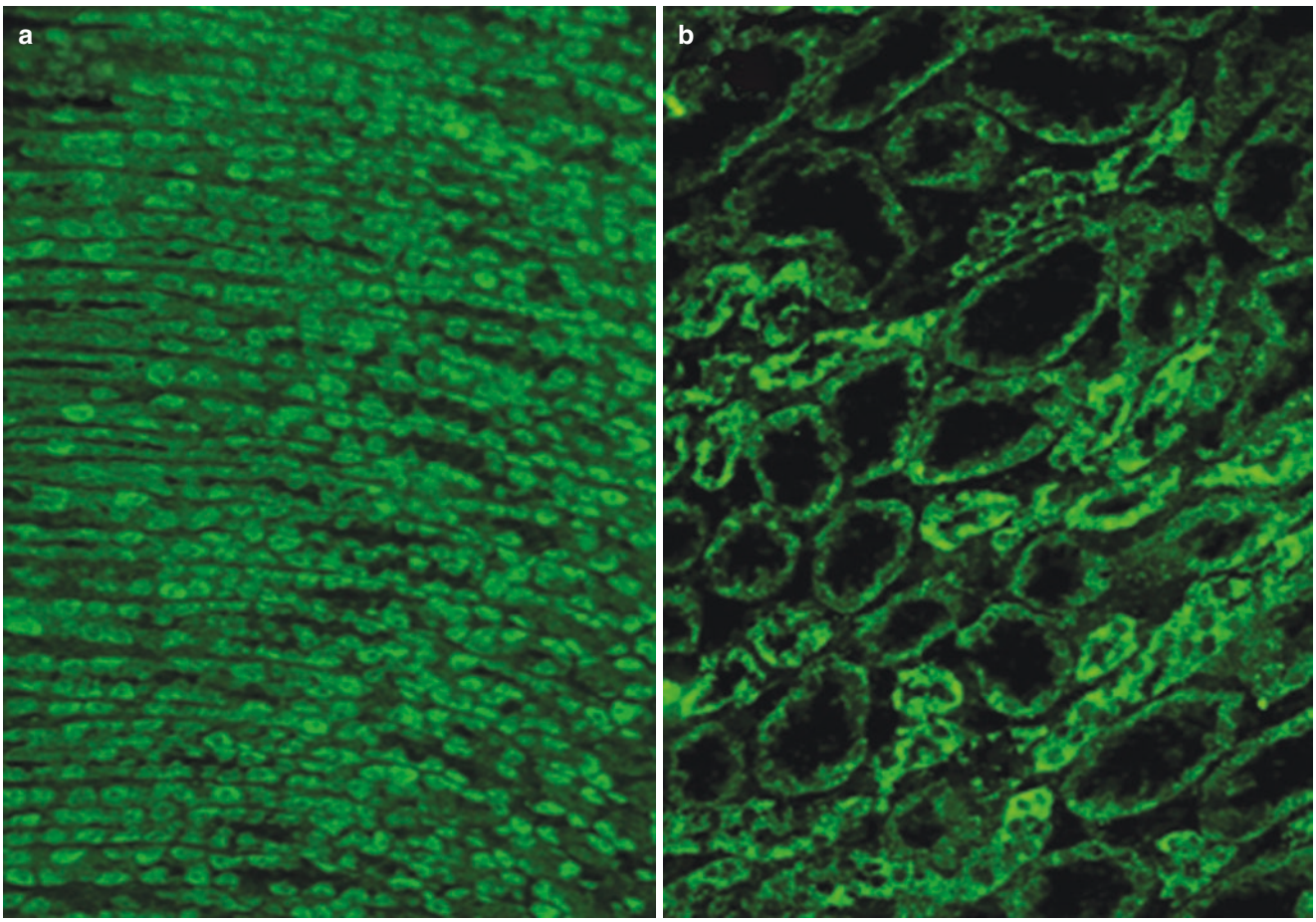

Fig. 4.11 Indirect immunofluorescence pattern on rodent stomach (panel **a**) and kidney (panel **b**) tissue of anti-mitochondrial antibody. On stomach, the antibody stains brightly the gastric parietal cells; on the kidney, it stains preferentially the smaller, mitochondria-rich distal tubules

assays are ELISA and dot-blot. They are based on purified or recombinant antigens. In this context, it is of interest, that is, the development by Eric Gershwin's group of a recombinant antigen called MIT3 co-expressing the three immunodominant epitopes of PDC-E2, BCOADC-E2, and OGDC-E2. Assays based on MIT3 are highly sensitive, leading to detection of AMA in PBC patients who are AMA-negative by IIF, but lose specificity, detecting AMA also in patients without PBC, such as those with chronic infections [12]. Many clinical laboratories use an assay based on MIT3 and purified sp100 and gp210 called PBC screen, which has proved to be highly specific and sensitive, giving positive results in as much as 67% of PBC patients who are AMA-negative by IIF [47].

Clinical Significance

AMA is the serological hallmark of PBC, being present, if tested with sensitive methods, in up to 95% of the patients. AMA is considered the most disease-specific autoantibody in human pathology [48]. While it has been known for decades that AMA may predate clinical overt PBC, warranting monitoring of AMA-positive patients without cholestasis, it has recently been shown that the majority of AMA-positive patients without biochemical cholestasis have PBC-compatible histological changes at liver biopsy, at times with advanced disease [49]. These data have to be confirmed before liver biopsy can be recommended in patients with isolated AMA positivity.

As mentioned above, if highly sensitive methods are used, AMA may be detected in non-PBC patients, for example, it can be present transiently in acute liver failure, emphasizing the notion that serology tests are not diagnostic on their own and need to be interpreted in the clinical context of the individual patient.

Practical Points for the Clinicians

Autoantibodies are an essential diagnostic tool in autoimmune liver diseases, provided they are tested according to dedicated guidelines (Table 4.2). The clinician needs to have an essential knowledge of the laboratory methods used for autoimmune serology testing, particularly to be aware of differences, advantages, and disadvantages of IIF compared to molecular-based assays. In addition, such knowledge allows cross-talk between the laboratory and the treating physician, which is crucial in order to maximize the clinical usefulness of autoimmune liver serology. If correctly tested, > 95% of patients with autoimmune liver diseases have at least one serological positivity [50].

The diagnostic work-up of an adult patient with acute or chronic liver disease of unknown origin should begin with a thorough medical history, including exposure to drugs and herbal products, risk factors for viral hepatitis, comorbidities, family history, and alcohol intake. Abdominal ultrasound is helpful in identifying vascular abnormalities, focal liver lesions, signs of portal hypertension and liver steatosis. At a laboratory level, a careful exclusion of viral hepatitis A, B, C, D in HBs-antigen positive subjects, and E is essential. Furthermore, the diagnostic work-up should include IIF on triple rodent tissue and a molecular-based anti-SLA assay (Fig. 4.12), allowing the detection of the most liver relevant autoantibodies, that is, ANA, SMA, anti-LKM1, anti-LC1 and AMA, and anti-SLA. In case of negative results, second-line autoantibody testing includes a MIT3-based AMA molecular test or a PBC-screen test, IIF on fixed human neutrophils to look for ANCA, molecular based F-actin, sp100 and gp210 assays. In rare cases, particularly in patients presenting with an acute hepatitic illness, autoantibodies may be negative at presentation and re-testing during follow-up is advisable. ANA-positive sera should be further examined on HEp2 cells in order to identify the nuclear staining pattern: a homogeneous pattern suggests type 1 AIH, whereas a rim-like/membranous or multiple dots patterns are diagnostic for PBC. The clinical significance of cytoplasmic staining patterns on HEp2 cells in AMA-negative sera remains to be investigated. If SMA is detected, it is important that the laboratory report includes the staining pattern on kidney tissue, since the VG and VGT patterns are typically seen in AIH patients, whereas the V pattern is less specific. IIF on VSM 47 or cultured fibroblasts can be used to confirm the VGT pattern observed on kidney substrate. Detection of anti-SLA, particularly outside HCV infection, should prompt a liver biopsy, being highly specific for AIH. Anti-LKM1 positivity, with or without coexisting anti-LC1, is virtually diagnostic of type 2 AIH, particularly in the clinical context of a child/adolescent presenting with acute hepatitis, provided that HCV infection has been excluded. Positive anti-LMK1 IIF results can be confirmed by ELISA or dot blot.

While AMA-positivity coupled with biochemical cholestasis is diagnostic of PBC, isolated AMA-positivity warrants long-term monitoring due to the high risk of development of PBC on the long term; according to recent data, reporting high frequency of PBC characteristic histological abnormalities in this clinical setting, a liver biopsy may be considered. ANCA-positivity, particularly for pANNA, in a

Table 4.2 Most relevant features of liver-relevant autoantibodies other than anti-nuclear antibody

Specificity	Antigenic targets	Methods of detection	Frequency	Diagnostic role	Other liver diseases	Comments
SMA	Filamentous actin Vimentin Desmin	IIF	85% in AIH-1 75% in ASC Up to 83% in PSC Unknown in PBC	VG and VGT IIF patterns specific for AIH-1 Concomitant ANA confers 99% diagnostic specificity	DILI NAFLD Viral hepatitis Wilson disease	V pattern is seen in 20% of type 1 AIH patients Titers correlate with disease activity
Anti-actin antibody	Actin	Molecular-based assays	60% in AIH-1	Specific for AIH-1 at high titers	DILI NAFLD Viral hepatitis Wilson disease	Sensitivity and specificity depend on the chosen cut off point Less specific for AIH than the VG/VGT IIF pattern
Anti-LKM1	Epitopes of CYP2D6	IIF Molecular-based assays	Up to 90% in AIH-2	Specific for AIH-2 in absence of hepatitis C	Hepatitis C	Titers correlate with disease activity Post-transplant reappearance predicts AIH-2 recurrence Atypical anti-LKM1 associated with de novo AIH
Anti-LC1	Formiminotransferase cyclodeaminase	IIF Molecular-based assays	60% of AIH-2 Anti-LC1 and anti-LKM1 co-occur in half of the patients with AIH-2	Specific for AIH-2 in absence of hepatitis C	Hepatitis C	Only serological marker in 10–30% of AIH-2 cases Titers correlate with disease activity
Anti-SLA	*O*-Phosphoseryl-tRNA (sec) selenium transferase (SEPSECS)	Molecular-based assays	Up to 58% in AIH-1 and AIH-2; 41% in ASC when tested with radioligand assays	Highly specific for AIH. Low disease sensitivity (20–30%) when tested with commercial solid phase assays	Rare in hepatitis C	Associated with worse outcomes May be the only autoantibody present
pANNA	Beta-tubulin isotype 5; HMG1; HMG2; other yet unknown autoantigens	IIF	40–96% in AIH-1 26–94% in PSC 74% in ASC	Specific for AIH-1, PSC and IBD		May be the only serological marker in AIH-1 or ASC Absent in AIH-2 IBD and sclerosing cholangitis should be excluded in pANNA-positive patients
AMA	E2 subunits lipoyl domains of PDC, OGDC and BCOADC, E3 binding protein of PDC	IIF Molecular-based assays	95% in PBC Virtually absent in AIH and PSC	Highly specific of PBC	PBC/AIH overlap Acute liver failure	Predicts development of PBC May be associated with histological PBC changes even in absence of biochemical cholestasis Suggests PBC/AIH overlap if present in AIH

Abbreviations: *SMA* smooth muscle antibody, *IIF* indirect immunofluorescence, *V* vessel, *G* glomerulus, *T* tubulus, *AIH* autoimmune hepatitis, *DILI* drug-induced liver injury, *PSC* primary sclerosing cholangitis, *PBC* primary biliary cholangitis, *NAFLD* non-alcoholic fatty liver disease, *LKM* liver kidney microsomal, *LC1* liver cytosol type 1, *SLA* soluble liver antigen, *pANNA* perinuclear anti-neutrophil nuclear antibody, *HMG1* high mobility group non-histone chromosomal protein, *AMA* anti-mitochondrial antibody; IBD, inflammatory bowel syndrome. *PDC* pyruvate dehydrogenase complex, *OGDC* 2-oxoglutarate dehydrogenase complex, *BCOADC* branched-chain 2-oxo acid dehydrogenase complex

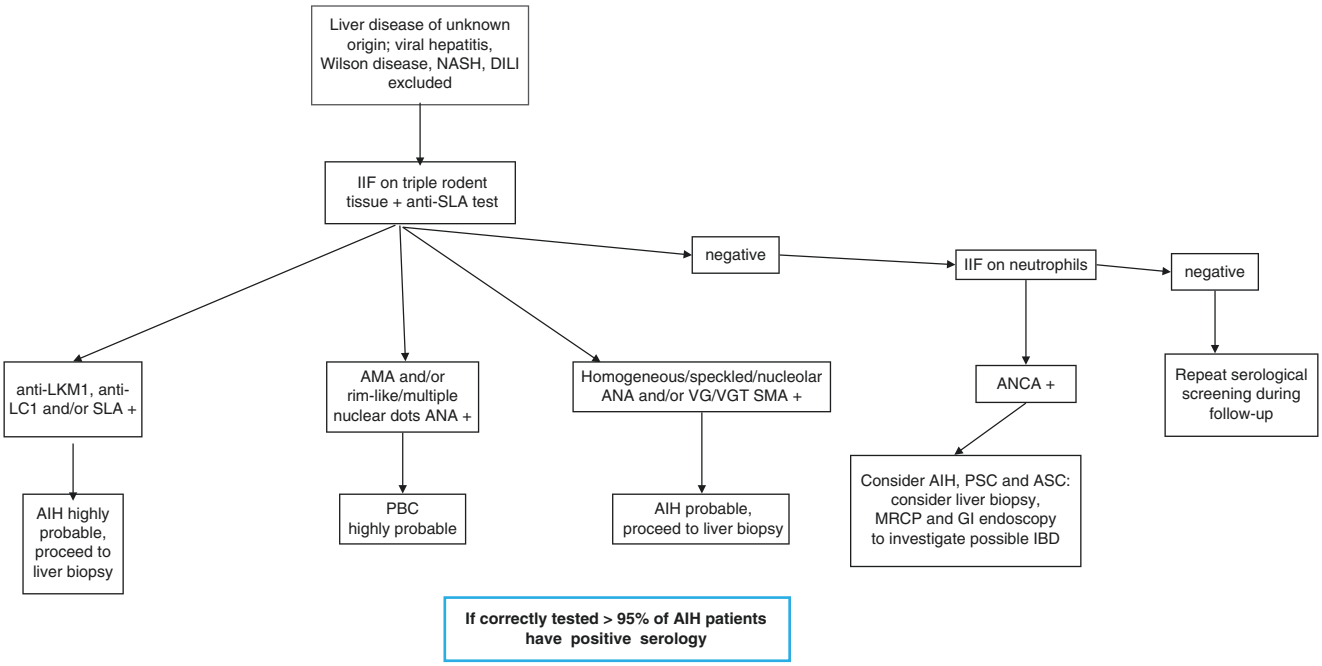

Fig. 4.12 Proposed diagnostic algorithm for the clinical usage of autoimmune liver serology. NASH, non-alcoholic steatohepatitis; DILI, drug-induced liver disease, IIF, indirect immunofluorescence; ANA, anti-nuclear antibody; AMA, anti-mitochondrial antibody, anti-LKM1, anti-liver kidney microsomal antibody type 1; anti-LC1, anti-liver cytosol type 1; SMA, smooth muscle antibody; anti-SLA, anti-soluble liver antigen; ANCA, anti-neutrophil cytoplasmic antibody; PBC, primary biliary cholangitis; AIH, autoimmune hepatitis; PSC, primary sclerosing cholangitis; ASC, autoimmune sclerosing cholangitis; IBD, inflammatory bowel disease; MRCP, magnetic resonance cholangio-pancreatography; GI, gastrointestinal

patient with acute hepatitis points toward a diagnosis of type 1 AIH or ASC, and there should be a low threshold for endoscopic screening for inflammatory bowel disease. Magnetic resonance cholangiography (MRCP) should also be considered in ANCA-positive AIH patients, due to the high frequency of ANCA positivity in sclerosing cholangitis.

In children/adolescents presenting with liver disease of unknown origin, the same serological diagnostic work-up used in adults should be applied, taking into account that autoantibody titers lower than in adults are significant and that PBC is virtually absent in this population. An MRCP is mandatory in every child/adolescent diagnosed with AIH owing to the high prevalence of ASC in the pediatric population [13, 51].

In patients having undergone liver transplant for PBC and type 1 AIH, the serological profiles usually remain unchanged and are not helpful on their own in diagnosing disease recurrence. On the contrary, in type 2 AIH patients, anti-LKM1 usually disappears after transplantation, and its reappearance is associated with disease recurrence. Appearance of autoantibodies after liver transplantation for non-autoimmune liver diseases is not rare, pointing toward a diagnosis of de novo *AIH* if coupled with raised IgG and transaminase levels and the histological picture of interface hepatitis. It is important that the laboratory reports atypical anti-LKM1 if detected, due to its association with de novo *AIH*.

Conclusion and Future Directions

Autoimmune liver serology is an essential clinical tool in the diagnosis and management of autoimmune liver diseases; however, the clinician needs to bear in mind that lack of standardization of the techniques employed may lead to discrepancies between laboratories. Moreover, autoimmune serology results must be interpreted in the clinical context of the individual patient and are not diagnostic on their own.

Efforts are being made to establish observer-independent and standardized assays, as well as to identify new, specific serological markers. In addition, new biomarkers of liver autoimmune diseases are highly needed; therefore, it is essential that the prognostic value of current and novel autoantibodies is thoroughly investigated.

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Genetics of Autoimmune Liver Diseases

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Key Points

- Autoimmune liver diseases (AILDs) are complex traits, which derive from the interaction of unknown environmental factors and genetic susceptibility.
- Linkage analyses are difficult to perform due to the rarity of these conditions.
- Genome-wide, case-control association studies have given a significant contribution to the understanding of their genetic architecture.
- The variants with higher odds ratios are located within the HLA region, but only a small proportion of subjects carry HLA-related variants.
- Non-HLA variants associated with AILDs are mainly related to genes involved in the regulation of immune functions, and their discovery has helped to enlighten novel pathogenic pathways.
- Only a small proportion of the heritability of AILDs has been explained, and this knowledge gap is defined "missing heritability."
- The role of epigenetics, gene–gene epistatic effects, and gene–environment interactions has been poorly characterized in AILDs, but these factors are likely to play a significant role in shaping phenotypes.
- Current strategies aiming to explain a broader proportion of heritability of AILDs should take advantage of novel bioinformatics tools, including machine learning.

Introduction

Autoimmune liver diseases (AILDs) have an important genetic background. This is supported by different lines of evidence, that is, clustering in families [1, 2], high concordance rates in monozygotic twins [3], increased risk of the disease in siblings compared to general population [4], and association with other autoimmune conditions [5–7]. From a genetic perspective, AILDs are considered "complex traits," a definition that refers to "any phenotype that does not exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus" [8].

Classical linkage studies require multiple families and are mostly effective in near-Mendelian complex traits, characterized by few susceptibility loci and high penetrance, while for rare and non-monogenic diseases association studies represent the best method to investigate genetic susceptibility. The theoretical assumption behind association studies is the "common disease/common variant" hypothesis, which postulates that many variants contribute for a little part to the overall susceptibility [9]. Multiple genes are involved, each one increasing or decreasing with a small effect size the risk of developing the disease.

Genome-wide association studies (GWASs) adopt a "hypothesis-free" approach, since there are no a priori hypothesis on the possible variants associated with the phenotype of interest. Strongly relying on large sample sizes, these studies employ a large scanning of the entire genome for specific polymorphisms (typically single nucleotide polymorphisms, SNPs) to identify those statistically associated with the disease [10]. GWASs take advantage of the

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linkage disequilibrium (LD), that is, the non-random association between alleles at two loci. Indeed, thanks to LD, it is not necessary to genotype all the SNPs in a genomic region, but only a bunch of "tag SNPs" through which haplotypes can be built. Nevertheless, this strategy carries also the clear disadvantage that it is not easy to determine which gene/variant in a specific region is causal.

GWASs have contributed hugely in the advancement of the knowledge of AILDs and their genetics [11]. The strongest statistical associations with AILDs and, more in general, with autoimmune diseases, have been mapped to the human leukocyte antigen (HLA) locus. This is a highly polymorphic region of the genome characterized by extreme levels of LD [12], encompassing an extended region of about 6.7 Mb on chromosome 6p21 [12]. In particular, the HLA locus is the most gene-dense region of the human genome, encoding hundreds of expressed loci, including histone and tRNA genes, several key immune response genes, as well as those of the major histocompatibility complex (MHC). The extended MHC region can be subdivided into class I (extended and classical, respectively, containing the *A, B, C* and *MICA* and *MICB* loci), class II (extended and classical, respectively, containing the *DPA1/DPB1* and *DQA1/DQB1* loci), and class III (containing the *DRA1/DRB1* loci) [12]. Most GWAS studies on autoimmune diseases focused on the role of genes coding for the HLA-DR and HLA-DQ molecules, that is, those able to present exogenous antigens for the recognition by CD4+ T helper cells.

Apart from the HLA region, a significant number of non-HLA genes have been identified, and many of the alleles included genes involved in innate immunity, suggesting novel perspectives on the role of this arm of the immune system in the immunogenetics of these conditions. In addition, likewise to other complex traits, most of the gene signatures identified in GWASs lie in non-coding regions of the genome, meaning that regulatory loci play a role and the network of interactions is multifaceted [11].

In this chapter, we will summarize current knowledge regarding each of the three most common AILDs (autoimmune hepatitis, AIH; primary biliary cholangitis, PBC; and primary sclerosing cholangitis, PSC); we will then propose a series of possible future lines of investigation to explore the amount of heritability which is still to be determined.

Genetics of Autoimmune Hepatitis (AIH)

Among AILDs, AIH is somewhat the prototypical autoimmune disease, being characterized by increased prevalence in women, clustering with other non-liver autoimmune diseases (Table 5.1), presence of specific autoantibodies, and response to immunosuppressive treatments. For type 2 AIH, the autoantigen implicated in the pathogenesis has been recognized (i.e., Cytochrome P450 2D6 (CYP2D6), the main target of autoantibodies against liver and kidney microsomal antigens (anti-LKM)), while in type 1 AIH research is still ongoing [13].

Table 5.1 Frequencies of autoimmune comorbidities in patients with AIH, PBC, and PSC

AIH		PBC		PSC	
disease	%	disease	%	disease	%
Any	40	Any	53	Any	70
AITD	10	Sjogren syndrome	25	IBD	70
Vitiligo	1.8	Raynaud	24	T1DM	10.1
Sjogren syndrome	1.4	Rheumatoid arthritis	17	Psoriasis	4.2
Ulcerative colitis	1.4	Scleroderma	8	Sarcoidosis	4.1
Conjunctivitis	1.4	Pernicious anemia	4	Rheumatoid arthritis	3.4
Coeliac disease	1.1	SLE	1	Nephritis	1.7
SLE	0.7	AITP	1	Vitiligo	1.7
T1DM	0.7			SLE	1.7
Multiple sclerosis	0.7			Coeliac disease	1.7

Abbreviations *AIH* autoimmune hepatitis, *PBC* primary biliary cholangitis, *PSC* primary sclerosing cholangitis, *AITD* autoimmune thyroid disease, *IBD* inflammatory bowel disease, *SLE* systemic lupus erythematosus, *AITP* Autoimmune thrombocytopenia, *T1DM* type 1 diabetes mellitus
Adapted from Mells et al. [134]

Genetic predisposition is confirmed by increased prevalence in family members, despite a very low risk and low concordance in monozygotic twins [2]. This supports probably the concept of a polygenic disease with low prevalence.

In genetic complex traits with autoimmune pathogenesis, HLA variants have typically been found as the strongest predisposing signals, and AIH makes no exception. A few non-HLA signals have also shown suggestive associations that deserve mention, in particular the *SH2B2* gene, which has been recognized as potential predisposing gene throughout all the three AILDs.

HLA Variants

First studies identifying a significant association between HLA variants and type 1 AIH date back to the 1970s. These pre-GWAS studies in North American and European patients documented *A1-B8-DR3* as risk haplotype, *DRB1*0401* as risk allele in DR3-negative patients, and *DRB1*1501-DQB1*0602* as protective haplotype [14–18]. Later on, several other alleles were identified in Japan (*DRB1*0405*) [19], Mexico (*DRB1*0404*) [20], and Argentina (*DRB1*0405*) [21].

In 2014, a multicenter GWAS from a mixed cohort of Dutch, German, and Swiss individuals confirmed the relevance of the HLA complex as genetic risk for AIH and identified novel potential SNPs at a suggestive threshold [22]. To

date, this GWAS is the only case-control, genetic association study performed in AIH.

Identified HLA variants, *DRB1*03:01* (primary susceptibility genotype) and *DRB1*04:01* (secondary susceptibility genotype), were in line with previous reports on Caucasians from pre-GWAS era. In this study, authors confirmed also the single amino-acid variant at position 71 of *DRβ*.

The associations between HLA variants and AIH, as well as HLA variants and most frequent extrahepatic autoimmune diseases shared also with AIH are summarized in Tables 5.2 and 5.3, respectively.

Non-HLA Variants

Among non-HLA loci, *SH2B3* and *CARD10* did not reach genome-wide significance, but the rs3184504 variant of the *SH2B3* gene still deserves attention, having being associated also with the risk of developing PBC [23], PSC [24], as well as other autoimmune diseases [25]. More recently, a study on 952 Japanese subjects found that the rs3184504 variant was non-polymorphic and hence excluded from further analyses; nonetheless, another variant mapping in the *SH2B3* gene (rs11065904) was significantly reduced in AIH patients compared to healthy controls [26]. Another study assessing frequencies of *SH2B3* and other gene variants in Tunisia confirmed the association in a small but probably neglected sample of cases from North Africa [27].

From a functional perspective, *SH2B3* is interesting in that it is highly expressed in monocytes and dendritic cells; it modulates hematopoiesis, cytokine signaling, inflammatory cascades, and controls different downstream pathways regulated by Janus and tyrosine kinases (e.g., JAK2) [28]. *SH2B3* codes for a protein that is involved in inhibition of T lymphocyte activation [28]. *Sh2b3−/−* mice have augmented reactivity to several cytokines (IL-15, GM-CSF); interestingly, *Sh2b3−/−* dendritic cells showed increased capacity to promote the differentiation of IFN-γ-producing Th1 cells from naive $CD4^+$ T cells, thanks to an elevated expression of *Il-12Rβ1* and increased production of INF-γ. There is preliminary evidence to support the concept that *SH2B3* may influence increased immune reactivity in peripheral lymphoid tissues [29].

In type 1 AIH, HLA alleles have also been used to improve risk stratification; indeed, if compared to patients carrying *DRB1*0401*, those with *B8* and *DRB1*0301* have a more severe phenotype of the disease, with higher liver enzymes and more prevalence of cirrhosis, translating also in worse treatment outcomes [15, 18]. In addition, subjects with *DRB1*0301* are more frequently positive for antibodies to soluble liver antigen/liver-pancreas (anti-SLA/LP), which have been associated with a more aggressive disease course [30].

Table 5.2 HLA haplotype associations with AIH

Type of AIH	HLA allele	Effect
Type 1	*A1-B8-DRB3*01:01-DRB1*03:01-DQA1*05:01-DQB1*02:01*	Susceptibility
	*DR4 (DRB1*04:01*, Europe; *DRB1*04:04*; *DRB1*04:05*, China, Japan, Mexico)	
	*DRB1*13:01-DQB1*06* (Latin America)	
	*DRB1*14* (India)	
	*DRB5*01:01-DRB1*15:01* (UK)	Protective
	*DQB1*04 DQB1*03:01* (Latin America)	
	*DQB1*04*	
Type 2	*DRB1*03:01-DQB1*02*	Susceptibility
	*DRB1*07-DQB1*02*	
	*DQB1*06*	

Adapted from Mells et al. [134]

Table 5.3 Extrahepatic autoimmune conditions associated with and HLA associations shared with AIH

Autoimmune conditions	HLA associations
Hashimoto's thyroiditis	*DR3, DR4*
Graves' disease	*DR3*
Type 1 diabetes mellitus	*DR3, DR4*
Addison's disease	*DR3*
Celiac disease and dermatitis herpetiformis	*DR3, DR7*
Localized lupus erythematosus	*DR3, DR4*
Pemphigus and pemphigoid	*DR4*
Myasthenia gravis	*DR3*
Rheumatoid arthritis	*DR4*
Polymyositis/dermatomyositis	*DR3*
Systemic lupus erythematosus	*DR3, DR4, DR7*
Sjögren's syndrome	*DR3*
Polymyalgia rheumatica and temporal arteritis	*DR4*
Vitiligo	*DR3, DR4, DR7*

Abbreviations: *AIH* autoimmune hepatitis, *HLA* human leukocyte antigen
Adapted from Grønbæk et al. [6]

Genetics of Primary Biliary Cholangitis (PBC)

The genetic component in the risk to PBC is supported by the family clustering of the disease [1], high sibling risk recurrence ratio [31], and high disease concordance in monozygotic twins [3]. Patients with PBC are typically affected by at least another autoimmune condition [5], which suggests a possible shared landscape of risk variants [32].

To date, GWASs completed in PBC have included cases and controls from Caucasians (Canada, Italy, the United Kingdom, and the United States) and East Asians (China and Japan) [33–38]. Many variants have been identified, with two fine-mapping studies [39, 40] and one genome-wide meta-analysis [41] having further expanded the list. Variants within the HLA region have been found as the strongest predisposing signals also in PBC, but several non-HLA signals

have been identified and replicated, mostly referring to genes directly or indirectly involved in immune regulation.

HLA Variants

Before the advent of the GWAS era, a few pioneer studies associated HLA loci *DRB1*08* with an increased risk of PBC, and *DRB1*11* and *B1*13* with a protective effect [42, 43]. A more recent meta-analysis from Li et al. [44] identified HLA *DR*07* and *08* as risk factors and *DR*11*, *12*, *13,* and *15* as protective. It is of note that the HLA signals *DR*08*, *DRB1*11*, and *13* have been confirmed across different ethnicities, except in Sardinians [45] (a summary of HLA findings is presented in Table 5.4). Unfortunately, the strong LD in this complex genomic region makes challenging the definition of causality for identified variants.

The association between HLA variants and PBC susceptibility is the strongest, but the effect size is small, and the frequency of the risk alleles in the population of patients is low (less than 15%); therefore, only few subjects among the total of PBC patients carry the "predisposing" haplotypes, meaning that environment and other non-HLA variants are likely to be relevant [46].

Non-HLA Variants

In the pre-GWAS era, some candidate gene studies have identified genes involved in autoimmunity, but, probably due to power issues of these studies, the signals that emerged were not replicated in GWASs. At present, GWASs have identified 44 non-HLA PBC predisposition loci at a genome-wide level of significance [47–52] (Table 5.5).

GWASs identified several non-HLA genes that are related to different immune pathways. Among the others, the IL-12 signaling pathway showed a strong signal and has a biological plausibility (Fig. 5.1). Indeed, IL-12 is composed of two subunits, IL-12 p35 and IL 12 p40. IL-12 p35 is encoded by the *IL12A* gene, while IL12 p40 is encoded by the *IL12B* gene; variants at both *IL12A* and *IL12B* loci have been associated with PBC in several GWASs. The IL-12 receptor, constituted by two chains (β1 and β2), is expressed on the cell membrane of CD4+ T cells and its interaction with IL-12 provokes activation of the Th1 response through Jak-STAT signaling. It is of note that variants at *TYK2* [40] and *STAT4* loci, which are parts of the Jak-STAT signaling, have also been found associated with PBC susceptibility. TYK2 belongs to the Janus kinase family and phosphorylates STAT transcription factors; heterozygotes for the variant at *TYK2* locus have reduced TYK2 activity [53]. Interaction between IL-12 and its receptor determines also interferon-γ production, causing the inhibition of Th17 cells through IL-23, which is also part of the IL-12 superfamily. IL-12 p35 and IL-12 receptor β2 are also part of IL-35, another member of IL-12 superfamily favoring proliferation of regulatory T cells [46].

Other relevant signals identified are interferon regulatory factor 5 (*IRF5*), transportin 3 (*TNPO3*), transcription factor Spi-B (*SPIB*), tumor necrosis factor superfamily, member

Table 5.4 HLA haplotype associations with PBC

Study	Year	Population	Cases	Controls	HLA allele	Effect	OR (95% CI)	*p*-value
Donaldson et al.	2006	UK Italian	412 80	236 95	*DRB1*08:01-DQA1*04*01-DQB1*04:02*	S	2.90 (1.45–5.82)	0.0027
Umemura et al.	2012	Japanese	229	523	*DRB1*08:03-DQB1*06:01* *DRB1*04:05-DQB1*04:01*	S	2.22 (1.53–3.20) 1.38 (10.2–1.87)	0.000025 0.044
Zhao et al.	2014	Chinese	145	500	*DRB1*08:03-DQB1*06:01* *DRB1*07:01-DQB1*02:02*	S	3.17 (1.91–5.23) 1.85 (1.20–2.83)	<0.0001 0.005
Clemente et al.	2017	Sardinian	20	89	*DRB1*03:01-DQB1*02:01*	S	3.02 (1.51–6.05)	<0.05
Yasunami et al.	2017	Japanese	1199	1193	*DRB1*08:03-DQB1*06:01* *DRB1*14:05-DQB1*04:01*	S	1.77 (1.43–2.18) 1.49 (1.24–1.79)	7.82 × 10–8 1.44 × 10–5
Donaldson et al.	2006	Italian	21	46	*DRB1*11:01-DQA1*05:01-DQB1*03:01*	P	0.47 (0.27–0.83)	0.086
			3	12	*DRB1*13:01-DQA1*01:03-DQB1*06:03*	P	0.28 (0.08–0.95)	0.0041
Umemura et al.	2012	Japanese	220	523	*DRB1*13:02-DQB1*06:04* *DRB1*11:01-DQB1*03:01*	P	0.27 (0.12–0.60) 0.37 (0.15–0.88)	0.00093 0.03
Zhao et al.	2014	Chinese	145	500	*DRB1*12:02-DQB1*03:01*	P	0.43 (0.22–0.86)	0.015
Yasunami et al.	2017	Japanese	1199	1193	*DRB1*13:02-DQB1*06:04* *DRB1*14:03-DQB1*03:01* *not (DRB1*14:03)-DQB1*03:01*	P	0.19 (0.13–0.27) 0.21 (0.09–0.49) 0.54 (0.43–0.68	1.84 × 10–22 5.1 × 10–5 8.54 × 10–8

Abbreviations *S* susceptibility, *P* protective
Adapted from Joshita et al. [135]

Table 5.5 Non-HLA risk loci associations with PBC

Chr	Locus	Study	SNP	OR	p-value	Candidate gene(s)	Disease(s) with shared risk loci
1	1p13	Qiu et al. [38]	rs2300747	1.30	4.54E−08	*CD58*	MS, neuromyelitis
	1p31	Liu et al. [40]	rs72678531	1.51	2.47E-38	*IL12RB2*	BD,MS
	1p36	Hirschfield et al. [34]	rs3748816	1.33	3.15E-08	*MMEL1*	MS
	1q31	Mells et al. [36]	rs12134279	1.34	2.06E-14	*DENND1B*	CD
2	2q12	Cordell et al. [41]	rs12712133	1.14	5.19E-09	*IL1RL1, IL1RL2*	
	2q32	Mells et al. [36]	rs10931468	1.50	2.35E-19	*STAT4,STAT1*	RA,SLE,SS,IBD,SSc,BD
	2q32	Qiu et al. [38]	rs10168266	1.31	5.82E-08	*STAT4,STAT1*	RA,SLE,SS,IBD,SSc,BD
	2q33	Qiu et al. [38]	rs4675369	1.37	6.56E-11	*CD28,CRLA4, ICOS*	
	2q33	Qiu et al. [38]	rs7599230	1.37	6.56E-11	*CD28,CRLA4, ICOS*	
	2q36	Cordell et al. [41]	rs4973341	1.22	2.34E-10	*CCL20*	
3	3p24	Mells et al. [36]	rs1372072	1.20	2.28E-08	*PLCL2*	RA
	3q13	Liu et al. [40]	rs2293370	1.39	6.84E-16	*CD80*	MS,SLE,CeD
	3q25	Liu et al. [40]	rs2366643	1.35	3.92E-22	*IL12A*	CeD
4	4p16	Cordell et al. [41]	rs11724804	1.22	9.01E-12	*DGKQ*	
	4q24	Mells et al. [36]	rs7665090	1.26	8.48E-14	*NFkB1*	UC
5	5p13	Liu et al. [40]	rs6871748	1.30	2.26E-13	*IL7R*	MS,UC
	5p13	Kawashima et al. [136]	rs6897932	1.52	5.72E-09	*IL7R*	MS,UC
	5q21	Cordell et al. [41]	rs526231	1.15	1.14E-08	*C5orf30*	
	5q33	Cordell et al. [41]	rs2546890	1.15	1.06E-10	*IL12B,LOC285626*	Psoriasis
6	6q23	Cordell et al. [41]	rs6933404	1.18	1.27E-10	*OLIG3, TNFAIP3*	RA
7	7p14	Mells et al. [36]	rs6974491	1.25	4.44E-08	*ELMO1*	RA,CeD
	7q32	Liu et al. [40]	rs35188261	1.52	6.52E-22	*IRF5*	RA,SLE,SSc,UC
9	9p32	Nakamura et al. [37] Kawashima et al. [136]	rs4979462	1.57	185E-14	*TNFSF15*	UC,CD
	9p32	Qiu et al. [38]	rs4979467	1.40	8.28E-12	*TNFSF15, TNFSF8*	UC,CD
11	11q13	Mells et al. [36]	rs538147	1.23	2.06E-10	*RPS6KA4*	IBD
	11q23	Nakamura et al. [37] Kawashima et al. [136]	rs4938534	1.38	3.27E-08	*POU2AF1*	
	11q23	Qiu et al. [38]	rs77871618	1.55	9.12E-14	*CXCR5,DDX6*	RA,IBD,CeD
	11q23	Liu et al. [40]	rs80065107	1.39	7.20E-16	*CXCR5,DDX6*	RA,IBD,CeD
	11q24	Kawashima et al. [136]	rs12574073	1.33	1.09E-07	*ETS1*	AS
12	12p13	Liu et al. [40]	rs1800693	1.27	1.18E-14	*TNFRSF1A, LTBR*	MS
	12q24	Liu et al. [40]	rs11065979	1.20	2.87E-09	*SH2B3*	RA,T1D, hyperthyroidism, celiac
13	13q14	Juran et al. [23] Liu et al. [40]	rs3862738	1.33	2.18E-08	*TNFSF11*	CD
14	14q24	Liu et al. [40]	rs911263	1.26	9.95E-11	*RAD51B*	RA
	14q32	Mells et al. [36]	rs8017161	1.22	2.61E-13	*TNFAIP2*	
16	16p12	Kawashima et al. [136]	rs3785396	1.35	7.10E-08	*PRKCB*	
	16p13	Liu et al. [40]	rs12708715	1.29	2.19E-13	*CLEC16yA, SOCS1*	MS,UC,TD1M
	16q24	Mells et al. [36]	rs11117432	1.31	4.66E-11	*IRF8*	MS,IBD,RA,SSc
17	17q12	Liu et al. [40]	rs 17564829	1.26	6.05E-14	*IKZF3*	UC,CD,RA,T1D
	17q12	Kawashima et al. [136]	rs 9303277	1.43	8.37E-11	*IKZF3*	UC,CD,RA,T1D
	17q12	Qiu et al. [38]	rs 9635726	1.38	6.42E-11	Multiple genes	
	17q21	Liu et al. [40]	rs 17564829	1.25	2.15E-09	*MAPT*	
19	19p12	Liu et al. [40]	rs 34536443	1.91	1.23E-12	*TYK2*	IBD,RA,SLE,psoriasis,T1D
	19q13	Liu et al. [35]	rs 3745516	1.46	7.97E-11	*SPIB*	
22	22q13	Liu et al. [40]	rs 2267407	1.29	1.29E-13	*SYNGR1*	

Abbreviations: *AS* ankylosing spondylitis, *BD* Behçet's disease, *CeD* coeliac disease, *CD* Crohn's disease, *Chr* chromosome, *IBD* inflammatory bowel disease, *RA* rheumatoid arthritis, *SLE* systemic lupus erythematosus, *SS* Sjögren's syndrome, *SSc* systemic sclerosis, *UC* ulcerative colitis, *T1D* type 1 diabetes mellitus

Adapted from Joshita et al. [135]

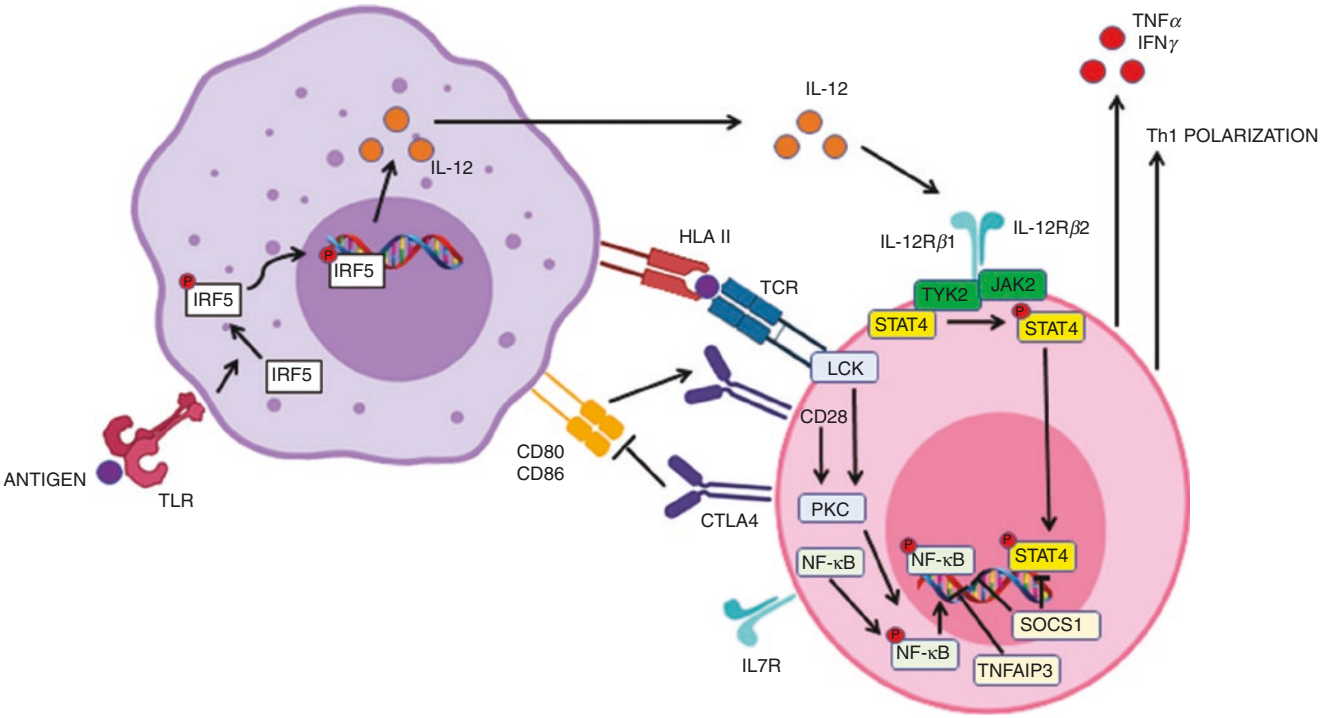

Fig. 5.1 IL-12 pathway in PBC. IL-12 pathway has been involved in PBC by GWASs. Antigen activates APC via TLR. Following phosphorylation of IRF5, APC produces IL-12. Antigen is presented to $CD4^+$ T cells by HLA II with co-stimulation through CD80 and CD86 to CD28. There is competitive inhibition of this co-stimulation by CTLA4. IL-12 activates a flow of signaling factors including NF-kB and STAT4 to stimulate the production of Th1-type cytokines including TNF-α and IFN-γ; the transcription factor IRF8 is implicated. IL7R backs lymphocyte development. Th1 cytokines exert a positive feedback to APCs. Arrows mean positive effects; barred lines mean negative effects. APC antigen-presenting cell, CD cluster of differentiation, CTLA4 cytotoxic T lymphocyte antigen 4, HLA II human leucocyte antigen class II, IFN-γ interferon-g, IFNgR interferon-g receptor, IL-12 interleukin-12, IL-12Rβ1/2 IL-12 receptor b subunits 1 and 2, IL7R interleukin-7 receptor, IRF5 and IRF8 interferon response factors 5 and 8, JAK2 Janus kinase 2, Lck lymphocyte-specific protein tyrosine kinase, NF-kB nuclear factor kappa-light-chain-enhancer of activated B cells, PKC protein kinase C, SOCS1 suppressor of cytokine signaling 1, STAT4 signal transducer and activator of transcription 4, TCR T-cell receptor, TLR Toll-like receptor, TNFAIP3 tumor necrosis factor alpha-induced protein 3, TNFRSF1a tumor necrosis factor receptor superfamily 1a, TNFα tumor necrosis factor alpha, TYK2 tyrosine kinase 2

15 (*TNFSF15*), and POU domain class 2-associating factor 1 (*POU2AF1*). *IRF5* codes for interferon regulatory factor 5 and interacts with NF-kB to modulate expression of Th1 cytokines. *IRF8* codes for a transcription factor that, interacting with the *IL-12* promoter, modulates IL-12 and IFN-γ production.

It is of note that *IL-12* loci were not replicated in the Japanese cohorts. Th1 and Th17 pathways were involved though, since TNFSF15 can interact with IL-12 and IL-18 to favor IFN-γ production. The biological role of IL-12 is further supported both by animal models [54] and by the presence of PBC-specific autoantibodies in patients with deficiency of IL-12 receptor [55]. Unfortunately, a trial assessing the efficacy and safety of an anti-IL-12 molecule (Ustekinumab) failed to show a benefit in patients with PBC [56]. Yet, as the autoimmune attack on cholangiocytes is an early event in the pathogenesis of PBC, and considering that patients included in the trial had already cholestasis, it could be speculated that the use of Ustekinumab could have been futile at this stage.

Finally, a risk locus near the *TNFSF11* gene at 13q14 with potential translational interest has been identified through a meta-analytic approach. This gene encodes for the RANKL protein, which regulates bone metabolism but has also been involved in immune-regulation. RANKL proteins regulate T-regulatory cells development, and blood levels of RANKL protein are reduced in patients with PBC. This region has been also associated to Chron's disease and may be implicated in the reduced bone density, which is frequently encountered in PBC [39].

Genetics of Primary Sclerosing Cholangitis (PSC)

PSC shows a marked heterogeneity in its phenotype, raising doubts whether different phenotypes of the disease might represent distinct conditions. Nonetheless, male prevalence and clear association with inflammatory bowel disease (IBD) (despite at a variable percentage in different popula-

tions) are constant elements. PSC, like AIH and PBC, is a complex trait and its genetic architecture is similar to other autoimmune diseases [57]. While different clinical aspects may go against the concept that PSC is an autoimmune condition (first of all the lack of efficacy of immunosuppressive drugs), the merit of GWAS studies has been to clearly back the notion that PSC is an immune-mediated condition, since most of the GWAS signals were found within the HLA region. There is a high degree of overlap between non-HLA genetic variants identified in PSC and other autoimmune diseases [32]. Interestingly, overlapping genes often do not match clinical classifications, that is, several genes can overlap between conditions that are not overlapping on the clinical side. Conversely, genetic analyses have clearly separated PSC from IBD, since only around 5% of risk variant in ulcerative colitis and Chron's disease have been confirmed in PSC [58]; this finding is in line with the clinical phenotype of colitis in PSC, which does show distinct and peculiar features [7]. Within the group of AILDs, the overlap between AIH and PSC in terms of non-HLA variants is small and limited to the non-HLA suggestive variants identified in the only GWAS study performed in AIH up to now [22]. Similarly, the genetic overlap between PBC and PSC is limited to few non-HLA variants [24], while the clinical overlap is exceptionally rare.

HLA Variants

PSC makes no exception in showing the strongest signals within the chromosome 6, HLA region. Ancestral Haplotype 8.1 (*AH8.1*), carrying the *HLA-B*08* and *DRB1*03:01* alleles, and the *DRB3*01:01-DRB1*13:01-DQA1*01:03-DQB1*06:03* haplotype are the most relevant haplotypes associated with increased risk of PSC [59–61]. The possibility to disentangle which variant is the causal among all the recognized ones is limited by LD; LD also prevents identification of the possible contribution of non-HLA genes within this region. However, in admixed African-American populations, LD between previously cited *HLA-B* and *DRB1* alleles is low and only *HLA-B*08* is associated with PSC [62]. This might possibly suggest the role of *HLA-B*08* in the *AH8.1* association with PSC. While this is suggestive of the role of MHC class I genes, there is also evidence of class II associations with protective effect [61]. Like for AIH and PBC, trigger peptides have not been identified yet. A chart of identified HLA variants in PSC is shown in Table 5.6.

Table 5.6 HLA haplotype associations with PSC

HLA allele	Effect
*B*08:01*	Susceptibility
*DRB1*03:01-DQA1*05:01-DQB1*02:01*	
*DRB1*13:01-DQA1*01:03-DQB1*06:03*	
*DRB1*15:01-DQA1*01:02-DQB1*06:02*	
*DRB1*01:01-DQA1*01:01*	
*DRB4*01:03-DRB1*04:01-DQA1*03-DQB1*03:02*	Protective
*DRB4*01:03-DRB1*07:01-DQA1*02:01-DQB1*03:03*	
*DRB4*02:02-DRB1*11:01-DQA1*05:01-DQB1*03:01*	
*MICA*002*	

Adapted from Mells et al. [134].

Non-HLA Variants

GWASs have identified 22 genome-wide association outside the HLA region and additional nine loci have been found associated at a suggestive threshold (Table 5.7). These latter genes deserve attention due to the rarity of PSC, since many of them have been reported in other autoimmune conditions and it cannot be excluded that lack of power in GWAS studies may have played a role.

The key to understand pathogenesis of PSC might rely within the cross-talk existing between cholangiocytes and immune cells (including both innate and adaptive arms) to promote stimulation of stellate cells and portal fibroblasts and fibrosis deposition [7] (Fig. 5.2).

Macrophage stimulation 1 (*MST1*) and hematopoietically expressed homeobox (*HHEX*) are two risk genes for PSC highly expressed in the liver. *MST1* controls cell proliferation, and variants of *MST1* have been associated with sporadic cholangiocarcinoma [63]. *HHEX* is a pleiotropic transcription factor; intriguingly, deletion of *HHEX* during mouse embryonic life causes severe abnormalities in intrahepatic bile ducts [64]. Unfortunately, specific data about the functional role of these genes in PSC are lacking.

Regarding innate immune pathways, LPS-TLR4-NF-kB axis is of paramount importance in biliary inflammation in animal models of cystic fibrosis [64]. Risk genes identified in PSC that might be related to LPS are Peroxiredoxin 5 (*PRDX5*), G-protein-coupled bile acid receptor, *GPBAR1* (also noticed as *TGR5*), and Proteasome Assembly Chaperone 1 (*PSMG1*). *PRDX5* exerts a protective role against oxidative stress in LPS-induced macrophages. In *Mdr2−/−* mice, there is higher periportal expression of *Prdx5* together with other antioxidant proteins compared to wild-type mice; cholestasis might induce increases in periportal oxidative stress responses through cell-specific upregulation of some antioxidant proteins, and *Prdx5* seems to play a pivotal role [65]. Lack of *Tgr5* in mice renders the bile epithelium much more susceptible to cholestatic damage [65]. *PSMG1* is a protein coding gene, associated with Down syndrome, which promotes assembly of the 20S proteasome as part of a heterodimer with PSMG2. In patients with IBD, mutations close to *PSMG1* might alter the formation of proteasome, impairing NF-kB signaling from bacterial LPS [66]. Variants

Table 5.7 Non-HLA risk loci associations with PSC

Chr	Locus	Study	SNP	OR	p-value	Candidate gene	Disease(s) with shared risk loci
1	1p36	Folseraas et al. [52]	rs3748816	0.79	2.1E-08	*MMEL1, TNFRS14*	CeD, MS, RA, UC
2	2q13	Melum et al. [49]	rs6720394	1.29	4.1E-08	*BCL2L11*	None
2	2q33	Liu et al. [24]	rs7426056	1.30	1.9E−20	*CD28, CTLA4*	AA, CeD, GV, MG, RA, T1D
2	2q36	Ellinghaus et al. [50]	rs7556897	0.85	4.7E-09	*CCL20*	PBC, IBD, UC
2	2q37	Ellinghaus et al. [51]	rs3749171	1.39	3.0E-09	*GPR35*	IBD, UC
3	3p13	Ji et al. (2017)	rs80060485	1.44	2.6E-15	*FOXP1*	None
3	3p21	Melum et al. [49]	rs3197999	1.39	1.1E-16	*MST1*	IBD,CD,UC
4	4q24	Ellinghaus et al. [50]	rs3774937	1.17	6.1E-09	*NFkB1*	UC,PBC
4	4q27	Liu et al. [24]	rs13140464	1.30	8.9E-13	*IL2, IL21*	AA, IBD, CeD,T1D
6	6q15	Liu et al. [24]	rs56258221	1.23	8.4E-12	*BACH2*	CeD, IBD, CD, T1D,VT
10	10p15	Srivastava et al. [48]	rs4147359	1.20	1.5E-08	*IL2RA*	AA,IBD, CD,MS,RA,T1D,VT
11	11q13	Ji et al. [47]	rs663743	1.20	2.2E-13	*CCDC88B*	IBD, CD, PBC, SARC
11	11q23	Liu et al. [24]	rs7937682	1.17	3.2E-09	*SIK2*	None
12	12q13	Liu et al. [24]	rs11168249	1.15	5.5E-09	*HDAC7*	IBD
12	12q23	Ellinghaus et al. [50]	rs12369214	0.85	1.3E-09	*RFX4,RIC8B*	None
12	12q24	Liu et al. [24]	rs3184504	1.18	5.9E-11	*SH2B3, ATXN2*	CeD,HT, IBD, RA, SLE, T1D, VT
16	16q12	Ellinghaus et al. [50]	rs11649613	1.19	1.3E-11	*CLEC16A, SOCS1*	CeD, IBD, CD, MS, PBC, SLE, T1D
18	18q21	Ellinghaus et al. [51]	rs1452787	0.75	2.6E-08	*TCF4*	None
18	18q22	Liu et al. [24]	rs1788097	1.15	3.1E-08	*CD226*	IBD, RA, T1D
19	19q13	Liu et al. [24]	rs60652743	1.25	6.5E-10	*PRKD2, STRN4*	T1D
21	21q22	Liu et al. [24]	rs2836883	1.28	3.2E-17	*PSMG1*	AS, IBC, UC
21	21q22	Ji et al. [47]	rs1893592	1.22	2.2E-12	*UBASH3A*	RA, T1D, VT

Abbreviations: *AA* alopecia areata, *AID* autoimmune diseases, *AS* ankylosing spondylitis, *MS* multiple sclerosis, *CD* Crohn's disease, *CeD* coeliac disease, *Chr* chromosome, *GV* Graves' disease, *HT* hypothyroidism, *IBD* inflammatory bowel disease, *MG* myasthenia gravis, *MS* multiple sclerosis, *OR* odds ratio, *RA* rheumatoid arthritis, *SARC* sarcoidosis, *SLE* systemic lupus erythematosus, *SSc* systemic sclerosis, *UC* ulcerative colitis, *T1D* type 1 diabetes mellitus, *VT* vitiligo

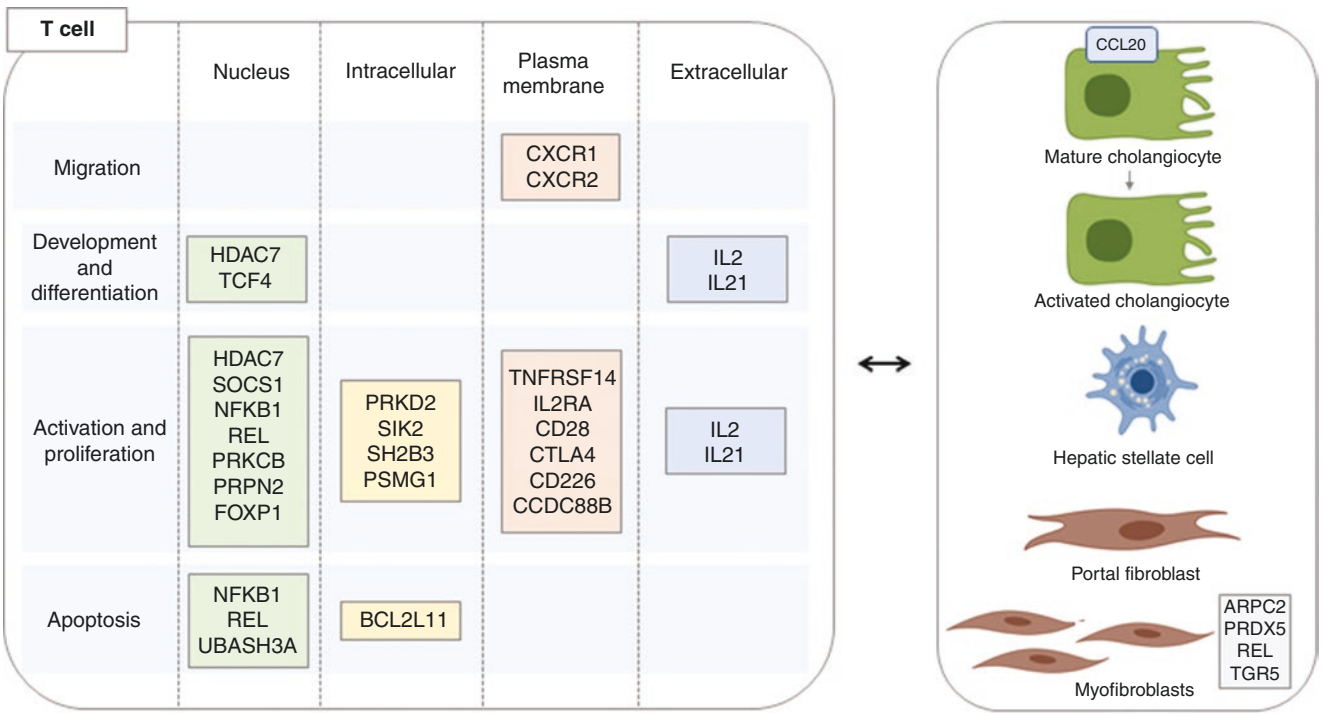

Fig. 5.2 Cross-talk among immune cells, stromal cells, and cholangiocytes. The integration of genetic data and disease models in primary sclerosing cholangitis (PSC) supports the concept that the interplay among T lymphocytes, cholangiocytes, and stromal cells is key to disease onset. In the above figure, candidate susceptibility genes in PSC are located according to these three cell types. The left rectangle shows the T-cell localization. The right rectangle shows the possible interactions between cholangiocytes, portal fibroblasts, and hepatic stellate cells in the development of typical fibrotic lesions of PSC. Cellular expression and localization are based on the literature

at *NFkB1* and *c-REL*, genes part of the NF-kB family, have been reported as susceptibility genes in PSC. Both are pleiotropic transcription factors involved in myriads of cellular functions. Little is known of specific abnormalities in this pathway in PSC.

GWASs found relevant associations between genes involved in adaptive immune pathways and PSC. One significant pathway relates to the biological cascade involving IL-2. IL2RA is one of the markers of regulatory T cells. Interestingly, inflammation in the liver and colon can be spontaneously found in *IL2RA*-deficient mice [54]. In patients with PSC, homozygosity for the rs10905718 SNP in the *IL2RA* gene predisposes to a reduced number of peripheral blood Treg [67].

Another important signaling pathway in inflammation and immunity involves tumor necrosis factor (TNF). The rs3748816 polymorphism tags a gene region where tumor necrosis factor receptor superfamily member 14 (*TNFRSF14*) is located. This receptor is involved in adaptive immunity (T-cell inhibition) [68] and fibrosis [69]. No experimental data potentially related to PSC are available yet.

Among genes involved in cytokine production, *SH2B3* is probably the most attractive, having been found in all three AILDs. Its function and possible translational speculations have been previously outlined in the AIH paragraph.

There is evidence that gut-primed lymphocytes contribute to liver and biliary inflammation in PSC [70]. The variant rs11676348 showed association with PSC below genome-wide significance and maps to a gene region including *CXCR1* and *CXCR2* genes, which are IL-8 receptors. IL-8 serum concentrations are higher in chronic liver diseases, especially cholestatic ones [71].

Limitations of GWASs and Future Perspectives

The Problem of Missing Heritability

GWASs promised to unravel the complexity of the genetics of multifactorial diseases but, despite several important merits, failed to explain a great portion of genetic heritability. The concept of "missing heritability" has been introduced to summarize the gap between the numerous variants that have been identified and the small effect size that these variants do carry, even when considered altogether and when their additive effects are taken into account [72]. The heritability ratio ($\pi_{explained}$) is the ratio between the heritability due to observed variants h^2_{known}, derived from SNPs found significant in GWAS studies, and the total heritability, h^2_{all}, determined from concordance studies on homozygous siblings. This ratio is now estimated to be around 20–30% [73].

Complexity is even more pronounced in diseases with rare prevalence [74], and research in this field has typically focused on rare monogenic diseases rather than rare multifactorial ones. Odd ratios for each variant identified in GWASs are typically lower than 1.5 and it has been estimated that <10% of the heritable risk of AILDs is explained by these variants [75, 76].

Several possible explanations of the missing heritability have been proposed [72, 77]. Earlier GWASs were unable to capture all the genetic variants involved in disease susceptibility (especially those with small effect size), and the increase of sample size in more recent studies has increased the number of discovered hits. Rare variants with high penetrance and mild deleterious effect could also be involved and typically underappreciated by classical study designs and statistical methods. Moreover, structural and copy-number variants have been ignored by first studies due to technical issues of the arrays, and more recent data including recurrent copy-number variants improved prediction.

While most of the efforts have been put toward finding new causal variants, it is possible that flaws in the estimates of the total heritability also do matter. Indeed, current estimators of total heritability h^2_{all} are not consistent, and this might determine that, even in presence of all explanatory variants, the proportion of explained heritability $\pi_{explained}$ would still not be 100%. Some authors called this the issue of "phantom heritability" [78]. Indeed, estimators of total heritability do not consider the potential gene–gene interaction among loci. When considered, the proportion of heritability explained becomes larger [78]. Similarly, gene–environment correlations and interactions do play a role too. Novel strategies are now under evaluation and will be elucidated in the next paragraphs (see also Fig. 5.3 for a summary list of future trajectories of genetic research in AILDs).

The Neglected Role of X Chromosome in PBC

The role of the X chromosome in PBC still remains largely unknown, with no association signals being reported at a genome-wide threshold of significance so far. This could also be explained by the fact that, normally, X chromosome polymorphisms have not been included in GWAS analysis, and also for the lack of ad hoc bioinformatics pipelines to be used in the analytic steps [79]. These limitations have indeed a more general impact on genetics of complex diseases: X chromosome constitutes 5% of the nuclear genome and mutations in genes mapping on this chromosome account for almost 10% of Mendelian disorders [80]; nevertheless, only 114 X chromosome susceptibility loci (0.8%) at $P \leq 5*10–8$ have been described, on a total of ~15,000 signals identified by GWAS studies for more than 300 traits [81].

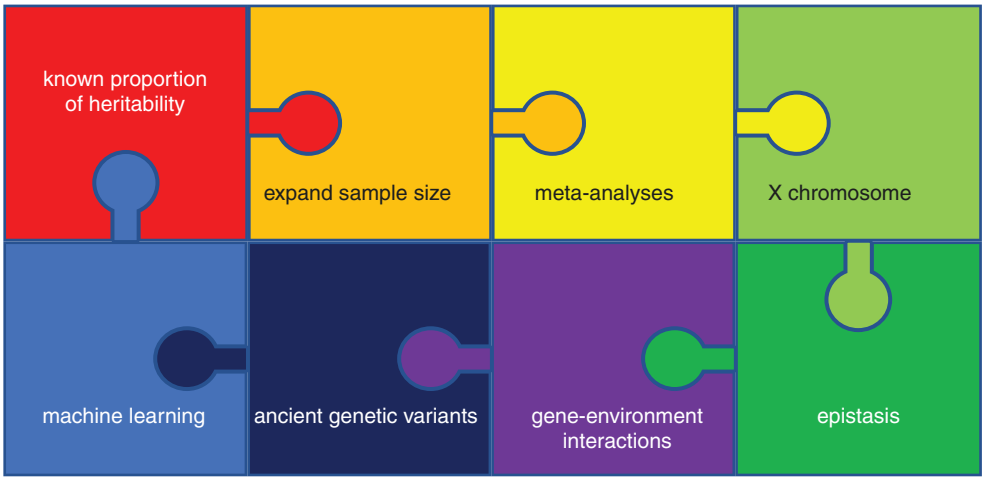

Fig. 5.3 Future strategies to find the missing heritability of autoimmune liver diseases

There is evidence behind the contribution of X chromosome in autoimmunity. Starting from physiology, males live shorter and are sicker compared to women, and this has been partly attributed to female immunological advantage, in turn questionably due to the role of sex chromosomes. Males have an evident drawback, since all mutations occurring on the X chromosome have to be phenotypically expressed, not having another copy of this chromosome. This concept is well shown by the case of X-linked primary immunodeficiencies. Affected patients are more likely to get infections because of the malfunction of key proteins involved in the host immune response located on the X chromosome; conversely, the matter is made more complex by the increased incidence of autoimmune diseases in these subjects.

There is a well-posed hypothesis, called the "haploinsufficiency hypothesis," stating that abnormalities in the number of chromosomes of a cell can contribute to the development of autoimmune phenomena [82]. Patients with Turner's syndrome have X monosomy due to a germinal defect and are more prone to develop autoimmune conditions [83]. A higher than expected rate of monosomy has been described in peripheral lymphocytes of patients with PBC [84]. This phenomenon has been described also in other autoimmune disorders [85]. It is speculated that, from a functional point of view, haploinsufficiency may alter immunity in PBC since immune-related genes in the pseudo-autosomal region of the X chromosome are not present [86].

The development of novel bioinformatics pipelines able to analyze X-chromosome can offer a tool to shed a light on the possible contribution of X chromosome to the genetic risk of AILDs [87].

Epistasis (Gene–Gene Interactions)

Different definitions of epistasis have been proposed, either looking at the biological side or focusing on statistical interaction. From a biological point of view, epistasis refers to the phenomenon where the effect of one gene is dependent on another gene, more broadly relating to the concept of gene–gene interactions [73]. Statistically, epistasis refers to any statistical derangement from the additive combination of different gene loci [88].

In estimates of heritability, most of the studies have assumed that no epistatic effects among loci were present; there is evidence that this postulation can have biased the interpretation of the results [78].

Despite the recognition of the importance of gene–gene interaction in complex genetic systems, few studies on epistatic interactions have been performed, probably due to the high computational burden that these analyses carry. Nonetheless, there is a growing interest in overcoming these obstacles also by using data-mining and machine-learning algorithms.

In AILDs, there are some findings showing the possible role of epistasis. In PBC, *IL12RB2* and *IRF5* loci do show an epistatic interaction [39]; similarly, the carriage of the *TNF2A* allele is increased in PBC patients with the *CTLA4* rs231725 A/A genotype compared to controls [89]. No epistatic interactions have been described between risk genes of AIH and PSC.

Research over the last several decades has accumulated vast amounts of biological information that is stored in public databases [90]. Interaction models should take advantage of the available information and build biology-driven epistatic analyses.

Gene–Environment Interactions

It is well established that multiple genetic and environmental components contribute to complex traits, possibly interacting with each other in an additive or even multiplicative mode. In other words, for some polymorphisms,

the effect of the specific variant depends on certain environmental exposures, or, likewise, the effect of an environmental factor relies upon an individual genetic makeup (bidirectional interactions). Studying gene–environment interactions, that is, including in the genetic statistical models (allelic, genotypic, dominant, recessive) also the environmental component, is of pivotal importance to increase the power to detect novel genes that influence the trait through an interaction, which can otherwise go unnoticed if the interaction is ignored. In addition, quantifying gene–environment interactions can help in developing predictive models, either for disease onset or even for response to treatments.

As for AILDs, and more in particular for PBC, the profound differences between the Japanese and European PBC GWAS results suggest that the identified risk variants could indeed be interlaced with environmental factors, even for simple reasons, like the differences in population history or environmental exposures.

More in general, several environmental factors have been recognized as more frequently associated with cases than controls in patients with AILDs. For example, in PBC, cigarette smoking and urinary tract infections have been implicated in increased susceptibility to the disease [91, 92]. Another interesting perspective regards molecular mimicry, that is, the structural similarity between the target of disease-specific autoantibodies (for PBC the human pyruvate dehydrogenase complex) and some environmental compounds (bacterial components, chemicals present in pesticides or cosmetics) [93]. To make things more intricate, environmental factors (such as air/water pollution) can also influence the DNA methylation process, so also epigenetics may play a role (see below). Changes in the microbiome might also be involved, with a complex relationship between microbiome, sex, and autoimmunity. The transfer of gut microbiota of adult male mice to female individuals susceptible to type 1 diabetes prevents the onset of the disease, calling in action sex hormones [94]. Observational findings have revealed more frequent abnormalities in the microbiome (dysbiosis) of patients with AIH, PBC, and PSC compared to healthy controls [95–97]. Whether these changes are causal or simply a consequence of other pathogenic phenomena is unknown. A pivotal study has shown that translocation of a gut pathobiont, *Enterococcus gallinarum*, to the liver triggers autoimmune responses in mice genetically predisposed to autoimmunity. More interestingly, *E. gallinarum*-specific DNA was found in liver biopsies of patients with AIH, supporting a similar process also in humans [98]. More mechanistic studies are needed to further dissect these initiation phases, and, more in general, more efforts are needed to unravel the gene–environment landscape of in AILDs, which is far from being painted.

Epigenetics: Beyond the Genes

Epigenetics refers to the study of changes not involving alterations in DNA sequence. There is a growing body of evidence that shows that epigenetic changes occur in autoimmune diseases and may account for the phenotypic variance, which is not explained by genotypic variance [99].

DNA methylation and histone modification are the most well-described mechanisms affecting gene expression. DNA methyltransferase is the enzyme involved in the addition of a methyl group to the DNA, in this way repressing gene expression. Eukaryotic DNA is typically organized in chromatin, and histones are the main proteins present. Acetylation, methylation, phosphorylation, and other post-translational modifications are the typical changes histones encounter in the process called chromatin remodeling. Other epigenetic mechanisms involve small or long non-coding RNAs that regulate post-transcriptional gene expression.

Epigenome-wide association studies (EWASs) are increasingly being performed, thanks to the improvement in high-throughput technology in DNA methylation profiling [100]. For example, whole-genome bisulfite sequencing investigates hundreds of thousands cytosine-guanine dinucleotides (CpG) sites, areas where DNA bases are typically enriched in methyl groups. Interesting reports have been described in systemic lupus erythematosus (SLE) [101], Sjogren's syndrome [102], and rheumatoid arthritis [103], while data in AILDs are still lacking.

Probably, X inactivation in females is the most cited example of epigenetic control [104]. X chromosome inactivation refers to the random silencing of one copy of the X chromosome during embryogenesis. The main aim of this process is to prevent redundant gene expression in females and favors cellular heterogeneity, since inactivation is different across different cell types. When this process does not happen at random, X-chromosome inactivation is "skewed" [105]. Despite this could arguably be a physiological phenomenon since skewing increase with age, it has been reported in several autoimmune conditions, including PBC [106].

Genes on sex chromosomes could also be differently expressed and this could generate some further degree of heterogeneity. This "incomplete" X-chromosome inactivation (XCI) may affect at least 23% of X-chromosomal genes, as a thorough survey of XCI has recently shown [107]. Escape from inactivation is more frequent on the short arm, since the centromere could act as physical barrier for the spreading of inactivation promoted by the long non-coding RNA X-inactive-specific transcript (*XIST*) gene, located on the long arm [105]. As far as immunity is concerned in this process, *DDX3X*, which is involved in production of type 1 interferons, has been reported to escape gene silencing in SLE and PBC. The functional consequence is a higher level of DDX3X in females and increased interferon activity [108].

When dendritic cells express only one set of possible self-antigens due to skewing, autoreactive T lymphocytes reacting to the other set of self-antigens can avoid negative selection and persist. This "loss of mosaicism" has been revealed in different autoimmune diseases, like SLE [109], systemic sclerosis [110], and autoimmune thyroid diseases [110], but not in PBC [111].

Another mechanism determining X-chromosome-related autoimmunity is the reactivation of silenced genes; this could translate in increased production of autoantibodies. For example, in PBC, the *CD40* promoter has been found less methylated in CD4+ T lymphocytes [112].

Other significant players in autoimmunity are microRNAs (miRNAs) [113]. They act in the post-transcriptional phase, being involved in several aspects of the cell function. It is of notice that the density of miRNAs on X chromosome is much higher than autosomes. Several autoimmune conditions have been shown to present abnormal expression of different miRNAs, including AIH [114], PBC [115], and PSC [116]. In particular, miRNA506 is upregulated in cholangiocytes and favors the shift toward a PBC-like phenotype [115, 117].

Ancient Genetic Variants

A novel approach in the complex trait field is to study the pathological role of archaic genetic variants, that is, those alleles that derive from interbreeding between modern humans and archaic humans, like Neanderthals and Denisovans.

Neanderthals are an extinct group of hominins who have been present in Eurasia before anatomically modern humans (i.e., humans with similar skeletal features to those of present day) moved from Africa [118]. The first evidence of genetic admixture between Neanderthals and Eurasian modern humans came up in 2010: the genome of three Neanderthal individuals was sequenced and compared to the genome of five present-day humans, revealing a higher overlap between SNPs of Neanderthals and present-day humans in Eurasia than that present between SNPs of Neanderthals and present-day humans in sub-Saharan Africa. After scanning the human genome for alleged Neanderthal genome segments, it has been estimated that 1–4% of the genome of non-African individuals is derived from Neanderthals in a positive selection process [119]. Later on, two seminal studies have investigated the persistence of Neanderthal genes on a big cohort of present-day humans. Both works are the result of an extensive effort to set tools to detect Neanderthal DNA by using computational methods [120, 121]. Denisovans are another extinct group of hominins who lived in an area ranging from Siberia to Southeast Asia [122]. From a genetic perspective, Denisovans interbred with Neanderthals [123] and interbred with the ancestors of some modern humans, with about 3–5% of the DNA of Melanesians and Aboriginal Australians and around 6% in Papuans deriving from Denisovans [122, 124]. More recent studies have shown that there are at least three different branches, one contributing an introgression signal in Oceania, another restricted to New Guinea, and a third in East Asia and Siberia [124].

Aside from the evident implications in evolutionary anthropology, there are also important and fascinating consequences for the biological field. Evidence is accumulating that some alleles derived from ancient hominins may have been beneficial in modern humans, while others detrimental. Genetic admixture between archaic humans and modern humans, and consequent introgression of archaic alleles, might have contributed to substantial immune advantage for modern humans. Researchers have identified three Toll-like receptors that carry archaic alleles with a well-defined functional role in present-day humans [125]. There are also proofs that introgressed Neanderthal DNA in modern humans helped them to adapt against viruses [126]. On the contrary, archaic alleles might also be implicated in predisposition to diseases, for example, type 2 diabetes [127]. The contribution of archaic genetic variants on the susceptibility to AILDs is currently unknown. Yet, a risk gene variant associated with PBC (rs12531711) was found to be Neanderthal-derived [120]. Intriguingly, in some regions of modern genomes, researchers have found "desert" areas, that is, areas with very few Neanderthal genes compared to others, and these deserts were mainly located in the X chromosome [120, 121]. Since X chromosome is somehow involved in PBC pathogenesis, it would be of interest to investigate whether the lack of introgressed Neanderthal DNA might play a role in disease risk.

Application of Machine Learning to Population Genetic Data

Research in population genetics has mostly focused on the formalization and validation of statistical models that describe patterns of variations and their application to experimental molecular data [128]. Similarly to what is already happening in other fields, machine learning (ML) has the potential to revolutionize population genetics reversing this approach: ML methods (especially unsupervised ones) can shed light on predictive features that would not be considered otherwise. Indeed, while classical population genetics has been mainly characterized by parameter estimation in the context of a predetermined probabilistic model (typically the Wright–Fisher model), the target of ML is optimization of the accuracy of predictions [128]. Polygenic risk scores predictions are fixed models that put together a set of risk alleles to predict the risk of a specific complex disease [129].

They are commonly based on a linear parametric regression model, with strict assumptions like additive effects, independent effects, normal distribution of the data, independence of observations, which could often be not valid in complex traits [129]. Supervised learning algorithms can build a mathematical model to determine how a set of variables (features), which constitute the input, relates to a target (output) [130]. In population genetics this output could be represented by the status (case or control), the features being individual sample genotype data [129]. Data feature selection is the key step to obtain an accurate ML model [131] and there are a few methods (embedded methods, wrappers) useful to select only informative SNPs as potential predictors [129].

A possible application of supervised ML in AILDs could be to identify novel predictive features (SNPs) associated with the phenotype, possibly integrating also biologically distinct sub-phenotypes of the disease (early vs. advanced disease, onset at younger age vs. older age, positivity for specific autoantibodies). In this way a predictive model is generated, taking advantage of the different contribution of variables within the "training" genotype data [129]. After the training phase, the models with the maximum predictive power are selected for validation. This stage is essential to avoid overfitting and is usually achieved by cross-validation (dividing original dataset in a training set and a test set). Nonetheless, external validation is still required for the final validation of the model [129]. These "ML disease prediction models" might represent a complementary tool, or even an evolution of current polygenic risk scores. Nonetheless, newer models have to be compared to current validated models to really prove their clinical utility.

Another peculiar and powerful feature of ML is its capacity to handle thousands of dependent variables, each characterized by a massive amount of information; this ability is of interest in genomics world, where increasing dimensionality of data is an issue [128]. Unsupervised learning typically aims to solve the classification problem (clustering) without a prespecified target, finding patterns in the data [130]. Among unsupervised learning ML techniques, principal component analysis (PCA) and hidden Markov models (HMMs) have been the most frequently used in genetics. When evaluating gene–gene interactions, a typical hurdle of classical statistic genetics is the impracticability of an exhaustive search of all interactions among more than two loci at genome-wide level, so that an exhaustive search of all two-locus interactions within a set of predefined loci is commonly performed [132]. Thanks to their non-linearity, ML algorithms allow us to account for complex interactive effects between associated alleles, being possibly able to scale up to higher-order interactions [131]. Whether part of the missing heritability of AILDs due to epistatic interactions will be elucidated by novel ML techniques is still to be ascertained.

Conclusions and Open Questions

Over the last decade, GWASs hugely contribute to the understanding of the genetic basis of AILDs. Many signals have been identified and related to genes that are, directly or indirectly, involved in regulatory functions within the immune system. Nonetheless, we are far from having a clear knowledge of the cascade of events that are behind the onset of the disease in a predisposed subject. It is likely that identification of variants through GWAS alone will probably not be sufficient to explain the missing heritability in AILDs and complex traits in general. Future studies should focus on sex chromosomes, as well as investigate gene–gene and gene–environment interactions [72]. In vitro biological validation of future findings will also be advisable, and integration with epigenomic, transcriptomic, and proteomic information might provide a multidimensional view of the functional relevance of the genes [32]. We believe that ML algorithms will probably be able to revolutionize the field and be a key player to this end [133].

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The Epigenetic Basis of Loss of Tolerance

6

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Key Points

- The main regulations for epigenetic modifications are DNA methylation, histone modification, and noncoding RNAs, and these processes regulate the gene expression, cell differentiation, proliferation, and survival.
- T cell differentiation is regulated by DNA methylation.
- Abnormal epigenetic modifications have been found in several autoimmune disorders, such as lupus, rheumatoid arthritis, type 1 diabetes, primary biliary cirrhosis, multiple sclerosis, and psoriasis.
- DNA hypomethylation is found in lupus T and B cells, which might contribute to the over-activated phenotype of T and B cells, and DNA methylation level on IFI44L can serve as a biomarker for lupus with high sensitivity and specificity.
- Noncoding RNA in circulation or urine might be a suitable source for disease biomarkers.
- Histone modification occurs in many biological processes, and it is a potential target for therapy.

Introduction

The immune system is a critical host defense system to prevent pathogens, such as virus and parasitic worms, to infect our body, or it can act as a surveillance to remove abnormal cells, such as tumor cells. However, the immune system can be also harmful when it responses to autoantigens, which are referred to the loss of immune tolerance. Once this balance is broken down, autoimmunity or tumors occur.

Autoimmune diseases are a complicated set of chronic and potentially also life-threatening diseases that are characterized by abundant autoantibodies and abnormal innate immune cells, such as dendritic cells and monocytes, and adaptive immune cells, such as T cells and B cells. In these diseases, immune cells cannot distinguish self-antigens and attack self-tissues, resulting in organ damages. The pathogenesis of autoimmune diseases has been intensively studied more than a century. However, the direct cause has still not been elucidated. Genetic susceptibilities can provide some explanations for the loss of immune tolerance. In genome-wide association studies (GWAS), over 60 genetic loci have been revealed as risk genes in lupus, and some of them have been found to be related to antibody production, complementary deficiency, and renal involvements [1]. However, genetic studies cannot completely explain the low incidence rate of SLE in homozygous twins, which ranges from 24% to 58% [2], indicating that besides genetics, other factors, such as environmental factors, also contribute to this disease. As one of the molecular mechanisms of environmental factors, epigenetics has been proposed as a critical player in the diseases, and it might provide additional explanation for the loss of tolerance.

Epigenetics refers to a modification which is inheritable and changes the gene expression without changing DNA sequence. DNA methylation, histone modification, and noncoding RNAs are the primary mechanism of epigenetic regulations. As the coming of the epigenetic era, accumulating evidence has shown the strong association between abnormal epigenetic regulations and autoimmune diseases, such as lupus, rheumatoid arthritis, type 1 diabetes, primary biliary cirrhosis, multiple sclerosis, and psoriasis. Therefore, in this chapter, we summarize the update progress of epigenetic modifications in the pathogenesis of autoimmune diseases, broadening our understanding of mechanisms for diseases etiopathogenesis, discussing the potential use of abnormal epigenetic modifications as biomarkers and therapeutic targets.

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Tolerance and Autoimmunity

In normal conditions, the immune system can distinguish between self- and nonself-antigens. Self-tolerance is achieved in T and B cell-positive and cell-negative selections by eliminating autoreactive T and B cells through central or peripheral tolerance. Central tolerance occurs in the bone marrow and thymus so that immature B and T cells that show high affinity to self-antigens are clonally deleted by the process of central tolerance. B and T cells that display low affinity to self may escape from the bone marrow and thymus and are induced to die by apoptosis in periphery by the process of peripheral tolerance [3, 4]. Receptor editing occurs in the immune system when B cells encounter an autoantigen in the periphery and induce B cell tolerance. T cells, which encounter autoantigens that are not found in the thymus in early T cell development, are suppressed by other cells, such as regulatory T cells [5] in the process of peripheral tolerance. When this tolerance breaks down, autoimmunity occurs.

In lupus, for example, the immune system recognizes and reacts to autoantigens. In SLE, apoptotic blebs, double-standard DNAs and RNAs, and nucleosomes are the main autoantigens and are formed during the apoptosis process. These are recognized and processed by immature myeloid dendritic cells (mDCs), and these cells can further become mature DCs with high-level expression of co-stimulatory molecules (CD86, CD40, and MHC-II) and pro-inflammatory cytokines including IL-6 and IL-12p70. Mature DCs activate T helper (Th) 1 and Th2 cells through CD86/80-CD28 and CD40-CD40L ligation, and they also polarize naive T cell to Th1 under the help of IL-12. In the presence of IL-6, regulatory T cells are inhibited, and pro-inflammatory Th17 cells are promoted. With the help from T cells, autoreactive B cells produce autoantibodies against self-antigens, and these antibodies further form immune complexes (ICs) via binding with autoantigens. In addition, after taking up ICs, plasmacytoid dendritic cells (pDCs) produce high amount of IFN-α which can further promote antibody production and isotype switching. Immune-complexes (ICs) also deposit in glomerular leading to immune cell infiltration and local tissue damage, which can induce more apoptosis [6] acting as a positive loop which can accelerate disease progression.

Breakdown tolerance in SLE may be triggered by many factors, such as infection due to molecular mimicry between self- and bacterial antigens [7], cryptic antigens and protein changes, superantigens and bystander activation of immune cells [8]. Some self-antigens and bacterial peptides share similar amino acid sequences and may show cross-reactivity. In tissue injury, cell death and reparative changes may lead to exposure of new self-antigens or modification of self-antigens which induces autoimmune response. Moreover, infective organisms act as superantigen and can bind to T cell receptor (TCR) regardless of antigen specificity and activate a large number of T cells with different antigen specificity. Besides the factors mentioned above, other environment factors which might result in epigenetic modifications will be elaborated in following text.

Epigenetic Mechanisms

All cells and tissues in our body share the same set of genomic DNA; however, cells display various morphology and phenotypes due to the gene transcription mediated by epigenetics. Epigenetics is a biological process that recruits or removes reversible and potentially heritable modifications in genomic DNA and/or chromatin but does not change DNA sequence. It is mainly comprised of DNA methylation, histone modifications, and noncoding RNA-mediated regulations. Epigenetic regulations participate in numerous biological processes, such as cell proliferation and differentiation, and increasing evidence has shown that dysregulated epigenetic modifications are involved in pathogenesis of several autoimmune diseases [9–12]. The influence of environmental factors, such as UVB, and disease predominance in female emphasize the importance of epigenetics in the pathogenesis of autoimmune disorders. In addition, 5-azacytidine and procainamide [13] are capable of inducing lupus via epigenetic alterations. Similar phenomena have been found in other autoimmune diseases: dysregulation of epigenetic modifications in RA synovial fibroblasts (RASF) leading to abnormal gene expression [14], Epstein-Barr virus (EBV) infection, sunlight [15, 16], and aberrantly expressed miRNAs [17] contributing to the pathogenesis of multiple sclerosis (MS).

DNA Methylation

DNA methylation refers to a well-known biological process which involves a recruitment of a methyl group to a cytosine or adenine residue at the 5th position on the pyrimidine ring, resulting in inhibition of binding of transcription factor on the promoter region of gene, leading to the end of gene transcription [18]. This process is mainly regulated by methyltransferase, including DNA methyltransferase 1 (DNMT1), DNMT3a, and DNMT3b. Each of the methyltransferase executes different functions. Generally, during cell replication, DNMT1 maintains the methylation levels, whereas DNMT3a and DNMT3b promote methylation process [19]. On the contrary, DNA hydroxymethylation and demethylation are processes that reactivate transcription of silenced genes [20]. DNA hydroxymethylation is an instable status and transient demethylation status. DNA hydroxymethylation is modulated by hydroxymethylation transferases, such

as ten-eleven translocation methylcytosine dioxygenase 1 (TET1), TET2, and TET3 [21].

Histone Modifications

Histone modification is a covalent posttranslational regulation that modulates gene transcription by altering the structure of chromatin. Histone modifications include methylation, acetylation, ubiquitination, phosphorylation, sumoylation, etc. [22]. Acetylation and deacetylation are intensively studied ones which can recruit or remove an acetyl group on histones, thereby activating or inhibiting gene transcription. Mechanically, acetylation activates gene transcription by opening the chromatin structure and facilitating the binding of transcription factors, while methylation converts opened chromatins into a restrictive structure, inhibiting the binding of transcription factors via stereo hindrance, thereby resulting in the repression of gene expression. Acetylation is mediated by histone acetyltransferases (HATs); deacetylation is regulated by histone deacetylases (HDACs) [23]. However, the effects of histone modifications vary depending on the modification positions and the number of modifications. For example, H3K4me3 promotes gene expression, whereas H3K9me3 and H3K27me3 repress gene transcription [24].

Noncoding RNAs

MicroRNAs (miRNAs) are small noncoding RNAs, which are usually 21–25 base pairs. It has been well established that miRNAs modulate gene expression at posttranscriptional and posttranslational level through binding to the 3′-UTRs of target mRNAs, resulting in blocking gene translation by mRNA cleavage and degradation [25–27]. Therefore, miRNAs are believed to be involved in several biological processes and disease pathogenesis. Indeed, accumulating evidence has shown that the abnormal miRNA-mediating regulations contribute to the pathogenesis of autoimmune disorders, implying the utilization of miRNAs as potential diagnostic and predicting markers, which are relatively more convenient and noninvasive compared with biopsy.

Besides, long ncRNAs are recently identified noncoding RNAs, with the length of greater than 200 nt. However, by now a few of them have been assigned functions. lncRNAs are usually divided into five subtypes: sense, antisense, intronic, intergenic, and bidirectional ones [28]. Differing from miRNAs, lncRNAs can either promote or repress gene expression. LncRNAs usually act by complexes of lncRNA:RNA, lncRNA:protein, or lncRNA:chromatin [29, 30]. Recently, lncRNAs become a rising star in the field of disease pathogenesis. Increasing evidence suggests that lncRNAs are involved in numerous human diseases, such as cancer, by changing the primary and secondary structure of DNA, thereby regulating gene expression [31, 32].

Abnormal Epigenetic Modifications in Autoimmune Diseases

Dysregulated Epigenetic Modifications in SLE

DNA Hypomethylation in SLE

DNA Hypomethylation in Lupus T Cells

The role of DNA methylation in pathogenesis of SLE was firstly proposed half a century ago [33]. The first evidence of epigenetic regulation in lupus is from the observation that two DNA methylation inhibitors, procainamide and hydralazine, have been revealed to induce a lupus-like manifestation in normal mice after a long-term administration. The symptom disappears after the withdrawal of procainamide and hydralazine. In addition, cells from the thymus and lymphatic nodules from MRL/lpr mice (spontaneous lupus mouse model) show lower DNA methylation level compared with cells from MRL/mpj normal mice [34, 35], which might provide an explanation for over-proliferated and over-activated immune cells in lupus mouse model.

In human studies, DNA demethylation has been observed in lupus $CD4^+$ T cells [36, 37]. DNA hypomethylation has been observed in $CD4^+$ T cells from active SLE patients, and overexpressed LFA-1 has been found on an autoreactive subset of T cells, which can produce perforin and granzyme B to lyse autologous immune cells [38, 39]. Epigenetic accessibility and transcriptional poising of interferon-regulated genes in naive CD4+ T cells from SLE patients have been shown in a genome-wide DNA methylation study [40]. In a consequent study, different DNA methylation patterns have been revealed in different organ involvements in lupus, such as renal involvement versus nonrenal involvement and malar versus discoid rash [41]. Interestingly, some proteins such as RFX1 [42], high mobility group box protein 1 [43], and DNA damage-inducible 45 alpha (Gadd45a) [44] have been revealed as upstream regulators.

In a large-scale DNA methylation study on SLE $CD4^+$ naive T cells, DNA hypomethylation is observed on interferon-regulated genes (IFI44L, IFIT1, IFIT3, MX1, STAT1, USP18, BST2, and TRIM22) which suggests that lupus T cell progenitors have abnormalities which can contribute to pathogenesis of SLE [40]. More interesting is that our recent studies have proposed DNA hypomethylation level on IFI44L promoter as a biomarker for the diagnosis of lupus, which has both high sensitivity and specificity [45]. Besides, in lupus $CD4^+$ T cells 5-hmC binds in transcriptional regulatory regions of lineage-specific signature genes,

such as IL-17 and IFN-gamma. Mechanically, TET2 protein, a hydroxymethylation transferase, is found to be recruited to 5-hmC-binding regions and then promotes the production of T helper cell signature cytokines, such as IL-17 and IFN-gamma [46]. We have recently observed that lupus $CD4^+$ T cells display an increased 5-hmC level on whole genomic DNA compared with normal subjects, with the enhanced expression of TET2 and TET3. As a consequence of DNA demethylation, transcription activator CTCF binds to the promoter region of SOCS1 and therefore promotes SOCS1 overexpression in SLE $CD4^+$ T cells [47].

It has been reported that lupus T cell autoreactivity is attribute to DNA hypomethylation [38]. This study is a hallmark of a beginning of a new era of epigenetics in the pathogenic study of lupus. These findings were further confirmed by the evidence of induction of autoreactive $CD4^+$ T cells from healthy controls by the administration of 5-azacytidine [38, 48], which followed prior evidence of the induction of IL-2 and IFN-γ by the same drug [49]. Accumulating evidence have revealed the regulatory effects of DNA methylation on individual genes during the T cell activation and differentiation. IFN-γ and IL-4 are signature cytokines for Th1 and Th2 program, respectively. During Th1 and Th2 differentiation processes, DNA hypomethylation level has been observed at *Ifng* and *Il4* loci [50, 51]. In addition, compared to naive T cells, decreased DNA methylation level is found at the key transcription factor FOXP3 locus in regulatory T cells (Treg) [52]. Furthermore, the key transcription factor Bcl6 in Tfh cell has been reported to be highly expressed but with a decreased level of 5hmC [53] during Tfh cell differentiation, suggesting that Tfh cell differentiation is also mediated by DNA methylation modification.

DNA Hypomethylation in Lupus B Cells

Besides, SLE is an autoantibody-mediated autoimmune disorder. As the main and unique origin of autoantibodies, numerous evidence have well documented that B cell plays an essential role in the pathogenesis of SLE. Preclinical studies and clinical trials of B cell-targeting treatments have proven to be effective to some extent. However, more effective and safe treatments are still in a great need. Not to our surprise, DNA hypomethylation has been also shown in lupus B cells [54], which might regulate B cell development, differentiation, and autoreactivity. For example, abnormally expressed HRES1/p28 by lupus B cells is reported to be regulated via DNA methylation [55]. DNA hypomethylation on *LINE1* gene has been shown in lupus B cells [56]. The regulatory effect of DNA methylation in B cells is further supported by the evidence that enhanced levels of antinuclear antibodies can be induced by adoptive transferring of DNMT1 inhibitor-treated B cells [57]. Although it is elucidated that antibody production is attributed to DNA hypomethylation in V(D)J region and Igh 3′-LCR [58], little has been revealed in this process in the lupus condition. Furthermore, DNA hypomethylation might be a consequence of decreased level of DNMT1 and DNMT3b or AID-mediated active DNA demethylation in autoreactive B cells [59].

Histone Modifications in SLE

Not to our surprise, histone modifications also play a critical role in the differentiation, activation, and function of T cells, contributing to pathogenesis of SLE. Lupus $CD4^+$ T cells show global histone H3 and H4 hypoacetylation [60]. Abnormal histone modifications have been found in the promoter region of *TNFSF7* in T cells, resulting in CD70 overexpression, which might lead to the autoreactivity of T cells [61]. Administrating normal T cells with HDAC inhibitors leads to decreased CD3ς chain expression, which results in abnormalities of T cells [62]. In addition, it has been shown that a transcription factor CREMα might be involved in the process of histone acetylation in active lupus T cells via inhibition of IL-2 production. This process might be mediated by recruiting HDAC to Cre binding sites in the promoter region of *Il2* [63]. Besides, in lupus PBMCs, altered H3K4me3 modification has been observed on lupus-related candidate genes [64]. Acetylation of global H4 has been reported to be changed in lupus monocytes. Among them, 63% of these H4-acetylated genes are potentially modulated by IFN regulatory factors [65], which are involved in the pathogenesis of SLE.

In addition to the whole genomic modifications, histone modification has been reported to modify specific gene expression, such as modulating cytokine expression. For example, increased H3 acetylation level has been found at the IL-17 locus, and enhanced IL-10 production has been revealed to be mediated by chromatin remodeling. This process is further revealed to be mediated by Stat3 [66, 67]. Moreover, histone hyperacetylation has been shown to be a reason for an increased serum level of TNF-α in lupus patients, as well as an enhanced maturation of SLE monocytes [68]. However, it is still unclear whether histone modifications are the initiator or results of immune disorders, even though the contribution of histone modifications in pathogenesis of lupus has been revealed in mouse studies.

Sirtuin-1 (Sirt-1) is a histone deacetylase, which has been observed to be overexpressed by T cells from MRL/lpr mice [69]. The silencing Sirt-1 in lupus mice leads to short-term enhancement of H3 and H4 acetylation, accompanied by impaired lupus symptoms such as reduced serum levels of anti-dsDNA and IgG deposition in glomerular and kidney damages [36]. Treating MRL/lpr mice with HDAC inhibitors also displays therapeutic effects, including attenuated renal damage and decreased level of lupus-related cytokines [70]. A recent progress has been made from a genetic and epigenetic mapping study which identifies candidate causal variants in 21 autoimmune diseases in different T cell sub-

types, including Th1, Th2, Treg, and Th17 cells [71]. In this study, unique H3K27 peaks are shown in the super-enhancer in *Il2RA* locus, particularly in Treg and Th17 cells.

In our previous study, we showed that RFX1 inhibits Th17 cell differentiation via increased histone H3 acetylation and decreased DNA methylation and H3K9 tri-methylation [72], thereby contributing to SLE pathogenesis. More recently, the downregulation of TNF-alpha-induced protein 3 (TNFAIP3), one of the major SLE susceptibility genes involved in the regulation of inflammatory responses through modulation of the nuclear factor-kappaB (NF-kappaB) pathway, has been observed in lupus patients. This downregulation may be mediated by reduced H3K4me3 in the gene promoter region [73], providing a promising target for the treatment of SLE in clinical practice.

Noncoding RNAs in SLE

Dysregulated Noncoding RNAs in Lupus T Cells

It has been well documented that miRNAs can bind to various regions but modulate the same gene expression. A large number of miRNAs have been reported to be aberrantly expressed by T cells. Some of these miRNAs have been found to target lupus-related genes, such as *Il10*, *Il17*, and *dnmt1*. It has been reported that the expression level of miR-21, miR-126, and miR-148a is observed to be reduced in lupus T cells and they are found to target DNMT1, although they bind to different regions of DNMT1 [74, 75]. Furthermore, the inhibition of miR-21, miR-29b, and miR-148a in SLE T cells has been found to be capable of attenuating lupus phenotypes, suggesting potential therapeutic roles in SLE [75, 76]. In addition, miR-21 has been found to inhibit the expression of PDCD4 on lupus T cells, thereby promoting T cell proliferation and the expression of CD40L and IL-10 [77]. Moreover, miR-142 [78] and miR-31 [79] have been demonstrated to modulate T cell activity by suppressing IL-4 and IL-10 production by T cells, inhibiting the expression of CD40L and ICOS and enhancing secretion of IL-2 by T cells. In addition to our previous studies on aberrantly expressed miR-146a and miR-241-3p/5p by lupus T cells, we have further found that mycophenolic acid, which has been commonly utilized in clinic for lupus treatment, attenuates the autoreactivity of lupus T cells through miR-146a and miR-241-3p/5p, suggesting the pathogenic role of these two miRNAs in SLE [80].

More recently, in short time-series expression miner analysis, some lncRNAs from lupus T cells have been found to be correlated with SLE disease activity [81], suggesting that the aberrant expression profile of lncRNAs may play a role in SLE pathogenesis. In addition, large intergenic noncoding RNAs (lincRNAs), a specific type of lncRNAs, can also modulate gene expression and are involved in various biological processes and diseases. For example, lupus PBMCs show lower level of linc0597and linc0949, compared to those from rheumatoid arthritis patients and normal subjects [82]. More importantly, the decreased level of linc0949 is correlated with the level of C3, SLE disease activity index (SLEDAI), and the appearance of lupus-specific organ damages. More interesting is that the levels of linc0949 can increase significantly depending on efficiency of treatment in lupus patients, suggesting a role as a biomarker for SLEDAI and drug response [82, 83].

Aberrantly Expressed MicroRNAs in Lupus B Cells

As critical regulators in B cell development and differentiation, miRNAs are also involved in the aberrant B cell expression and functions. Lupus B cells show increased levels of miR-30a. The level of miR-30a in lupus B cells negatively correlates with Lyn, which negatively regulates B cell activation [84]. It has been found that miR-155 and miR-181b negatively regulate AID expression, thereby modulating antibody diversity [85, 86]. In lupus-prone mice, the levels of miR-15a in regulatory B cells positively correlate with the serum level of anti-dsDNA antibodies [87]. In our recent studies, increased expression of miR-1246 has been observed in lupus B cells, and it has been found to regulate EBF1 expression, thereby promoting the expression of CD40 and antibody production [88]. Moreover, enhanced levels of miR-17-92 and miR-21 have been found in SLE B cells [89, 90]. More interestingly, miRNA profiling of B cell subsets has been proposed as a biomarker for lupus [91], indicating a critical role of miRNAs in lupus abnormal B cells. Moreover, miR-150 is found to be decreased in B cells from MRL-lpr mice, which might be a result of a decreased acetylation level and inhibition expression of the miR-150 host gene [92].

Dysregulated Epigenetic Modifications in Signaling Pathways

Besides, epigenetic modifications may also contribute to the various signaling pathways involved in pathogenesis of SLE. It has been revealed that reduced DNMT1 activity and downregulation of DNA demethylation have been found in daughter cells by blocking the ERK signaling pathway in lupus $CD4^+$ T cells [93]. The hydralazine (ERK inhibitor)-treated $CD4^+$ T cells show autoreactivity which has been confirmed by the findings that it can induce lupus-like manifestations by adoptive transferring of hydralazine-treated $CD4^+$ T cells. This symptom is similar to 5-azacytidine treatments [94]. In addition, the inhibition of ERK pathway (PKC-ras–raf-MEK–ERK) by targeting PKCδ can induce DNA hypomethylation on CD70 promoter and therefore promote CD70 expression, resembling that seen in 5-azacytidine-treated T cells or lupus T cells [95, 96]. More convincing evidence is that PKCδ knockdown mice can spontaneously develop lupus-like manifestations [97, 98]. dnMEK mouse model is another autoimmune mouse model

which is mediated by blocking ERK signaling [99], suggesting an important role of ERK signaling pathway and epigenetic changes in the pathogenesis of autoimmune disorders.

Environmental Factor-Induced Epigenetic Alterations in SLE

It has been well established that DNA methylation or demethylation in disease progress does not occur spontaneously. An external factor is required to trigger the changes of DNA methylation status. Numerous evidence have revealed that environmental factors are implicated as triggers in lupus. It was a mystery how the environmental factors affect the body biology. However, as the coming of the epigenetic era, more and more evidence have shown that epigenetic regulations might be a reasonable explanation. For example, an intensively studied one, oxidative stress, which is capable of reducing DNMT1 expression and changing the DNA methylation level in T cells, is usually induced by external factors such as UV light, smoking, infections, mercury exposure, and even air pollution [100]. Other environmental factors which can act synergistically, such as dietary deficiencies in folate, vitamin B, choline, methionine (Met), and Zn [101], are demonstrated to be required to maintain a normal level of DNMT1 [102].

Aberrant Epigenetic Modifications in Psoriasis

DNA Methylation in Psoriasis

Psoriasis is a chronic inflammatory autoimmune skin disease, which is characterized by hyperproliferation of keratinocytes and dysregulated T cells, especially Th17 cells [103]. Similar with SLE, genetic susceptibility is not the only factor for the onset of this disease; due to that the concordance of psoriasis in monozygotic twins is 35–72% [104], suggesting that epigenetic regulations might be an additional factor. Increased evidence has shown the critical role of DNA methylation in the hyperproliferated keratinocytes.

In our previous study, abnormal DNA methylation pattern has been observed in skin lesions and PBMCs of patients with psoriasis vulgaris [105, 106]. On the gene-specific level, the abnormal methylation pattern on the promoter of p16INK4a gene has been reported in psoriatic epidermis [107]. Increased DNA methylation level on promoter of secreted frizzled-related protein (Sfrp4) has been observed in inflamed psoriatic skin and in the IL-23-induced psoriatic mice, thereby reducing the expression of Sfrp4, a negative regulator for keratinocyte proliferation [108]. Hypomethylation of LINE-1 has been found in psoriatic keratinocytes. More importantly, manipulating LINE-1 methylation may change the gene expression, thereby resulting in a phenotypic alteration of psoriatic skin [109]. In addition, aberrant DNA methylation pattern has also been revealed in $CD4^+$ T cells from psoriatic patients [110], indicating that the epigenetic regulations on immune cells also attribute to psoriasis pathogenesis.

Dysregulated MicroRNA Mediating Modulation in Psoriasis

miRNAs have been reported in psoriatic patients. In our previous studies, miR-210 is found to be overexpressed by T cells filtrating in the dermis of psoriatic lesions. Further, miR-210 is capable of inducing T helper (Th) 17 and Th1 cell differentiation but inhibiting Th2 differentiation by repressing expression of STAT6 and LYN [111]. In addition, the upstream regulation has revealed that TGFbeta and IL-23 enhance miR-210 expression by inducing HIF-1-alpha, which recruits P300 and promotes histone H3 acetylation in the miR-210 promoter region [111]. As Th17 cells play a critical role in pathogenesis of psoriasis, targeting miR-210 might provide potential therapeutic strategies for psoriasis patients. Besides, miR-17-92 cluster has been revealed to promote the proliferation and the chemokine production of keratinocytes [112]; miR-let-7b has been shown to inhibit keratinocyte differentiation by targeting IL-6-mediated ERK signaling in psoriasis [113]; miR-194 has been demonstrated to regulate keratinocyte proliferation and differentiation via grainyhead-like 2 in psoriasis [114].

Abnormal Epigenetic Regulations in RA

DNA Methylation in RA

RA is an autoreactive immune cell-mediated inflammation which primarily affects joints. Autoreactive immune cells and synovial fibroblasts (SF) are well defined as the critical players in the pathogenesis of RA. Heterogeneity in RA patients is a hindrance for rheumatologists and dermatologists to diagnose and treat patients. The treatment of RA is always delayed due to the current criteria that in addition to meeting all diagnostic criteria, RA patients need to consistently display arthritic symptoms for at least 6 months [115]. Early intervention is necessary because a clinical trial on BeSt have shown that BeSt can delay the onset of RA on several patients [116].

Increasing evidence has shown that DNA methylation contributes to the pathogenesis of RA. Increased DNA methylation variability has been observed in rheumatoid arthritis-discordant monozygotic twins [117], indicating the importance of DNA methylation in the pathogenesis of RA. Abnormal genome-wide DNA methylation patterns have been revealed in $CD4^+$ T cells from Chinese Han patients with rheumatoid arthritis [118]. In PBMCs from RA patients, decreased DNA methylation levels have been found at the promoter regions of Il6 and ERa, which may be associated with overproduction of IL-6 and hyperactive ERa signaling [119]. Global DNA hypomethylation is also

found in T cells from RA patients [38, 120]. On the gene-specific level, CD40L gene has found to be demethylated on $CD4^+$ T cells from RA patients [121]. Moreover, DNA hypomethylation on promoter region of L1 retrotransposon gene has been observed in RA fibroblast-like synoviocytes [122]. Further, DNA hypomethylation on CXCL12 gene has been shown in synovial fibroblasts that may result in cell infiltration in joints [123, 124]. More interestingly, DNA methylation status has been proposed as biomarkers to predict the drug responses [125].

Histone Modifications in RA

Although histone modifications have been included in the mechanism for the pathogenesis, the reports are restricted to the expression of histone-modifying enzymes in RA samples. Conflicting data has been published regarding the expression of HDACs in PBMCs and synovial tissues in RA patients, partially due to the diverse HDAC activities influenced by disease activity and therapies in patients [126–129]. In RA synovial fibroblasts, enhanced levels of H3K4me3 have been found in the promoter regions on *MMP-1*, *MMP-3*, *MMP-9*, and *MMP-13* genes, whereas decreased levels of H3K27me3 have been observed in the promoter regions on *MMP-1* and *MMP-9* genes [130]. Moreover, increased levels of histone acetylation have been reported in the *MMP-1* and *Il6* genes, resulting in accumulation of MMP-1 and IL-6 proteins in RA synovial fibroblasts [131, 132].

Aberrantly Expressed MicroRNAs in RA

The screening study of differentially expressed miRNAs has identified abnormally increased miRNA-155 and miRNA-146a in synovial fibroblasts from RA patients [133]. A recent study revealed that PU.1 is a target of miRNA-155. PU.1 is a transcription factor in early B cell commitment, which is downregulated during B cell maturation. The inhibition of miRNA-155 expression in B cells from RA patients results in the upregulation of PU.1 expression and the reduction of the antibody secretion [134]. As components in Toll-like receptor pathway, IRAK1 and TRAF6 are targets of miRNA-146a. In this study, there is no difference of IRAK1 and TRAF6 expression in PBMCs from RA patients and healthy controls [135], indicating that increased miRNA-146a might regulate to other unclear targets to contribute to inflammation in RA. Compared to OA synovial fibroblasts, miRNA-124a, which targets monocyte chemoattractant protein 1 (MCP1) and cyclin-dependent kinase 2 (CDK2), has been found to be decreased in RA patients, resulting in decreased proliferation rate of synovial fibroblasts [136]. Moreover, miRNA-223 is increased in the peripheral of RA patients, and it is positively correlated with rheumatoid factor titers [137]. Furthermore, miRNA-223 has been found to suppress the insulin-like growth factor 1 receptor-mediated IL-10 production in T cells from RA patients [138].

Abnormal Epigenetic Modifications in SSc

Dysregulated DNA Methylation in Systemic Sclerosis (SSc)

SSc is a relatively rare disease which is characterized by damages of connective tissues mediated by autoreactive immune cells. Its etiopathogenesis remains unclear. Abnormal epigenetic modifications have been shown in SSc. Lower levels of DNMTs have been observed in $CD4^+$ T cells from SSc patients compared to normal controls [139]. DNA demethylation on promoter regions of *CD11a*, *CD70*, and *CD40L* genes has been found in $CD4^+$ T cells from SSc patients [139–142]. However, hypermethylated genes, such as *PRF1*, *CDKN2A*, *Foxp3*, *CD11a*, and *CD70*, have been found in whole blood from black South African patients with SSc [143], which might contradict to our previous reports. The differences in cell origin and race might be an explanation. Moreover, *RORC1* and *RORC2*, which are key transcription factors for Th17 cells, have been found to show demethylation and be correlated with inflammatory parameters in SSc PBMCs [144]. Furthermore, in dermal fibroblasts from SSc patients, an enhanced DNA methylation level has been found in *FLI1* and TGF-beta-related genes, which are Wnt pathway antagonist genes [145–147], accompanied by increased levels of DNMT1 [148] and TET1 [149].

Dysregulated Epigenetic Modifications in T1D

DNA Methylation Status in T1D

T1D is well documented as an autoimmune disease, which is mainly mediated by T cells by attacking beta cells. In an epigenome-wide association study (EWAS) in 52 monozygotic twins, epigenetic modification patterns have been mapped in CD4+ T cells, CD19+ B cells, and CD14+ monocytes [150]. This study has identified a substantial enrichment of differentially variable CpG positions [150], suggesting the involvement of DNA methylation in T1D. In addition, differential DNA methylation status on 88 CpG sites has been found in lymphoblast cell lines which are derived from 6 pairs of monozygotic twins concordant for T1D and 3 pairs of monozygotic twins discordant for T1D, separately. In these cell lines, the altered expression of genes, including Hla, Ins, and Il2rb, are involved in immune responses [151]. Furthermore, dysregulated DNA methylation has been found in Pdchb16, Magi2, and Fancc in T1D-discordant monozygotic twins [152]. DNA demethylation on transcription factor HOXA9 has been observed in T1D patients [153]. DNA hypermethylation has been found in the promoter region of Foxp3, which represses the binding of transcription factor IRF-7 to Foxp3, resulting in the reduced number of regulatory T cells in the peripheral blood from T1D patients [154]. More interesting, the serum levels of unmethylated preproinsulin DNA might serve as a biomarker for T1D [152].

Histone Modifications in T1D

Studies have revealed that HDAC expression is dysregulated in T1D patients. Decreases in H3K9Ac in promoter regions of *HLA-DRB1* gene and an increase in H3K9Ac at the promoter/enhancer region of *HLA-DQB1* gene have been reported in patients with T1D, and both genes are highly associated with T1D [155]. Upregulated acetylated histone H4 levels have been observed to be positively associated with T1D patients without vascular complications, indicating a protective role of acetylated histone H4 against vascular injury [156]. In addition, a significant increase in methylation levels of H3K9me2 has been found in several high-risk genes for T1D, such as *CTLA4* gene [153].

MiRNAs in T1D

Accumulating evidence has shown a pathogenic role for miRNA in the initiation and development of T1D. miR-326 has been found to be significantly increased in PBMCs from patients with T1D and positively correlated with disease severity [157]. Downregulated expression of miR-21a and miR-93 has been noticed in the PBMCs from T1D patients in the presence of glucose [158]. Moreover, global miRNA profiles in PBMCs from newly diagnosed T1D patients have revealed that the most downregulated miRNA, miR-146, is associated with the ongoing autoimmune imbalance in T1D patients [159].

Aberrant Epigenetic Modification in PBC

PBC is a chronic, cholestatic autoimmune liver disorder which resulted from both genetic and environmental factors [160]. It is a life-threatening disease which may further progress to liver cirrhosis and eventually liver failure.

DNA Methylation in PBC

DNA methylation profiles in 60 differentially methylated regions corresponding to 51 genes on the X chromosome and 9 genes on autosomal chromosomes have been revealed in PBC twins and normal twins. DNA hypermethylation has been observed in specific gene families, such as ATP12A, ATP5A1, and HOXD4 [161]. DNA hypomethylation at the CD40L promoter has been observed, and the methylation level is negatively correlated with IgM serum levels in $CD4^+$ T cells from PBC patients [145], indicating the involvement of methylation modifications of CD40L in the development of PBC.

Histone Modifications in PBC

Dysregulated histone modifications of genes have been also reported in autoreactive T cells with PBC patients, including upregulated histone H4 acetylation in the promoter regions of *CD40L*, *LIGHT*, *IL17*, and *IFNG* genes and downregulated histone H4 acetylation in the promoter regions of *TRAIL*, *Apo2*, and *HDAC7A* genes [162].

MicroRNAs in PBC

A total of 35 independent miRNAs has been identified to be differentially expressed in the tissues from PBC patients. There miRNAs are predicted to targeting genes, which belong to cell proliferation, apoptosis, inflammation, oxidative stress, and metabolism. Among these miRNAs, the reduced expression of miR-122a and miR-26a and the increased expression of miR-328 and miR-299-5p have been further validated in PBC patients [163]. miR-26a has been further investigated as a posttranscriptional regulator and contributor of the overexpression of a polycomb-group protein EZH2 in PBC patients [164, 165].

All dysregulated epigenetic modifications are listed in Tables 6.1 and 6.2.

Table 6.1 Dysregulated DNA methylation levels in autoimmune diseases: SLE, RA, SSc, T1D, and PBC

Disease	Cell types	Modified genes and DNA methylation status	References
SLE	Whole blood	*IFI44L*: hypomethylation FOXP3 TSDR: hypermethylation	[47] [45]
SLE	PBMCs	Global, *Era*: hypomethylation	[36, 119]
SLE	T cells	X chromosome genes, *IL4*, *IL6:* hypomethylation	[166–168]
SLE	$CD4^+$ T cells	Global, IFN-regulated genes, *perforin*, *PP2Aca*, *KIR2DL4*, *CD11a*, *CD70*, *CD40L*, *IL10*, *IL13:* hypomethylation	[169–176]
SLE	Naive $CD4^+$ T cells	IFN-regulated genes, *MIR886*, *TRIM69*, *CHST12*: hypomethylation	[40, 41, 177]
SLE	B cell	IFN-regulated genes: hypomethylation *LINE-1:* hypomethylation	[170] [56]
SLE	Monocytes	IFN-regulated genes: hypomethylation	[170]
Psoriasis	Keratinocytes	*Sfrp4:* hypermethylation *LINE:* hypermethylation	[108] [109]
RA	PBMCs	*IL6, Era*: hypomethylation	[119, 121, 178]
RA	T cells	Global: hypomethylation	[38, 120]
RA	$CD4^+$ T cells	*CD40L*: hypomethylation	[121]
RA	Fibroblast-like synoviocytes	Global, *L1 retrotransposon*: hypomethylation	[122, 179]
RA	Synovial fibroblasts	Global, *CXCL12*: hypomethylation	[123, 124]
SSc	$CD4^+$ T cells	Global, *CD40L CD11a*, *CD70*: hypomethylation	[139–142]
SSc	Dermal fibroblasts	*FLI1*, TGF-beta-related genes: hypermethylation	[145–147]
T1D	PBMCs	*HOXA9*: hypomethylation	[153]
T1D	Treg cells	*Foxp3*: hypermethylation	[154]
PBC	PBMCs	*ATP12A*, *ATP5A1*, and *HOXD4: hypermethylation*	[161]
PBC	$CD4^+$ T cells	*CD40l: hypomethylation*	[145]

Table 6.2 Dysregulated miRNA levels in autoimmune diseases: SLE, RA, SSc, T1D, and PBC

Disease	Origins	Levels of miRNAs	Target genes	References
SLE	PBMCs	miR-155: + miR-146a: –	*PP2Ac IFN-a and IFN-b*	[180, 181]
SLE	T cells	miR-21: + miR-31: –	*PDCD4 RhoA*	[77, 79]
SLE	$CD4^+$ T cells	miR-142-3p/5p: – miR-21, miR-148a, miR-126, and miR-29b: +	*SAP, CD84, and Il10 DNMT1*	[74–76, 78]
SLE	B cells	miR-30a: + miR-1246: –	*Lyn EBF1*	[84, 88]
Psoriasis	T cells	miR-210: + miR-17-92 cluster, miR-let-7b, miR-194	*HIF-1-alpha* –	[111] [112–114].
RA	T cells	miR-223: –	*IGF-1R*	[138]
RA	$CD4^+$ T cells	miR-146a: +	*FAF1*	[182]
RA	Synovial fibroblasts	miR-155: +	*MMP-3*	[133, 183]
SSc	Fibroblasts	miR-21: + miR-29a: – miR-196a: +	*Smad7* Type I and III collagen Type I collagen	[184–187]
T1D	Plasma	MicroRNA-16-5p, MicroRNA-17-5p, and MicroRNA-20a-5p: +	–	[188]
T1D	Plasma-derived exosome	miRNA signature	–	[189]
T1D	Treg	miR-125a-5p: +	*CCR2*	[190]
T1D	Beta cell	MicroRNA-503: +	*mTOR pathway*	[191]
T1D	Plasma	miRNA profile, miRNA-320a, and miRNA-486	–	[192, 193]
T1D	Urine	miRNA profile	–Predict disease	[194]
PBC	Liver tissue	miR-122a, miR-26a: – miR-328, miR-299-5p: +	–	[163]
PBC	Liver tissue	miR-26a: +	*EZH2*	[164, 165]

+ increased, – decreased

Conclusion

As the epigenetic era approaches, more and more evidence have demonstrated a key role of epigenetic regulations in the pathogenesis of autoimmune diseases. Newly discovered lncRNA, extra RNAs, and circle RNAs have begun to undergo significant research into their roles in disease pathogenesis. The specific epigenetic regulations in autoimmune diseases might provide potential biomarkers for diseases. For example, in our previous study, the DNA methylation level of the IFI44L promoter is both sensitive and specific in lupus patients and lower in nephritis patients than in patients without renal damage [165], indicating an organ-specific biomarker to predict LN. Another urgent need is to be able to translate research findings into clinical application. The most significant challenges include complex techniques, time consuming and the high cost of both DNA methylation arrays and bisulfite next-generation sequencing. To solve this problem, as in our study of IFI44L, rather than pyrosequencing of IFI44L DNA methylation levels, we have developed a high-resolution melting (HRM) analysis for detecting IFI44L DNA methylation levels, which can be easily completed with QPCR. This new technique may be more available for clinical use in the future. With regard to treatment, as our new findings on miR-210 in mouse psoriasis treatment, miRNAs might provide alternative options to currently used drugs. The application of CRISPR-Cas9 may shed light by guiding epigenetic modifications on specific genes. Together, epigenetic modifications provide additional tools for broadening the understanding of autoimmune diseases, as well as development of potential biomarkers and therapies to provide alternative strategies.

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Bile Acids and Bilirubin in Liver Immunology

7

Ci Zhu, Thierry Claudel, and Michael Trauner

Key Points

- Bile acids (BAs) are cholesterol-derived molecules circulating within the enterohepatic circulation via specific transport systems allowing them to pass the membranes of hepatocytes and enterocytes.
- Historically, BAs have been viewed as emulsifying agents facilitating the digestion and absorption of dietary lipids, lipophilic vitamins, and steroids.
- Recent studies have uncovered the hormone-like signaling functions of BAs that modulate a myriad of metabolic and inflammatory pathways in multiple cell types and tissues via dedicated BA receptors in the nucleus (e.g., FXR) and plasma membrane (e.g., TGR5).
- Accumulating evidence suggests that bilirubin also has active signaling roles in immune cells beyond its well-established antioxidant effects.
- Interactions of BAs and bilirubin with the hepatic and mucosal immunological microenvironment and gut microbiome modulate inflammation and immunity in the liver and intestine.
- Deregulated BA and bilirubin metabolism and/or transport has been implicated in the pathogenesis of a variety of diseases including cholestatic and metabolic liver diseases, hepatic malignancies, and inflammatory bowel disease.
- Although the functions of BAs and bilirubin in the regulation of inflammation and immunity are only beginning to be appreciated, targeting BAs and their cellular receptors (e.g., FXR, TGR5) represents an important and highly promising area of drug discovery.

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Introduction

Bile was first documented as an effective remedy and detergent in Ebers Papyrus back to circa 1550 B.C [1]. Bile acids (BAs) are amphipathic molecules and the major constituents of bile. BAs are exclusively synthesized by hepatocytes from cholesterol as primary BAs, i.e., cholic acid (CA) and chenodeoxycholic acid (CDCA) in humans, and conjugated with either glycine (human) or taurine (rodents) to become negatively charged before secretion into bile, where they form mixed micelles with phospholipids and cholesterol. In addition to these biliary lipids, bile also contains conjugated bilirubin, glutathione, bicarbonate, electrolytes, proteins, and water [2].

Much of our original concepts on BAs have centered on pro-inflammatory and cytotoxic actions due to their detergent-like chemical properties (required for lipid digestion and absorption) disrupting cell membranes and promoting the secretion of cytokines and chemokines. BAs are major driver of cell damage, hepatic inflammation, fibrogenesis, and carcinogenesis (reaching concentrations up to 200 μM in humans) particularly in cholestatic liver disease [3]. Furthermore, systemic BA accumulation may also damage extrahepatic organs and tissues such as the kidney (e.g., cholemic nephropathy) [4, 5]. Tissue inflammation and injury triggered by BAs have been closely related to the level of hydrophobicity which correlates their potentials to attack cell membranes [6, 7]. Conversely, BA-based therapeutics have so long mainly focused on hydrophilic less cytotoxic BAs, such as ursodeoxycholic acid (UDCA) which has been broadly tested for a myriad of cholestatic and metabolic liver diseases over the past decades [7, 8].

Apart from these chemical detergent actions, BAs are increasingly recognized as signaling molecules and ligands for a range of nuclear hormone receptors (NRs) as well as extracellular G protein-coupled receptors (GPCRs) [9]. Through ligand binding to these receptors, BAs can control not only their own synthesis and transport but also lipid, glucose, and energy metabolism [10] as well as immune responses in the liver and gut or even systemically [11].

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Therefore, direct modulation of BA receptor activity by naturally occurring or synthetic receptor agonists or antagonists represents a promising approach to managing metabolic and inflammatory disorders of the liver, intestine, and perhaps also beyond [12–14].

Bilirubin is another important bile component that has antioxidant and immunomodulatory properties. Bilirubin has been identified as a ligand-activating nuclear receptor, linking BA, lipid, and xenobiotic metabolism.

In this chapter, we summarize recent advances in the understanding of BA signaling in modulation of liver and gut inflammation and immunity in preclinical and clinical studies, with a special focus on BA-modulated signaling pathways through dedicated BA receptors and subsequently derived BA-based therapeutics. In addition, this chapter also covers the emerging immunomodulatory and anti-inflammatory properties of bilirubin.

Overview of BA Homeostasis

Biliary excretion of BAs is the driving force for bile flow and facilitates hepatobiliary secretion of lipids, endogenous metabolites, and xenobiotics [15]. Bile is enriched in bicarbonate in the bile ducts and concentrated (by absorption of water) during storage in the gallbladder, from where it reaches the intestine at the level of the duodenum [16]. After meal ingestion, bile is released from the gallbladder into the small intestine to facilitate intestinal digestion and absorption of dietary lipids, lipophilic vitamins, and steroids [17]. The majority (>95%) of BAs is efficiently reabsorbed in the terminal ileum and recycled back to the liver via the portal vein (where they reach concentrations of 20–50 μM) in a process termed "enterohepatic circulation" which depends on active transport systems expressed in enterocytes and hepatocytes (Fig. 7.1) [2]. Bilirubin is also actively trans-

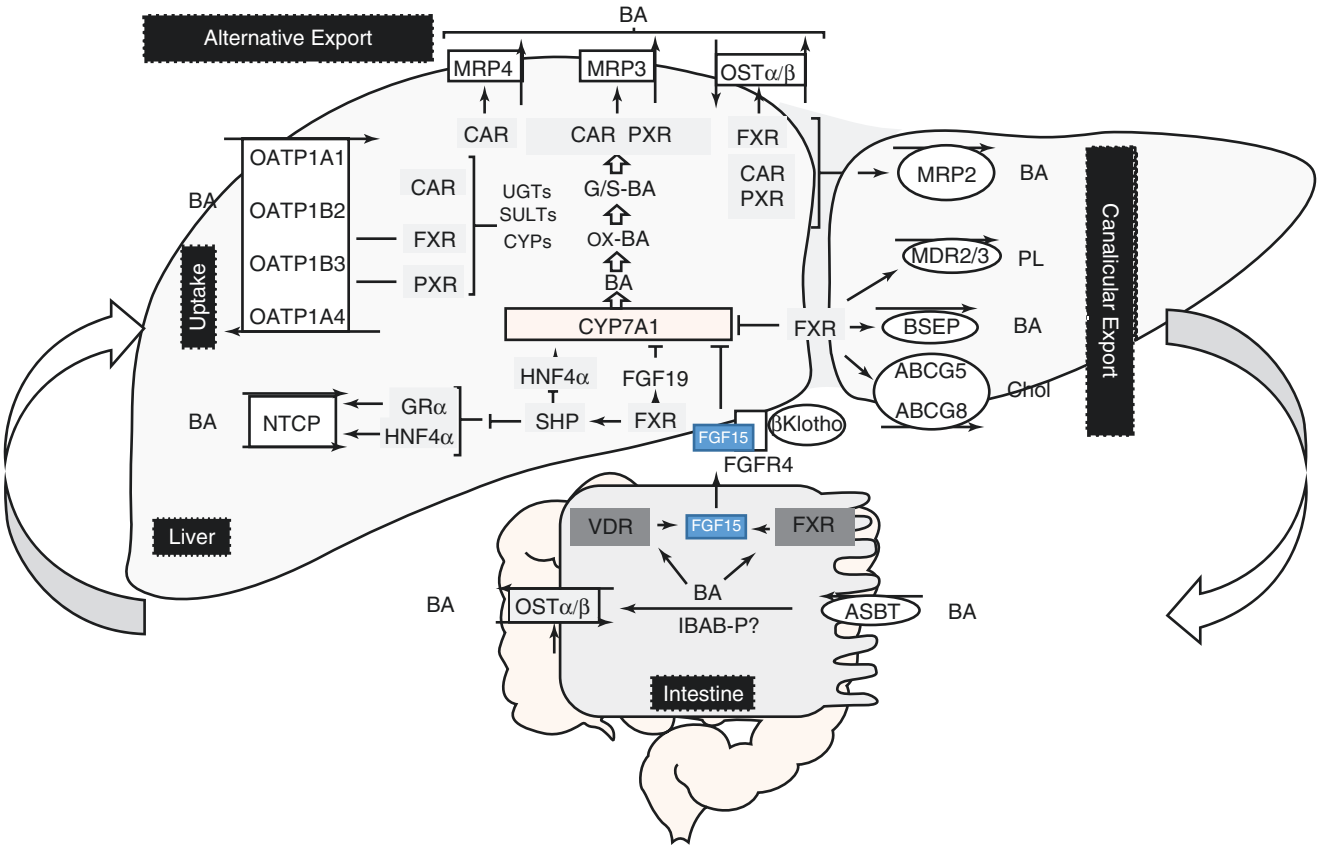

Fig. 7.1 Transcriptional regulation of hepatic BA homeostasis. Bile acids (BAs) are taken up in hepatocytes by NTCP and OATP1 family of transporters. FXR and CAR upregulate the OATP1 transporter expression, while GR (in human) and HNF4α in mouse upregulate NTCP expression. BA-activated FXR increases the expression of the nuclear receptor SHP, which can inhibit GR and HNF4α, thus lowering NTCP and CYP7A1. FXR can also activate FGF19 expression which upon binding to FGFR4 and β-Klotho represses CYP7A1 expression. BA can be oxidized by CYPs, conjugated by SULT, or glucuronidated by UGTs which are regulated by the nuclear receptors FXR, CAR, and PXR. BA in excess can be excreted into the blood via the transporters MRP3 (controlled by CAR and PXR) or MRP4, controlled by CAR, or OSTα/OSTβ which is controlled by FXR. In normal conditions, BA is secreted into the biliary canaliculus via the transporters BSEP and MRP2 which are controlled by FXR and FXR/CAR/PXR, respectively. FXR also promotes the expression of the phospholipid transporter MDR2/3 and the cholesterol transporters ABCG5/ABCG8. In the intestine bile acids are absorbed by the transporter ASBT and sent to the basolateral membrane via IBAB-P at least in female mice, where they can be secreted to the portal blood via OSTα/OSTβ. In the intestine bile acids can activate the nuclear receptors VDR and FXR which in turn upregulate the expression of FGF19 in human and Fgf15 in mice. FGF19/fgf15 will reach the hepatocytes by the portal circulation and activate FGFR4 and β-Klotho to repress CYP7A1 expression. BA bile acid, Chol cholesterol, PL phospholipids, G/S-BA glucuronidated or sulfated bile acids; block arrows, inhibition; arrows, activation

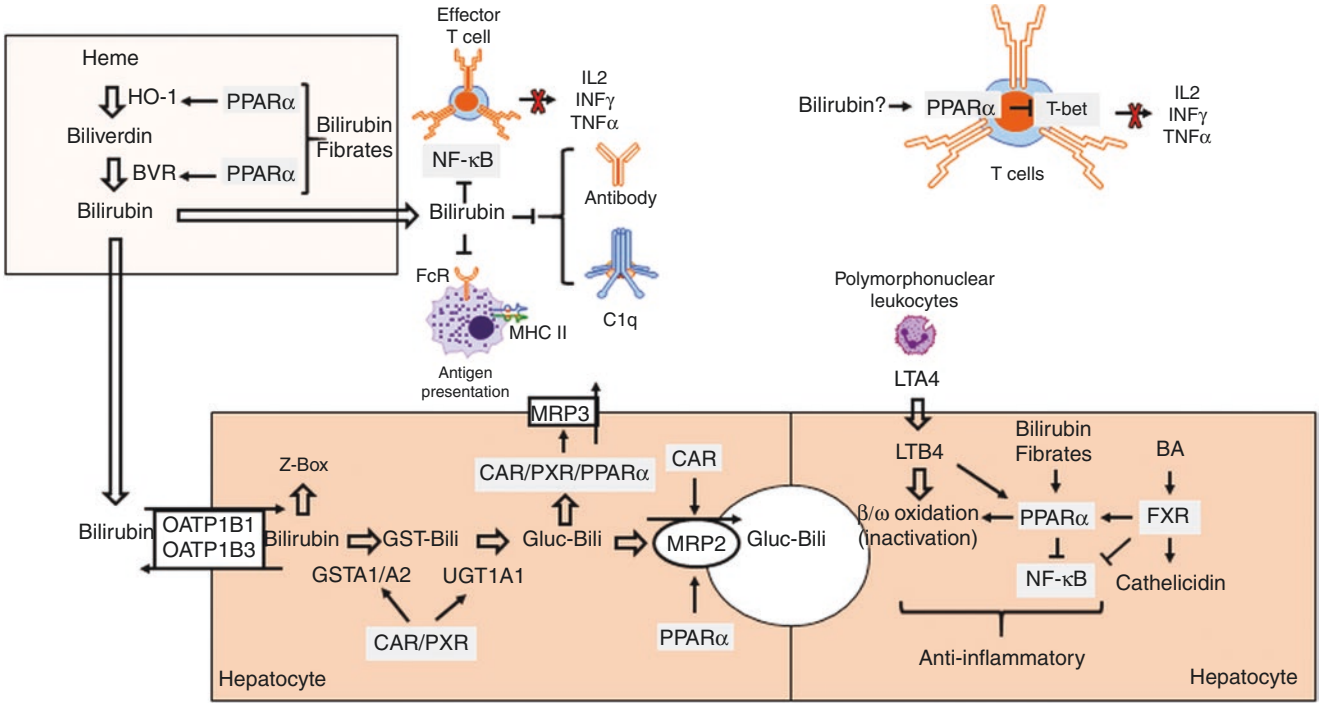

Fig. 7.2 Bilirubin synthesis, transport, and regulation of immunity. Heme is oxidized into biliverdin via HO-1 a target of the nuclear receptor PPARα, which can be activated by fibrates and bilirubin. Biliverdin is oxidized into bilirubin by BVR which is controlled by PPARα. Bilirubin in the blood can inhibit NF-κB in T cells and its target genes (IL2, IFN-γ, TNFα). Bilirubin blocks the interactions between antibodies and the complement protein C1q. Bilirubin also inhibits FcR and the MHC class II complex expression in antigen-presenting cells. Bilirubin can be taken up in hepatocytes by the transporters OATP1B1 and OATP1B3. Bilirubin is then either conjugated with glutathione by GSTA1 and GSTA2 and subsequently glucuronidated by UGT1A1 or metabolized into Z-BOX. The nuclear receptors CAR and PXR control the expression of UGT1A1 as well as GSTA1 and GSTA2. Glucuronidated bilirubin can be excreted into the blood via the transporter MRP3 which is controlled by the nuclear receptors CAR, PXR, and PPARα; alternatively, glucuronidated bilirubin can be excreted into the bile by the transporter MRP2 which is controlled by the nuclear receptors CAR and PPARα. Polymorphonuclear leukocytes generate LTA4 which is subsequently metabolized in hepatocytes into LTB4 an endogenous ligand activating the nuclear receptor PPARα, which inhibits its NF-κB activation and promotes LTB4 degradation by inducing ω and β oxidation. BA-activated FXR upregulates PPARα expression and promotes the antimicrobial peptide cathelicidin expression and inhibits NF-κB activation. In T cells the unliganded nuclear receptor PPARα inhibits the transcription factor T-bet which upregulates Il2, IFN-γ, and TNFα. Whether bilirubin activating PPARα can also inhibit T-bet is currently unknown. HO-1 heme oxygenase-1, BVR biliverdin, PPARα peroxisome proliferator-activated receptor α, OATP1B1 solute carrier organic anion transporter family member 1B1, OATP1B3 solute carrier organic anion transporter family member 1B3, GSTA1 glutathione S-transferase A1, GSTA2 glutathione S-transferase A2, UGT1A1 UDP glucuronosyltransferase family 1 member A1, CAR constitutive androstane receptor, PXR pregnane X receptor, MRP2 multidrug resistance-associated protein 2, LTB4 leukotriene B4. Block arrows, inhibition; arrows, activation

ported by hepatocytes (Fig. 7.2) and can also undergo an enterohepatic circulation (see below). During the postprandial state, BAs can escape the hepatic uptake resulting in systemic exposure of BAs at low (5–10 μM) physiological signaling levels [18]. BAs, which are glomerularly filtrated, are reabsorbed in the kidney, thus limiting the renal loss of BA into urine under normal conditions [18]. Once they have entered the ileum and the colon, BAs are transformed by gut microbiota by deconjugation and dehydroxylation to generate secondary BAs, such as deoxycholic acid (DCA) and lithocholic acid (LCA), thus expanding the molecular and biological repertoire of the BA pool (Table 7.1) [19].

BA Synthesis

Primary BAs are synthesized from cholesterol in hepatocytes via two tightly regulated pathways, namely, the "classical/neutral" leading to CA and the "alternative/acidic" leading to CDCA synthesis. BAs are obtained after several oxidation steps catalyzed by cytochrome P450 (CYPs), where CYP7A1 is the rate-limiting step [2]. CDCA is the most potent human agonist activating the nuclear receptor farnesoid X receptor (FXR/NR1H4) [20–22]. FXR heterodimerizes with the retinoid X receptor alpha (RXRα/NR2B1), activating the transcription of the nuclear receptor small heterodimer partner

Table 7.1 Bile acid nomenclature, biological activities, and key target genes

Bile acid	Full name	Origin	Biological activity	Target gene
CA	Cholic acid	Liver (Cyp7a1)	FXR agonist	NF-κB
CDCA	Chenodeoxycholic acid	Liver (Cyp27a1 and Cyp7a1)	Most potent FXR agonist in human but not in mouse; FPR receptor antagonist	NF-κB COX2
LCA	Lithocholic acid	Intestine: 7α-dehydroxylation of CDCA and/or 7α-dehydroxylation of UDCA	FXR, PXR, CAR VDR, TGR5 agonist	NF-κB MRP3 MRP4 FGF19 NF-κB
UDCA	Ursodeoxycholic acid	Intestine: hydroxysteroid dehydrogenization of CDCA	FXR partial agonist GRα agonist	NF-κB NTCP
DCA	Deoxycholic acid	Intestine: 7α-dehydroxylation of CA	FXR, TGR5, S1PR2, FPR receptor antagonist	NF-κB NF-κB FXR COX2
3-OxoLCA	3-Oxolithocholic acid		RORγ	IL17A

(SHP/NR0B1) [23], mediating CYP7A1 suppression by suppressing hepatocyte nuclear factor 4 alpha activity (HNF4α/NR2A1) (see Fig. 7.1) [24]. Moreover, BA-dependent activation of FXR in the ileum also downregulates hepatic BA synthesis via the secretion of fibroblast growth factor 15/19 (FGF-15 in mice; FGF-19 in humans), which binds to its receptor on hepatocytes (FGFR4/βKlotho) suppressing CYP7A1 transcription [25] via HNF4 [24] (see Fig. 7.1). LCA can also activate the vitamin D receptor (VDR; NR1I1) in the intestine [26], and VDR also increases FGF15/19 expression (see Fig. 7.1) [27]. Primary BAs are conjugated with taurine or glycine by the BA-CoA amino acid N-acyltransferase enzyme, making them suitable for a controlled transport across membranes (see Fig. 7.1).

BA Transporters and Detoxification

BA uptake is mediated by an Na^+-dependent bile acid transporter NTCP (SLC10A1) and various family members of multi-specific organic anion transporters (OATPs; SLC21A) mediating Na+-independent uptake of amphipathic organic compounds, like conjugated/unconjugated BA, or bilirubin. Quantitatively Na+-independent bile acid uptake is less important and mediated by facilitated exchange with intracellular anions (e.g., glutathione (GSH), bicarbonate HCO_{3^-}) (see Fig. 7.1) [28]. NTCP regulation by BA differs among humans, mice, and rats [29]. FXR/SHP-dependent and FXR/SHP-independent mechanisms limit hepatocellular BA uptake by NTCP in rats and mice [2] via HNF4 [30] (see Fig. 7.1), while in human SHP suppresses the activation of NTCP mediated by the glucocorticoid receptor (GR/NR3C1) [31] (see Fig. 7.1) a mechanism explaining NTCP downregulation in cholestasis (for review see [2]). In humans, FXR also represses OATP1B1 expression – the major sodium-independent BA transporter – via SHP and HNF4α [32] but upregulates OATP1B3 which transports xenobiotics and probably BA [33].

BAs are excreted from the hepatocytes into the bile at the canalicular membrane by the bile salt export pump (BSEP/ABCB11) and form mixed micelles with phosphatidylcholine secreted by the multidrug resistance protein 3 (rodents Mdr2/human MDR3/ABCB4), while cholesterol is excreted by the two half- transporters ABCG5/ABCG8 (see Fig. 7.1) [2]. BSEP [34], ABCB4 [35], and ABCG5/G8 [36] are all upregulated by FXR. While BSEP transports monovalent BAs (CA, UDCA, CDCA, and their tauro- and glyco-conjugated bile salts), MRP2 transports bilirubin (see Figs. 7.1 and 7.2) and organic anions, conjugated with glutathione, glucuronidate, and/or sulfate as well as divalent BAs with two negative charges (sulfated tauro- or glyco-LCA) [37]. In addition to FXR, other promiscuous nuclear receptors activated by LCA such as pregnane X receptor (PXR; NR1I2) [38, 39] and constitutive androstane receptor (CAR; NR1I3) [40, 41] upregulate MRP2 expression (see Fig. 7.1) [42].

Since BA in excess can be cytotoxic, they can be oxidized by CYP3A4 via FXR, PXR, and CAR induction [43–46] to become oxo-BA and subsequently conjugated with sulfate by the sulfotransferase SULT2A1 upregulated by FXR [47], PXR [48], and CAR [41] or glucuronidated by UDP-glucuronosyltransferase 2B4 (UGT2B4) after FXR induction [49] (see Fig. 7.1).

Alternatively at the basolateral side, these BAs can be excreted into the blood and eliminated in urine by a variety of transporters such as MRP3 upregulated by CAR and PXR [45, 50], MRP4 upregulated by CAR [51], or the organic solute transporter α/β (OSTα/β/-SLC51A-51B) a heterodimer

upregulated by FXR [52, 53] (see Fig. 7.1). Notably, bilirubin is preferentially excreted via MRP3 (see Fig. 7.2), while MRP4 transports preferentially BAs. Altogether, these redundant transporters have low expression in normal conditions but are highly inducible and represent a safety mechanism protecting the liver from BA overload and explain the appearance of sulfated or glucuronidated BAs in the serum of cholestatic patients, which will then be eliminated after renal filtration [2].

Biliary BAs are reabsorbed in the terminal ileum by the apical sodium-dependent BA transporter (ASBT/SLC10A2). Cytoplasmic BAs bound to ileal BA-binding protein (IBABP) are then chaperoned to the basolateral surface – at least in female mice [54] – where they are exported into the portal blood by the heterodimer OSTα/β (see Fig. 7.1). Hepatic reuptake of BAs by NTCP and OATP (see Fig. 7.1), which takes place predominantly in periportal hepatocytes [55], completes the enterohepatic circulation of BAs characterized by less than 5% of loss at every cycle.

BAs can escape from ileal reabsorption and enter the colon, where they are unconjugated by bile salt hydrolase (BSH) and dehydroxylated by hydroxysteroid dehydrogenase (HSDH) bacterial enzymes converting them into secondary BA metabolites [19]. There are more than 20 secondary BA species identified in humans and murine by mass spectrometry, the most abundant being DCA, the 7-dehydroxylation product of CA; and LCA, the 7-dehydroxylation product of CDCA, in humans (see Table 7.1) [56]. Secondary BAs produced by gut flora via dehydroxylation can also be epimerized at the C-7 position to form UDCA (see Table 7.1), epimerized at the C-3 position to form "iso"-BAs, oxidated at various positions to generate "oxo"-BAs, or shortened of the C24 alkyl side chain to generate C23 "nor"-BAs [57–59] activating different receptors in a cell-specific manner (see below and Fig. 7.3).

Such critical and integrated control over BA biosynthesis, circulation, and transport contributes to a functional and circulating BA pool without inducing cytotoxicity. Moreover, direct modulation of the various BA receptors by natural or synthetic receptor modulators represents a promising approach to manage metabolic and inflammatory disorders of the liver, intestine, and perhaps also beyond [12–14].

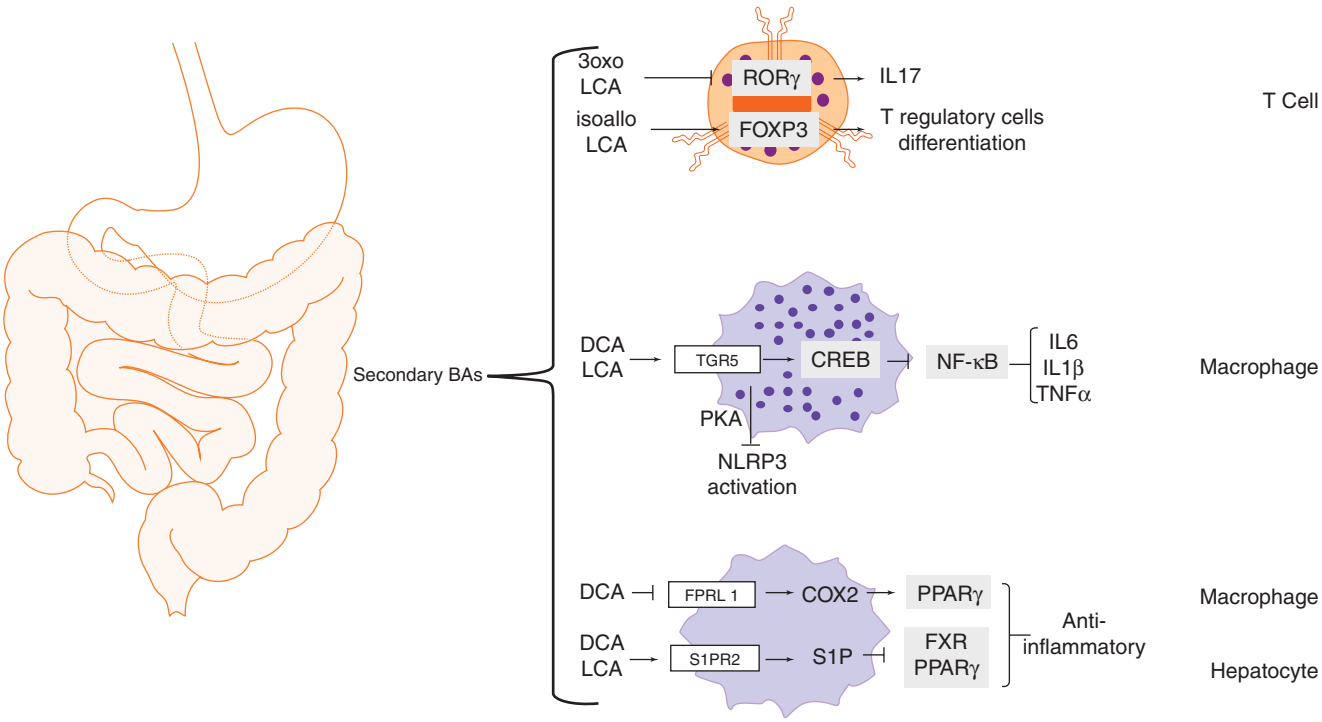

Fig. 7.3 Secondary bile acid metabolites as regulators of immunity. 3-OxoLCA obtained after bacteria metabolism inhibits RORγ, thus blocking IL17 secretion in T cells. IsoalloLCA obtained after bacteria metabolism activates FOXP3, thus increasing T cell differentiation. DCA and LCA activate the G-coupled receptor TGR5 in macrophages; TGR5 activation inhibits NRLP3 activation via PKA phosphorylation. TGR5 also increases the activity of the transcription factor CREB in the nucleus, which inhibits NF-κB and its target genes (IL6, IL1β, TNFα). DCA inhibits the G-coupled receptor FPRL1 in macrophages which controls the expression of COX2, the enzyme generating ligands activating the anti-inflammatory nuclear receptor PPARγ. DCA and LCA activate the G-coupled receptor S1PR2 leading to S1P accumulation in the nucleus of hepatocytes which inhibits the activities of the anti-inflammatory nuclear receptors FXR and PPARγ. LCA lithocholic acid, DCA deoxycholic acid, PKA protein kinase A, CREB cAMP response element-binding protein, PPARγ peroxisome proliferator-activated receptor γ, S1PR2 sphingosine-1-phosphate receptor 2, S1P sphingosine-1-phosphate. Block arrows, inhibition; arrows, activation

Cholestasis

Cholestasis may be due to mechanical obstruction of the bile ducts or impaired bile synthesis and formation [5]. BA-activated FXR, VDR, PXR, and CAR are induced in cholestasis, when both intrahepatic and systemic BA levels are rising, in order to coordinate a counter regulatory, potentially/partly protective response [60]. This response lowers BA uptake and de novo synthesis, while increasing BA oxidation, sulfation, glucuronidation, and BA excretion into the blood [2]. In addition, the same nuclear receptors will be activated in the kidney, intestine, and bile ducts [18]. In the kidneys, BA export will be increased in proximal tubular cells, and conversely BA reabsorption is diminished, therefore increasing BA loss in urine [61]. In the intestine ASBT is downregulated and limits BA reabsorption in the ileum [62]. Nevertheless, all these coordinated regulations fail to avoid cholestatic injury; therefore, further therapeutic approaches such as UDCA and other more novel therapies are applied. Apart from its hydrophilic and choleretic actions, UDCA seems to work at least in part by weakly activating GR [63] and PXR after bacterial LCA generation [38, 39] and by antagonizing FXR [64]. Alternatively, FXR agonists/modulators are another new therapeutic option (see below). Activating FXR reduces BA synthesis and inhibits NTCP (see Fig. 7.1) but also promotes BA excretion in the bile duct by BSEP and MRP2 induction, while protecting the bile duct by also inducing ABCG5/G8 and MDR3 expression, thus making micelles (see Fig. 7.1). Finally, FXR activation also increases oxidation (CYP3A4), sulfation (SULT2A1), and glucuronidation (UGT2B4) and via OST promotes alternative export resulting in decreased intracellular BA levels (see Fig. 7.1). Such FXR agonist like obeticholic acid (6-ethyl-CDCA) already showed promising results in primary biliary cholangitis (PBC) patients not responding to UDCA [65] or as monotherapy [66].

GR ligands can transactivate BA transporters in human (e.g., ASBT, NTCP, MRP2, BSEP) as well as CAR and as such may improve cholestasis by changing BA distribution and increasing their detoxification [67, 68]. Since UDCA was reported to activate GR [63], a combination of UDCA and dexamethasone may have synergistic beneficial effects perhaps together with obeticholic acid or other FXR activators, if the drugs are carefully designed and evaluated to avoid deleterious effects due to shared pathways involving their oxidation/conjugation via PXR/CAR/FXR target genes.

BAs and Intestinal Microbiome

Bile also has potent antimicrobial properties that can contribute to the selection or exclusion of certain species resulting in profound alterations of the composition of gut microbiome (see Chap. 8). Since large amounts of BAs have already been actively reabsorbed in the ileum, the magnitude of BA concentrations decreases from 5 to 20 mM to 400 μM in the colon, thus generating a niche favoring bacterial colonization and bacterial BA metabolism into secondary BAs [69]. The constitutively high BA concentration of the small intestine reaches the critical micellar concentration; this not only assists lipid emulsification but also promotes direct lysis of the bacteria that are sensitive to bile. Consequently, a reduction of intestinal BAs – due to liver injury in both clinical and experimental settings – is associated with excessive bacterial growth in the small intestine [70–73]. Intriguingly, the antimicrobial activities of BAs observed in vitro is not as pronounced as it was observed in vivo [74–77]. One proposed mechanism could be that BAs exist in mixed micelles in bile in vivo, together with other components including phospholipids, bilirubin, and long-chain fatty acids whose presence strengthens the direct bacteriostatic effects of BAs [78, 79]. Another important mechanism includes FXR activation by BAs and prompts secretion of antimicrobial peptide, such as defensins and cathelicidin by IECs [78–80], which could add to direct bacteriostatic BA effects.

Although BAs have potent antimicrobial properties, some BAs, on the other hand, can expand the growth of certain gut microbiota. Thus, BAs not only control but may also promote the (out) growth of pathobionts. Accordingly, one study reported that IL10-deficient mice receiving milk-fat diet upregulated synthesis of taurine-conjugated BAs, such as TCA, which in turn specifically promotes the expansion of *Bilophila wadsworthia*, a pathobiont associated with development and severity of colitis. Similar to *Bilophila wadsworthia*, some other intestinal bacteria such as *Helicobacter hepaticus* and *Listeria monocytogenes* are also highly favored by the presence of bile [81, 82]. The metabolic byproducts of these bacteria, such as H2S or secondary BAs, may further compromise mucosal integrity leading to enhanced inflammatory cell infiltration and thus act synergistically with bacterial antigens that stimulate specific immune response to induce tissue injury. Furthermore, a "leaky gut" may promote hepatic bacterial translocation through the gut-liver axis, which may be relevant for the pathogenesis and progression of many liver diseases, such as primary sclerosing cholangitis (PSC). As such, in the Mdr2 (Abcb4)-deficient murine model of PSC, cholestasis induced gut dysbiosis and promoted endotoxin translocation into the portal vein subsequently resulting in NLRP3 inflammasome activation increasing cholestatic liver injury [83]. Notably, disruption of gut integrity resulting by pore-forming *Klebsiella pneumonia* driving a Th17 response has recently received considerable attention [84], although the role of BAs in this context has not yet been clarified [85].

Intriguingly, BAs can also serve as signaling molecules of the intestinal microbiota to regulate chemokine levels in the

liver [86, 87]. As such, manipulating gut commensal bacteria in mice reduces conversion into secondary BAs and stimulates production of primary BAs, such as CDCA. Relatively higher amount of CDCA upregulate CxCL16 expression on LSECs, which in turn specifically recruited CXCR6+ natural killer T (NKT) cells, thus inducing antitumor activities in the liver [86, 87].

Dynamic Interplay Between BAs and the Immune Cells

The liver is exposed to gut bacterial metabolites and products via portal blood from the intestine which comprises 70% of the entire liver blood supply. Approximately 80% of the liver volume is composed of hepatocytes that fulfill the metabolic and detoxifying demands of the body, while the remaining cells are populated with non-parenchymal cells including liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), Kupffer cells (KCs), dendritic cells (DCs), monocytes, T cells, and NKT cells (see Chap. 2) providing a dynamic niche for close interactions among immune cells and hepatic metabolites, such as BAs.

BAs as Mediators Driving Hepatic Injury and Inflammation

BAs exert varying biological actions depending on their hydrophobicity and concentration spectrum [3]. High-level hydrophobic BAs have long been considered as key players driving hepatobiliary injury due to their well-known cytotoxic properties attacking liver cell membrane and inducing necrosis [88]. It still remains controversial whether BAs in cholestatic hepatocytes ever reach concentrations that are directly toxic. Actually the most abundant BAs under cholestatic condition are non toxic BAs, such as TCA and GCA, while the major toxic BAs, GCDCA, only present at concentration of less than 30 μM in the serum [6, 89]. The level of toxic BAs within the liver of most cholestatic animals and patients is usually below 10 μM, and serum BA level in cholestatic patients rarely exceed 150–200 μM [89–91]. However, studies reporting that BAs can damage mitochondria, induce oxidative stress to the endoplasmic reticulum (ER), and activate Fas or TRAIL-dependent cell death signaling are based on the observations primarily derived from in vitro culture system incubating isolated hepatocytes with BAs whose concentrations exceeded those normally found in the serum or liver of cholestatic animal models or patients with cholestatic liver diseases [92–94]. It has been shown that BAs at pathophysiological concentration are unlikely to initiate necrosis in cholestatic hepatocyte unless their concentrations reach extremely high level at mM range [89], suggesting BA-induced liver cell injury during cholestasis may not result from their direct cytotoxic effects but from other alterative mechanisms. It has been shown that BAs at concentration of 100 μM can already trigger a hepatocyte-specific inflammatory response initiated by ER stress and mitochondrial damage, resulting in release of mitochondrial DNA and proteins which in turn stimulates an innate immune response by activating Toll-like receptor 9-dependent and possibly other independent signaling pathways, thus inducing inflammatory chemokine CCL2 and CXCL2 expression which recruits neutrophils, and the neutrophil-mediated inflammatory response results in hepatocyte necrosis [95] (Fig. 7.4). Other studies have shown that BAs can also act as inflammagens directly upregulating hepatocellular expression of early growth response factor-1, a transcription factor required for inflammation to occur in the liver during cholestasis, to stimulate production of pro-inflammatory mediators in cholestatic mice [96] (see Fig. 7.4). Dead hepatocytes release alarmins that activate the inflammasome in macrophages/Kupffer cells, orchestrating an immune response that contributes further to the hepatocyte injury [97] (see Fig. 7.4). Another study revealed that BAs, in particular CDCA, DCA, and their taurine conjugates, can directly act as critical danger-associated molecular patterns to stimulate NLRP3 inflammasome activation in macrophages by inducing prolonged calcium influx synergistically with LPS under cholestatic condition, which can be directly counteracted by FXR activation [98] (see Fig. 7.4).

BAs Promote Neutrophil-Driven Hepatic Immunopathology

Hydrophobic DCA and CDCA activate Erk1/2 and Egr-1 signaling which upregulate expression of neutrophil chemoattractant CxCL1 and adhesion molecule ICAM-1 in hepatocytes [99]. LCA also enhances superoxide production by neutrophils during cholestasis [100], which can contribute to hepatocyte and LSEC damage under cholestatic liver injury such as bile duct ligation (BDL) [101].

BAs Abrogate Immunological Tolerance Induced by KCs and DCs

In addition to scavenging endotoxin, clearing apoptotic cells, and defending against pathogens, liver-resident KCs and DCs have been implicated in hepatic tolerance by priming FoxP3$^+$ T regulatory cells (Tregs) to prevent the establishment of effector T lymphocyte response against self-expressing antigens in the liver [102]. Hepatic inflam-

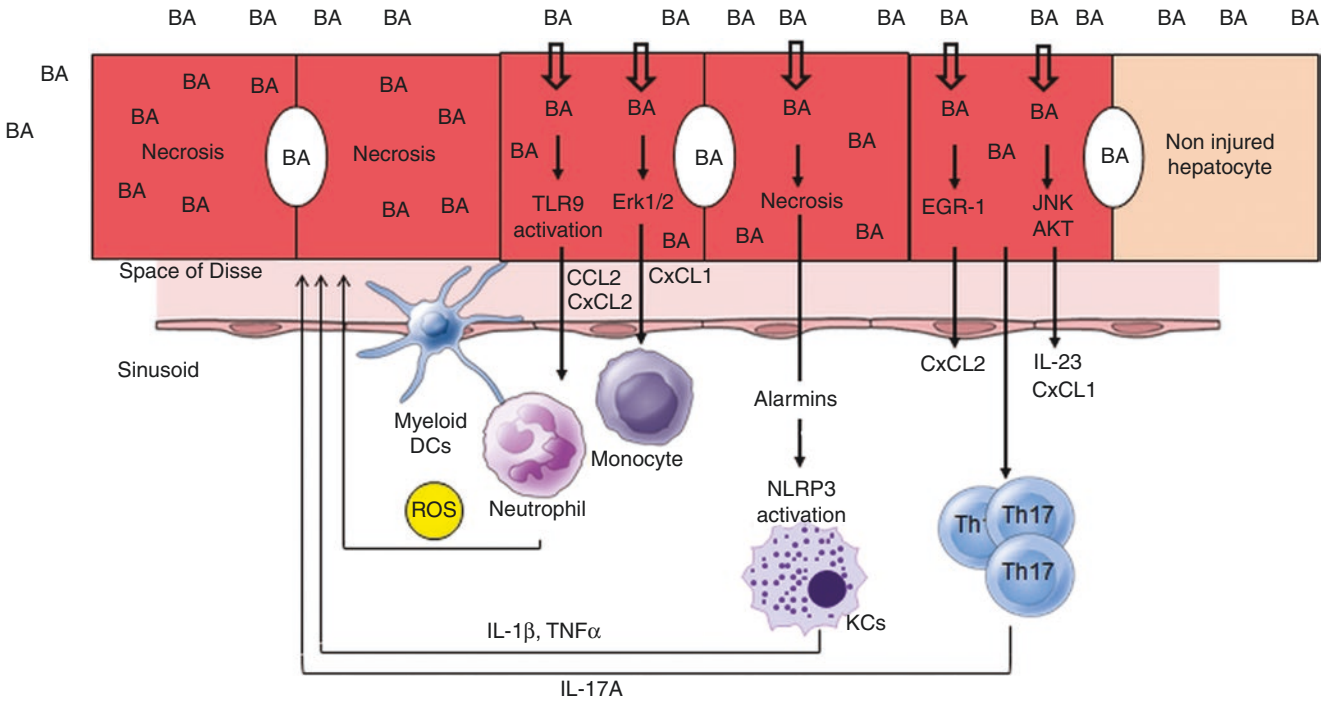

Fig. 7.4 BAs trigger hepatic inflammatory responses. BAs stimulate an innate immune response by activating TLR-9-dependent and possibly other independent signaling pathways, thus inducing inflammatory chemokine CCL2 and CXCL2 expression which recruits neutrophils and monocytes which mediate inflammatory response results in hepatocyte necrosis. Hydrophobic DCA and CDCA activate Erk1/2 and Egr-1 signaling which upregulates expression of neutrophil chemoattractant CXCL1. BAs act as inflammagens directly upregulating hepatocellular expression of EGR-1, a transcription factor required for inflammation to occur in the liver during cholestasis, to stimulate production of pro-inflammatory mediators in cholestatic mice. Necrotic hepatocytes release alarmins that activate the inflammasome in macrophages/Kupffer cells, orchestrating an immune response that contributes further to the hepatocyte injury. BAs, in particular CDCA, DCA, and their taurine conjugates, can directly act as critical danger-associated molecular patterns to stimulate NLRP3 inflammasome activation in macrophages by inducing prolonged calcium influx synergistically with LPS under cholestatic condition, which can be directly counteracted by FXR activation. BA retention may amplify Th17 cell infiltration and response by inducing hepatic production of MIP-2/CxCL2 and other cytokines via upregulating Egr-1. In addition, BAs activate AKT- and JNK-associated pathways in hepatocytes leading to IL23 upregulation, which fuels Th17 expansion and strengthens IL17A expression. BA bile acid, TLR-9 Toll-like receptor 9, EGR-1 early growth response factor-1, KC Kupffer cell, ROS reactive oxygen species

mation abrogates the immune-tolerant microenvironment established by KCs and DCs, resulting in immunogenic reprogrammation of antigen-specific $CD4^+$ T cells [103, 104]. Infiltration of monocyte-derived macrophages in cholestatic livers contributes to abrogate the tolerogenic KC phenotype [105, 106], and KC cytokines upregulate expression of LSEC adhesion molecules, such as ICAM-1, ICAM-2, and VCAM-1, thus enhancing monocyte recruitment in a vicious cycle [106]. BAs have been shown to convert the tolerogenic phenotype of liver-resident DCs into a hyper reactive phenotype [107] by selective expansion of CD11c $^+$CD11b$^+$ CD8α$^-$ liver myeloid DCs [107] and by increasing antigen presentation, thus making allogeneic and syngeneic T lymphocytes pro-inflammatory [108–115]. Moreover, excessive BAs in obstructive cholestasis impaired phagocytosis activity of KCs and DCs in mice, hence compromising bacteria clearance by APCs [106, 116]. In addition, the phagocytic capacity of monocytes was also hampered by BA retention [116]. This might explain why cholestasis followed by gut-derived microbiota translocation can give rise to sepsis.

BAs and T lymphocytes

BA retention during cholestasis may transform hepatic APCs from tolerogenic state to immunogenic state. BA retention directly alters hepatic T cell immune response [117, 118]. In obstructive cholestasis, liver bulk T cell increases intrahepatic PD-1 expression, which leads to expanded hepatic Th17 cell and neutrophil infiltration [118]. High serum BA level prompts biliary epithelial cells to secrete IL6 and IL1β contributing to the induction of Th17 cell responses [118]. BA retention may amplify Th17 cell infiltration and response by inducing hepatic production of MIP-2/CxCL2 and other cytokines via upregulating Egr-1 (see Fig. 7.4) [117]. In addition, BAs activate AKT- and JNK-associated pathways in hepatocytes leading to IL23 upregulation, which fuels

Th17 expansion and strengthens IL17A expression (see Fig. 7.4). In turn, IL17A synergistically increases hepatic MIP-2/CXCL2 and IL-23 expression in response to high level of BAs generating a positive feedback loop, which further elicits inflammation during cholestasis [117]. Thus, some of the inflammatory changes seen in cholestatic liver diseases may be due to BA retention per se and may cause confusion with autoimmune hepatitis. It is noteworthy that from the abovementioned studies, BAs can act as pro-inflammatory signaling molecules at μmolar concentrations (in the 100 μM range) far below tissue-damaging micellar concentrations viewed traditionally as a key mechanism of BA toxicity [3].

Impact of Immune Cells and Immune Response on BA Metabolism

While most studies have focused on the potential impact of BAs on immune cell function as outlined above [119], the potential impact of immune processes/inflammation on BA metabolism also needs some consideration. An interesting study using a novel mouse model combining chronic and acute T cell-driven hepatic biliary injury demonstrated that liver-infiltrating $CD8^+$ T cells targeting bile ducts dramatically alter BA metabolism [120]. This was achieved by suppression of de novo hepatic BA biosynthesis and uptake, while enhancing BA export. Liver-infiltrating CD8+ T cell lowered intrahepatic levels of unconjugated BAs as indicated by a profound inhibition of enzymes regulating both the "classical" and "alternative" BA biosynthesis pathways [120]. Additionally, T cell transfer decreased the expression of basolateral BA uptake transporters (NTCP and OATP) and increased expression of the canalicular transporter BSEP, which resulted in increased serum levels of conjugated BAs and reduced intrahepatic levels of toxic, unconjugated BAs, suggesting a possible mechanism of how hepatic pro-inflammatory CD8+ T cells may protect the liver from additional injury during cholestasis [120].

Inflammation-induced cholestasis in sepsis is predominantly seen in infections with Gram-negative bacteria. Lipopolysaccharide (LPS) derived from Gram-negative bacterial cell walls (also known as endotoxin) is a potent trigger of local and systemic inflammation. Endotoxinema and sepsis in mice impair the expression of hepatocellular transporter proteins at the basolateral or canalicular membrane [121] by cytokine-mediated pathways [122] interfering with the activity of key regulatory transcription factors [123] including FXR [123] and RXR [5, 124, 125]. Pro-inflammatory cytokines in sepsis alter not only hepatic transporter expression like NTCP [123, 126] but also trafficking, such as MRP2 retrieval from the canalicular membrane [127]. Additionally, disruption of cellular tight junctions observed in sepsis also lead to the loss of osmotic gradients between portal blood and bile canaliculi further contributing to cholestasis [128]. If positive acute-phase proteins are generally involved in direct host protection by neutralizing damaging agents in order to limit tissue destruction [10], the role of negative acute-phase genes is unclear. By lowering RXR heterodimers [124], the acute-phase response promotes hypertriglyceridemia and lowers HDL cholesterol, BA synthesis, and transport. Hypertriglyceridemia enriches HDL particles with triglycerides making them less anti-inflammatory and as such promoting a response fighting infections [129].

Role of BA-Activated Receptors in Modulation of Liver and Gut Immunity

Although the fundamental roles of BAs in cholesterol homeostasis and lipid emulsification had been recognized for decades, the novel function of BAs as active signaling metabolites with hormonal actions has been discovered more recently. As such, BAs control a broad range of metabolic processes including hepatic BA transport and metabolism, lipid and glucose metabolism, drug disposition, as well as liver regeneration, inflammation, fibrosis, cell differentiation, and tumor formation [130]. As stated above BA can activate FXR, PXR, CAR, VDR, and the Takeda G protein-coupled receptor 5 (TGR5) [131].

FXR

In addition to BA and lipid/glucose homeostatic actions, BA-dependent FXR activation has anti-inflammatory effects in the liver via repressing nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) activity by inhibiting nuclear co-receptor clearance from NF-κB-binding sites in the TNFα and IL1β locus [132, 133]. Besides interaction with NF-κB, FXR activation also triggers GR activation, a potent anti-inflammatory receptor of corticosteroids [134]. In addition, BAs behave like danger-associated molecular patterns (DAMPs) activating both the signal 1 (involved in IL1β induction) and signal 2 (involved in Ilβ activation) pathways required to activate NLRP3 in macrophages. Interestingly, FXR physically interacts with NLRP3 and caspase 1, thus repressing NLRP3 activity in a transcriptionally independent manner, suggesting that restoring FXR expression in macrophages could help to fight [98]. Moreover, deficiency in FXR signaling has been associated to inflammation-mediated hepatic carcinogenesis, while FXR ligands prevent HCC development in experimental murine models [135]. Pharmacological agonists for FXR, such as obeticholic acid (OCA; also known as 6-ethyl-chenodeoxycholic acid), and

non steroidal FXR agonists (e.g., cilofexor, tropifexor) have been clinically tested in a wide range of liver diseases including autoimmune cholestatic liver diseases, such as PBC (see above) and PSC, as well as metabolic disorders, such as NASH [65, 136, 137]. Recent clinical trials in patients with NASH showed that OCA reduced liver enzymes and improved liver fibrosis histologically [138, 139].

FXR expression has been detected in various murine and human immune cells and has attracted significant attention in immune regulation and innate immunity [140–142]. Recently, FXR has also been implicated in activation of hepatic NKT cells and hepatic accumulation of myeloid-derived suppressor cells, counteracting immune-mediated liver injury in murine [143]. One study has shown that in vitro activation of FXR and its target SHP inhibited c-Jun binding to the osteopontin promoter in murine hepatic NKT cells, therefore reducing production of osteopontin, a potent pro-inflammatory mediator, as well as effector cytokines such as IFNγ and IL1β [144]. In line with this, in the model of concanavalin A-induced autoimmune hepatitis, pharmacological activation of FXR attenuated acute liver injury by suppressing NKT cell activity and hepatic infiltration [144]. Interestingly, in another immune-mediated hepatic injury model induced by alpha-galactosylceramide, FXR activation expanded $CD11b^{+}Ly6C^{high}$ myeloid-derived suppressor cells and enhanced MDSC suppressive function through increasing paired Ig-like receptor B by binding the PIR-B promoter [142]. FXR activation also promoted homing of MDSCs to the inflamed site in the liver through upregulation of S100A8 [142].

Besides anti-inflammatory effects, FXR activity is important for maintaining mucosal immune homeostasis and barrier function, as well as preventing excessive bacterial growth under physiological conditions through transducing multiple genes involved in intestinal mucosa defense against inflammation and microbes and in mucosal protection [145]. Consistent with these beneficial effects of FXR in the intestine, FXR transcriptional activity and single-nucleotide polymorphisms (e.g., NR1H4 SNP rs3863377) are frequently impaired and down regulated during chronic mucosal inflammation, in both murine and human IBD patient biopsies [133, 146, 147]. Inflammatory cytokines such as IFNγ, IL1β, and TNFα can trigger physical interaction between FXR and the NF-κB p50 and p65 subunits, thus limiting FXR transcriptional activity [133]. Treatment with FXR agonists, such as OCA, exerts systemic anti-inflammatory effects including limiting expressions of pro-inflammatory cytokine and chemokine, increasing serum IL10 levels, and inhibiting DC from the spleen and expanded Tregs [141] in murine colitis models chemically induced by either dextran sulfate sodium (DSS) or 2,4,6-trinitrobenzene sulfonic acid (TNBS) [148]. Recent studies reported that FXR activation in IECs lowered Madcam1 expression, a ligand for α4β7 integrin-dependent leukocyte extravasation into the colonic lamina propria, as well as CXCL3, the ligand for CCR2 expressed on inflammatory macrophages and DCs, and mediated leukocyte homing to inflamed peripheral tissues [141, 149]. OCA administration was shown to promote colonic expression of CCL25 instead of CXCL3, creating a microenvironment favoring CCR9-dependent recruitment of Tregs to the inflamed mucosal tissues [141]. In addition, FXR activation-induced transcriptional responses in IECs enforced gut integrity and antimicrobial peptide secretion that restricted bacterial translocation across the epithelial barrier [148]. Interestingly, further in vitro studies have shown that FXR activation by OCA also decreases ileac transcription of pro-inflammatory gene expression induced by TLR4 and pro-inflammatory cytokine and chemokine expression in ex vivo isolated human monocytes and DCs, indicating FXR impacts on gut inflammatory response might be synergistic in both IECs and immune cells.

Apart from GI and liver, FXR agonist also improved autoimmune inflammation in the central nervous system in murine experimental autoimmune encephalomyelitis (EAE) model by reducing IFNγ production and modulating both T and B migration [150]. Collectively these data indicate that FXR has broad anti-inflammatory and immunomodulatory actions which could be used therapeutically to treat immune-mediated disorder within and outside the liver and contribute to counteract inflammation as important consequence of cholestatic and metabolic liver cell injury.

PXR, CAR, and VDR

In addition to FXR, PXR, CAR, and VDR serve as low-affinity BA "sensors" to promote BA detoxification (see above). PXR agonist feeding reduced liver inflammation in SJL/J mice which constitutively develop severe hepatic portal inflammation as reflected by lower transcriptional level of TNFα and IL1β. Although PXR is primarily expressed in the gastrointestinal tract and liver, it is also detected on immune cells, such as $CD4^{+}$, $CD8^{+}$ T lymphocytes, B cells, and monocytes [151, 152]. Mechanistically, PXR activation represses pro-inflammatory signaling in immune cells, for example, PXR activation decreased LPS-induced NF-κB activity and TNFα expression in Kupffer cells [153]. Another study reported increased PXR expression in both murine and human T lymphocytes upon immune activation, and pharmacological activation of PXR inhibits T lymphocyte proliferation and anergizes T lymphocytes by reducing the expression of CD25 and IFN-γ and decreasing phosphorylation level of NF-κB and MEK1/2 [154]. Conversely, T cells lacking PXR exhibit a strong pro-inflammatory phenotype with exaggerated proliferation, increased CD25 expression, and IFN-γ production [154].

Recent studies reported that PXR is able to specifically bind to LCA and LCA-dependent PXR activation in mice with experimental necrotizing enterocolitis-triggered TLR4 mRNA instability, thereby silencing TLR4 signaling [155]. This suggests that PXR effects on the immune system could at least in part be stimulated by BAs.

CAR is a PXR-associated xenobiotic-sensing nuclear receptor and as such is also recognized for its role in hepatic BA and drug detoxification (see above). Currently no experimental evidence has been found that BAs can directly bind to CAR, but other biliary components such as bilirubin have been discussed as potential ligands. CAR can be activated by various pharmaceutical compounds such as phenobarbital and hydrophobic BAs, such as LCA [40, 156–161]. A number of studies showed that activation of CAR was beneficial for protecting against BA toxicity during cholestasis in mice [41, 45, 162, 163]. In line with this, mice lacking CAR had more severe hepatic injury than WT mice upon LCA challenge or cholestasis in BDL setting, and liver damage was aggravated in mice deficient in CAR and PXR, suggesting that PXR and CAR may play complementary roles in BA detoxification [162]. Polymorphisms in the CAR locus have been linked with pathogenesis of IBD, and inflamed mucosal biopsies from IBD patients showed lower expression of CAR and a number of its target genes [164].

VDR is expressed on a wide array of innate and adaptive immune cells, such as APCs, T cells, B cells, and monocytes. In addition to classical endogenous VDR agonist vitamin D (1,25-dihydroxyvitamin D3), LCA has been identified as a natural ligand that can activate VDR with regulatory potencies on immune responses. Unconjugated LCA impaired $CD4^+$ T cell activation and inhibited Erk activities in Jurkat T cells through VDR, while VDR shRNA abrogated these immunomodulatory effects [119]. Furthermore, VDR signaling negatively modulated DC and macrophage activation, maturation, and functions. CD8+ T cells deficient in VDR proliferated spontaneously due to excessive IL2 production [165] and adopted exhausted phenotype with altered homing patterns and reduction in granzyme B production in LCMV model [166]. VDR KO CD8+ T cells transferred to leukopenic hosts were able to induce strong production of IL17 and IFN-γ in vivo and cause more severe colitis compared with mice transferred with wide-type CD8+ T cells [167]. VDR also plays an essential role in the development of iNKT cells due to its regulation of survival of maturing iNKT cells in the thymus [168].

Vitamin D deficiency and VDR polymorphism are extrinsic factors commonly associated with autoimmunity, as well as cholestatic liver diseases [169–171]. The local activation of VDR suppressed the development of pro-inflammatory effector T cells, while expanding the frequency and suppressive function of Tregs [172–178]. In PSC patients a negative correlation between serum vitamin D (25[OH]D) concentration and frequency of peripheral T cells lacking CD28 has been observed, an activated memory phenotype that exhibited strong pro-inflammatory profile [179]. Interestingly, pharmacological intervention with vitamin D (colecalciferol) among PSC patients who had vitamin D insufficiency reduced frequency of peripheral T cells lacking CD28 surface expression [179].

In addition to immune cells, VDR is also expressed on primary human hepatocytes at very low level [166]. VDR activation in hepatocytes decreased CYP7A1 transcription and thus inhibits BA synthesis [180]. However, 1α,25-dihydroxyvitamin D3 treatments did not affect BA levels in mice upon BDL challenge, indicating a minimal role of VDR in modulating BA metabolism under cholestatic condition [181]. However, VDR activation in BDL mice reduces pro-inflammatory cytokines, suggesting that the anti-inflammatory properties of VDR may provide certain beneficial anti-inflammatory effects in cholestatic liver diseases.

Vitamin D insufficiency presents an established risk factor in IBDs, and reduced VDR expression is commonly observed in mucosal biopsies from IBD patients [182]. Activation of VDR regulates cathelicidin antimicrobial peptide production in cholangiocytes [183].VDR can also act as a BA sensor in the intestine and protect the gut from BA-induced toxicity [184]. Accumulation of LCA in the gut may activate the VDR to convert LCA to less toxic intermediates for excretion. LCA-dependent VDR activation is found to accelerate BA metabolism in IECs through the induction of CYP2B, CYP2C, and CYP3A, suggesting a role for VDR in drug and BA detoxification [185].

BA-Activated G Protein-Coupled Receptors: TGR5, FPRs/FPRL1, and S1PR2

Conjugated BAs can also transduce signals via a dedicated transmembrane BA receptor TGR5 the Takeda G protein-coupled receptor (TGR5; GPBAR1, M-BAR, BG37) [186, 187], which is highly expressed on monocytes and macrophages but also in several hematopoietic cell lineages as well as cholangiocytes and IECs [131, 188], thus allowing BAs to signal in cells lacking specific transport/uptake systems [186, 187]. TGR5 expression was originally detected on hematopoietic cell lineages, most notably circulating monocytes and KCs; TGR5 has been reported to be highly expressed in cholangiocytes (but not in hepatocytes), sinusoidal endothelial cells, and hepatic stellate cells [131, 188]. Unlike FXR, TGR5 shows the highest affinity for binding with the secondary BAs, LCA, and DCA, whereas primary BAs which act as potent FXR agonists display lower affinities for TGR5 [78, 186, 187]. BA-dependent TGR5 signaling potently represses pro-inflammatory activity in macro-

phages, both systemically and locally in the liver or intestinal mucosa [189, 190]. Like BA-dependent FXR, liver injury/inflammation during disease states may downregulate TGR5 activation limiting its counter regulatory mechanisms in inflammation [191, 192].

TGR5 activation by either endogenous BAs or the synthetic TGR5 agonist 6α-ethyl-23(S)-methylcholic acid (S-EMCA/INT-777) decreased lipopolysaccharide (LPS)-induced inflammatory cytokine production, whereas these responses remained unaffected or even exaggerated by BAs in TGR5-deficient macrophages [193, 194]. Mechanistically, TGR5 activates adenylate cyclase, which enhances synthesis of cyclic AMP (cAMP) subsequently leading to activation of the cAMP response element-binding protein (CREB). As a consequence of BA- and pharmacological TGR5-dependent CREB stimulation, TLR4-mediated NF-κB transcriptional activation of multiple pro-inflammatory cytokine genes (e.g., TNF, IL1A, IL1B, IL6, and IL8) is repressed [194]. TGR5-dependent cAMP synthesis also interferes with the activation of NLRP3. cAMP overproduction further leads to activation of protein kinase A (PKA) which subsequently phosphorylate NLRP3 (Ser291), ultimately resulting in its ubiquitination and degradation [195].

Using in vitro system of human monocyte-derived DCs (MDDCs), it was shown that BA-dependent TGR5 activation was able to promote differentiation of IL12 hypo-producing MDDCs via TGR5, suggesting that TGR5 could be a novel therapeutic target for Th1-driven chronic inflammatory disorders, such as Crohn's disease and psoriasis [196]. Chemically induced colitis mice treated with TGR5 selective small molecule agonist, BAR501, demonstrated preference for developing tissue-protective (M2) macrophage over pro-inflammatory (M1) macrophages in the mucosa [197]. TGR5 signaling in addition also enhances epidermal growth factor receptor (EGFR)-SRC kinase (SRC) axis, as well as STAT3 phosphorylation/activation, which inhibited expression of pro-inflammatory cytokine, such as IL12, IFN-β, and IL6, and promoted Treg cell migration to inflamed colonic tissue [197]. Mucosa-associated macrophages isolated from biopsies of inflammatory bowel disease patient express high levels of inactive TGR5, and ex vivo treatment of these cells with TGR5 agonists leads to reduced pro-inflammatory cytokine expression [193]. Similar to FXR, TGR5 activation showed beneficial effects in a murine model of EAE prolonging survival and improving clinical scores via reducing inflammatory immune cell infiltration to the central nerve system and blocking activation of myeloid cells [150, 198]. These findings suggest that BA may exert systemic immune modulatory effects through activation of TGR5, thus complementing the effects of nuclear receptors.

Additionally, BAs are ligands antagonizing of formyl peptide receptors (FPRs) and formyl peptide receptor-like 1 (FPRL1) which also belong to G-coupled receptors and critically participate in sensing of bacteria and chemotaxis [199, 200]. N-Formyl-methionyl-leucyl-phenylalanine (fMLP) is known as one of the most potent ligands binding to FPR and FPRL1 with high affinity [201]. Primary BAs such as DCA and CDCA can serve as antagonists for FPR and FPRL1 [199] through competing with fMLP by steric hindrance, resulting in impaired FPR and FPRL1 activities under experimental or clinical cholestatic conditions [199, 200]. FPRL1 regulates COX2 expression, the enzyme generating prostaglandins, a key player inhibiting fibrosis and promoting hepatocellular carcinoma proliferation [202] and generating ligands activating the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ/NR1C3) [203], a receptor controlling inflammation in macrophages [204]. Taken together, BA antagonism of FPRL1 might represent a key pathway modulating inflammation in different liver diseases ranging from sepsis to cholestasis and cancer.

Moreover, it was reported that BAs such as DCA serve as ligands for the GPCR sphingosine-1-phosphate receptor 2 (S1PR2), which is mainly expressed in the liver, kidney, heart, brain, lung, and vascular smooth muscle cells (see Table 7.1) [205]. S1PR2 activation triggers the activation of the phospholipase Cβ that subsequently activates membrane metalloproteinase (MMP) to release EGF from the membrane allowing EGF to bind to EGFR ultimately resulting in the activation of EGFR [205, 206] and downstream signaling of ERK, AKT, JNK, and SPHK [205]. Increasing activity of the kinase SPHK2 results in S1P accumulation in cell nuclei, which inhibits histone deacetylase and nuclear receptor activity such as FXR and PPARγ [207], therefore affecting inflammatory response. Interestingly, EGFR activation leads also to PKC activation which phosphorylates FXR and SHP increasing their transcriptional activities [208].

BAs and Their Immunomodulatory Therapeutic Potential Beyond FXR and TGR5

UDCA

Early BA-based therapies before the development of therapeutic FXR ligands have focused mostly on hydrophilic and less toxic BAs, such as UDCA, which has been widely used in treating cholestatic and metabolic liver diseases over the past decades and now is an established first-line treatment of PBC and intrahepatic cholestasis of pregnancy [209]. Traditional Chinese medicine recognized the therapeutic value of dried bile from black bear for treating cholestasis more than a thousand years ago at Tang dynasty in China. UDCA constitutes around 60% of the total BA pool in black bears [210]. In humans, UDCA is considered as a minor secondary (tertiary) BA as it is transformed by 7β epimerization of CDCA by intestinal commensal and presents less than 3%

of the human total BA pool. Although UDCA has mild affinity binding to FXR [211] in vitro, when it behaves as antagonist in vivo [64] and is also a weak PXR agonist [191] and GR agonist [63], it has multiple beneficial actions. As such UDCA stimulates bile flow by virtue of its own biliary secretion, stimulating targeting of canalicular transporters, by inducing "biliary HCO_3 umbrella" and phospholipid excretion to protect biliary epithelial cells against cytotoxicity of hydrophobic BAs, ameliorating ER stress, protecting against oxidative stress, and inhibiting apoptosis [212–214]. Additional studies have unraveled some immune regulatory effects of UDCA by negatively regulating immunoglobulin synthesis, cytokine secretion by lymphocytes, decreasing hepatic expression of MHC class I, and impairing activation and degranulation of eosinophil and mast cells [215–217]. UDCA can also activate GR in a ligand-independent way and suppress NF-kB transcription via GR-p65 [218]. Recent evidences indicate that UDCA is also protective in murine IBD models. For example, UDCA ameliorates DSS-induced colitis in mice by promoting anti-inflammatory enteric bacterial species, including cluster *XIVa Clostridium* and *Akkermansia muciniphila*, which are generally missing in IBD patients [219, 220]. Beyond the gastrointestinal tract, UDCA exerted immune suppressive potency against eosinophilic airway inflammation in an asthma mouse model, by shortening the time of physical interactions between DCs and T cells [221]. In this study UDCA promoted BMDCs to secrete polarizing cytokine production in favor of IL12, thus repressing the potential of BMDCs to prime for Th2-dependent eosinophilic airway inflammation. Additionally, UDCA enhanced migration of BMDCs to reduce interaction durations between BMDCs and T cells, leading to reduction of T cell cytokines [221].

NorUDCA

24-Norursodeoxycholic acid (norUDCA) is a side chain-shortened derivate of UDCA and lacks a methylene group in its side chain. This side chain shortening results in relative resistance to amidation with taurine or glycine compared with UDCA with profoundly different pharmacokinetic and therapeutic properties. Consequently, norUDCA undergoes cholehepatic shunting (instead of undergoing a full enterohepatic circulation) resulting in "ductular targeting" to inflamed bile ducts/ductules and hepatic enrichment [222, 223]. Importantly, cholehepatic shunting also results in a bicarbonate-rich hypercholeresis which counteracts bile acid toxicity and potently reinforces the biliary "bicarbonate umbrella." As such, norUDCA (but not "conventional" UDCA) reverses sclerosing cholangitis in the experimental Mdr2/Abcb4 knockout mouse (Mdr2/Abcb4$^{-/-}$) cholangiopathy model for PSC, while UDCA aggravates bile infarcts in cholestatic conditions with (complete or partial) biliary obstruction [224]. Notably, neither norUDCA nor its parent compound UDCA has relevant affinities for dedicated BA receptors such as FXR or TGR5. Preclinical studies have shown that norUDCA has potent anti-inflammatory properties in cholangiocytes and macrophages, inhibiting NF-kB and mTOR signaling, alleviating ER stress, and restoring abnormal cell cycle regulation [225, 226]. NorUDCA stimulates autophagy which resulted in reduced alpha-1-antitrypsin (a1AT) protein accumulation and attenuated liver injury in a mouse model of a1AT deficiency [227]. Moreover, norUDCA (but not UDCA) reduces granuloma size and hepatic fibrosis in a mouse model of *Schistosoma mansoni* infection as world-leading cause of hepatic fibrosis and portal hypertension [228]. The anti-inflammatory properties of norUDCA were directed to MHC class II protein expression on dendritic cells and macrophages, and norUDCA reduced T lymphocyte proliferation and serum levels of pro-fibrogenic Th2 cytokines IL13 and IL4 [228]. These properties may also contribute to anti-inflammatory and anti-fibrotic effects of norUDCA [228]. Based on these encouraging experimental data in preclinical models [229, 230], norUDCA had been tested in phase II clinical trials for PSC and nonalcoholic fatty liver disease and demonstrated promising effect on improving liver enzymes [231, 232] and is currently undergoing phase IIb and III trials for studying its long-term effect on disease progression of NASH and PSC, respectively.

Other BA Metabolites

In addition to UDCA and norUDCA, another BA species, namely, tetrahydroxylated bile acids, may have critical immunomodulatory functions. They have been recognized as suppressors of RORγ (patent no. WO201304159A1). Since its isoform RORγt is known to be involved in differentiation of the pro-inflammatory T cell subpopulation Th17 cells [233], it is tempting to speculate that these BA species may have potential immune modulatory effects counteracting inflammation-driven liver disease. Accordingly, mice generating such tetrahydroxylated BAs were shown to be protected from acquired cholestasis induced by either bile duct ligation or DDC feeding [234]. In addition, the recently reported 3 oxoLCA and isoalloLCA (see Table 7.1) also attract therapeutic attentions in treating immune-mediated liver and gut disorders due to their discovered potentials in suppressing Th17 differentiation and expanding Treg differentiation via modulating RORγt activity [235]. Recent studies have revealed the anti-inflammatory potential of BA signaling, particularly in the innate immune system by repressing NF-κB-dependent signaling networks [236, 237] and by restricting NLRP3-dependent inflammasome activity via the TGR5-cAMP-PKA axis or FXR [98, 195]. One recent

study uncovered the anti-inflammatory role of two microbial metabolites of LCA present in the feces of patients and mice with colitis that could directly modulate CD4+ T helper cell development: as such, 3-oxoLCA suppresses Th17 cell differentiation, and isoalloLCA promotes Treg cell differentiation by modulating RORγt activity [235] (see Fig. 7.3).

Bilirubin and Regulation of Immunity

Bilirubin is the end product of heme catabolism mainly obtained from the breakdown of heme released from senescent red blood cells engulfed by phagocytes in organs structured with reticuloendothelial system, such as the bone marrow, spleen, and liver. Heme oxygenase 1 (HO-1; see Fig. 7.2) catalyzes heme degradation to generate carbon monoxide, ferrous iron, and biliverdin [238]. Biliverdin is then reduced to bilirubin via biliverdin reductase (BVR; see Fig. 7.2). Following uptake of unconjugated bilirubin (UCB) by a bidirectional transporter OATP1B1 and/or OATP1B3 [239], UCB is bound to a complex of glutathione S transferase A1 and A2 (GSTA1/A2; see Fig. 7.2) [240] and then conjugated with glucuronic acid via UDP-glucuronosyltransferase 1-A1 (UGT1A1; see Fig. 7.2) and excreted into the bile by MRP2 (see Fig. 7.2). Alternatively, MRP3 transport glucuronidated compounds in vitro [241] and bilirubin in vivo [242].

Upon deconjugation by the gut microbiome, namely, *Clostridium ramosum*, *Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides fragilis* [243–245], conjugated bilirubin is converted into urobilinogen and urobilin and excreted into the feces and urine. Unconjugated bilirubin is partially reabsorbed in the gut and recycled back to the liver following enterohepatic circulation. During this process bilirubin and its metabolites can also signal and modify the immune system. Approximately 80% of urobilinogens are continuously catabolized by the intestinal bacteria, mainly stercobilin, to form stercobilin to be excreted in feces, while 2% of urobilinogens are excreted in the urine as urobilin and 18% of urobilinogens are reabsorbed in the gut and recycled back to the liver following enterohepatic circulation [243–245].

Biliverdin and bilirubin were previously considered as end products of heme catabolism; however there is now increasing evidence that bilirubin can be further degraded into diverse bioactive metabolites in health and cholestatic disease across species [246]. Unconjugated bilirubin can be oxidized to higher-order degradation products such as two major bilirubin oxidation end products (BOXes), in particular the regioisomers Z-2-(4-methyl-5-oxo-3-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene) acetamide (Z-BOX A) and Z-2-(3-methyl-5-oxo-4-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene) acetamide (Z-BOX B) [246]. Z-BOX A and B arise upon oxidation and impair hepatocellular integrity and might mediate intra- and extrahepatic cytotoxic effects previously attributed to hyperbilirubinemia indicating that cytotoxic effects so far solely attributed to bilirubin might, at least in part, be mediated by higher-order degradation products of heme [246, 247].

Bilirubin has long been considered a cytotoxic waste product over last past decades, particularly in the case of neurotoxicity upon extreme brain accumulation in infants [248] until its beneficial anti-oxidative properties have been identified [249]. In vitro evidences have shown that bilirubin can directly scavenge reactive nitrogen species, a well-known hallmark driver of many inflammatory disorders and aging-associated pathologies [250]. Therefore, beneficial effects of bilirubin in these conditions have been reported in several studies [251]. Elevated total bilirubin levels are shown to be protective in rheumatoid arthritis [252], and increased HO-1-induced bilirubin formation dampened the inflammation in a murine arthritis model induced by collagen [253]. Further support for the immune regulatory effects of bilirubin comes from clinical observations indicating improvement of inflammatory/autoimmune conditions associated with high endogenous levels of bilirubin [254]. Cholestatic patients with concomitant ulcerative colitis and PSC seem to manifest milder or asymptomatic colitis compared with patients with normal bilirubin levels [255]. Similarly, patients with Gilbert syndrome, who have higher levels of UCB because of defective UGT1A1, are less likely to develop IBD, further supporting the immune protective role of UCB in this condition [256, 257].

It has been shown that UCB is able to interact with macrophages by altering the surface expression of Fc receptor, therefore modulating macrophages' phagocytic and antigen-presenting function (see Fig. 7.2) [258]. In addition, UCB showed inhibitory effects on expression of MHC II class molecules on LSECs suggesting a potential impairment of antigen presentation to lymphocytes (see Fig. 7.2) [259]. UCB inhibits the complement cascade by blocking the binding between antibodies and the C1 complex (see Fig. 7.2) [260]. UCB inhibits also IκB phosphorylation, thus inhibiting NF-κB activation and blocking IL2, IFN-γ, and TNFα pro-inflammatory cytokine synthesis in a dose-dependent manner (see Fig. 7.2) [261, 262]. A recent study expanded our understanding about bilirubin in regulation of adaptive immunity. In a T cell-driven EAE model, bilirubin showed powerful immunomodulatory properties by significantly inhibiting $CD4^+$ T cell immune response through multiple actions, including inhibition of co-stimulatory activities, suppression of immune transcription factor activation, and down regulation of inducible MHC class II expression (see Fig. 7.2), while other similar antioxidants completely lacked

this effect [261]. In vivo administration with bilirubin profoundly suppressed experimental autoimmune encephalomyelitis in SJL/J mice; conversely, depletion of endogenous bilirubin dramatically exacerbated the disease suggesting an important immunomodulatory role of bilirubin against autoimmunity and therapeutic potential in the treatment of immune disorders [261].

In addition, in mouse, bilirubin binds to peroxisome proliferator-activated receptor alpha (PPARα/NR1C1) [263] a nuclear receptor activated by fibrates [264]. PPARα also upregulates HO-1 and BVR, thus promoting bilirubin synthesis but also bilirubin excretion by increasing MRP3 expression in hepatocytes, like CAR and PXR activators [265] and MRP2 together with CAR [266] (see Fig. 7.2). This PPARα effect is also seen in human cells [267], and PPARα was identified as a master controller of bilirubin metabolism [268]. Leukotriene A4 (LTA4) is produced in leukocytes, and COX2 metabolizes it into LTB4 in the liver, a ligand activating PPARα [269] (see Fig. 7.2). PPARα is an anti-inflammatory receptor inhibiting NF-κB signaling and also inducing ω- and β-oxidation inactivating LTB4 (see Fig. 7.2) [270]. Therefore, it is tempting to speculate that bilirubin and PPARα are working in a loop to control bilirubin concentrations and lower inflammation (see Fig. 7.2). It is also important to note that BA-activating FXR increase PPARα expression [271]. Therefore, in cholestasis and jaundiced patents, FXR and PPARα can work together to detoxify BA and bilirubin, to lower inflammation, and to adjust lipid metabolism (see Fig. 7.2).

Finally, unliganded PPAR is also suppressing the phosphorylation of P38 MAPK in CD4 T cells, thus inhibiting the transcription factor T-bet and lowering IL2, IFN-γ, and TNFα production, while highly potent PPAR ligand such as fibrates increased T-bet (see Fig. 7.2) [272]. It would be of interest to know whether in this context bilirubin which is a rather weak agonist would be anti-inflammatory.

Interestingly, it was reported that bilirubin serves as endogenous ligands of aryl hydrocarbon receptor (AhR) which is defined as a crucial transcriptional regulator involved in immune response and adaptive xenobiotic response [273, 274]. In a DSS-induced colitis model, UCB exerted strong immunosuppressive properties via ligand activation of AHR to upregulate expression of CD39, an ectoenzyme catalyzing the conversion of extracellular ATP and ADP into AMP, thus promoting Th17 cell transition to a less pathogenic phenotype and preferentially boosting IL10 production by colonic intraepithelial $CD4^+$ T cells [275]. Genetic deletion of CD39 or AHR in mice abrogates the UCB salutary effects in experimental colitis [275].

Collectively, recent data suggest that bilirubin is a molecule of immunologic significance. In addition to its antioxidative properties, bilirubin and its downstream signaling targets therefore may represent an interesting player in management of autoimmune diseases.

Conclusion

BAs and bilirubin have emerged as critical signaling molecules which exert pleotropic functions in the regulation of metabolism and immunity by interacting with dedicated receptors and gut microbiota. The emerging diagnostic, prognostic, and therapeutic potentials of BAs and bilirubin for immune-driven disorders warrant further investigations.

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The Microbiota-Gut-Liver Axis: Implications for the Pathophysiology of Liver Disease

8

Eamonn M. M. Quigley

Key Points

- Interactions between the gut and the liver in relation to immunological responses and metabolic functions are well recognized.
- The advent of high-throughput molecular techniques now enables the detailed description of microbiota and their products.
- As our understanding of the gut microbiome and its homeostatic functions has developed the concept of the microbiota-gut-liver axis has emerged.
- A general hypothesis has emerged to explain how interplay between these factors might initiate or perpetuate various liver diseases.
- In this framework, disrupted microbiota and their products gain access to the gut-associated immune system via a permeable gut barrier and generate inflammatory responses which then impact on the liver.
- Though considerable evidence in support of these microbiome-gut-liver axis interactions has emerged from animal models, data from human studies is more limited, and several challenges confront clinical studies on this issue.
- There is hope that from research on the microbiome-gut-liver axis, new therapeutic avenues might emerge.

By virtue of its anatomical location, the unique nature of its blood supply, and its critical metabolic and immunologic functions, the liver is strategically positioned to confront and interact with those microbes, microbial components, and products of microbe-gut interactions that traverse the gut barrier and gain access to the portal circulation (Fig. 8.1) [1]. Conversely, liver disease or shunting of portal blood through various collaterals so that it bypasses the liver will have serious consequences.

The idea of an interactive, bidirectional axis between the gut and the liver is not new, and hints of an enterohepatic circulation of bile can be found in literature dating back to the nineteenth century only to be clearly identified in the 1920s [2] and described in greater detail in the 1970s [3, 4]. Other molecules were also identified as undergoing an enterohepatic circulation: estrogen, thyroxine, and bilirubin being early examples [5–7] with many others being added to the list since then. It is interesting to note, as we will see later, that the gut microbiome is now seen to be an active participant in the interactions between the gut and the liver in the regulation of bile secretion [8].

The idea of a microbiota-gut-liver axis is also far from new. That the gut microbiota was relevant to the natural history of liver disease was recognized over 60 years ago when relationships between gut bacteria, their metabolic products, and hepatic coma were first described [9–12]. In these studies, the importance of coliforms was emphasized, and these same bacteria and the inflammatory response that they evoke have since been incriminated in the pathophysiology of portal hypertension as well as in such infectious complications of chronic liver disease as spontaneous bacterial peritonitis, systemic sepsis, and hemostatic failure [1, 13, 14].

While the role of gut bacteria in the aforementioned complications of liver disease is now widely appreciated and is appropriately the subject of considerable research interest and clinical import [1, 15–19], research efforts have also begun to focus on the possibility that the gut microbiota may be fundamental to the pathogenesis of various liver diseases. Indeed, evidence accumulates to support a role for microbiota in alcoholic liver disease, nonalcoholic fatty liver disease (NAFLD), total parenteral nutrition (TPN)/intestinal failure-associated liver disease (IFALD), and even in immune-mediated diseases such as primary biliary cholangitis and primary sclerosing cholangitis [20–22].

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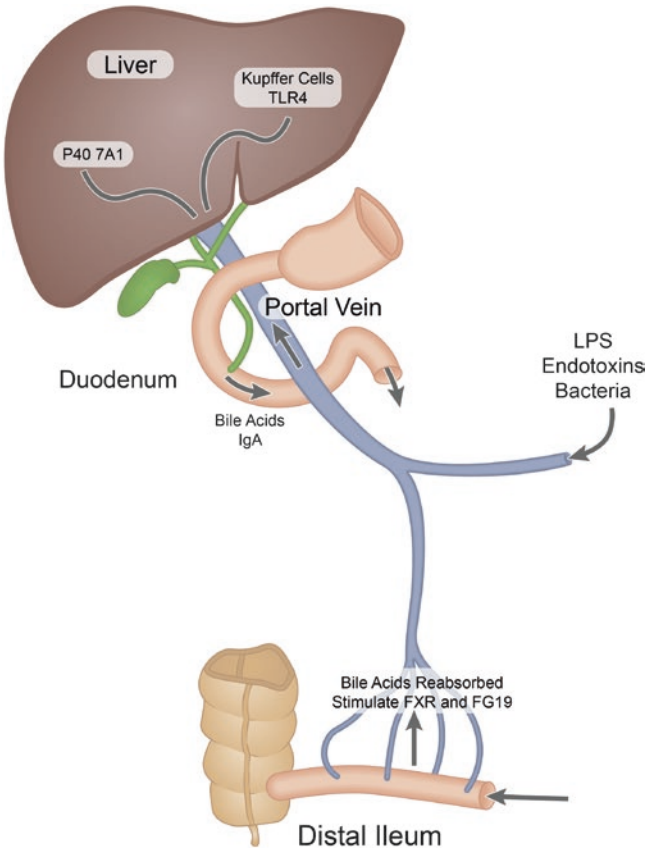

Fig. 8.1 An overview of the elements of the microbiome-gut-liver axis. Bile acids synthesized in the liver with the cytochrome P450 7A1 enzyme as the rate-limiting step are excreted into the small intestine where with immunoglobulin A (IgA) they exert microbiome modulating effects. The arrival of bile salts in the distal ileum, their site of active absorption, stimulates the secretion of FGF 19 via FXR activation which through a negative feedback loop effect on P450 7A1 inhibits bile acid synthesis in health. LPS and other endotoxins as well as bacteria or bacterial components, if they enter the portal circulation from the gut, will be cleared by Kupffer cells in the liver following engagement with Toll-like receptors such as Toll-like receptor 4

It is also interesting to note that the model developed to explain the pathophysiology of HE those many years ago, that is, the convergence of small intestinal bacterial overgrowth (SIBO) and/or an abnormal microbiota, impaired gut barrier function, a pro-inflammatory state, and the appearance in the systemic circulation of neuroactive molecules generated by bacterial metabolism has become virtually ubiquitous as the template to explain the role of the microbiota-gut-brain axis in the pathogenesis of several liver diseases [1, 15–18] (Fig. 8.2). The following players are considered key to the development and/or progression of several liver diseases, be they metabolic, inflammatory, or neoplastic in nature: the gut microbiome and its interactions with luminal contents (including those originating from our diet), the gut barrier, the mucosal immune response, and the metabolic and immune responses of the liver itself [1, 15–18].

The Gut Microbiome: An Overview

The human microbiome refers to the collection of all microorganisms (bacteria, archaea, viruses, fungi, and protozoa), including their genes and genomes, that live in a complex relationship with the human body, in various niches (e.g., skin, oral cavity, gut, vagina). This term is often used interchangeably with the term "microbiota" which, strictly speaking, refers to the microorganisms alone [23]. While these organisms may be microscopic, their sheer number is such that they comprise 1–3% of our total body mass. The bacterial population in an average human body includes ten times more cells than human cells and includes a thousandfold more genes than are present in the human genome [23]. Our unique relationship to these organisms is termed "symbiotic," consonant with the intimate relationships that characterize highly evolved ecological niches. This relationship can be commensal, wherein the interaction is beneficial for one, or mutualistic, wherein both organisms benefit. When certain species or strains in the microbiota disproportionately bloom, our symbiotic relationship with these organisms may be lost, and this imbalance may result in functional changes that are ultimately detrimental to our health [24].

Many of the recent developments in this field have centered on the largest and most diverse commensal bacterial community on or in the human body – the gut microbiome. Not only is the gut microbiome thought to be involved in diseases related to the gastrointestinal tract, but its influence may extend beyond the gut to diseases of the lungs, joints, endocrine system, vascular system, and nervous system [25–30]. These microorganisms that reside in our gastrointestinal tract participate in biologic activities and produce various metabolic products which are highly likely to influence liver function. The advent of new technologies such as high-throughput sequencing, metagenomics, metabolomics, metabonomics, and metatranscriptomics now permit a detailed interrogation of the composition, function, and metabolic products of bacterial communities [31].

While there is some evidence from studies of mother-infant pairs to suggest that an infant's gut may be colonized by bacteria in utero, this has not been consistently demonstrated [32, 33]. For the most part, the infant's microbiota is initially acquired by vertical transmission from the mother during birth and continues to develop over the first 2–3 years of life. During this critical period, the ultimate composition of an infant's microbiota is influenced by mode of delivery (vaginal birth vs. cesarean section), source of nutrients (breast milk vs. formula), maternal weight, prenatal diet, geography, and exposure to antibiotics [34–42]. Human and mouse studies have demonstrated that antibiotics in early life alter bacterial metabolism and gene expression and transiently perturb the gut microbiota, thereby predisposing the infant to the later development of inflammatory and meta-

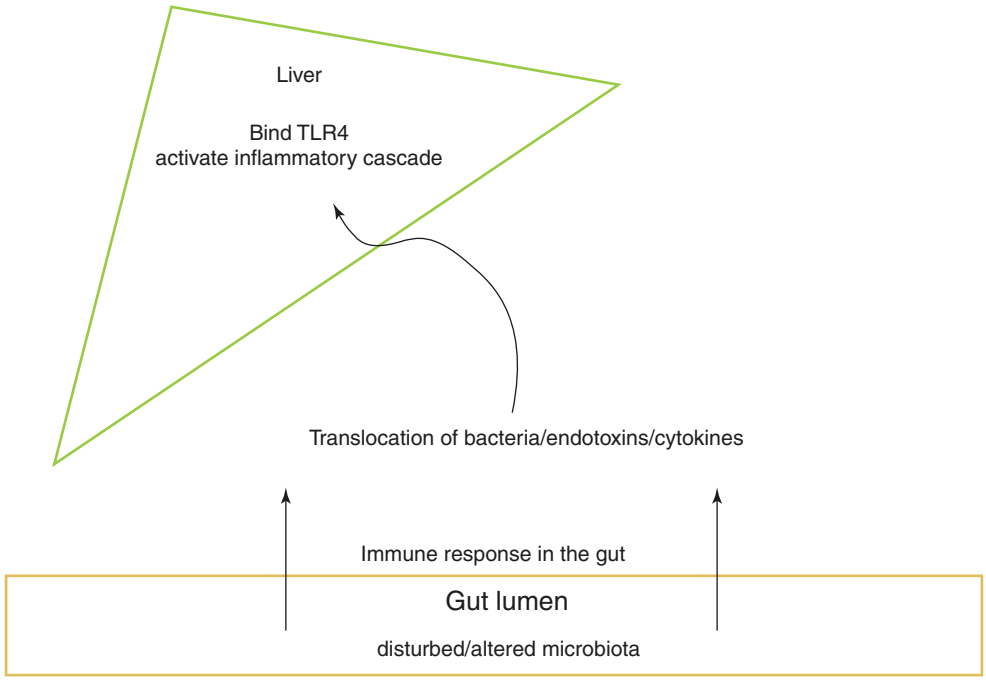

Fig. 8.2 A general model for microbiome-gut-liver involvement in liver disease. A disturbed/altered microbiota (e.g., small intestinal bacterial overgrowth or what is referred to as "dysbiosis") gains access to the intestinal submucosa via a permeable gut barrier and provokes an inflammatory response. The arrival of excessive loads of bacteria, endotoxin, and pro-inflammatory cytokines in the liver initiates inflammatory cascades following Toll-like receptor (TLR) engagement. Note that this axis is bidirectional, as liver disease (through effects of portal hypertension on the gut barrier or of bile salt depletion or an activated inflammasome on microbiota) per se can also impact on the gut lumen and barrier

bolic disorders [43–48]. These early years represent a vulnerable period wherein alterations of the microbiome may have far-reaching impacts on childhood development [49–52]; the Canadian Healthy Infant Longitudinal Development study [50, 51] and others [52] have illustrated the impacts of perinatal factors, as well as feeding practices and antibiotic exposure in early childhood on the later development of obesity, a major factor in the development of what is rapidly becoming the most common liver disease worldwide – NAFLD [19].

During infancy, there is a rapid expansion in bacterial diversity that slows in early childhood. Over time, the composition of gut microbiota becomes more stable, with multiple members of Bacteroidetes, including those with butyrate-producing capacity, establishing a presence. By preadolescence (7–12 years of age), the number of bacterial taxa and functional genes present in the gut microbiome have matured to what will persist throughout most of adulthood [30, 34, 39]. The adult microbiome is largely dominated by two phyla, Firmicutes and Bacteroidetes, which some have subclassified into so-called enterotypes based on the prominence of one of the following genera: *Prevotella*, *Bacteroides*, and *Ruminococcus* [53]. Later in life, the gut microbiome seems to undergo some age-related changes characterized by a proliferation of opportunistic Proteobacteria at the expense of symbionts Firmicutes and Bacteroidetes, phyla that include species with recognized anti-inflammatory properties [54]. It is fair to state that the precise nature and origin of aging-related changes in the gut microbiota remain to be defined [55]. Moreover, diet may well be a critical determinant of age-related changes in the elderly microbiota, and if inadequate or poor in diversity and quality, it may lead to a reduction in species richness and a distorted gut ecology [56, 57].

The functions of the microbiome continue to be revealed and include the development and maturation of the mucosal immune system [29, 58, 59], maintaining the integrity of the gut barrier [60], modulating gut neuromuscular function [61–63], and performing a number of key metabolic functions [64, 65]. These functions include both the metabolism of dietary fibers and starches into short-chain fatty acids (SCFAs), which support the proliferation of microbial species and are beneficial to host health [66], the metabolism of bile acids, and the biosynthesis of neurotransmitters and other potentially neuroactive molecules [30, 67]. The role of the microbiome in the metabolism of drugs and xenobiotics is increasingly recognized [68] and of considerable relevance to liver function in health and disease given the many roles of this organ in drug metabolism. Through a variety of reactions and calling upon an array of enzymes, the microbiome can variably activate prodrugs, detoxify drugs or their metabolites, or, in other circumstances, enhance drug effects and toxicity before they even reach the liver [68].

Diet has been found to substantially influence the microbiota throughout all phases of life and is an important confounder in studies of the gut microbiota in health and disease. The features (i.e., total calories, consumption of highly processed foods vs. vegetable and fruit-based diet) [56, 69–71] as well as the individual components of our diet such as carbohydrate [72, 73], protein [74], fat [75], fiber [76–78], and vitamins [79] all influence the composition of the microbiota. While dietary changes generally take effect over long periods of time [80], dietary changes, if sufficiently drastic and if introduced at an appropriate time, can evoke alterations in microbiota composition in the short term [34, 37, 70, 71, 81–83]. Many other factors play a role in microbiome composition and diversity, including genetic predispositions [84], environmental stressors [85, 86], and medications such as antibiotics, proton pump inhibitors, and metformin [87–91]. It is critical that every one of these factors be borne in mind when evaluating studies that associate a given hepatic malady with a microbial signature; confounders must be accounted for [92].

Changes in Gut Microbiota in Liver Disease

Historically, two alterations in gut microbiota populations have been described in individuals with liver disease or its complications: small intestinal bacterial overgrowth (SIBO) and changes in the fecal microbiome.

Small Intestinal Bacterial Overgrowth (SIBO)

A link between the gut microbiota and chronic liver disease was first reported by Hoefert over 80 years ago [93]. By virtue of well-documented changes in gut motility and transit, on the one hand, and intestinal permeability, on the other, subjects with chronic liver disease are predisposed, firstly, to intestinal stasis and, secondly, to bacterial translocation from the gut lumen to the portal circulation [1, 13, 15–18]. It should come as no surprise, therefore, that SIBO been shown to be common across a broad spectrum of chronic liver diseases [94–99]. SIBO has been documented in approximately 41% of subjects with cirrhosis, is more common among those with decompensated disease, and has been linked to such important clinical complications as ascites, encephalopathy, and spontaneous bacterial peritonitis [95]. Other factors also contribute. For example, the incidence of SIBO has been shown to be up to three times higher in alcoholics than in nonalcoholic controls [100], perhaps reflecting the effects of alcohol, alcoholism, and alcoholic liver disease on gut motility, gastric acid secretion, local immune responses, and gut barrier function [101–103].

SIBO has also been demonstrated in NAFLD and nonalcoholic steatohepatitis (NASH) [96, 99, 104–106], and its role in the pathogenesis of steatohepatitis among some individuals who had undergone the jejunoileal bypass procedure for morbid obesity has been well documented [107].

The major issue with SIBO is its very definition – the techniques to assess small intestinal bacterial populations, be they invasive such as aspiration and culture or noninvasive and based on breath hydrogen analysis, lack sufficient reproducibility and accuracy [108]. The small intestinal microbiome has, unfortunately in view of its interaction with the liver, been somewhat of a terra incognita [109]; fortunately, advances in sampling methods and microbiota analysis suggest that this gap in our knowledge may soon be filled [110].

Quantitative or Qualitative Changes in the Microbiota

Molecular techniques are now being directed at the investigation of the microbiota in liver disease. Up until very recently most were based on sequence divergences of the small subunit ribosomal ribonucleic acid (16S rRNA) of bacteria [31] which provides relatively limited detail on microbial composition and will not, of course, provide information on bacterial metabolites. As metagenomics, metabolomics, and other ‘omics [31] come to be applied to those who suffer from liver disease, a more complete picture of the role of the microbiome should emerge, and we should move forward from purely descriptive associative studies to gain insights into causation. It is also important to emphasize that the vast majority of microbiome studies, in man, have been based on the evaluation of fecal samples which will fail to accurately enumerate juxtamucosal populations, microbiota that may be highly relevant to certain microbe-host interactions [111].

Mindful of the reservations noted above, studies using high-throughput 454 pyrosequencing of the 16S ribosomal RNA variable 3 (V3) region followed by real-time quantitative polymerase chain reaction (qPCR) of fecal samples have identified changes in cirrhosis [112–114] which have been linked to inflammation in the liver [113] as well as disease severity [112] and complications of liver disease such as hepatic encephalopathy (HE) [113], spontaneous bacterial peritonitis, and bacteremia [114, 115].

From animal models have emerged a variety of plausible hypotheses to explain how the gut microbiome might initiate or perpetuate liver disease. Several mechanisms have been identified relevant to the involvement of microbiota in the pathogenesis of NAFLD/NASH [15, 20, 106, 116]. First, a role for gut microbiota and their metabolites in the pathogenesis of obesity per se, as well as the metabolic syndrome, has been identified [117–122]. Second, the activation, by the microbiota, of pro-inflammatory cytokines (e.g.,

tumor necrosis factor α (TNFα)) via Toll-like receptor (TLR) engagement appears relevant to progression from steatosis to NASH [105, 123–125]. Pathogenic, endotoxin-producing species, present in the small intestinal microbiome of obese individual, have been shown to induce steatosis [126], and genes encoding for inflammatory bacterial products, such as lipopolysaccharide (LPS), were found to be enriched in children with NASH [127]. Complex interactions between inflammasomes and the microbiota may also play a role; as a consequence of defective/deficient inflammasome sensing, intestinal microbial populations change leading to translocation and the appearance of increased amounts of bacterial products (microbe or pathogen-associated molecular patterns – MAMPs or PAMPs) in the portal circulation, substances that are known to lead to the progression of NAFLD to NASH [128, 129].

Any consideration of alcoholic liver disease must account for the direct effects of alcohol on gut microbiota and the intestinal barrier [17, 103, 130]. Not only are microbiota qualitatively and quantitatively different, in alcoholics, from that of nonalcoholics, but are also capable of converting ethanol to acetaldehyde [17, 103, 130]. Parenthetically. It should be noted that certain bacterial species are capable of generating endogenous ethanol from the fermentation of carbohydrates [131], an occurrence that may have implications for NAFLD as well. Other effects of alcohol conspire to increase permeability, promote transfer of endotoxin across the intestinal epithelium, and impair the host immune response [17, 103, 130].

Interactions with Luminal Contents: The Many Roles of Bile Acids

The primary bile acids (BAs), cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the liver, conjugated with the amino acids taurine and glycine, and excreted into bile [8]. In the small intestine, BAs play a central and critical role in the digestion and absorption of fat and fat-soluble vitamins. A highly efficient enterohepatic circulation ensures the conservation of secreted BAs with less than 10% being lost in feces and less than 5% of secreted BAs being composed of newly synthesized BAs, at any given time [8]. Though a fraction of BAs is absorbed passively, the primary means of BA conservation is active absorption via the apical sodium-dependent ileal BA transporter (ASBT or IBAT) located on the apical surface of enterocytes in the terminal ileum. BA absorption from the terminal ileum and secretion in the liver are closely linked through a feedback loop mediated, in part, by the hormone fibroblast growth factor 19 (FGF 19) secreted by the ileal enterocyte in response to high intracellular concentrations of BAs. FGF 19 secretion is, in turn, mediated by the nuclear farnesoid X receptor (FXR) [132], one of a family of nuclear receptors that BAs bind to. FGF 19, in turn, binds to the FGF 4 receptor and its co-receptor, Klotho-β (KLB), on hepatocytes to inhibit cholesterol 7α-hydroxylase (cytochrome P450 7A1), the rate-limiting enzyme in BA synthesis [133]. In this manner, bile acid physiology acts as a paradigm of the gut-liver axis (see Fig. 8.1). As primary BAs traverse the small intestine, approximately 15% are deconjugated by the microbiota; the small fraction of primary BAs that enters the colon is deconjugated by colonic bacteria and CA and CDCA transformed by bacterial 7α-hydroxylase into the secondary BAs deoxycholic acid and lithocholic acid, respectively [8]. While lithocholic acid is minimally absorbed, up to 50% of deoxycholic acid is reabsorbed and reconjugated in the liver to enter bile.

The traditional focus on bile acids is related to their critical role in fat and fat-soluble vitamin digestion; it is now clear that bile acids have several other physiological functions. These include not only local effects on gut motility, sensation, fluid secretion, and permeability but also signaling/hormonal effects which impact on several targets and cell types and influence such activities as energy expenditure, insulin sensitivity, and lipid metabolism [134, 135]. Through the activation of FXR in the intestinal epithelium, bile acids promote intestinal protection and gut barrier and gut vascular barrier integrity and prevent the development of potentially pathogenic microbiota [136]. Bile acids, indeed, enjoy a complex interaction with gut microbiota and the mucosal immune system [137–139]. Certain bile acids are bacteriostatic (to *Clostridioides difficile*, for example) [140]; in response, commensal gut bacteria adapt to the presence of bile acids by possessing bile salt hydrolase and other enzymes involved in the metabolism of bile acids [141].

The Gut Barrier and the Mucosal Immune Response

Various definitions have been applied to the term gut barrier – to some, this is limited to the single-cell thick epithelial layer; to others, it incorporates all elements that contribute to gut defense and integrity. The latter include the commensal microbiota and the mucus layer, the columnar epithelium itself, the lamina propria along with its constituent blood and lymph vessels, immune cells, as well as intrinsic and extrinsic nerve terminals (Fig. 8.3a). In the small intestine, the mucus layer, secreted by goblet cells, is approximately 100 μm thick and plays an important role by virtue of its hydrophobicity, as well as its bacteriostatic effects, in gut defense. At the juxtamucosal surface and within the mucus are found important antibacterial molecules, such as defensins that are secreted by Paneth cells. Between individual enterocytes in the epithelium are tight junctions that play a critical role in the move-

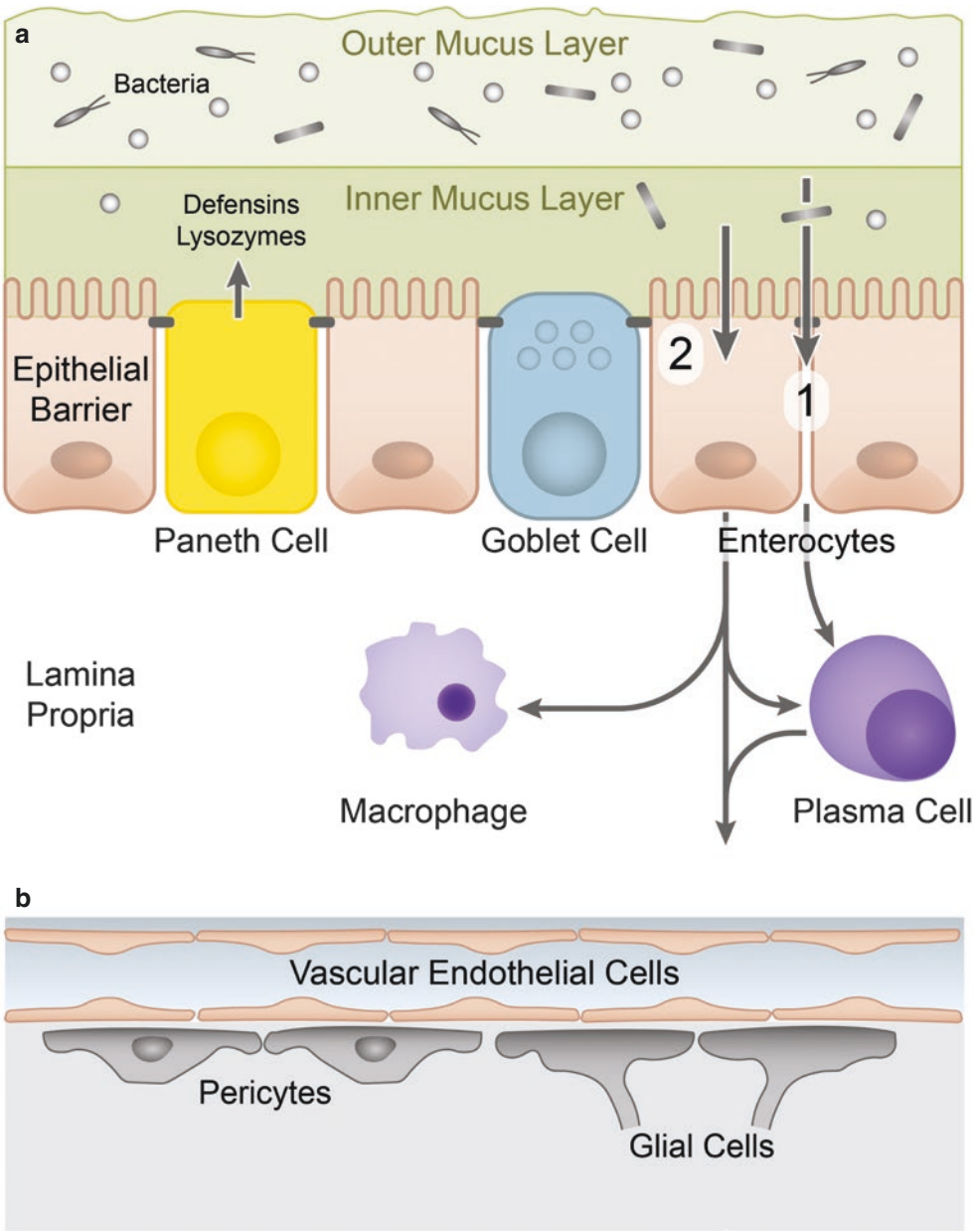

Fig. 8.3 The gut barrier and the gut-vascular barrier. (**a**) The components of the gut barrier. Components include the mucus layer secreted by goblet cells, bacterial defenses such as defensins and lysozymes elaborated by Paneth cells, the single-cell layer of epithelial cells bonded together by tight junction at their apices, and mucosa-associated immune tissue (MALT) in the lamina propria. In liver disease, it is thought that bacteria, bacterial products, or components gain access, via the paracellular (1) or transcellular (2) routes, to the lamina propria where they engage with macrophages and plasma cells to initiate an immune response. (**b**) The gut-vascular barrier. The various cell types which comprise this barrier are illustrated

ment of water and electrolytes via the paracellular pathway. At the apical surface, the tight junction complex is composed of tight junctions (Fig. 8.3a), adherens junctions, and desmosomes. Intracellular (zonula occludens (ZO)-1, ZO-2, and ZO-3 and cingulin) and surface-membrane proteins (occludin, claudins, and junctional adhesion molecules) are major components of tight junctions. Of these, occludin seems to play a role in the regulation of integrity of tight junctions, while their strength, size, and ion selectivity are determined by claudins and junctional adhesion molecules (JAMs) are involved in construction and assembly [142–145].

A number of factors, relevant to the pathogenesis of liver disease, can disrupt gut barrier integrity (Table 8.1). These include ethanol, inflammatory mediators such as interferon gamma and TNFα, proteases released from mast cells and neutrophils, and a number of drugs [102, 146, 147].

Many diseases have been linked to impaired gut barrier function – the "leaky gut" hypothesis. In relation to liver disease, it has been postulated that an overgrowth of Gram-negatives, allied to impaired gut barrier function, allows whole organisms, through the process referred to as translocation, and/or the Gram-negative bacterial component lipopolysaccharide (LPS), endotoxins, and other bacterial products to gain access to the portal system [148]. While translocation has been repeatedly demonstrated in a host of animal models, its demonstration in man has proven much more challenging due, in large part, to limitations of available assays [149]. It also needs to be emphasized that techniques

Table 8.1 Factors that impact on the microbiota-gut-liver axis in liver pathophysiology

Gut microbiota
Changes in gastric acid secretion, gut motility, and gut defenses promote small intestinal bacterial overgrowth, as well as qualitative and quantitative changes in microbiota
Alcohol
Medications, e.g., antibiotics, proton pump inhibitors, and lactulose
Diet
Reduced bile acid secretion in cirrhosis impacts on microbiota composition
Gut barrier
Alcohol (exogenous and endogenous) effects
Acetaldehyde
Portal hypertension
Inflammatory mediators (e.g., TNF α, interleukin-6, interferon γ)
High fat diet
Antibiotics
Inflammatory bowel disease
Immune deficiency/suppression

that assess gut permeability in man and that have demonstrated changes in various disease states measure paracellular permeability, a process that given the dimensions of this pathway may have little to do with the passage of intact bacteria, their components, or products [150]. Caution needs to be exercised, therefore, in imputing gut "leakiness" in the pathogenesis of liver disease until more reliable methods are available to accurately measure gut permeability in man and detect the passage of bacteria or their components or products into the portal circulation for presentation to the liver. It is likely that bacterial passage across the gut barrier occurs via mechanisms and routes other than the paracellular pathway [150].

The next line of defense is presented by the mucosa (or gut)-associated lymphoid tissue (MALT or GALT) which includes Peyer's patches as well as immune cells in the lamina propria and epithelium (Fig. 8.3a). Microbiota-MALT interactions are critical to the induction of tolerance to the commensal microbiome and the detection and management of pathogens in health and the generation of disordered responses to these same commensals in disease states, such as inflammatory bowel disease [151]. A more complete description of interactions between the microbiome and the immune system of the gut is beyond the scope of this chapter.

A further layer has recently been added to this defense – the gut vascular barrier [152] (Fig. 8.3b). This shares many features with the much better-known blood-brain barrier and involves close contact between enteroglial cells and pericytes and vascular endothelial cells which are, in turn, linked by tight junctions [152]. The gut vascular barrier seems much more permissive than its central nervous system equivalent allowing molecules as large as 4Kd to cross [152]; it has been suggested that by permitting access of dietary and commensal bacterial antigens to the liver, tolerance is promoted [153]. This should preclude the translocation of bacteria; however, certain pathogens, such as *Salmonella typhimurium*, can circumvent this obstacle by directly disrupting this barrier [152]. Already evidence is accruing to indicate that disruption of this gut-vascular barrier by other components of the microbiome, by inflammatory mediators, as well as by signaling molecules involved in bile acid physiology may be involved in the pathogenesis of liver injury and disease [136, 154–157].

The Immune Response in the Liver

In relation to the immune response in the liver to bacteria or bacterial components or products, TLRs and TLR-4 in particular appear to play a key role [158–160]. The role of these factors is exemplified by how lipopolysaccharide (LPS), a glycolipid derived from the outer membrane of Gram-negative bacteria, is handled in health and disease. In health, the integrity of the intestinal barrier, comprising the adherent mucus layer, the enterocyte with its microvilli, the tight junctions between epithelial cells, as well as the secretion of various factors such as defensins, prevents translocation allowing only minute amounts of microbial products to reach the liver [161]. Those that do reach the liver are immobilized or destroyed before they can access the systemic circulation [162]. For example, the systemic circulation is normally protected from endotoxemia by the binding of LPS by LPS receptors and TLR-4 in particular located on the surface of Kupffer cells [163, 164].

In liver disease, an overgrowth of Gram-negative bacteria allied to impaired gut barrier function allows whole organisms, through the process referred to as translocation, and/or LPS to gain access to the portal system. If translocation occurs, bacterial components such as LPS arriving in the portal system activate the inflammasome complex resulting in a cascade of pro-inflammatory cytokine production which ultimately leads to liver injury and can progress to fibrosis [160]. Activation of inflammasome pathways is initiated via binding of LPS to TLR-4 receptors located on the cell surface of Kupffer cells in particular. It should be noted that TLRs are present not only on macrophages and dendritic (antigen-presenting) cells but also on hepatic stellate cells and endothelial cells. The primary step in all inflammasome-mediated reactions is cleavage and activation of caspase-1 which, in turn cleaves and activates the pro-cytokines pro-IL-1β and pro-IL-18. TLR4 activation initiates inflammation through activation of MyD88-dependent and MyD88-independent pathways, the former proceeding via NF-κB and the latter via phosphorylation of the interleukin-regulating factor 3 and leading to the production of type 1 interferons. The ultimate result of these pathways is the production of TNFα,

IL-6, IL-1β, and interferon γ that induce liver injury [165]. Kupffer cells appear to play a major role in inflammasome-mediated pathways as animal studies that involve the depletion of Kupffer cells result in an attenuation of experimental liver damage [162].

Immune responses involving the microbiota have been invoked in the pathophysiology of liver diseases thought to have an immunological or "autoimmune" basis.

Primary sclerosing cholangitis (PSC), a disease characterized by inflammation and fibrotic destruction of the intra- and/or extrahepatic biliary ducts, features marked expression of TLR4, the pathway activated by bacterial lipopolysaccharides (LPS) [22, 158, 166]. It has been postulated that a loss of immune tolerance to endotoxin plays a role in the initial immunologically mediated injury to biliary epithelial cells and subsequent progression in PSC [158]. PSC is commonly associated with ulcerative colitis involving the entire colon (pancolitis); the extent of involvement in the colon in ulcerative colitis has been shown to correlate with levels of bacterial-derived endotoxin concentrations in the portal vein [167]. A further microbiota-mediated mechanism has been proposed in the case of PSC: cross-reactivity between microbial antigens and human tissue components. In PSC, atypical perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) recognize both tubulin beta isoform 5 in human neutrophils and the bacterial cell division protein FtsZ [158, 168, 169].

Cross-reactivity has also been implicated in the pathogenesis of primary biliary cholangitis (PBC) and related autoimmune liver diseases [170]. Toll-like receptors, specifically TLR4, have again been invoked in the pathogenesis of PBC [171]. PBC is characterized by the presence of anti-mitochondrial antibodies (AMA) and, particularly, the M2 component of AMA. AMA cross-react with bacterial components, specifically *E. coli* pyruvate dehydrogenase-E2 [172, 173]. Half of PBC patients also have IgG3 antibodies that cross-react with *Lactobacillus delbrueckii* and other fecal bacteria [174].

It needs to be stressed that microbiota-liver interactions are not all negative. Indeed, the liver has also evolved what has been referred to as a "slow track" of bacterial handling whereby a small fraction of bacteria is allowed to induce antibacterial, T-cell-mediated immunity which is lasting [16]. In a similar manner, low levels of exposure to dietary antigens in portal blood allow tolerance to develop via regulatory T cells.

Gut Microbiota and Hepatic Carcinogenesis

Hepatocellular carcinoma (HCC) is a common and much feared complication of several liver diseases such as those related to hepatitis B and C, hemochromatosis, alcoholic liver disease, and NAFLD. In experimental animals, components of the gut microbiota have been shown to potentiate the hepatic carcinogenic effects of both carcinogenic chemicals and hepatitis viruses [175, 176], and microbial production of deoxycholic acid has been shown to promote obesity-associated HCC through the induction of a senescence-associated secretory phenotype (SASP) in hepatic stellate cells. These cells, in turn, secrete inflammatory and tumor-promoting factors in the liver [177]. Conversely, manipulation of the microbiota to enhance the production of propionate reduces malignant transformation [178]. In NAFLD, progression to HCC has been linked to a distinct change in the microbiome featuring a deficit in anti-inflammatory species and linked to elevated levels of calprotectin in the feces [179]; recent work suggests that microbiota analysis may assist in the early detection of HCC [180]. It has been suggested that intestinal venous congestion occurring during liver resection or transplantation promotes translocation and through TLR4 activation and contributes to the recurrence of HCC; in an experimental animal model, gut decontamination and the inhibition of TLR signaling reduced the likelihood of cancer recurrence [181].

Future Directions

The importance of the microbiome-gut-liver axis in the genesis of important complications of liver disease has long been recognized clinically. More recent explorations of this axis have revealed its complexities and exposed its potential relevance to inflammatory/immune-mediated, metabolic, cholestatic, and neoplastic liver diseases [15, 20, 22, 103, 106, 182–186]. In coming years, studies of the microbiome in human disease will rapidly move from being purely descriptive to being mechanistic and will tell us what a given bug or what a bug-derived molecule actually does and how it can be manipulated. We should also learn more about the contributions of nonbacterial members of the microbiome [187] and other immune cell populations [188, 189] to homeostasis of the microbiome-gut-liver axis in health as well as in liver disease. Only then will the promise of interventions such as diet, prebiotics, probiotics, and fecal transplantation [19, 190–193] be realized, and precise microbial manipulations employed in the prevention and treatment of liver disease and its complications.

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Diagnostic Liver Immunology

9

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Key Points

- Most forms of both acute and chronic liver diseases involve at least a component of an immune response which often is central to diagnosis.
- Even with the widespread use of molecular virology in clinical practice, serologic markers of immune responses to hepatitis viruses and other infectious agents remain relevant to the clinical practice of hepatology.
- Abdominal ultrasound is the first-line radiological technique to assess acute and chronic liver diseases.
- Liver histology remains central in the diagnosis of many acute and chronic liver diseases, particularly metabolic dysfunction-associated fatty liver disease and autoimmune hepatitis.
- Anti-mitochondrial antibodies remain one of the key diagnostic hallmarks of primary biliary cholangitis with extremely high sensitivity and specificity.
- Autoantibodies, provided they are tested according to dedicated guidelines, are a key feature of autoimmune hepatitis and of the pediatric condition termed autoimmune sclerosing cholangitis.
- Autoantibodies are common in primary sclerosing cholangitis but of lesser clinical significance due to lower diagnostic accuracy.
- IgG4-related sclerosing cholangitis is a newly recognized disease that may mimic primary sclerosing cholangitis but is responsive to immunosuppression.

Introduction

The vast majority of liver diseases involve, at least in part, an immunologic reaction either as a primary cause of liver injury or in response to an infectious agent or to a toxic xenobiotic. Primary biliary cholangitis (PBC) and metabolic dysfunction-associated fatty liver disease (MAFLD) are representative of the former, while viral hepatitis B and drug-induced liver injury are typical of the latter. Historically, the diagnosis of a liver disease was based primarily on histology and in large part on the types and locations of inflammatory cells within the liver parenchyma. This remains the case for alcoholic liver disease, autoimmune hepatitis (AIH), and the now epidemic fatty liver disease. However, increasingly liver disease diagnoses are made noninvasively based upon specific immune responses signified by the presence of specific antibodies and other laboratory findings, such as identification by polymerase chain reaction (PCR) of infectious agent-specific genome. Currently, the role of genetic tests in the diagnosis of liver diseases is becoming more important, well beyond the classical HLA class II alleles predisposing to AIH and the variants in the HLA class I-like *HFE* gene resulting in dysregulation of the antimicrobial peptide hepcidin and subsequent iron overload. Examples of genetic tests that recently entered clinical practice include *ABCB4* in cholestatic conditions and genetic variations in drug-metaboliz-

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Table 9.1 Etiologies of several immune-mediated liver diseases

Infectious liver diseases	Autoimmune liver diseases	Granulomatous liver diseases
Hepatitis A	Primary biliary cholangitis	Primary biliary cholangitis
Hepatitis B	Primary sclerosing cholangitis	Sarcoidosis
Hepatitis C	Autoimmune hepatitis	Common variable immunodeficiency
Hepatitis D	IgG4-related sclerosing cholangitis	*Mycobacterium*
Hepatitis E		*Leishmania*
Brucellosis	**Posttransplant liver diseases**	*Schistosoma*
Entamoeba histolytica	Allograft rejection	*Listeria*
Echinococcus	De novo autoimmune hepatitis	*Yersinia*
Schistosoma		Tularemia
		Psittacosis
	Graft-versus-host disease	*Bartonella*
		Cytomegalovirus
		Epstein-Barr virus
		Hepatitis A, B, and C
		Histoplasma
		Coccidioides
		Cryptococcus
		Nocardia
		Candida
		Coxiella burnetii
		Allopurinol
		Diltiazem
		Interferon alpha
		Anti-CTLA-4 drugs

ing enzymes associated to increased susceptibility to drug-induced liver injury. In this chapter, we will review the more common liver diseases with an immunologic basis with an emphasis on the diagnostic laboratory and imaging tools in current use (Table 9.1).

Liver Biochemistries

Central to the diagnosis of any liver disease are the liver biochemistries, particularly the hepatocellular damage markers aminotransferases, encompassing alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and the cholestasis markers alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT). Except in severe cases of acute hepatitis or advanced disease, the majority of chronic liver diseases cause few, if any, symptoms and often nonspecific signs. Thus, chronic liver disease is often diagnosed inadvertently on routine liver biochemistries and less frequently in response to specific complaints. Conversely, it has been recognized that even minor elevations of liver biochemistries, particularly the ALT which may be reported as within the normal clinical laboratory reference range, are associated with increased liver-related mortality [1–3]. Evaluation of the pattern of liver biochemistry abnormality is the first step in the diagnosis of any liver disease, bearing in mind that a normal liver biochemical profile is not infrequent in patients with cirrhosis or fatty liver disease [4]. Elevation primarily of the ALT suggests an injury to the hepatocyte due to a viral hepatitis, AIH, or other infectious or toxic causes. Outside alcoholic liver disease, ALT levels exceeding AST levels are suggestive of absence of advanced fibrosis, explaining why the ALT and AST levels are included in many scores used to discriminate between early and advanced liver fibrosis [4]. Of note, the clinician should be aware that ALT and AST elevation may indicate cellular damage outside the liver, particularly in skeletal muscles and myocardial tissue. Therefore, measurement of creatine kinase serum levels should be included in the diagnostic workup of elevated transaminase levels.

A cholestatic pattern of liver biochemistries, i.e., elevation of ALP and GGT coupled with bilirubin elevation in case of advanced disease, is typical of disorders affecting the bile ducts such as PBC and primary sclerosing cholangitis (PSC) but may also be present in granulomatous disease such as sarcoidosis or *Coxiella burnetii* infection.

An isolated raised GGT level is often encountered in clinical practice, the differential diagnosis being broad, which can be caused by alcohol consumption, metabolic dysfunction-associated fatty liver disease (MAFLD), obesity, hyperglycemia, exposure to certain drugs, and cholestasis, and is a marker of increased cardiovascular risk [5]. While in adults concomitant GGT and ALP elevation is highly suggestive of cholestasis, in children, there is a physiological elevation of ALP of bone origin, so GGT levels are key to the diagnosis of cholestasis in the pediatric population.

Platelet count should also be part of the laboratory tests performed in patients with suspected chronic liver disease, since thrombocytopenia is an early indicator of advanced liver fibrosis [4].

Serum bilirubin and albumin levels, as well as the international normalized ratio (INR), are markers of the liver synthetic function, rather than of liver damage, and are part of the Child-Pugh score, which is widely used in clinical practice for prognostic purposes. Similarly to what was discussed above concerning transaminase levels and cholestatic markers, extrahepatic causes of perturbation of these tests must be ruled out. Concerning bilirubin, it is helpful to measure unconjugated and conjugated fractions, whereby increased levels of unconjugated bilirubin are suggestive of an extrahepatic cause, mostly reflecting overproduction (e.g., during hemolysis), while increased conjugated bilirubin levels are more suggestive of a liver dysfunction, being due to impaired hepatic or biliary secretion. The most common extrahepatic causes of decreased albumin serum levels include malnutrition and proteinuria. INR may be increased in case of malnutrition, particularly dietary vitamin K deficiency, as well as

Table 9.2 Proposed first-line laboratory diagnostic workup in adult immunocompetent patients presenting with chronic liver disease of unknown cause in Western countries. Hepatitis A serology is included in order to assess the need of vaccination

Infectious serology	Anti-HAV HBsAg, anti-HBc, anti-HBs Anti-HCV Anti-HDV if HBsAg is positive HIV
Metabolic	Thyroid-stimulating hormone Ferritin, transferrin saturation; serum lipids and glucose
Genetic	Ceruloplasmin Alpha-1 antitrypsin
Autoimmune	Antinuclear, anti-smooth muscle, anti-liver kidney microsomal 1, anti-soluble liver antigen, anti-mitochondrial, antineutrophil cytoplasmic antibodies, total IgG and IgM Anti-transglutaminase antibody

Abbreviations: *HAV* hepatitis A virus, *HB* hepatitis B, *HDV* hepatitis delta virus, *IgG* immunoglobulin G, *IgM* immunoglobulin M

in case of liver dysfunction, reflecting impaired hepatic synthesis of vitamin K-dependent clotting factors, i.e., factors II (prothrombin), VII, IX, and X.

A list of laboratory tests useful in assessing the cause of chronic liver disease in adult patients is presented in Table 9.2.

Imaging

In addition to laboratory tests, imaging is key in the diagnostic workup of patients presenting with acute or chronic liver disease. The first-line radiological technique is ultrasound, which is widely available and inexpensive, can be done at bedside, and does not expose patients to radiation. A liver parenchymal echogenicity higher than kidney tissue is suggestive of steatosis; however, hyperechogenicity ("brighter liver") may also be seen in cirrhosis [6]. Surface nodularity, hypertrophy of the caudate lobe, and reduced liver size are suggestive of cirrhosis, whereas splenomegaly (spleen volume > 200 cm^3), ascites, and peri-splenic portosystemic collateral vessels are signs of portal hypertension. The liver parenchyma should be carefully evaluated for the presence of focal lesions. Doppler imaging is used to assess the blood flow of the portal vein, of the hepatic veins, of the inferior vena cava, and of the hepatic artery. Second-line imaging techniques include cross-sectional, multiphasic, and contrast-enhanced techniques. Computer tomography, which has the main disadvantage of exposing patients to radiation, is the preferred technique to study the blood vessel patency and anatomy. Magnetic resonance imaging is the technique of choice to investigate focal liver lesions, often detected by screening ultrasound. Magnetic resonance contrast agents which are taken up by normal hepatocytes and excreted into the bile are particularly useful in characterizing malignant and benign focal liver lesions [7].

Assessment of Liver Fibrosis

Fibrosis stage is the most important prognostic factor in chronic liver diseases [8–10], whereby the gold standard method for its assessment is liver histology, which also allows to get insights into the fibrosis pattern. Due to the invasiveness and to the high costs of liver biopsy, noninvasive diagnostic tools are increasingly entering clinical practice, being particularly useful to rule out advanced fibrosis. They include tests based on serologic markers and liver elastography and are validated in several chronic liver diseases. The former are divided into direct markers of fibrosis, reflecting ongoing fibrogenesis or fibrinolysis and including, for instance, hyaluronic acid, metalloproteinases, and collagen IV, and into indirect markers of fibrosis, reflecting the consequences of liver fibrosis, encompassing routine laboratory tests and clinical parameters. Direct markers of fibrosis are included in patented algorithms, limiting their availability. Among the most widely used indirect markers of fibrosis are the AST to platelet ratio index and the FIB-4 score, along with the NAFLD fibrosis score which is specific for nonalcoholic fatty liver disease [11]. Elastography harnesses the physical law stating that the velocity of propagation of shear waves in a tissue depends on the tissue elasticity [11]. Fibrosis increases the stiffness of the liver, leading to higher propagation velocity, which can be measured by ultrasound or by magnetic resonance, providing a noninvasive estimate of liver fibrosis. The most widely used and better validated ultrasound-based technique is transient elastography; other newer methods include point shear wave elastography and two-dimensional shear wave elastography [12]. Importantly, liver stiffness is increased in the presence of parenchymal inflammation, of non-fasting status, and of impaired blood outflow, such as Budd-Chiari syndrome and right heart failure. A transient elastography value <20 kPa associated with a platelet count >150 G/l is now considered a reliable indicator of absence of large varices in patients with compensated cirrhosis, allowing to avoid screening endoscopies in patients meeting these criteria [13]. Moreover, combined ultrasound elastography of liver and spleen is increasingly used as a diagnostic tool to assess non-cirrhotic portal hypertension [12].

Infectious Liver Diseases

Serum antibodies, whether directed to foreign agents or to self-antigens, are key to the diagnosis of immunologic liver diseases. Antibodies to viral, bacterial, and parasitic antigens are central to diagnosing infectious liver diseases as well as determining immunity status. Similarly, autoantibodies and elevated levels of immunoglobulins are characteristic of autoimmune liver diseases. Importantly, the absence of immunoglobulins such as occurs in common variable immunodeficiency (CVID) is also associated with a variety of liver

diseases. Moreover, decreased or absent immunoglobulin A is associated with juvenile AIH, particularly type 2 [14].

Hepatitis A Virus

Hepatitis A virus (HAV) is generally transmitted via a fecal-oral route and typically presents as an acute illness with abdominal symptoms and jaundice, though the infection may be completely asymptomatic, particularly in children, and may take a relapsing cholestatic course in about 10% of the cases. The diagnosis is dependent upon the presence in serum of anti-HAV IgM, which appears within 2–4 weeks of infection [15]. Recently, detection of anti-HAV IgM in saliva has also been reported, at times remaining positive for longer as compared to serum [16]. Although IgM is lost in the majority of cases 6 months after infection, its persistence beyond 9 months has been reported [17, 18]. Positive tests for anti-HAV IgM have also been reported in individuals with no signs of an acute infection leading to the recommendation that this test only be performed when there is clinical suspicion of an acute illness [19]. The IgG class of anti-HAV is present early in infection and thus cannot distinguish an acute infection from a resolved infection or prior vaccination. After vaccination or, most importantly, after viral exposure, delayed seroconversion is possible in immunocompromised or very young subjects: in this case, the diagnosis relies on detection of HAV RNA in plasma by PCR, which is performed only in specialized laboratories.

Hepatitis B Virus

In contrast to HAV infection where the diagnosis is based upon the presence of a humoral response to viral epitopes, hepatitis B virus (HBV) infection is typically diagnosed by the presence of the HBV surface antigen (HBsAg). Nevertheless, antibodies to HBV antigens are critical for the accurate diagnosis, staging, and treatment decisions (Table 9.3). During acute infections acquired in adolescence or adulthood, as typically occurs in North American and European populations, HBsAg may be absent by the time of presentation with clinical symptoms. In this "window" period, the presence of IgM antibodies to the core antigen (anti-HBc IgM) indicates an acute infection in most cases. The diagnosis is helped by molecular detection of HBV DNA. It is important to recognize that during reactivation of chronic HBV infection, anti-HBc IgM may become positive [20, 21]. The mere presence of anti-HBc, often referred to as anti-HBc only status, is not infrequently encountered in clinical practice and can represent a false-positive reactivity (particularly in low-prevalence countries), an association with HCV or HIV coinfection, or, lastly, a recovered infection with undetectable anti-HBs (see Table 9.3) [22]. Further, detectable levels of HBV DNA can be found in up to 15% of individuals with anti-HBc alone status, this status corresponding to occult HBV infection [23]. Immunosuppression, particularly with anti-CD20 antibody, can lead to severe HBV reactivation even in patients with occult HBV infection or anti-HBc only [24, 25].

In chronic HBV infection, which is a dynamic process with varying serological findings over time and typically dates back to the neonatal period or early childhood in non-immunocompromised individuals, a clinically important milestone is the loss of HBV e antigen (HBe) and the appearance of anti-HBe which often signifies a transition to inactive disease, associated with a good prognosis. However, viral mutations in the basal core promoter and pre-core regions can lead to active disease in the absence of HBe production and disease progression. Nevertheless, a primary outcome of treatment in patients with HBe-positive infection is seroconversion to anti-HBe [26, 27]. Anti-HBs that developed either through natural infection or immunization are protective against infection. The titer of anti-HBs after vaccination wanes over time, and booster immunization, particularly needed in at-risk patients such as health workers, is probably effective in eliciting an anamnestic response. The small subgroup of patients not responding to standard vaccine doses requires repeated vaccination with higher doses, which, however, not always leads to effective immunization [28].

Table 9.3 Interpretation of hepatitis B serum tests

	HBs	Anti-HBs	Anti-HBc IgG	Anti-HBc IgM	HBV DNA	HBe	Anti-HBe	ALT
Acute infection	+	–	–	+	+/–	+/–	+/–	Elevated
"Window" period	–	–	–	+	+/–	+/–	+/–	Elevated
Chronic infection								
HBe-positive chronic infection	+	–	+	–	+++	+	–	Normal
HBe-positive chronic hepatitis	+	–	+	–	+++	+	–	Elevated
HBe-negative chronic infection	+	–	+	–	–/+	–	+	Normal
HBe-negative chronic hepatitis	+	–	–	+	++	–	+/–	Elevated
Past exposure/vaccination								
Convalescent infection	–	+/–	+	–	–	–	+/–	Normal
Vaccinated	–	+	–	–	–	–	–	Normal
Occult infection	–	+/–	+	–	+	–	+/–	Normal

Abbreviations: *HBV* hepatitis B virus, *HBs* hepatitis B surface antigen, *HBc* hepatitis B core antigen, *HBe* hepatitis B e antigen, *ALT* alanine aminotransferase

Hepatitis C Virus

Although antibodies to hepatitis C virus (HCV) antigens develop as early as 2 months after infection, they do not differentiate acute, chronic, and resolved infections; thus, the diagnosis of ongoing HCV infection is dependent upon the detection of viral nucleic acids in serum. Nevertheless, prevalent cases of HCV infection are still detected primarily by the presence of anti-HCV IgG, and screening for HCV has been recommended by the US Centers for Disease Control and Prevention for all persons born between 1945 and 1965 [29]. Since the cloning of the HCV genome and identification of B-cell epitopes, several generations of immunoassays have been developed. First-generation assays were based only on the nonstructural 4 (NS4) antigen. These assays detected approximately 80% of post-transfusion hepatitis but lacked sensitivity and specificity [30]. Second-generation assays incorporated epitopes from the core and NS3 regions followed by the addition of epitopes from NS5 in third-generation assays [31]. Although these assays have a diagnostic accuracy of >99%, they can yield false-negative results in immunocompromised patients and have a low positive predictive value in populations with a low prevalence of HCV infection. Anti-HCV seroconversion after spontaneous or therapy-induced viral clearance is rare but possible [32].

Hepatitis D Virus

Hepatitis D virus (HDV) is a defective RNA virus which requires infection with HBV for HDV to replicate [33]. HDV RNA can be detected in serum by reverse transcription (RT)-PCR methods or in liver tissue by in situ hybridization, and HDV antigen can be detected in serum by either enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). In the USA, HDV antigen tests are not available; on the contrary, HDV RNA tests are offered by commercial laboratories but are qualitative and not approved by the Food and Drug Administration. Anti-HDV IgM and IgG antibodies should be tested in every HBsAg-positive subject. Anti-HDV antibodies usually persist after spontaneous or treatment-induced HDV clearance.

Hepatitis E

Similar to HAV infections, short-lived anti-HEV IgM is detectable in serum within 2–6 weeks of infection and is followed by long-lived IgG antibodies. Assays for both IgM and IgG classes of anti-HEV varied considerably in their performance, leading to considerable variation of reported seroprevalence. After becoming aware of this problem, more sensitive and specific assays are increasingly used; nevertheless, none of them are currently licensed in the USA [34]. Although initially characterized as a waterborne disease of developing countries with both endemic infections and sporadic outbreaks, there has been an increasing recognition of autochthonous infections in developed countries, where HEV is a zoonosis, mainly transmitted via consumption of undercooked pork meat [34]. Perhaps due to the lack of easy access to testing and/or high frequency of asymptomatic infections, HEV is rarely reported in the USA despite a reported seroprevalence of 21% and an annual incidence of 0.7% [35, 36]. A recent meta-analysis estimated a similar HEV seroprevalence in Europe and the USA of about 9% [37].

Bacterial and Parasitic Infections

Nonviral infections of the liver span bacterial, mycobacterial, parasitic, and, in immunocompromised subjects, fungal organisms and are often difficult, if not impossible, to diagnose by culture. Brucellosis is caused by a small Gram-negative coccobacillus, which is the most common zoonotic infection worldwide and often causes a granulomatous hepatitis. Culture of *Brucella* is time-consuming and insensitive, leaving the diagnosis to serologic tests including serum agglutination testing and ELISA, the latter being able to measure IgM, IgG, and IgA titers. Hepatic amebiasis and amebic abscess from disseminated infections caused by *Entamoeba histolytica* are typically diagnosed based upon the appropriate travel history, symptoms, and imaging with confirmation by serologic testing for antibodies to *E. histolytica*. Imaging and serologic testing for antibodies are the basis for diagnosis in the majority of cases of infection with *Echinococcus*, a zoonotic infection in humans as a result of ingestion of eggs of the tapeworm resulting in hepatic cysts. Focal liver infections may also be caused by pyogenic bacteria or fungi, mostly in immunocompromised patients. In this setting, the diagnosis is based on culture of an abscess specimen.

Schistosomiasis, a parasitic infection caused by blood flukes which is endemic in large areas of Africa, South America, and Asia, is typically diagnosed by microscopical identification of parasitic eggs in urine or stool. Several serological assays are available with variable sensitivity and specificity, being particularly useful to rule out schistosomiasis in endemic areas or as a diagnostic tool in patients with a low parasite burden [38].

Autoimmune Liver Diseases

The major autoimmune liver diseases have historically included primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), and autoimmune hepatitis (AIH).

More recently, a pediatric condition referred to as autoimmune sclerosing cholangitis (ASC), referring to an overlap of juvenile AIH with sclerosing cholangitis, has been recognized [39]. Another newly recognized autoimmune liver disease is a variant of autoimmune pancreatitis with biliary involvement associated with elevated levels of serum and tissue IgG4, thus the term IgG4-related sclerosing cholangitis. PBC, PSC, and IgG4-related sclerosing cholangitis must be distinguished not only from each other but also from other causes of cholestasis, including genetic cholestatic diseases, a clinical entity increasingly recognized also in adults with chronic cholestasis [40, 41]. In addition to the clinical setting, the diagnosis of autoimmune liver diseases is based upon autoantibodies, imaging studies, and liver histology (Table 9.4). Recently, a quantitative PCR protocol analyzing the IgG4/total IgG RNA ratio in blood has been proposed as a valuable tool in distinguishing PSC from IgG4-related sclerosing cholangitis, which is a highly relevant clinical issue [42]. Similarly, AIH may present with all the features typical of an acute or chronic viral hepatitis: indeed, it is well known that autoantibodies are frequently detected in patients with viral hepatitis [43]. Imaging is less useful in this setting, the diagnosis of AIH being established by serologic and histologic findings. In rare cases, patients may present with features of two autoimmune liver diseases, particularly PBC and AIH or PSC and AIH, either simultaneously or sequentially. These so-called "overlap" syndromes have been poorly defined, and agreement on the criteria is lacking. Half of the children presenting with AIH have radiological evidence of concomitant sclerosing cholangitis, thus meeting the definition of autoimmune sclerosing cholangitis [39].

Primary Biliary Cholangitis

Diagnosis of PBC is based primarily on the highly sensitive and specific anti-mitochondrial antibody (AMA) reacting to the precisely defined epitope of lipoic acid of the E2 subunit of pyruvate dehydrogenase located on the inner mitochondrial membrane. AMA is present in up to 95% of cases, and its presence is one of the three key criteria to PBC diagnosis, the other two being an elevated serum ALP level and a liver biopsy with features consistent with PBC. In addition, even in the absence of elevated alkaline phosphatase, the presence of AMA has been associated with histological changes in the liver and perhaps portends the future development of PBC [44]. In addition to AMA, PBC is also associated in about one-third of the patients, with specific antinuclear antibodies (ANAs), namely, anti-gp210 and anti-sp100, giving a nuclear rim and multiple nuclear dot pattern, respectively, in indirect immunofluorescence on HEp2 cells. In cases of AMA-negative PBC, these ANAs can assist in making the diagnosis [45].

Elevated levels of serum IgM are found in some 70% of the PBC patients and appear to be related at least in part to genetic polymorphisms in Toll-like receptor 9 (TLR9) leading to hyperresponsive memory B cells to bacterial CpG [46, 47]. Serum IgM levels in PBC have also been inversely correlated with methylation of the CD40L promoter in CD4+ T cells suggesting a mechanism involving the cross talk of CD40 and CD40L which is involved in CD4+ T-cell priming, B-cell terminal maturation, and Ig class-switch recombination [48]. Gene methylation leads to gene silencing, and anti-CD40 ligand monoclonal antibody has shown to ameliorate cholangitis in a PBC mouse model, suggesting that

Table 9.4 Diagnostic features of autoimmune liver diseases

	Age	Sex	ALP	AST/ALT	AMA	ANA	pANCA	SMA	Ig
Primary biliary cholangitis	>40 years	90% female predominance	Mildly to markedly increased	Normal to mildly increased	Positive in 90–95%	Nuclear rim or multiple nuclear dot	Negative	Negative	Elevated IgM
Primary sclerosing cholangitis	Any age, peak incidence 30–40 years	Male predominance	Normal to markedly increased	Normal to moderately increased	Negative	Positive in 8–77%, no specific pattern	Positive in 26–94%	Positive in 0–83%	Elevated IgM in 45% Elevated IgG4 in 10% (poor prognosis)
Autoimmune hepatitis	Any age	Female predominance	Normal to mildly elevated	Elevated	Negative	Positive in up to 80% (type I)	Positive in up to 90%	Positive in up to 85% (type I)	Elevated IgG in >90%
Autoimmune sclerosing cholangitis	Children and adolescents	Equally distributed	Normal to markedly increased	Normal to markedly increased	Negative	Positive in up to 77%	Positive in up to 74%	Positive in up to 77%	Markedly elevated IgG
IgG4-related sclerosing cholangitis	>50 years	>80% male predominance	Elevated	Normal to markedly elevated	Negative	Positive in up to 40%	Unknown	Unknown	Elevated serum IgG4 and/or histological staining for IgG4

Abbreviations: *ALP* alkaline phosphatase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AMA* anti-mitochondrial antibody, *ANA* anti-nuclear antibody, *pANCA* peripheral anti-neutrophil cytoplasmic antibody, *SMA* anti-smooth muscle antibody, *Ig* immunoglobulin

blocking of the CD40-CD40 ligand interaction may be of benefit in PBC [49]. While an elevated IgM may be a useful diagnostic tool in AMA-negative cases, its clinical significance remains unclear. In patients transplanted for PBC, raised IgM levels have been associated with disease recurrence [50].

Primary Sclerosing Cholangitis

A host of autoantibodies have been detected in PSC patients, but none has been shown to be of clinically significant prevalence and specificity to warrant inclusion as a major diagnostic criteria [51]. Perinuclear antineutrophil cytoplasmic antibodies (pANCA) have been found in approximately 80% of PSC subjects, but they are also frequently found in patients with ulcerative colitis and AIH, in addition to the extra-gastrointestinal conditions microscopic polyangiitis and eosinophilic granulomatosis with polyangiitis [52–54]. Although a specific atypical pANCA, also termed peripheral antineutrophil nuclear antibody (pANNA) or nuclear antineutrophil antibody (NANA), has been associated with the above-mentioned autoimmune gastrointestinal conditions and a putative self-antigen has been reported, confirmation of the antigen and identification of the epitope remain unresolved [55, 56]. Other autoantibodies including ANA and anti-smooth muscle antibody (SMA) are less frequently present in PSC patients, and their clinical significance has yet to be determined. Thus, in PSC, autoantibodies play only a minor diagnostic role leaving the diagnosis to typical cholangiographic findings in the absence of secondary causes of sclerosing cholangitis, often coexisting with inflammatory bowel disease. In a minority of cases, diagnosis is made on liver biopsy findings typical of PSC including bile duct injury and obliterative fibrosis. Such cases are designated as "small duct" PSC when the cholangiogram is normal [51, 57].

Autoimmune Hepatitis

Specific diagnostic criteria for AIH have been established by an international panel of experts and revised twice [58–60]. All three versions of the International AIH Group scoring system have included the presence of specific autoantibodies reflecting their importance in the diagnosis of AIH, a definite diagnosis being not reached in autoantibody-negative patients, according to the most recently published scoring system [59]. Nevertheless, it remains clear that autoantibodies are neither necessary nor sufficient to establish the diagnosis of AIH. Thus, autoantibodies may be detected in a variety of acute and chronic liver inflammatory conditions, including viral hepatitis, drug-induced liver injury, acute liver failure, and MAFLD. In this context, it should be reminded that diagnostic laboratory assays are not standardized, and, if specific methodological guidelines are followed, more than 95% of AIH patients have at least one serological positivity [52]. In addition to ANA and SMA, liver-kidney microsomal type 1 (LKM-1), anti-liver cytosol type 1 (LC1) antibody, and anti-soluble liver antigen (SLA) have been the primary autoantibodies used in the diagnosis and classification of AIH [61]. SMA along with ANA is typical of type 1 AIH. Although the antigens of SMA have not been completely characterized, anti-F actin ELISA-based tests are often used by large commercial laboratories rather than indirect immunofluorescence even though 20% of type 1 AIH patients with SMA are negative for F-actin [62]. In contrast, the molecular target of anti-LKM-1 antibody has been identified as the cytochrome P450 2D6 subunit, and reliable commercial immunoassays utilizing the antigen are available. The presence of anti-LKM-1 indicates type 2 AIH, which typically presents in children and young adults, with or without coexisting anti-LC1 antibody. Anti-LKM-1 titers correlate with disease activity in children. Antibodies to SLA were initially thought to represent a third type of AIH but more recently have been identified in typical cases of type 1 and 2 AIH. The target of this antibody has been identified as UGA tRNA suppressor-associated antigenic protein, and the presence of anti-SLA is associated with severe disease and poor outcomes [63–65]. Anti-SLA is detected also in 41% of children with autoimmune sclerosing cholangitis [66]. Hypergammaglobulinemia is present in greater than 90% of AIH cases and is a major diagnostic criterion [59]. The mechanism underlying this phenomenon is unclear, bust the level does correlate with disease activity making the serial testing of IgG levels useful for monitoring disease activity, even in patients with IgG levels within the normal range at diagnosis. Hypergammaglobulinemia may also be useful in distinguishing AIH from MAFLD in which ANA and SMA are frequently present [67].

Autoimmune Sclerosing Cholangitis

Autoimmune sclerosing cholangitis (ASC) is a clinical entity described in 2001 referring to an overlap between juvenile AIH and sclerosing cholangitis with strong autoimmune features [39, 68]. It is diagnosed by cholangiography in children/adolescents presenting with AIH, who are found to have abnormal cholangiographic findings in half of the cases [39]. Autoantibodies are invariably positive, ANA and/or SMA being detected in the vast majority of the ASC patients and anti-LKM-1 being rare. ANCA are positive in three quarters of the cases [68]. Therefore, in contrast to adult PSC, detection of serum autoantibodies plays a key

diagnostic role in ASC [39, 68]. Total IgG serum levels are elevated, often at particularly high levels, in 90% of the patients [39]. Association with inflammatory bowel disease is frequent, being present in half of the patients [39]. Response to the same immunosuppressive treatment as AIH is satisfactory in a high proportion of the patients, but the transplant-free survival is significantly shorter as compared to juvenile AIH [69].

The relationship between the classical, adult PSC and the pediatric ASC remains to be established [68].

IgG4-related Sclerosing Cholangitis

IgG4-related sclerosing cholangitis has been recognized as one of the many systemic sclerosing diseases associated with elevated levels of serum IgG4 and tissue lymphoplasmacytic infiltration of IgG4-positive cells. IgG4-related sclerosing cholangitis is often associated with autoimmune pancreatitis and can resemble PSC with sclerosing lesions of the bile ducts [70–75]. Differentiating IgG4-related sclerosing cholangitis from PSC can be problematic and is made more difficult by the lack of sensitivity of raised serum IgG4 for IgG4-related sclerosing cholangitis, which can be normal in up to one-third of the patients. As mentioned above, an RNA PCR helping in differentiating PSC from IgG4-related sclerosing cholangitis has been recently reported but needs to be validated before entering clinical practice [42]. Additionally, elevated levels of serum IgG4 are present in approximately 10% of PSC patients, a group noted to have more rapid progression of disease and less frequent inflammatory bowel disease [76, 77]. It remains unclear if these PSC patients actually represent IgG4-related sclerosing cholangitis or a true subgroup of PSC [78–80]. However, making this distinction may be clinically relevant because like a host of other IgG4-related disorders and in contrast to PSC, IgG4-related sclerosing cholangitis is usually responsive to immunosuppression with steroids and azathioprine [71, 73, 75]. It should be noted that very high (i.e., >5.6 g/l) serum IgG4 levels have 100% specificity for IgG4-related sclerosing cholangitis. A history of allergy/atopy and elevated serum IgE levels are found in 40–60% of patients with IgG4-related sclerosing cholangitis. To add more complexity in the differential diagnosis, ANA is positive in about half of the patients with IgG4-related SC [70].

Granulomatous Liver Diseases

Granulomas represent a specific form of tissue inflammation triggered either by a nonspecific inflammation or by an antigen, being composed of aggregates of modified macrophages, T lymphocytes, and dendritic cells [81]. Involved triggers are manifold, including infective agents, xenobiotics, malignancies, and systemic inflammations. Macrophages are the predominating cell type within granulomas, whereby two subtypes can be distinguished, i.e., M1 macrophages, having pro-inflammatory function, and M2 macrophages, having immunoregulatory functions. One type of macrophages tends to predominate in a specific granulomatous condition [81].

Relatively rare, granulomas are found in only 2–15% of liver biopsies either as an isolated granulomatous disease or as part of a systemic disease [82–88]. Biochemically, hepatic granulomatous diseases typically present with elevated serum levels of ALP and GGT, although the liver biochemical profile may be normal [89]. Rarely do these diseases result in non-cirrhotic portal hypertension or cirrhosis. Although the list of potential causes of liver granulomas is too numerous to include here, the most common causes can be classified as immunologic disorders, infectious diseases, or drug reactions. In Europe and North America, the most common identifiable causes include PBC and sarcoidosis, with drug reactions and infectious diseases responsible for a small minority of cases [83, 84, 86]. In contrast, infectious causes including *Mycobacterium tuberculosis*, visceral leishmaniasis, and schistosomiasis are common in the Middle East and South Asia [85, 87, 88]. However, in the past decades, infectious diseases were the predominant etiology of hepatic granulomas also in Europe [82].

Sarcoidosis

Sarcoidosis is a systemic granulomatous disorder defined by the presence of noncaseating granulomas in the tissues involved; most frequently, involvement of the lungs is diagnosed. However, autopsy studies suggest that the liver is frequently involved with granulomas being found in 67–70% of cases, with a greater percentage found in African-Americans compared to Caucasians [90]. Up to one-quarter of patients can have liver without lung involvement [91]. While the majority of patients are asymptomatic, the clinical presentation can include hepatomegaly, pruritus, and rarely jaundice or non-cirrhotic portal hypertension [92–94]. Sarcoidosis may even present with biliary obstruction mimicking PSC [95].

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) is a heterogeneous disease characterized by impaired B-cell differentiation resulting in defective immunoglobulin production and therefore low IgG serum levels (<5 g/l in adults), leading to chronic infections, as expected of an immunodeficient state.

CVID frequently manifests with autoimmune disorders, including autoimmune cytopenia, rheumatoid arthritis, inflammatory bowel disease-like conditions, and vitiligo [96]. Twenty-four to 90% of CVID patients develop epithelioid granulomas in the liver which may be isolated or involve multiple organs [97–99]. Typically, these patients present with elevated serum ALP, with or without raised GGT and transaminases serum levels. Given that granulomas are also typical of PBC, it is not surprising that cases of PBC in CVID have been reported: importantly, PBC in the context of CVID may be AMA negative due to immunoglobulin deficiency [94, 96]. In addition, nodular regenerative hyperplasia (NRH) has been reported in 84% of CVID patients [99].

Conclusion and Future Directions

Establishing the diagnosis of immune-mediated liver diseases requires a comprehensive knowledge of liver immunology and pathology. Through combination of a careful medical and family history, serologic testing, imaging, and liver biopsy histology, a diagnosis can be reached in the majority of cases. In the setting of infectious liver diseases, the diagnosis is relatively straightforward with highly sensitive and specific tests available. In contrast, the autoimmune and granulomatous liver diseases, with the exception of PBC, lack tests which are both sensitive and specific, thus requiring the interpretation of a multitude of tests and the judgment of the clinician.

As complex as diagnostic liver immunology is presently, the future will likely see additional complexity as genetic markers are added to the diagnostic armamentarium. In particular, the role of primary immunodeficiencies in autoimmunity is increasingly recognized, but the heterogeneous clinical features, the lack of knowledge about the immunological mechanisms, at times the complex and polygenic genetic defects, and limitations in the immunological diagnosis are factors contributing to the current underdiagnosis of these conditions. Efforts have to be made also in the field of autoimmune liver serology, in order to achieve standardization of the assays and therefore a better exploitation of the high diagnostic value of liver-related autoantibodies.

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The Pathologist's Approach to Reviewing Liver Histology

10

Kenichi Harada

Key Points

- The differentiation of acute vs. chronic and hepatitic vs. cholestatic is fundamental to a pathological diagnosis.
- There is always a risk of sampling error with liver biopsy when diagnosing chronic liver diseases.
- Overlaps of more than two liver diseases, especially non-alcoholic fatty liver disease (NAFLD) and drug-induced liver injury (DILI), always should be considered.
- It is difficult and risky to render a diagnosis solely based on the presence of bile duct lesions, including chronic nonsuppurative destructive cholangitis (CNSDC).
- Hepatitic changes are closely associated with the developmental stage of primary biliary cholangitis (PBC).
- The primary histological feature of autoimmune hepatitis (AIH) is chronic active hepatitis with nonspecific findings.
- The pathological diagnosis of acute-onset AIH is challenging to differentiate from DILI.

Introduction

The pathological diagnosis of hepatobiliary diseases, particularly inflammatory diseases, is based on clinicopathological features. It is relatively easy to arrive at a definitive diagnosis of some hepatobiliary diseases, such as viral hepatitis, based on the presence of hepatitis virus in sera. However, other immune-mediated hepatobiliary diseases, such as autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC), with an atypical subtype often coexist or are complicated with other liver diseases. In particular, non-alcoholic fatty liver disease (NAFLD) and drug-induced liver injury (DILI) are generally caused by lifestyle factors, and the prevalence of cases has continued to increase. Moreover, the complications of these diseases are often encountered in AIH and PBC patients. Therefore, the pathologist must consider the association and complication of additional diseases in patients with liver dysfunction, even if the clinical possibilities of NAFLD and DILI are not substantial. In this chapter, the pathological approach to examine liver specimens is reviewed, particularly for arriving at a diagnosis of PBC and AIH.

Determination of Normal, Nonspecific Reactive, Original Liver Diseases Versus Overlapping Liver Diseases

The hepatic parenchyma is composed of small lobules with roughly hexagonal shapes and portal tracts at the apices. Within the lobules, hepatocytes are arranged as cords of cells connecting the portal tracts in the periphery to the central veins (terminal branches of hepatic veins). In the observation of liver histology, first of all, the pathologist must differentiate normal from abnormal hepatic tissues. In the normal liver, the lobular architecture consists of portal tracts with a well-preserved central vein. The presence of fibrous expansion with septal and bridge formation, in addition to abnormal location of portal tracts and central veins, indicates distortion of the lobular architecture. Surgical specimens, including wedge biopsies, should be sufficient to evaluate the lobular architecture, as it is often difficult to evaluate distortions of needle-acquired liver biopsy samples (Fig. 10.1). Sometimes, a few portal tracts are contained in needle specimens because the peripheral portal tracts are small and sampling errors easily occur. In contrast, in chronic liver diseases, the portal tracts have cellularly and fibrously enlarged, sometimes with the fibrous septa formation; thus, it is relatively

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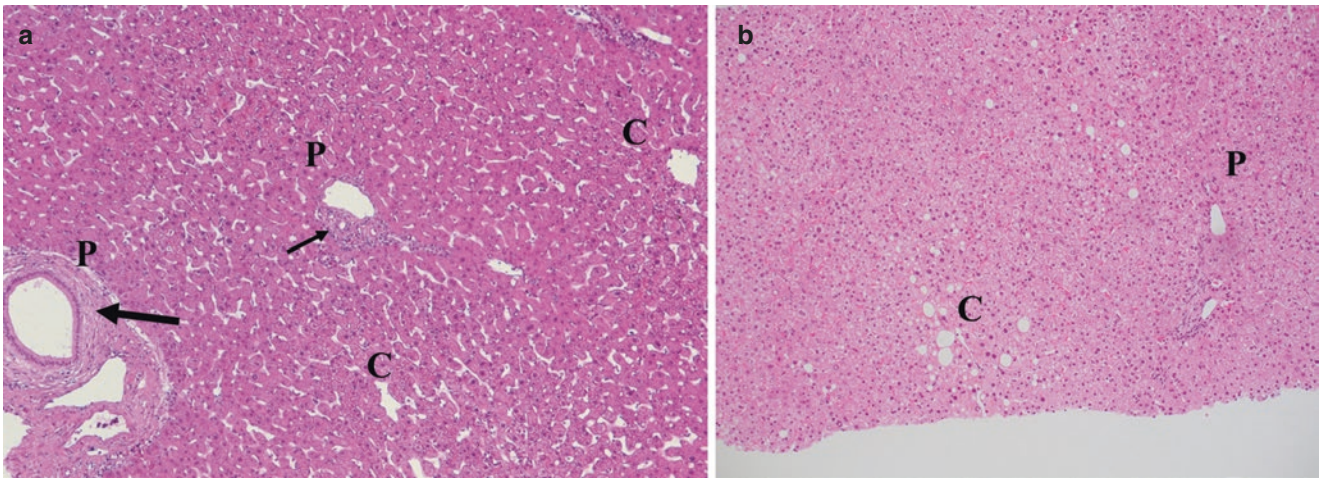

Fig. 10.1 Normal architecture. (**a**) Surgical specimens. It is easy to confirm the normal lobular architecture. Portal tracts (P) and central vein (C) are located in order. Small and large arrows denote interlobular and septal bile ducts, respectively. (**b**) Needle biopsy. It is necessary to evaluate whole the lobular architecture from a part of hepatic lobule by prolific imagination

easy to identify abnormal architecture, as compared with the normal lobular architecture of the normal liver. Moreover, normal variations that occur with aging, such as mild fibrosis, steatosis, and polymorphisms of hepatocytes, should also be considered. Even the livers of candidate transplant donors are not always completely normal. For example, mild/occasional steatosis of the parenchyma, mild/occasional fibrosis, and inflammatory cell infiltration of the portal tracts are often observed in otherwise healthy candidate donors. Therefore, clinical laboratory data are needed to confirm the histology of a suspected clinical diagnosis. Most often, biopsy samples are screened to indicate compatibility and a definitive histology of a clinical diagnosis. Several nonspecific and mild findings, such as necroinflammatory changes and fibrosis, are found, but in some cases, it is not possible to arrive at a definitive diagnosis clinically. Such cases are diagnosed as nonspecific reactive changes, or if necroinflammation is present, a diagnosis of nonspecific reactive hepatitis is made to avoid overdiagnosis of suspected diseases. These include the minimal change, a recovery phase, or an inactive phase of proper liver diseases, as well as the hepatic phenotypes of systemic diseases, such as diabetes mellitus, in addition to sampling errors.

The presence of hepatitis virus in sera is sufficient to arrive at a diagnosis of viral hepatitis, as the purpose of liver biopsy is to evaluate the degree of disease activity (grading) and fibrosis (staging). However, the presence of autoantibodies alone is not sufficient to reach a definitive diagnosis of PBC or AIH, which requires liver biopsy to exclude other diseases, such as NAFLD and DILI. Moreover, the possibilities of an overlap of NAFLD and DILI, and, more rarely, acute-onset AIH, should be pathologically considered. When disease overlap is suspected, a combination of different histological findings of more than one etiology is complex; thus, each finding must be relatively evaluated to arrive at a pathological diagnosis. However, it is relatively easy to make a diagnosis of overlapped diseases, such as NAFLD, because the histology of this entity consists of specific findings, including macrovesicular fatty change and sites of this fatty change and pericellular fibrosis in zone 3, which are thought to be associated with malnutrition. In contrast, it is difficult to evaluate overlapping of DILI or AIH with the suspected liver disease.

Diagnosis of Hepatitis Versus Cholangitis

The pathological differentiation between hepatitis and cholangitis is based on clinical data, but cholangitis-related diseases are often accompanied by hepatitic changes and sometimes overlapping is encountered. Of course, this differentiation affects the clinical treatment strategy. For example, steroid/ursodeoxycholic acid (UDCA) combination therapy is recommended for so-called PBC–AIH overlap syndrome, while UDCA alone is primarily recommended for PBC with marked hepatitic change. Even if typical AIH features are present, severe bile duct damage resembling chronic nonsuppurative destructive cholangitis (CNSDC) is rarely encountered with typical AIH histological findings. Hence, the pathologist should encourage the clinician to consider the possibility of an overlap with PBC. However, it is difficult or risky to make a diagnosis of CNSDC based solely on the presence of bile duct lesions (Fig. 10.2).

In the early stages of biliary diseases, bile duct lesions, including periductal inflammation and periductal fibrosis, are highlighted because of unnoticeable hepatitic changes, such as portal inflammation and interface hepatitis. However, based on the extent of portal inflammation with biliary dis-

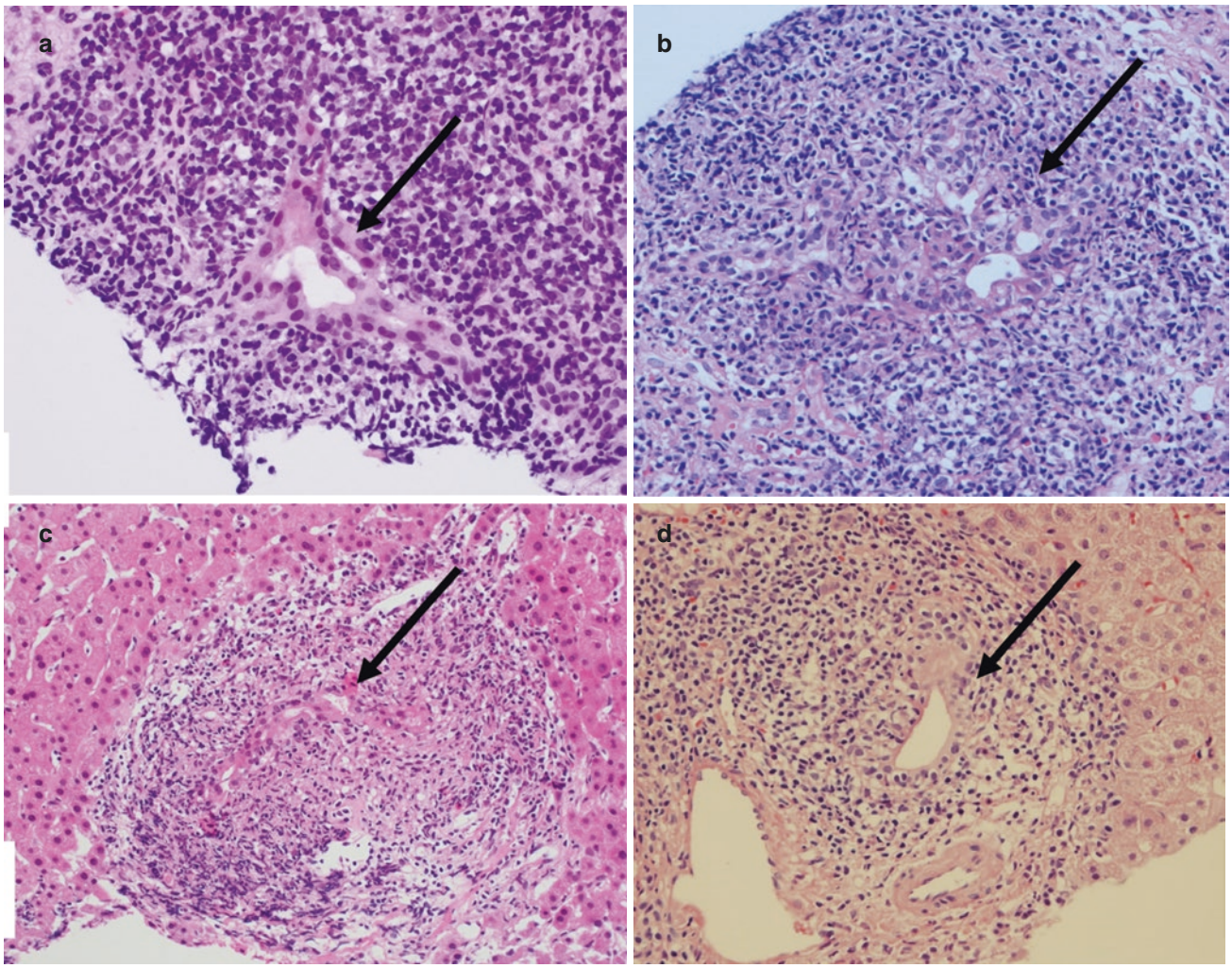

Fig. 10.2 Marked bile duct damage resembling CNSDC (arrows). (**a**) HCV-related chronic hepatitis. (**b**) AIH. (**c**) AMA-positive PBC. (**d**) AMA-negative PBC

eases including PBC, portal inflammation and interface hepatitis are also usually found. Therefore, the differentiation between hepatitic and biliary diseases is especially challenging in some cases.

Determination of Chronic Versus Acute Liver Injury

The presence of portal and/or parenchymal inflammation is an important finding during the initial survey of liver specimens. In particular, diffuse parenchymal necroinflammatory change present in most lobules histologically indicates acute liver injury, including acute hepatitis (Fig. 10.3). In contrast, heterogeneous distribution of necroinflammation, characterized by prominent changes to some portions of the parenchyma, indicates chronic hepatocellular damage, which mostly occurs with chronic hepatitis (see Fig. 10.3). However, portal and parenchymal changes are similar, as some portal tracts are inflamed and fibrously enlarged, while the degree of inflammation and fibrosis differs in others, especially specimens with relatively normal appearances. Such changes to the portal tracts indicate chronic hepatitis. When differentiating the clinical aspects of chronic hepatitis defining liver dysfunction with a duration of more than 6 months, the pathological definition of chronic hepatitis is based on the degree of heterogeneous parenchyma and portal necroinflammatory change and fibrosis.

Acute vs. chronic cholestasis is also characterized by a diffuse vs. heterogeneous phenotype, respectively (see Fig. 10.3). The etiology of acute cholestasis includes extrahepatic biliary obstruction and DILI, while bile stagnation is directly visualized as a bile plug in a centrilobular canaliculus. A bile plug is also diffusely found in other lobules, which is pathologically characteristic of acute change. In contrast, chronic cholestasis is not histologically characterized by direct stasis of bile.

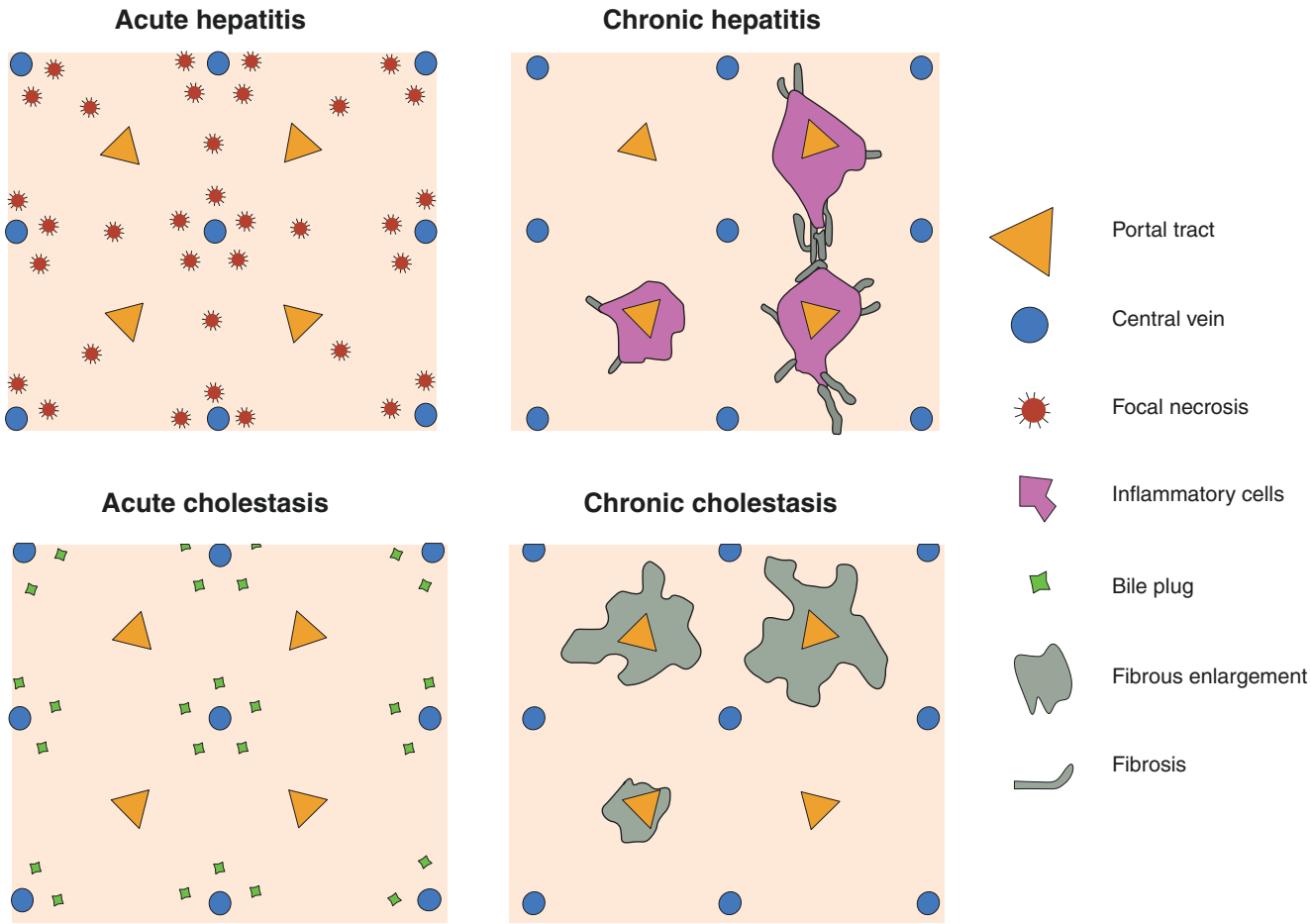

Fig. 10.3 Schema of acute vs. chronic hepatitis and acute vs. chronic cholestasis

Copper deposition in hepatocytes is a collateral evidence of chronic cholestasis. As the most sensitive staining option, copper-binding proteins are visible as Orecein-positive black granules. In PBC, because localized cholestasis occurs with the loss of small bile ducts, Orecein-positive granules in the periportal area result from the nonhomogeneous distribution of the portal area accompanied by bile duct loss.

Histological findings reflecting chronic liver diseases are characteristically heterogeneous, as mentioned above. Therefore, the possibility of sampling errors in liver needle biopsies, such as no characteristic finding of a suspected chronic liver disease, should be kept in mind. In contrast, most biopsy samples of acute liver diseases are characterized by diffuse findings. However, most cases of acute hepatitis lead to regression to various degrees and the histology changes according to the degree of clinical severity and the clinical course. Liver biopsy specimens obtained in the off-peak phase of acute hepatitis sometimes lack characteristic etiological findings. In some cases, necroinflammatory changes in the parenchyma completely disappear. However, even in these cases, pigmented macrophages occasionally accumulate in the parenchyma and portal tracts. Pigmented, or pigment-laden, macrophages result from the phagocytosis of necrotic hepatocytes by resident Kupffer cells and/or non-resident macrophages, which remain within necrotic areas for prolonged periods, although some transfer to the portal tracts of the same lobule. Therefore, the presence of these cells indicates that of past hepatocellular necrosis. Some liver specimens of clinically acute hepatitis cases, especially those obtained from patients at the stable phase after the peak phase, have pigmented macrophages within the portal tracts, but no necroinflammatory change in the parenchyma. These histological findings do not necessarily directly demonstrate the presence of "hepatitis" but rather are diagnostic of the convalescent stage of acute hepatocellular damage, including hepatitis.

Common Histological Findings of AIH and PBC

Most histopathological findings of the diseased liver are nonspecific, irrespective of the etiology. However, AIH and PBC are characterized by the combination and degree of the fol-

lowing findings. CNSDC is thought to be a histological hallmark of PBC [1], but similar bile duct damage is also encountered in chronic viral hepatitis and AIH.

Necroinflammatory Changes to the Parenchyma

Necroinflammation of the parenchyma reflecting lobular activity primarily consists of hepatocellular necrosis/apoptosis and infiltration of various types of inflammatory cells. The formation of acidophilic (Councilman) bodies is a manifestation of apoptotic cell death (Fig. 10.4a). Acidophilic bodies are found in areas with severe necroinflammation, but no significant inflammatory cell infiltration around the acidophilic bodies (see Fig. 10.4a). In contrast, spotty and focal hepatocellular necrosis accompany inflammatory cell infiltration to sites of necrosis (Fig. 10.4b). With an increased degree of hepatocellular necrosis, confluent necrosis, including zonal and bridging necrosis, appears (Fig. 10.4c). Such confluent necrosis usually occurs in acute hepatitis and also acute on chronic hepatitis, irrespective of etiology. Upon the observation of confluent necrosis of AIH or PBC specimens, the possibility of acute exacerbation of AIH or PBC overlapped with acute onset of AIH or DILI should be considered. Moreover, if there is an accumulation of spotty and/or focal necrosis in zone 3 (perivenular necroinflammatory activity) in PBC cases, the possibility of PBC overlapped with classical AIH (hepatitis form of PBC) should be considered.

Portal Inflammation and Interface Hepatitis

Chronic liver diseases are histologically characterized by portal inflammation and fibrosis. The primary location of hepatitis is the liver parenchyma, which mostly consists of hepatocytes. However, the link between portal inflammation

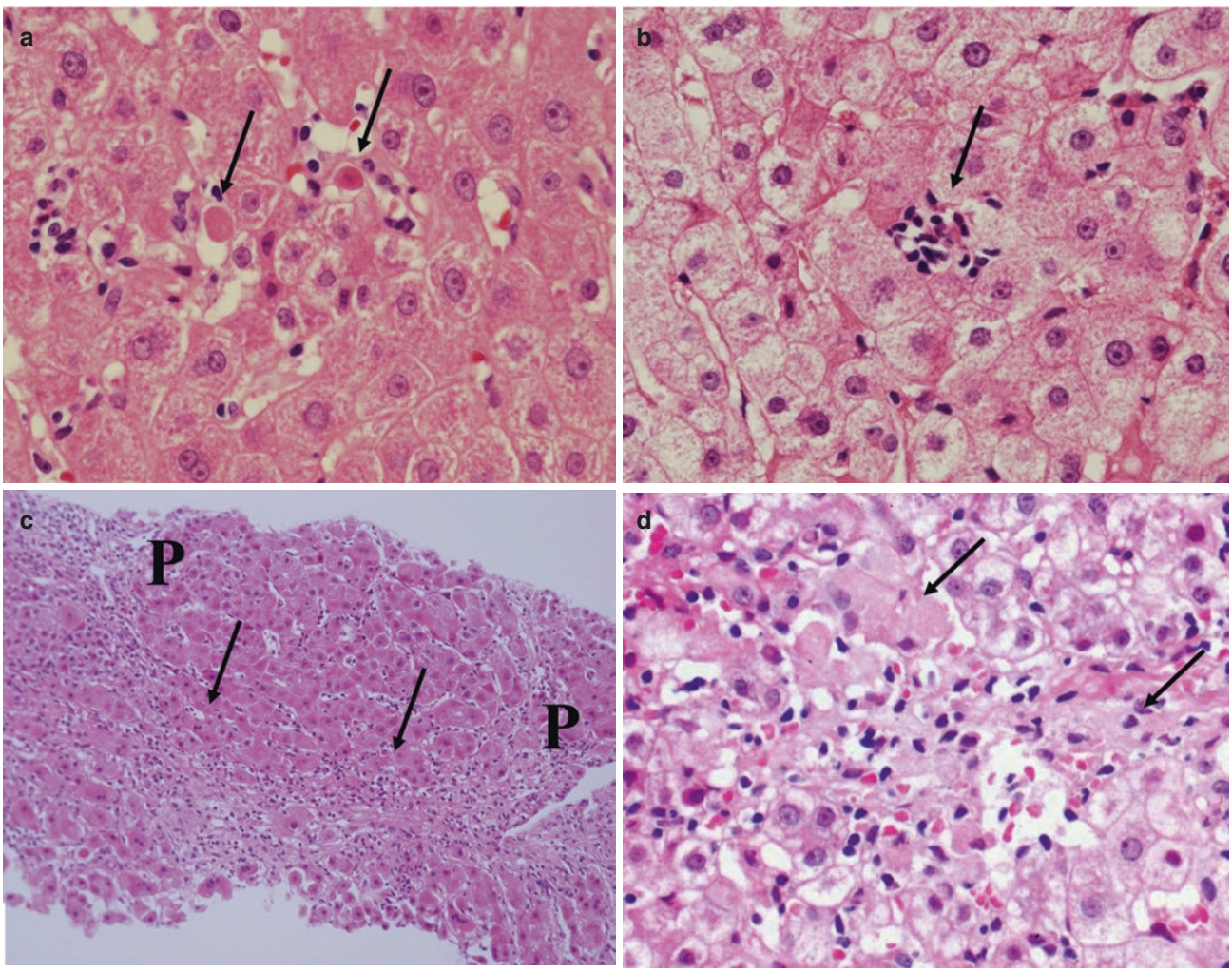

Fig. 10.4 Findings of lobular hepatitis. (**a**) Acidophilic body (arrows). (**b**) Spotty necrosis (arrow). (**c**) Bridging necrosis (arrows) connecting neighboring portal tracts (P). (**d**) Pigmented macrophages (arrows) in focal necrosis

and chronic hepatitis remains unknown. As a possible explanation, consider the function of macrophages including Kupffer cells. Necrotic hepatocytes promote proximal inflammation, and marcophage/Kupffer cells phagocytize necrotic cells via chemotaxis. The phagocytized cells (i.e., pigmented macrophages) appear microscopically as large cells with brownish, rich cytoplasm by staining with hematoxylin and eosin (Fig. 10.4d) and highlighted by periodic acid–Schiff staining with diastase. These pigmented macrophages move from necrotic areas to regional portal tracts where they could act as causal cells of inflammation or antigen-presenting cells, thereby possibly promoting an inflammatory milieu in the portal tracts.

Conventionally, chronic viral hepatitis is classified as either the active or inactive (persistent) type based on the presence or absence of interface hepatitis (formerly called "piecemeal necrosis"), respectively. The presence of interface hepatitis indicates the destruction of the limiting plate and progression of hepatocellular necrosis around the portal tracts, causing cellular and fibrotic enlargement of the portal tracts, which are associated with the progression of chronic hepatitis. Although chronic viral hepatitis and AIH are histologically characterized by these portal changes, similar periportal findings are closely associated with the progression of PBC [2–4]. Moreover, similar inflammatory changes in the portal tracts, including the interface areas, are sometimes observed in acute hepatitis. In the early phase of acute hepatocellular damage, necroinflammatory changes are limited to the parenchyma with infiltration of pigmented macrophages to areas of hepatocellular necrosis. In the later stage of disease, pigmented macrophages move to the portal tracts and may cause portal inflammation resembling chronic hepatitis. For example, in liver biopsy specimens obtained at 1 or 2 weeks after the clinical peak phase of liver dysfunction, portal inflammation is found to various degrees. Interface hepatitis-like irregularity of the limiting plate is also found, which is referred to as "spill-over" in acute hepatitis. This distinction is due to the term "interface hepatitis" used in chronic hepatitis.

Pathology of PBC

Appearance of Cholangitis

The principal finding of PBC is the irreversible loss of intrahepatic small bile ducts, especially the interlobular bile ducts [5], although cholangitis, including CNSDC, is thought to be the main histological hallmark of PBC [1]. Although several experimental animal models of PBC have already been reported, relatively few result in bile duct loss. In the process of bile duct loss, the transition from chronic cholangitis to CNSDC, especially a florid duct lesion accompanied by severe periductal inflammation and bile duct injury, results in damage to the bile ducts, which then become embedded and disappear in areas of inflammation [1] (Fig. 10.5). This destructive process is a pathological hallmark of PBC along with the characteristic periductal microenvironment and autoimmune-mediated responses. Prior to the development of bile duct lesions, portal inflammation is conspicuous, but intact bile ducts are found in the original location of the portal tracts, indicating the lack of an immune phenomenon toward the bile ducts. This histology is often found in chronic viral hepatitis and AIH but does not directly indicate the presence of a biliary disease, including PBC. In contrast, chronic cholangitis is characterized by mild epithelial damage with severe lymphocytic infiltration into the biliary layer, although the bile duct diameter remains unchanged (see Fig. 10.5). Eosinophilic infiltration, which is often observed around cholangitic features, is useful for the diagnosis of

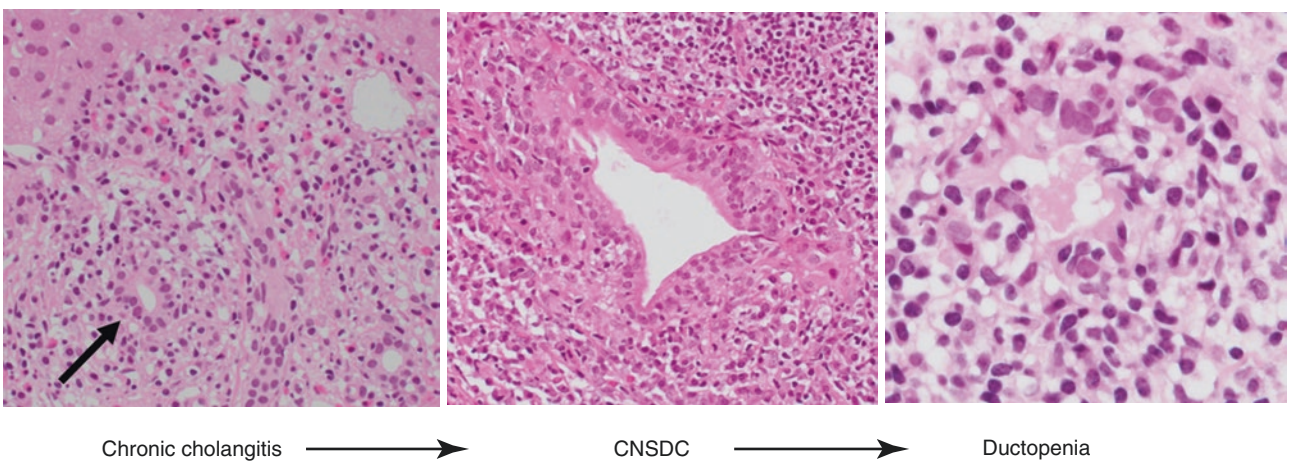

Fig. 10.5 Transition of PBC cholangiopathy. PBC cholangiopathy consists of three steps. In chronic cholangitis, damaged bile ducts are normal in size, and severe lymphocytic infiltration into the biliary layer is found (arrow). Moreover, many eosinophils are found in the portal tracts. CNSDC consists of proliferative and destructive features of biliary epithelial cells and the formation of complicated tubular structures with increasing size. In the final stage of cholangiopathy, an imbalance of cell kinetics causes bile duct loss

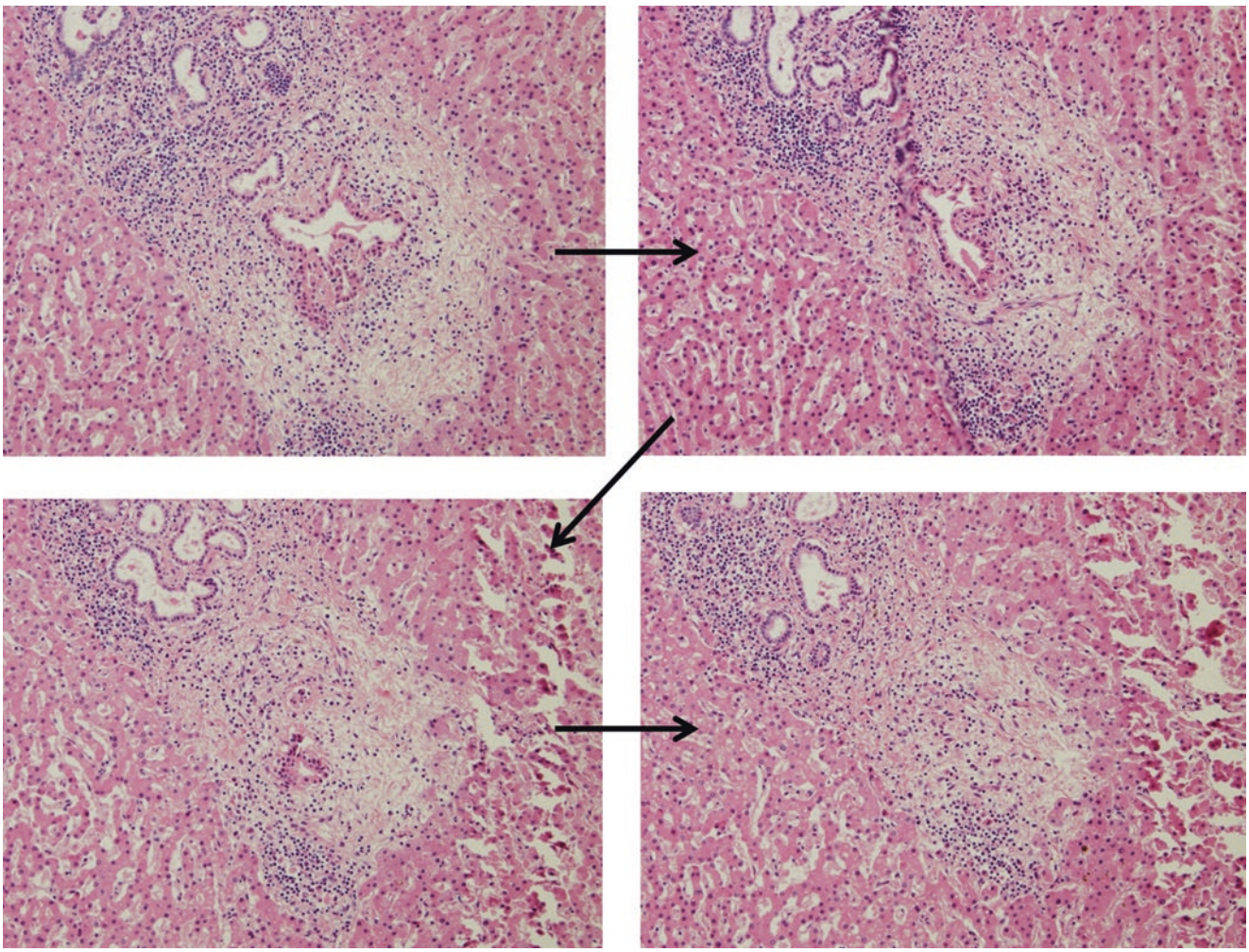

Fig. 10.6 Tracking of cholangitis using serial sections. Limited periductal inflammation and bile duct loss are found in scant inflamed portal tracts

PBC, especially early stage (see Fig. 10.5) [6]. Although the detailed mechanisms of this chronic cholangitis remain unclear, two possibilities could be speculated (1) an immune phenomenon involving the bile ducts and (2) "spill-over" of portal inflammation into the bile ducts. In contrast, even though portal inflammation is not significant, cholangitis with limited periductal inflammation and bile duct loss are often observed, which are definitely indicative of bile duct damage specifically caused by an autoimmune phenomenon (Fig. 10.6). Different stages of the development of bile duct lesions in the same liver needle specimen include bile duct loss and intermingling of the remaining intact bile ducts. This heterogeneity of bile duct lesions is characteristic of the chronicity of PBC.

Appearance of CNSDC and Bile Duct Loss

In the healthy liver, the interlobular bile ducts are tubular structures with clear lumens. However, CNSDC is distinguished by both proliferative and destructive features of biliary epithelial cells [7, 8]. Proliferative findings include torsion and deformation of tubular structures with increased diameters of the bile ducts, increased density and multistratification of nuclei, and low papillary proliferation of the biliary layer, while destructive findings include hydrophilic swelling, acidophilic degeneration, and apoptosis of biliary epithelial cells, resulting in damage to the biliary layer (see Fig. 10.5).

Biliary epithelial cell death is a principal autoimmune reaction of PBC that induces bile duct loss due to the infiltration of effector cells, such as cytotoxic T cells and natural killer cells [9]. Nevertheless, similar histological features of bile duct injury and loss and similar immune-mediated mechanisms have been proposed in vanishing bile duct syndrome caused by DILI and rejection after liver transplantation. However, CNSDC intermingled by both proliferation and destruction is limited in PBC. This imbalance of cell kinetics causes the loss of targeted tissues in various autoimmune diseases including PBC [7, 8], and hepatic stem cell failure has also been associated with bile duct loss in PBC [10, 11]. Granulomatous cholangitis, which is characterized

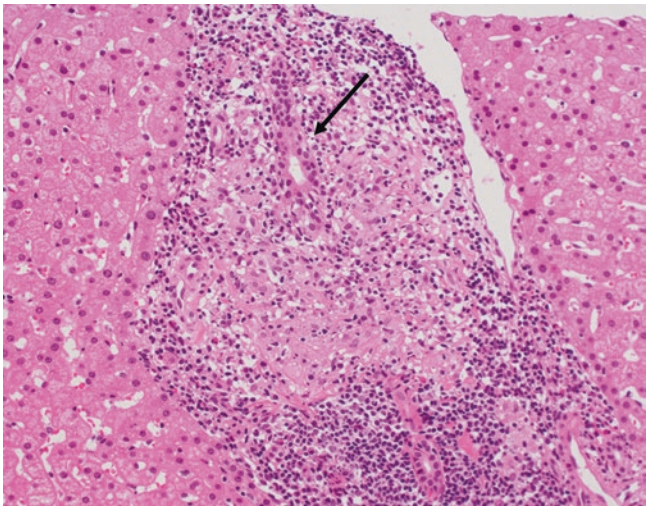

Fig. 10.7 Granulomatous cholangitis. A damaged bile duct (arrow) is surrounded by an epithelioid granuloma

by the accumulation of epithelioid cells and granuloma formation, is an additional distinctive finding of CNSDC (Fig. 10.7). Granuloma formation is a valuable indicator for the diagnosis of PBC [12], although sarcoidosis should be considered as a differential diagnosis [13].

Similar to chronic cholangitis, bile duct lesions, called hepatitis-associated bile duct injuries or hepatitic bile duct lesions, are often found in chronic viral hepatitis and AIH. In addition, hepatitis-associated bile duct injury sometimes accompanies destructive changes (up to 12% of biopsies) [14], which resembles CNSDC of PBC (see Fig. 10.2a, b) [15]. Therefore, the observation of bile duct lesions alone is insufficient to differentiate AIH from PBC [16], as bile duct loss is exceedingly rare in AIH. Hence, the pathologist must confirm other findings of PBC, such as the bile duct loss on serial sections and chronic cholestasis by Orcein staining, to arrive at a diagnosis of PBC.

Changes After Bile Duct Loss

Because immune-mediated targets are missing with increasing bile duct loss in PBC, portal inflammation with bile duct loss is diminished or resolves (Fig. 10.8a, b). The size of the portal tract is also restored to normal, as if nothing had happened, just that bile duct is then missing (see Fig. 10.8b). However, in advanced cases and UDCA-refractory PBC cases, portal inflammation remains, and interface hepatitis involves the portal tracts with bile duct loss (see Fig. 10.8c), and fibrous enlargement of the portal tracts is associated with disease progression.

Localized cholestasis occurs around the portal tracts, which is accompanied with bile duct loss, although there is no bile plug reflecting cholestatic bile in PBC. The formation of a bile plug is a morphological finding of acute cholestasis, but not chronic cholestasis. The histological features of hepatocytes in chronic cholestasis include the presence of Orcein-positive granules and small cell dysplasia-like changes (Fig. 10.9a, b). Copper-binding proteins are stained with Orcein as black granules, which are actually metallothionein in the lysosomes of hepatocytes and reflect the deposition of copper granules associated with chronic cholestasis (see Fig. 10.9a) [2, 17]. Small cell dysplasia is characterized by acidophilic cytoplasm and nuclear atypia and thought to be a precursor lesion of hepatocellular carcinoma. Similar hepatocellular changes, but scant nuclear atypia, are regionally found and reflect chronic cholestasis in PBC (see Fig. 10.9b). A ductular reaction, especially the atypical type, also reflects cholestasis and is characteristic of Scheuer classification stage II. The ductular reaction is histologically classified as typical, which is found in a number of pathological liver diseases, or atypical, which mainly occurs in biliary diseases and alcoholic liver fibrosis. The typical bile ductule is composed of normal biliary epithelial cells with a clear tubular structure and lumen, which is often continuous with interlobular bile ducts and is presumed to reflect the proliferation of preexisting biliary epithelial cells. In contrast, an atypical bile ductule has a dendritic shape and no clear lumen and is thought to be derived from hepatic stem cells or ductular metaplasia/transformation of periportal hepatocytes (see Fig. 10.9c). Neutrophil infiltration is a feature of atypical bile ductules, but not associated with biliary infection (see Fig. 10.9d). An atypical ductular reaction with scant mesenchyme is difficult to identify as a biliary component (see Fig. 10.9d). Because these morphological findings of chronic cholestasis are also found in other advanced liver diseases, such as cirrhosis, irrespective of etiology, the diagnostic value is limited to early stage PBC. Moreover, aberrant expression of biliary-type cytokeratin, keratin 7, in hepatocytes has been proposed as a marker of chronic cholestasis and progression of PBC [18]. The presence of keratin 7-positive periportal hepatocytes can be used as a surrogate finding of Orcein-positive granules and atypical ductular reaction.

Emergence of Hepatitic Changes

In early PBC, portal tracts are inflamed with cellular expansion accompanied with bile duct lesions (Fig. 10.10). In contrast to chronic hepatitis, the limiting plate is preserved in most portal tracts with no interface hepatitis (see Fig. 10.10). Although a portal inflammation in addition to bile duct injury is also prominent, the absence of interface hepatitis and lobular hepatitis is characteristic of early PBC. However, during disease progression, hepatitic change in addition to cholangitis, especially interface hepatitis, is closely associated with fibrous enlargement of the portal tracts, even those that lack

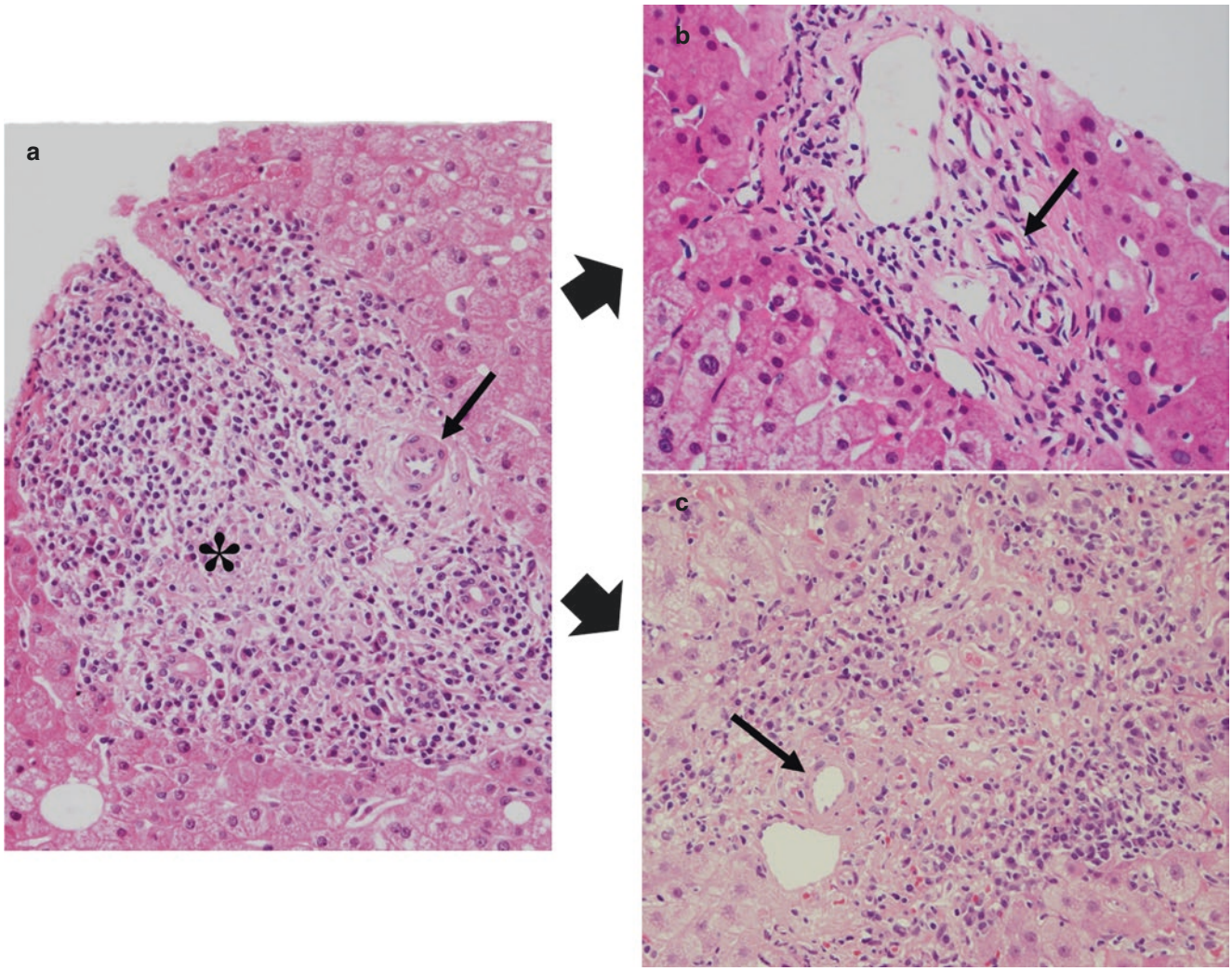

Fig. 10.8 Portal tracts with bile duct loss in PBC. (**a**) Immediate aftermath of bile duct loss. In the center of the portal tract, trace of cholangitis is found (asterisk). Interface hepatitis is not observed. (**b**) The size of the portal tract is normal, and inflammation is nearly resolved. As if nothing had happened, only the bile duct is missing. (**c**) In advanced PBC, portal inflammation and interface hepatitis are found in the portal tracts with bile duct loss. Arrows indicate the accompanying arteries

bile ducts, which serves as an immunological target (see Fig. 10.8c). The histogenesis of interface hepatitis includes an immune-related reaction against periportal hepatocytes, similar to AIH, and/or cholestasis-related hepatocellular damage (biliary piecemeal necrosis). Interface hepatitis is closely associated with the progression of PBC. According to the findings of our previous studies of a new PBC staging and grading system [2–4], hepatitis onset is important in the progression of PBC, and associated prehension is useful for pathological diagnosis and treatment selection.

Moreover, lobular hepatitis is also prominent in some PBC cases, indicating a hepatitic form of PBC or so-called PBC–AIH overlap syndrome. T cells are dominant infiltrating lymphocytes during the formation of lobular hepatitis and interface hepatitis, as compared with B cells, irrespective of PBC or AIH, indicating a similar pathogenesis of cell-mediated immunity in hepatocellular injuries associated with PBC and AIH [19].

Pathology of AIH

Basic Histology

The basic histological features of AIH include chronic active hepatitis, which is characterized by portal inflammation with interface hepatitis and follicle-like aggregation of lymphocytes, with rare findings of lymph follicles with germinal centers in the portal tracts (Fig. 10.11). As compared with chronic viral hepatitis, hepatitic changes, including

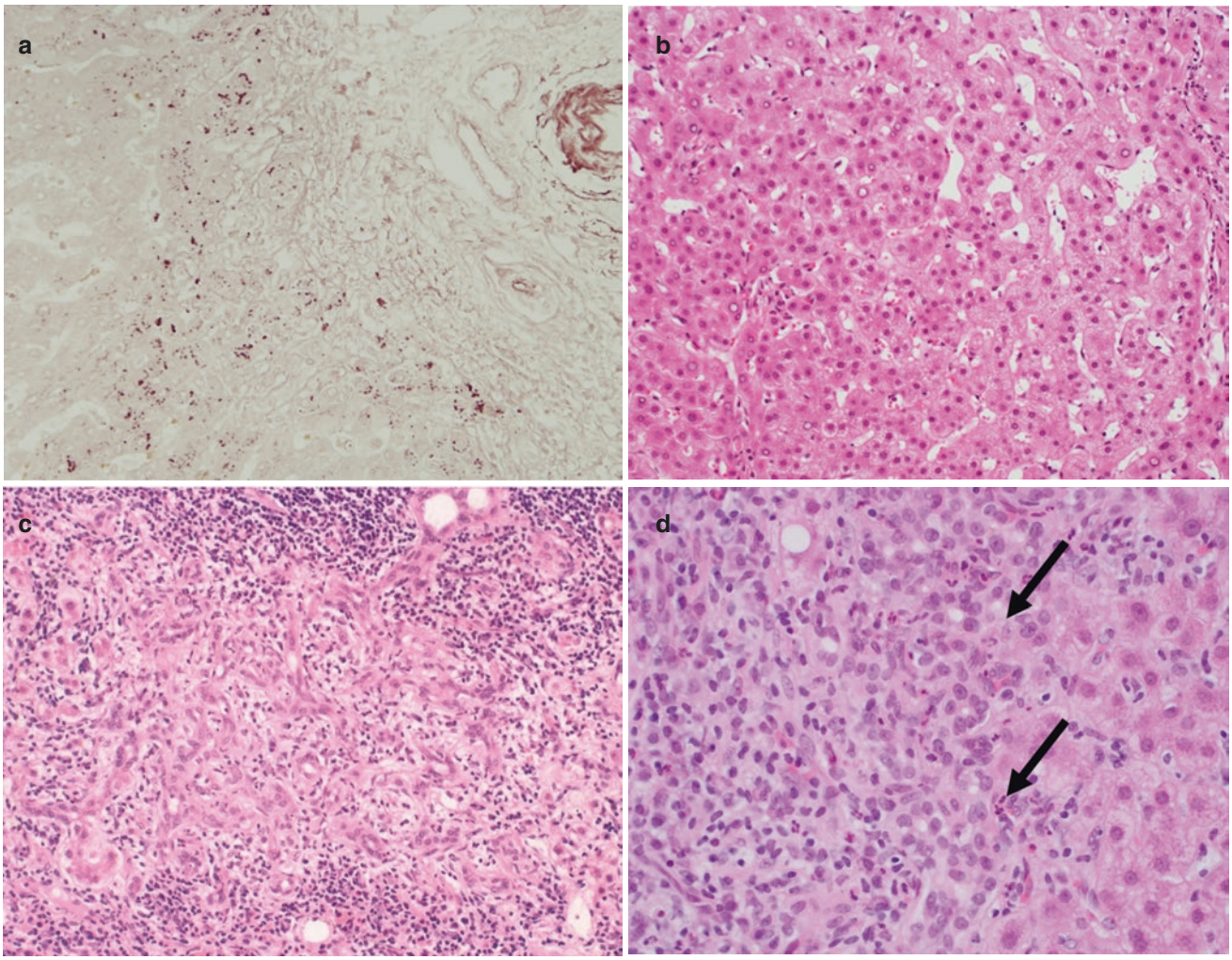

Fig. 10.9 Histological findings indicating chronic cholestasis. (**a**) Orcein stain. Copper-binding proteins appear as black granules. (**b**) Small cell dysplasia-like change of hepatocytes (lower left) with acidophilic cytoplasm and no nuclear atypia. (**c**, **d**) Ductular reaction. Dendritic shape, no clear lumen, and neutrophil infiltration are features of atypical bile ductules. Atypical bile ductules with scant mesenchyme are difficult to identify (arrows)

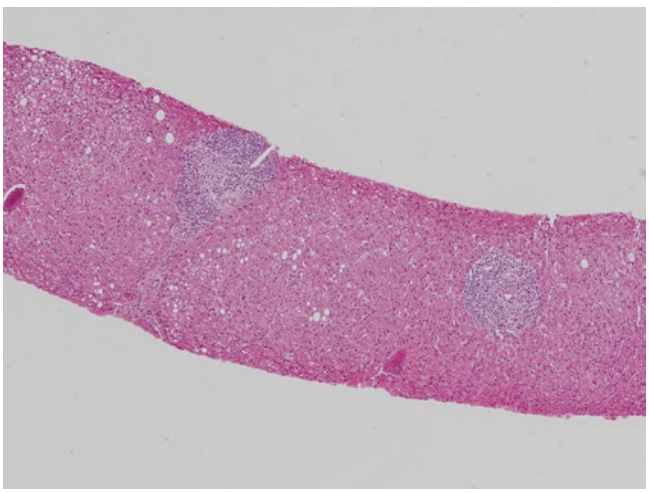

Fig. 10.10 Early PBC. Portal tracts are cellularly enlarged, but there is no interface hepatitis. In the parenchyma, no necroinflammatory change is evident

lobular hepatitis and interface hepatitis, are prominent in typical AIH. Sites of spotty and focal necrosis tend to accumulate around central veins, which promote perivenular necroinflammatory activities in AIH (see Fig. 10.11c) and sometimes confluent necrosis (bridging and zonal necrosis), rosette formation, and emperipolesis of hepatocytes. Marked plasma cell infiltration is found in about two-thirds of patients with AIH [14], supporting the diagnosis of AIH. The formation of giant syncytial multinucleated hepatocytes, broad hepatocellular collapse, and multiple sites of confluent necrosis consisting of zonal and bridging necrosis, are observed in acute exacerbation of AIH. Fulminant hepatitis is histologically characterized by submassive and massive necrosis. In addition to severe lobular necrosis, including massive hepatocyte necrosis and dropout, regeneration of hepatocytes may be present and sometimes mimic the parenchymal nodules of established cirrhosis in the recovery phase of fulminant AIH.

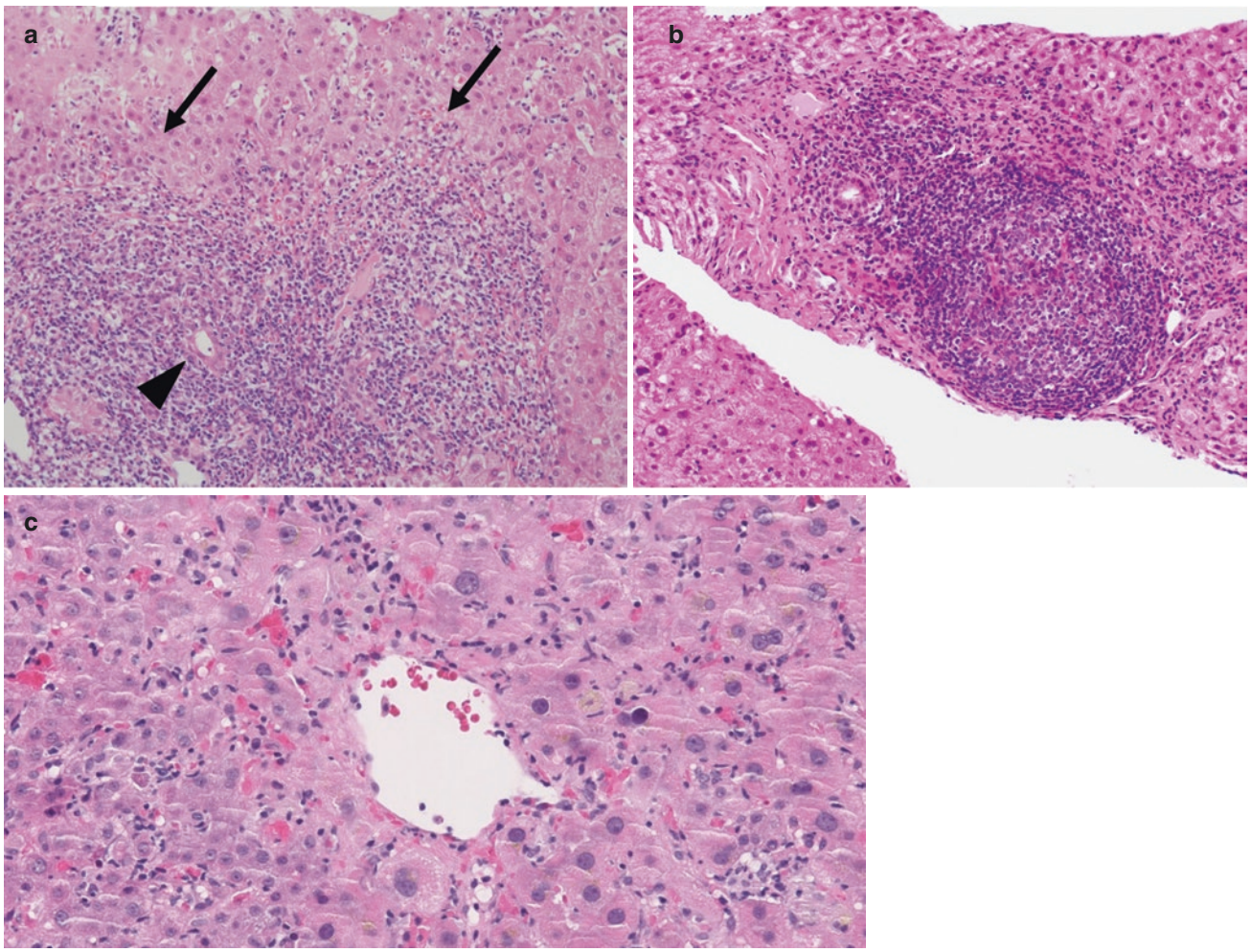

Fig. 10.11 Basic histology of AIH. (**a**) Portal inflammation with interface hepatitis (arrows) is observed. Arrowhead denotes bile duct lesion (hepatitis-associated bile duct injury). (**b**) A lymph follicle in a portal tract. (**c**) Perivenular necroinflammatory activity (perivenulitis). Inflammation, spotty necrosis, and pigmented macrophages scattered around the central vein

Pathological Diagnosis: Histological Components of the AIH Diagnostic Scoring System

The clinicopathological diagnosis of AIH requires the exclusion of other causes of liver disease, including viral hepatitis, alcohol and drug abuse, NAFLD, and other autoimmune diseases. In particular, the pathological differentiation of AIH from chronic viral hepatitis and the presence of AIH superimposed on hepatitis virus-infected patients are difficult or impossible in most cases because the histological difference is dependent on the relative evaluation of histological findings. At present, the modified criteria (1999) [20] and simplified criteria (2008) [21] proposed by the International Autoimmune Hepatitis Group are used. Although the former consist of many complex items, these criteria are sufficient to adequately distinguish AIH from other liver diseases. Pathological items consist of interface hepatitis (+3), predominant lymphoplasmacytic infiltrate (+1), rosette formation (+1), and biliary changes (−3): each of which is given a score of 5 out of a definitive score >15 before treatment. The most important point is that biliary changes suggestive of PBC and primary sclerosing cholangitis (PSC) are assigned negative points for the accurate identification of AIH alone. "Biliary changes" refer to bile duct changes that are typical of PBC or PSC (i.e., granulomatous cholangitis or severe concentric periductal fibrosis), a substantial periportal atypical ductular reaction, and/or the accumulation of Orcein-positive copper-binding proteins (see Figs. 10.7 and 10.9).

In contrast, the simplified criteria [21] have been proposed for the rapid diagnosis and treatment of AIH and are also useful for nonspecialized hepatologists. Regarding the pathological items in these criteria, the following three categories for histological grading are assigned a score of 0–2 (possible total score = 8): atypical histology (0 points), histologically compatible with AIH (1 point), and typical histology (2 points). In addition to evident hepatitis as a necessary condition (interface hepatitis and lymphocytic/

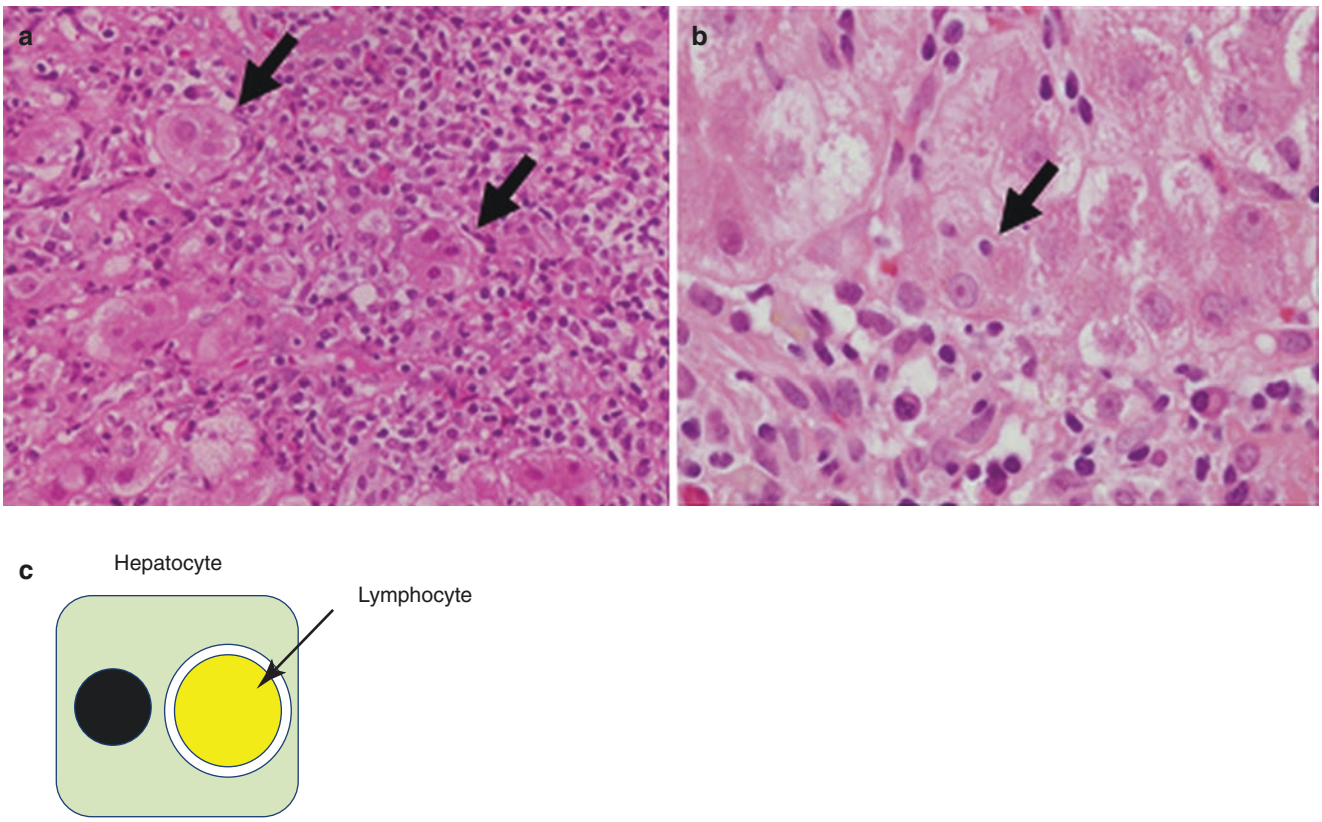

Fig. 10.12 Rosette formation of hepatocytes (**a**) and emperipolesis (**b**) (arrows). (**c**) Scheme of emperipolesis. Active penetration by a lymphocyte (effector cell) into a hepatocyte (target cell)

lymphoplasmacytic infiltrates in portal tracts that extend into the lobule), emperipolesis and hepatic rosette formation are regarded as typical characteristics for the diagnosis of AIH. To be considered typical, each of these three features of typical AIH histology must be present. As points to remember, hepatic rosette formation and emperipolesis only reflect severe hepatitic activities in chronic hepatitis including viral hepatitis as well as AIH [22]. Hepatic rosette formation results from the development of bile canaliculi, which are composed of surviving isolated hepatocytes in sites of severe interface hepatitis (Fig. 10.12a). Emperipolesis is defined as the active penetration by one cell into and through a larger cell and is immunologically the strongest pattern of cell-to-cell contact (see Fig. 10.12b, c). Compatible features are chronic hepatitis with lymphocytic infiltration but have only either one of rosette and emperipolesis or none of them. Histologically, a feature is considered atypical when other hepatobiliary diseases are suspected. In particular, steatohepatitis is clinically considered difficult to differentiate from AIH because antinuclear antibody is detected in approximately one-third of cases of NAFLD, particularly non-alcoholic steatohepatitis [23, 24]. Although the pathological differentiation between AIH and NAFLD is relatively easy based on liver histology, overlapping cases are often encountered. Moreover, because atypical AIH cases, such as acute-onset AIH (see below), are probably ruled out as being non-AIH, as mentioned below, rather than using the simplified criteria, the modified criteria (1999) should be applied in these cases.

Acute Presentation of AIH

AIH is usually defined as a chronic liver disease (classical AIH), but some cases with clinical features resembling acute hepatitis (acute presentation) have been reported [25]. These AIH cases have mostly acute exacerbation from chronic AIH, but genuine newly developed acute-onset AIH (acute hepatitis phase) without preceding clinical findings of chronic liver disease is also encountered. Moreover, although preceding clinical symptoms and liver dysfunction may not be clear, the liver histology sometimes includes centrilobular changes, including mild necroinflammatory changes and fibrosis/fibrous scar formation (Fig. 10.13), which pathologically indicate preceding subclinical and inactive AIH features.

The diagnostic criteria for classical AIH are generally applicable to acute exacerbation because the preceding clinicopathological features of chronic hepatitis are present. However, in acute-onset AIH, the serum levels of immunoglobulin G and γ-globulin and autoantibody titers are not generally high, and the histological findings of chronic active hepatitis are also lacking. Therefore, it is difficult to diagnose acute-onset AIH using the international criteria mentioned above; thus, liver biopsy is necessary to arrive at a diagnosis. Centrilobular necrosis is a type of confluent necrosis that is thought to characterize the presentation of acute-onset AIH (Fig. 10.14a, c). However, centrilobular necrosis is also a feature of DILI; thus, there are no known histological characteristics exclusive to acute-onset AIH. Moreover, the possibilities of drug-induced AIH and immune-mediated DILI further complicate a differential diagnosis [26]. In addition to centrilobular necrosis, bridging necrosis among portal tracts and central veins, and rarely periportal zonal necrosis, may accompany lobular disarray in acute-onset AIH [27–29]. A specific cell death pattern, i.e., the collapse of hepatocytes forming a centrilobular necrosis, is common in acute-onset AIH (Fig. 10.14c) [30, 31]. Around sites of necrosis, a cobblestone appearance reflecting hepatocellular regeneration is usually observed (see Fig. 10.14c). Of course, plasma cell infiltration (see Fig. 10.14b), emperipolesis (see Fig. 10.12), and rosette formation (see Fig. 10.12) are also found in most cases, which are common features of classical AIH [30, 31] and have been reported to favor AIH over DILI [32–34]. In contrast, several histological features suggestive of DILI should be noted, including the infiltration of polymorphonuclear leukocytes and eosinophils, granuloma formation, irregular fatty change, canalicular cholestasis, and bile duct/ductule damage with scant inflammation. At present, liver biopsy is mandatory for the diagnosis of acute-onset AIH, but careful consideration of all clinicopathological signs is necessary for a differential diagnosis.

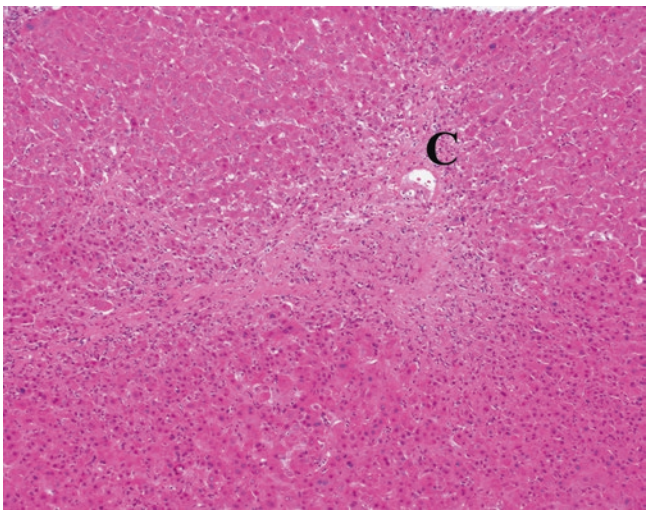

Fig. 10.13 Scar-like fibrosis in zone 3 (C, central vein). The presence of this pattern of fibrosis indicates preceding liver disease, including AIH

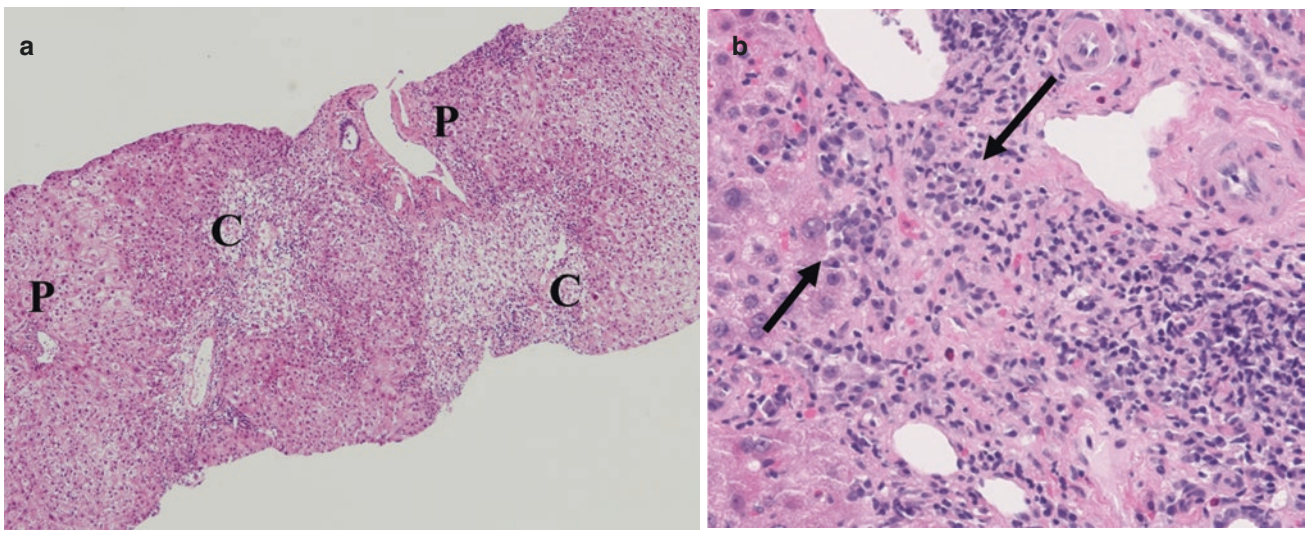

Fig. 10.14 Typical acute-onset AIH. (**a**) Centrilobular necrosis (zonal necrosis in zone 3) is found around the central vein (C). Portal tracts (P) are almost normal. (**b**) Many infiltrating plasma cells (arrow) in the portal tracts. (**c**) Dropout of hepatocyte resembling the punch-out form of centrilobular necrosis around the central vein (C). Cobblestone appearance of hepatocytes (arrows) is observed surrounding the necrotic area

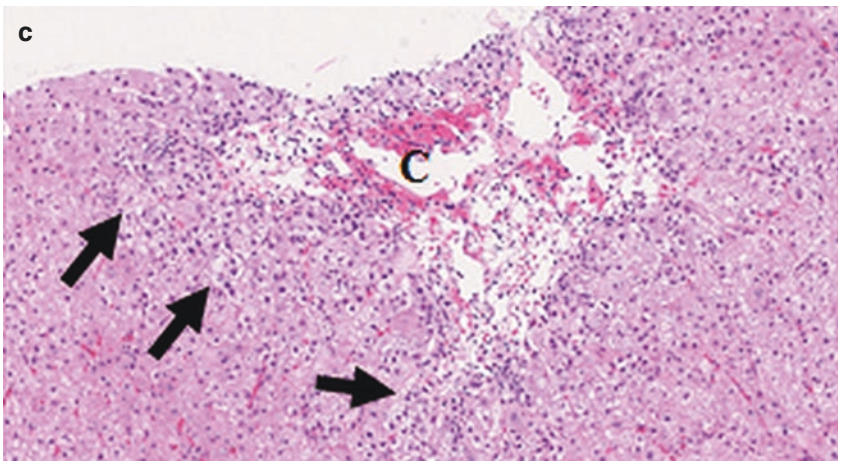

Fig. 10.14 (continued)

Conclusion

Liver biopsy is an essential procedure for the diagnosis of autoimmune liver diseases because of the nonspecificity of serological and clinical features. Histological examinations are useful to exclude other potential causes of liver diseases. However, there are no histological hallmarks for the definitive diagnosis of any liver disease, and a pathological diagnosis is derived from a combination of nonspecific findings based on the pattern classification of acute vs. chronic and hepatitic vs. cholestatic.

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Geoepidemiology of Autoimmune Liver Diseases

11

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Key Points

- The liver is a unique organ that plays a vital role in the defense against pathogens and the maintenance of tolerance against autoantigens.
- Despite its central role in immune tolerance, the liver, the largest lymphoid organ, is targeted by tissue-specific inflammatory responses in autoimmune liver diseases (AILD) including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC).
- The etiopathogenesis of AILD remains unclear but is multifactorial with genetic and environmental factors.
- Overlap syndromes with liver involvement in systemic autoimmune diseases are common and poorly understood or defined.
- AILD are relatively rare with wide geographic variations and aggregation in family members.
- The prevalence of AILD is low, but the health burden of these disorders is substantial.
- Considerable work needs to be done on both the genetic and environmental contributors to these diseases.

Introduction

The liver is a unique organ that plays a vital role in the defense against pathogens and the maintenance of tolerance against autoantigens [1]. As the largest lymphoid organ, the liver is targeted by tissue-specific inflammatory response, observed in primary autoimmune liver diseases (AILD) including autoimmune hepatitis (AIH), primary biliary cholangitis (PBC, formerly known as primary biliary cirrhosis), and primary sclerosing cholangitis (PSC). AILD is characterized by peculiar histopathological change and chronic course, progressively developing into cirrhosis or even malignancy. The etiopathogenesis of AILD remains unclear, but it is believed to be multifactorial with genetic and environmental factors involved. The clinical presentations vary in individuals and are usually atypical. In some cases, liver biopsy is required for the definite diagnosis. Of note, overlap syndromes and liver involvement of systemic autoimmune diseases also account for part of liver dysfunction in an autoimmune setting.

AILD is a relatively rare disease with geographic variations and aggregation in family members. Although the prevalence is low, the health burden of these disorders to both individuals and society is substantial. The incidence and prevalence are reported to be increased in AIH, PBC, and PSC during the past few decades. More and more attention has been paid to the AILD these years, and several population-based researches fill the vacancy of epidemiology of AILD. In this chapter, we are going to describe the epidemiological features of AILD and to discuss the impact of genetic and environmental factors on the development of these complex diseases.

Autoimmune Hepatitis

AIH is a chronic progressive inflammatory liver disease, clinically manifested as elevated alanine aminotransferase (ALT)/aspartate aminotransferase (AST), hyperglobulin-

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emia, and the presence of autoantibodies. If left untreated, AIH can lead to liver cirrhosis and hepatic failure, even hepatocellular carcinoma (HCC). The etiology of AIH is unclear, and it is hypothesized that unknown triggers result in autoimmune response to hepatocytes. Serologically, AIH can be divided into two subgroups: type 1 AIH which is characterized by antinuclear antibodies (ANAs) and/or anti-smooth muscle antibodies (ASMAs) and type 2 AIH which manifests anti-LKM-1 and anti-LC1. Exclusion of other liver disease and the correlation of clinical and histological presentations helps the diagnosis of AIH. The typical histologic features of AIH are interface hepatitis, emperipolesis, and hepatic rosette formation [2]. The treatment of AIH mainly depends on immunosuppressants, especially glucocorticoids and azathioprine.

AIH in the General Population

There is limited information regarding epidemiology on AIH. Previous population-based studies in Western countries revealed the annual incidence rates from 0.67 to 2.2/100,000 persons and a point prevalence from 11 to 26.9/100,000 persons [3–8]. The incidence and prevalence of AIH in Asia are relatively low, with an overall prevalence of 4–5.61 per 100,000 [9, 10]. AIH displays a female predominance (up to 95%), and most patients are middle-aged [4]. AIH may present at any age from childhood to elderly. Type 1 subtype of AIH mainly affects adults, while type 2 occurs frequently in younger patients. Type 1 AIH is more common than type 2 AIH, which is mostly a pediatric condition and more aggressive [11]. In Canada, the annual incidence of type 2 AIH is reported to be 0.23/100,000 children [12]. The 10-year cumulative mortality is estimated to be 26.4% in Northern Europe, at least twofold higher than the general population, especially patients with cirrhosis [5, 13]. Male gender and cirrhosis are associated with higher risk for HCC [14, 15]. Steroid treatment induces clinical, laboratory, and histological improvement in approximately 80% of patients [16], and the combination of steroids and azathioprine is associated with less side effects of steroids. However, a minority of patients will not respond to steroids and require alternative immunosuppressants such as mycophenolate mofetil.

Family Occurrence

Family occurrence has been rare. It has been reported that AIH accumulates in twins, siblings, parents, and children [17–19]. Recently, a Danish nationwide population-based study revealed that first-degree relatives of AIH patients have a fivefold increased likelihood to develop AIH, and the 10-year cumulative risk was 0.1% for the relatives [20]. Regarding the concordance of AIH in twins, no comprehensive studies have been reported previously. Nolte et al. described an acute hepatitis of unknown etiology, possibly with AIH origin in a monozygotic twin pair [18]. An epidemiological study in the Netherlands reported the concordance in monozygotic twins and discordance in dizygotic twins [17]. The Danish nationwide registry study also demonstrated a significantly higher risk of AIH in co-twins, and the probandwise concordance rate is higher in monozygotic than in dizygotic twins [20].

Risk Factors

Multiple factors contribute to the etiopathogenesis of AIH, including genetic predisposition (Table 11.1). Several genes have been reported to confer susceptibility to type 1 AIH, the strongest association of which is within the HLA-DRB1

Table 11.1 Main risk factors and comorbidities in AIH, PBC, and PSC

	AIH	PBC	PSC
Risk factors	HLA alleles	Genetic factors	Concomitant IBD, mainly UC
	Autoimmune polyendocrine syndrome type 1 with AIRE mutations	History of recurrent urinary tract infections	Continuous exposure to endogenous and exogenous toxins
	Environmental factors	First-degree relatives	Ischemic injury
	Intestinal dysbiosis	Past smoking	Bile toxicity
		Hormone replacement therapies	HLA alleles A1, B8, and DR3
		Frequent use of hair dye and nail polish	
		Pathogenic intestinal microbiota	
Comorbidities	PSC	Sjögren's syndrome	Ulcerative colitis
	Systemic autoimmune diseases such as SLE	Scleroderma	Colorectal and hepatobiliary malignancies
	IBD	Rheumatoid arthritis	
	Viral infections	Connective tissue disease	
		Autoimmune thyroiditis	
		Celiac disease	
		Increased risk of overall cancer	

locus, a class II MHC locus. In 2014, a genome-wide association study identified two relevant HLA alleles: HLA-DRB1*0301 as a primary susceptibility genotype and HLA-DRB1*0401 [21]. The study also demonstrated association between the AIH and variants of SH2B3 (rs3184504, 12q24) and CARD10 (rs6000782, 22q13.1) [21]. A number of other factors may trigger autoreactive response in AIH. The female predominance suggests a role for sexual hormones in AIH. Wei et al. demonstrated the dysbiosis in Chinese AIH population and identified several associated intestinal microbiota, suggesting the potential role of intestinal microbiome in the pathogenesis of AIH [22]. Administration of drugs could result in hepatic autoimmune responses. Drug-induced autoimmune hepatitis (DIAIH) is an increasingly recognized phenomenon, which has been reported to make up less than 10% of AIH case cohort in 2014 and increase to 18% in 2019 [8, 23, 24]. Notably, DIAIH differs from drug-induced liver injury by positive autoantibodies and response to immunosuppressants [24, 25]. Increasing usage of biological compound may contribute to the growing number of DIAIH.

Comorbidities

Some diseases have been reported to be associated with AIH, including systemic autoimmune diseases (i.e., systemic lupus syndrome, multiple sclerosis) [26], inflammatory bowel diseases (IBD) [27], celiac disease [28], and viral infections (i.e., hepatitis C virus (HCV), Epstein-Barr virus (EBV)) [29]. A subgroup of patients manifest signs of both AIH and PSC, named as autoimmune sclerosing cholangitis (ASC). Notably, IBD is a common comorbidity in ASC patients, the prevalence of which closely mirroring that in PSC patients in a population-based study [30]. The coexistence of AIH and IBD ranges from 4.5% to 18%, less common than that in ASC patients [31, 32]. AIH is also prevalent in HCV patients, in which the viral antigen is a mimicry of smooth muscle [33]. Thus, a mechanism of molecular mimicry is implicated in AIH patients with HCV infection. In addition, AIH patients have a higher risk to develop osteopenia secondary to prolonged usage of steroids as well as metabolic syndrome. Hematopoietic risks also increase as the side effects of azathioprine.

Primary Biliary Cholangitis

PBC is a chronic cholestatic liver disease characterized by nonsuppurative destructive inflammation of small and medium-sized bile ducts. Intrahepatic cholestasis and peribiliary fibrosis can culminate over time in an end-stage cirrhosis, eventually resulting in HCC. The majority of PBC cases arise insidiously, and the diagnosis is based on the presence of serum autoantibodies and the elevation of cholestatic enzymes (i.e., alkaline phosphatase, gamma-glutamyltransferase) [34]. Anti-mitochondrial antibody (AMA) reactive against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) is a specific serum marker in PBC. Serum antinuclear antibodies (ANAs), such as anti-gp210 and anti-Sp100, are accepted as PBC-specific markers during diagnosis. Liver biopsy is unnecessary unless either serum autoantibodies or elevation of cholestatic enzymes is absent. The pathogenesis of PBC remains obscure, but the detection of autoreactive T cells and autoantibodies suggests autoimmune humoral responses against mitochondria [35, 36]. Ursodeoxycholic acid (UDCA) is the first-line therapy, and obeticholic acid (OCA) is optional for those UDCA-unresponsive or non-tolerant cases.

PBC in the General Population

The incidence and prevalence of PBC vary widely in different regions and seem to be increasing over time (Table 11.2). In 2012, systemic review of epidemiological studies worldwide reported that the incidence rate ranges between 0.9 and 5.8 per 100,000 inhabitants, with 92% of female patients, and the prevalence of PBC ranges from 1.91 to 40.2 per 100,000 inhabitants [37]. A recent meta-analysis of epidemi-

Table 11.2 Incidence and prevalence rates reported for PBC

P	Country/region	Incidence (per 100,000)	Prevalence (per 100,000)
1980	UK	5.8	54
1980	UK	10.6	40.2
1983	UK	10	37–144
1984	Sweden	4–24	28–92
1984	Western Europe	4	23
1985	Sweden	14	128
1987	UK	11–15	70–93
1990	Sweden	13.3	151
1990	Canada	3.26	22.4
1990	Northern England	19	129–154
1995	Australia	N/A	19.1
1995	Estonia	2.27	26.9
1997	UK	14–32	240
2000	USA	27	402
2004	Australia	N/A	51
2005	Spain	17	195
2009	Canada	30	227
2012	Southern Israel	20	238
2012	Iceland	22.5	383
2016	South Korea	8.75	47.5
2017	Hong Kong	8.4	56.4
2019	Japan	N/A	33.8

N/A not available
Adapted from [37] and [38]

ology of PBC in the Asia-Pacific region demonstrated a pooled overall incidence as 8.55 per 100,000 people. The pooled overall prevalence was estimated to be 118.75 per 100,000 people with a respective pooled prevalence of 36.24 and 146.47 cases per 100,000 during pre-UDCA era and post-UDCA era [38]. Of note, large population-based study reported the incidence and prevalence rates increase over time with a mean annual incidence of 1.1 between 2000 and 2007. It stated that the net growth of PBC patients in the Netherlands was attributed to increase in incidence instead of decrease in the number of deaths [39]. Another study in Sweden mentioned an increased prevalence of PBC during 30 years although incidence remained stable [40]. It is worth mentioning that countries, ethnicity, and variable criteria for case inclusions may explain the wide range of incidence and prevalence rates between different countries. However, the increase in prevalence may probably attribute to the increased recognition, better data capture, improved laboratory detection methods, and increased survival after UDCA treatment.

PBC has a female predominance with a female to male ratio of about 10 to 1 [34]. A cohort study in the USA estimated 12-year prevalence of PBC with a highest adjusted prevalence value among women (42.8 per 100,000) [41]. The symptoms are similar in men and women, but men may have a worse disease progression with a higher risk to develop HCC. PBC is closely associated with a higher risk of HCC [42]. Male sex and advanced liver stage are independent risk factors for the development of HCC in patients with PBC, suggested by the representative cohort in China and Japan [43, 44].

An international meta-analysis in Western countries reported that the 5-year, 10-year, and 15-year transplant-free survival rates were 90%, 77.5%, and 65.6%, respectively [45]. The 5-year death/liver transplantation in PBC patients is 4.02% in the Asia-Pacific region [38]. Before the availability of UDCA, PBC patients usually develop to an advanced stage with a subsequent median survival of 6–8 years [34]. In the UDCA era, the introduction of UDCA at early stage improves the survival rate of PBC patients [46–48]. The survival rate of patients who respond to UDCA treatment is similar to that of an age-matched and sex-matched healthy people [46]. The favorable effects of UDCA are probably attributed to the delay of histological progression and the development of esophageal varices.

Family Occurrence

The studies of familial PBC revealed a fundamental role played by genetic factors and environmental influences on the pathogenesis of PBC. The first-degree relatives of PBC patients have a higher risk of developing PBC [49]. A recent nationwide study with genealogical database has defined the relative risk of the first-, second-, and third-degree relatives of PBC patients as 9.13, 3.16, and 2.59, respectively. The fourth- and fifth-degree relatives also had a slight increase in the relative risk [50]. Apart from the familial aggregation of occurrence, the AMA aggregate among first-degree relatives as well, which recommends a close follow-up of these relatives for early diagnosis [51]. However, in AMA-negative first-degree relatives and AMA-positive first-degree relatives with normal alkaline phosphatase levels at initial assessment, the risk of developing PBC in the subsequent 8 years is low [52]. A recommendation for a standardized follow-up approach for family members of PBC patients requires further investigation. By comparing eight monozygotic and eight dizygotic twin pairs, concordance rate for PBC is estimated to be 63% in monozygotic twins and null in dizygotic twins [53]. Of note, the monozygotic concordance rate is the highest reported for autoimmune disease. However, the sibling relative risk, namely, the odds ratio for PBC of an individual with a sibling affected by PBC, is 10.5 among the lowest for autoimmune disease. Genome-wide analysis of epigenetics in monozygotic twins and sisters discordant for PBC has revealed particular differences in DNA methylation profiles, copy number variation, and gene expression which explains the different phenotypes in siblings [54].

Risk Factors

Although the etiopathogenesis of PBC remains to be determined, several risk factors have been identified (see Table 11.1). The familial occurrence suggests the genetic predisposition of PBC, like many other autoimmune diseases. HLA class II alleles are believed to be associated with the development of PBC, especially HLA-DRB1*08 allele family [55]. In recent years, high-throughput technologies such as genome-wide association studies (GWAS) have revealed more risk loci associated with PBC. Forty non-HLA alleles possibly contributing to PBC susceptibility are discovered according to the GWAS analyses from different countries. Even though it differs among different studies and populations, the identified genes participate in certain pathways including antigen presentation and production of interleukin (IL)-12 (i.e., IRF5, SOCS1, IL-12A, etc.), activation of T cells and interferon (IFN)-γ secretion (i.e., IL12R, TYK2, STAT4, etc.), and activation of B cells and production of immunoglobulins (i.e., ARID3a, POU2AF1, IKZF3, etc.) [56–58].

The environmental factors including urinary tract infections, cigarette smoking, and the use of hormone replacement therapies are associated with increased risk of PBC [59]. A strong relationship lies between smoking and PBC, demonstrated by studies from the UK and France [60, 61]. Molecular mimicry is considered to be the underlying mechanism by which pathogens and xenobiotics trigger autoim-

mune responses [62]. It is well established that humoral and cellular autoimmune responses in PBC are associated with pyruvate dehydrogenase complex (PDC-E2). The homozygous enzyme of PDC-E2 in microbiota or chemical xenobiotics can induce serological and histopathological changes in PBC [63]. Recent studies have revealed a correlation between the intestinal microbiome and PBC, suggesting the potential risk of dysbiosis in the pathogenesis of PBC [64].

Comorbidities

PBC frequently coexists with rheumatic disorders in up to 30% of cases. A monocentric study demonstrated the co-occurrence in 61.2% of cases of PBC patients, with the most common comorbidity as Sjögren's syndrome, followed by Raynaud's phenomenon and Hashimoto thyroiditis [65]. Other extrahepatic autoimmune diseases that might occur include Graves' thyroiditis, systemic lupus erythematosus, scleroderma, rheumatoid arthritis, vasculitis, and celiac disease [65, 66]. Interestingly, extrahepatic autoimmune diseases commonly coexisted with PBC have a tendency to be less severe. For example, systemic sclerosis (SS) most commonly associated with PBC is limited to cutaneous tissue, and the disease progression is much slower compared with matched patients with PBC alone [67–69]. Similar to other chronic liver diseases, PBC is associated with a higher risk of HCC. The risk of HCC is reported higher in PBC, ranging from 6 to 18.8 times that of general population [42, 70, 71]. An internationally representative cohort study has reported that the incidence of PBC-HCC is significantly greater in male, patients with advanced disease, and 12-month UDCA non-responders [72]. Osteoporosis with an increased fracture risk is frequently encountered in PBC patients, largely driven by deficient bone formation [73–75]. Thus, vitamin D and calcium supplementation should be addressed in the clinical management of PBC patients.

Primary Sclerosing Cholangitis

PSC is a complex chronic cholestatic autoimmune disease with unknown causes. Unlike PBC, PSC is characterized by fibrotic obstructive cholangitis involving intra-/extrahepatic bile ducts and forms "onion-skin" fibrosis. Classically, PSC affects large bile duct while some may involve small ducts or overlap AIH. In a retraspective study, 89.9% patients had classical or large duct disease [76]. PSC is often associated with IBD, suggesting the important role of gut-liver axis in the pathogenesis of PSC. The recent guidelines for PSC suggest that all patients with IBD should receive an assessment for PSC [77]. The diagnosis is mainly based on abnormal cholestatic enzymes and distinctive radiological manifestations: segmental stenosis and dilation in magnetic resonance imaging (MRI). Liver biopsy is unnecessary unless in the case of small-duct PSC. There is no effective medical therapy for PSC, and many patients progress to end-stage liver disease that requires liver transplantation (LT) or even cholangiocarcinoma (CCA). PSC patients usually have a higher risk of developing CCA, and the annual incidence of developing CCA ranges between 0.5% and 1.5%, and the lifetime risk is between 6% and 12% [78].

PSC in the General Population

The epidemiological information of PSC is poorly described. The incidence rate of PSC ranges from 0.07 to 1.3 per 100,000 inhabitants per year, and the prevalence ranges from 0.22 to 16.2 (Table 11.3) [79]. A meta-analysis in 2011 reported a pooled incidence rate of 1.0 (0.82–1.17) per 100,000 person-years in six population-based studies in western countries. The pooled incidence rate ratio for males versus females was 1.7 (1.34–2.07), correlating with the susceptibility of males [80]. The incidence of PSC seems to be higher in Northern Europe and Northern America, but relatively low in Asia and Africa. The widely variable incidence and prevalence might be attributed to the ethnical diversity, and genetic background may play a role in the etiology and natural history of PSC [81]. Besides the genetic background, the study design and the inclusion criteria may partly explain the differences. A recent retrospective cohort study in the UK revealed an incidence of 0.68 per 100,000 person-years and a prevalence of 5.58 per 100,000 person-years, which is the highest incidence and prevalence reported ever in the UK [82]. It has been proposed that the incidence of PSC is increasing. Two cohort studies revealed a significant increase

Table 11.3 Incidence and prevalence rates reported for PSC

Year	Country/region	Incidence (per 100,000)	Prevalence (per 100,000)
1994	Spain	0.07	0.22
1996	Canada	N/A	6.5
1998	Norway	0.7	5.6
1998	Norway	N/A	6.5
2002	Singapore	N/A	1.3
2003	USA	0.9	13.6
2004	UK	0.91	12.7
2007	Canada	0.92	N/A
2008	UK	0.41	3.85
2010	Sweden	1.22	16.2
2011	USA	0.41	4.15
2013	Netherlands	0.5	6
2008, 2016	Japan	N/A	0.95
2019	Japan	N/A	1.8

N/A not available
Adapted from [146]

in incidence ratio of PSC over time with an average annual percent change of 3.06 in one study [83, 84]. A questionnaire-based survey conducted in Japan reported the point prevalence of PSC was 1.8 in 2016, indicating an increasing trend compared to the prevalence of 0.75 in 2007 [85]. Most patients with PSC have serum antibodies such as ANA, anti-SMA, and antineutrophil cytoplasmic antibody (ANCA) but are not specific. Recent studies identified zymogen granule glycoprotein 2 (GP2) as the first autoimmune mucosal target in PSC, the detection of antibody against which could be used for risk stratification [86]. Contrary to PBC, PSC has a male predominance, with a male/female ratio of 2/1 [87]. Female patients are usually associated with a lower risk of LT or death or malignancies [76]. The median transplant-free survival time of PSC is 14.5 according to an international retrospective study. The occurrence of hepatopancreatobiliary malignancies, mainly CCA, is associated with a significantly increased risk of patient mortality [76].

Family Occurrence

Unlike PBC, data on family occurrence of PSC is limited. A case report in 2005 described two brothers diagnosed with PSC who were positive for the susceptibility HLA haplotypes DR3-DQ2 and DR6-DQ6, suggesting a genetic origin of PSC [88]. In a monocentric study in Sweden, first-degree relatives of PSC patients have a PSC prevalence of 0.7%, nearly 100-fold increased risk compared to that of general population, indicating a potential role of genetic disposition [89]. Another study from Sweden also confirmed an increased risk of PSC in first-degree relatives of PSC patients. The offspring, siblings, and parents of PSC patient cohort had a significantly higher risk of cholangitis with the hazard ratios and 95% confidence intervals, 11.5 (1.6–84.4), 11.1 (3.3–37.8), and 2.3 (0.9–6.1), respectively [89].

Risk Factors

The etiology of PSC is unclear, but several genetic and non-genetic predispositions have been identified (see Table 11.1). Early serological studies documented the association between HLA complex and PSC. The following GWAS confirmed the importance of HLA as a risk locus. HLA-B*08 and DRB*03 have a strong association with PSC, with an odds ratio of 4.9 and 3.8, respectively [90]. Recently, the largest GWAS of PSC has identified a new significant locus which affects the expression of UBASH3A, a gene involved in the regulation of T cell signaling [91]. As for the genetic contribution to the disease severity and progression, genetic variant rs853974 outside the HLA complex is reported to be relevant to the disease progression of PSC [92]. In accordance with the strong association between PSC and IBD, PSC shares some susceptibility loci with PSC. However, most of these loci have failed to show a genetic link to PSC, suggesting that PSC-IBD might be a unique phenotype. As for environmental factors, smoking is considered to be a protective factor for PSC, independent of its protective effects on UC [93]. Like PBC and AIH, dysbiosis occurs in PSC patients, including bacteria and fungi [94, 95]. The identification of PSC marker genera either relevant to intestinal inflammation severity or biliary obstruction also suggests the association between PSC and microbiome [96].

Comorbidities

As mentioned above, PSC has a strong correlation with IBD, mostly ulcerative colitis (UC). The comorbidity of Crohn's disease (CD) is less common than UC, and PSC patients usually show milder symptoms in the setting of CD than UC [76, 97]. Approximately 75% of PSC patients have concomitant IBD, while the prevalence of PSC is 8.1% in IBD patients [98, 99]. More and more studies demonstrated that IBD patients associated with PSC are identical to patients with IBD alone with a relatively mild clinical course but an increased risk of developing colorectal carcinoma [100, 101]. The presence of PSC symptoms at PSC diagnosis in IBD patients is the only factor related with this increased risk of colorectal carcinoma [102]. Whether PSC coexisting with IBD differs from PSC alone remains unclear and requires further investigation. CCA is another common comorbidity in PSC with a 398-fold increased risk of developing CCA in PSC patients compared to the general population in a population-based multicenter study [84]. And the risk of CCA is significantly higher in patients with concomitant IBD and PSC than general population in a clinical study with 20-year follow-up [103].

Overlap Syndromes

Coexistence of clinical features of at least two different AILDs is defined as overlap syndromes. In overlap syndromes, shared clinical, immunological, and histological features exist between AIH, PBC, and PSC. In most cases, overlap syndromes are between AIH and PBC or AIH and PSC, but a few cases have reported the overlap syndrome of PBC and PSC [104–106]. The epidemiological information of overlap syndromes is limited due to the diagnosis and publication bias.

AIH-PBC overlap syndrome is more common than AIH-PSC, largely due to the relative frequent occurrence of PBC and AIH in the spectrum of AILDs. The prevalence of AIH-PBC overlap syndrome is estimated to be 4.3–9.2% among patients with PBC and 2–19% among patients with AIH [107, 108]. The adjusted prevalence of AIH-PBC overlap

syndrome by eliminating score for female gender or the presence of other autoimmune disorders is 4% [109]. AIH-PBC overlap syndrome seems to aggregate in Hispanic patients, with a significantly higher prevalence to develop overlap syndrome than that of non-Hispanic patients (31% vs. 13%, respectively) [110]. The frequency of cirrhosis and cirrhotic complications (i.e., gastrointestinal bleeding, portal hypertension, esophageal varices, etc.) are reported significantly higher in the overlap group than PBC alone [111]. A recent study compared the natural history of patients with PBC alone to those with overlap syndrome, and a decreased 5-year adverse event-free survival was observed in overlap patients [112]. The treatment of AIH-PBC overlap depends on the combination of steroids and UDCA, more effective than UDCA monotherapy according to a meta-analysis [113].

AIH-PSC overlap syndrome is a rare syndrome that has been described in both children and adults. AIH-PSC overlap is more common in children, adolescents, and young adults. The diagnosis is made upon the overt cholangiographic or histologic findings of PSC together with robust histologic features of AIH [108, 114]. The prevalence of characteristic cholangiographic appearance suggesting PSC found in adult AIH patients varies between different studies, ranging from 2% to 10% [115, 116]. The prevalence to develop PSC is much higher in children with AIH, up to 50% [31]. AIH is rarely diagnosed in patients with an original diagnosis of PSC, the prevalence of which ranges from 7% to 14% [117, 118]. The adverse outcome-free survival of patients with PSC/AIH overlap syndrome is reduced [119]. Interestingly, AIH-PSC overlap patients seem to have a better outcome than straightforward PSC patients with the combination treatment of UDCA and immunosuppressants [120, 121]. AIH-PSC overlap patients are still regarded to have a poorer prognosis than patients with classical AIH and AIH-PBC overlap [122].

Liver Involvement in Systemic Rheumatic Diseases

Liver involvement in systemic rheumatic diseases is common even though the liver is not a common target organ. The epidemiology of these liver autoimmune conditions is largely correlated to the prevalence of systemic rheumatic diseases and the susceptibility of liver involvement. Several common conditions will be discussed in detail in the following part.

IgG4-Related Diseases

IgG4-related disease is a systemic inflammatory condition that can affect multiple organs. Involvement of nearly every anatomic site has been reported, but the most commonly affected organs are pancreas, biliary tract, major salivary glands, lacrimal glands, retroperitoneum, and lymph nodes [123]. IgG4-related diseases share similar histological appearances: lymphoplasmacytic infiltration, storiform fibrosis, and obliterative phlebitis with variable presence of eosinophils [124, 125]. With regard to IgG4-related hepatobiliary disease, characteristic imaging features of segmental or diffuse biliary strictures with thickened bile duct walls are required to support the diagnosis apart from histopathological features [126]. The prevalence of IgG4-related hepatobiliary disease remains unclear. A nationwide survey in Japan identified 43 IgG4 sclerosing cholangitis (IgG4-SC) without autoimmune pancreatitis (AIP). The male to female ratio was 3.3 to 1 in IgG4-SC with an average age of onset of 69.3 years [127]. A novel concept of IgG4-realted AIH has been proposed [128, 129]. Patients who met the diagnostic criteria for AIH had a high serum IgG4 level, and abundant IgG4-positive plasma cells were reported to be diagnosed as IgG4-related AIH. The prevalence of IgG4-SC and IgG4-AIH is lacking due to the scarce reports.

Sarcoidosis

Sarcoidosis is a chronic granulomatous inflammatory disease that can affect any organ. Liver involvement is relatively common in sarcoidosis with prevalence ranging from 5% to 30% [130]. It has been found that 50–65% of sarcoid patients have hepatic involvement as per liver biopsy [131]. A population-based study reported a prevalence of 6%, and cholestatic enzymes are elevated in the majority of patients [132]. A close association has been demonstrated between sarcoidosis and hepatitis C virus infection [133]. It has also been reported that a link lies between sarcoidosis and PBC or AIH [32, 134]. The histological abnormalities of hepatic sarcoidosis include non-caseating granulomas, intrahepatic cholestasis, periportal fibrosis, etc. For patients with end-stage hepatic sarcoidosis who require liver transplantation, the 10-year survival rate is estimated to be 51.3%, lower than matched PSC/PBC group (61.5%) in a monocentric study [135].

Connective Tissue Diseases

Connective tissue diseases (CTDs) are composed of a large and heterogeneous group of immunological disorders with unknown etiology. Liver, as the largest lymphoid organ, is frequently involved in CTDs in the form of abnormal biochemical indexes.

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease that can cause damage to almost every organ. It has been reported that patients with SLE have a 9.3–59.7% chance to develop liver dysfunction during follow-up [136, 137]. With the criteria of liver disease as two-fold elevation of liver enzymes, a monocentric study revealed

20.7% of SLE patients have liver disease and the prevalence to develop liver dysfunction is increased in males, indicating that male patients with SLE are more susceptible to liver involvement [138]. SLE-associated hepatitis, termed lupus hepatitis, occasionally occurs. It has been reported that 4.7% of SLE patients develop AIH and 19.4% of SLE patients have liver enzyme abnormalities [139].

Sjögren's syndrome (SS) mainly affects salivary and lacrimal glands, manifested by keratoconjunctivitis sicca, xerostomia, and swelling of salivary glands. Liver involvement is commonly seen in SS. About 27–49% of SS patients have abnormal liver function with 11–21% found to develop hepatomegaly [140]. Of note, a group of SS patients have positive serum AMA [141]. AMA is considered to be associated with pathogenesis of SS. In both PBC and SS, the autoantibodies can target bile duct and salivary gland, partly explaining the presence of AMA in SS patients. SS patients have a higher risk of developing AILD with 9% PBC and 4% AIH [142, 143]. It is worth mentioning that liver function assessment should be conducted in SS patients regularly.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by joint involvement and extra-articular manifestations. Liver involvement is not a typical extra-articular manifestation in RA. The presentation of liver damage in RA is a cholestatic pattern with predominantly elevated ALkaline Phosphatase (ALP) and gamma-Glutamyl Transpeptidase (γGT). Abnormal liver function test results are present in between 18% and 50% of cases [144]. A recent cross-sectional study identified 44% liver involvement in RA patients with most of the cases asymptomatic [145]. Notably, the liver involvement in RA may be attributed to the hepatotoxicity of medications.

Besides the CTD mentioned above, systemic sclerosis, myopathies, antiphospholipid syndrome, and many other systemic autoimmune diseases can involve liver, characterized by abnormal liver enzymes or hepatomegaly. The prevalence of liver damage caused by systemic autoimmune disease varies between different diseases and ethnic groups. Liver function should be well-monitored once the diagnosis of CTD is made.

Conclusion

The increased annual incidence and prevalence have been drawing attention to the management of AILDs during the past decades. AIH, PBC, PSC, and overlap syndromes are the most recognized ones that affect liver in situ. Liver involvement of systemic rheumatic diseases usually does not display specific biochemical nor histological features. Although the prevalence is increasing, AILDs remains rare. The epidemiological features of these kinds of diseases are limited. Most AILDs have a female predominance with the exception of PSC and IgG4-related diseases. Ethnic and sexual factors usually play an important role in the occurrence and pathogenesis. Genetic predisposition is considered to have a strong association with the onset of AILDs. The management of these kinds of diseases usually relies on immunosuppressants, including glucocorticoids and immunosuppressive drugs. To sum up, AILDs should be considered in patients with liver dysfunction when the infectious and metabolic causes are ruled out.

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Immune Responses to Bacterial Infections

12

Eirini I. Rigopoulou, M. Eric Gershwin, and Dimitrios P. Bogdanos

Abbreviations

DC	Dendritic cell
HSC	Hepatic stellate cell
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NK	Natural killer
NKT	Natural killer T cell
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PD	Programmed death
PPR	Pattern recognition patterns
Th	T-helper
TLR	Toll-like receptor

Key Points

- Systemic infections affecting the liver greatly vary leading to acute hepatitis, pyogenic abscesses, and granulomatous liver diseases.
- The extent of innate and adaptive immune responses against bacterial antigens depends on the agent and its ability to circumvent host's immune system.
- The exact mechanisms used by bacteria to accomplish invasion, replication, and survival within the liver are ill-defined.
- The decisive elements regulating innate and adaptive immune responses against bacteria of the liver are poorly understood.
- Organisms commonly cultured in jaundice or sepsis include *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, *Salmonella*, *Bacteroides*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.
- Direct bacterial infections of the liver include *Mycobacteria*, *Listeria monocytogenes*, *Brucella* species, *Legionella pneumophilia*, *Burkholderia psuedomallei*.
- *Mycobacteria* affecting the liver include *Mycobacterium tuberculosis*, *bovis*, *kansasii*, *gordonae*, *leprae*, and *avium-intracellulare.*

Introduction

Direct infection or bacterial-induced indirect inflammation of the liver may cause hepatocyte or biliary epithelial cell destruction, and subsequent liver failure. A wide variety of systemic infections affect the liver leading to acute hepatitis, pyogenic abscesses, and granulomatous liver diseases. Liver diseases of bacterial cause comprise a wide clinical spectrum that involves asymptomatic patients, cases with elevated transaminases and/or cholestatic liver enzymes, patients with fulminant hepatitis and acute liver failure, and chronic liver disease with formation of abscesses or granulomas.

When direct hepatic bacterial infection occurs, the organisms commonly isolated include but are not limited to *Brucella melitensis/abortus/suis*, *Listeria monocytogenes*, *Legionella pneumophilia*, *Burkholderia psuedomallei*, *Francisella tularensis*, *Treponema pallidum*, *Neiserria gon-*

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orrhoae, and *Mycobacteria*. Liver destruction can be the outcome of direct cytotoxic effects on infected parenchymal cells and/or Kupffer cells or indirect bystander injury likely caused by cytokines, their production being largely influenced by specific bacterial infections. At times, it may be difficult to determine whether liver destruction is due to direct, indirect effects or a combination of both. This chapter will not cover aspects of bacterial infections complicating patients with liver cirrhosis. Cases at greater risk to develop such infections are immunosuppressed patients with alcoholic cirrhosis or decompensated chronic liver disease of any underlying cause. Spontaneous bacterial peritonitis, bacteraemia (accounting for more than 30% of infections), pneumonia, urinary tract infection, and to a lesser extent infectious endocarditis and meningitis can be noted. Bacterial infections of such a kind are relatively common and represent one of the most important causes of frequent hospitalizations, impairment of health-related quality of life, outstanding healthcare costs, and enhanced mortality rates in cirrhotic patients. In fact, bacterial infections may be a triggering factor for the occurrence of decompensated liver cirrhosis complications such as gastrointestinal bleeding, hypervolemic hyponatremia, hepatic encephalopathy, kidney failure, and acute-on-chronic liver failure. Bacterial infections fundamentally develop as a consequence of immune dysregulation that gradually develops during the course of cirrhosis. The hierarchy of events leading to the establishment of bacterial infection in cirrhotic patients is quite distinct to that noted in liver-disease-free bacterial infections and assists efforts to better understand the distinction between the incriminating bacteria as well to better appreciate differences of immune responses against the respective infectious agents. A close interplay of host-pathogen interactions is driving immune responses, which may lead to total control of the infection or its persistency (Fig. 12.1). In a significant proportion of patients, Gram-negative bacteria cause infections in cirrhotic patients from intestinal origin, but Gram-positive bacteria are also causing infection and inflict damage in numerous occasions, predominantly in hospitalized patients [1].

Jaundice per se is not a prerequisite for a high index of suspicion of bacterial infection, as its absence is noted in several occasions, while its presence is often noted in septic patients, toxic shock syndrome, leptospirosis, and even in pneumococcal pneumonia. In the right clinical context and with the assistance provided by serologic, microbiologic, noninvasive (abdominal ultrasound/transient elastography, computerized tomography – CT, magnetic resonance imaging – MRI) and invasive (liver biopsy) imaging techniques, the diagnosis of bacterial infection can be confirmed in most cases, as the underlying cause of the established features.

The subsequent paragraphs of this chapter give emphasis focusing on the immunological alterations noted due to bacterial infection involving the liver. There is no intention to discuss in great detail the plethora of infectious causes inflicting the liver. Priority is given to those the readers may find of interest because of the complexity and the interplay of

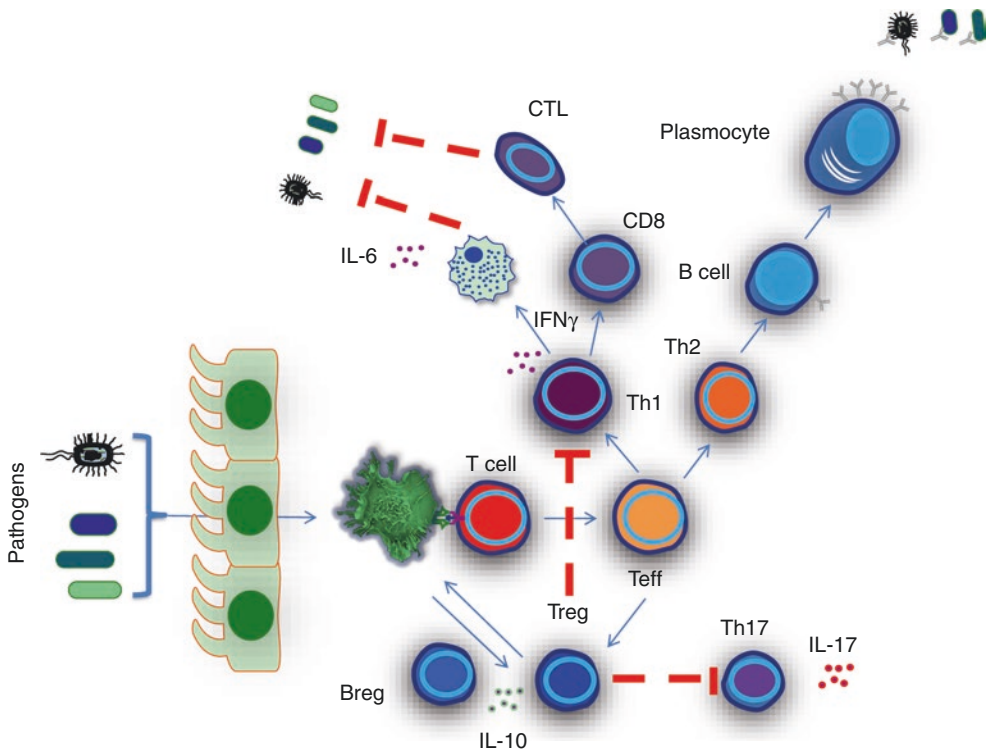

Fig. 12.1 Immune responses against extracellular and intracellular pathogens are initiated upon antigen presentation of antigenic epitopes to T cells. A plethora of immune events will then take place, depending on the close interplay between effector cells and pro-inflammatory or anti-inflammatory cell subsets, which will either lead to the elimination of the pathogen or its persistency

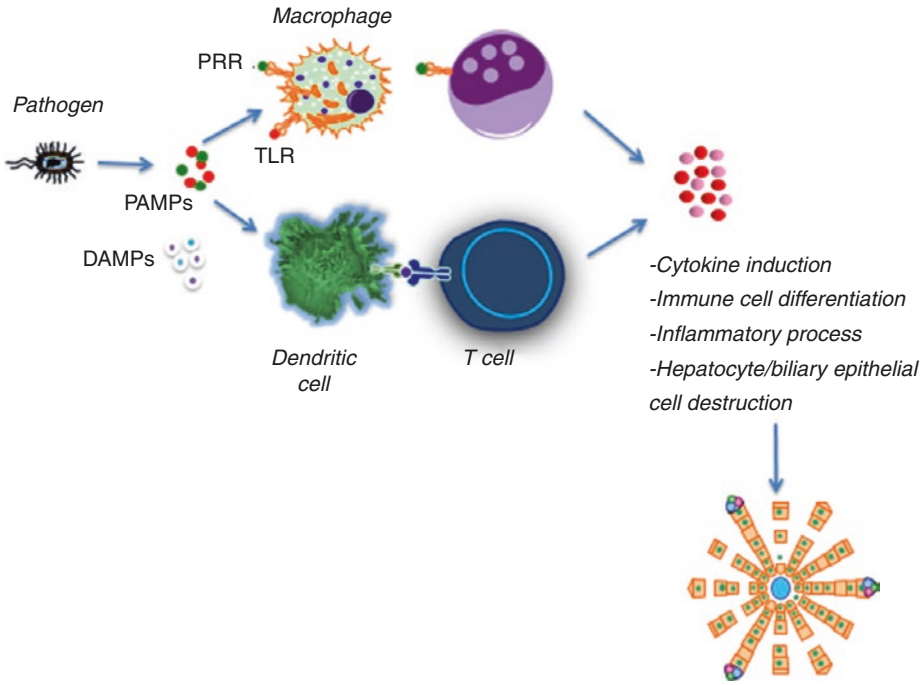

Fig. 12.2 Pathogen related or danger signals initiate through the release of PAPMs and DAMPs the activation of pathogen-host interaction, which through the activation of innate and adaptive immune responses and the contribution of the respective immune cells lead to cytokine release and the induction of inflammatory processes and the ultimate hepatocyte and biliary epithelial cell destruction

innate and adaptive immune system and bacteria. Immunological aspects discussed are those attempting to address how, when, and why infection circumvents host's immune capacity to eliminate the foreign agent and why liver disease is established. The interested reader is referred to previous versions of this book or other textbooks of liver and infectious diseases, in case he/she is eager to learn more on the diagnostic and clinical aspects (including treatment approaches) of bacterial infections of the liver, as a cause of liver damage or in patients with established liver failure.

Anti-Bacterial Innate and Adaptive Immune Dysregulation in Cirrhotic Patients

Cirrhosis is a dynamic state correlated with systemic inflammation documented as enhanced immune cell activation and circulating inflammatory mediators. These pro-inflammatory cellular and cytokine mediators can participate in the exacerbation of clinical features of cirrhosis, such as renal failure and hemodynamic imbalance. Disease progression is accompanied by a state of immunosuppression and diminished antimicrobial competence and resistance to infection. One major mechanism responsible for the induction of an infectious state is the translocation of intestinal bacteria to extraintestinal sites, which initiates a systemic inflammatory process, chronic activation of immune cells and pro-inflammatory cytokine production, and ultimately leads to immunosuppression, which rises susceptibility to intestinal Gram-positive bacteria or bacteria of nonintestinal origin including *Enterobacteriaceae,* such as *Escherichia coli* and *Klebsiella pneumoniae*. *Staphylococcus aureus* and *Enterococci* are among the most common Gram-positive infections [2].

Macrophages, neutrophils, and monocytes are important innate immune cells, which respond to invading pathogens in an attempt to control/eradicate the pathogens' infection. In patients with liver disease, these cells are critical inflammatory mediators, responding to the damage-associated molecular patterns (DAMPs), which are released from the destroyed hepatocyte and biliary epithelial cells initiating hepatic stellate cell activation and fibrogenesis (Fig. 12.2) [3].

Evidence obtained from studies in pre-cirrhotic and early cirrhotic disease documented an inflammatory continuum of monocytes and inflammatory cytokines trafficking through the liver and a tenacious release of DAMPs into the circulation providing constant inflammatory stimuli. Decompensated cirrhosis is accompanied by small intestinal bacterial overgrowth and increased gut permeability, gut dysbiosis, and microbiome changes leading to increased systemic exposure to gut microbial products, which provide the impetus for further chronic stimulation of innate immune cells. This increase in microbial products has been considered a "fine tune" switching stage from the pre-cirrhotic proinflammatory immunological state to a predominant hyporesponsive immunodeficient phenotype to the extreme end of the spectrum observed in decompensated cirrhosis. This can be explained by the continuous priming of monocytes and mac-

rophages with lipopolysaccharide (LPS) and other bacterial products through toll-like receptor (TLR) 4 that diminishes subsequent responses to stimuli (immunodeficient state/tolerance) (see Fig. 12.2) [4].

LPS-mediated immunoparalysis occurring after Gram-negative sepsis is a fundamental mechanism of tolerance which largely explains the immunodeficient status of innate immune cells noted in cirrhotic patients. Pro-inflammatory cytokine production by monocytes following microbial encounter is pivotal to innate immune defense against pathogens. In case TNF, IL-6, and other pro-inflammatory cytokines are reduced (despite LPS stimulation), antimicrobial activity is severely impaired, another hallmark of the LPS-tolerant immunosuppressed state in sepsis. The genetic make-up of these patients also participates to increased infection risk as common polymorphisms in genes encoding innate immune pattern recognition receptors including IL-1, TLR2, TLR4, TLR9, CD14, and NOD2 have been associated with the acquisition of infections [5].

When gut-derived bacteria escape surveillance by gut immune cells, they reach the liver via the portal vein. This organ acts as a filter for gut bacteria and bacterial products. This explains why liver-resident macrophages is the largest population of tissue macrophages with direct access to the blood stream. However, it still remains uncertain whether macrophage phagocytic capacity is impaired in patients at pre-cirrhotic or cirrhotic stages [6].

Recent murine data in a model of Listeria infection demonstrate, though, that gut bacterial translocation impairs antibacterial immunity causing loss of infection control through the induction of type 1 interferon expression in the liver and the production of myeloid cell-mediated IL-10 production [7]. Also, neutrophils of cirrhotic patients consistently demonstrate impaired neutrophil phagocytic capacity and/or elevated ROS production at steady state and diminished neutrophil killing of intracellular bacteria, as well as dysregulated neutrophil endothelial adhesion and chemotaxis [8–10]. Remarkable reduction of neutrophil migration and phagocytosis of heat-killed *E. coli* in vivo occur in cirrhotic patients with previous episodes of bacterial infection compared to cirrhotic patients with no evidence of prior infection [8]. Over the years, data have convincingly shown that neutrophils in patients with cirrhosis are chronically activated, exhibiting high resting ROS production but are severely impaired in their trafficking potential to infectious sites being unable to mount efficient antibacterial responses. Their impairment appears, at least in part, reversible as data demonstrate improved function in vitro with interventions such as TLR7/8 agonism [11].

In addition to augmenting tissue macrophage pools via to inflammatory sites, circulating monocytes are important innate immune effector cells, because they mediate recruitment of circulating macrophages and activation of tissue-resident macrophages in inflammatory sites. In addition, they are key elements of potent adaptive immune responses, via antigen presentation and the production of immunoregulatory cytokines. Human "classical" CD14high/CD16− monocytes (comprising ~80% of peripheral blood monocytes) exhibit strong phagocytic activity, while the nonclassical CD14+CD16+ monocytes, subsets, and in particular the intermediate CD14HighCD16+ subsets, have shown pro-inflammatory potential [12].

The most intriguing feature of phenotypic analysis of monocyte populations from cirrhotic patients is the reduced HLA-DR expression, which dampens antigen presentation and the development of adaptive immune responses and is a feature of LPS-mediated immunoparalysis [13, 14]. Ex vivo monocyte-derived dendritic cells from cirrhotic patients appear to be equally capable of upregulating co-stimulatory molecules and stimulate expansion of antigen-specific T cells as those of healthy controls [15].

Data have also been obtained reporting that attenuated antigen-specific T cell responses in cirrhotic patients are associated with elevated serum interleukin-10 levels and downregulation of HLA-DR on monocytes [16]. Adaptive immune dysfunction is also a feature of cirrhotic patients. Roger Williams's group was the one of the first to demonstrate the existence of impaired T cells function and hyperactive B cells in peripheral blood of patients with alcoholic liver disease [17]. Reduction of memory of $CD27^+$ B cells in the peripheral blood of cirrhotic patients, independent of the underlying cause of cirrhosis and functional impairment (reduced TNF-β production) has been documented, likely explaining at least in part the vaccine hyporesponsiveness and susceptibility to bacterial infection in these patients. Cirrhotic patients appear to have elevated CD8+ T cells and reduced $CD4^+/CD8^+$ T cell, which has been considered to favor pro-fibrotic processes [18]. Profound alterations in the peripheral blood monocyte and T cell compartments of cirrhotic patients consistent with a state of monocyte and T lymphocyte activation, with the presence of an increased population of both $CD4^+$ and $CD8^+$ T cells committed to apoptosis and with an increased population of effector $CD8^+$ T cells with characteristics of senescent cells have been shown [19].

Various routes accomplish access of bacteria to the liver: direct inoculation, haematogenous, by the biliary tract, or contiguous spread. Haematogenous spread of bacterial infection of the liver is achieved via the portal vein or the hepatic artery. Except for viral hepatitides, bacterial infections causing acute hepatitis are those attributed to *Brucella spp*., *Neisseria meningitidis*, and *Salmonella typhi*, *Coxiella burnetii*, *Rickettsia spp*. and mycobacterial infections can cause granulomatous liver disease. Tables 12.1, 12.2, 12.3, 12.4, and 12.5 provide an overview of morphological classifications of granulomas and the most frequent infectious and

Table 12.1 Morphological classifications of hepatic granulomas used for diagnostic purposes

Morphology	Cause
Epithelioid granulomas (necrotizing)	Infectious agents
Fibrin ring granulomas	Bacterial Infectious agents (*Coxiella burnettid, Leishmania*, *Mycobacterium avium-intracellulare*) Other bacterial infections (toxoplasmosis, typhoid fever, Boutonneuse fever) Viral infections (i.e., human cytomegalovirus infection, Epstein-Barr virus) Noninfectious causes: drug-induced liver injury, Hodgkin's lymphoma
Foamy macrophage aggregates (lacking of significant inflammatory infiltrates)	Infectious agents in immunosuppressed patients
Lipogranulomas	Mineral oils in foods
Microgranulomas	No specific etiological association
Granulomatous inflammation/poorly formed granulomas	Various infectious agents
Stellate abscesses (with associated granulomatous inflammation)	Infectious etiologies (i.e., *Bartonella henselae*)

noninfectious causes of granulomatous disease that must be included in the differential diagnosis.

Immune responses to some of those factors determining the outcome of these responses will be further discussed.

Brucella

General Aspects

Brucella spp. are Gram-negative, nonmotile, nonspore-forming coccobacilli that belong to the Brucellaceae family and alongside *Bartonella*, *Rickettsia,* and *Ehrlichia* in the class Alphaprotobacteria of the phylum Proteobacteria [20].

Brucella melitensis was initially identified in 1887 by David Bruce as the cause of disease in British soldiers stationed in Malta. In 1905, Themistocles Zammit, a Maltese doctor, recognized unpasteurized goat's milk as the major source of the pathogen in humans. Up to the present, several *Brucella spp*. have been identified in different hosts, that is, *Brucella abortus* in cattle, *Brucella canis* in dogs, *Brucella suis* in swine, *Brucella ovis* in sheep, and *Brucella neotomae* in wood rat of the desert. *Brucella cataceae* and *Brucella pinnipediae* have been recently identified [20].

Human brucellosis has been attributed to *Brucella melitensis*, *B. abortus*, *B. canis,* and *B. suis*, though the predominant cause is *B. melitensis* worldwide. Brucellosis is the commonest zoonotic infection worldwide with 500,000 reported cases annually. On the whole, the disease has minimal mortality, though it is related to considerable lasting disability due to severe complications and travel-associated morbidity. The epidemiology of human brucellosis has considerably changed over the last two decades mainly as a result of socioeconomic changes, sanitary measures, and evolving international travel [21].

Infection by *Brucella spp.* results via ingestion of contaminated food, that is, unpasteurized, infected milk, and animal products via direct contact with infected animals, where the pathogen is inoculated through ruptured skin or mucosal surface. This is also the reason why brucellosis is considered an occupational disease affecting livestock workers, veterinarians, and persons working with dairy products. In addition, inhalation of Brucella containing aerosolized particles is another, less common, source of infection, as manifested by airborne spreading of disease in laboratory workers. Airborne spreading has been exploited in the use of Brucella as biologic weapon [20].

Brucella can replicate within a variety of cells, from macrophages, dendritic cells (DCs), and trophoblasts to epithelial and endothelial cells. This intracellular tropism determines unique pathology characteristics in the infected host. In detail, three phases are recognized during the infection process: the incubation phase where no symptoms are present, the acute phase where Brucella after encountering with local tissue lymphocytes is transferred in the circulation and towards different organs with tropism to the reticuloendothelial system, including the liver and the spleen, and the chronic phase where severe organ damage may occur leading to death of the host.

Brucellosis manifests with a vast variety of symptoms, ranging from fever and malodorous sweat, that is, almost pathognomonic, to symptoms related to individual organ involvement [20]. Osteoarticular disease is the most common manifestation of localized infection followed by epididymoorchitis in men, while central nervous system involvement and endocarditis are less common. Liver involvement presenting as hepatomegaly is reported in up to 55% of patients or even more. Granuloma formation is the typical histopathological feature in brucellosis, and it is attributed to the immune system's effort to restrict (localize) the infection [22].

Immune Responses

In vivo, *Brucella* is found in association with phagocytic cells, most prominently macrophages, in which a subset of bacteria is able to evade killing in phagolysosomes and replicate successively with an endoplasmic reticulum–associated compartment and a modified autophagosome. *Brucella spp*. have developed a stealth strategy to avoid PAMPs recognition and evade innate immune responses. In this way,

Table 12.2 Major infectious causes of hepatic granulomas (in gray boxes) in relation to their morphological classification

Type of granuloma / **Infectious cause (alphabetic order)**	Epithelioid granuloma	Fibrin granulomas	Stellate microabcesses with granulomatous inflammation	Micro-granulomas	Lipo-granuloma	Foamy macrophage aggregates	Predominantly suppurative/ Granumolatous inflammation
Actinomycetes			■				
Bartonella			■				
Brucella	■						
Burkholderia psudomallei (Melioidosis)							■
Candida (and other fungi)			■				
Chlamydia	■						
***Coxiella burnetii* (Q fever)**		■					
Epstein-Barr virus		■					
Histoplasmosis						■	
Human cytomegalovirus		■					
Leishmania		■				■	
Listeria				■			■
Mycobacterium avium-intracellulare	■					■	
Mycobacterium tuberculosis	■						
Tuberculoid leprosy	■						
Lepromatous leprosy						■	
Nocardia			■				
Salmonella		■					
Schistosomiasis	■						
Toxoplasma		■					
Treponema pallidum (Tertiary syphilis)	■						
Tularemia			■				■

Brucella can reach its maximum replication capacity before adaptive immunity mechanism is activated. In addition, *Brucella spp*. are able to survive within phagocytic cells and manipulate the host's immune responses by restraining apoptosis of infected mononuclear cells, inhibiting DC maturation, reducing antigen presentation and activation of naive T cells [23].

By producing virulence factors, *Brucella* spp. can modify phagocytosis, phagolysosome fusion, cytokine secretion like TNF-a and apoptosis, and in this way, evade innate immune responses. At initial stages of infection, Brucella aims at curtailing recognition by PAMS. Brucella can bypass recognition by TLRs and NLRs and this closely relates to the modified structure of lipid A moiety of its LPS that enables

Table 12.3 Major noninfectious causes of hepatic granulomas

Autoimmune diseases
Primary biliary cholangitis
Primary sclerosing cholangitis
Systemic lupus erythematosus
Vasculitides (polyarteritis nodosa)
Inflammatory bowel diseases
Sarcoidosis
Idiopathic eosinophilic gastroenteritis
Cancerous diseases
Hepatocellular carcinoma
Metastatic liver tumors
Hodgkin's disease
Drug-induced liver injury (DILI)
Allopurinol
Isoniazid
Inherited diseases
Chronic granulomatous disease
Foreign material
Mineral oil
Starch
Silicone
Metal toxicity
Copper
Beryllium

Table 12.4 Infectious causes of liver abscesses

Escherichia coli
Klebsiella pneumoniae
Enterococci (S. faecalis, S. faecium)
Streptococcus milleri
Bacteroides species
Fusobacteria
Yersinia enterocolitica and *Y. pseudotuberculosis*
Staphylococcus aureus
Listeria monocytogenes
Brucella suis
Proteus vulgaris
Pseudomonas aeruginosa
Salmonella
Clostridium perfringens
Francisella tularensis
Actinomycetaceae (Acitonomyces israelii, Nocardia spe cies)
Bartonella henselae
Burkholderia pseudomallei
Campylobacter jejuni
Fusobacterium nucleatum
Legionella pneumophila

Table 12.5 Nosological causes of liver abscess

Portal vein (inflammatory bowel disease, pelvic inflammatory disease, appendicitis, diverticulitis, rectal ulcer, perianal abscess)
Hepatic artery (dental infections, bacterial endocarditis), bile ducts (stones, strictures, cholangiocellular carcinoma)
Per continuity (gallbladder empyema, perforated peptic ulcer, perforation of the stomach by foreign bodies, subphrenic abscess)
Posttraumatic
Iatrogenic (liver biopsy, occluded biliary stent, arterial embolization, endoscopic or surgical interventions on bile ducts)
Infectious cysts or intrahepatic malignancy
Cryptogenic/idiopathic causes

avoidance of TLR4 detection. In fact, except for having a longer lipid A moiety compared to other pathogens (i.e., enterobacteria), the glycosylation pattern of its core oligosaccharide constituent, as far as *Brucella abortus* concerns, prevents from binding to the TLR4 co-receptor MD-2. In addition, existing data demonstrate Brucella's flagellin being able to avoid recognition by TLR5 [24]. Also, evasion of TLR2 and TLR4 recognition is being accomplished by a Brucella-encoded protein (Btp1/BtpA in *B. abortus* and TcpB in *B. melitensis*) that degrades the MyD88 adaptor like (MAL) required for TLR2 and TLR4 signaling [25].

Brucella's LPS O-antigen is also involved in survival and persistence of the pathogen, as interaction with specific cell surface receptors is able to decrease macrophage activation [26].

In addition, through its interaction with MHC class II molecules on antigen presenting cells, LPS-O-antigen downregulates T cell activation [27].

In addition, the outer membrane protein 25 (Omp25) of *B. suis* has been shown to negatively regulate TNF-a production in infected human macrophages [28]. This finding is of outmost importance because the defect in TNF-a may facilitate the development of *Brucella* spp. at different levels, as this proinflammatory cytokine promotes the bactericidal activity of phagocytes and stimulates macrophages and antigen-presenting cells.

Studies on innate and adaptive immune responses to *Brucella spp.* have been mainly conducted in cell or cell lines from mice or domestic ruminants. However, the existing data show vast differences among immune responses between human and mice, and the obtained data lack credibility. It appears that, after entering the host, up to 90% of *Brucella spp*. is being killed within the first hours by macrophages that exert multiple phagocytic and inducible bactericidal functions [29]. The minority survives avoiding phagolysosomal fusion and replicate intracellularly within a membrane compartment, the Brucella-containing vacuole (BCV) (Figs. 12.3 and 12.4) [30]. Persistence of *Brucella spp.* for prolonged time in the phagosomal part of the phagocytes is the basis for the pathogen persistence and establishment of chronic infection.

Similar to other pathogens, *Brucella spp*. express the type IV secretion system (T4SS), encoded by the virB operon, which is crucial for its intracellular survival and multiplication. VirB operon's expression is induced inside macrophages and regulated by environmental signals like phagosome acidification [31]. The importance of this system in Brucella's survival is emphasized by the fact that VirB mutant strains lose their replicative capacity in cultures of primary and THP-1 macrophages.

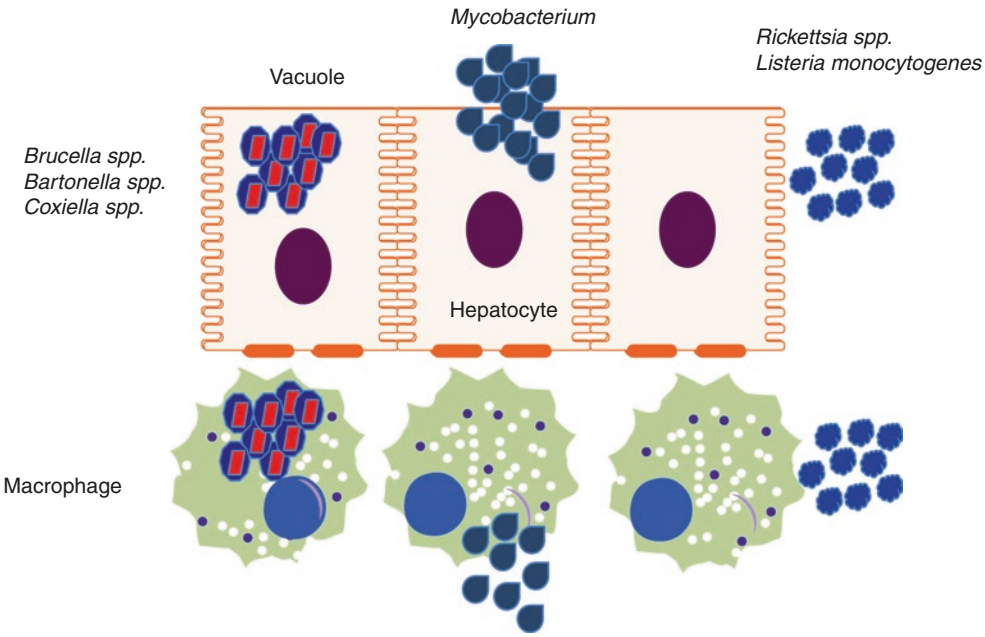

Fig. 12.3 Representative examples of intracellular bacterial infection replicating either in specialized vacuoles within hepatocytes (in blue-red), or in cytosol (in blue), or in both

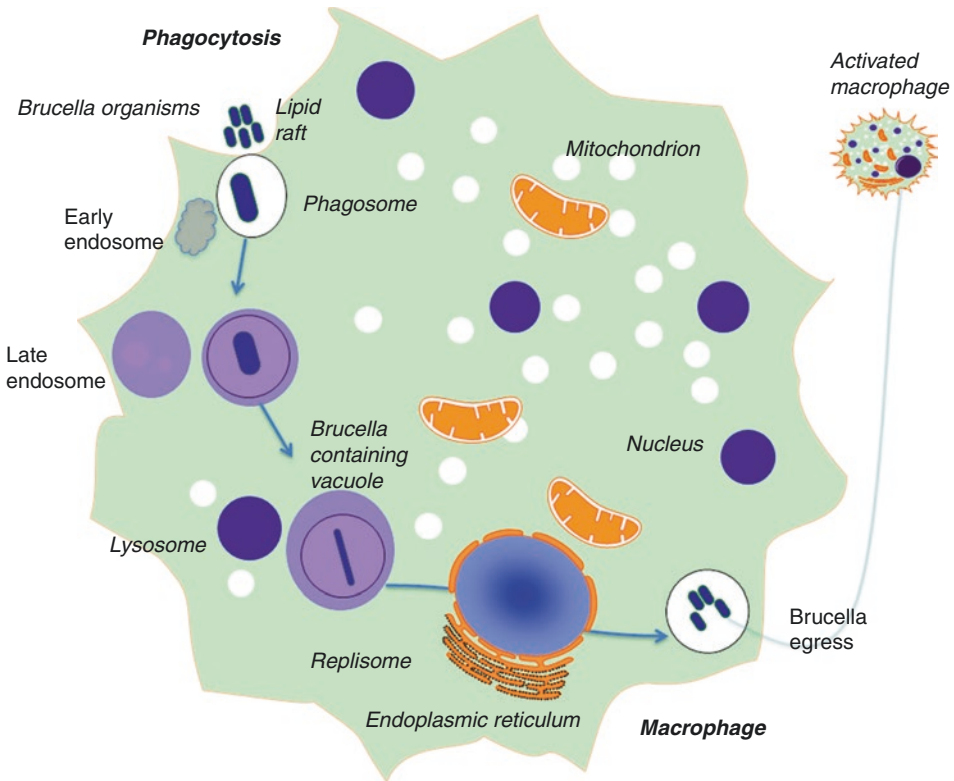

Fig. 12.4 Following phagocytosis, *Brucella organisms* reside within the Brucella containing vacuole (BCV). This organism-specific vacuole undergoes interactions with early endosomes, late endosomes, and partially fuse with lysosomes to transform to BCV. Following replication in the ER, BCVs are converted into autophagic BCVs, promoting completion of the *Brucella* intracellular cycle and formation of bacterial egress

Brucella has also established several mechanisms to defeat adaptive immune responses and facilitate progression to chronic stages of infection. In line with this, brucellosis has been also associated with inhibition of DC maturation, as attested by decreased expression of surface markers (CD40, CD80, CD86, and MHC-II) and low cytokine concentrations in DCs infected by *B. abortus* [25]. In addition, impaired IL-12 production attenuates antigen presentation by DCs leading to poor induction of T lymphocytes.

As demonstrated in mice, bacteria are detected in the Kuppfer macrophages resident in liver sinusoids during acute phases of infection, where they continue to replicate. Granulomas, composed of macrophages, DCs, plasma cells, and lymphocytes are detected in liver parenchyma early dur-

ing the first week post infection. Other histopathologic features are varying degrees of cellular infiltration of liver parenchyma and portal tracts, parenchymal necrosis, and Kupffer cell hyperplasia [22].

Except for Kupffer cells, infected hepatocytes can play a vital role in innate immune responses against *Brucella* spp., contributing to recruitment of inflammatory cells to the site of infection.

Infection of human hepatoma cells HepG2 by *Brucella abortus* was shown to mediate inflammation via production of IL-8, which is a potent chemoattractant of neutrophils. In addition, Brucella-infected neutrophils induced expression of ICAM-1 leading to further exacerbation of neutrophil adhesion to hepatocytes [32]. In this model, *B. abortus* has been shown to promote a profibrogenic response via a TGF-b dependent activation of hepatic stellate cells, leading to inhibition of matrix metalloproteinase-9 and collagen deposition. This fibrotic phenotype induces apoptosis of hepatoma cells.

More recent data indicated that *B. abortus* infection to induce cleavage of Beclin-1, which is a dual regulator of both autophagy and apoptosis. This model suggested *B. abortus* to promote a profibrotic response that occurs at the same time with activation of autophagy, and subsequently results in apoptotic cell death of HSCs. This hypothesis is in line with the observation that human brucellosis has been rarely observed as a cause of liver cirrhosis [33].

Defected T-cell responses have been reported in patients with chronic brucellosis. In humans, Th1 immune responses prevail during early stages of brucellosis and diminish during progression of the disease to chronic stages [34]. In addition, NK cells from patients with acute brucellosis have an impaired cytotoxic function, which is being reverted after in vitro incubation with IL-2 or antibiotic treatment [35].

During chronic brucellosis, immature CDs are thought to induce the immunoregulatory action of Tregs after contact with CD4+ T cells that results in diminished Th1 immune responses in a TGF-b-dependent fashion [36]. Weak CD4+ T cell responses after stimulation with Brucella cell extract antigens or nonspecific stimulation have been reported during chronic infection, while an increase in cytotoxic CD8+ T cells is thought to occur in compensatory manner in these cases [37]. In addition, the number of CD4+CD25+ and CD4+ CD28+ T cells is significantly decreased in cases of chronic compared to acute infection [37].

Bartonella

General Aspects

Bartonella spp. are Gram-negative intracellular bacteria belonging to the a2-subgroup of the proteobacteria. More than 30 *Bartonella species* have been identified until now, though only 10 are pathogenic for humans. *Bartonella bacilliformis*, *Bartonella quintana,* and *Bartonella henselae* are the most frequent causes of Bartonellosis in humans [38].

A large variety of mammals, including domestic and wild animals, consist Bartonella's reservoir, while bloodsucking arthropods are vectors of the pathogen that is mainly transmitted by flea feces and superficial scratching. In humans, some species may be transmitted from companion animals through scratch or bite [39].

In 1909, *Bartonella bacilliformis* causing Oroya fever and verruga peruana was the first to be described causing disease in humans. *Bartonella henselae* is the cause of cat scratch disease (CSD) and bacillary peliosis (or hepatic peliosis) and *Bartonella quintana* is the cause of trench fever. In healthy people, the infection can have mild and self-limiting clinical course suggesting adaptation of the pathogen to the infected host and evasion of its immune responses. On the contrary, immunosuppressed individuals are prone to develop severe and often life-threatening diseases.

The most frequently encountered form of human Bartollenosis is CSD manifesting with general symptoms, fever, and lymphadenopathy near the site of the bite or scratch. Hepatosplenomegaly with granulomatous hepatitis and bacteremia are less common, while local disease can manifest as encephalitis, endocarditis, osteomyelitis, and various ocular manifestations [39].

Immune Responses

Similar to other infections, phagocytes and dendritic cells are the first line of defense. *Bartonella spp.* employ several mechanisms to subvert innate immune responses. Along this line, the pathogen has a LPS with unique surface structure that avoids recognition by TLRs and specific by TLR4 on dendritic cells [40]. In addition, LPS from *B. henselae* has been shown to be significantly less active compared to LPS from *Salmonella enteritica* in inducing TLR4 activation, which is consistent with the fact that we don't observe LPS-associated septic shock in bacteremia due to *Bartonella spp*. Of interest, LPS of *B. quintana* antagonizes efficiently TLR4 activation, and this feature has been used as potential therapeutic weapon to block this pathway in a mice model of experimental arthritis [41]. Flagella, a rod-like structure with a central role in bacterial motility, acts as a TLR5 recognition site [24]. *B. bacilliformis* one of the spp. expressing flagellin, the main constituent of flagella, has been shown to be a TLR5 agonist.

The first step during Bartonella infection is inoculation of the derma by the pathogen (dermal niche). Interaction with components of extracellular matrix is another centrally conserved characteristic of *Bartonella spp. B. henselae* through Bartonella adhesin A (BadA) has been shown to bind to vitronectin, laminin, hyaluronic acid, fibronectin (both cellular

and plasma forms), and collagens I, II, and IV. Analogously, *B. quintana* binds to collagen IV through highly conserved adhesins (Vomp A and C). Along this line, intradermal infection of a Vomp null mutant of *B. quintana* in a rhesus macaque model was unable to cause bacteremia. These data suggest extracellular matrix interaction to play a decisive role during the early stages of the infection [42].

Current knowledge on pathogenetic mechanism leading to Bartonellosis is based on progress in bacterial genetics and animal and cell culture infection models. In a rat model intravenously infected with *B. tribocorum*, the pathogen is being rapidly cleared from the circulation. Five days post inoculation, the bacteria reappear in the circulation, a phase called hemotropism characterized by long-lasting intraerythrocyte bacteremia. Bartonella infections are characterized by cyclic bacteremia in their natural reservoir host. Infection is eventually cleared by specific antibody responses after a prolonged period of switching between bacteremic and abacteremic [43]. These data along with others indicate that after inoculation of the derma, *Bartonella* spp. inoculate dendritic and endothelial cells (blood-seeding niche), where they replicate, persist, and seed into the bloodstream [43]. At this stage of infection, *Bartonella spp*. apply several mechanisms to survive. Specifically, *Bartonella spp*. are utilizing a VirB/VirD4 type-IV-secretion (T4SS) to translocate a mixture of Bartonella effector proteins (BEPs) inside host cells. BepE is protecting infected DCs from injury triggered during migration from the derma site of inoculation to the blood [44]. Deletion of BepE in an in vivo experimental model was sufficient to impair cell migration and induce cell fragmentation. Other Beps (BepC, BepF, BepG, and BepA) are required for the pathogen to effectively colonize endothelial cells [45]. Subsequently, *Bartonella spp.* share the characteristic ability to invade and persistently colonize mature erythrocytes [46]. They use different factors to attach and subsequently invade erythrocytes, where they replicate for a period of time and persist inside the erythrocyte for the rest of the cell's life [46]. Erythrocytes are a compartment where Bartonella can remain protected from humoral and cellular immune responses, and this is closely related to the fact that these cells lack MHC molecules on their surface. Uptake of Bartonella by erythrocytes is actively triggered by the pathogen. Adherence and invasion of erythrocytes is being mediated by utilization of T4SS Trw expressed by bartonellae of lineage 4, while lineage 2 and 3 species most probably use flaggela for this function [47]. Of relevance, antibodies against the flagellin subunit appear to be able to inhibit binding to erythrocytes and almost entirely eliminate erythrocyte invasion [48]. Bacteria enter erythrocyte by a process called forced endocythosis. As shown in infection by *B. bacilliformis* and *B. henselae*, a hydrophilic molecule called deformin, facilitates erythrocyte invasion by induction of invaginations. Invasion-associated locus proteins A (IalA) and B (IalB) have been identified as virulence factors with putative implication in erythrocyte invasion during *B. bacilliformis* infection.

Another striking feature of Bartonellosis, especially caused by *B. bacilliformis*, *B. quintana,* and *B. henselae,* is the formation of vasoproliferative tumors particularly in the skin as a result of multiple mechanisms acting directly and indirectly on endothelial cells [43]. Such lesions are verruga peruana as manifestation of *B. bacilliformis* and bacillary angiomatosis in *B. quintana* and *B. henselae* infection. *B. henselae* can trigger vasoproliferative lesions in the liver and spleen, called bacillary peliosis (or hepatic peliosis). Immunosuppression is considered the basic prerequisite for these lesions at least in cases of *B. quintana* and *B. henselae* infection [49]. These lesions are characterized by abnormal angiogenesis resulting from pre-existing capillaries. Histologically, these lesions consist of a mixture of endothelial cells, bacteria, and infiltrates of macrophages and polymorphonuclear neutrophils, indicating a chronic inflammatory process [43]. Indeed, consistent with this proinflammatory phenotype is the activation of the transcription factor nuclear factor (NF)-κB, which is responsible for upregulation of adhesion molecules and recruitment of neutrophils.

Interaction of Bartonella with vascular endothelial cells of the infected host elicits also other signaling processes. Rho, a small GTPase that controls actin reorganization, is activated, which subsequently results in cytoskeleton rearrangement and finally in bacterial internalization. After internalization, *Bartonella henselae* forms a vacuole called Bartonella-containing vacuole (BCV), where it can avoid lysosomal fusion and acidification [43].

Subsequently, these *Bartonella spp*. can inhibit apoptosis of vascular endothelial cells via translocation of BepA and BepA2 (a T4SS), which bind to endothelial membrane receptors. Inhibition of apoptosis is related to increased cAMP levels. In this way, BepA protects endothelial cells from apoptosis triggered by cytotoxic T cells [50].

Existing data have demonstrated *Bartonella spp*. to stimulate the expression of growth factors and angiogenic cytokines in vitro leading to endothelial proliferation and formation of vasoproliferative tumors in a paracrine and/or autocrine way.

Clinical and in vitro studies have highlighted the implication of Th1 immune responses in the pathogenesis of Bartonellosis. Immunocompetent individuals with CSD demonstrate upregulation of proinflammatory cytokines, like IL-2 and IL-6 in conjunction with IL-10, known for its anti-inflammatory role. On the contrary, elevated IL-10 levels in patients with low CD4 numbers may contribute towards persistence of acute infection [51]. Increased INF-α and IL-4 levels are characteristic features of chronic infection in both animal and human studies.

Coxiella burnetii

General Aspects

Coxiella burnetii, an intracellular, Gram-negative bacterium, is the responsible agent for Q fever. Since its first description in Australia in 1937, a significant amount of work has changed our perception on *C. burnetii* and its associated infections. One major progress in *C. burnetii* research was the development of a system that permitted axenic culturing in artificial media in 2009 [52].

Based on phylogenetic analysis of its genome, *C. burnetii* is now classified in the gamma subgroup of the proteobacteria in the Legionellales order and Coxiellaceae family. The pathogen circulates in two forms, representing a diphasic development cycle: the large-cell variant (LCV) being the replicating form and the small-cell variant (SCV) a nonactive, nonreplicating form that is resistant to environmental stimuli. After entering the host, SCV changes to LCV.

Q fever is a zoonosis, and the reservoir host consists of a wide range of vertebrate and invertebrate. The main reservoirs are sheep, cattle, while other domestic mammals, birds, and reptiles have been also reported. Most frequent mode of transmission to humans is inhalation of aerosolized bacteria stemming from birth products, urine, and feces that are spread to the environment. Alternative transmission modes are ingestion of unpasteurized milk or via tick bites. Human-to-human transmission is considered a rare phenomenon [53]. *C. burnetti* has a worldwide distribution. Reported prevalence varies depending on the geographic area and on whether the disease is reportable or not in each country/region. Q fever can present either as sporadic cases in areas of high endemicity, or as outbreaks (the example of Netherlands) [53].

Clinical presentation of primary infections varies largely from asymptomatic disease in approximately 60% of patients to flu-like symptoms, pneumonia, and hepatitis [54].

Hepatic involvement in the form of hepatitis is a frequent event in acute Q fever and has general good prognosis. Cases of acute liver failure are reportedly rare and are more common in patients with pre-existing history of viral hepatitis and alcoholic liver disease. Histologically, the typical feature is granulomatous hepatitis with typical "doughnut" granulomas, characterized by a clear central space and fibrin ring, while epithelioid granulomas with eosinophilic infiltrates and acute cholangitis without granuloma have been also reported [55].

Determinants of acute Q fever infections are host factors, the most important being sex, age and also the strain involved. Regarding persistent (chronic) Q fever infection, endocarditis is the most frequent manifestations of disease. Vascular infection, usually in preexisting lesions, such as an aneurysm or vascular graft, bone/joint infections, and persistent lymphadenopathy are less frequent manifestations of chronic infection.

Immune Responses

During in vitro infection, monocytes, macrophages as well as epithelial, endothelial cells, and fibroblast get infected [56]. The avb3 integrins and the OmpA invasin mediate the process of uptake into phagocytic and nonphagocytic cells, respectively [57, 58]. After *C. burnetii* enters the phagocytic cells, the phagosome interacts and fuses with the autophagosome, leading to development of a phagolysosome-like compartment, termed the *Coxiella*-containing vacuole (CCV). CCV permits intracellular replication of the pathogen that expands into the host's cytoplasm. A Dot/Icm T4SS facilitates the release of effector proteins into the cytoplasm and enables the intracellular survival of the pathogen [59].

Chronic Q fever is characterized by deficient lymphocyte proliferation, impaired cytokine, and failure of granuloma formation [54]. Central players for persistent infection with *C. burnetti* are LPS and other virulence factors. Molecular variations in LPS determine the virulence potential of *C. burnetii*. Two phase variants of *C. burnetii* have been identified that have different LPS structure. The phase I variant is the naturally occurring variant, that is virulent, and phase II is the result of multiple passage through cell cultures. Phase I *C. burnetii* has a complete LPS with an O chain, in contrast to phase II that lacks the O chain and additional sugar residues. In fact, LPS from virulent phase I *C. burnetii* species (vLPS) induce modification of cell cytoskeleton, which results in protrusions and projections. In contrast, LPS from avirulent phase II species (avLPS) don't stimulate any morphologic changes [60]. In addition, vLPS can antagonistically engage TLR-4, and in this way, block p38a-MAPK phosphorylation that is essential for the targeting of pathogens to lysosome compartments [61]. This vLPS cytoskeleton distribution leads to reorganization and redistribution of TLR-2 and -4 at the surface of the macrophage.

Existing data in TLR4 knockout mice report a defect in cytokine production and formation of granuloma after encounter with *C. burnetii* [62]. However, in vitro cytokine production is not affected by TLR4 in human PBMCs after encounter with the pathogen [63]. In line with this, no difference between TLR4−/− mice and wild mice in their efficacy to control *C. burnetii* infection could be recognized [62].

On the contrary, TLR2-deficient macrophages were highly vulnerable to phase II *C. burnetii* pathogens and this was related to impaired IL-12 and TNF-a production, indicating the vital role of TLR2 in *C. burnetii* recognition [64]. Analogously, virulent *C. burnetii* phase I strains (Nine Mile

RSA493 (NM) and Dutch outbreak isolate *C. burnetii* [3262]) induced efficiently cytokine production via TLR2 [63].

In the same work, TLR1/TLR2 and TLR2/TLR6 heterodimers were reported to recognize *C. burnetii* 3262 strains [63]. Additionally, in humans, TLR1 and NOD polymorphisms have been also linked with deficient cytokine production after stimulation with *C. burnetii* phase I strains [63].

TLR10, known for his inhibitory effect on TLR2, was shown to suppress cytokine production by mononuclear cells after contact with *C. burnetii*. It is, though, of interest that TLR10 polymorphisms were not associated with persistent Q fever infection [65]. These data propose that deficient TLR-mediated signaling might contribute to evasion of the host immune response during early stages of *C. burnetii* infection.

Usually, acute Q fever is asymptomatic in over half of cases, indicating adequate innate and adaptive immune responses. Granulomas, a major characteristic of *C. burnetii* infection consisting of macrophages at various maturation levels, epithelioid cells and neutrophils, reflect functioning immune mechanisms of the infected host. While formation of granulomas is a feature of healthy immune responses during acute Q fever, in chronic Q fever, defective immune responses are associated with absence of granulomas. This suggests that granuloma formation has a central role in containment of Q fever, as they prevent pathogens from spreading to other sites [54].

During the initial stages of *C. burnetii* infection, encounter of macrophages with the pathogen polarizes them towards an atypical M2 program, similar to that induced by IL-4 and IL-10. In contrast, survival of the pathogens is potentiated by resting monocytes that have an M1-type program, as is seen in cases of IFN-γ stimulation [66].

In an in vitro model, granulomas were formed some days after infection, while they were disintegrated in 3 weeks, following a pattern similar to those reported in patients with acute Q fever and Coxiella-infected mice [67]. Of interest, a virulent phase I *C. burnetii* strains fail to produce granulomas, whereas virulent phase II strains do. As previously reported, this relates to differences in LPS structure and its interaction with TLR4 receptor [62]. In general, it is postulated that interaction of monocytes with various ligands or bacterial extracts from *C. burnetii* strains (as, e.g., abv3 intergrin in phase I and abv3 and a2b2 integrins in phase II strains) might lead to granuloma formation or not.

Except for macrophages and monocytes, dendritic cells are also targets of *C. burnetii*. In detail, in phase I, *C. burnetii* were shown to infect and also partially arrest maturation of dendritic cells and subsequently dampen type I IFN production, while this didn't happen in cases of phase II *C. burnetii* [68].

Adaptive immune responses play also significant roles in the control of *C. burnetii* infection, as indicated by the studies in nude and SCID mice that were highly prone to infection by the pathogen [69].

In fact, CD8+ T cells were more competent in terms of achieving control of *C. burnetii* infection compared to CD4+ T cells [70]. Polarization towards Th1 immune responses is accomplished via the production of IFN-γ from T cells and NK cells that stimulate the microbicidal activity of macrophages. IFN-γ is implicated in phagosome maturation and apoptosis promotion of infected macrophages via TNF-a production [71]. However, the role of IFN-γ in controlling Q fever has been recently disputed, as overlapping IFN-γ levels between patients with chronic Q fever and those with persistent disease were reported [72]. It has been postulated that chronic Q fever is characterized by an immunosuppressive environment, as evidenced by altered distribution of immune cells and upregulation of immunoregulatory factors, such as IL-10. Along this line, Q fever endocarditis, the archetype of chronic infection, was associated with expansion of regulatory T cells ($CD4^+CD25^+Foxp3^+$) [73]. An in vitro study of patients with Q fever, endocarditis has revealed an altered distribution of monocyte subsets. In fact, the number of classical monocytes ($CD14^+$, $CD16^-$) remained unaltered, while intermediate ($CD16^+$) and nonclassical monocytes were decreased in this population. In addition, intermediate monocytes and CD4+ T cells and Treg cells were characterized by overexpression of PD-1 that was linked to overproduction of IL-10 [74].

The role of antibodies has been considered dispensable in *C. burnetii* infection. Contrary to early studies, demonstrating a protective role of passively transferred antibodies to guinea pigs from subsequent challenge with the pathogen, subsequent work has shown antibodies to play a regulatory role, even if the pathogen's clearance is not affected [69].

Antibodies to *C. burnetti* were shown to play a role in tissue damage via formation of immune complexes and also in development of autoimmunity, as formation of autoantibodies, including anticardiolipin antibodies, has been reported during acute Q fever.

Leptospira

General Aspects

Leptospirosis in humans is caused by the Gram-negative spirochete *Leptospira interrogans*. Leptospirosis is a zoonosis, where the pathogen is transmitted via direct or indirect exposure to wild and domestic animals, representing the infected reservoir host. The most important reservoir host for human leptospirosis is the brown rat (*Rattus norvegicus*). The reservoir host carries the pathogen in its renal tubules. Transmission occurs via bacteria excreted in the urine of infected host that contaminate soil or water. High-risk

individuals are those with occupations at risk, including direct contact with animals (farm workers, veterinarians), hunters, and in cases of recreational exposures [75]. Human infection has a worldwide distribution with at least one million cases diagnosed every year. Outbreaks have been reported in areas of poor sanitary measures and during periods of heavy rain and flooding.

Leptospira enters the body through the skin or mucous membranes and spreads into the circulation. Except for kidneys and the lungs, the liver is one of the major target organs. Pathology specimens from fatal cases of Weil's disease have shown congested liver sinusoids and distention of the space of Disse. Disruption of the liver cord, enlargement of Kupffer cells, and bile stasis in biliary canaliculi are some of the basic histopathological characteristics in liver involvement in cases of leptospirosis [76]. Apoptotic features in hepatocytes are another finding. A study in a hamster model of Weil's disease demonstrated Leptospiras to infiltrate the space of Disse, move between the hepatocytes and detach the intercellular junctions. This resulted in destruction of bile canaliculi, which coincided with elevation of bilirubin.

In vivo studies have demonstrated that leptospires could avoid uptake by the liver reticuloendothelial system and reach the biliary canaliculi after penetrating the endothelial lining of the liver sinosoids [77]. The clinical spectrum of leptospirosis in humans varies from asymptomatic or mild disease (80–90% of infections) to Weil's disease presenting with jaundice, renal failure, and hemorrhage or pulmonary hemorrhage syndrome [75].

Immune Responses

Leptospira is one of many organisms to use multiple strategies to circumvent or inactivate all pathways of the complement cascade. One of these strategies is acquisition of host complement regulators, including Factor F (FH) that acts as a regulator of the alternative pathway, C4b-binding protein (C4BP) which is a regulator of the classical and lectin pathway and vitronectin (Vn) that is terminal pathway regulator. Additionally, Leptospira acquires host proteases that cleave complement proteins on the bacterial surface, while it also secrets proteases that inactivate complement in the pathogens surroundings [78]. Additional data show that leptospiral LPS that has atypical features can escape recognition by human TLR4 receptor, contrary to what has been observed in murine models, and in this way has a crucial role in the outcome of human infection in humans [79]. Though leptospiral LPS is recognized by TLR2 human cells [80]. Additionally, in a murine model, Leptospirosis avoids sensing of its peptidoglycan through the NOD proteins and this way protects from degradation to muropeptides. Responsible for this protective effect is a conserved outer membrane lipoprotein, named LipL21 that is tightly bound to the PG [81]. It is of interest that this protective role of LipL21 is specific to *Leptospira* spp., since no homology with this lipoprotein from other species has been identified up to the present [81].

Listeria

General Aspects

Listeria monocytogenes (Lm) is a Gram-positive intracellular pathogen known to cause listeriosis in humans. Until now, 17 Listeria species have been identified, though only two, *Listeria monocytogenes* and *Listeria ivanovii*are, are pathogenic for humans and ruminants, respectively.

Epidemiological data suggest the incidence of listeriosis to have declined in most industrialized countries in the past years, though outbreaks have been reported in North America and several European countries [82]. Lm is transmitted by ingestion of contaminated food, such as fairy and meat products, while transmission via coleslaw and vegetables has been also reported.

A wide range of symptoms has been recognized, ranging from a self-limiting form of the disease affecting immunocompetent population and presenting as febrile gastroenteritis, to invasive forms, such as meningoencephalitis, blood stream infection, or maternofetal listeriosis with an average case-fatality rate of 20–30%. Identified risk factors for invasive listeriosis are immunosuppressive diseases and treatments, immunocompromised conditions, including kidney, autoimmune diseases, alcoholism, and diabetes, as well as older age. Listeriosis can also present as focal infection, involving the endocardium, joints, peritoneum, and the eyes.

Immune Responses

After entering the susceptible host, Lm faces a number of natural barriers. Its unique ability to cross several natural barriers of the susceptible host and its intracellular lifecycle has showcased Lm as a model to study host-pathogen interactions [83].

Indeed, expressing various bacterial effectors, Lm is characterized by its potency to invade and replicate in phagocytic and nonphagocytic cells (enterocytes, hepatocytes, fibroblasts, and endothelial cells), by disrupting cell receptors of the host, modulate cellular and organelle functions, and influence gene expression and DNA stability [84].

Lm enters nonphagocytic cells via different cell receptors, using the receptor-mediated endocytosis pathway. In detail, Internalin (Inl) A and IntB are considered major invesins, acting by binding to membrane receptors of eukaryotic cells and specific to E-cadherin and Met (the receptor of the

hepatocyte growth factor), respectively [84]. Recently, it was suggested that the high affinity immunoglobulin gamma Fc receptor (FCGR1A) can serve as an additional receptor for the entry of Lm in fibroblasts and monocytes [85].

After uptake of host cells, Lm is restricted inside a vacuole or a phagosome. Subsequently, internalized bacteria are released into the cytosol, after disruption of the phagosomal membrane by two phospholipases (PI-PLC and PC-PLC) and a secreted pore-forming toxin, called listeriolysin O (LLO). Following multiplication, they infect other cells by cell-to-cell spreading. Of interest, a recent study suggested LLO to act in a cell type-dependent fashion, as shown by internalization of Lm into hepatocytes, though not into cytotrophoblasts and endothelial cells [86]. Replication of Lm inside the cytosol is driven by the actin assembly-protein ActA, a surface-anchored virulence protein that induces host-cell actin polymerization and facilitates direct cell-to-cell spreading [85]. Moreover, ActA exerts a crucial role in escaping antibacterial autophagy [87]. In addition, to those mentioned above, Lm utilizes several other surface and secreted molecules that either modulate adhesion and entry into cells, act as adhesins or activate different pathways resulting in actin assembly and remodeling, bacterial engulfment, and subsequently infection of neighboring cells.

Also, additive evidence has shown Lm to escape from autophagy, which is one of the defense mechanisms against external pathogens and a regulator of host immune responses to microbial and autoantigenic targets [88].

Since its first description in 1926, a significant amount of work has established Lm as a model pathogen in elucidating immune response mechanisms against intracellular pathogens. Seminal studies dating back to early 1960s have hinted on the important role of innate and adaptive immune responses in clearing Lm [89]. Early studies have highlighted the pivotal role of innate immune responses in detecting and containing Lm, while adaptive immune responses are necessary for the clearance of the pathogen. Still, only recent data have shed light into events taking place early during the time Lm is entering the liver, where the pathogen replicates actively. Studies on immune responses to Lm were mainly performed in mice, where infection via the oral route is ineffective as attested by poor interaction between InlA and E-cadherin on murine IECs. In this model, investigation of Lm infection is achieved intravenously (i.v.) and focuses on the liver and spleen [90].

In general, immune responses are elicited after binding of pathogen-associated molecular patterns to their respective pattern recognition receptors (PPRs), including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (retinoic acid-inducible gene 1), and C-type lectin receptors, which are differentially expressed on the cell surface membrane or in the cytosol [90]. This binding elicits signaling cascades resulting in secretion of cytokines and chemokines that direct the ensuing immune responses.

Regarding Lm recognition by IECs, accumulating evidence suggest NOD2 to play a crucial role, as NOD2−/− mice failed to mount adaptive responses to the pathogen [91]. Accordingly, activation of TLR2 and TLR10 has been shown to enhance the phagocytic ability of macrophages and upregulate TNF-a, IL-12, and NO production in vitro and in vivo. A recent study elucidated the role of TLR2 in Lm infection further by demonstrating that monocytes/macrophages recruited by hepatocytes via TLR2-dependent secretion of CCL2, which is considered the prototype monocyte attracting CC chemokine. Results from this study highlighted also the contribution of TLR2 on macrophage motility, though polymerization of F-actin, and subsequently on liver microabscess formation during the initial stages of Lm infection [92].

Retinoic acid-induced gene 1 (RIG-1) has been shown to recognize small RNAs secreted by Lm during active infection and trigger type I IFN production by IECs. Still, existing data hint that the ability of RIG-1 may by cell type specific.

Cells participating in early eradication of Lm are neutrophils, macrophages, natural killer (NK) cells, and dendritic cells (DCs). In mice models, neutrophils are among the first to migrate to the site of inflammation, that is liver and spleen, as result of chemokines secreted by hepatocytes.

In fact, in mice infected i.v. with sub-lethal doses of Lm, 90% of the inoculum is detected 10 min after injection in the liver. During the first 6 h, the number of Lm decreases significantly in the liver as a result of the pathogens destruction by neutrophils. During the following 2–3 days, existing Lm multiply in the liver and spleen in an exponential manner before being eradicated as a result of immune responses [90].

Neutrophils kill intracellular bacteria effectively during the first 1–2 days by producing reactive nitrogen and oxygen species, while they also contribute to immune response by secreting inflammatory cytokines, including IFN-γ [93]. The pivotal role of neutrophils in encountering Lm infection is supported by studies showing neutrophil-deficient mice or mice lacking G-CSF or its receptor to be susceptible to severe Lm infection compared to wild type mice.

Extensive work performed in i.v. models of Lm infection has highlighted also the importance of macrophages as primary defense mechanism. After i.v. Lm infection, the pathogen is being taken up by marginal zone macrophages of the spleen's red pulp. In the absence of these macrophages, the pathogen is able to replicate and disseminate systematically [94]. Subsequently, Lm is translocated to the T cell zone that is essential for the initiation of antigen presentation to CD8 T cells.

In the liver, Kupffer cells have a pivotal role in restricting early replication in the respective organ. Specifically, Kupffer

cells secrete TNF-a and IL-12 upon encounter with Lm and subsequently induce NK activation. In addition, NK cells are activated by infected DCs in an IL-18 dependent mode and are a major source of IFN-γ production during early stages of listeriosis [95]. Additionally, in listeriosis, NK cells exert an important regulatory function by producing IL-10 as IFN-γ production wanes. Increased IL-10 production has been shown to be associated with suppression of activation and accumulation of inflammatory myeloid cells, rendering the host more susceptible to Lm infection [96].

Moreover, IFN-γ aids macrophages to obtain bactericidal activity and induces maturation of a subset of DCs, called Tip-DCs, that produce TNF-a and inducible nitric oxide synthase. DCs are key players in linking innate and adaptive immune responses and have the ability to circulate to areas where T cells are resident, that is, the white pulp of the spleen or the T cell zone of lymph nodes. Specifically, the CD8a(+) DC subpopulation has been found to be the required cell type for robust Listeria infection, as they are required for the pathogens efficient entry into the spleen [97].

Adaptive immunity to Lm is the main determinant of listeriosis outcome, as higher mortality in immunocompromised people has been associated with deficits in this part of immune response mechanisms [98]. Adaptive immunity is T-cell mediated, develop rapidly, and confer sterilizing immunity [99].

In contrast to other pathogens where priming of DCs by CD4+ T cells is required, in Lm infection, DCs can be directly activated by bacterial microbial associated molecular patterns to subsequently prime CD8+ T cells. Secretion of Lm proteins in the cytosol allows bacterial antigens to be processed and presented to CD8+ T cells via the endogenous MHC class I pathway. Along this line, the MHC class I-restricted CD8+ T cells epitope recognizing a listeriolysin O epitope (LLO91-99) is considered the most immunodominant [100]. An additional epitope in p60 protein (p60217-255) has been also identified [101]. In BALB/c mice, CD8+ T cells specific for these epitopes were shown to confer significant protection after Lm infection. Moreover, CD8+ T cell responses can be also primed by nonsecreted Lm antigens.

Memory CD8 T cells with distinct phenotypic markers and functions have been identified in various organs infected by Lm (spleen, liver, intestine). This probably suggests adaptation of functional properties of CD8+ T cells according to the environment so that maximum contribution to protective immunity is achieved.

During CD8+ T cell proliferation, expression of CD11a, PD-1, and CD69 becomes upregulated. Based on CD11a expression in the BALB/c spleen, 30% of total T cells were specific for Lm 7 days post i.v. infection [102]. Indeed, CD8 T cell responses peak 7–8 days after i.v. Lm infection, while this is identified later (8–9 days post infection) during oral Lm infection. The pivotal role in CD8+ T cell effector function has been emphasized by data showing CD11a-deficient mice to exert reduced primary CD8+ T cell responses, while older mice demonstrated impaired T cell expansion.

Both CD8+ and CD4+ T cells exert several functions, the most important being cytotoxicity (especially for CD8+ T cells), secretion of Th1 (IFN-γ and TNF-a), and IL-17A type cytokines, while some CD4+ T cells act as regulatory T cells (high expression of Foxp3) by inhibiting T cell function.

In the early phase of Lm infection, antigen-specific CD8 T cells acquire cytotoxic effector function, which declines steadily while the total CD8 T cells are still expanding. This could possible reflect a protective mechanism against excessive tissue damage in various infected organs [103]. IL-17A acts as a chemoattractant of neutrophils within the liver and is mainly produced by memory γδ T cells. IL-17A is considered essential for orchestrating innate immune responses against Lm and contributes in clearing the pathogen in both the liver and intestine [104].

Taking advantage of robust immunostimulatory responses, elicited during listeriosis, has led researchers to study Lm as vaccine vector for cancer immunotherapy with promising results, while Lm-based vaccines could be developed against difficult-to-immunize pathogens.

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Immunity of Parasitic Infections of the Liver

13

Shyamapada Mandal, Eirini I. Rigopoulou, Manisha Mandal, and Dimitrios P. Bogdanos

Abbreviations

IL1R	IL-1 receptor
IRBC	Infected red blood cells
PAMP	Pathogen-associated molecular pattern
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PRR	Pathogen-recognition receptor
PSG	Promastigote secretory gel
TLR	Toll-like receptor
VL	Visceral leishmaniasis

Key Points

- Parasites that can infect the liver and biliary tract are classified into protozoans and helminths.
- A complex host–parasite interplay, involving key pathogen and self-molecules, determine the clinical pattern and the outcome of parasite's infection.
- Impaired innate immune responses and compromised activation of neutrophils, macrophages, NK, and NKT cells play an important role in driving parasite's invasion and multiplication.
- An imbalance of Th1/Th2 and Treg/Th17 immunity has been considered important not only for the establishment of the infectious disease but also for its progression.

Introduction

Parasitic infections are prevalent mainly in developing countries and constitute a major public health problem [1]. Parasites form a heterogeneous group of pathogens, from intracellular protozoa to visible helminths. In developed countries, protozoa cause more often gastrointestinal infections compared to helminths.

Parasites that can infect the liver and biliary tract are classified into protozoans and helminths, including nematodes (roundworms), trematodes (flatworms or flukes), and cestodes (tapeworms) (Table 13.1).

The spectrum of histological lesions related to liver and bile duct parasitic infections is wide and involves mainly hepatocellular manifestations, reticuloendothelial disease, and biliary disease. Granulomatous hepatitis is the most commonly encountered finding reported in cases of schistosomiasis, toxocariasis, fascioliasis, strongyloidiasis, and hepatic capillariasis. Hepatic microabscesses or necrosis is characteristic of amoebic liver disease, while echinococcosis and amoebic liver disease present as cystic lesions of the liver. Portal fibrosis is a specific feature of schistosomiasis.

The protozoa *Toxoplasma gondii* (*T. gondii*) and *Entamoeba histolytica* (*E. histolytica*) are the causative agents for toxoplasmosis and amebiasis, respectively, and are contracted from contaminated food and/or water. *Leishmania donovani* (*L. donovani*) and *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. knowlesi*) being the causative agents of visceral leishmania-

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Table 13.1 Major pathophysiological features, clinical manifestations, and diagnostic tests of parasites

Disease (agent)	Pathophysiology	Manifestations	Diagnosis
Protozoans			
Amebiasis (*Entamoeba histolytica*)	Hematogenous spread Abscess formation	Fever, right upper quadrant pain	Cysts in stool Serology (anti-lektin antibodies by ELISA) Liver imaging
Malaria (*Plasmodium falciparum, P. vivax, P. ovale, P. malariae*)	Replication and maturation in the liver	Hepatomegaly, splenomegaly Seldom acute liver failure (*P. falciparum*)	Blood smear Rapid test PCR
Leischmaniasis (*Leischmania donovani*)	Infection of reticuloendothelial cells	Fever, hepatomegaly, splenomegaly, hyperpigmentation	Amastigote in tissue specimens or cultures Serology (direct agglutination test, ELISA, IIFL, WB, PCR
Toxoplasmosis (*Toxoplasma gondii*)	Inflammation/necrosis of liver (replication)	Fever, hepatomegaly, splenomegaly, lymphadenopathy	Serology
Helminths			
Nematodes			
Ascariasis (*Ascaris lumbricoides*)	Larval migration to the liver and bile ducts	Fever, jaundice, abdominal pain	Eggs in stool
Toxocariasis (*Toxocara canis, T. cati*)	Larval migration to the liver	Hepatomegaly, granuloma formation, hepatic abscess	ELISA (*T. canis* excretory or secretory (TES) antigens), liver imaging
Hepatic capillariasis (*Capillaria hepatica*)	Larval migration to the liver	Hepatomegaly, splenomegaly, hepatitis, eosinophilia	Larvae (tissue biopsies) Serological tests (ELISA, IIF)
Trichinosis (*Trichinella spiralis*)	Hematogenous spread to the liver	Occasionally jaundice	Eggs or adult worms in liver biopsy, serological tests (excretory/secrteroy antigens)
Trematodes			
Schistosomiasis *(Schistosoma mansoni, S. japonicum*)	Host immune response to eggs causing fibrosis	Acute: fever, headache Chronic: hepatosplenomegaly, portal hypertension (pre-sinusoidal)	Eggs in the stool, urine, tissue biopsies, serological tests (acute cases), PCR
Fascioliasis (*Fasciola hepatica*)	Larval migration to the liver	Acute: fever, hepatomegaly, occasionally jaundice	Eggs in stool, adult worms in endoscopic/surgical specimens
Cestodes			
Echinococcosis (*Echinococcus granulosus, E. moltilocularis*)	Larval migration to the liver, encystment	Hepatomegaly, fever, cyst rupture	Serological tests, liver imaging

sis (VL) and malaria, respectively, are caused by vector-borne parasites (Fig. 13.1).

Among nematode infections affecting the liver, toxocariasis is the sequel of zoonotic spread of the round worms, *Toxocara canis* (*T. canis*) of dogs and *Toxocara cati* (*T. cati*) of cats.

Ascaris lumbricoides (*A. lumbricoides*) infection is attributed to obstruction of the intestine and bile ducts by ingested embryonated *A. lumbricoides* eggs. Echinococcosis is the most serious parasitic disease caused by a larval cestode (*phylum Platyhelminthes*) affecting the liver.

Several trematode species can infect humans. Liver disease is observed in cases of clonorchiasis and opisthorchiasis (family Opisthorchiidae: *Clonorchis sinensis, Opisthorchis viverrini,* and *Opisthorchis felineus*) that share same pathophysiology and disease manifestations.

This chapter highlights key clinical and immunological aspects involving the human liver during parasitic infections, giving an emphasis to two of the major protozoan diseases, malaria and VL, and also schistosomiasis, which is considered a model of helminthic infection.

Malaria (Asymptomatic Liver Stage)

Malaria is one of the most significant causes of morbidity worldwide, accounting for almost one million deaths yearly [2–6].

In humans, malaria is caused by five species of intracellular protozoa: *P. falciparum, P. vivax, P. ovale, P. malariae,* and the recently identified *P. knowlesi*, normally infecting apes, which are transmitted by mosquito bites [7] . After entering the host, the malaria sporozoites migrate via the circulation to the liver that represents the initial replication site of the parasite (hepatic schizogony) (Fig. 13.2). The liver stage is critical in the life cycle of the parasite. In fact, the

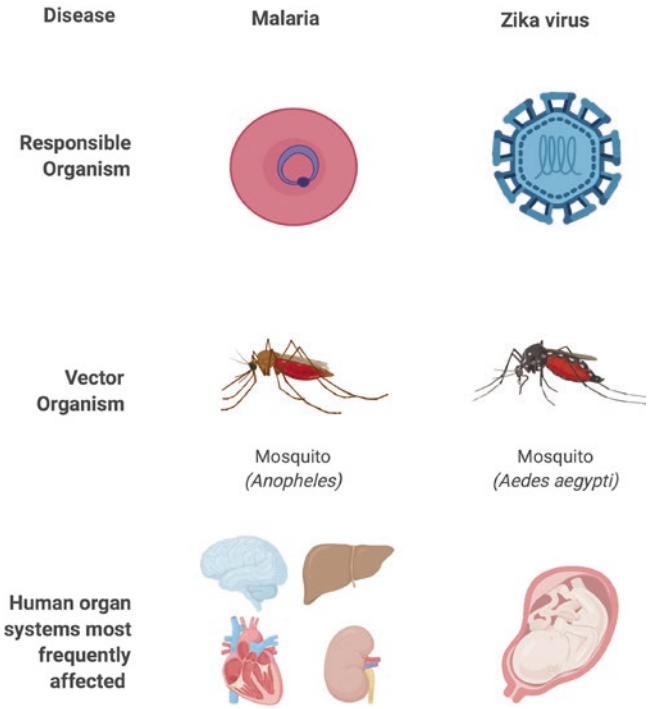

Fig. 13.1 Examples of zoonotic diseases transferred via mosquitos. (Created with Biorender (under license))

liver has a unique role in the life cycle of *Plasmodium spp.,* as it is the only organ required for their maturation [8].

Accumulating data suggest the path of the sporozoites to the hepatocytes is complex [9–12]. The hepatocytes are infected after the sporozoites are crossing the sinusoidal wall using various proteins (Fig. 13.3). Endothelial and Kupffer cells are utilized by sporozoites to traverse the sinusoidal cell wall, though other cell transversal-independent pathways have been suggested for hepatocyte crossing [13]. Of interest, a sporozoite model liver infection in rodents has revealed cell traversal to exert a principal role in inhibition of sporozoite clearance by Kupffer cells [13–15].

It has been suggested that after entering the Kupffer cells, sporozoites form a nonfusogenic vacuole that allows them to pass and exit them undamaged towards the space of Disse. Then they migrate to several hepatocytes causing necrosis and settle into a last hepatocyte (the hepatocyte invasion phase) that results in hepatic schizogony, which involves asexual replication of parasites and production of thousands of merozoites infecting the erythrocytes [15]. After erythrocyte invasion, merozoites transform to trophozoite that grow and lead subsequently to multiple asexual replications and production of more merozoites. After rapture of red cells, merozoites are released and infect new red cells. In cases of *P. vivax* and *P. ovale*, liver cells function as a reservoir for hypnozoites, a dormant stage responsible for relapses occurring weeks to years following the initial infection.

Symptoms of malaria infection range from asymptomatic to uncomplicated disease to severe malaria with increased mortality. The asexual blood stages are considered responsible for malaria symptoms, while the liver stage is often stated as silent, asymptomatic phase.

Hepatic involvement during malaria has been long recognized. Still, lack of consensus in definition of hepatopathy during malaria hinders the accurate estimation of its incidence. Liver involvement in malaria is considered a multifactorial process. One of the mechanisms leading to liver damage is the phagocytic uptake of hemozoin and fragments of infected erythrocytes by liver cells, which results into resident macrophage activation and iron storage problems. Main histology features are cholestasis, bile stasis and granulomatous lesions, and focal hepatocyte necrosis [16]. In jaundiced patients, congestion of hepatocytes, inflammatory infiltrates, centrizonal necrosis, and cholestasis, as well as hyperplastic Kupffer cells and iron deposits have been reported.

Microscopy for the examination of a stained thick and thin blood smear for malaria detection and species identification, respectively, is the standard method for malaria diagnosis. The rapid diagnostic tests are recommended for malaria diagnosis. In addition, the infection can be diagnosed either by the detection of antibodies to malaria parasites and by PCR-based detection of parasite DNA.

Immune Responses to Malaria Parasites

P. falciparum and *P. vivax* are the most common causes of malaria. The malaria parasites enter the skin as sporozoites, circulate in blood, crossing the liver sinusoidal endothelium to home the liver, residing in hepatocytes, and they parasitize the erythrocytes, termed infected red blood cells (IRBCs) [7]. In the case of *P. falciparum*, IRBCs bind to certain cell surface proteins of vascular endothelia. The binding of IRBCs to the endothelial cell surface proteins in the microvasculature of vital organs and chondroitin 4-sulfate in the placenta is mediated by a family of antigenically variant parasite proteins collectively called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [17]. PfEMP1 confers virulence to *P. falciparum* through IRBC binding to endothelial cells and enhance local inflammatory processes and immune cell infiltration, endothelial damage, tissue damage, and organ failure. *P. vivax* is less virulent because it lacks PfEMP1 ortholog [17].

Immune responses defend the host against infectious agents by detecting specific signature structures of pathogens, the so-called pathogen-associated molecular patterns (PAMPs), which include microbial DNA and RNA, bacterial LPS, peptidoglycan, and fungal glucans [18]. Host identifies PAMPs through receptors called pathogen-recognition receptors (PRRs). Noticeable among transmembrane PRRs

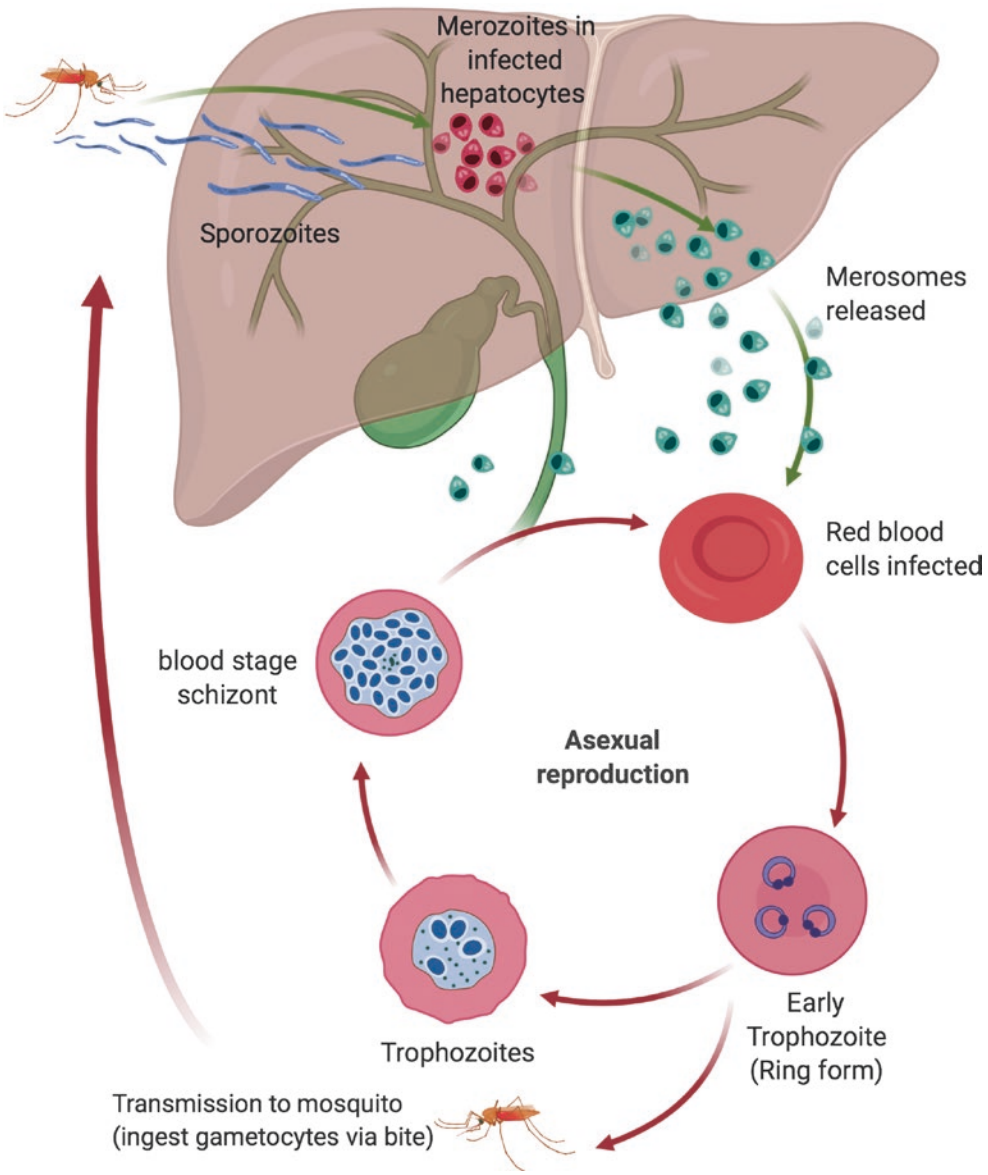

Fig. 13.2 Plasmodium's blood stage asexual preproduction cycle in human host. Plasmodium is entering the human host by the bite of an infected mosquito and releases sporozoites contained in the salivary fluid directly into the bloodstream or the skin tissue, whereby the sporozoites can infiltrate blood vessels. In the liver stage, sporozoites access through Kupffer cells the space of Disse and penetrate the hepatocytes. Within the infected hepatocytes, the sporozoites mature to schizont and when they rupture release merosomes, which in turn are trafficking to the blood circulation. The merozoites released invade unaffected red blood cell cells, which become infected initiating the blood stage of the parasite. (Created with Biorender (under license))

are the toll-like receptors (TLRs). The innate immune system is furnished with a variety of PRRs. Host also senses certain endogenous factors freed during infection named danger-associated molecular patterns (DAMPs), such as the high mobility box 1 (HMGB1), HSP70, and the SP100 family of proteins [19]. Following recognition of PAMPs and DAMPs by PRRs, innate immune cells are activated and release various cytokines [20]. Because the liver stage of malaria infection is clinically silent, contrasting that of the blood stage, which is characterized by remarkable symptomatology, it has long been thought that parasites inside hepatocytes mature totally ignored by the host's immune system. However, recent studies have shown that the developing parasites are recognized by cytosolic PRRs of hepatocytes, initiating type I IFN responses [21].

It has also been shown that the parasites in infected hepatocytes are recognized by the interaction of parasite RNA with a RIG-I family of proteins homolog called melanoma differentiation-associated protein 5 [22]. This leads to a series of innate immune responses, which include the production of IFN-γ and chemokines by NK and NKT cells [23]; penetration of resident NK and NKT cells to the liver and CD1d-restricted removal of the infected hepatocytes by NKT cells. It also includes more generic innate immune phenomena, such as the expression of interferon-related genes by hepatocytes, the induction of hepatocyte-related chemokines, and chemotaxis-mediated recruitment of resident neutrophils and lymphocytes macrophages, to the surrounding infected hepatocytes [24].

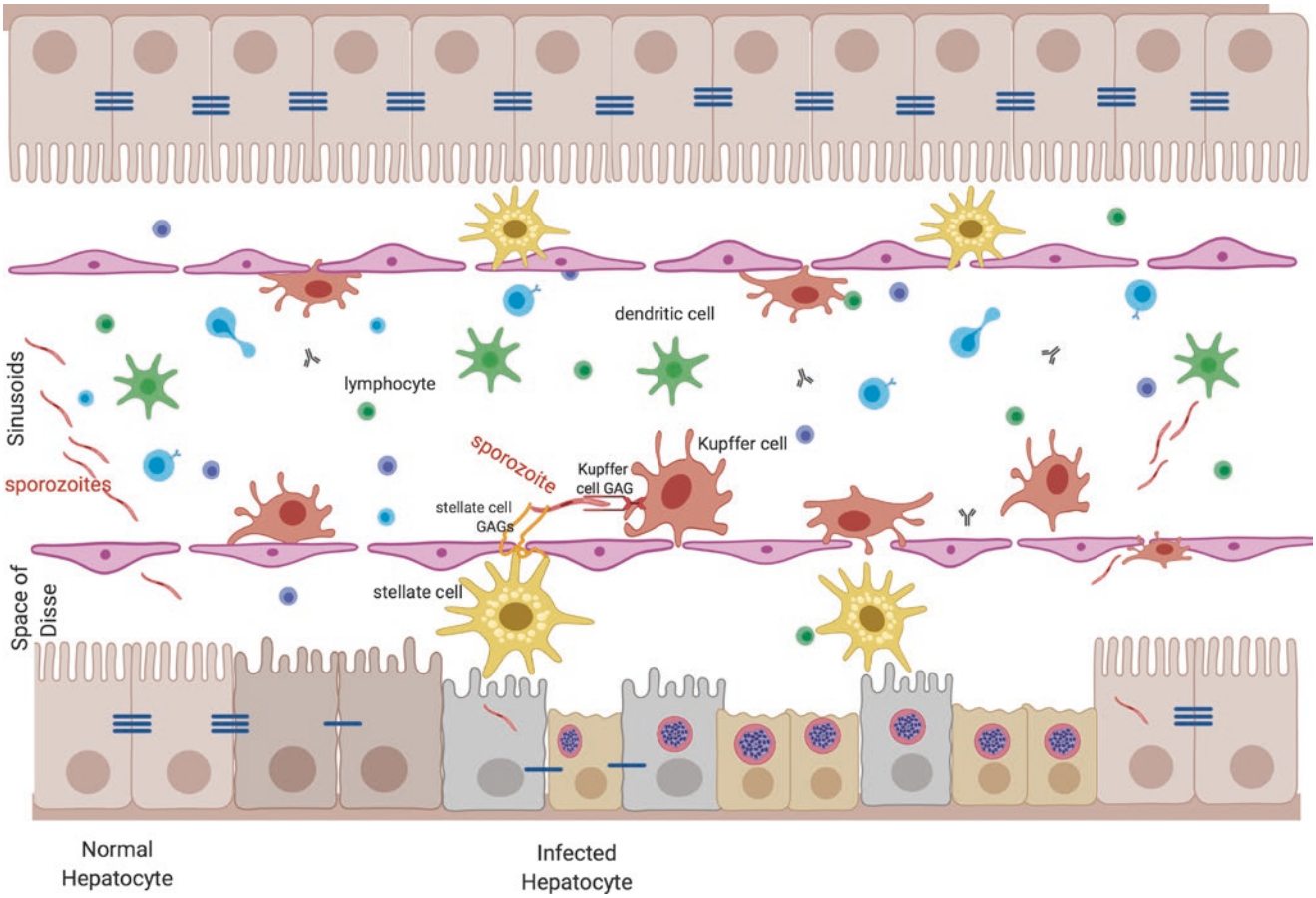

Fig. 13.3 A close interplay between the liver microenvironment and its cell subsets and the parasite is responsible for the infection of hepatocytes. Sporozoites can make Kupffer cells unresponsive to inflammatory stimuli, supporting their apoptosis, which promotes hepatocytes infection [11]. (Created with Biorender (under license))

Parasites exist inside a parasitophorous vacuole, and their RNA is exported to the cytosol but not to phagolysosomes. It appears that cytosolic sensors are the only PRRs that interact with parasite factors in infected hepatocytes. DNA appears to be an apparent PAMP at the blood stage of the parasite infection, but its role at the liver stage is not clear.

Innate Immune Responses

Thus, during the liver stage, malaria-infected hepatocytes produce type I IFNs through cytosolic sensing of RNA, leading to killing of parasite-infected hepatocytes by NKT cells. However, because in natural infections the parasite load in the liver is very low, the innate immune responses are likely to be diminished. During the blood stage infection, efficient induction of innate immunity is achieved because parasites grow dramatically, through recurring erythrocytic cycles. Both DCs and resident macrophages are critical for the initiation of innate immune system. However, macrophages appear crucial during the blood stage infection. These macrophages become dysfunctional upon internalization of infected erythrocytes, merozoites, or hemozoin [25].

On the other hand, efficient induction of type I IFNs in response to malaria parasites is achieved by DCs, which also regulate adaptive immunity targeting parasites. Cumulative data suggest that type I IFNs promote IFN-γ-dependent anti-parasitic immunity, delivering resistance against severe infection. Overall, the available data indicate that type I IFNs acquires contrasting functions during malaria infection subject to the timing and amount of production and the relative compositions of lymphocyte cell subsets [21]. DCs produce type I IFNs and various other pro-inflammatory cytokines, including IL-12, IL-6, and TNF-α. Among chemokines, CXCL1, CXCL2, CCL2, CCL5, CXCL9, and CXCL10 are produced by DCs upon encounter with malaria parasites, promoting the recruitment of various immune cells to mount efficient cell-mediated anti-parasitic properties [26]. Type I IFNs prime DCs and activate proinflammatory NK, NKT, γδT, and T cells to induce IFN-γ and other inflammatory responses. IL-12 produced by DCs stimulates IFN-γ induction of NK cells, provoking Th1 responses and effector T cell

responses [27]. The accelerated induction of IFN-γ and other cytokines permits structured parasitemia control and promotes neutrophil activation, which exert efficient phagocytic activity in an attempt to achieve clearance of the parasitic load [28]. In *P.falciparum* infection, strong pro-inflammatory responses contribute to an effective control of parasitemia [29]. However, such immune responses ultimately lead to tissue destruction, organ damage, and severe illnesses.

Adaptive Immune Responses

Work on infected mice with radiation-attenuated *P. falciparum* sporozoites has demonstrated the involvement of protective innate (NK cells) and adaptive (CD8+ T CD4+ T cells) immune responses and participation of IL-12 and IFN-γ cytokines [30]. Genetically attenuated parasites (GAPs) can generate concrete immune defense in animals and humans [31, 32]. Efficient CD8+ T cells kill parasitized hepatocytes in vitro and CD4+ and CD8+ T cells secrete large amounts of IFN-γ [33]. In vivo studies have shown that CD8+ cytotoxic T lymphocytes killing of the malaria-infected cells are the prevailing mechanism of host defense rather than cytokine-mediated control. Of relevance, depletion of CD4+ T cells at the time of challenge does not alter anti-malaria immunity during the liver stage [34]. However, CD4+ T cells are crucial for expansion and survival of protective CD8+ T cells [35, 36].

Schistosoma Infection

Schistosoma infection, known also as bilharzia, infects more than 250 million people worldwide [37, 38].

Schistosomiasis in humans is caused by three main species of the trematode parasites of the genus *Schistosoma* (*S. mansoni*, *S. japonicum,* and *S. haematobium*), while three more species have been reported to have a local distribution.

In terms of geographic distribution, *S. mansoni* has been mainly reported in sub-Saharan Africa, parts of South America, and the Caribbean, *S. haematobium* in Africa and parts of the Middle East and *S. japonicum* is found in Asia (China and Philippines).

Scistosomiasis is considered a human parasitic infection where various animals (dogs, cats, rodents) serve as reservoirs and snails as intermediate hosts [38].

Schistosomas enter the human host via the skin in the form of cercariae and become schistosomulae and via the venous circulation migrate towards the lungs, the heart, and subsequently to the liver. Inside the liver, parasites progress into mature life stages. It is suggested that the liver's vasculature provides the suitable environment for the parasite to achieve maturity. Female and male parasites pair inside the liver before migrating into the portal vein and mesenteric veins. Females produce eggs that are either excreted in stool or move via the hepatic vessels to the liver. This applies to all human *Schistosoma* species except for *S. haematobium* that is lodged inside the bladder and urogenital system [39].

Accumulating evidence over the years has shown signs and symptoms of schistosomiasis to result from the host's immune responses against the parasite's eggs in various tissues. Acute schistosomiasis (also known as Katayama syndrome), usually occurs in older than usual people who travel to endemic areas and are exposed for the first time to schistosome antigens. The typical features are fever of abrupt onset, headache, myalgia, abdominal pain, and bloody diarrhoea presenting 4–8 weeks after the infection (*S japonicum, S mansoni*). One of the key features of chronic schistosomiasis is the development of granulomas and fibrosis leading to presinusoidal block of portal blood flow – also called Symmer's pipe-stem fibrosis – that finally leads to presinusoidal portal hypertension. As a result of the portal system congestion, splenomegaly and development of oesophageal and gastric varices ensues. Contrary to patients with cirrhosis, these individuals maintain normal synthetic liver function and seldom develop ascites.

The diagnosis of shistosomiasis relies on epidemiologic data, symptoms, presence of eosinophilia, and detection of living eggs in stool (*S japonicum, S mansoni*), urine (*S haematobium),* or tissue biopsies. Serological tests for the detection of antibodies to schistosomal antigens are valuable in cases of acute infection. PCR-based techniques are capable of detecting DNA released from *S. mansoni*, *S. haematobium,* and *S. japonicum*.

Immune Responses to Schistosoma Infection

Th1 and Th2 Cells

It is generally believed that hepatic fibrosis is primarily instigated by hepatic inflammation triggering activation of hepatic stellate cells (HSCs). These cells can transdifferentiate into collagen-producing myofibroblasts, and this also appears to be the case for liver fibrosis caused by *Schistosoma* infections [40–42]. An imbalance of Th1/Th2 and more recently Treg/Th17 immune responses has been considered important not only for the establishment of schistosomiasis but also for the development and staging of liver fibrosis during infection [43]. Most research has been performed in animals but work in humans appears to share several common denominators with the murine disease.

The current view, regarding the role of adaptive immune responses in schistosomiasis, is that Th1 responses are mainly induced and prevail during the early phase by larval

worms, succeeded by Th2 responses, which are developed by deposited eggs in the tissue, both during *S. mansoni* and *S. japonicum* infection, further indicating that the role of such responses is of paramount importance for the parasite's establishment [44].

Regulatory T Cells

High % Tregs levels have been found during *S. mansoni* infection and elevated Tregs in the periphery have been associated with the severity of hepatic fibrosis in *S. japonicum*-infected patients [45, 46]. It is not clear, though, whether this is a circumstantial or causative association.

Th17 Cells

Recent work has shown that Th17 responses are important for the infectivity of schistosome infections. Th17 downregulation inhibits the progression of schistosomiasis fibrosis [47]. These data indicate that Th17 cells may actively participate in the early anti-infection immunity and late immunopathogenesis for granuloma formation and fibrosis [36, 48, 49].

Cytokines and Cytokine Receptors

Th2 overproduction (mainly IL-4, IL-5, and IL-13) exceeds that of Th1 cytokines (IFN-γ, IL-6) and (regulatory) IL-10 in hepatic fibrosis related to human *Schistosoma mansoni* infection [50]. IFN-γ decline is associated with deterioration of liver fibrosis during human schistosomiasis and vice versa [51]. Also, genetic studies have revealed several risk associations with SNPs within the IFN family, such as the IFNGR1 gene translating into reduced gene transcription of IFN-γ, which diminishes its functionality and strongly downregulates cytokine-induced anti-fibrotic effect [52–55]. The exact mechanism, by which Th1 and NK cells produced IFN-γ, exerts an anti-fibrogenic effect, is under intense investigation. It appears that this cytokine acts on macrophages driving M1 differentiation leading to inhibition of the trans-differentiation of HSCs into myofibroblast. This diminishes the production of extracellular matrix proteins and increases collagenase's activity of the liver. IL-6 acts similarly.

Th2 responses and their respective cytokines appear to modulate liver fibrotic processes during hepatosplenic schistosomiasis [56]. In a chronic murine schistosomiasis model, transgenic reduction of IL-4 receptor alpha-mediated signalling leads to anti-fibroproliferative pathology [55]. High levels of IL-4 and IL-13 (secreted primarily by type-2 immune cells such as Th2 cells to act toward the alternatively activation of macrophages into M2 and the activation of Hepatic stellate cells) are associated with periportal fibrosis progression during schistosomiasis [50]. In addition, an rs1800925 of IL-13 promoter confers higher risk of hepatic fibrosis in *S. japonicum*-infected individuals [57].

IL-17 and its receptor IL-17RA are upregulated in hepatic fibrosis. IL-17 directly induces the production of collagen type I (Col-I) in murine HSCs. This induction is mediated by Stat3 molecular signalling pathways [58]. This is of importance because during *S. japonicum* infection in mice, IL-17 is elevated as early as 3 weeks reaching its top at 7 weeks, but cytokine elevation is still evident at 10–12 weeks following infection [59]. More recent work has shown that the inhibition of Rho-Kinase (ROCK) downregulates Th17 cells induction and improves hepatic fibrosis caused by *S. japonicum* infection. ROCK is expressed in hepatic tissues in hepatocellular carcinoma and suppresses the cell cycle and the p53 or NF-κB-mediated apoptosis pathway in these cancerous diseases, a finding which may indeed underline the role of p53 or NF-κB pathways in *S. japonicum*-related hepatic fibrosis and the potential role of ROCK inhibitors in the treatment of the disease [49].

HLA Class I and Class II

Positive associations between HLA Class II alleles and the risk for developing severe or moderate liver fibrosis following *S. japonicum* have been reported, as well as negative associations. HLA-DRB1*0901, DRB1*1202, HLA-DRB1*1302; DRB1*1404, and DRB1*1405 and HLA-DQB1*0303 and HLA-DQB1*0609 are associated with rapid progression of hepatic fibrosis [60]. HLA-DRB1*1501; HLA-DQB1*0601, DRB1*11011, DRB1*0409, DRB1*0701, HLA-DPA1*0103, and DPB1*0201 haplotypes confer protection from developing liver fibrosis [60].

IgG4 and IgE

IgG4 levels are significantly more elevated in patients with chronic schistosomiasis compared to non-infected controls [61]. Schistosome-driven liver fibrosis is associated with IgG4 [62]. Immunoglobulin E (IgE) elevated levels are also associated with the severity of liver fibrosis [63]. Such data are implicating IgG4 and IgE in liver fibrogenesis driven by human schistosome infections [63].

Visceral Leishmaniasis

VL, known also as Kala-azar, is caused by two species of the intracellular parasite *Leishmania*. *L. donovani*, prevalent in South Asia and East Africa, where humans are the main reservoir, and *L. infantum*, prevalent in Latin America and the Mediterranean region, with the domestic dog being the main reservoir [64, 65].

The disease is transmitted by the female phlebotome sandflies that inoculate the parasite in the form a promastigote into the skin, where they are being phagocytozed by monocytes and macrophages. Inside these cells, after being transformed into the amastigote stage, the parasite multiplies

and spreads to new cells. Triggered cellular and humoral immune responses are crucial for the outcome of the infection.

Along this line, immunocompetent individuals can elicit a successful immune response associated with prevention of clinical disease, though without eliminating the infection [66]. On the contrary, immunosuppressed patients, including HIV positive patients, solid organ transplant recipients, and patients on new biologic therapies are prone to develop clinical overt disease, even long after initial infection [1, 66].

Additionally, the parasite per se has the ability to develop strategies to evade the host's immune response contributing to its persistence [67].

The liver is one of the principal organs targeted by *Leischmania spp*. Granuloma formation in liver tissue is T-cell-mediated and signifies resolution of infection. Accordingly, chronic granulomatous lesions are the most prevalent histological characteristic. Acute hepatocellular injury has been rarely reported either in the acute or in the chronic phase of the disease.

Clinical presentation of VL ranges from asymptomatic to full blown disease (kala azar). The main characteristics are persistent fever, hepatomegaly, and splenomegaly. Other features are weight loss, pancytopenia, and hypergammaglobulinemia [64, 65].

Considering that symptoms of patients with VL lack specificity, laboratory confirmation is mandatory for the diagnosis. Detection of the parasite in the amastigote stage in tissues (lymph nodes, liver, bone marrow) or blood by light microscopy is considered the classic diagnostic method. Serological techniques (such as ELISA, direct agglutination tests, immunofluoeresence) have high sensitivity, though they lack specificity and can't discriminate between different disease stages. Molecular techniques such as PCR can be also used for the diagnosis of VL.

Immune Responses to Leishmania

Leishmania parasites infect dendritic cells and fibroblasts but mainly professional phagocytes such as macrophages, neutrophils, and monocytes and macrophages [68].

The major target cell is the macrophage. There, the parasite multiplies, ultimately rupturing the cell membrane, and consequently spreading to neighbouring uninfected cells [69].

As macrophages migrate to all mammalian tissues, *Leishmania* parasites have a great potential for damaging bodily functions. In the dermis, they cause the cutaneous form of the disease (which can be localized or diffuse); in the mucosa, they result in mucocutaneous leishmaniasis; and the metastatic spread of infection to the spleen and liver leads to VL. One of the major factors determining the type of pathology is the species of *Leishmania* [66]. However, the transmitting vector, as well as genotype, nutritional status of the host, and environmental and social factors also have a large impact on the outcome of the disease [66]. That is why even patients, infected by the same species of *Leishmania*, develop different symptoms and may differ in their response to therapy [1, 66]. The basis of this heterogeneity is not well understood, but part of this variation is likely genetic. Numerous potentially relevant genes were reported [65, 66, 70].

The disease's features are probably caused by the absence of antigen-specific immune responses, which can control the parasite, leading to active disease [71].

In active VL, proinflammatory cytokines, including IFN-γ and TNF-α, are increased both in plasma and in tissues such as the spleen, bone marrow, and lymph node [72–74]. However, suppressor IL-10 and TGF-β cytokines are also increased in lesional tissues from human VL patients, further indicating that the ratio of Teff/Treg cytokine milieu may indeed govern the fate of the infection [73, 74].

The Immunobiology of Leishmaniasis

The complex host–parasite immunological interactions initiated during *Leishmania* infection are yet to be delineated. A plethora of data has emerged from experimental models of leishmaniasis that reproduce only some of the immunopathological features of the human disease [67]. Animal and human studies concluded that a close interplay between innate and adaptive immune responses govern the fate of disease progress over time [67].

Innate Immunity

In early stages, once inoculated into the host dermis by an infected sandfly, infective metacyclic promastigotes of *Leishmania* are engulfed by resident dermal dendritic cells, infiltrating neutrophils and macrophages. This is the first line of defense exercised mainly by neutrophils [29, 75–77]. The gigantic recruitment of neutrophils to the site of parasite inoculation is a phenomenon well documented over the years [76]. The insertion of the sandfly's proboscis into the dermis initiates neutrophils-mediated inflammatory responses, resulting in tissue damage [78].

The mechanisms responsible for the prompt, enormous, excessive, rapid, and constant infiltration of neutrophils at the site of the bite are under intense investigation. It appears that vector-derived saliva plays an important role. It contains vasodilators and anticoagulants, which play an anti-hemostatic role [79].

Of great interest for its immunomodulatory properties is the promastigote secretory gel (PSG) [80]. PSG is a proteophosphoglycan (PPG)-rich, mucin-like gel formed by promastigotes, which accumulates in sandfly's gut and mouthparts and blocks the vector mouthparts pushing the infected sandflies to regurgitate several times during blood feeding. This acts in favour of the parasite, as both parasites and gel are co-transmitted, enhancing the chances of parasite transmission and parasite-mediated inflammation. Experimental murine work has shown that PSG aggravates cutaneous leishmaniasis and VL and provokes chronic infection [80, 81].

Gut microbiota of the sandfly's midgut that are co-egested with *Leishmania* parasites into the skin promote the early recruitment of neutrophils and the activation of the inflammasome in these cells, leading to a rapid induction of IL-1β [82]. The egested microbes trigger the inflammasome, leading to a rapid production of IL-1β, which sustains neutrophil infiltration. Antibiotic treatment leading to the sharp reduction of midgut microbiota abolishes neutrophil recruitment, a phenomenon, which is also noted when *Leishmania*-infected, sandflies-bitten experimental mice are treated with an IL-1 receptor (IL1R) antagonist.

Depletion of neutrophils in self-contained cutaneous lesions in mice with *Leishmania* infection increase the production of IL-1α and IL-1β pro-inflammatory cytokines and decreases the quantity of viable parasites at the site of the infection [75]. *L. mexicana* alters neutrophils making them able to prevent the initiation of a protective immune response, which has a negative impact in the control of lesion development; infected neutropenic or antibody-mediated neutrophil-depleted mice had better control of the disease [83, 84].

It has been proposed that neutrophils facilitate *Leishmania* infection by having better access to parasites than other phagocytic cells in extracellular spaces and by promoting their safe transition to mononuclear phagocytes [75, 76].

On the other hand, neutrophil extracellular traps (NETs) from human neutrophils can kill *L. amazonensis* parasites, and such formatted NETs are also reported in cutaneous lesions of patients with leishmaniasis. However, such an effect was not seen for *L. mexicana*, *L. donovani,* or *L. infantum* parasite-related NETs, suggesting that the role of neutrophils may differ among different *Leishmania* species [83].

Several days after the infection, parasite replication is mainly achieved within infected macrophages and monocytes. Recruitment of monocytes is achieved through release of chemokines such as CCL3 and macrophage inflammatory protein (MIP)-1β from degranulated infected neutrophils upon stimulation with released IL-8, TNF-α, and other cytokine mediators [85].

Within macrophages, the main host cells of *Leishmania* parasites, internalized promastigotes differentiate into non-motile amastigotes. These amastigotes replicate and persist for long in phagosomes. Their persistence is the cause of latent infections through reactivation [86].

The key role of immature inflammatory monocytes as major facilitators of *L. major* expansion and persistence in vivo during primary infection and parasite internalization seems to delay the maturation of these cells [46].

In naturally resistant mouse strains, such as C57BL/6 or C3H, IL-12, secreted mainly by DCs, has the essential role of inducing a Th1 immune response (Fig. 13.4). The Th1 effector cytokine IFN-γ leads to an activation of infected macrophages and parasite killing. Conversely, the susceptibility of BALB/c mice has been attributed to a Th2 immune response characterized by the secretion of IL-4, IL-5, and IL-13. Accordingly, IL-10 appears to be also critical for both disease's development and *Leishmania*'s persistence [72, 73].

Adaptive Immunity

Resolution of the infection is attained by activated T lymphocytes that first, induce cytokine production (IL-12p40, IL-18, TNF-α, IL-6, and IL-23) and, second, activate infected mononuclear phagocytes that eliminate the parasite [87]. Newer cytokines such as IL-32γ play also an important role [88].

The predominance of an anti-inflammatory Th2 response characterized by the overproduction of IL-10, TGF-β, IL-4, and IL-13 is associated with disease persistence and progression and intracellular proliferation of *Leishmania* parasites [89].

In immunosuppressed patients or BALB/c mice, infection with *Leishmania* results in predominant Th2 immune responses with increased levels of IL-4 and IL-10. The early appreciation that a shift of Th1 to Th2 responses may account for the persistence of the parasite has been followed by the emerging role of Tregs and Th17 cells [90].

Murine work has shown that BALB/c IL-17A$^{-/-}$ mice are resistant to *L. major* infection. This has led to the appreciation that IL-17A plays an important role for disease's fate. Since IL-17A is mainly produced by Th17 cells, the role of these cells has been considered pivotal [91]. A consensus as to whether IL23/IL17A axis is important for the clearance of Leishmaniasis in resistant animal models has not yet been reached [92]. Impairment of Th17 development in IL-23p19-deficient BALB/c mice appears to confer protection against progressive cutaneous L [93]. This is important given that IL-23 provokes the expansion of IL-17A producing CD4+ Th17 cells and that the Th17/IL-23 axis is not involved in clearance of *L. major* infections in resistant C57BL/6 mice. Both IL-23p19$^{-/-}$ and

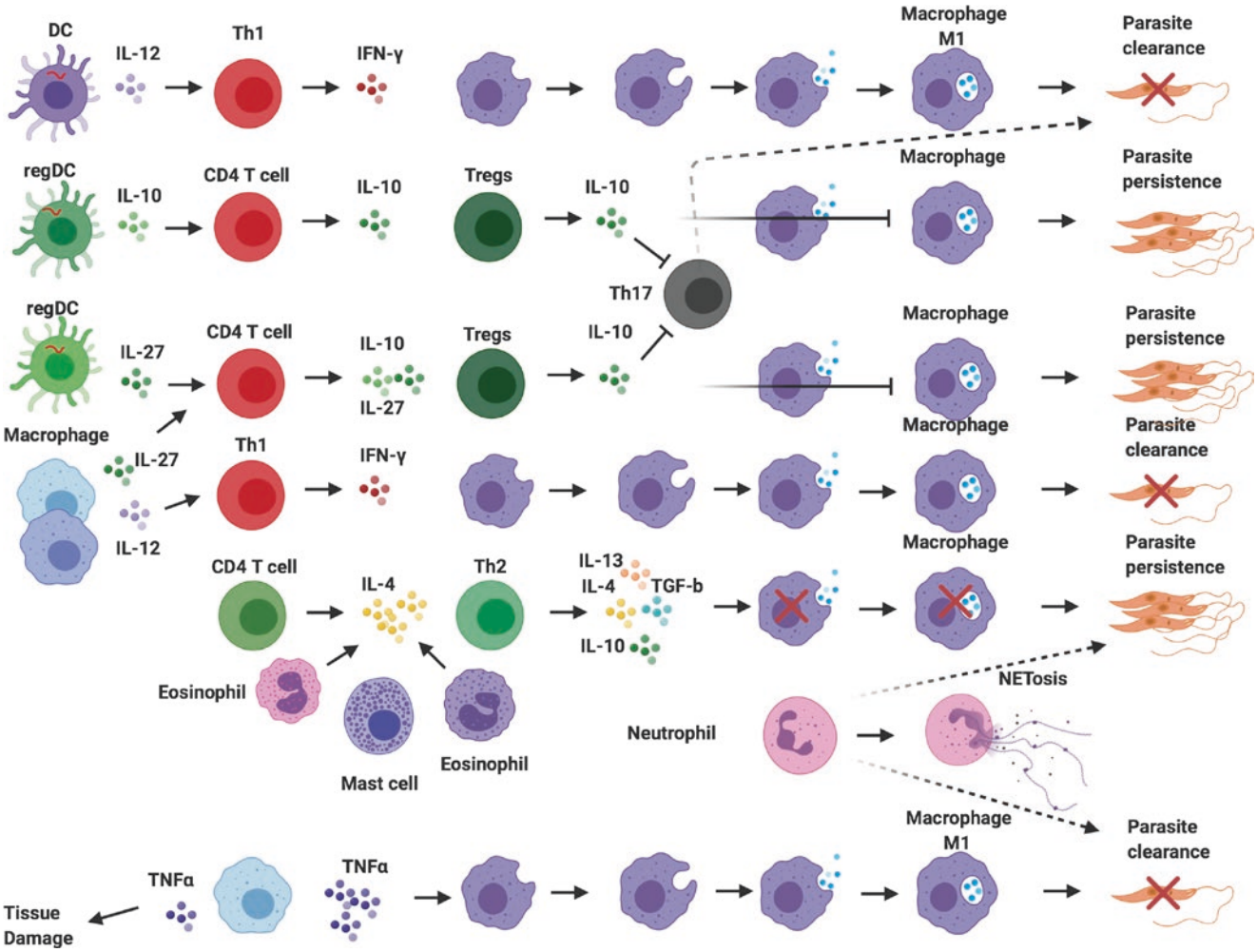

Fig. 13.4 Cell-mediated mechanisms for L. donovani's clearance, survival or persistence. In general, dendritic cells (DCs) promote via IL-12 an IFN-γ mediated Th1 response, leading to macrophage activation and parasite's clearance. A Th2 response also promoted by IL-4 production from eosinophils, baseophils, and mast cells has the opposite effect. A similar influence is provided by IL-10 producing Tregs; Th17, a pro-inflammatory cell subset, which drives parasite clearance, diminishes Tregs' effect. A subpopulation of regulatory DCs produces IL-10 or IL-27, which have a regulatory role, promoting IL-10 or IL-27 production, inhibiting Th17, and endorsing parasite's survival. Neutrophils play a dual role; they can control the parasite but can also be used by the parasite to escape immune system as they are used by some *Leishmania* species that evade neutrophils and target them to achieve silent transmission. (Created with Biorender (under license))

IL-17A$^{-/-}$ C57BL/6 mice have similar cytokine patterns and developed similar skin lesions. IL-17A appears mainly to be produced by CD4+ T cells and neutrophils [29]. In a similar vein, IL-10, a regulatory cytokine mainly produced by Tregs, has been proved to be crucial in the development of Leishmania infection and the progression of the disease in humans. In general, patients with CL, VL, and post-kala-azar dermal leishmaniasis have increased levels of IL-10 [73, 94–96].

Recent work in animals has shown that IL-10-deficient C57BL/6 animals showed enhanced IFN-γ and IL-4 expression in the lymph nodes but not in macrophages or neutrophils. T cell-specific dendritic cell-based vaccination against Leishmaniasis professionally overpowers the primary secretion of IL-10, participating in the regulation of parasite's spread. IL-10-producing T cells appear to exert a significant impact on immune activation early after infection and are per se sufficient to render BALB/c mice susceptible to an unrestrained infection by *L. major* [97].

The persistence of *L. major* in the skin after healing in resistant C57BL/6 mice is controlled by an endogenous IL-10 producing and IL-10-independent Tregs [98, 99].

Data have demonstrated that *L. donovani* is able to provoke expansion of IFN-γ producing CD4+ Th1 and CD8+ T cells at an early stage of the infection, but the frequency of these cells decreases at a later stage, despite persistence of parasites. Persistent infection induces expansion of interleukin-10+ FOXP3+ Treg and CD4+ and CD8+ T cells expressing PD1 and blocking of PDL-1 signalling leads to the restoration of protective Th1 CD4+ and CD8+ T cell responses, which have an impact in significantly diminishing the parasite's burden [100].

Th17 cells and IL-17A are also implicated in the immunopathology of murine models of CL [91, 101–103]. Susceptible BALB/c mice have higher IL-17A levels in *L. major* lesions compared to resistant C57BL/6 mice. Importantly, the levels of IL-17A produced by draining LN cells from a C57BL/6 4 weeks post-single *L. major* infection group in response to whole promastigote *L. major* antigen were comparable to the levels observed from the draining LN cells of *L. major*-infected C57BL/6 mice in response to soluble *Leishmania* antigen [91]. Of interest, vitamin D deficient mice are more prone to control *Leishmania*-induced lesions and have more increased CD4+ IFN-γ+ T cells percentages compared to control mice [104].

Conclusion

Parasites infecting the liver consist of those that can be transmitted by vectors, by food consumption, or by direct environmental transmission. They are totally mostly preventable by simple measures of improved hygiene, good health and sanitation conditions, and proper food process that sharply decrease the risk of infection from food-borne zoonoses.

Early studies revealing the pivotal role of cellular immunity focusing on the role of neutrophils, macrophages, and the Th1/Th2 imbalance have now been updated by more recent focusing on the decisive role of Treg/Th17 disparity in the regulation of parasite's invasion, clearance, or progression of the disease.

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The Treatment of Bacterial and Parasitic Diseases of the Liver

14

Christopher Rombaoa and Ke-Qin Hu

Abbreviations

ACLF	Acute-on-chronic liver failure
ALA	Amebic liver abscess
ALD	Acute liver disease
ALF	Acute liver failure
CLD	Chronic liver disease
DILI	Drug-induced liver injury
ESLD	End-stage liver disease
MDSC	Myeloid-derived suppressor cell
MRSA	Methicillin-resistant *Staphylococcus aureus*
PLA	Pyogenic liver abscess
SAB	*Staphylococcus aureus* bacteremia
SBP	Spontaneous bacterial peritonitis
SSTI	Skin and soft tissue infection
UTI	Urinary tract infection
VL	Visceral leishmaniasis

Key Points

- The dynamic interaction between type and severity of hepatic infection, underlying liver disease, host immunity, and antibiotic treatment has a significant impact on clinical presentation, course, management, and prognosis.
- Patients with underlying chronic liver disease have a compromised immune response. This leads to increased frequency of infection as well as predisposition to certain types of infection.
- Multiple factors contribute to malnutrition in cirrhotic patients, which has a reported prevalence as high as 80%. Nutrition is a significant predictor of morbidity and mortality, and improvement in nutritional status is associated with better outcomes.
- Drug-induced liver injury should be avoided in patients with bacterial and parasitic infection, especially cirrhotic patients, given their relatively low hepatic reserve and high risk for decompensation.
- Infection is the most common precipitating factor for acute-on-chronic liver failure, and the most common types of bacterial infection in this setting are pneumonia, urinary tract infection, and bacteremia.
- The risk of spontaneous bacterial peritonitis increases with the progression of liver disease, and prophylaxis should be considered if patients meet certain criteria.
- Patients with chronic liver disease due to alcohol are more vulnerable to specific pathogens that cause pulmonary infections such as *Legionella pneumophila* and *Mycobacterium tuberculosis*.
- The most common bacteria that cause pyogenic liver abscesses in the United States are streptococcus species and *E. coli*. Management mainly consists of a combination of antibiotics and drainage.
- Patients with chronic liver disease are considered immunocompromised and susceptible to a wide spectrum of parasitic infection, such as *G. lamblia*, *Cryptosporidium*, and *Leishmania*. Conversely, some parasitic infections may not require immunosuppression to affect the liver such as schistosomiasis, amebiasis, echinococcus, and clonorchiasis.

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Introduction

Infections of the liver can be caused by viruses, bacteria, and parasites. Chapters 12 and 13 in this book have discussed in detail the immune response to bacterial infections and diagnosis and classification of parasitic diseases of the liver, respectively. This chapter will focus on treatment of the most common bacterial and parasitic pathogens related to the liver.

The dynamic interaction between type and severity of infection, underlying liver disease/condition, host immunity, and antibiotic treatment has a significant impact on clinical presentation, course, management, and prognosis (Fig. 14.1). Patients with chronic liver disease (CLD) are in an immunocompromised state. Thus, the presence or absence of underlying liver disease significantly influences a patient's susceptibility to certain pathogens. Both bacterial and parasitic infections pose a major risk for morbidity and mortality in patients with various CLDs such as cirrhosis. There is increased potential for complications such as septic shock, acute-on-chronic liver failure (ACLF), and multi-organ failure. Thus, early recognition and timely treatment are crucial to reduce the risk of complications.

Traditionally, other review articles and textbook chapters on this topic are organized by listing individual pathogens, or into certain types of liver disease, and focused antibiotic regimens. To better review this topic from a hepatology standpoint, the following chapter is organized according to the most common bacterial and parasitic pathogens associated with normal liver function as well as those which occur in the setting of acute liver failure (ALF), chronic liver disease (CLD), and alcoholic liver disease (ALD). We will also explore the principles of managing underlying CLD in the setting of liver infection.

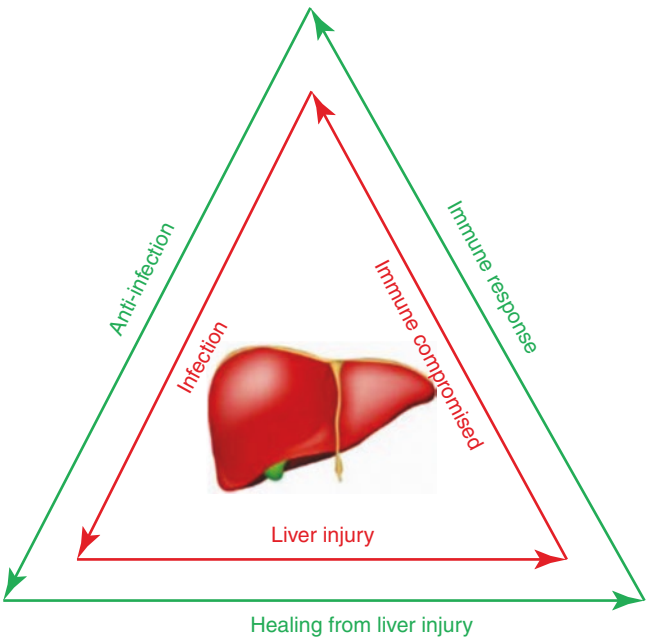

Fig. 14.1 Dynamic interaction of liver with infection and host immune response. Dynamic interaction between infection type, underlying liver disease/condition, and host immunity has the potential to perpetuate continued liver injury. These factors must be considered when choosing appropriate antibiotics and supportive therapy because they have a significant impact on clinical presentation, course, management, and prognosis

Principles in Managing Bacterial and Parasitic Diseases of the Liver

Although antibacterial and parasitic treatments are the mainstay of etiologic treatment for hepatic infection, the following principles should be exercised in managing these patients. A full assessment should always include the state of hepatic function and whether underlying CLD is present because this can significantly impact the clinical course, management, and prognosis of hepatic infection. Hepatic dysfunction is a common presentation of CLD in the setting of bacterial and parasitic diseases of the liver. Clinicians must consider the dynamic interaction of the liver with infection and the host immune response (see Fig. 14.1).

Managing Underlying CLD while Treating the Infection

Infections may induce liver injury or functional deterioration. For instance, bacterial infection can trigger a rapid deterioration of liver function and multi-organ failure in patients with cirrhosis. Acute kidney injury following infections develops in 27–34% of patients with advanced cirrhosis. Pulmonary complications are commonly observed in cirrhotic patients with infections. Prognosis of cirrhotic patients with respiratory failure is poor with a mortality rate up to 33–60%. Thus, effective supportive care is essential for those with infection and underlying chronic liver disease. This includes providing adequate nutritional support and avoiding further liver injury with hepatotoxins. In addition, it is important to consider screening for possible concomitant liver conditions that may be overlooked such as nonalcoholic fatty liver disease, viral hepatitis infection, autoimmune hepatitis, and iron or copper overload.

Compromised Immune Response

From an immunology perspective, patients with ACLF become predisposed to infections due to several factors which ultimately results in a state of immune paresis (Table 14.1) [1]. This defective immune response then leads to increased

Table 14.1 Factors for increased risk of infection in cirrhotic patients

Barrier failure	Volume overload leading to edematous, vulnerable skin Increased bacterial translocation
Altered microbiome	Altered small bowel motility with delayed transit Increased small intestine bacterial overgrowth
Decreased cellular defenses	Reduced number of leukocytes Suppressed T cell function Reduced phagocytosis activity
Clinical factors	Malnutrition Alcohol use Iatrogenic related infections – invasive procedures and catheters
Genetic factors	NOD2 mutation Toll-like receptor 2 polymorphisms

frequency of infection. The overall number of leukocytes tends to be reduced in cirrhotic patients due to hypersplenism. In addition, neutrophils involved in the innate immune response show reduced phagocytosis of opsonized bacteria [2].

Myeloid-derived suppressor cells (MDSCs) have been described in many pathologic conditions and have the ability to suppress T cell proliferation and responses. A particular mononuclear subtype called M-MDSCs has been shown to be markedly expanded in ACLF patients. In turn, these M-MDSCs appear to play a role in the impairment of T cell antimicrobial responses. The mechanism includes suppression of both proinflammatory cytokine secretion and phagocytosis of bacteria [3].

ACLF patients also have altered microbiomes with intestinal bacterial overgrowth due to a decrease in small bowel motility and delayed transit. This plays a key role in increased intestinal bacterial translocation in cirrhotic patients [2, 4]. The bacterial translocation also helps to trigger release of endotoxins, cytokines, and nitrous oxide which all contribute to development of systemic infection [5]. One study illustrated that bacterial translocation of gut microbiota triggered tonic type I interferon (IFN) expression in the liver. This in turn leads to interleukin (IL)-10 production that works to suppress immune function. Blockade of this pathway in mice with liver fibrosis led to reconstitution of antibacterial immunity [6].

There are also genetic factors that seem to play a role. Patients with variants in the NOD2 (nucleotide-binding oligomerization domain-containing protein 2) gene have impaired immune recognition of muramyl dipeptide produced by both Gram-positive and Gram-negative bacteria, which in turn increases the risk for infection and death [7]. Cirrhotic patients with Toll-like receptor (TLR) 2 polymorphisms have also been shown to have increased risk for SBP [8].

An additional factor which significantly affects a patient's ability to recover from infection is nutritional status. Unfortunately, this tends to be compromised in those with end-stage liver disease.

Diminished Nutrition Status

Nutrition is a significant predictor of morbidity and mortality in cirrhotic patients, and it has been shown that improvements in nutritional status are associated with better outcomes [9]. In cirrhotic patients, there are many factors that contribute to malnutrition, which has a reported prevalence as high as 80% [10]. Studies have shown that protein-calorie malnutrition in patients with cirrhosis complicated by portal hypertension results in a greater prevalence of ascites (65% vs. 48%) and hepatorenal syndrome (5.1% vs. 2.8%). There is also significantly greater inpatient mortality in malnourished patients when compared to those with adequate nutritional status (14% vs. 7.5%) [11].

Unintentional consumption of a low-calorie diet is common in cirrhotic patients and contributes to inadequate dietary intake. The increased presence of cytokines such as TNF-α in these patients has been shown to reduce appetite [12]. Early satiety accompanied with nausea may also be related to increased intra-abdominal pressure and decreased gastric accommodation due to ascites [13].

Patients also often have issues with digestion and absorption [14, 15]. Altered digestion of fats is of particular importance in the setting of end-stage liver disease. Portosystemic shunting causes nutrients to bypass the liver without being metabolized [16–18]. Reduced bile salt output and intraluminal bile acid deficiency from cholestatic liver disease can also impair the absorption of fat-soluble vitamins (A, D, E, and K) [16].

Cirrhotic patients also have altered glucose metabolism. The rates of protein catabolism and gluconeogenesis are increased when compared to non-cirrhotic patients [17, 18]. This is likely due to diminished capacity of hepatocytes to synthesize and store glycogen. Thus, the body must rely on alternate sources of energy such as catabolism of fats and protein. In addition, studies have shown end-stage liver patients to have higher insulin resistance [19]. This leads to decreased glucose utilization in the peripheral tissues and decreased glycogen production which furthers reliance on lipid oxidation.

Cirrhotic patients at higher risk for malnutrition include those with BMI <18.5 kg/m^2 and Child-Pugh class C disease [13]. These patients should undergo a more detailed nutritional assessment, and referral to a registered dietician can be considered.

Guidelines available from the European Association for the Study of the Liver (EASL) and the European Society for Clinical Nutrition and Metabolism (ESPEN) recommend that for nonobese patients (BMI < 30) the optimal daily energy intake should not be lower than 35 kcal/kg (actual body weight). For obese patients (BMI > 30), caloric intake should be reduced by 500–800 kcal/day. Optimal daily protein intake for both groups should be 1.2–1.5 g/kg daily [20, 21].

In addition to accounting for a compromised immune response and providing supportive nutritional care, it is important to avoid further iatrogenic liver injury which could push a cirrhotic patient toward liver failure.

Precautions Against Drug-Induced Liver Injury (DILI)

Drug-induced liver injury is the most common cause of acute liver failure in the United States. Acute DILI is defined as abnormal liver enzymes for less than 3 months, while chronic injury is greater than 3 months. The most commonly implicated drugs in the United States are acetaminophen and antibiotics. Amoxicillin-clavulanate is the most common antibiotic worldwide [22, 23]. It is important to be vigilant for DILI, especially in cirrhotic patients given their relatively low hepatic reserve and high risk for decompensation.

Many drugs and herbal supplements are associated with DILI, and an online searchable database maintained by the National Institutes of Health is a valuable resource and available online (www.livertox.nih.gov). DILI can be categorized into the type of liver injury: hepatocellular injury, cholestatic injury, and mixed injury. Hepatocellular injury is characterized by more pronounced elevations of serum aminotransferases and alkaline phosphatase. Cholestatic injury is characterized by an accentuated increase in alkaline phosphatase compared to serum aminotransferases. Both types can also feature elevated bilirubin. Examples of drugs associated with the different types of liver injury are summarized in Table 14.2.

Table 14.2 Examples of drugs associated with different types of liver injury

Hepatocellular injury	Cholestatic injury	Mixed injury
Acetaminophen	Amiodarone	Amitriptyline
Allopurinol	Azathioprine	Captopril
Ethanol	Carbamazepine	Clindamycin
Isoniazid	Erythromycin	Ibuprofen
Phenytoin	Ketoconazole	Phenobarbital
Pyrazinamide	Naproxen	Sulfonamides
Rifampin	Sulfonylureas	Verapamil
Sertraline	Terbinafine	–
Statins	Trimethoprim-sulfamethoxazole	–
Valproate	Tricyclics	–

Bacterial Infections

The clinical presentation and prognosis of bacterial infections are significantly impacted by the liver's condition. For instance, bacterial infection in patients without underlying CLD may present only with infection-induced liver injury; however, infection is the most common precipitating factor for ACLF in patients with cirrhosis from any etiology [24]. Among hospitalized cirrhotic patients, 30% will have an infection on admission or develop one while inpatient. Cirrhotic patients who are diagnosed with a bacterial infection are at an elevated risk for short-term mortality. This is likely related to a much higher tendency to develop multiorgan failure [25, 26]. One study examining the survival in infection-related ACLF showed that a majority (61%) of infected cirrhotic patients also develop organ failure in at least one other system [27]. As mentioned earlier, the presence or absence of underlying liver disease can significantly influence the presentation, clinical course, prognosis, and management of a particular infection. Thus, the following section will discuss the most common bacterial pathogens associated with normal liver function, acute liver failure (ALF), chronic liver disease (CLD), and alcoholic liver disease (ALD).

Bacterial Infections in Patients with Acute Liver Failure (ALF) and Acute-on-Chronic Liver Failure (ACLF)

Infection is one of the main causes of mortality in patients with ALF [28, 122]. The most common types of bacterial infection in ALF patients are pneumonia, urinary tract infection, and bacteremia. These common infections overlap with those seen in patients with CLD discussed later in this chapter. Intravenous catheters can also be a major source of infection in hospitalized ALF patients [29]. The most common causative organisms are Gram-positive cocci (*Staphylococci* and *Streptococci*) as well as enteric Gram-negative cocci [30, 31]. It is common for ALF patients to not exhibit typical signs of infection. In one study, clinical presentation such as fever and leukocytosis was absent in 30% of patients [30]. Thus, vigilance and early recognition are key to improving outcomes.

Broad-spectrum coverage for Gram-positive bacteria with vancomycin is recommended in all patients at increased risk for methicillin-resistant *Staphylococcus aureus* infection. This includes previously hospitalized patients and those with IV catheter infections. In addition, third-/fourth-generation cephalosporins or piperacillin-tazobactam are recommended for broad-spectrum Gram-negative coverage depending on bacterial culture results. An antifungal agent should also be considered for any patient that does not improve with antibiotics. In particular, *Candida* can be present in up to one third of ALF patients [32].

Infection is the most common precipitating factor for ACLF. Karvellas et al. showed that in 184 patients with ACLF, 36% had bacteremia, of which 36% were Gram-positive bacterial infection, 58% were Gram-negative bacterial infection, and 6% were fungal infection. The median time of bacteremia onset was 8 days. Patients with infection showed higher MELD and APACHE scores, more severe coma, higher ratio in renal replacement treatment and artificial ventilation, longer ICU stay, and higher mortality rate. Another study reported 28.5% and 22.5% of patients with ACLF had urinary tract infection and spontaneous bacterial peritonitis (SBP), respectively. Secondary infections could develop in 21.6% of patients with ACLF that have been significantly associated with the 30-day mortality [33].

While ACLF presents a significant risk for the pathogens discussed above, patients with stable CLD are also at risk for an additional set of potential infections.

Common Bacterial Infections in Patients with Underlying Chronic Liver Disease

Cirrhosis results in an immunocompromised state and predisposes patients to spontaneous bacterial infections, hospital-acquired infections, and a variety of infections from pathogens that are uncommon in immunocompetent patients. Bacterial infection accounts for about 30–50% mortality in patients with cirrhosis. Once infection develops, it may induce severe complications, such as septic shock, acute-on-chronic liver failure (ACLF), multiple organ failure, and death. Thus, effective prevention, early recognition, and timely and proper management are essential for minimizing morbidity and mortality in these patients [34].

The most common types of infections in patients with cirrhosis include spontaneous bacterial peritonitis (SBP, 25–31%), urinary tract infection (UTI, 20–25%), pneumonia (15–21%), bacteremia (12%), and soft tissue infection (11%) [34–36]. The majority of infections (75%) in cirrhotic patients are caused by Gram-negative organisms such as *Escherichia coli*, *Klebsiella spp*., *Enterobacter spp*., and *P. aeruginosa*. Gram-positive infections account for 20%, while anaerobes occur in approximately 3% of cases [37]. Of note, cirrhotic patients who have been hospitalized are at a much higher risk for infection with Gram-positive organisms (38–70%), especially methicillin-resistant *Staphylococcus aureus* (MRSA) [38, 39]. Tables 14.3, 14.4, and 14.5 summarize the treatment of common bacterial liver infections in the setting of CLD as discussed below.

Spontaneous Bacterial Peritonitis

The most common infection causing sepsis-induced ACLF is SBP [40]. SBP is observed in 10–30% of hospitalized patients with cirrhosis and ascites [41]. The risk of SBP increases with the progression of liver disease. One study showed that for every point increase in MELD score (model for end-stage liver disease), the risk for developing SBP increases by 11% [44].

Table 14.3 Treatment for SBP and indications for prophylaxis

Indication	Antibiotic regimen
SBP treatment	Ceftriaxone 2 g IV daily ×5 days or cefotaxime 2 g IV q8 h ×5 days
	Albumin 1.5 g/kg IV on day 1 followed by 1 g/kg on day 3
Primary SBP prophylaxis	Ciprofloxacin 500 mg PO daily or trimethoprim-sulfamethoxazole one double-strength table (160 mg/800 mg) PO daily [42]
Secondary SBP prophylaxis	Ciprofloxacin 500 mg PO daily or trimethoprim-sulfamethoxazole one double-strength table (160 mg/800 mg) PO daily
Gastrointestinal bleed	Ceftriaxone 1 g IV daily ×7 days total
	Consider switching to oral therapy after bleeding is controlled and patient is stable for total 7-day course of antibiotics: Norfloxacin 400 mg PO daily or ciprofloxacin 500 mg PO daily or trimethoprim-sulfamethoxazole one double-strength table (160 mg/800 mg) PO daily [43]

Table 14.4 Empiric antibiotic coverage in patients with cirrhosis and bacterial infection

Infection	Common pathogens	Empiric antibiotic regimen
Urinary tract infection	*Escherichia coli* *Klebsiella pneumoniae* *Enterococcus*	*Community-acquired infection* Third-generation cephalosporin IV
		Healthcare-associated infection Piperacillin/tazobactam IV or meropenem IV vs. linezolid IV if high prevalence of MDR
Pneumonia	*Streptococcus pneumoniae* *Staphylococcus aureus* *Haemophilus influenza* *Moraxella catarrhalis*	*Community-acquired infection* Amoxicillin 1 g TID or amoxicillin-clavulanate XR 2 g BID ×5 days PLUS azithromycin (500 mg day 1; 250 mg day 2–5)
		Healthcare-associated infection Piperacillin/tazobactam IV PLUS vancomycin IV if risk factors for MRSA
Skin and soft tissue infection	*Staphylococcus aureus*	
	Streptococcus pyogenes	Third- or fourth-generation cephalosporin IV
	Escherichia coli	or piperacillin/tazobactam IV
	Klebsiella spp.	or meropenem IV
	Pseudomonas aeruginosa	PLUS vancomycin IV if risk factors for MRSA

MDR multidrug resistant

Table 14.5 Common bacterial infections in patients with underlying CLD

Infection	Antibiotic regimen
Legionellosis	Levofloxacin 750 mg IV daily ×7–10 days or azithromycin 500 mg IV daily ×7–10 days *Consider a 21-day course in severely immunosuppressed patients
Staphylococcus aureus bacteremia	*Empiric MRSA coverage* Vancomycin 15–20 mg/kg IV q8–12 h, not exceeding 2 g/dose or daptomycin 6–10 mg/kg IV daily
	If MSSA, consider de-escalation to a beta-lactam antibiotic Nafcillin 2 g IV q4 h Cefazolin 2 g IV q8 h
	Repeat surveillance blood cultures within 72 h of antibiotic initiation. Patients should be treated with a 14-day course starting from the day of first negative surveillance blood culture
Streptococcus pneumoniae bacteremia	*Empiric therapy* Vancomycin 15–20 mg/kg IV q8–12 h, not exceeding 2 g/dose PLUS concurrent pneumococcal meningitis coverage: Ceftriaxone 2 g IV q12 h or cefotaxime 2 g IV q4–6 h *Therapy should be narrowed pending culture results. Duration 10–14 days.
Salmonellosis	Levofloxacin 500 mg PO daily or ciprofloxacin 500 mg PO daily or azithromycin 1 g PO on day 1 followed by 500 mg daily for 5–7 days
Mycobacteria tuberculosis	First-line drugs include: Isoniazid, rifampin, pyrazinamide, and ethambutol (also known as "RIPE therapy")
	Intensive phase with four drugs ×8 weeks: isoniazid, rifampin, pyrazinamide, and ethambutol Continuation phase with two drugs ×16 weeks: isoniazid and rifampin

MRSA methicillin-resistant *S. aureus*, *MSSA* methicillin-susceptible *S. aureus*

SBP typically presents with abdominal pain and fever. The diagnosis is confirmed by paracentesis and subsequent analysis of the ascitic fluid. Greater than 250 polymorphonuclear cells/mm^3 (also known as neutrophils) present in the ascitic fluid is diagnostic and is an indication to administer antibiotics [45]. It is common for the gram stain of ascitic fluid to be negative in SBP due to the low concentration of bacteria. Third-generation cephalosporins such as cefotaxime and ceftriaxone are the first choice for treatment of SBP. The duration of treatment is typically 5–8 days. In addition, intravenous albumin (1.5 g/kg at diagnosis and 1 g/kg on day 3) should be given to reduce the risk of hepatorenal syndrome (33% vs. 10%) and improve short-term survival [46].

There are three main indications for antibiotic prophylaxis against SBP [47, 48]. Primary prophylaxis to prevent the first episode of SBP is indicated in patients with low protein concentration in ascites (<10–15 g/L) and markers of severe liver failure (Child-Pugh score ≥9 points with serum bilirubin ≥3 mg/dL) or circulatory dysfunction (serum creatinine ≥1.2 mg/dL, blood urea nitrogen ≥25 mg/dL, or serum sodium ≤130).

After the first episode of SBP, secondary prophylaxis is indicated to prevent recurrent SBP. Following the initial SBP infection, the probability of recurrent SBP is very high without prophylaxis (43% at 6 months, 69% at 1 year, and 73% at 2 years) [47, 49].

Lastly, antibiotic prophylaxis is indicated to prevent SBP in the setting of upper gastrointestinal bleeding. Antibiotic prophylaxis in this setting has been shown to significantly reduce the rebleeding rate within 7 days (7% vs. 34%) [50]. In addition, studies have shown that prophylaxis lowers the 28-day mortality rate after an upper GI bleed (13% vs. 35%) [24]. Table 14.3 summarizes the recommended antibiotic therapies and scenarios for prophylaxis.

Urinary Tract Infection (UTI)

The second most common type of infection in cirrhotic patients is that of the urinary tract [36]. In one study, the prevalence of bacteriuria in cirrhotic patients has been shown to be twice that of non-cirrhotics (15.6% vs. 7.5%) [51]. This may be related to immunosuppression in addition to a tendency toward significant post-voiding volume in cirrhotics with ascites [52]. Additional risk factors include female sex and higher Child-Pugh grade. The most common organisms involved are Gram-negative bacilli such as *E. coli* and *Klebsiella spp*. Treatment commonly consists of cephalosporins or quinolones (see Table 14.4).

Pneumonia

Pneumonia is the third most common type of infection in cirrhotic patients. Of note, patients with end-stage liver disease (ESLD) are more likely to have pulmonary infections associated with bacteremia, multi-lobar involvement, renal failure, and septic shock with an overall higher mortality of 14.4% vs. 7.4% [53]. The pathogens most commonly identified in community-acquired pneumonia in cirrhotic patients are similar to those in the general population and include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Group A streptococci* [54]. In addition, those with alcoholic cirrhosis are at an increased risk for aspiration pneumonia with anaerobic bacteria such as *Peptostreptococcus*, *Fusobacterium*, and *Bacteroides spp*. [55]. These patients require empiric treatment with beta-lactams plus a macrolide [54]. Cirrhotic patients who are hospi-

talized are more likely to be infected with resistant organisms such as methicillin-resistant *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Thus, empiric antibiotics of choice for these patients may include IV vancomycin or linezolid plus antipseudomonal cephalosporin, carbapenem, or piperacillin-tazobactam (see Table 14.4).

Skin and Soft Tissue Infection (SSTI)

Cirrhotic patients can have an increased risk for skin and soft tissue infections due to fragile, thin, edematous skin, poor hygiene, malnutrition, frequent hospitalization, and invasive procedures [35]. In addition, patients with ESLD have a higher mortality rate from severe cellulitis and necrotizing fasciitis (6–76%) depending on the specific pathogen, extent, and severity of cirrhosis [56]. Although Gram-positive cocci such as *S. aureus* and beta-hemolytic *streptococci* are commonly isolated in SSTIs, cirrhotic patients have an increased incidence of Gram-negative pathogens such as *E. coli*, *Klebsiella spp.*, *P. aeruginosa*, and *Aeromonas spp.* [35, 57]. Similar to pneumonia, nosocomial SSTIs also have a higher incidence of MRSA and *P. aeruginosa*.

Compared with non-cirrhotic patients, those with underlying liver disease have been known to develop necrotizing fasciitis without an obvious portal of entry in the lower extremities. This may suggest an alternative mechanism for bacterial translocation leading to bacteremia and ultimately to soft tissue infection [35, 56]. In necrotizing fasciitis, early recognition and surgical intervention significantly reduces morbidity and mortality [57]. The antibiotic therapy of choice usually includes broad-spectrum coverage with third- or fourth-generation cephalosporins, piperacillin-tazobactam, and carbapenems (see Table 14.4).

A notable subtype of chronic liver disease is alcoholic liver disease. Patients with chronic alcoholic liver disease also tend to be at risk for infections which favor the lungs.

Bacterial Infection in Patients with Alcoholic Liver Disease

Bacterial infections in patients with alcoholic liver disease have significant overlap with the infections seen in patients with underlying cirrhosis of any etiology. Patients with alcohol use disorder (AUD) and resulting alcoholic liver disease (ALD) are vulnerable to specific pathogens that take advantage of alcohol's ability to increase susceptibility to pulmonary infections and impair immune response. This ultimately can result in systemic dissemination and infection of the liver. However, some types of bacterial infection are more commonly seen in patients with ALD, as discussed below.

Legionellosis

Legionellosis, also known as Legionnaires' disease, is caused by *Legionella pneumophila*, which is an anaerobic Gram-negative coccobacillus. In 1976, Legionella famously contaminated the air-conditioning system at the Bellevue-Stratford Hotel in Philadelphia, Pennsylvania. This led to a major outbreak of respiratory disease at the American Legion 58th Annual Convention. The Centers for Disease Control (CDC) discovered the culprit bacterium 1 year later, naming it *Legionella pneumophila*.

Pulmonary legionellosis is the most common manifestation given transmission is via inhalation or aspiration of Legionella-containing aerosols [58]. However, there can be widespread dissemination to other organs such as the heart, kidney, pancreas, and liver. Patients with alcoholic liver disease are particularly at an increased risk, especially given alcohol use and immunosuppression are major risk factor for legionellosis [59]. There have also been case reports of patients developing acute hepatitis due to legionellosis. Approximately 10% of patients initially presenting with pulmonary infection also develop jaundice [60, 61].

Legionella pneumophila's virulence stems from its ability to replicate within monocytes and alveolar macrophages via the type IV Dot/Icm secretion system which is responsible for trafficking bacterial proteins to the host cytosol and inducing apoptosis. This system facilitates the creation of *Legionella pneumophila*-containing vacuoles which help the pathogen to escape from the endocytic maturation process and to avoid fusion with the host lysosome. Studies have demonstrated that *Legionella pneumophila* with mutations within the Dot/Icm gene are defective in their cytotoxicity to macrophages [62].

Treatment of legionellosis typically entails an antibacterial regimen involving levofloxacin 750 mg IV daily (or other fluoroquinolone such as moxifloxacin) or azithromycin 500 mg IV daily [63]. The total duration of therapy is usually 7–10 days; however, longer courses are recommended for immunosuppressed patients.

Mycobacterium Tuberculosis

Alcohol use has negative effects on pulmonary infections with *Mycobacterium tuberculosis* and can increase the risk of systemic dissemination [55, 59, 64]. Patients with cirrhosis have an increased incidence of extrapulmonary involvement (11–31%) [65, 66]. ALD is frequently linked to TB peritonitis. In Western countries, greater than 50% of TB peritonitis cases have underlying cirrhosis related to alcohol [35, 67]. Granuloma-related infection due to mycobacteria and its treatment is also discussed later in this chapter.

Common Bacterial Infections in Patients Without Underlying CLD

Although CLD significantly increases the risk for infection, there are also pathogens that affect the liver of patients who have no underlying liver issues.

Hepatic Involvement of Systemic Infection with Bacteremia: *Staphylococcus aureus* and *Streptococcus pneumoniae*

Parainfectious hepatitis with abnormal liver function is well described in patients with severe bacterial infections. Past studies have described this phenomenon with as much as 54% of bacteremic patients experiencing an elevation in bilirubin [68]. Other studies have shown transaminase elevations in as much as 53% of patients [69]. Numerous infectious organisms in a wide variety of primary infection sites have been implicated. Two of the most common offending organisms in this setting are *Staphylococcus aureus* and *Streptococcus pneumoniae*.

S. aureus is a leading cause of hospital-acquired bacteremia. Rates of *Staphylococcus aureus* bacteremia (SAB) in the United States are estimated to be approximately 50 per 100,000 population [70]. Risk factors include advanced age (>65), male gender, frequent healthcare contact, and hemodialysis [71]. Of note, comorbidities such as alcoholism and cirrhosis have been associated with increased mortality in the setting of SAB [72, 73].

S. pneumoniae is a well-known cause of bacteremia in both cirrhotic and non-cirrhotic patients. The incidence of invasive pneumococcal disease in the United States in 2017 was 9.5 cases per 100,000 population [74]. However, when broken down by age, patients >65 and infants <1 years old are at highest risk with incidences of 26 and 11.6 per 100,000, respectively [74]. In addition to age, risk factors include male sex, smoking, alcohol abuse, and chronic disease of the heart, lungs, liver, and kidneys.

Empiric therapy for these bacteria includes vancomycin IV. For invasive pneumococcal disease, ceftriaxone is added for the initial empiric treatment. Empiric therapy should be narrowed as soon as cultures reveal antibiotic sensitivities.

Bacterial Hepatitis: Salmonellosis

Numerous bacterial infections have been described to affect the liver directly. A prime example of this is acute salmonellosis or enteric fever which is caused by *Salmonella enterica* serotype Typhi (formerly *S. typhi*). Infection is endemic in areas of Southern Africa and South Asia [75]. In the United States, 80% of cases occur among travelers [76].

Infection occurs via ingestion of contaminated food or water. *S. typhi* organisms are able to survive gastric acid exposure and penetrate small bowel epithelium, through which they enter lymphoid tissue. Dissemination of *S. typhi* can occur via the lymphatics and bloodstream. This can lead to infection of the liver, spleen, and bone marrow, which also increases the risk for relapsing infection. Patients typically present with fever and abdominal pain [77]. Hepatosplenomegaly is common.

For uncomplicated disease, fluoroquinolones are the drugs of choice in most regions. However, infections acquired in South Asia have high risk for resistance to fluoroquinolones and are recommended to be treated with azithromycin.

Bacterial hepatitis can also be caused by several other infections that infect the liver directly. These bacteria include *Clostridium perfringens*, *Burkholderia pseudomallei*, *Yersinia enterocolitica*, *Brucella abortus*, *Coxiella burnetii*, and *Neisseria gonorrhoeae*. Table 14.6 summarizes common features of these infections.

Pyogenic Liver Abscesses (PLA)

In the United States, pyogenic liver abscesses have an overall incidence of 3.6 per 100,000 population [78]. PLAs are associated with significant mortality of 5.6%. Significant risk factors for developing a PLA include diabetes mellitus, immunodeficiency, and underlying hepatobiliary disease [79]. Bacteria can infect the liver through five main routes including the biliary tract, hepatic artery, portal vein, direct extension from an intra-abdominal source, and penetrating trauma. Although there are many bacteria that have been described in PLAs, the most common bacteria in the United

Table 14.6 Pathogens involved in bacterial hepatitis

Bacteria	Common features
Clostridium perfringens	Abscess formation Gas in portal vain or biliary tract Jaundice in 20% of patients is mainly due to intravascular hemolysis from toxin release
Burkholderia pseudomallei	Causes melioidosis which can affect multiple organs, including the liver Hepatic inflammatory infiltrates, abscess formation, granulomas, and focal necrosis
Yersinia enterocolitica	Hepatic abscess formation Granulomatous hepatitis
Brucella abortus	Hepatosplenomegaly Noncaseating or necrotizing granulomas
Coxiella burnetii	Causes Q fever Hepatosplenomegaly and jaundice
Neisseria gonorrhoeae	Fitz-Hugh-Curtis syndrome in 10% of women with acute pelvic inflammatory disease – extension of infection to the liver capsule causing perihepatitis Elevated LFTs

Table 14.7 Pyogenic liver abscesses

Infection	Common pathogens	Empiric antibiotic regimen
Pyogenic liver abscess	*Streptococcus spp.* *Escherichia coli* *Enterococcus* *Klebsiella pneumoniae*	*Empiric therapy with broad-spectrum antibiotics* Piperacillin-tazobactam 4.5 g IV q6 h or Ceftriaxone 2 g IV daily plus metronidazole 500 mg IV q8 h
		Consider MRSA coverage if patient is at increased risk

States are streptococcus species and *E. coli*. However, in Asia, studies have shown a higher prevalence of the enteric Gram-negative bacilli, *E. coli* and *Klebsiella pneumoniae* [78]. Table 14.7 summarizes the treatment of empiric therapy for pyogenic liver abscess.

Management of PLAs mainly consists of antibiotics with drainage. Methods of drainage include computed tomography (CT)-guided or ultrasound-guided percutaneous drainage, drainage with endoscopic retrograde cholangiopancreatography (ERCP), and surgical drainage (laparoscopic vs. open) [80]. Surgical drainage is usually reserved for patients who have inadequate response to percutaneous drainage [124]. Drainage with ERCP has been shown to be useful in treating abscesses that communicate with the biliary tree [80]. The initial approach to antibiotics should be broad-spectrum coverage of streptococci, Gram-negative bacilli, and anaerobes. Aspiration of the abscess is critical to guide antibiotic selection.

Granuloma-Related Infection: Mycobacteria

Mycobacteria implicated in liver disease include *Mycobacterium tuberculosis*, *bovis*, *kansasii*, *gordonae*, *leprae*, and *avium-intracellulare* [81–83]. Patients at increased risk include those who are HIV positive, on immunosuppressive therapy, IV drug users, alcohol use disorder, and diabetes mellitus. Approximately 50–80% of patients with pulmonary *Mycobacterium tuberculosis* have been noted to have hepatic involvement [84]. The most common scenarios for hepatic mycobacterial involvement include military tuberculosis, primary hepatic infection, or nodular tuberculoma/abscess. Patients typically present with fever, hepatomegaly, and increased ALP and GGT. Diagnosis is confirmed via ultrasound or CT-guided biopsy.

The antibiotic regimen usually includes rifampicin, isoniazid, pyrazinamide, and ethambutol for 8 weeks, followed by isoniazid and rifampin for an additional 16 weeks [85]. Of note, isoniazid, rifampin, and pyrazinamide have been associated with hepatotoxicity. Thus, baseline liver function tests should be measured prior to starting therapy and monitored throughout the antibiotic course. Therapy should be discontinued if serum bilirubin becomes elevated ≥3 mg/dL or serum transaminases are >5 times the upper limit of normal [86]. Due to space constraints, this chapter will not discuss in detail the various regimens available.

Parasitic Infections

Parasitic liver infections cover a wide range of organisms from simple intracellular protozoa to complex helminths with complicated life cycles. In this chapter, we will focus on the most common pathogens that are seen in patients with underlying CLD and those pathogens encountered in patients without previous liver issues. Patients with CLD can be considered immunocompromised, which makes them susceptible to a wide spectrum of parasitic infection such as those outlined in the following section. This includes pathogens such as *G. lamblia*, *Cryptosporidium*, and *Leishmania*. Conversely, there are many parasitic infections that do not require immunosuppression to affect the liver. These pathogens can cause primary disease in those without underlying CLD and include schistosomiasis, amebiasis, *Echinococcus*, and clonorchiasis. Table 14.8 summarizes the treatment of common parasitic infections of the liver as discussed below.

Parasitic Infections That Commonly Occur in Patients with Underlying CLD

Giardia lamblia

Giardia lamblia is a protozoan parasite that causes giardiasis, a common diarrheal illness. Most people infected by *G. lamblia* are asymptomatic. However, infection of the small intestine can potentially cause acute explosive, foul-smelling diarrhea, abdominal bloating, and cramping. Diagnosis is confirmed by antigen detection assays, nucleic acid amplification, or stool microscopy with findings of trophozoites or cysts [87]. Spontaneous resolution is common in patients with mild symptoms; however, patients who are immunocompromised or with underlying CLD are at higher risk for developing extraintestinal manifestations such as chronic cholecystitis, cholangitis, and granulomatous hepatitis. These patients present with right upper quadrant pain, fever, and jaundice.

Preferred agents for symptomatic patients with giardiasis include tinidazole and nitazoxanide [88]. Tinidazole is approved in the United States for patients ≥3 years old. It is often the preferred first-choice therapy due to good adherence with a single-dose oral regimen and >90% efficacy is and relatively well tolerated [89]. Common side effects are metallic taste, nausea, fatigue, headache, and dizziness [90]. Nitazoxanide is an alternative option; however, it requires

Table 14.8 Summary of treatments for parasitic infections of the liver discussed in this chapter

Infection	Antibiotic regimen
Giardia lamblia	Tinidazole 2 g PO, one dose Nitazoxanide 500 mg PO BID ×3 days
Cryptosporidium	Most immunocompetent hosts recover spontaneously with supportive care. If symptoms are severe or last greater than 14 days: Nitazoxanide 500 mg PO ×3 days (FDA approved)
Visceral leishmaniasis	*First line* Liposomal amphotericin B 3 mg/kg IV on days 1–5, 14, and 21 for a total dose of 21 mg/kg
	Alternative 2.5 mg/kg/day PO ×28 days
Hepatosplenic schistosomiasis	Praziquantel 40 mg/kg in one or two divided doses
Amebiasis	*Initial therapy* Metronidazole 500 mg PO TID ×7–10 days or tinidazole 2 g PO daily ×3 days
	Followed by intraluminal agent Paromomycin 25–30 mg/kg/day PO divided in three doses ×7 days or diloxanide furoate 500 mg PO TID ×10 days
Echinococcosis	*Start 1 month prior to surgery, and continue for 1 or 3 months, respectively afterward*: Albendazole 15 mg/kg/day, divided into two doses daily or mebendazole 40–50 mg/kg/day, divided into three doses daily
Clonorchiasis	Praziquantel 25 mg/kg/dose PO TID ×2 days or albendazole 10 mg/kg PO daily ×7 days

twice-daily dosing for 3 days. Common side effects of nitazoxanide are usually mild and related to the gastrointestinal system including nausea, abdominal pain, diarrhea, and headache [91]. Table 14.8 summarizes the most common treatment regimens.

Cryptosporidium

Cryptosporidium parvum is a protozoan parasite that also causes severe diarrhea as well as biliary tract disease. Transmission occurs via ingestion of food or water fecally contaminated with *Cryptosporidium* oocysts. In the United States, infection rates are highest among children as well as those who are immunocompromised such as patients with AIDS. Immunosuppression is the main risk factor for severe or prolonged disease [92]. More severe clinical manifestations can include cholecystitis, cholangitis, pancreatitis, and hepatitis. Up to 30% of AIDS patients can have biliary tract involvement [93]. Diagnosis is confirmed by stool microscopy or an immunofluorescent assay.

Most immunocompetent patients will recover spontaneously within weeks without requiring therapy. However, those with severe symptoms or are immunocompromised are treated with nitazoxanide or paromomycin [94, 95].

Leishmaniasis

Visceral leishmaniasis (VL), also known as kala-azar, is a disseminated infection that can affect the liver, spleen, and bone marrow caused by different species of the protozoan *Leishmania* [96]. VL involving the liver usually results from infection with *L. donovani* and *L. infantum*. In endemic areas such as the Mediterranean basin, Indian subcontinent, and East Africa, *Leishmania* is typically transmitted to humans via sandflies, while canines and rodents act as reservoir hosts [97]. *Leishmania* can also be transmitted through IV drug use, sexual contact, and transplantation of infected organs. Immunocompromised patients, especially those with HIV, organ transplantation, and those receiving immunosuppressive treatments, are at highest risk [98]. Patients with organ transplantation have a four-fold increased risk of VL, and kidney and liver transplant patients have the highest incidence. In addition, a relationship between VL and chronic liver disease has been reported and is suspected to be due to increased susceptibility to infection by intracellular and capsulate bacteria. One study showed that the incidence of VL in patients with cirrhosis living in the Campania area was 8- to 17-fold higher than that of the general population in the same area [99].

First-line treatment for leishmaniasis is liposomal amphotericin B because it is highly effective with a relatively low toxicity [100, 125]. Other drugs include deoxycholate amphotericin B, paromomycin, and pentamidine; however, their use is limited by side effects. Miltefosine is also considered when relapse or failure occurs.

Parasitic Infections of the Liver That Commonly Occur in Patients Without Underlying CLD

Hepatosplenic Schistosomiasis

Schistosomiasis is caused by parasitic blood flukes which live in different types of freshwater snails. Infection occurs by ingestion of freshwater contaminated with eggs from feces of infected humans or animal reservoirs. Hepatosplenic schistosomiasis typically results from *S. mansoni* and *S. japonicum* egg migration to the liver [101]. This is a common cause of non-cirrhotic portal hypertension, and most patients have well-preserved liver synthetic function [102]. The mechanism for portal hypertension occurs in two phases and is a result of the patient's immune response to schistosome eggs. The initial early phase is a granulomatous reaction to antigens secreted by the eggs trapped within the presinusoidal periportal spaces of the liver [103]. While the early granulomatous reaction results in destruction of the egg, chronic infection ultimately results in perisinusoidal inflammation and periportal fibrosis [101]. Activated hepatic stellate cells (HSCs) are mainly responsible for deposition of collagen leading to fibrotic liver disease [104]. Esophageal

and gastric varices resulting from severe portal hypertension can lead to gastrointestinal bleeding as the most common complication.

Praziquantel is the mainstay of treatment for schistosomiasis and has cure rates as high as 100% [105]. Hepatic fibrosis resulting from infection also has the potential to improve after treatment [106, 107]. Surgical treatment options also include splenectomy with esophagogastric devascularization and portosystemic shunt, which have been shown to improve portal hypertension and liver function tests [108, 109].

Amebiasis

Entamoeba histolytica is the underlying organism that causes amebiasis and amebic liver abscess (ALA). *E. histolytica* exists in two forms: as a cyst in its infective stage and as a trophozoite in its invasive stage. Infection occurs through ingestion of cysts via contaminated food and water. Trophozoites in the colon then invade the colonic mucosa and spread hematogenously via the portal circulation to the liver, which is the most common extra-intestinal manifestation of *E. histolytica*. Pulmonary involvement is the second most common. Patients commonly present with fever and right upper quadrant and epigastric abdominal pain [110]. Liver abscesses are usually detected by abdominal imaging such as ultrasound, computed tomography, or magnetic resonance imaging.

Treatment of invasive disease consists of metronidazole and tinidazole. Aspiration of liver abscesses is also indicated in the following situations: no improvement after 48–72 h, left lobe abscess, thin rim of liver tissue (<10 mm) around the abscess, and seronegative abscess [111].

Patients who are asymptomatic with confirmed infection should be treated with intraluminal agents to prevent development of invasive amebiasis and ALA. These agents include diloxanide furoate and paromomycin [112].

Echinococcosis

Echinococcus multilocularis and *E. granulosus* are the etiological agents behind cystic echinococcosis or hydatid disease which can result in significant destruction of hepatic parenchyma. Infection occurs via ingestion of food contaminated with echinococcus eggs. Dogs and other canines such as foxes, coyotes, and wolves act as the reservoir [113]. *Echinococcus* will then penetrate into the duodenal wall and enter the portal vein to reach the liver and lungs. Once in the liver, *E. multilocularis* will cause small, interconnected multilocular cysts 1–10 mm in diameter that resemble alveoli [114, 115]. Patients typically present with abdominal pain if the cysts become large. The liver cysts are detected by abdominal imaging and have the potential to rupture.

Treatment includes percutaneous drainage vs. surgical removal of the liver cysts depending on the size, location, and symptoms. Chemotherapy with anti-helminthic agents mebendazole or albendazole is recommended as an adjunct treatment [116, 123]. These medications are usually recommended to start 1 month prior to the procedure and extend to 6 months afterward [117].

Clonorchiasis

Clonorchis sinensis is a liver fluke and member of the family Opisthorchiidae that causes clonorchiasis. It is contracted through ingestion of undercooked freshwater fish containing infective metacercaria. Infection is endemic in East Asian countries such as China and Thailand [118]. *C. sinensis* attaches itself to intrahepatic bile duct epithelium and can have a life span of more than 10 years. Bile ducts then become dilated and irregular before patients present with abdominal pain, fever, and peripheral eosinophilia [119]. Diagnosis of clonorchiasis is confirmed by identification of eggs in the feces vs. a PCR-based fecal test.

Treatment consists of anti-helminth therapy with praziquantel and albendazole. The standard dose of praziquantel is 25 mg/kg orally three times daily for 1–2 days [120]. This regimen is highly efficacious with cure rates >90%. As an alternative, one can consider albendazole dosed at 10 mg/kg orally twice daily for 7 days [121]. This regimen also shows good efficacy with cure rates >90%. Common side effects of both praziquantel and albendazole include headache, abdominal pain, nausea, and vomiting.

Table 14.9 briefly summarizes additional parasitic infections (protozoans, nematodes, and trematodes, respectively) not included in the above discussion.

Table 14.9 Additional parasitic infections of the liver

Parasite	Liver related presentation	Antibiotic regimen
Protozoan infections		
Malaria *Plasmodium vivax*, *P. ovale*, *P. falciparum*	Plasmodium life cycle has a hepatic stage and RBC stage Relapses from the liver are more likely with *P. vivax and P. ovale* Liver is the initial site of replication and serves as reservoir for hypnozoites during the liver stage, which may present with hepatosplenomegaly, jaundice, and icterus The asexual blood stages (rings, schizont, and trophozoite) cause symptoms of fever, chills, malaise, and myalgias	Uncomplicated infection with chloroquine-sensitive *Plasmodium spp.* *For P. falciparum:* Chloroquine phosphate 600 mg PO immediately, followed by 300 mg PO at 6, 24, and 48 h For *P. ovale* and *P. vivax* Chloroquine phosphate as above PLUS Primaquine phosphate 30 mg base PO daily × 14 days [126, 127]

(continued)

Table 14.9 (continued)

Parasite	Liver related presentation	Antibiotic regimen
Toxoplasmosis *Toxoplasma gondii*	Hepatomegaly, hepatitis, hepatic granulomas	Immunocompetent adults are not treated unless symptoms are severe or persistent
		For immunocompetent patients: Pyrimethamine 100 mg daily ×1–2 days and then 25–50 mg daily PLUS Sulfadiazine 1 g q6 h daily ×2–4 weeks PLUS Folinic acid 10–25 mg daily
		Alternative: Trimethoprim-sulfamethoxazole (5 mg/kg and 25 mg/kg, respectively) IV or PO BID ×2–4 weeks [128, 129]
Nematode infections		
Ascariasis *Ascaris lumbricoides*	Biliary ascariasis with adult worms and eggs in the bile ducts Symptoms of hepatomegaly, biliary colic, acute cholangitis, acute pancreatitis due to mechanical obstruction Liver abscess	Albendazole 400 mg PO once or mebendazole 500 mg PO once [130]
Hepatic capillariasis *Capillariasis hepatica*	Fever, hepatomegaly, RUQ abdominal pain	Drugs of choice include thiabendazole or albendazole (no current consensus on dosage or duration) [131]
Trematode infections		
Fascioliasis *Fasciola hepatica*	Fever, RUQ abdominal pain, hepatomegaly, biliary obstruction	Triclabendazole 10 mg/kg PO daily ×2 days [132]
Opisthorchiasis *Opisthorchis viverrini*	Fever, RUQ abdominal pain, liver abscess, cholangitis	Praziquantel 75 mg/kg divided into 3 doses PO ×1 day [131]

Conclusion

Patients with CLD are at an increased risk for both bacterial and parasitic infections due to a combination of mechanisms including compromised natural barriers, altered microbiome, decreased cellular defenses, malnutrition, and genetic factors. Cirrhotic patients are essentially in an immunocompromised state, and a full assessment of hepatic dysfunction must be factored into the treatment of infections. It is also important to offer supportive care such as adequate nutrition and avoidance of drug-induced liver injury.

Patients with end-stage liver disease should also be aware of their own risk for infection and be vigilant regarding initial signs and symptoms. They should be advised to seek medical attention early in the course of infection. The most common bacterial infections in those with underlying liver disease are SBP, UTI, pneumonia, and skin and soft tissue infection. Those with alcoholic liver disease may also be predisposed to pathogens that take advantage of alcohol's ability to increase susceptibility to pulmonary infections such as aspiration pneumonia, legionellosis, and tuberculosis. Many bacterial and parasitic infections are avoidable by improving sanitation conditions, good hygiene, avoiding sick contacts/at-risk animals, and proper preparation of raw foods. Physicians must also be attentive to opportunities for other preventative measures such as SBP prophylaxis and immunizations (e.g., hepatitis A and B viruses, influenza, and pneumococcus) [130]. Ultimately, morbidity and mortality related to infections in cirrhotic patients should be minimized through a combination of preventative measures, early recognition, supportive liver care, and careful consideration of antibiotics.

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Hepatitis A and Other Viral Infections

15

Yuval Ishay and Yaron Ilan

Abbreviations

CMV	Cytomegalovirus
EBV	Epstein-Barr virus
HAV	Hepatitis A virus
HHV	Human herpesvirus
HSV	Herpes simplex virus
IM	Infectious mononucleosis
PCR	Polymerase chain reaction
PTLD	Posttransplant lymphoproliferative disorder
VZV	Varicella-zoster virus
XLP	X-linked lymphoproliferative disorder

Key Points

- Hepatitis A virus activates all arms of the immune system. A profound humoral response assists both in diagnosis and in the protective immunity to vaccination.
- In addition to the hepatotropic hepatitis viruses A to E, a variety of viruses can affect the liver. These include Epstein-Barr virus, cytomegalovirus, herpes simplex virus, varicella-zoster virus, human herpesviruses (6, 7, and 8), human parvovirus B19, adenoviruses, influenza virus, and others.
- The clinical presentation of infections with these viruses may be indistinguishable from that associated with the "classic" hepatotropic viruses and can range from transient elevation of aminotransferases to severe liver failure.
- Both the innate and adaptive parts of the immune system play a role in the pathogenesis of virus-mediated target organ involvement.
- In most immunocompetent patients, an asymptomatic or mild disease occurs, while immunosuppressed patients and organ transplant recipients are at high risk for the development of severe infections and associated complications.
- Antiviral agents, as well as immune-based therapies, have a role in the treatment of immunocompromised patients and in immunocompetent patients who present with severe life-threatening disease.

Introduction

Viral-mediated liver injury can result from infections with the classic hepatotropic viruses, hepatitis A through E, or by other viruses [1]. In the present chapter, we review the immune-based pathogenesis and liver-related manifestations of hepatitis A virus as well as several additional viruses that affect the liver including Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), varicella-zoster virus (VZV), human herpesviruses (HHV 6, 7, and 8), human parvovirus B19, adenoviruses, and influenza virus (Table 15.1). The clinical presentations range from mild and transient elevation of aminotransferases to severe chronic

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liver disease and liver failure [1]. These viruses should be considered as possible etiologic agents in patients who manifest liver injury and whose serologic markers for the classic hepatotropic viruses are negative [1] (Table 15.2).

Table 15.1 Non-hepatotropic viruses that affect the liver

Herpesviruses: Epstein-Barr virus, cytomegalovirus, Varicella-zoster virus, human herpesvirus 6, human herpesvirus 7, and human herpesvirus 8
Erythrovirus: Parvovirus B19
Adenoviruses
Orthomyxoviruses: Influenza
Arenaviruses: Guanarito virus, Junín virus, Lassa fever virus, Machupo virus, and Sabiá virus
Bunyaviruses: Crimean-Congo hemorrhagic fever virus, Dobrava virus, Hantaan virus, Puumala virus, Rift Valley fever virus, and Seoul virus
Coronavirus: Severe acute respiratory syndrome virus
Filoviruses: Ebola virus and Marburg virus
Flaviviruses: Dengue, Lujo virus, Kyasanur Forest disease virus, Omsk hemorrhagic fever virus, and Yellow fever virus
Picornaviruses: Echovirus
Reovirus: Colorado tick fever virus and Reovirus 3

Hepatitis A Virus

Hepatitis A virus (HAV) is a member of the Picornavirus family, with a genome consisting of 7.5 kbs single-strand positive-sense RNA [2]. This single strand of RNA is translated into a polypeptide, cleaved to structural and nonstructural proteins, mostly via the viral protease 3Cpro, the only viral protease elaborated by the virus [3]. These proteins play a major part in the typical cellular membrane rearrangement observed in HAV-infected cells. This membranous complex has a role in further amplifying viral RNA replication [4]. Probably working within or proximally to these membranous complexes, cellular poly(rC) binding protein 2 (PCEP2), ATP binding cassette transporters, and FK506 binding proteins were shown to be essential for viral replication and translation [5, 6]. Once structural proteins are translated and the viral capsid is constructed, HAV virions are secreted in a non-cytopathic manner from the cell [7]. Both naked and quasi-enveloped virion are released from infected cells, and during infection, they may be found in the blood, feces, and hepatocytes [8, 9]. Quasi-enveloped

Table 15.2 Clinical features, diagnosis, and treatment summary table

Virus	Population at risk	Clinical and laboratory features	Acute liver failure	Unique complications	Diagnostic tests	Treatment	Active antivirals
EBV	IC Age >30 (esp. >60) XLP Solid organ transplant recipients (especially pediatric)	Lymphocytosis Monocytosis Splenomegaly Gradually rising liver enzymes	Rare More common in immunosuppressed patients (60% in patients with XLP)	Splenic rupture PTLD HLH AIH exac.	Monospot EBV VCA IgM PCR (blood/tissue) Liver biopsy – rarely	ICP: For severe complications Steroids Antivirals (if steroid refractory) IC: Anti-EBV CTLs Antivirals	Ganciclovir Valganciclovir Valacyclovir Famciclovir Foscarnet
CMV	IC Solid organ transplant recipients Neonates (congenital CMV) IBD	Hepatosplenomegaly Aminotransferases lower than in "classic viral hepatitis" Leukopenia Thrombocytopenia	Rare More common in IC	Graft loss, encephalitis, pneumonitis, hepatitis, uveitis, retinitis, and colitis	CMV IgM PCR (blood and tissue) Liver biopsy important	ICP – only in severe end-organ disease IC – antivirals ± IVIG Organ transplant – prophylactic versus preemptive treatment	Ganciclovir Valganciclovir Foscarnet Cidofovir CMV hyperimmune globulin Leflunomide
HSV	IC Pregnancy (3rd trimester) Neonates	Leukopenia, thrombocytopenia, relatively mild elevation in bilirubin Mucocutaneous lesions (50%)	Rare More common in pregnancy, IC, and neonates	Esophagitis Pneumonitis	HSV PCR (blood and tissue) Liver biopsy essential	Early high-dose acyclovir	Acyclovir
VZV	Adults IC Liver transplant recipients	Cutaneous rash	Rare More common in IC	Graft loss	Viral isolation from skin lesions HSV PCR (blood/tissue) Liver biopsy	Early therapy with acyclovir in severe disease or IC patients	Acyclovir

Abbreviations: *IC* immunocompromised, *ICP* immunocompetent, *XLP* X-linked lymphoproliferative disorder, *PTLD* posttransplant lymphoproliferative disorder, *HLH* hemophagocytic lymphohistiocytosis, *AIH* autoimmune hepatitis, *CTLs* cytotoxic T lymphocytes

virions are wrapped in cellular membranes, imparting resistance to neutralizing antibodies during acute HAV infection [10, 11].

Following ingestion of HAV virions, the initial site of replication and bloodstream entry is unclear. Initial infection and replicating have been posited to occur within gastrointestinal mucosal epithelial cells by direct invasion and replication at these sites, this hypothesis being supported by better virus replication when introduced to apical membranes of epithelial cells [12]. Replication or direct transcytosis via M cells located in Peyer's patches has been demonstrated in poliovirus [13], with which HAV shares several protein homologies [14]. Within this lack of clarity, further hypotheses have been made including amplification of viral uptake by IgA-mediated endocytosis [15]. It is likely these mechanisms work in cohort and need not be viewed as mutually exclusive.

Once infection has occurred, HAV localizes to hepatocytes, inside of which its replication is nearly exclusive. Entry into hepatocytes has also not been clearly elucidated as a single mechanism. A receptor for HAV intake into hepatocytes in humans has been recognized [16], and here also, the IgA-HAV complex has been demonstrated to take part in the virion endocytosis into hepatocytes [17], likely via the basolateral part of the hepatocyte [18].

With viral replication taking place mostly within hepatocyte, the mechanism for HAV-induced hepatitis also remains unclear. Liver biopsies performed during clinical and biochemical acute hepatitis reveal necroinflammation and ballooning degeneration of hepatocytes, accompanied by an inflammatory infiltrate [19]. HAV is largely agreed to be non-cytopathic and does not seem to greatly interfere with intracellular homeostasis. In tune with this assumption, peak HAV replication and shedding occur before maximal ALT elevation in infected patients [8].

HAV acts through nonstructural proteins 3ABC and 2B to inhibit cellular production of type 1 interferons [20]. Plasmacytoid dendritic cells have been shown to be recruited to the liver early in the process of infection and to be robustly stimulated to secrete interferon by infected hepatocytes. However, the presence of type 1 interferons remains low, and its peak seems to far predate the peak of hepatitis, and is thus unlikely to be the direct cause of hepatitis or directly aid in viral defense [21, 22]. Interferon-γ (IFN-γ) has been shown to be robustly secreted from infected hepatocytes in culture. These cells also secrete chemokines with the ability to attract other immune cells, but this secretion has been shown to be unrelated to IFN-γ secretion [23]. With no clear model of type 2 interferon (i.e., IFN-γ) secretion in vivo, it is difficult to allocate specific activity to these findings.

Chemokine secretion is likely an important part in the attraction of adaptive immunity cells to the liver. CD8+ T cells from HAV-infected patients have been shown to become activated after ex vivo reintroduction of HAV-infected cells and HAV-related peptides [24, 25]. Regulatory T cells (Tregs) have also been shown to be reduced in number and activity during acute HAV hepatitis [26], possibly driving CD8+ cell activation. While this mechanism was never directly demonstrated in vivo, it is likely CD8+ cell activity and regulation play a major part in both HAV-induced hepatitis and viral clearance.

Further studies have illuminated the role of other adaptive immunity cell in HAV infection. In HAV-infected chimpanzees, CD4+ T cells were demonstrated to be more robust in comparison to CD8+ cells and were characterized by cytokine production and a course of activity and proliferation related to HAV activity [27]. It is interesting to speculate whether this evidence for more robust CD4+ than CD8+ activation has to do with the function of hepatocytes as antigen-presenting cells [28].

While classically adaptive immunity cells have been associated with immune-mediated apoptosis, innate immunity mechanisms have been shown to take a large and active part in this process. In a model of HAV hepatitis, NK cells demonstrated strong lytic activity against infected cells, augmented by anti-HAV antibodies [29].

The innermost layer of innate immunity may be localized to the hepatocyte themselves. Despite HAVs ability to disrupt cellular immunity and type 1 interferon pathways, it appears intrinsic hepatocyte mechanisms including activation of mitochondrial-associated antiviral signaling (MAVS) and basal expression of interferon regulatory factors (IRFs) act to drive both hepatocyte immunity to RNA virus invasion and hepatocyte apoptosis [30, 31]. These mechanisms may have a heretofore unrecognized significance in both viral clearance and clinical hepatitis.

The most widely referred to layer of HAV immunity is humoral immunity. Robust IgM secretion appears concomitantly with the appearance of symptomatic hepatitis and aids in diagnosis [32]. Class switching later becomes the dominant response and IgG provides lifelong immunity against reinfection, with possible rare exceptions in the case of severe immunosuppression and lymphocyte depletion [33, 34]. A highly effective HAV vaccination has been licensed since the mid-90s and has been recommended to all US children since 2006. These developments have seen a drastic fall in epidemiological reports of HAV hepatitis cases in vaccinating countries. Predictions have the length of immunity at ~25 years, less than the lifelong immunity of persons previously infected with HAV. The clinical relevance of this predicted gap has yet to be encountered, though further booster shots may conceivably be needed to prevent waning immunity during middle age [35].

Epstein-Barr Virus

EBV Infection (Fig. 15.1)

Epstein-Barr virus (EBV), also known as human herpesvirus 4 (HHV-4), is a member of the Herpesviridae family and is a double-stranded DNA virus [1]. Its genome consists of a lin-

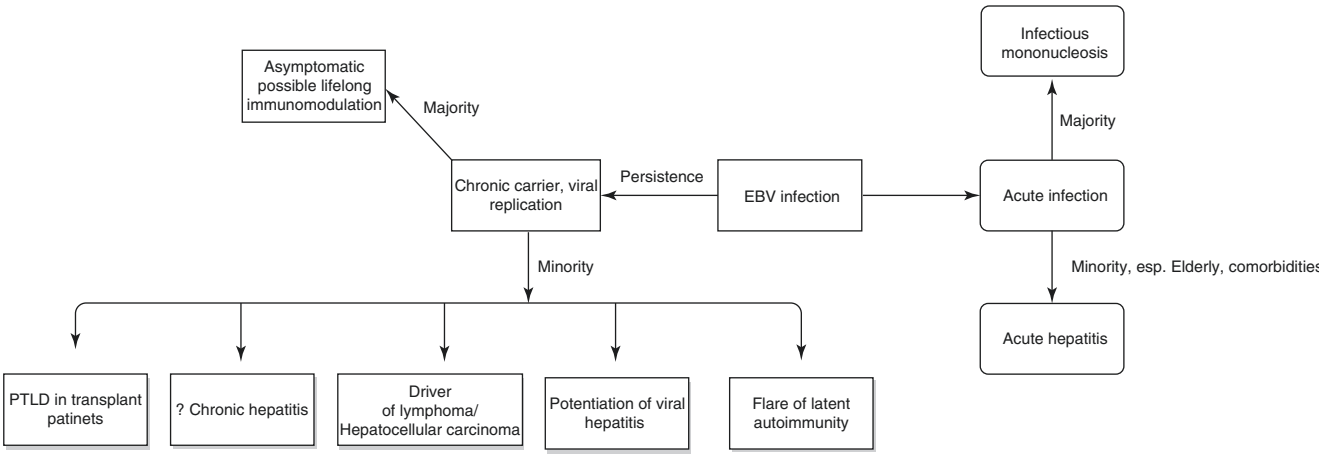

Fig. 15.1 Natural history and outcomes of EBV infection

ear DNA molecule that encodes nearly 100 viral proteins. Expression of different combinations of these proteins allows the virus to establish different forms of infection [36]. Cell entry and translocation of EBV particles to the nucleus are confirmed by the detection of the EBV genome in isolated nuclei [37]. EBV infection is a common and lifelong infection affecting over 90% of humans worldwide [38]. In the United States, EBV affects 95% of the young population between 35 and 40 years of age. The virus replicates in nasopharyngeal epithelial cells, and seropositive persons actively shed the virus in saliva [1, 39]. Transmission of EBV usually occurs by contact with oral secretions.

Diagnosis of EBV infection is based on clinical features and on laboratory and serological findings indicative of a recent infection. The most common is leukocytosis, which appears in 70% of cases, predominantly as lymphocytosis and monocytosis, and mild thrombocytopenia in up to 50% of affected individuals. EBV-specific IgG and IgM antibodies directed against the viral capsid antigens (VCA), the early antigens (EBV anti-D and anti-R), the nuclear antigen (EBVNA), and soluble complement-fixing antigens (anti-S) are used for viral detection [1]. The "monospot" test that detects heterophilic antibodies is sensitive but not specific. The diagnosis of EBV-associated hepatitis is established based on a combination of elevated aminotransferases, serology compatibility with active EBV infection, typical findings in liver biopsy, and the presence of the viral genome in liver tissue. A liver biopsy shows portal and sinusoidal mononuclear cell infiltration with focal hepatic necrosis or fatty infiltration [1, 40].

The Role of the Immune System in EBV Infection

Both the innate and the adaptive parts of the immune system play a role in anti-EBV immunity [41, 42]. B cells in the oropharynx may be the primary site of infection; resting memory B cells are thought to be the sites of persistent infection with EBV throughout the body. EBV infection of B cells triggers activation of several signaling pathways which are critical for cell survival, virus latency, and growth transformation [43]. Consequently, EBV has evolved several strategies to evade immune system recognition and to establish latent infection in memory B cells, where it resides lifelong without any ill effects in a majority of individuals [41].

After infecting B lymphocytes, the linear EBV genome becomes circular, forming an episome, which usually remains latent in these B cells. Several of the viral proteins are expressed in latently infected B cells in vitro. In immunocompetent individuals, EBV establishes in B cells as an asymptomatic lifelong latent infection controlled by the immune system. CCR1/CCR2B is involved in clearing latently infected B cells in immunocompetent individuals via directing migration of these cells and attracting chemokines-expressing immune cells [44]. However, limited gene expression during latency ensures successful escape of the infected B cells from cytotoxic T-cell (CTL) recognition [36]. Viral replication is spontaneously activated in only a small percentage of latently infected B cells [45]. Thus, imbalances in equilibrium between the virus and the host's immune system lead to the development of liver damage in EBV-infected patients.

Innate sensing and the resulting innate immune responses against EBV impact viral transmission between epithelial cells and B cells and their life cycle stages. Innate recognition and the resulting innate immune responses against EBV also involve myeloid cells, dendritic cells, monocytes, macrophages, neutrophils, and natural killer cells. Posttranscriptional gene regulatory factors are required for EBV lytic replication [46].

The tonsils are a primary site for EBV infection. EBV triggers monocyte toll-like receptors (TLRs) inducing maturation of dendritic cells (DCs), which activate CD16−CD56 bright NK cells via IL12. NK cells hamper pathogen entry at mucosal sites, restricting EBV infection until adaptive

immunity establishes control on the virus [47]. NK cells respond against EBV-infected B cells in the lytic cycle and control the viral infection by the involvement of IFN-γ secretion. IFN-γ secreted by DC-activated NK cells is associated with delayed expression of latent EBV antigens. It inhibits B-cell transformation, decreasing their proliferation during the first week postinfection [41, 48]. IFN-γ also promotes an EBV-specific adaptive immune response by favoring a Th1 polarization. NK cell Ab-dependent cellular cytotoxicity (ADCC) is triggered via FcgammaR-IIIA (CD16) in the response to EBV. Serum from EBV(+) individuals triggered vigorous NK cell degranulation and cytokine production (TNF-alpha and IFN-gamma) against EBV-infected cells, enhancing NK cell activation [49].

In an early phase after a primary viral infection, NK cells limit the viral burden until virus-specific T cells eliminate the infection or maintain viral titers at low levels. Innate immunity uses several "pattern recognition" receptors to sense pathogen-associated molecular patterns (PAMPs) [41]. TLR activation has downstream effects during primary EBV infection that favor viral latency or reactivation and facilitate immune control. Intact viral particles are recognized by the membrane surface receptor, TLR2 [50]. Following viral entry into cells, viral DNA is recognized by TLR9. Dual interactions via TLR2 on the cell membrane and intracellular TLR9 lead to a rapid production of IL-8, initiating effective antiviral immunity. Programmed death ligand 1 (PD-L1) is a membrane immunomodulatory protein, whose overexpression on the surface of tumor cells and antigen-processing cells (APCs) impairs T-cell-mediated killing. EBV infects monocytes using HLA-DR and induces a strong upregulation of PD-L1 expression on their surface. EBV activated TLR signaling, increased intracellular reactive oxygen species (ROS), and phosphorylated STAT3. Targeting these molecules reverted PD-L1 upregulation, altering cytokine production, and reduced monocyte cell survival, impairing the antiviral immune response [51].

EBV expresses several viral noncoding RNAs (ncRNAs) during latent infection, which have regulatory functions and can posttranscriptionally regulate viral and/or cellular gene expression. EBV-encoded RNAs (EBERs), the BamHI-A rightward transcripts (BARTs), a small nucleolar RNA (snoRNA), and viral microRNAs (miRNAs) are expressed during EBV infection in a variety of cell types [52]. EBV counteracts or exploits innate immunity in its latent and lytic life cycle stages via TLRs, EBERs, and microRNAs [53]. EBV encodes 25 viral precursor microRNAs within its genome that are expressed during lytic and latent infection. These viral miRNAs regulate the expression of viral and host genes. EBV infection induces the expression of cellular oncogenic miRNAs, such as miR-155, miR-146a, and miR-21, which contribute to the persistence of latently infected cells [54]. Several miRNAs, such as miR-BHRF16, show higher expression levels during primary infection [55]. Moreover, type I IFNs play critical roles in orchestrating the antiviral defense. It is observed that EBV-encoded miR-BART16 interferes with the type I IFN signaling pathway and directly targets CREB-binding protein, a key transcriptional coactivator in IFN signaling. Additionally, it abrogates the production of IFN-stimulated genes by inhibiting the antiproliferative effect of IFN-alpha, thus facilitating latency of EBV infection and enhancing viral replication [56]. EBERs are released from EBV-infected cells and induce biological changes in cells via signaling from TLR3. EBER-1 and EBER-2 are excreted from infected cells in exosomal fractions and are found to be present in the purified exosome fractions of EBV-infected cells [57].

An increase in neutrophils is observed during the initial phases of EBV infection, whereas a transient episode of acute neutropenia is often observed in infectious mononucleosis (IM) during the third week of illness [58]. Infected neutrophils rapidly die by apoptosis [59]. Secretion of various cytokines and chemokines (e.g., IL-1, IL-8, MIP-1α, LTB4, and reactive superoxide anion) promotes the development of EBV-specific immunity, whereas upregulation of IL-1R and induction of apoptosis in neutrophils inhibit anti-EBV immune responses [60].

Episodes of monocytopenia are observed during the acute phase of IM [41]. EBV impairs monocyte differentiation into DCs and reduces their survival. These effects correlate with macroautophagy/autophagy, ROS, and reduction of mitochondrial biogenesis. By inhibiting autophagy, EBV reduces ROS negatively, thereby affecting autophagy. It was revealed that reduction of autophagy correlated with the downregulation of RAB7 and ATG5 expression and STAT3 activation, thus upregulating the antioxidant response, reducing ROS, and further inhibiting autophagy [61]. By inhibiting the differentiation of monocytes into mature DCs, EBV temporarily halts the onset of immune responses during primary infection, enabling efficient viral replication. This permits the accumulation of a large pool of virus-infected B lymphocytes, allowing viral access to memory B-cell compartment, interfering with the functions of DCs during the initiation of virus-specific immunity, and modifying the profile of secreted cytokines, thus creating a favorable environment for viral propagation [37, 41]. Patients with EBV-associated malignancy show a deficiency in monocyte-mediated ADCC, along with a reduced phagocytic activity of EBV-infected monocytes [37]. In addition, EBV infection inhibits the functional ability of macrophages to respond to bacterial challenge by reducing their phagocytic potential [62].

CTLs are major determinants in the control of acute EBV infection and are directed against both lytic and latent antigens [63]. EBV induces strong CD8+ T-cell responses in primary infection yet persists for life, continually challenging T-cell memory through recurrent lytic replication and potentially influencing the spectrum of antigen-specific responses [64]. About half of the total CD8+ T cells in an acute infection are specific for a single lytic EBV epitope, and most of these epitope-specific cells have an activated/memory phe-

notype. In the late stages of infection, the frequency of epitope-specific CD8+ T cells directed against latent EBV proteins increases, confirming that CTLs are important for limiting infection in the convalescent phase of virus infection. Long-term EBV carriers generate robust polyfunctional T-cell (PFC) responses against lytic and latent EBV antigens. EBV antigen-specific CD4+ and CD8+ PFC responses emerge during the first year of primary EBV infection, with the greatest responses toward immunodominant epitopes in both lytic and latent proteins, correlating to a steady decline in peripheral blood mononuclear cells (PBMCs) and plasma viral loads. Both IM and asymptomatic (AS) patients had elevated PBMCs and plasma viral loads, which declined steadily during a 12-month period from the time of diagnosis. There was a decrease in the magnitude of CD8+ T-cell responses toward EBV lytic peptides, in contrast to an increase toward latent peptides [65]. An interferon-gamma (IFN-gamma) enzyme-linked immunospot (ELISPOT) assay using isolated CD8(+) and CD4(+) T cells stimulated with mRNA-transfected DCs showed that the frequency of latent membrane protein 1 (LMP1)-specific IFN-gamma-producing CD4(+) T cells was higher than that of LMP2a. Furthermore, the frequency of IFN-gamma producing CD4(+) T cells correlated with that of CD8(+) T cells in LMP1-specific immune responses. It was observed that CD8(+) and CD4(+) T cells from EBV-seropositive donors secreted only the Th1 cytokines – IFN-gamma, TNF-alpha, and IL-2 [66].

In lytic infections, the virus expresses a full complement of immediate early, early, and late lytic cycle proteins and is capable of replicating within the host cell [63]. In latent infection, the virus expresses fewer proteins, does not replicate, and persists within the host cell. EBV has developed an ability to rapidly promote the expression of its own genes while simultaneously shutting down the transcriptional program of its host cell [41]. TNF-α levels are increased in IM patients, indicating its importance in ongoing antiviral response. However, the virus inhibits TNF-α secretion by monocytes and macrophages [37] and downregulates TNF-α mRNA transcripts via suppressive action at the transcriptional level [41]. Besides, EBV proteins are also known to modulate IFN signaling [41, 67].

The life cycle of EBV is dependent on many viral proteins but also regulates a number of endogenous proteins. The 7TM receptor encoded by EBV *BILF1* downregulates cell surface MHC class I expression as part of the immune evasion strategy of EBV [68]. An EBV tegument protein, BGLF2, activates members of the mitogen-activated protein kinase signaling pathway. The protein is delivered to cells upon infection, activating signaling pathways to enhance viral production and reactivation from latency. Expression of *BGLF2* increased expression of EBV *BZLF1*, which in turn activates a switch from latent to lytic virus infection, and increased production of EBV [43].

EBV nuclear antigen 1 (EBNA1) is an EBV-encoded nuclear antigen and sequence-specific DNA binding protein required for viral binding and episome maintenance during latency. It binds directly to the promoter regulatory regions and upregulates the transcription of host genes that are important for the survival of EBV-infected cells [69].

Long-term virus carrier state along with a low-level virus replication and lytic antigen release is associated with a reshaping of the virus-specific response [64]. Screening against each of 70 EBV lytic cycle proteins in combination with HLA class I alleles revealed multiple reactivities against immediate early (IE), early (E), and late (L) lytic cycle proteins. Primary responses targeted IE and a small group of E proteins, in line with their presentation on the infected cell surface before late-expressed viral evasions occur.

EBV reactivation associated with increased specific CTL response to a lytic EBV epitope can lead to EBV-associated chronic hepatitis [70]. EBV reactivation in these patients is based on an increased percentage of terminally differentiated CD28−CD27−CD8+ T cells, suggestive of chronic antigen stimulation [70]. Diminished expression of co-stimulatory molecules, CD28 and CD27, compromises CD8+ reactivation, making cells more resistant to apoptosis [36, 71, 72].

While cellular immunity is fundamental for controlling both the primary and persistent phases of EBV propagation, the humoral response controls viral spread in late phases of infection [73]. EBV stimulates strong humoral responses to lytic cycle proteins. IgM and developing IgG responses to nucleocapsid and envelope proteins are detectable in primary EBV infections [41]. IgG responses to immediate-early and early lytic cycle proteins and to the latent proteins, EBNA1 and EBNA 2, are also detectable, together with neutralizing antibodies directed against gp350 [73].

EBV makes more than 12 glycoproteins, providing flexibility in the mode by which it colonizes its human host. Some of these are associated with transporting the virus through the cell membrane and toward the nucleus, and some glycoproteins help the virus to exit and infect the next cell in the same or a new host. They also weaken host defenses, helping the virus persist for a lifetime [74].

EBV represents a potentially important factor in the pathogenesis of several T-cell-mediated autoimmune disorders, with molecular mimicry as a likely mechanism. T-cell cross-reactivity reinforces the molecular mimicry in which microbial peptides sharing structural features with host peptides stimulate T cells that cross-react with self-peptides, initiating autoimmune disease. Natural presentation of a self-peptide is cross-recognized in the context of self-HLA by EBV-reactive CD8(+) T cells. As reported in a study, a human self-peptide (DELEIKAY) is a homolog of a highly immunogenic EBV T-cell epitope (SELEIKRY) presented by HLA-B*18:01. This self-peptide binds to HLA-B*18:01 and is presented by this HLA molecule on the surface of

human cells. A significant proportion of CD8(+) T cells raised in some healthy individuals against this EBV epitope cross-reacted with the self-peptide [75].

Role of the Immune System in EBV-Mediated Malignancy

EBV is a contributory factor in 1–2% of all cancers and is associated with the development of tumors such as lymphoproliferative disorders, Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma [41, 76].

Upon primary infection, EBV transiently undergoes a short lytic cycle and then predominantly establishes a latent infection. Only a small percentage of infected cells switch from the latent stage to the lytic cycle to produce progeny viruses. EBV in cancer cells is mostly in the latent state; however, the lytic cycle of the virus also contributes to tumorigenesis via the secretion of cytokines or growth factors [77]. Transforming growth factor beta 1 (TGFβ1) contributes to the pathogenesis of EBV-mediated cancer [78]. EBV is latent in lymphocytes and can detach from the cytoplasm to form a circular DNA molecule integrating into cellular the chromosomes. The interaction between EBV latent genes and oncogenes leads to host cell cycle disturbances, including the promotion of G1/S phase transition and inhibition of cell apoptosis, promoting the development of EBV-associated neoplasms [79]. The latent genes of EBV modulate cell death associated with growth transformation and lymphomagenesis and also regulate cell death pathways in Burkitt's lymphoma and lymphoblastoid cell lines (LCLs) [76]. Reactivation of the virus from latency is dependent on expression of the viral BZLF1 protein. The BZLF1 promoter (Zp) exhibits low basal activity but is activated in response to chemical or biological inducers. These mechanisms control the EBV lytic switch and contribute to the oncogenesis [77]. EBV-associated malignancies and LCLs express latent viral proteins and maintain an ability to grow indefinitely through the inappropriate activation of telomere-specific reverse transcriptase (TERT) – a catalytic component of a telomerase. BATF, a transcription factor activated by NOTCH2, the major NOTCH family member in B cells, negatively affects the expression of BZLF1, the master regulator of viral lytic cycle. High levels of endogenous TERTs are associated with high NOTCH2 and BATF expression levels, contributing to the preservation of EBV latency in B cells via the NOTCH2/BAFT pathway [80]. In EBV-induced cancers of epithelial origin, including nasopharyngeal carcinomas (NPCs) and gastric carcinomas, the latent EBV genome expresses high levels of a cluster of 22 viral pre-miRNAs. miR-BARTs exert an antiapoptotic effect in EBV-infected epithelial cells [81].

Epigenetic modifications of the viral and host cell genomes occur in EBV-associated lymphomas and carcinomas. Viral oncoproteins interact with the same epigenetic regulators and alter their cellular epigenotype and gene expression patterns. Hypermethylated promoters are unique EBV-associated epigenetic signatures in EBV-positive gastric carcinomas. EBV-immortalized B-lymphoblastoid cell lines are characterized by genome-wide demethylation and loss and rearrangement of heterochromatic histone marks [82]. In the initial stages after EBV infection, B cells undergo a transient period of hyper-proliferation, which results in replicative stress and DNA damage, activation of the DNA damage response (DDR) pathway, and, ultimately, senescence. Arrested EBV-infected B cells manifest an increase in the presence of telomere dysfunction-induced foci. Increasing human TERT expression permitted early EBV-infected B cells to overcome cellular senescence and enhanced transformation [83].

Epstein-Barr virus nuclear antigens (EBNA3A, EBNA3B, and EBNA3C) are latency-associated proteins expressed in B cells that are induced to proliferate by the virus. Together with other nuclear antigens, they are expressed from a polycistronic transcription unit that is unique to B cells. EBNA3s are required for the persistence of EBV in the B-cell system and in modulating B-cell lymphomagenesis, restraining the oncogenic capacity of EBV [84].

Mutations in SAP (signaling lymphocyte activation molecule (SLAM)-associated protein) are associated with a loss of EBV-specific immune control [41]. During EBV latency, the virus develops mechanisms of immune escape from innate immunity-dependent mechanisms, including the inhibition of NK cell activation through EBV-induced gene 3 (EBI3) [41]. EBV-transformed B lymphocytes express high levels of EBI3 protein, which has an immunosuppressive activity [60]. The expression of viral antigens by malignant cells makes them suitable targets for immune therapy. The demonstration that immunotherapeutic approaches are effective for some of these cancer patients further supports a role for the immune system in limiting the pathogenesis of EBV virus [41]. Infusion of EBV-specific cytotoxic T lymphocytes has proved to be safe and effective and induces protective antivirus immunity, which is lacking in EBV-associated malignancy [41]. Innate lymphocytes also play a role in resistance to EBV-associated malignancies. EBV type II latency tumors, such as Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), and nasopharyngeal carcinoma, express a limited array of EBV antigens including Epstein-Barr nuclear antigen (EBNA)1, latent membrane protein (LMP)1, LMP2, and BamH1-A right frame 1 (BARF1). Adoptive immunotherapy for these malignancies has focused on EBNA1, LMP1, and LMP2. BARF1-specific T-cell lines contain CD4- and CD8-positive T-cell subpopulations. Targeting BARF1, in addition to EBNA1, LMP1, and LMP2, improves the efficacy of T-cell immunotherapy against these malignancies [85]. Epstein-Barr virus LMP1 is an oncoprotein required for immortaliz-

ing B lymphocytes and transforms nonlymphoid tissue. Over 1000 proteins with direct or indirect relationships to LMP1 are discovered, some of which are involved in signal transduction and protein or vesicle trafficking [86]. Latent membrane protein 2A (LMP2A) promotes activation and proliferation of infected B cells and is expressed in many types of EBV-associated cancers and reduces the reactivity of CD8+ T cells against EBV-infected cells [87]. LMP2A mediates a rapid onset of lymphoma by allowing B cells to bypass apoptosis mediated by the p53 pathway in mice [88]. Overexpression of human MutS homologue 2 (hMSH2), a stress-inducible protein ligand for human gammadelta T cells, was shown in EBV-transformed B lymphoblastic cell lines (B-LCLs) and EBV-positive B lymphoma cell lines. Consequently, its overexpression can serve as a potential target for establishing gammadelta T-cell-based immunotherapies [89]. Besides, COX-2, a key mediator of the inflammatory processes, is frequently overexpressed in EBV-positive cancer cells. Upregulated COX-2 levels modulate the events in EBV life cycle related to latency-lytic reactivation through its downstream effector PGE2 [90]. It is observed that EBV-positive lymphoproliferative disorders express PDL1. PD1-positive tumor-infiltrating lymphocytes are found in these tumors. An active engagement between PD1 and PDL1 and EBV-positive LPDs that are positive for PDL1 may be suitable for PD1/PDL1 antibody therapies [91].

Clinical Manifestations Affecting the Liver in Acute EBV Infection

EBV infects up to 95% of the adult human population, with a primary infection typically occurring during childhood, and is usually asymptomatic. However, EBV infection can result in infectious mononucleosis, as well as in various and often fatal clinical sequelae, including fulminant infectious mononucleosis, hemophagocytic lymphohistiocytosis, lymphoproliferative disease, organomegaly, and/or malignancy. Such clinical outcomes are typically observed in immunosuppressed individuals [92, 93]. Various additional clinical conditions have been associated with EBV, including chronic infections, Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, peripheral T-cell lymphoma, and post-transplant lymphoproliferative disease (PTLD) [94, 95]. Proteins produced by EBV in latent infections suppress cytokines or upregulate PD-1 in B cells to repress the cytotoxic T-cell response. Many malignancies, including Hodgkin lymphoma and non-Hodgkin's lymphomas, occur at a much higher frequency in EBV-positive individuals during HIV infection [96].

Transmission of EBV generally occurs not only through oral secretions but also via blood transfusions and organ transplantations. A primary EBV infection takes place in the oropharyngeal region; the virus is transported by saliva droplets from infected individuals. The primary infection leads to transient viremia followed by a strong T-cell adaptive immune response that retains the infection in a latent stage in immunocompetent individuals [94, 97]. If the infection occurs in adolescence or adulthood, it can cause infectious mononucleosis (IM), a self-resolving lymphoid disorder largely resulting from an uncontrolled T-cell reaction directed against EBV-infected cells. In IM patients, EBV is found in blast cells that proliferate under the influence of latent genes [41]. Following resolution of the primary infection, EBV establishes a lifelong persistence in memory B cells, in which the virus remains clinically silent. In this B-cell reservoir, viral expression is repressed, a process described as "true latency." Short episodes of spontaneous reactivation and consequent viral replication normally occur in healthy individuals [97]. Manifestations affecting the liver in immunocompetent hosts range from mild self-limiting acute hepatitis to occasional reports of fatal acute fulminant hepatitis. Abnormal liver blood tests are common in EBV infection and occur in more than up to 90% of patients, but symptomatic hepatitis is rare [95]. Jaundice is present in only 5–10% of cases. Typically, the rise in aminotransferases is gradual, reaching a peak that is lower than that encountered in acute viral hepatitis [1]. The diagnosis of EBV infection is confirmed by the presence of a lymphocytosis and/or splenomegaly [95].

Compared with IM, which usually affects young patients, EBV hepatitis usually affects older people. In a review reporting a large cohort of patients, 59% were aged >30, and 41% were ≥60 years [95]. While 88% had clinical or biochemical jaundice, 100% had lymphocytosis and 88% had splenomegaly; only 12% manifested the classic symptoms of IM. Symptoms lasted for a median of 8 weeks, and only a minority of patients required brief hospitalization. However, severe cholestatic jaundice and right upper quadrant abdominal pain, which could be mistaken for bile duct obstruction, may occur in elderly patients [98]. In this setting, indirect hyperbilirubinemia resulting from EBV-associated autoimmune hemolytic anemia is more commonly the cause of jaundice than viral-induced cholestasis. Other occasional clinical settings for the involvement of EBV in manifesting liver disease include posttransfusion hepatitis, granulomatous hepatitis, and fatal fulminant hepatitis [1, 99]. Primary EBV infection accounts for <1% of adult acute liver failure (ALF) cases but is associated with a high case fatality rate. Liver transplantation (LT) is associated with favorable short- and long-term outcomes. Among the 1887 adult ALF patients enrolled in the US ALF Study, there were four patients (0.21%) with EBV-related ALF. All patients were treated with antiviral agents – two died, one underwent LT, and one survived with supportive care [100]. EBV superinfection may occur in patients with preexisting autoimmune hepatitis,

resulting in severe hepatic decompensation [101]. Cases of liver failure were described both in immunocompromised and immunocompetent hosts [99, 102, 103].

Viral replication may cause significant clinical symptoms and severe complications in patients with diminished cell-mediated immunity [36, 104].

EBV DNA in blood can be quantified in PBMCs, in circulating cell-free (CCF) DNA specimens, or in whole blood. CCF viral DNA may be actively released or extruded from viable cells, packaged in virions, or passively shed from cells during apoptosis or necrosis. In infectious mononucleosis, viral DNA is detected in each of these specimens [105]. In a population survey, anti-EBV capsid (VCA; IgG and IgM), nuclear (EBNA; IgG), and early (EA-D; IgG) antigens were studied. DNA was extracted from the buffy coat and subjected to EBV-DNA quantification using qRT-PCR. It was observed that 97.9% of the samples were seropositive for VCA-IgG, while 52.6% had detectible EBV-DNA. EBV seroprevalence and viremia rates increased with age [106]. A high level of HEV, EBV, and CMV IgM cross-reactivity was demonstrated, indicating that serology is unreliable in the diagnosis of acute viral hepatitis. Thus, it is suggested that the diagnosis of viral hepatitis should be based on clinical features, raised transaminases, serology, and confirmatory PCR testing [107]. Quantification of EBV copy numbers is a useful diagnostic marker. Furthermore, 25% of EBV viral DNA was detected in plasma or PBMCs, which was clinically significant. When EBV was detected in the absence of an EBV(+) disease, it was present only in the PBMCs in 69% of cases. Immunocompromised patients were less likely to have EBV in plasma than in PBMCs in the absence of EBV(+) disease. In patients with active, systemic EBV(+) disease, EBV was detected in plasma in 99% of the cases but was present in PBMCs in only 54% cases. EBV in plasma had higher specificity and sensitivity for EBV(+) disease than those with EBV in PBMCs [108].

EBV-Mediated Chronic Liver Damage

Persistent infection by EBV is explained by the germinal center model (GCM). The virus persists quiescently in resting memory B cells for a lifetime of the host in a nonpathogenic state that is undetectable to the immune response elements. EBV infects naive B cells in the lymphoepithelium of the tonsils and activates these cells using the growth transcription program. These cells migrate to the GC and switch to a more limited transcription program, holding them into a memory compartment where the virus persists. Infected memory cells return to the lymphoepithelium and differentiate into plasma cells, thereby activating viral replication. The released virus infects more naive B cells or is amplified in the epithelium for shedding. This cycle of infection and the quiescent state in memory B cells allows for a lifetime persistence of EBV at very low levels and is stable over a period of time [109].

Chronic active EBV infection (CAEBV) may result from a disturbance in the host-virus balance and Th1/Th2 imbalance, associated with an aggressive clinical course. CAEBV is defined by chronic severe illness, which begins as a primary EBV infection manifested by elevated transaminases, abnormal EBV serology, suggestive histopathological features, serological profile, and detection of viral genome in the liver tissue. Evidence of recurrent EBV reactivation, increased circulating EBV-specific CTLs, and increased CD38 B-cell expression, along with increased LDH levels, mild splenomegaly, and thrombocytopenia, supports the diagnosis [36, 110]. Severe CAEBV disease is defined as a severe progressive illness lasting 6 months or longer with infiltration of tissues with EBV-positive lymphocytes, markedly elevated levels of EBV DNA in the blood, and no known immunodeficiency. These patients usually have fever, splenomegaly, and lymphadenopathy and may have markedly elevated EBV antibody titers to viral capsid antigen. However, for most cases of severe CAEBV, the cause is unknown [111]. Specific latent antigens, as well as EBER transcripts, were detected in infiltrating CD8+ CTLs [36]. Chronic hepatitis can be induced by soluble Fas-ligand, TNF-α, and IFN-γ. Activated CD8+ cells are trapped in the liver via specific adhesive molecules expressed by Kupffer cells and sinusoidal endothelial cells [112–114]. Reactivation of infection leading to liver damage may occur whether the infected lymphocytes are incidentally or intentionally present in the liver. CAEBV may progress to a chronic or recurrent IM-like disease [115]. In Western countries, CAEBV is milder than in Asian countries [36]. The mild form is characterized by intact immune control of B cells, low viremia, and EBV-specific CTL expansion comparable to those of seropositive patients.

Patients with iatrogenic, congenital, or acquired immunodeficiency are at increased risk for EBV-associated lymphomas and CAEBV. Immune senescence in the elderly is also associated with both reactive and neoplastic EBV-driven lymphoproliferative disorders. EBV may also trigger autoimmune hepatitis [116], chronic granulomatous hepatitis [117], and vanishing bile duct syndrome [118]. Chronic EBV hepatitis in immunocompetent patients was suggested in several studies [110]; however, EBV was not detected in human hepatocytes [36]. EBV in this setting may be referred to as an "incidental virus," reflecting a coinfection with other hepatotropic viruses that are a more likely cause of chronic liver disease; moreover, they cause amplification of the EBV genome in circulating B cells rather than the liver [36].

In some patients with chronic liver disease caused by a major hepatotropic virus, a co-EBV infection was suggested. In a cohort of patients with chronic hepatitis B and C, patients

with reactivated EBV infection had lower levels of HBV DNA and higher mean values of serum hepatitis C virus (HCV) RNA, respectively, than those in EBV patients without reactivated infection [36]. Moreover, EBV reactivations may precede HBV flares. Reactivation of EBV-specific T cells promotes production of several cytokines such as interferon-γ (IFN-γ), interleukin (IL)-1, IL-2, and IL-10. EBV BCRF1 shares a high-sequence homology with IL-10, and it is known exogenous IL-10 enhances HCV replication. In addition, EBNA1 can also promote HCV replication. However, IFN-γ inhibits HBV replication in the absence of cell necrosis. Furthermore, studies have revealed that T-cell cross-activation may also explain HBV or HCV reactivation [36].

Epstein-Barr virus-associated T-/natural killer cell lymphoproliferative diseases (EBV-T/NK-LPDs) are a group of rare diseases resulting from ectopic infection of T or NK lymphocytes with EBV. EBV-T/NK-LPDs include chronic active EBV infection, EBV-associated hemophagocytic lymphohistiocytosis, hydroa vacciniforme-like lymphoproliferative disease, and severe mosquito bite allergy [119]. CAEBV of T-cell or NK-cell type is an EBV+ polyclonal, oligoclonal, or often a monoclonal LPD with different clinical presentations, including systemic and cutaneous disorders, hydroa vacciniforme-like T-cell LPD, and mosquito bite hypersensitivity. The systemic form of the disease is characterized by fever, persistent hepatitis, hepatosplenomegaly, and lymphadenopathy, which shows varying degrees of clinical severity depending on the immune response of the host and the EBV viral load [120].

Posttransplant Lymphoproliferative Disorder

Posttransplant lymphoproliferative disorder (PTLD) is a spectrum of lymphoproliferative diseases occurring in a posttransplantation setting. Most PTLDs are caused due to activation of B cells, whereas two-thirds of the cases showed an EBV infection of the neoplastic cells [121]. The incidence of PTLD ranges from 0.5% to 30% [122]. Risk factors include EBV seronegativity at the time of transplantation, the type of organ transplanted (being highest in lung and heart and lowest in liver and kidney recipients), and the level and type of immunosuppression (specifically anti-T-cell immunosuppression) [123]. PTLD causes complications of up to 10% in pediatric liver graft recipients, with a mortality of up to 50%. In the pediatric population, posttransplant primary infection within 3 months of orthotropic liver transplantation (OLT) was associated with sustained EBV detection and increased the risk of the late occurrence of PTLD [124]. PTLD emerges either from a recipient or donor origin depending on the type of transplant. Bone marrow transplant (BMT) patients develop PTLD of donor origin when EBV-infected B cells derived from the donor marrow proliferate into a lymphoma. Conversely, solid organ transplant patients develop PTLD of recipient origin, in which the EBV released from the transplanted organ infects the recipient's B cells [41, 123].

The spectrum of PTLD ranges from polymorphic lymphocyte proliferation to high-grade life-threatening monoclonal lymphomas [123]. The interplay between the EBV life cycle, latency, and nonviral factors determines the histology and clinical presentation of the disease. In vitro transforming abilities of EBVs, distinctive latency, and clonality within the malignant cells determine the biology of the disease [123]. Measurement of viral load by quantitative PCR can assist in the surveillance and diagnosis of PTLD [123]. Posttransplantation patients should be monitored for EBV PCR levels in the peripheral blood to detect active EBV infection early, and preemptive therapy should be instituted prior to the development of overt PTLD.

In transplanted patients, miR-BART22 serum levels in patients with positive EBV PCR were significantly higher than those in patients with negative EBV PCR and served as a potential biomarker for EBV reactivation [125]. A total of 304 patients with PTLD were followed, of whom 103 tested seronegative for EBV at transplantation. Following transplantation, 48% of seronegative patients initially developed EBV infection (based on PCR assays for EBV DNA), several of whom ultimately reverting to the negative state. Among the 201 seropositive patients, only 19% presented a reactivation of EBV. Having a maximum peak of EBV viral load above the median value was an independent predictor of PTLD [126]. NF-kappa B signaling components were present in a majority of PTLD-derived B cells. Subgroups related to EBV infection, mainly latency type III and mostly lacking CD19; upstream B-cell signaling and NF-kappa B constituents related to EBV infection with expression of the alternative NF-kappa B pathway compounds, RelB, CD10, FOXP1, or MUM1; and compound p65m unrelated to virus infection with expression of the classic NF-kappa B pathway were identified [121]. In a study of 176 adults with PTLD, 33% were EBV negative and 67% EBV positive. EBV-negative PTLD had distinct characteristics (monomorphic histology, longer latency) though high-risk features (advanced stage, older age, high lactate dehydrogenase, central nervous system involvement) were not common compared to EBV-positive PTLD. EBV negativity was not significantly associated with a weak response to initial therapy. The likelihood of achieving a complete remission (CR) was not significantly different for EBV-negative versus EBV-positive PTLD including when therapy of immunosuppression was reduced either alone or with rituximab. EBV negativity was also not associated with poorer overall survival [127].

Management options for PTLD include reduction of immunosuppression, biological therapy with anti-B-cell antibodies, combination chemotherapy, and adoptive immu-

notherapy using EBV-specific CTLs [128]. Surgery may be considered for localized PTLDs. Reduction of immune suppression alone results in clinical remission in 25–63% of adults and in 40–86% of pediatric PTLD patients by restoring EBV-specific immunity [123]. These patients should be monitored closely for acute allograft rejection. Newer immunosuppressants, including mycophenolate mofetil and sirolimus, appear to be associated with fewer posttransplant malignancies. Out of patients with X-linked lymphoproliferative disorder (XLP), approximately 60% may develop a severe form of IM with hemophagocytic lymphohistiocytosis and fulminant hepatitis. Treatment consists of etoposide-based chemotherapy and hematopoietic stem cell transplantation. Early treatment of primary EBV infection in these patients (prior to development of HLH) may comprise treatment with anti-CD20 antibodies in combination with antivirals (acyclovir or ganciclovir), IVIG, or steroids.

Pretransplant administration of rituximab is an effective and nontoxic intervention that drastically reduces EBV reactivation and PTLD in high-risk patients. Among 147 patients who did not receive rituximab, the cumulative incidence of posttransplant EBV reactivation and of EBV PTLD was 13% and 8%, respectively. Among 51 who received pretransplant rituximab, the incidences were 2% and 0%, respectively [129]. Adoptive transfer of EBV-specific CTLs was suggested as an immunotherapy to effectively prevent or treat these complications. Identifying HLA-A*03:01-restricted EBV-CTL epitopes as immunodominant targets was performed for improving the efficacy of these therapies [130].

EBV-Mediated Liver Cancer

EBV or infected cell clones can promote the replication of HCV and have been suggested to be involved in the development of hepatocellular carcinoma (HCC). EBV-infected cells support HCV replication better than uninfected cells, suggesting that EBV may act as a helper virus to promote HCV replication in HCV-positive HCCs. A higher amount of EBV DNA was reported in HCV-positive HCC compared to that in HBV-associated HCC. In some studies, up to 30% of liver cancers were found to harbor EBV DNA [131]. This finding, however, was not confirmed in other studies. A possible source of the detected EBV DNA could be the infiltrating lymphocytes [36]. The weak positivity of EBV DNA in some liver tissues was explained by amplification of EBV DNA in the lymphoid infiltrate or blood, reflecting a high EBV DNA load in these patients. A retrospective analysis of 15 studies containing a total of 918 cases of HCC, cholangiocarcinoma, and gallbladder carcinoma and 157 controls showed that the infection rate of EBV was 23% among all the patients. Comparable EVB infection rates were observed in hepatobiliary system cancer [132].

Treatment of EBV Hepatitis

Primary EBV infection is subclinical in the majority of immunocompetent individuals; and it may lead to IM in adolescents and adults and is generally self-limiting. Therefore, in immunocompetent individuals, symptomatic treatment alone is recommended. In patients suffering from IM, avoiding exertion and participation in sports is recommended for at least 3 weeks due to the rare risk of splenic rupture. A few patients who suffer from severe complications of acute EBV are usually treated with corticosteroids even though there is little evidence to support their use [133, 134]. The use of antivirals in the management of severe EBV infections in immunocompetent hosts is debatable. However, it is suggested as an adjunct to steroid treatment [135] and mainly for refractory disease [136]. Several antiviral drugs, including acyclic nucleoside and nucleotide analogues and pyrophosphate analogues, inhibit replication of EBV in cell culture via inhibition of EBV DNA polymerase. Acyclovir inhibits in vitro EBV replication and transiently reduces viral shedding in the oropharynx but does not reduce viremia or symptoms. Ganciclovir was effective in the treatment of EBV hepatitis in a small number of children and in adults [137]. Valganciclovir, the oral pro-drug of ganciclovir, has been successfully used in the treatment of severe acute EBV hepatitis (900 mg × 2, daily for 15 days) [136]. Additional drugs with antiviral activity against EBV include valacyclovir, famciclovir, and foscarnet. Patients with acute liver failure should be considered for urgent liver transplantation as the likelihood of spontaneous recovery is small [138]. Patients with immunodeficiencies are at an increased risk of liver failure and development of lethal lymphoproliferative diseases. The major pathogenic causes thought to be important in the development of lymphoproliferative disorders/lymphomas are primary immunodeficiency [X-linked lymphoproliferative syndrome (XLP), ataxia telangiectasia syndrome, Wiskott-Aldrich syndrome, Chediak-Higashi syndrome, SCID, CVID, and others], immunosuppressive therapy, and HIV/AIDS. In these patients, primary EBV infection should be treated preemptively with ex vivo-generated EBV-specific CTLs or with effective antiviral medication. In seronegative patients with XLP, monthly prophylaxis with IVIG is recommended [139]. Several experimental therapies are being evaluated. Heat shock protein 90 (HSP90) inhibitors have been shown to kill EBV-infected cells by reducing the level of EBV EBNA-1 and/or LMP1. Ganetespib is an HSP90 inhibitor evaluated in clinical trials for cancer and was demonstrated to kill EBV-positive B and T cells and reduce the levels of both EBV EBNA-1 and LMP1. Treatment of cells with ganetespib also reduced the level of pAkt. Treatment of a patient with T-cell chronic active EBV with ganetespib reduced the percentage of EBV-positive cells in the peripheral blood [140]. Herpesvirus entry into cells requires a coordinated action of

multiple virus envelope glycoproteins, including gH, gL, and gB. Regarding EBV, the gp42 protein assembles into complexes with gHgL heterodimers and binds HLA class II to activate gB-mediated membrane fusion with B cells. EBV tropism is dictated by gp42 levels in the virion. The gHgL and gB proteins are targets for neutralizing antibodies and potential candidates for subunit vaccine development. Anti-gHgL neutralizing antibodies block gHgL-mediated activation of gB through different surface epitopes and mechanisms [141]. Ideally, prophylactic EBV vaccines should be capable of priming the immune system against lytic and latent proteins. In one study, immunogenic particles that contained antigens from both these cycles were prepared. These particles enabled the ex vivo expansion of cytolytic EBV-specific T cells that efficiently control EBV-infected B cells, preventing their growth. It was observed that particles containing the latent protein, EBNA1, provided protection against wild-type EBV in a humanized mouse model [142]. Furthermore, linear and conformational B-cell epitopes as well as CTL epitopes were predicted by using Web servers for EBV proteins (GH, GL, GB, GN, GM, GP42, and GP350). A panel of epitopes that could be used for immunization against multiple diseases caused by EBV were detected [38].

Cytomegalovirus

CMV Infection and Diagnosis

Human cytomegalovirus (CMV) is a ubiquitous virus that causes chronic infection and, thus, is one of the causes of the most common infectious complications of immunosuppression. CMV both evades and shapes the immune responses [143]. CMV is a double-stranded DNA virus, the largest member of the beta Herpesviridae family. CMV infection is characterized by a spectrum of clinical syndromes ranging from asymptomatic infection to life-threatening congenital CMV syndrome in neonates, to infectious mononucleosis syndrome in young adults, and to severe pulmonary, retinal, neurological, gastrointestinal, and hepatic diseases in immunocompromised hosts [1]. Infection can be acquired in the perinatal period and infancy or in adulthood through sexual contact, blood transfusion, or organ transplantation [1].

Serologic studies of CMV-IgM antibodies are helpful for the diagnosis of primary infections. Viral culture techniques have been largely superseded, making way for molecular techniques to detect early antigen or CMV DNA, thus increasing sensitivity for detecting CMV infection in blood and end-organ tissue. However, to establish the diagnosis of active CMV infection, it is necessary to have histological evidence of cellular injury associated with the infection. Distinct pathologic findings of liver biopsy are important for the diagnosis of CMV hepatitis, especially in immunocompromised hosts. Giant multinucleated cells with an associated inflammatory response, multifocal necrosis, and biliary stasis are common. Large nuclear inclusion-bearing cells, so-called "owl's eye" inclusions, can be detected in hepatocytes or bile duct epithelium.

The Immune Response to CMV

The immune response to CMV is characterized by extremely elevated T-cell and antibody responses that persist for a lifetime but do not prevent superinfection with other CMV strains [144]. CMV shapes both innate and adaptive immunity in humans [145]. Changes in the T-cell pool caused by CMV infection contribute to immunosenescence, but CMV may also have beneficial effects in young individuals, improving the immune response to other pathogens [146]. The CD8 T-cell response is the most important effector response. However, CD4 T cells and also gamma/delta T cells and NK cells are involved in the response [145]. CMV-specific CD4(+) T cells possess antiviral functions and participate in anti-CMV humoral/cellular responses [147]. Subjects with effective CMV control, evidenced by low CMV IgG titers, have effective responses to CMV driven by either NKG2C+ NK cells or CMV-specific T cells [148]. It is ascertained that regulatory T cells (Tregs) have divergent control of CMV infection in a mouse model. In the spleen, Tregs antagonize CD8+ effector function and promote viral persistence, while in the salivary gland, Tregs prevent IL-10 production and limit viral reactivation and replication [149].

Tissue T-cell reservoirs for CMV control are shaped by both viral and tissue-intrinsic factors. T-cell differentiation is enhanced in sites of viral persistence with age. CMV-specific T cells were found to be present in the blood, bone marrow (BM), or lymph nodes (LN). CMV genomes were detected predominantly in the lungs and also in spleen, BM, blood, and LN [150].

Adoptive transfer of CMV-specific T cells has emerged as an effective method to reduce the risk of infection and/or reactivation by restoring immunity in transplant recipients. A majority of CMV-specific CD8(+) T-cell population is made up of terminally differentiated effector T cells with effector functions. Self-renewing memory T cells within the CMV-specific population retain the capacity to expand and differentiate upon rechallenge and are important for long-term persistence of the CD8(+) T-cell response. Mucosal organs, the sites of CMV reactivation, are primarily inhabited by tissue-resident memory T cells, which do not recirculate [151]. NK cells also play a role in the control of CMV; the virus developed immunoevasion mechanisms targeting these cells [143]. CMV infection is associated with the presence of a population of CD16(+) CD56(dim) NKG2C(+) NK cells in both acutely and latently infected individuals. An accumula-

tion of NKG2C(+) NK cells over a period of time, which preferentially expressed CD57, was shown during the infection. This accumulation is particularly prominent in elderly. Latent CMV infection is sufficient for NKG2C(+) CD57(+) NK cells to persist in healthy individuals but is not necessarily required in old age [152]. A study reported that CMV is associated with autoimmune diseases. CMV cross-reactive autoantibodies that recognize CIP2A on NK possibly impact their function in autoimmune patients [153].T cells expressing CD56 (NKT-like cells) are cytotoxic effector cells. The percentage of NKT-like cells increases with the combination of both CMV and age. The response to Staphylococcal enterotoxin B (SEB) and polyfunctional index of NKT-like cells increases with age in CMV-seropositive individuals [146].

CMV encodes numerous proteins and microRNAs that assist in evading the immune response, enabling the virus to replicate and disseminate in the face of a competent immune system. A latent infection by CMV, if quiescent at the level of viral gene expression, represents an ultimate strategy in immune evasion but is not sufficient for lifelong persistence and dissemination of the virus. CMV needs to reactivate and replicate in a lytic cycle of infection in order to disseminate further in the face of a primed immune response. Therefore, there is a balance between virus immune evasion and host immune recognition over a lifetime [154]. CMV affects T-cell subset composition and exhaustion and can cause large expansions of CMV-specific T cells, particularly in older people. This phenomenon undermines immunity to other pathogens, accelerating immunosenescence.

Thus, in the elderly, CMV infection impairs immunity to other viruses and is associated with T-cell senescence, while in younger people, CMV confers a degree of protection from other pathogens [147]. Polyfunctionality is a property of central memory CD4(+) T cells in CMV-seronegative individuals. Following CMV infection, polyfunctional T cells become highly differentiated, enabling eradication of infections. CD57 is a polyfunctionality marker of T cells which shows an increase after CMV infection. CD4(+) T cells that coexpress CD57 and CD154 are exclusively present in CMV-positive individuals and belong to the most polyfunctional CD4(+) subset. Conversely, the frequency of CD4(+) CD28(+) T cells correlates with higher polyfunctionality of CD4(+)CD57(−) T cells from CMV-seronegative individuals and CD4(+)CD57(+)CD154(+) T cells from CMV-seropositive individuals [147]. Chronic infection with CMV, along with aging, is associated with the expansion of highly differentiated CD4+, CD4(hi)CD8(lo), and CD8+ T cells, which express T-bet and Eomes that may promote effector memory and effector T lymphocytes involved in conferring protection against chronic CMV. The percentage of CD4+ T cells expressing T-bet or Eomes was low in CD4+ T cells from young CMV-seronegative individuals and higher in CMV-seropositive older individuals, in both CD57 T cells and CD57+ CD4+ T cells. CD4(hi)CD8(lo) T cells expressing T-bet are associated with CMV seropositivity, and coexpression of Eomes, T-bet, and CD57 in CD4(hi)CD8(lo) T cells is observed in CMV-seropositive donors [155].

The numbers of classical, intermediate, and nonclassical monocytes slightly increased with age, while the numbers of myeloid (mDC) and plasmacytoid DC (pDC) did not vary significantly. A decrease in the numbers of pDC with age was noted in CMV-positive individuals [156]. Aging and CMV persistence impact DN and CD8+TCRgammadelta+ T cells. A progressive decrease in absolute numbers of total TCR gammadelta+ T cells in blood, affecting the predominant Vgamma9/Vdelta2 population, was noted with aging. Aged TCR gammadelta+ T cells shift from naive to late-stage effector phenotypes and are more prominent in cases of persistent CMV infections [157].

CMV primary infection and periodic reactivation of latent virus are controlled by T-cell responses in healthy people. In healthy aged donors, CMV-specific changes in the T-cell compartment were not affected by age and were effective as viremia is a very rare event in healthy donors. In older donors, overt CMV disease is not generally seen despite the association of CMV infection with increased risk of mortality. Increases in CMV DNA in urine of older people suggest that, although the immune response retains its functionality, immunomodulation due to a lifelong viral carrier state may alter its efficacy. IFN gamma responses by CD4(+) and CD8(+) T cells to all CMV proteins were detected, with no age-related association [158].

CMV reactivation is under the control of the cellular immune response; however, both the humoral and innate arms play a role in this process. CMV displays an array of several Fcgamma-binding glycoproteins with cell surface disposition and incorporation into the virion. The virus-encoded Fcgamma receptors differ in their Fcgamma-binding mode but function as adversaries of host FcgammaRs to prevent IgG-mediated triggering of the activating host FcgammaRs, such as FcgammaRI, FcgammaRIIA, and FcgammaRIIIA [159].

A recent study demonstrated a CMV immune response in immunodeficient CMV-positive human leukocyte antigen (HLA)-matched bone marrow recipients after immunoablative conditioning, showing a decrease in immunity. Reconstitution of marrow-derived B and NK cells was noted prior to that of thymic origin T cells. In this study, the lowest levels of CMV-IgG were found just prior to CMV viremia. The sole factor in this CMV-specific immune response is a residual recipient antibody class IgG which corresponds to the increase of NK cells and undetected CMV-specific CD8 cells. In an immunocompetent adult who was CMV negative, the cellular and humoral immune response increased in a parallel manner, but symptoms of CMV mononucleosis per-

sisted until the increase of specific IgG. During infancy, decrease in maternal CMV-IgG levels was followed by detectable sequelae, such as CMV replication. Before development of a primary cellular immune response, high levels of residual CMV-IgG (about >100 R/mL) from the mother prevents virus reactivation [160].

CMV Infection in the Immunocompetent Host

The seroprevalence for CMV, worldwide, ranges from 60% to 100% [161]. In a large cohort of adults, the overall CMV seroprevalence was 56.7%, with a higher seroprevalence in women (62%) than in men (51%). Seroprevalence increased with age: from 31% to 63% in men and from 44% to 77% in women when comparing the 18- to 29- with the 70- to 79-year-old groups, respectively. Factors associated with CMV seropositivity were age, country of birth, smoking status, education, number of household members, and having resided in child care homes [162].

Most primary CMV infections in immunocompetent adults are asymptomatic or associated with a mild IM syndrome. Symptomatic CMV infections in non-immunocompromised hosts display a benign self-limited course resembling EBV-IM syndrome. Most primary infections resolve and enter a lifelong latency period, in which viruses are sequestered in a nonreplicative state. Persons with latent infections and intact immune systems have no symptoms but exhibit antibodies to CMV. Circulating lymphocytes, monocytes, and polymorphonuclear leukocytes may serve as the reservoir sites of viral latency [1]. Nevertheless, the virus can be reactivated in the case of immunosuppression. The risk of CMV recurrence is dependent on the level of incompetency of the immune system, manifested as an impairment of T-cell immunity, including the presence and functioning of CMV-specific cytotoxic T lymphocytes [163].

Liver dysfunction is commonly associated with CMV mononucleosis. It is usually mild and rarely symptomatic in an immunocompetent patient. Hepatosplenomegaly and laboratory evidence of mild to moderate elevations of liver enzymes are the predominant features, with increased aminotransferases and alkaline phosphatase in majority of cases but lower than those encountered in acute hepatitis due to "classic" hepatitis viruses [1, 164]. Rare manifestations of CMV hepatitis include tender hepatomegaly, granulomatous hepatitis, anicteric or icteric cholestatic hepatitis, and acute hepatitis with massive necrosis [164].

Severe CMV infections may occur in immunocompetent hosts affecting many organs. The gastrointestinal tract (duodenitis, ileitis, colitis) and the central nervous system (meningitis, encephalitis, transverse myelitis, nerve palsies) are most frequent [165, 166]. In addition, hematological manifestations (hemolytic anemia and thrombocytopenia), ocular (uveitis, retinitis), liver (hepatitis), pulmonary (pneumonitis), and thrombosis of the arterial and venous systems may occur [165, 167]. Several cases were treated with ganciclovir or valganciclovir, some with fatal outcome despite therapy.

A special population afflicted by CMV disease consists of patients with preexisting inflammatory bowel disease [168]. TNF-α and IFN-γ are frequently elevated in these patients, an environment of chronic inflammation promoting reactivation of a latent CMV infection, further driving additional cytokine release, mainly IL-6. This in turn leads to a vicious circle of exacerbation of the inflammatory bowel disease. This sequence of events may be observed in patients with inflammatory bowel disease who have not recently received any steroid treatment. CMV colitis in patients with underlying inflammatory bowel disease has the potential to lead to severe complications including toxic megacolon and perforation.

Perinatal infection with CMV may promote bile duct damage in biliary atresia (BA). A decreased Treg percentage associated with BA further contributes to bile duct damage. In mice, autoimmune-mediated and inflammatory responses induced by CMV infection in Treg-depleted mice resulted in increased intrahepatic and extrahepatic bile duct injury and contributed to disease progression [169].

CMV Infection in the Immunocompromised Host

In immunocompromised patients, CMV disease results from either a primary infection or, more commonly, from reactivation of a latent infection [1, 165]. Disseminated CMV infections in immunocompromised patients, including HIV-infected patients, transplant recipients, and congenitally infected patients, are associated with increased morbidity and mortality. Anti-CMV antibodies are detected during episodes of reactivation. The incidence and severity of CMV disease closely parallel the degree of cellular immune dysfunction, characterized by decreased numbers of CTLs and NK cells [170].

The median rate of CMV recurrence in hematopoietic stem cells transplantation (HSCT) recipients was estimated as 30–40% after allogeneic HSCT or solid organ transplant and 5–20% during active HIV replication, primary immunodeficient patients, and patients receiving chemotherapy or immunotherapy. In perinatal infections, recurrence rates near 0.5%. The highest risk of CMV recurrence and CMV disease is reported for HSCT CMV-seropositive recipients, regardless of donor serostatus [163]. A negative correlation between CMV+ and CD4:CD8 ratio was shown for HIV patients. This correlation was observed among patients dis-

playing optimal CD4 recovery, suggesting that the CMV+ serostatus antagonizes normalization of the CD4:CD8 ratio [171].

CMV infections in HSCT recipients cause substantial morbidity and mortality. A strong association between low CMV cell-mediated immunity and progression to clinically significant CMV infection is seen in HSCT recipients [172]. Clinical syndromes observed in these patients include encephalitis, pneumonitis, hepatitis, uveitis, retinitis, colitis, and graft rejection. CMV infection affecting the human embryo, a host with immature immunologic responses, may lead to serious neurological, hematological, and hepatic complications [165].

In AIDS patients, CMV is the most common opportunistic viral infection. Most HIV-infected persons are CMV seropositive and retain latent virus prone to reactivation. Humoral and T-cell responses to CMV remained elevated in HIV patients >12 years on ART. A report indicated that age and presence of CMV disease influenced CD8 T-cell phenotypes. CMV antibody titers were higher in HIV patients, and levels of soluble B-cell activating factor (sBAFF) were elevated and correlated with levels of CMV antibodies. CD8 T-cell IFN-gamma responses to the IE1 peptide, related to early viral activation, remained elevated in the HIV patients [173]. CD4(+) T cells specific for CMV are elevated in HIV(+) CMV(+) subjects [174]. Clinically, patients may develop retinitis, central nervous system infections, esophagitis, and colitis. CMV can also invade the hepatobiliary tract in AIDS patients, causing hepatitis, pancreatitis, and acute acalculous cholecystitis [175]. In AIDS patients, CMV manifestations in other organs increase the risk for a cholestatic syndrome caused by papillary stenosis and sclerosing cholangitis (AIDS cholangiopathy), which does not usually respond to antiviral therapy.

CMV in Liver Transplant Recipients (Fig. 15.2)

Overall, 18–29% of liver transplant recipients develop CMV disease [176]. Hepatitis is the most frequent organ-specific complication of CMV infection following liver transplantation, affecting 10% of recipients albeit with a higher incidence among seronegative recipients than among seropositive patients (26% vs. 9%, respectively). In these cases, infection occurs as a consequence of reactivation rather than primary infection [1, 170]. CMV evades the immune system resulting in a state of latency in host cells. Cellular sites of viral latency become reservoirs of reactivation during periods of stress and cytokine release and serve as vehicles for transmission to susceptible hosts. Pharmacologically induced impairment of immune response to "endogenously reactivated" or "allograft-transmitted" CMV leads to febrile and tissue-invasive diseases in liver transplant recipients [170]. Viral "blips" reflecting polymerase chain reaction (PCR) artifacts or transient low-level replication are frequent when the viral load of the first positive PCR analysis is <910 IU/mL and serostatus risk is intermediary/low [177].

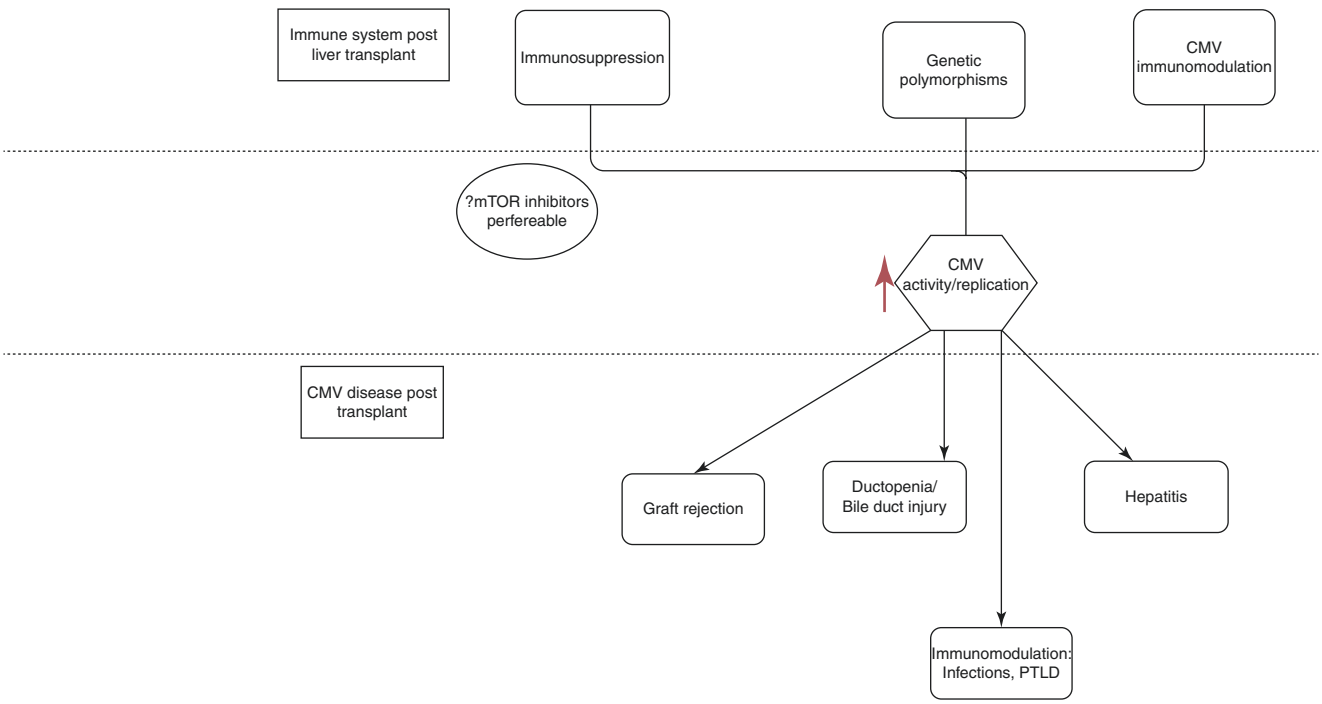

Fig. 15.2 Contributing factors and outcome of CMV activity post liver transplant

Knowledge regarding serostatus of donor and recipient (D/R) cytomegalovirus (CMV) is critical for risk stratification of CMV infection and disease in transplant recipients. However, up to 20% of seropositive recipients, classically considered at intermediate risk, develop episodes of CMV infection and disease after transplantation. CMV-specific T-cell-mediated immunity, neutralizing antibodies, and host genetics impact the risk of CMV infection and disease [178]. Pretransplant CMV serology is currently the only tool for assessing the risk of CMV infection although cellular immune responses driven by CMV-specific CD4 and CD8 T lymphocytes are important for controlling viral replication [179]. Defects in innate immunity and in CMV-specific cell-mediated immunity predispose these patients to severe infections. Mutations in innate immunity-associated genes increase the risk of CMV disease after liver transplantation. TLR2, expressed in innate immune cells, senses the glycoprotein B of CMV, thereby signaling immune cells to produce cytokines and antiviral peptides. A genetic polymorphism in the TLR-2 gene was associated with a higher CMV replication and a higher incidence of CMV disease by decreasing cellular recognition of CMV by TLR2-expressing cells. Programmed death-1 receptor expression and immune evasion genes have also been assessed as prognostic indicators of CMV disease following liver transplantation.

Pretransplant assessment of CMV immunity in organ transplant recipients, in which CMV-seropositive recipients had undetectable cell-mediated responses despite past immunity, showed that they were at a higher risk of developing CMV reactivation. Posttransplant CMV immune monitoring can act as a guide to predict the duration of antiviral prophylaxis, identify recipients at risk of post-prophylaxis CMV disease, and predict recurrent CMV reactivation [180]. A lack of a preexisting CMV-specific immunity in CMV-seronegative recipients of liver allograft from CMV-seropositive donors (CMV D+/R−) exposes these patients to the highest risk of CMV disease and its complications (44–65% in CMV D+/R− vs. 8–19% in CMV-seropositive [CMV R+] recipients) [181]. The CD8 responses to IE-1 antigen were absent at the pretransplant stage in patients who developed CMV infection posttransplant. Nonspecific and CMV-specific CD8+ T-cell functions were found to correlate with the course of CMV, and measuring these has the potential to assist in its clinical management [182]. Assessment of CMV-specific CD8+ response is recommended in all R+ candidates and is suggested to be essential in patients with a lower probability of being reactive, such as nonrenal transplant candidates, candidates less than 50 years of age, or those with non-HLA-A1/non-HLA-A2 alleles [183]. Assessment of IE-1-specific CD8 T-cell frequencies can identify seropositive patients at risk of developing CMV infection at the posttransplant stage [179]. Having CMV-specific CD8(+) IFN-gamma(+) cells ≥0.25% before transplant, 0.15% at 2 weeks, or 0.25% at 4 weeks after transplantation identifies patients that may spontaneously control CMV infection and may require less monitoring [184]. Solid organ transplant recipients with a positive pretransplant serology for CMV (CMV-R+) are at intermediate risk for CMV infection posttransplantation. Only one-third of R+ recipients had CMV-specific T-cell immunity [CD8(+)CD69(+)INF-gamma(+) T cells >0.25%] before transplantation. Patients with negative pretransplant immunity had more CMV infections and received more antiviral therapy. A study revealed that having CMV-specific immunity was an independent factor for protection from developing viremia ≥2000 IU/mL. Only patients with no pretransplant CMV-specific T-cell response were diagnosed with CMV disease [185]. The prevalence of CMV disease increased with increasing diagnostic PCR load of CMV and with screening intervals >14 days. Despite weekly screening intervals, patients can present with CMV disease at the time of diagnosis of CMV DNAemia [186]. Even in the absence of the disease, antigenic exposure may shape the CMV-responsive T-cell population posttransplantation. Transplant recipients have reduced memory T-cell function due to chronic immunosuppressive therapies. The frequency of CMV-responsive CD8(+) T cells, defined by the production of effector molecules in response to CMV peptides, increased during a course of 1 year posttransplantation. The increase commenced after the completion of antiviral prophylaxis, and these T cells were terminally differentiated effector cells [156].

Despite a trend toward immunity, 22% of patients developed symptoms in spite of having pretransplant CD8+IFNG+ response, suggesting that other immunological parameters may be involved [187]. ELiSpot IFN-gamma (CMVspot) is an additional method for establishing a treatment strategy that includes regular monitoring for risk stratification of reactivation [188]. In R(+)D(−) patients, immunity against CMV is mediated by recipient T cells. The donor CMV serostatus affects the clinical severity of CMV reactivation due to the CMV-specific memory T cells transferred with the graft, despite the formation of primary donor-derived CMV-specific T-cell responses in R(+)D(−) patients [189].

The use of highly potent pharmacologic immunosuppression severely impairs the ability of liver transplant recipients to mount an effective immune response against reactivating CMV, thereby predisposing them to increased risk of CMV disease [181]. The drug Sirolimus acts selectively on human naive and memory T cells and improves CMV-specific T-cell function. Sirolimus improved CMV-specific effector memory T-cell function and negatively influenced naive T cells. This unique mechanism is characterized by increased secretion of interferon-gamma (IFN-gamma) and granzyme B (GzB) and enhanced target-cell-dependent cytotoxic capacity of activated CMV-CTLs. IL-2 receptor (IL-2R)-driven signal transducer and activator of transcription-5 (STAT-5)

signaling under mammalian target of rapamycin (mTOR) inhibition allowed the fine-tuning of T-cell programming for enhanced antiviral response [190]. In a cohort of high-risk CMV D+/R− kidney transplant recipients receiving treatment with rabbit antithymocyte globulin (rATG) and tacrolimus, the use of mTOR inhibitors showed delayed CMV infection and less recurrences, with no difference in overall disease or acute rejection [191].

CMV disease in liver recipients manifests with fever, bone marrow suppression, and organ-invasive diseases. These direct clinical effects are classified as CMV syndrome (fever with myelosuppression) or as tissue invasive CMV disease, which most often involves the gastrointestinal tract, although any other organ may be involved. CMV hepatitis is common in liver transplant recipients compared to other than in solid organ transplant recipients and manifests with symptoms indistinguishable from acute allograft rejection [170]. The availability of sensitive tests for the rapid detection of CMV in the blood may obviate the need for a liver biopsy to differentiate between CMV infection and graft rejection. However, in many cases, a liver biopsy is required to differentiate or demonstrate a coexistence of CMV disease and allograft rejection.

Several indirect outcomes in these patients are mediated by the ability of the virus to modulate the immune system [170]. CMV is a potent upregulator of alloantigens, increasing the risk of acute rejection and chronic allograft dysfunction. CMV infection may promote tolerance to liver allografts, and CMV status should be considered when tapering or withdrawing immunosuppression. CMV positivity was associated with the expansion of peripheral effector memory T-cell subsets. Patients with CMV primary infection showed donor-specific CD8(+) T cell hyporesponsiveness. While terminally differentiated effector memory cells comprised a majority of peripheral donor-specific CD8(+) T cells in CMV primary infection patients, they were rarely present in liver allografts. R(−)D(+) serostatus was an independent protective factor for late acute rejection. CMV primary infection patients showed the highest Vdelta1/Vdelta2 gammadelta T cell ratio, which has been shown to be associated with operational tolerance after liver transplantation (LT) [192]. CMV is associated with the vanishing bile duct syndrome and ductopenic rejection, leading to chronic cholestasis and allograft failure and a higher incidence of hepatic artery thrombosis. The immunomodulatory effects of CMV predispose to other opportunistic infective agents, including fungi, other viruses, and bacteria such as *Nocardia*. CMV infection in liver transplant recipients may potentiate hepatitis C infection and increase the risk of posttransplant lymphoproliferative disease [193, 194]. Such recipients are more likely to develop EBV-associated PTLD or to develop coinfections with other viruses, such as human herpesvirus, HHV-6, and HHV-7 [195].

CMV infection is an independent predictor of mortality after solid organ transplantation. An analysis of 437 liver transplant recipients demonstrated that CMV disease occurred in 8.5% of the patients and that its occurrence was independently associated with a 5-fold increased risk of all-cause mortality and an 11-fold increased risk of infection-related mortality. The use of anti-CMV drugs, either through antiviral prophylaxis or preemptive therapy, led to reduction in the overall mortality [196]. Allograft rejection can promote CMV reactivation and is a risk factor for CMV disease following liver transplantation [170]. Cytokines released during acute rejection, particularly TNF-α, are potent activators of latent CMV. Therapy for allograft rejection, which involves intensification of the immunosuppressive regimen, further increases the risk of CMV disease [197].

There are two strategies for prevention of CMV disease after liver transplantation: preemptive therapy and antiviral prophylaxis [170]. For preemptive therapy, CMV reactivation is monitored by sensitive assays; upon detection, antiviral drugs are administered early to halt progression of the asymptomatic infection to full-blown clinical disease [198]. Preemptive therapy with oral ganciclovir, intravenous ganciclovir, or valganciclovir resulted in reduction of the disease by 70% [199] and, unlike antiviral prophylaxis, was not associated with a late onset of the disease. Valganciclovir is the most commonly used drug for preemptive therapy. However, this therapy may not be completely effective in CMV D+/R− liver transplant recipients because the replication kinetics of CMV in immune-deficient individuals is very rapid [197]. It was demonstrated that oral valganciclovir was effective as a preemptive treatment for CMV infection in transplant recipients with stable graft function [200].

CMV prophylaxis is efficacious and can safely prevent direct and indirect effects of CMV infection in CMV-seropositive liver transplant recipients. Independent factors associated with CMV reactivation were an absence of CMV prophylaxis, CMV serological status of the donor, cold ischemia time, and HLA A + B + DR compatibility [201]. For antiviral prophylaxis, drugs such as ganciclovir and valganciclovir are administered to patients at risk of CMV disease after transplantation [202–207]. It is offered by the majority of transplant centers for prevention of primary CMV disease in high-risk CMV D+/R− transplant recipients [208, 209]. Several clinical trials have demonstrated its effectiveness in preventing direct and indirect effects of CMV after liver transplantation [199]. Compared to placebo, patients who received antiviral prophylaxis had a 58–80% reduction in CMV disease and a 40% reduction in CMV infection [199]. The use of acyclovir as anti-CMV prophylaxis after liver transplantation has been supplanted by ganciclovir and valganciclovir because of their superior efficacy [204, 210, 211]. The incidence of CMV is reduced in liver transplant recipients who receive antiviral prophylaxis with valganci-

clovir or oral ganciclovir for the first 3 months following liver transplantation. CMV disease rates of 12–30% in high-risk CMV D+/R− and less than 10% in CMV R+ were reported in patients who received antiviral prophylaxis [176, 207]. A randomized control trial showed that 200 days of prophylaxis are more effective than 100 days of therapy in high-risk (D+/R−) patients [212]. In individuals who received antiviral prophylaxis, CMV disease may occur 3–6 months after completing antiviral prophylaxis, hence the term "delayed-onset" or "late-onset" CMV disease [170]. The effects of different immunoprophylaxis regimens on CMV infection in liver transplant recipients was studied in a cohort of CMV-seropositive recipient (R+) and seronegative donor/recipient (D−/R) patients. Such regimens included steroid-only, steroids plus rATG, and steroids plus basiliximab. The use of rATG immunoprophylaxis increases the risk of CMV infection in CMV-seropositive recipients, mainly in the CMV D−/R+ group. However, prophylaxis with valganciclovir in this group, for at least 6 weeks, decreased the risk of CMV infection [213]. A 14-day delay in CMV prophylaxis in D+/R− recipients was safe and could reduce the incidence of late CMV end-organ disease [214]. Primary CMV infections after cessation of prophylaxis were common but were successfully treated with valganciclovir or ganciclovir [215]. In prospective long-term follow-up of CMV (D+/R−) adult liver transplant recipients after 3 months of valganciclovir prophylaxis, 13% were CMV D+/R− and received antiviral prophylaxis up to 3 months after transplantation. No breakthrough CMV infections were recorded during the prophylaxis period. After cessation of valganciclovir prophylaxis, 90% of patients demonstrated CMV-DNAemia following a posttransplantation mean interval of 165 days and were treated successfully [215].

Prophylactic versus preemptive therapy for intermediate- and low-risk groups (D+/R+, D−/R+ and D−/R−) is based on the local expertise of each transplant center. However, the general approach for D−/R− patients is that only seronegative blood products are used, and no prophylaxis is administered. In contrast, D+/R+ or D−/R+ patients are monitored for CMV reactivation and treated preemptively for 7 days. Where available, "protective matching" of donor and recipient based on CMV serological status is advocated because it has been shown to reduce the risk of posttransplant CMV disease [202]. The current recommendation for antiviral treatment of CMV disease after liver transplantation is intravenous ganciclovir along with a reduction in the degree of pharmacologic immunosuppression [216]. Besides, valganciclovir is a possible oral treatment for mild to moderate diseases [216]. In cases of ganciclovir-resistant CMV disease, treatment options include foscarnet, cidofovir, CMV hyperimmune globulins, or leflunomide [202].

Compartmentalized CMV disease refers to clinical syndromes wherein the virus is detected in the affected tissues but is minimally detectable or undetectable in blood [170, 202]. In the gastrointestinal system, "compartmentalized" CMV disease in the form of gastritis, esophagitis, enteritis, or colitis constitutes a vast majority of tissue-invasive conditions [181].

Treatment of CMV Infection

CMV infection in immunocompetent patients does not require treatment [165]. Data on a need for antiviral treatment in immunocompetent patients with severe CMV infection is conflicting. The improvement observed in some treated patients may have been related to the typically self-limiting course of the disease and thus cannot be attributed with certainty to the effect of treatment [135]. Nevertheless, in severe cases, particularly in patients with impaired cell-mediated immunity, therapy can be lifesaving [1]. Drugs used for the treatment of CMV disease include antivirals, such as ganciclovir, valganciclovir, foscarnet, and cidofovir. Ganciclovir is considered as the antiviral agent of choice against CMV. The duration of therapy is guided by repeated measurements of CMV in blood samples. Emerging strains resistant to ganciclovir pose a therapeutic challenge for which foscarnet or cidofovir may be alternative antiviral agents [217]. Ganciclovir can lead to myelosuppression, central nervous system disorders, hepatotoxicity, irreversible infertility, or teratogenesis, whereas foscarnet can cause disturbances in mineral and electrolyte homeostasis and nephrotoxicity. Additionally, long-term administration of these agents may lead to an emergence of resistant viral strains [135].

Intravenous administration of hyperimmunoglobulins (HIGs) was applied to women with primary CMV infection as "off-label use" in some countries. All HIGs and standard intravenous immunoglobulins (IVIGs) showed similar CMV-neutralizing capacity following CMV IgG normalization [218]. Adoptive transfer of CMV-specific T cells has shown promising results in preventing pathological effects caused by opportunistic CMV infection in immunocompromised patients following allogeneic hematopoietic stem cell transplantation. CMV-specific CTLs can be efficiently isolated from G-CSF mobilized samples and are able to express activation markers and produce cytokines in response to antigenic stimulation. However, this antiviral functionality is moderately reduced when compared to non-mobilized products [219].

Herpes Simplex Virus

Herpes simplex viruses, HSV-1 and HSV-2, commonly infect humans and produce a wide variety of illnesses. The clinical manifestations and course of HSV infections depend on the sites involved and the patient's age and immune status

[1]. Defects in interferon (IFN) responses can result in lethal herpes simplex virus 1 (HSV-1) infections, such as encephalitis. IFN-alphabetagammaR(−/−) mice are susceptible to liver infection following corneal infection with HSV-1. An inability of IFN-alphabetagammaR(−/−) immune cells to control liver infection in IFN-alphabetagammaR(−/−) mice manifested as profoundly elevated aspartate transaminase (AST) and alanine transaminase (ALT) levels was observed in a mouse model [220].

HSV viremia results in visceral involvement, affecting mainly the esophagus, lungs, and liver. Liver involvement occurs in neonatal infections, pregnancy, and immunocompromised hosts, in which it is frequently a fulminant disease [1]. HSV is not a common cause of hepatitis in immunocompetent patients. A mild asymptomatic elevation of aminotransferase levels can be detected in 14% of healthy adults with genital infection [221]. In neonates, hepatitis occurs with multi-organ involvement and carries a high mortality rate. HSV during pregnancy is rare. It occurs as a disseminated primary infection during the third trimester and presents as fulminant hepatitis. Mucocutaneous lesions are present in half of the cases; thus, many cases are not diagnosed until autopsy [1]. It was reported that maternal death did not occur in patients administered with acyclovir (ACV) as empiric therapy [222]. The incidence of HSV hepatitis was reported to be up to 6% of fulminant hepatitis cases and could be associated with a favorable outcome after antiviral therapy [223].

In immunocompromised hosts, HSV hepatitis occurs during primary and, rarely, during recurrent infection, with a triad of fever, leukopenia, and markedly elevated liver enzymes, as well as thrombocytopenia and a relatively mild increase in bilirubin [1]. Liver biopsy is required for the diagnosis, manifesting focal or sometimes extensive hemorrhagic or coagulative necrosis of the hepatocytes with limited inflammatory response. Typical intranuclear inclusions (Cowdry type A) are often identified at the margins of the foci of necrosis. The diagnosis is confirmed by detection of HSV DNA sequences by molecular techniques [1]. The treatment of choice for HSV is an early high dose of acyclovir [224, 225]. With this treatment, recurrence is not observed, suggesting that disseminated HSV infection should not be an absolute contraindication for transplantation in certain clinical settings [1, 226, 227].

The importance of additional human herpesviruses (HHV6 and HHV 7) has been debated in recent years. According to some reports, HHV6-infection may be associated with higher rates of acute and chronic allograft rejection, bacterial and opportunistic infections, CMV disease, and shorter graft survival [228]. While HHV6 reactivation is common after solid organ transplantation, a clinical disease is rare. Reactivation may manifest as fever, myelosuppression, and end-organ disease including encephalitis and hepatitis. Treatment is indicated for end-organ disease and includes foscarnet, ganciclovir, and cidofovir [229].

Varicella-Zoster Virus

Varicella-zoster virus (VZV) is a causative agent of both chickenpox (varicella) and shingles (zoster). VZV survives host defenses, even with an intact immune system, and disseminates in the host before causing disease [230]. Several immunomodulatory strategies used by VZV to undermine host immunity have been identified. Expression of CD59, a member of host regulators of complement activation (RCA), is upregulated in response to VZV infection in human T cells and dorsal root ganglia (DRG) [230].

Primary varicella infection is usually benign with mild transient elevation in liver enzymes in up to 25% of children; however, it can cause severe acute hepatitis and even ALF in immunocompetent adults. In transplanted patients, primary infection can present with an aggressive liver disease. It may occur in the immediate postoperative period or up to several months after liver transplantation and is usually associated with rapid-onset and fatal hepatitis [231]. Serologic testing is of little value in immunocompromised patients. Confirmation of diagnosis is made through isolation of VZV from skin lesions or from the affected organs. Liver biopsy often shows foci of coagulative necrosis and intranuclear inclusions with an inflammatory response [1]. Early administration of intravenous acyclovir is critical in treating VZV hepatitis, especially in immunocompromised patients [1, 232].

Parvovirus (B19)

Parvovirus (B19), a small DNA virus, is a member of the Parvoviridae family. B19V infection exhibits high tropism for human erythroid progenitor cells (EPCs) in the bone marrow and fetal liver. The virus can only replicate in pronormoblasts and hepatocytes and in other cells that have globosides and glycosphingolipids in their membranes due to persistence of nonstructural protein 1 and indirectly by immune-mediated injury [233]. The exclusive restriction of B19V replication to erythroid lineage cells is partly due to the expression of receptor and co-receptor(s) on the cell surface of human EPCs and partly depends on the intracellular factors essential for virus replication [234]. Hypoxia, erythropoietin signaling, and STAT5 activation facilitate viral replication. The B19V infection-induced DNA damage response and cell cycle arrest at late S-phase promote its replication. It causes G2 arrest, followed by extensive cell death of EPCs, leading to anemia. B19V encodes a single precursor mRNA (pre-mRNA), which undergoes alternate splicing and alternative polyadenylation to generate at least 12 different species of mRNA transcripts. The posttranscriptional processing of B19V pre-mRNA is regulated via cis-acting elements and trans-acting factors, flanking the splice donor or acceptor sites [234, 235]. According to a study, phosphorylated STAT5 specifically interacted with viral DNA replica-

tion origins and was recruited within the viral DNA replication centers. STAT5 facilitates viral DNA replication by recruiting the helicase complex of the cellular DNA replication machinery to viral DNA replication centers [235]. During infection, B19V expresses three nonstructural proteins (NS1, 11-kDa, and 7.5-kDa) and two structural proteins (VP1 and VP2). NS1 is essential for B19V DNA replication, and the 11-kDa protein enhances viral DNA replication. The 11-kDa protein is tightly associated with cellular growth factor receptor-bound protein 2 (Grb2) during infection. The interaction of the 11-kDa protein with Grb2 disrupts the extracellular signal-regulated kinase (ERK) signaling that mediates upregulation of B19V replication [236].

B19 is pathogenic to humans and causes bone marrow failure diseases and various other inflammatory disorders. Infection is usually benign and self-limiting, and symptomatic therapy alone is recommended [1]. Its clinical manifestations may include erythema infectiosum, hydrops fetalis, and fetal death in children and arthritis in adults. Leukopenia, thrombocytopenia, and aplastic crisis in patients with chronic hemolytic anemia are additional features. Rare manifestations include neurological, cardiac, and hepatic end-organ damage and vasculitis. Hepatic manifestations range from mild transient hepatitis to acute liver failure with or without associated aplastic anemia. Sudden drop of hemoglobin and onset of transient aplastic anemia in immunosuppressed or immunocompetent patients can be the first manifestation, and the virus can be identified in bone marrow aspiration, confirmed either by IgM- and IgG-positive serology, PCR analysis, or in situ hybridization in biopsy specimens [233]. In adults, skin lesions were common along with mild to moderate abnormalities in liver enzymes which resolved spontaneously in immunocompetent patients [237]. B19 infections can cause a spectrum of liver diseases, from elevation of transaminases to acute hepatitis to fulminant liver failure and even chronic hepatitis. It can also cause fatal macrophage activation syndrome and fibrosing cholestatic hepatitis [233]. Severe aplastic anemia associated with human parvovirus B19 infection is a rare complication following liver transplantation [238].

There is no specific treatment for parvovirus B19-related liver diseases, but triple therapy regimen may be effective, consisting of immunoglobulin, dehydrohydrocortisone, and cyclosporine [233]. The FDA-approved drug pimozide dephosphorylates STAT5, thereby inhibiting B19V replication in ex vivo-expanded human erythroid progenitors [235].

Adenoviruses

Adenoviruses (AdVs) are DNA viruses that typically cause mild infections involving the upper or lower respiratory tract, gastrointestinal tract, or conjunctiva, which are usually self-limiting. Rare manifestations of AdV infections include hemorrhagic cystitis, hepatitis, hemorrhagic colitis, pancreatitis, nephritis, or meningoencephalitis. AdV infections are more common in young children due to a lack of humoral immunity. Epidemics of AdV infection may occur in healthy children or adults in closed or crowded settings (particularly military recruits). Different serotypes display different tissue tropisms that correlate with clinical manifestations of the infection. The disease is more severe, and dissemination is more likely in patients with impaired immunity (e.g., organ transplant recipients and human immunodeficiency virus infection) [239].

In the immunocompromised host, they can cause severe infections involving multiple organs, including the liver [240, 241]. Fatal cases of adenovirus infection with fulminant hepatitis were reported in immunosuppressed adults [242]. In a study of twelve cases of severe adenovirus hepatitis, there were eight pediatric patients, seven of whom had received orthotropic liver transplants and one of which was receiving chemotherapy for leukemia. There were four adult patients, of which one was actively receiving chemotherapy for leukemia and two had undergone hematopoietic stem cell transplantation. In all cases, histologic sections showed nonzonal coagulative hepatocyte necrosis and characteristic intranuclear inclusions. Hepatocyte necrosis ranged from spotty to massive. Most cases had no associated inflammation. However, in some cases, the inflammation was focal and lymphohistiocytic. Among the pediatric patients, 63% died secondary to organ failure, while there was 100% mortality in the adult population [240]. In a study of 89 cases of adenovirus-related hepatitis, 48% were liver transplant recipients, 21% were bone marrow transplant recipients, 12% had received chemotherapy, 6% had severe combined immunodeficiency, and 4% were HIV infected. Ninety percent of patients presented clinical symptoms within 6 months following transplantation, of which fever was the most common initial symptom. Abdominal CT scan revealed hypodense lesions in eight of nine patients. Diagnosis was made by liver biopsy in 48%, and on autopsy in 52% of the patients. Only 27% survived [243].

The mechanisms underlying the pathogenesis of severe adenovirus infections in non-immunocompromised individuals remain unclear. The host immunologic response determines the severity of adenoviral infection. Presence of parapneumonic effusion was associated with a longer febrile duration and a higher risk of hepatitis. Alterations of CD4+, CD8+, and CD20+ T cells were associated with more severe disease courses [244]. Human adenovirus type 5 drives the antiviral immune system to enter polarized epithelial cells. Blood-derived macrophages facilitate epithelial infection, which can occur in the absence of macrophages and in the presence of chemotactic cytokine CXCL8 (interleukin-8). In polarized cells, CXCL8 activates a Src-family tyrosine

kinase via the apical CXCR1 and CXCR2 receptors. This activation relocates the viral co-receptor alphanubeta3 integrin to the apical surface, allowing apical binding with the adenovirus, depending on the primary adenovirus receptor, CAR [245]. Cidofovir is the drug of choice for severe AdV infections, although not all patients require treatment. Live oral vaccines are highly efficacious in reducing the risk of respiratory AdV infection and are in routine use in the military in the United States; however, they are currently not available to civilians [239].

Influenza Virus

Elevation of liver transaminase levels may occur during systemic infections with influenza viruses. Serum levels of aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transpeptidase were significantly higher in patients with pandemic A/H1N1 influenza compared to those with seasonal influenza, which were correlated with the degree of hypoxia [246]. The pandemic of influenza A/H1N1 was associated with a significant immune response to the infection associated with liver damage. Avian influenza A(H7N9) virus were reported to affect the liver in 29% of patients. Hypoxic hepatitis (HH) manifested by acute severe liver injury and characterized by an abrupt, massive increase in serum aminotransferases resulting from anoxic centrilobular necrosis of liver cells was described in 1.8% of infected subjects. HH patients presented with severe liver impairment, accompanied by multiple organ failure (MOF) involving respiratory, cardiac, circulatory, and renal failure. Liver biopsy showed centrilobular necrosis, and real-time reverse transcription polymerase chain reaction of A(H7N9)-specific genes was negative, which excluded A(H7N9)-related hepatitis suggesting that the liver damage was associated with the hemodynamic changes [247].

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Hepatitis B Virus

16

Antonio Bertoletti and Hongming Huang

Key Points

- HBV epidemiology and geographical distribution
- HBV morphology
- HBV viral cycle
- Host immunity in HBV infection: innate immunity
- Host immunity in HBV infection: NK and NKT cells
- Host immunity in HBV infection: adaptive immunity
- How T and B cells control HBV
- Immune modulatory role of HBV antigens
- Natural history of HBV infection
- Present and future therapies in chronic HBV infection

Introduction

Hepatitis B virus (HBV), a hepatotropic, non-cytopathic DNA virus, apparently present in *Homo sapiens* from the dawn of its evolution [1, 2], represents a very important health problem worldwide. Despite the availability of an efficient prophylactic vaccine, it is calculated that HBV still infects approximately 300 million people and causes more than half a million death per year for hepatic diseases (HCC and liver cirrhosis) that develop as a consequence of its persistent infection [3]. In contrast to most communicable diseases, morbidity and mortality rates related to infection with both hepatitis B and C viruses have increased over the last 20 years [4]. However, while new therapies for HCV have delivered remarkable results [5], with more than 90% of patients achieving viral clearance with directly acting antivirals (DAA), the therapy options with curative intent for HBV are still a distant future [6].

In this chapter, after a brief summary of HBV epidemiology, we will mainly describe the virological and immunological features of HBV that make it difficult to eradicate from the infected host. We will also discuss the natural history of infection with a final paragraph focused on the therapeutic strategies that are currently under development to achieve a better cure.

Epidemiology and HBV Genotypes

The global burden of hepatitis B virus (HBV) infection is estimated to be around 260 million people [3]. The prevalence of chronic HBV in the population varies between different countries and appears endemic in most parts of Asia, Pacific Islands, Africa, Southern Europe, and Latin America, but it has been calculated that two thirds of HBV-infected people live in Asia, and in China, the burden of disease is considerable [7, 8].

Chronic HBV infection present in different geographical areas can be classified based on the presence of distinct serotypes or genotypes. Before the advent of molecular methods, HBV was differentiated based on diverse serological reactivities against the envelope protein (HBsAg) of the virus and classified into four serotypes, adw, adr, ayw, and ayr [9, 10]. Nowadays, analysis of the viral sequence differentiates ten major genotypes (A to J) with 4–8% of genetic distinctions [11, 12].

Figure 16.1 shows the geographical distribution of these genotypes: genotype A is highly prevalent in Europe, Africa, India, and America. Genotypes B and C are instead common in the Asia-Pacific region. Genotype D is prevalent in Africa,

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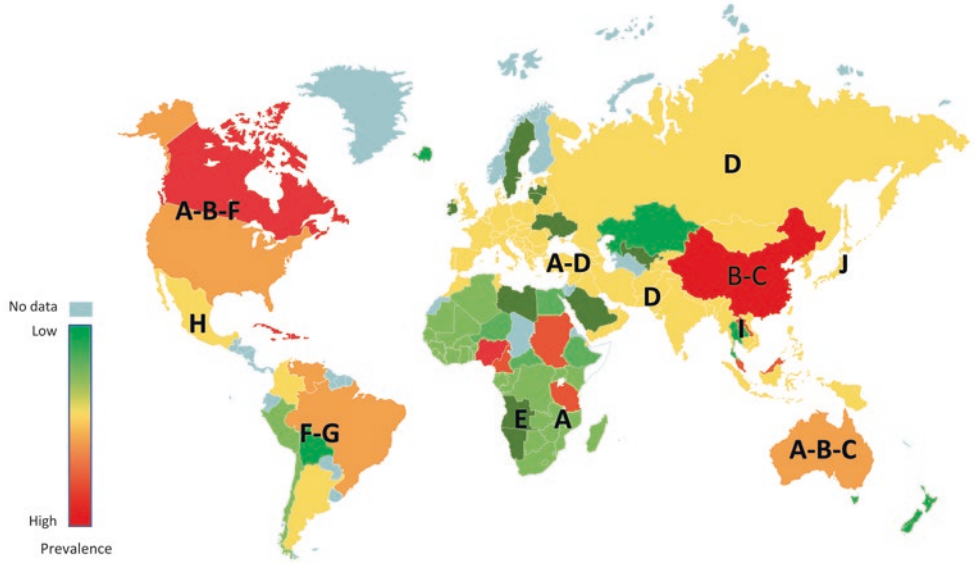

Fig. 16.1 1 HBV prevalence and HBV genotype localization

Europe, the Mediterranean region, and India. Genotype E is restricted to West Africa. Genotype F is found in Central South America but also Alaska.

Other genotypes like G have been reported in America [13] but also in Europe, while genotype H has been found in Central America [12]. New isolates of genotypes I and J were identified in Vietnam and in Japan, respectively [14]. HBV genotypes, present in different geographical area, are associated with particular infection modalities. For example, HBV genotypes B and C, prevalent in East Asia, are associated with vertical transmission from mother to child, which is the prevalent transmission way in this part of the world. In contrast, genotypes A and D are associated with horizontal transmission (close personal contact between young children, blood, or sexual interactions between adults), which is more frequent in Africa and Europe. A large quantity of studies have also linked HBV genotypes with different courses of infection, rate of chronicity, and response to treatment [15, 16].

The heterogeneity of the infected populations has made it extremely difficult to compare such associations between all the different genotypes. However, data, especially from Asia, where similar populations of patients can be infected by HBV genotypes B or C, have shown that genotype C-infected patients have more severe disease, higher level of HBV replication, and lower response to interferon therapy (reviewed in [16]).

HBV Morphology and Genome Organization

HBV belongs to the family of hepadnaviruses, a group of para-retroviruses found in different animal species (birds and different mammals like bats, woodchucks, and squirrel) [17], which replicates a DNA genome via reverse transcription of an RNA intermediate [18].

It is a small virus of approximately 42 nm diameter and contains a 3.2 k relaxed circular DNA genome, packaged together with a DNA polymerase (reverse transcriptase) in an icosahedral nucleocapsid of approximately 30 nm diameter that is wrapped by an envelope containing three related proteins and probably lipid [17, 19] (Fig. 16.2). The production of mature infectious virions starts when the pre-genomic HBV RNA (pgRNA) gets packaged with the viral reverse transcriptase (RT, or polymerase) [20] by approximately 240 copies of the viral capsid or core protein (HBcAg) that form the icosahedral nucleocapsid. Inside the nucleocapsid, the pgRNA is converted by the reverse transcriptase into a single-stranded DNA and then in a partial double-stranded circular DNA, called relaxed circular DNA (rcDNA). These nucleocapsid particles can either enter the nucleus of the host cell and replenish the pool of HBV-DNA (cccDNA) or they can be enveloped in the endoplasmic reticulum by the surface proteins (collectively called HBsAg) and form the mature virions that are then secreted [21]. The three related surface proteins forming the viral envelope are called small (S), medium (M), and large (L) and are present at a ratio of 4:1:1 in the complete viral particles [22]. The gene encoding for the S protein constitutes the 3′ end of the envelope gene open reading frame that is divided into Pre-S1, Pre-S2, and S regions. The protein M is encoded by the Pre-S2 and S regions, while the L protein is encoded by Pre-S1, Pre-S2, and S regions [23] (see Figs. 16.2 and 16.3).

An important and unique trait of HBV infection is that infected hepatocytes not only secrete complete viral particles (made of envelope proteins and a nucleocapsid which contains a rcDNA) but also a large quantity of incomplete subviral particles (SVP) made of only surface proteins or viral particles with an empty nucleocapsid (without genome) [24]. The schematic representation of these different viral SVP secreted by infected hepatocytes and their relative quantity

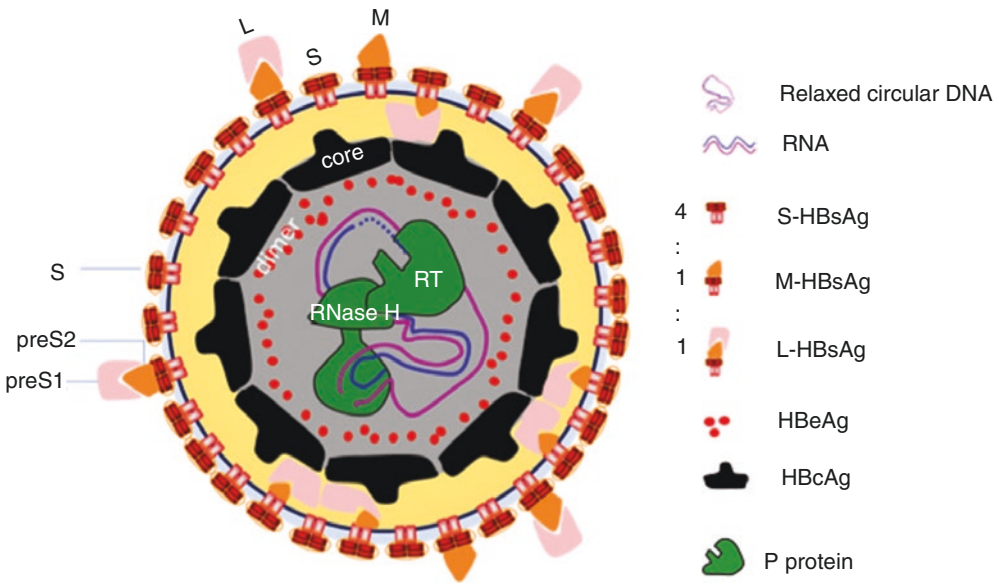

Fig. 16.2 HBV morphology: a 42 nm diameter virion contains a 3.2 k relaxed circular DNA genome, packaged together with a DNA polymerase (reverse transcriptase, RT) in an icosahedral nucleocapsid of approximately 30 nm diameter formed by HBcAg dimers and wrapped by an envelope containing three related proteins – small (S), medium (M), and large surface proteins

are presented in Fig. 16.3. The most abundant population of SVP is the spherical or filamentous lipoprotein particles of 20 nm diameter, which are present in the serum of infected patients at high quantity (~10^{14} × ml), and about 100.000-fold in excess of complete viral particles. Natural spherical SVP contain mainly S proteins and some M proteins but only small amounts of L, while filamentous SVP carry more L proteins. Historically, they were called Australian antigen due to their initial identification in the blood of Aboriginal Australians [25], and their presence in the serum is quantified as HbsAg [26].

HBV-infected cells also secrete genome-empty viral particles that are quite abundant since they are present in quantities (~10^{11} × ml) that are about 100 times more than mature complete virions (~10^{9} × ml) (see Fig. 16.3). Less abundant are particles similar to complete virions with nucleocapsid and envelope proteins containing viral RNA instead of DNA (100–1000 lower than complete HBV ~10^{6} × ml) [24] (see Fig. 16.3). The evolutive advantages provided by the production of incomplete viral particles and their effect on immunity will be discussed later [27].

HBV-infected cells secrete also a soluble, dimeric protein called hepatitis B e antigen (HBeAg) (see Fig. 16.3). HBeAg is derived from the so-called PreCore (PreC) protein [23, 28, 29]. Most of its amino acid residues are shared with HBcAg but have a C-terminal deletion of 34 amino acid residues and an N-terminal extension of 10 amino acids (unique to the PreC region). HBeAg is secreted by the infected cells, and it has been shown in animal studies to exert immunoregulatory effects [30, 31]. Historically, serum HBeAg has been used to check viral production, since its level is associated with high levels of HBV-DNA [32].

The production of complete and incomplete virions, the synthesis of the different HBV proteins, the secretion of all HBV particles and proteins, and viral replication are ultimately directed by a viral DNA episome, the covalently closed circular DNA (cccDNA) present in the nucleous of infected hepatocytes. CccDNA is synthesized in the nuclei of infected hepatocytes from rcDNA present in the nucleocapsid, derived from complete virions infecting the hepatocyte or from the nucleocapsid particles produced in the cytoplasm (schematic representation in Fig. 16.4), the so-called intracellular cccDNA amplification pathway [33, 34].

The cccDNA of HBV contains four genes with extensively overlapping open reading frames that can produce different RNAs coding for seven distinct proteins: envelope proteins (with the three forms of large, middle, and small), the nucleocapsid protein (HBcAg and its truncated form HBeAg), the polymerase protein, and the transcriptional transactivator protein X, which controls HBV transcription from cccDNA. A schematic representation of the HBV genome and its open reading frames is presented in Figs. 16.3 and 16.4.

HBV Viral Cycle

The HBV replication cycle initiates when the complete and mature HBV virion docks to human hepatocytes through binding heparin sulfate proteoglycans located on their membrane (see Fig. 16.4). HBV then enters the cells through high-affinity binding with sodium taurocholate cotransporting polypeptide (NTCP) receptor [35, 36]. The specific binding is mediated by the PRE-S1 protein (present in the large

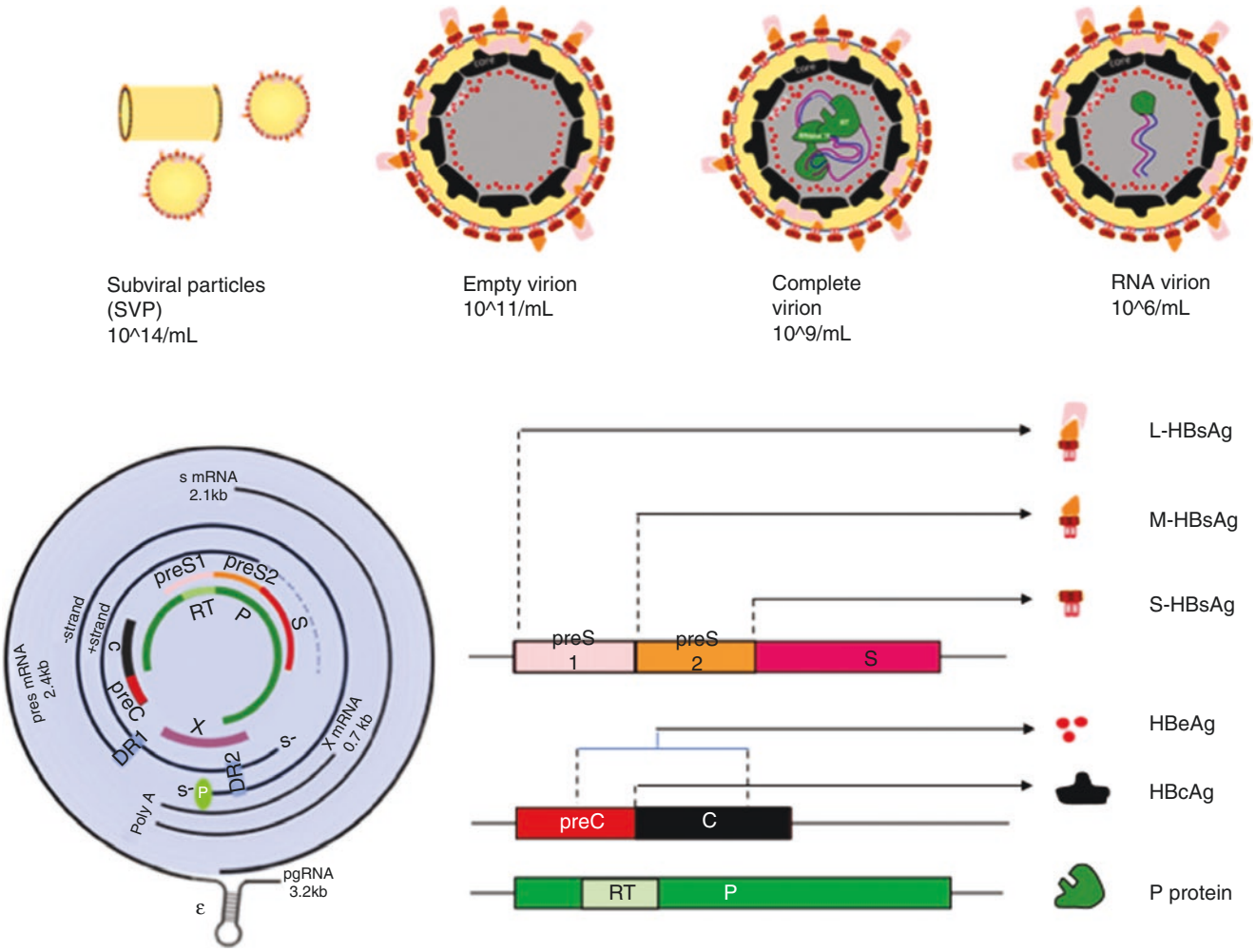

Fig. 16.3 Schematic representation of the different vial particles produced during HBV infection and HBV genome organization

envelope protein) that is budding out from the envelope of mature virions [37]. Interestingly, the location of PRE-S1 protein is changing during the maturation of HBV. Newly secreted HBV virions have their PRE-S1 protein located in the interior side of the viral envelope, and this might diminish unspecific docking of viral particles immediately after excretion.

After entering the hepatocytes, the viral particles are uncoated and the whole nucleocapsid reaches the nucleus, where they release their partially double-stranded rcDNA [38]. The host DNA repair machinery then converts the rcDNA to cccDNA, which gets packaged by histone and nonhistone proteins and forms a viral minichromosome [34]. The cccDNA is the HBV template for the transcription of HBV mRNA producing the different HBV proteins and of the pgRNA. This process is regulated mainly by HBx protein that, through the block of the function of host chromosome protein 5 (SMC5) and SMC6 complexes, governs cccDNA expression. The SMC5/6 complex is a host restriction factor for extracellular DNA that silences cccDNA. HBV X, by destroying this complex, relieves this inhibition and allows gene expression of HBV [39].

As we already described in the paragraph related to HBV morphology, HBV pgRNA is encapsulated by core proteins in the cytoplasm of the cells together with the polymerase protein (see Fig. 16.4). The polymerase protein reverse transcribes pgRNA to rcDNA [18]. This is the process that is inhibited by the nucleoside or nucleotide inhibitors (nucleoside analogs or NA), the drugs currently used to treat HBV [40]. Note, therefore, that NA drugs inhibit the production of HBV-DNA (and therefore the production of new mature virions) but do not alter the quantity of cccDNA already present in the nucleous of infected cells [41] and the quantity of HBV mRNA and HBV protein expression. The HBV viral cycle is terminated by the process of production of enveloped HBV: the mature nucleocapsid (containing rcDNA) gets enveloped by HBV surface proteins (large, medium, and small) and secreted outside [38].

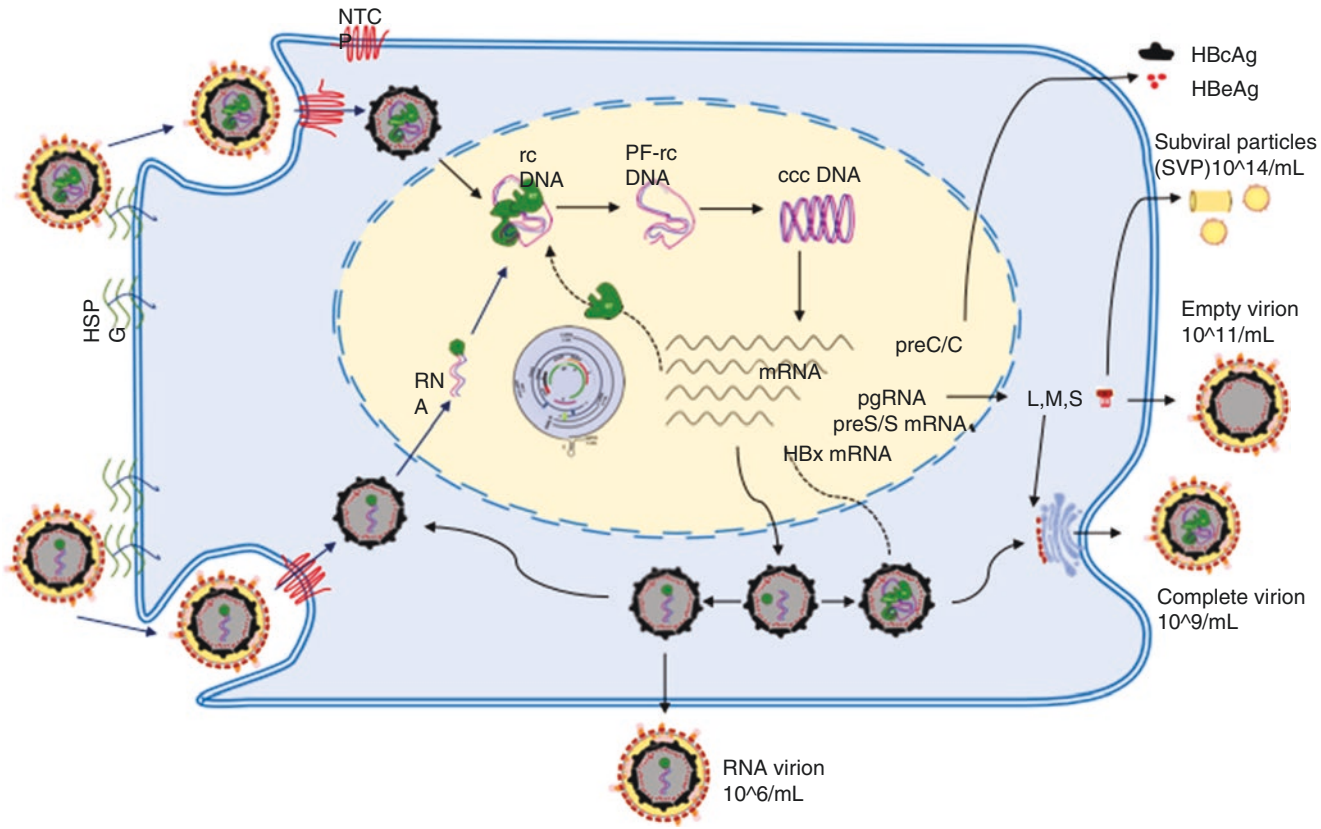

Fig. 16.4 Schematic representation of HBV viral cycle

One other important part of the HBV viral cycle is its ability to integrate into the host genome. It is common in all the hepadnaviruses [42], and HBV-DNA integration is detected also in hepatocytes that have cleared HBV cccDNA [43]. While initially HBV-DNA integration was described preferentially in patients with HBV-related HCC [44], it is now clear that HBV-DNA integration is present even in the early phases of HBV infection [45] and occurs very early during HBV replication cycle [46], within 5 days from infection [46], generally in 1 out of 10^5 infected cells [42]. The mechanism of HBV-DNA integration into the host genome derives from the production of mature particles with double-stranded linear HBV-DNA (dsl DNA) [47]. The production of dslDNA is occurring in about 10% of pgRNA containing capsids. Like the rcDNA containing capsids, dslDNA can recirculate back into the nucleous, and it might more easily integrate into the host genome, particularly in genome zones with double-stranded genomic breaks [48].

Despite woodchuck hepatitis virus (WHV) (a virus of hepadnavirus family infecting woodchucks) integrations were frequently detected in the Myc oncogenes [49], integrated HBV-DNA sequences in hepatocytes are heterogeneous, without any real hot spots of integration sites. They can present high complex rearrangements and deletions, but the whole dslDNA can also be integrated [47]. However, due to its reduced length, the integrated dslDNA cannot produce the entire pgRNA and thus HBV-DNA integration is not a source of whole mature virions [47]. Instead, it can produce whole or fractions of the different HBV proteins. For example, since the ORF of HBsAg is often intact in the integrated HBV-DNA, HBsAg can be produced, and a large part of HBsAg present in sera of adult chronic HBV-infected patients (particularly with anti-HBe+ infection or hepatitis) is produced from HBV-DNA integration [50]. HBx protein can also be produced from HBV integration, but it is classically fused to other host proteins since the stop codon of HBx is lacking in the integrated HBV-DNA form [51]. Other HBV proteins are not produced in the complete form, but short sequences or chimeric proteins can be synthesized [52]. The evolutive advantage for HBV to integrate into host genomes is not clear. However, its conservation in all hepadnaviruses infecting different animals (duck and woodchucks) suggests that it has a natural role in HBV persistence. The pathological consequences of HBV integration are also not clear. Historically, HBV-DNA integration has been studied mainly in the context of its role in hepatocarcinogenesis [44, 53, 54]. The first demonstration of HBV-DNA integration into the host genome was shown in HCC lines and HCC tissue [53–55] and leads to the suggestion that HBV-DNA integration was the cause of tumorigenesis. Many different

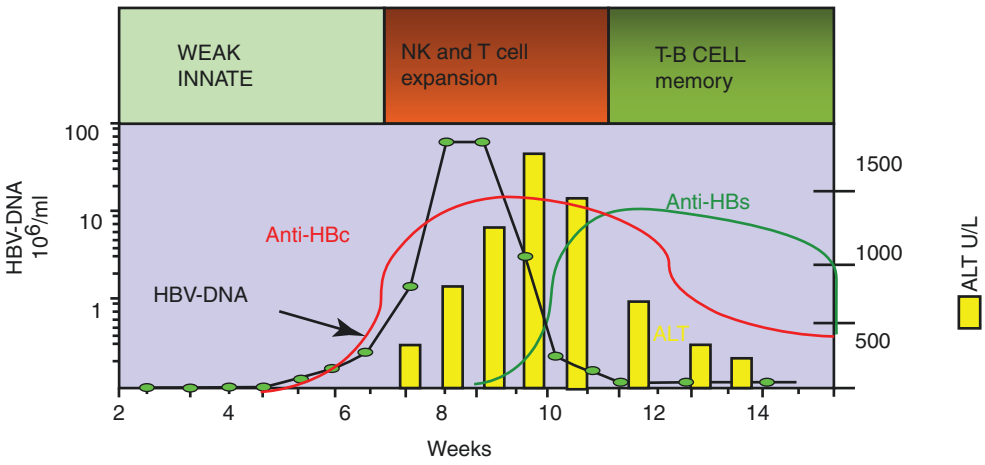

Fig. 16.5 Kinetics of HBV-DNA and host immunity in acute HBV infection

mechanisms were reported like insertional mutagenesis, induction of chromosomal instability, or production of aberrant proteins, but the mechanism of HBV carcinogenesis remains still poorly elucidated [56].

Host Immunity in HBV Infection: Innate Immunity

Host immunity against pathogens evolved in separate branches defined as innate and adaptive immunity. They perform different tasks to efficiently limit infections. The innate immunity branch has the scope to limit pathogen spread. It is activated by recognition of nucleic acids or proteins of the pathogen or by tissue damage. Activation is mediated by different families of cellular receptors (i.e., TLRs, RIG-1, DHX9, AIM2) located within the infected cells and classically leads to rapid intracellular production of cytokines like IFN-alpha or IFN-beta and to an activation of NK cells [57]. Innate immune activation triggers then adaptive immunity that acts through the maturation and expansion of distinct pathogen-specific B and T cells.

One of the peculiar characteristics of HBV in comparison with other viral infections is its poor activation of the innate immune system. The quantity of pro-inflammatory cytokines in the serum of patients with acute HBV infection is of lower magnitude and with delayed kinetics compared, for example, with HCV- and HIV-infected patients [58, 59]. The lower quantity of cytokines present in patients' sera is in line with the lack of flu-like symptoms experienced by most HBV infection patients. These observations are supported by the demonstration of limited induction of IFN-related genes in experimentally infected chimpanzees during the initial phases of HBV infection [60]. One reason for the inability of HBV to trigger innate immunity might be its delayed viral replication kinetics. Indeed, while after infection most viruses enter a logarithmic phase of propagation (e.g., HCV, HCMV, HIV, influenza), HBV, after acute infection, displays a delayed amplification of virions and spread (Fig. 16.5). HBV-DNA levels reach a maximum only 6–8 weeks after infection [59–62]. The lack of induction of type I IFN genes is, however, not only observed during acute infection but also during chronic viral reactivation [63] and in the livers of woodchucks chronically infected with woodchuck hepatitis virus (WHV) [64].

Whether HBV escapes or actively inhibits innate immune recognition has been highly debated [65–67]. A list of recent reviews discussing in detail the relation between HBV and innate immune is provided [68–70]. However, briefly, even though a minimal level of HBV recognition by RIG-1 [71] and production of type I IFNs were detected in in vitro infection systems [72] and in HBV chimeric mice [73], respectively, more recent data performed in HBV-infected primary hepatocytes [74] and in liver biopsies of HBV-infected patients [75] suggests that HBV mainly escapes intracellular innate immune recognition and does not exert any robust suppressive effect. Such lack of detection could result from the replication strategy of HBV, which uses a limited amount of transcription template (cccDNA/2–4 copies × cell) sequestered within the nucleus of infected cells. This produces polyadenylated viral mRNA that resembles the normal cellular transcripts and transcribes pgRNA to rcDNA within viral capsids shielding this process from the RNA-detection machinery of cells [65].

HBV is however susceptible to innate immune triggering. Intracellular activation of retinoic acid-inducible gene-I (RIG-I) [71] or APOBEC [76] pathways or cytokines such as IFNα [77], IFNγ, TNFα, and IL-1β [78, 79] produced by non-parenchymal cells of the liver suppresses HBV replication and reduces the pool of cccDNA [77]. The efficacy of innate cytokines to inhibit HBV is also supported by data of coinfection with HCV and HDV that activate innate immunity [80, 81].

The antiviral efficiency of type I IFNs against HBV is, however, weak. Only high doses of IFN-alpha are able to directly inhibit/clear HBV in vitro [76], and the antiviral effi-

ciency of IFN-alpha therapy is higher in HCV than HBV-infected patients [82]. For example, whereas in HCV-infected patients, IFN-a-based therapy results in a sharp decrease in viremia within the first 48 hours [82], a HBV-DNA reduction is observed only after 3–4 weeks of therapy in CHB patients. In studies conducted in humanized chimeric mice repopulated with human hepatocytes, HBV limited the direct antiviral effect of IFN-alpha by inhibiting nuclear translocation of STAT-1 and thus interfering with transcription of interferon-stimulated genes (ISG) [73].

Efficient HBV suppression and even complete clearance can be also mediated by more classical T cell cytokines like IFN-gamma and TNF-alpha [78, 83, 84] or through triggering of lymphotoxin beta/alpha receptor that induces activation of nuclear deaminases capable of destabilizing cccDNA [76].

Yet, it is however important to note that the ability of cytokines to suppress HBV was demonstrated in experimental systems devoid of chronic inflammatory events. The intrahepatic environment of chronic HBV patients is often altered by the presence of IL-10 [85], TGF-beta [86], and arginase [87], and high levels of suppression of cytokine signaling 3 (SOCS3), a negative regulator of cytokine signaling, can be detected [64, 88]. Thus, the efficacy of cytokines might be different in a liver microenvironment characterized by chronic inflammatory events.

Host Immunity in HBV Infection: NK and NKT Cells

NK and NKT cells are the cellular arm of innate immunity and can recognize and kill viral infected cells. NK cells are activated by target cells that express low level of MHC class I along with upregulation of host- or pathogen-encoded ligands signaling cell stress. NK cells respond also to cytokines induced by viral infections, such as type 1 interferons, IL-12, and IL-18 [89].

Other cells at the crossroad between innate and adaptive immunity that are, together with NK^{bright} cells, extremely abundant in the liver are iNKT cells and MAIT cells.

iNKT cells are a lymphocyte population that gets activated after recognition of lipid antigen associated with MHC class I like molecule CD-1. Their impact in HBV control has been shown in elegant models, but their role during natural infection is controversial, since CD-1-restricted NKT cells are abundant in mouse but extremely rare in human livers [90]. In human livers, different types of NKT cells, such as MAIT cells, are abundant [91], but these cells are not activated by lipids. MAIT cells are known to play a major role in antibacterial immunity, and their role in HBV infection is not clear. So far, a specific activation of MAIT cells has been only shown in HDV-HBV coinfection [92].

The impact of NK cells during HBV infection remains controversial, with possible protective or pathogenic roles [93]. The efficacy of IFN-α therapies has been linked with activation of NK cells [94], which can be also detected in patients who controlled acute HBV infection [61, 95]. A possible role of NK cells in the viral control was shown in woodchucks acutely infected with extremely high WHV doses (10^{11}) [96], and activation of NK cells is detectable in acute patients [61, 95]. However, at present it is not clear whether NK cells recognize HBV-infected hepatocytes. HBV has never been demonstrated to induce cellular stress or down-regulation of HLA-class molecules. This is why the regulatory capacity of NK cells on HBV-specific T cells has also been analyzed, which showed in chronically infected patients that NK cells can actually promote HBV persistence, since they contribute to HBV-specific T cell deletion by a mechanism of direct T cell killing [97].

Host Immunity in HBV Infection: Adaptive Immunity

The adaptive immunity is recognized as a crucial player in the clearance of HBV infection. Numerous reviews have summarized their different aspects [68, 98, 99]. Briefly, while data have shown that CD8+ cytotoxic T cells can clear HBV-infected hepatocytes through cytolytic and non-cytolytic mechanisms, CD4+ helper T cells are necessary for the efficient maturation of HBs-specific B cells (producing protective antibodies) and for the induction and maintenance of efficient CD8+ cytotoxic T cells.

This coordinated collaboration between the different components of adaptive immunity (T and B cells) is occurring in adults after acute HBV infection and leads to HBV control, but the kinetic of such induction is peculiar in HBV [98] in comparison with other viral diseases. Following the slow HBV expansion after infection (the so-called incubation period), HBV-specific CD4+ and CD8+ T cells are detectable in the blood at around 4–7 weeks after infection at the time of exponential increase in HBV replication [61, 95] (see Fig. 16.5). In other viral infections (i.e., HIV, influenza), activation of adaptive immunity occurs only 1–2 weeks after infection.

The detection of HBV-specific T cells in the blood is temporally associated with a decline of more than 90% of the HBV-DNA that precedes the peak detection of liver damage [84, 100, 101] (see Fig. 16.5), a kinetic that suggests that a large quantity of virus elimination is caused by a non-cytopathic process mediated by IFN-γ and TNF-α, secreted by CD8 T cells. We also know that, during successive HBV control, intrahepatic recruitment of HBV-specific cytotoxic T cells is facilitated by the secretion of chemokines (i.e., CXCL-10) and by platelet activation [102–105]. Platelets

help the docking of HBV-specific T cells to liver endothelial cells and facilitate the recognition/killing of infected hepatocytes. Recruitment of non-antigen-specific cells (monocytes, non-HBV-specific T cells) that amplify hepatocellular damage [106, 107] occurs after.

Quantitatively, the frequency of HBV-specific T cells in patients with acute HBV infection is low in comparison to other viral infection, with frequencies in blood that rarely exceed 1–2% of total T cells [108]. However, such numbers might not perfectly represent the size of the HBV-specific T cell repertoire, since HBV-specific T cells are enriched in the liver [109, 110] and an analysis of intrahepatic HBV-specific T cell frequency during acute hepatitis has so far not been performed in humans.

Antibodies are also produced during acute HBV infection. Their kinetics of production reveal a difference between antibodies specific for envelope (anti-HBs Ab) and nucleocapsid (anti-HBc Ab) (see Fig. 16.5). Anti-HBc antibodies are detected at very early stage of the infection, while anti-HBs antibodies only appear at later time points, after HBV-DNA declines [27, 111]. Such differential kinetics are not only explained by the fact that large quantity of HBsAg is produced and secreted into the circulation during acute HBV infection and thus can block anti-HBs detection [27, 112] but also by the maturation defects present in HBs-specific and not HBc-specific B cells in the presence of HbsAg [113, 114]. Such dysfunctionality of HBs-specific B cells was not only demonstrated in chronically infected patients but also during the early phase of acute HBV infection [115].

The profile of adaptive immunity in patients with chronic HBV infection is instead radically different. If we exclude anti-HBc-specific B cells that are functional during chronic HBV infection [114], T cells specific for different HBV proteins and HBs-specific B cells are present in lower frequency, upregulate exhaustion markers (mainly PD-1, but also TIM-3, Lag-3, and CD160) [115, 116], and have deep metabolic and energetic impairments [117–119], making them unable to exert antiviral functions and more susceptible to killing by NK cells [97]. Similarly, HBs-specific B cells, both in periphery and within the liver, display defects in the maturation toward antibody-producing cells [113, 120].

The causes of the functional defects of adaptive immunity in chronic HBV infection are heterogeneous. Dose of HBV infection and age and genetic background of the host play roles in the ability of the immune system to control the virus and mount a coordinated activation of T and B cell responses [121–123]. It is however likely the protracted presentation of large quantity of viral antigens can drive T and B cells toward progressive functional exhaustion. In this prospect, it seems logical that preferentially HBsAg, whose quantity exceeds other HBV antigens, appears to affect both B and T cells. Functional impairment is exclusively detected in HBs- and not in HBc-specific B cells [114], and envelope-specific T cells (both CD8 and CD4) are preferentially deleted in adult patients with chronic HBV infection [116, 124].

The Antiviral Mechanisms of T and B Cells

During HBV infection, B cells can produce antibodies specific for all the different HBV proteins, but only antibodies specific for envelope proteins (S and Pre-S1) have protective values [112]. Antibodies against HBcAg (anti-HBc Ab) have been hypothesized to be responsible for some form of fulminant hepatitis [125], and they are an immune marker of ongoing or progress of HBV infection. Antibodies against HBeAg (anti-HBe Ab) are used to differentiate clinical phases of HBV-induced disease, and their pathogenic role is unknown. It is important to remember that since HBV spreads to noninfected hepatocytes through an HBV-receptor-dependent mechanism [126], protective antibodies (specific for HBs and PreS1) have importance not only in prevention of the infection but can also modulate HBV spread during chronic infection. The protective ability of anti-HBV antibodies was not fully elucidated until the recent discovery of the sodium-taurocholate cotransporting polypeptide (NTCP) as the HBV receptor [35, 36], along with the establishment of easily infectible in vitro cell lines. This allowed precise mapping of HBV regions essential for infectivity, which are the pre-S1 domain and the antigenic loop region (known as the "a-determinant") of the HBsAg (reviewed in [127]). The pre-S1 domain (in particular amino acids 2–48) interacts directly with NTCP [35, 36], whereas the "a-determinant" mediates the initial docking of HBV to heparin sulfate proteoglycans on hepatocytes [128].

HBV-specific T lymphocytes act instead as the principal effector mechanism of viral clearance and liver inflammation. HBV-specific CD8 T cells recognize directly HBV-infected hepatocytes through recognition of HBV peptides presented at the surface of infected cells [100]. HBV-specific T cells can lyse HBV-infected hepatocytes [100, 129] and secrete cytokines (IFN-gamma, TNF-alpha) that trigger a process of non-cytolytic HBV clearance [83] and recruitment of inflammatory immune cells [106, 107]. HBV-specific CD4 T lymphocytes regulate the intensity of these processes.

While it is clear that HBs-specific but not HBc-specific B cell functionality has a protective function in HBV infection, the hierarchy of protective ability of T cells specific for different HBV antigens is still not clear. Multi-specificity has been associated with resolution [130], and CD4+ and CD8+ T cells recognizing different viral determinants are present in different quantities and establish a hierarchy of dominant and subdominant epitopes, but their comparative protective effect is unknown [98]. Instead, it seems established that

core- and polymerase-specific T cells persist more easily in chronic HBV patients, while HBs-specific T cells appear deleted in adult chronic HBV patients, likely caused by the continuous impact of HBsAg presentation [116, 124].

Immunomodulatory Roles of HBV Antigens

The persistent production of the soluble forms of HBV surface antigen (HBsAg) and e antigen derived from the core protein (HBeAg) in excessive amounts over whole virions is likely to play a role in the inhibition of host immunity.

Data in animal models have elegantly defined the mechanisms that enable HBeAg to suppress HBV-specific T cells in newborns [30, 31]. Moreover, experimental evidences demonstrated the ability of HBsAg to block the protective efficacy of anti-HBs antibodies [131] and to alter HBs-specific B cell function [113, 120].

Other effects are much more controversial. Persistent exposure to circulating HBsAg was suggested to impair the frequency and function of myeloid cells [132, 133] and plasmacytoid dendritic cells [134, 135]. It was also suggested that soluble viral antigens can inhibit antigen presentation, by altering the ability to produce cytokines, and thus prevent the induction of HBV-specific T cells [136]. However, the inhibitory effect of HBsAg on dendritic cell function has not been confirmed [137], and such defects are not compatible with the fact that chronic HBV patients were never shown to be more susceptible to opportunistic infections. In contrast, for example, reports have shown that in patients with malaria, HBsAg positivity is associated with lower parasitemia [138]. There are also data derived from newborns of HBV-infected mothers that show a beneficial effect of maturation of host immunity by HBV infection [139]. It is therefore likely that the effect of HBsAg on host immunity is specific for HBs-specific B and T cells and not for global immunity. The evidences of the preferential persistence of polymerase and core T cells in adults with HBV infection support such conclusions [116, 124].

Natural History of HBV Infection

The natural course of HBV infection can be modified by variables such as viral load, virus genotype, route of infection, and the age, sex, and genetics of the infected hosts [15, 123]. The majority of symptomatic acute HBV infections that occur in adults (after sexual or iatrogenic infection) do not develop chronic HBV infection but are able to control the virus and become HBsAg negative with the development of anti-HBs antibodies [6]. Such state is not associated with the presence of liver diseases despite HBV, like other human DNA viruses (HCMV, EBV), is not completely eliminated. Small quantities of infected hepatocytes persist in all subjects that were productively infected by HBV [140]. HBV-DNA can be detected in biopsies of individuals after resolution of HBV infection or in patients with chronic hepatitis but with HBsAg negativity with or without presence of anti-HBc antibodies. Such profile has also been defined as "occult HBV infection." The pathological consequences of the presence of such low quantity of infected hepatocytes are controversial [141, 142]. Patients with "occult HBV infection" are at risk of HBV reactivation after immunosuppressive treatment [143]. Yet the risk of HCC development in patients with "occult HBV infection," in the absence of other pathological processes of the liver, is low in comparison with chronically infected patients with productive HBV infection and detectable HBsAg and HBV-DNA in the blood [144]. This is also the reason why the current goal of therapies in chronic HBV patients is to obtain "functional HBV cure," a clinical, virological, and immunological status which coincides with "occult HBV" [145]. A few reviews discussing these definitions and the different pathological consequences of HBV infection are listed [6, 41, 144–147].

HBV infection occurring at birth and during childhood is instead more frequently developing into chronic HBV infection [121]. However, neonates/children are not completely unable to mount an efficient antiviral immunity against HBV, and evidences of vertically infected patients able to control HBV spontaneously or after early treatment are increasing [148]. Nevertheless, a large quantity of patients with chronic HBV infection acquired the viral infection at birth, without any associated symptoms of acute hepatitis. The establishment of chronic HBV infection is then characterized by different levels of HBV replication and occurrence of inflammatory events in the liver that have been used to schematically divide the infection into four clinical phases [147]. Figure 16.6 shows the different phases using the definition accordingly to EASL 2017 [149] and AASLD nomenclatures [147]. The EASL 2017 nomenclature divides clinical phases of natural chronic HBV infection in relation to the presence/absence of HBeAg and serological signs of liver inflammation (hepatitis/infection). AASLD uses a definition based on the concept that the initial phase of infection, characterized by a high level of viral replication and absence of serological signs of liver inflammation, is a more "immunotolerant phase" than later phases when liver inflammatory events are evident (thus defined "immune active" and immune reactivation). Patients with low level of HBV replication and normality of ALT are instead defined as "inactive chronic HBV." These divisions have been the cornerstone of clinical management of chronic HBV, since also pathological processes (i.e., cirrhosis or HCC) are more likely to develop while patients are in the inflammatory phases [147]. Nevertheless, these divisions do not always provide a correct representation of the clinical or immunological features. For

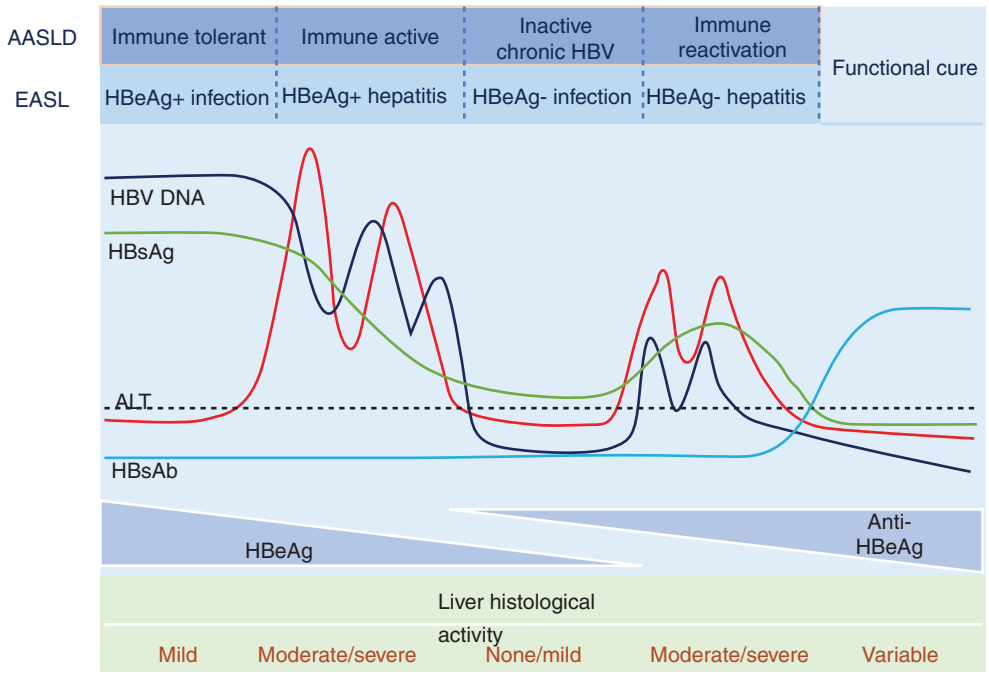

Fig. 16.6 Natural history of hepatitis B. Representation of the four clinical/virological phases (AASLD 2018 or EASL 2017 nomenclatures) of chronic hepatitis B, followed by functional cure

example, the demonstration that patients defined as "immunotolerant" have HBV-specific T and B cells at similar frequency and with comparable functionality as patients defined with chronic active hepatitis has challenged the use of his term [150]. Similarly, patients without serological evidences of hepatitis (thus with normal ALT level) have shown the presence of inflammatory events in the liver [151]. Nevertheless, it seems clear that chronic HBV infection is characterized by phases of infection in which levels of HBV replication, production of HBV antigens (HBsAg and HBeAg), and inflammatory responses in the liver vary, and they are differentially associated with pathological consequences. It is, for example, controversial whether treatment of chronic HBV infection should only be started in subjects with elevated ALT levels (thus with clear signs of hepatitis) or whether patients in the initial phase of disease (HBeAg+ infection or immunotolerant phase) should be targeted in order to prevent pathological consequences [152, 153].

Therapy of Chronic HBV Infection

The best therapy of HBV infection is its prevention that can be efficiently achieved with a vaccine based on HBsAg protein that elicit high titers of anti-HBs antibodies in the majority of immunized individuals [154]. Vaccination and immunoglobulin enriched with anti-HBs antibodies diminishes also the rate of the vertical infection of newborns from HBV-infected mothers [155]. These preventive strategies have contributed to reduce drastically HBV infection and its pathological complications in countries with high endemicity [156, 157].

When chronic HBV infection is already established, the current standard therapies are nucleotide/nucleoside analogs, thus drugs that targeted the reverse transcriptase, and IFN-alpha, a cytokine that has both antiviral and immunomodulatory effects. Nucleotide/nucleoside analog (NA) therapies are very effective in reducing both viral replication and liver inflammation. They suppress the development of hepatic failures and hepatocellular carcinoma in chronic HBV patients [158], and they diminish the risk of vertical infection by pregnant women [159]. Inhibition of viral replication is robust, with HBV-DNA becoming undetectable in sera after 2–4 weeks of therapy, and the emergence of resistant strains of HBV is rare particularly with the last generation of compounds [160]. However, since NAs do not target the HBV cccDNA, treatment needs to continue over time to avoid the risk of HBV reactivation and associated hepatic flares. In addition, NAs do not target the production of HBV proteins, and thus HBsAg levels are not altered even after long-term treatments [161]. As such, the frequency of patients achieving functional HBV cure, a condition characterized by sustained HBsAg negativity and anti-HBs positivity, is negligible. IFN-alpha treatment can instead achieve functional HBV cure through mechanisms that involve a direct inhibition of viral replication and a boost of antiviral immunity, but this occurs only in about 5% to 8% of the treated patients and is linked with side effects that can be severe.

To increase therapy efficacy, new drugs targeting different steps of viral life cycle or the antiviral immune response (Fig. 16.7) have been developed, and recent reviews have described the current efforts to provide better HBV treatment [6, 40, 69, 99, 162].

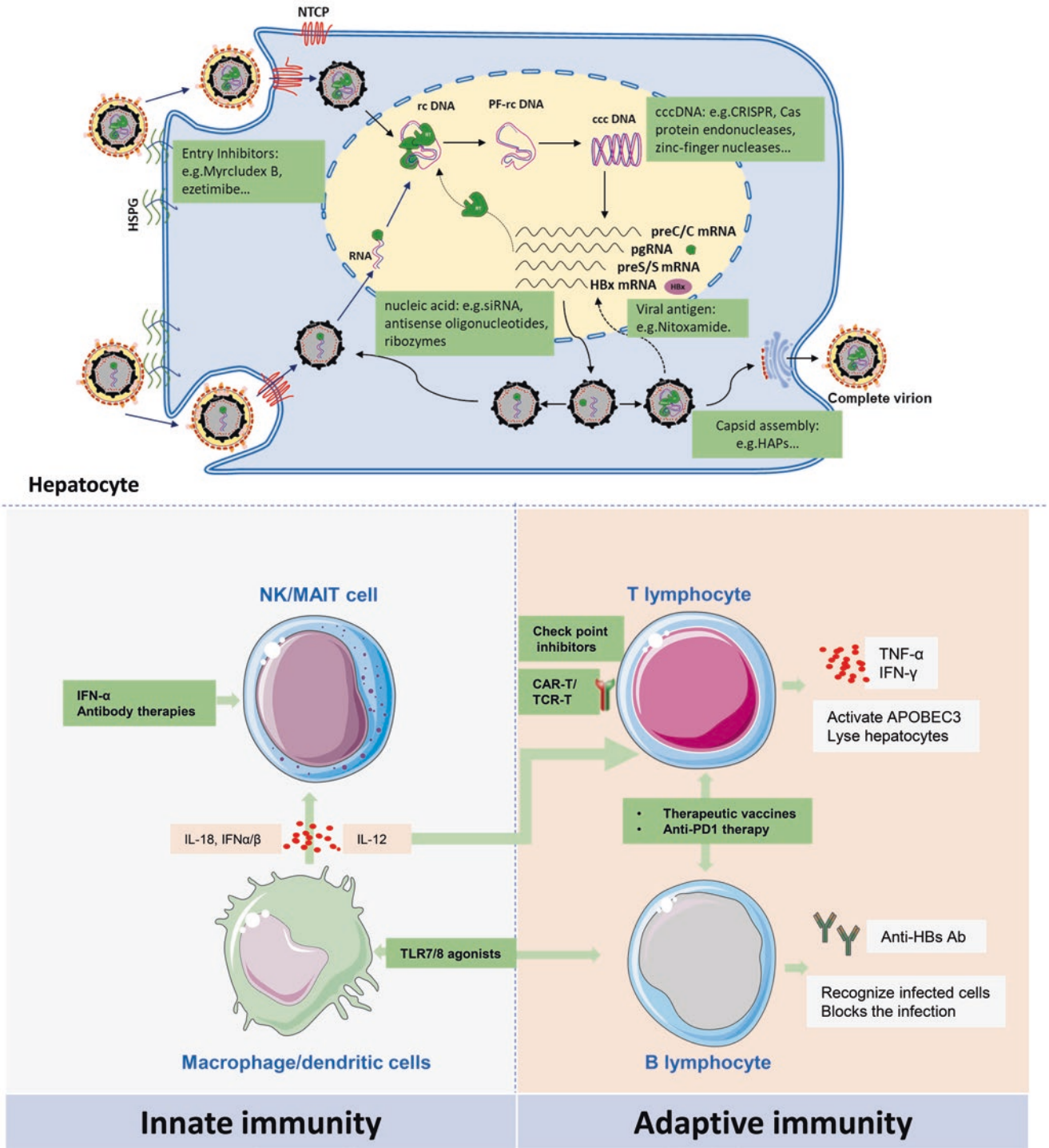

Fig. 16.7 Schematic representation of the therapeutic strategies targeting directing HBV natural cycle in hepatocytes and innate or adaptive immunity

The holy grail of HBV treatment is to eliminate HBV cccDNA, either directly or indirectly by affecting intracellular cccDNA recycling or blocking new rounds of infection or silencing its transcriptional activity.

Since the release of complete HBV virions results in infection of new hepatocytes with an increase of the cccDNA pool, blocking infection can progressively reduce the pool of infected hepatocytes [163]. Strategies to prevent HBV infection include the use of a peptide (Myrcludex B, also known as bulevirtide) that derived from the sequence of the Pre-S1 domain that binds to the NTCP receptor or monoclonal antibodies [126, 164]. A clinical trial of Myrcludex associated with IFN-alpha therapy showed encouraging results in HBV-HDV coinfection [165]. Other compounds such as ezetimibe

and cyclosporine derivatives have also been evaluated in experimental models for their ability to inhibit HBV viral entry [166, 167].

Direct inhibition of cccDNA formation using small molecules is attractive, but since cccDNA formation requires the use of many host nuclear enzymes, nuclear histones, and other components of host chromatin [168], such task might have severe side effects and has been so far unsuccessful. cccDNA can possibly be targeted by zinc-finger nucleases or transcription activator-like effector nucleases, which were used with success in vitro [169]. CRISPR and Cas protein endonucleases have been also used to inactivate cccDNA [170]. Nevertheless, before these gene editing approaches can reach the clinic, problems of hepatocyte-specific delivery, off-target effects, cleavage of integrated HBV-DNA, and the unpredictable consequences of chromosomal DNA recombination need to be addressed.

Other new therapeutic strategies are instead trying to target viral gene expression with the aim to reduce not only mature virion production but also the expression of viral antigens (see Fig. 16.7).

Blocking HBx activity that regulates HBV transcription through degradation of the transcriptome repressor SMC5/6 can lead to an inhibition of viral transcription. Nitazoxanide, an anti-protozoa drug, demonstrated, in cultured HBV-infected hepatocytes, to inhibit the HBx-DDB1 interaction and to restore SMC5 levels, therefore obtaining a suppression of viral transcription and protein productions [171].

Numerous nucleic acid-based strategies (RNA interference, antisense oligonucleotides, and ribozymes) to control posttranscriptionally the production of HBV proteins and mature virions were developed [172]. The suppression of HBsAg production derived from both cccDNA and HBV-DNA integration might facilitate the recovery of HBV-specific immunity. HBsAg presence is indeed altering HBs-specific B cell function [113, 120], while its general effect on the functionality of global host immunity is controversial. Data in animal models and trials in humans have shown the efficacy of such strategies to reduce HBV protein and HBV replication levels, but there has been no demonstration of a spontaneous recovery of HBV host immunity [50]. Compounds that block HBsAg release have been also developed. Nucleic acid polymers have shown clinical efficacy in combination with IFN-alpha, but the mechanism of action, the specificity of the "release inhibition" to HBV viral protein, and its overall toxicity need to be better evaluated [173].

Finally, a numerous number of small molecules have been developed to interfere with the formation of capsid [40, 174].

Many different therapeutic interventions are instead trying to achieve functional cure through direct stimulation or restoration of antiviral host immunity [69, 99, 175].

Therapies targeting the innate immunity exploit the antiviral effect of cytokines but can also restore adaptive immunity. IFN-alpha therapy, for example, acts by directly inhibiting viral replication in HBV-infected hepatocytes and stimulating NK cell activity [94], but a recovery of HBV-specific B and T cell immunity is detected only in patients who reach "functional cure" [176].

Therapies with antiviral cytokines do not only inhibit viral replication but can also clear cccDNA [76]. The cytokines can be delivered in their native form or pegylated to increase their half-life, or they can be bound to antibodies targeting HBV-infected hepatocytes for more targeted delivery [177].

Activation and production of antiviral cytokines able to suppress HBV replication can be also obtained with molecules, which are targeting pattern recognition receptors present in different cell types and that have been modified to have a preferential intrahepatic delivery.

TLR (TLR-7, TLR-8) and RIG-I agonists are the major representatives of such class of immune therapy. A Rig-I agonist (Inarigivir) that has been shown to directly inhibit HBV replication and to activate type IFN-I within the hepatocytes has demonstrated efficacy in animal models and in patients [162]. Similarly, a TLR-7 agonist (GS-9620), which preferentially induces IFN-alpha production in liver resident plasmacytoid dendritic cells, induced a very robust but transient antiviral effect in woodchucks [178] and chimpanzees [179]. A recent phase I/II clinical trial in chronic HBV patients has however shown little antiviral efficacy [180], but this is likely due to the fact that the dose used in human was low (~40 times less than in animals) to avoid the triggering of hepatic flares that were detected in some treated animals. The potential induction of inflammatory events in the liver caused by immune-based therapies is however a general problem of all these strategies, which requires to be better rationalized. It is difficult to think that, for example, compounds like TLR-8 agonists will not induce any inflammatory events in the liver. TLR-8 agonists have been designed to activate, through production of IL-18 and IL-12 by intrahepatic myeloid cells, NK and MAIT cells [181], and they can possibly recover exhausted HBV-specific CD8 T cells [182] (see Fig. 16.7). Interesting new TLR7/8 agonists tested in woodchucks have been also suggested to restore HBV-specific B cell responses [183].

The different therapeutic strategies that are designed to restore adaptive immunity are also likely to trigger inflammatory events in the liver in relation to their ability to restore HBV-specific T cell immunity. Strategies like vaccine therapies [184, 185] or the use of antibodies blocking checkpoint inhibitors (anti-PD-1 antibodies) on T and B cells have shown some efficacy in animal models [186] and in few patients [187, 188]. However, the success of anti-PD-1 therapy in chronic HBV patients was linked with the triggering of a hepatic flare that coincides with the recovery of HBV-T

cell responses [188]. Other new strategies are also in development and utilize the possibility to restore HBV immunity through engineering HBV-specific T cells using different constructs able to recognize HBsAg (chimeric antigen receptor, CAR) [189] or classical HBV epitopes (through T cell receptors) [190]. These new therapies are efficient in animal models, where they can achieve even complete HBV clearance, but control of HBV is linked with the triggering of hepatitis [190, 191].

Even though different strategies have been developed to limit such liver inflammation [192], it seems logical that all the therapeutic approaches designed to restore immunity will trigger liver inflammatory events. Even antibody therapies, which have shown success in some mouse models [193], act not only by blocking infection, but they can also facilitate NK cell recognition of HBV-infected hepatocytes through antibody-dependent cellular cytotoxicity. Therefore, one of the next challenges in the development of HBV therapies will be to understand the optimal doses and the combination of therapies that can achieve functional cure with triggering a level of hepatic inflammation that is safe and easily controlled [194, 195]. In addition, the possibility to treat patients at initial phases of infection will be taken into consideration, when immune recovery can be potentially better achieved [194, 196]. It is an exciting time for HBV research and therapies. A better understanding of the HBV life cycle and of the host immunity linked with advancements in biological technologies gives us the opportunity to explore different variables that could, in a not so distant future, achieve a functional cure of HBV in the large worldwide population of infected people.

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Hepatitis C

17

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Abbreviations

CTLs	Cytotoxic T lymphocytes
DAAs	Direct-acting antiviral agents
DCs	Dendritic cells
GWAS	Genome-wide association study
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hepatic stellate cell
IFN	Interferon
IL	Interleukin
IRES	Internal ribosomal entry site
ISG	Interferon-stimulated gene
KIR	Killer inhibitory receptor
LDL	Low-density lipoproteins
MAIT	Mucosal-associated invariant T cells
MDSC	Myeloid-derived suppressor cells
MSM	Men who have sex with men
NK	Natural killer
NKT	Natural killer T
NS	Nonstructural
PBMC	Peripheral blood mononuclear cells
PWID	People who inject drugs
SCID	Severe combined immunodeficiency
SNPs	Single-nucleotide polymorphisms
SVR	Sustained viral response
TCR	T cell receptor
Tfh	Follicular helper T cell
Tfr	T follicular regulatory
Th	Helper T cell
TLR	Toll-like receptor
Treg	Regulatory T cell
VLDL	Very-low-density lipoproteins

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Key Points

- Hepatitis C virus (HCV) infection is a global health burden and one of the main causes of liver-related death.
- Development of potent direct-acting antivirals (DAAs) against HCV has improved the clearance rate of HCV; however, new or reinfection of HCV has been on the rise in the world.
- Pervasive and moderately compromised immune dysfunction against HCV is a hallmark of chronically infected patients, the fundamental mechanisms of which are still undisclosed.
- HCV clearance from patients with chronic HCV infection by DAAs partially, but not completely, restores the function of immune cells.
- In order to achieve the goal of HCV elimination from the globe, the development of a prophylactic HCV vaccine is urgently needed.

Introduction

Both the hepatitis C virus (HCV) and hepatitis B virus (HBV) contribute to the global health burden [1]. Estimated numbers of individuals who are chronically infected with HCV or HBV worldwide are 71 and 235 million, respectively [2]. Both viruses are hepatotropic, but not directly cytopathic, and they elicit progressive liver injuries resulting in end-stage liver disease, unless effectively controlled. It is well known that the relative percentages of acutely

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infected patients developing chronic hepatitis are different when comparing HBV and HCV infections. Less than 10% of HBV-infected patients develop chronic hepatitis, while more than 80% of HCV-infected ones do so [3, 4]. One of the major determinants in the clinical course of viral hepatitis is the host immune response. It has been proposed that the ability of infected hosts to mount a vigorous and sustained cellular immune reaction to HBV and HCV is required for controlling the primary infection. Once HBV or HCV survives the initial interaction with the host immune system, it uses several means to nullify the selective immunological pressure during the later phases of infection.

Eradication of hepatitis virus from infected hosts, or at least the suppression of the viral replication, is critical for the prevention of the development of liver cirrhosis and hepatocellular carcinoma (HCC). Owing to the improvement of anti-HCV therapy, HCV clearance from patients is attainable in more than 95% of cases in the practical, clinical practice [1]. The WHO set the target for global HCV elimination by 2030; however, several issues still remain to be resolved in order to achieve such a goal [5, 6]. First, the number of new HCV infections has been robustly increasing in high- and middle-income countries. Annually, approximately 1.75 million new infections occur, 23% of which are caused by intravenous drug usage or people who inject drugs (PWID) [7]. Of particular importance, such an increasing trend of new HCV infections in a PWID population is significant in younger generations, spanning from 20 to 40 years of age [8]. Second, the risk of HCV reinfection has been increasing because of the diversity of social consciousness regarding sexual behavior. The meta-analysis has demonstrated that the percentages of HCV reinfection in patients who once cleared HCV by interferon (IFN)-based therapy were 10% in the PWID or incarcerated population and 15% with HIV coinfection or men who have sex with men (MSM) [9]. And, third, the risk of HCC development cannot be eliminated, even after HCV clearance by direct-acting antiviral agents (DAAs), in patients with advanced liver fibrosis at baseline. Furthermore, in patients who underwent treatment for HCC before DAA, the recurrence rates of HCC are 39% at 1 year and 60% at 2 years after a sustained viral response (SVR). These observations indicate that the extensive research on immune responses against HCV is warranted to establish the strategy for prevention of new cases of HCV or reinfections and occurrence or recurrence of HCC.

In this chapter, we discuss the current understanding of the roles of innate and adaptive immunity in the pathogenesis of acute and chronic HCV infections as well as their resolution after treatment-induced HCV clearance.

Life Cycle of HCV: Models

HCV generally is spread through parenteral routes and reaches the liver via the bloodstream. Virions may circulate as free particles or as particles bound to immunoglobulins and low-density or very-low-density lipoproteins (LDL or VLDL) [10]. The viral surface protein E2 can bind cellular CD81 (tetraspanin) as well as scavenger receptor class B type I (SR-BI) [11], both of which are found on hepatocytes and appear to function in the viral entry. The LDL receptor [12] and the lectins L-SIGN and DC-SIGN [13] may also facilitate entry of the virus into the hepatocyte. Further entry is dependent on the presence of claudin-1, claudin-6, and/or claudin-9 [14], occludin (OCLN) [15], the cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1) [16], epidermal growth factor receptor (EGFR), and ephrin receptor type A2 [16], and the virion is endocytosed into clathrin-coated pits. Viral surface membrane and endosomal fusion occur in the context of acidification, and the nucleocapsid is released into the cytoplasm where uncoating occurs. The positive strand RNA initiates translation by means of an internal ribosomal entry site (IRES) that is located in the 5′ noncoding region and binds the 40S ribosome. Junctions between structural proteins are processed by the host signal peptidase from the endoplasmic reticulum. This leads to the formation of a single polyprotein that is processed into individual peptides in a co-translational and posttranslational fashion, using both viral and cellular protease activities (Fig. 17.1). A replication complex then arises from a combination of viral nonstructural proteins and cellular material. Viral NS4B, NS5A, NS5B, and the NS3 helicase-NTPase domain are known to be important components of this structure, and the cellular substrate is referred to as a "membranous web," which is a perinuclear vesiculo-membranous aggregate thought to be derived from the endoplasmic reticulum. At this site, active RNA synthesis occurs. The assembly and release of mature virions is not completely understood. Assembly likely occurs in lipid raft structures, and secretion may be dependent on the ion channels formed by the p7 protein. Gastaminza et al. showed that the virus hijacks the host machinery for assembly, maturation, degradation, and secretion of VLDL, thereby partly explaining the tropism for hepatocytes [17]. HCV nucleocapsid is built from units of the core protein with RNA, surrounded by a membrane derived from the human cell with embedded heterodimers of the envelope glycoproteins E1 and E2. The virions associate with LDL and VLDL forming lipo-viro-particles.

In the liver, in situ hybridization shows up to 50% of hepatocytes contain HCV infection [18]. The proportion of infected hepatocytes were from 1% to 54% and correlated with viral load, but not with HCV genotypes, as reported

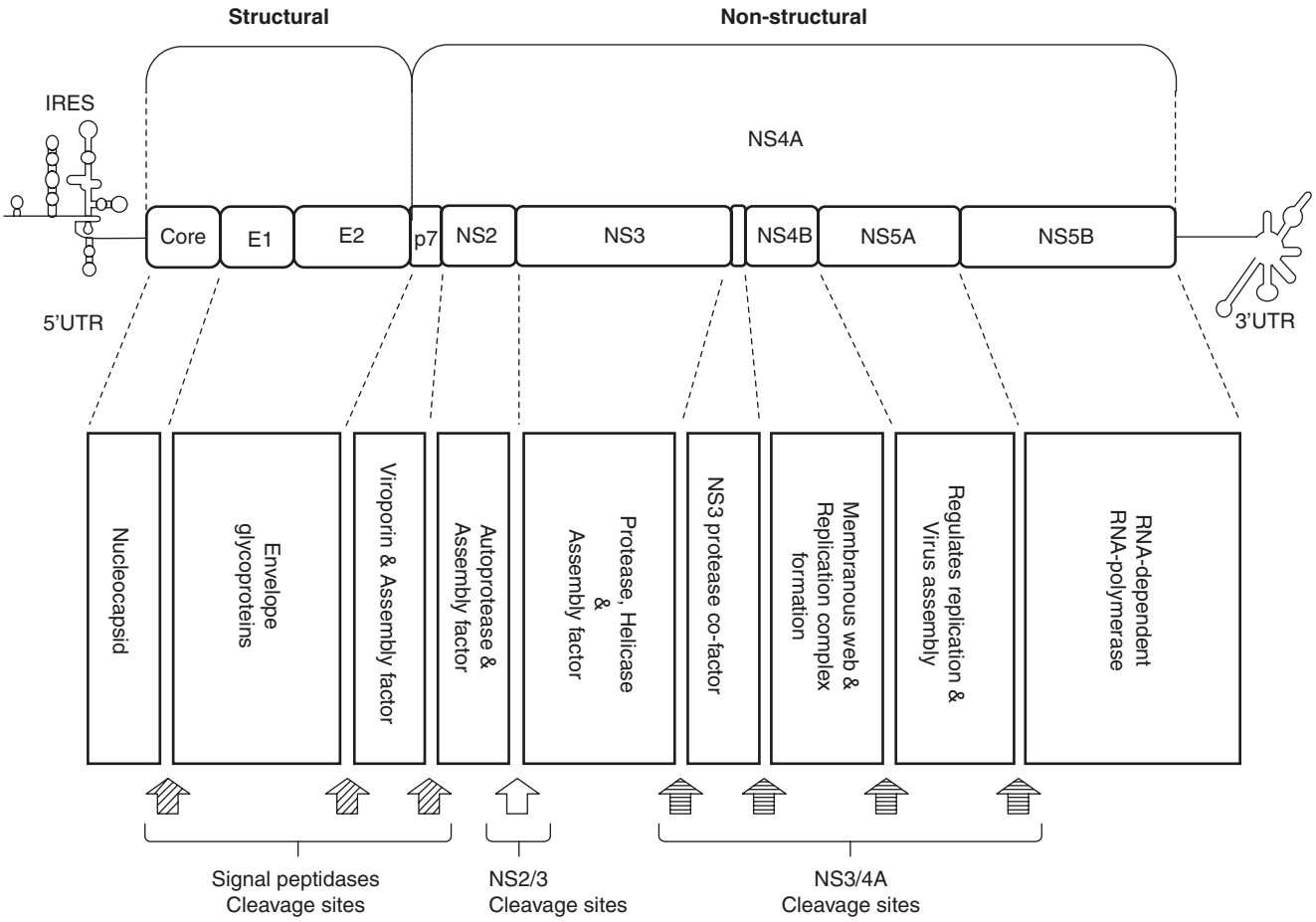

Fig. 17.1 HCV genome and polyprotein. The HCV genome is composed of an open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs). IRES-mediated translation of the ORF leads to the formation of a polyprotein that is processed into ten viral proteins. Cleavage of the core protein from E1 involves cellular signal peptidases, which also cleave E1, E2, and p7 from the polyprotein. The NS2-NS3 protease auto-cleaves itself. The NS3 protease located in the first one-third of NS3, assisted by its membrane-bound cofactor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A, and NS5B

elsewhere [19]. Using RT-PCR, HCV has also been detected in lymph nodes, pancreas, bone marrow, spleen, thyroid, brain, and adrenal glands [20, 21]. It is not known whether the virus replicates in hematopoietic cells.

Hepatitis C virus replicates poorly in tissue culture. The development of permissive cell culture models has long been awaited, and the first in vitro replicating HCV strain was isolated in 2005 from a Japanese patient with fulminant hepatitis termed JFH-1 virus, a genotype 2a [22]. In this system, very few cell lines were able to replicate the virus, often having adaptive mutations within the viral genome or impairment of cellular antiviral mechanisms. Actually, these models have greatly contributed to the understanding of innate antiviral mechanisms against HCV and the development of DAAs.

The in vivo chimpanzee model is the only one that mimics most, but not all, aspects of the human infection. However, the research on chimpanzees is now strictly restricted. In order to establish a small animal model that mimics HCV replication in humans, homozygous uPA-SCID mice with human chimeric liver were developed to provide susceptibility to HCV infection [23]. Importantly, the mice not only can be infected with JFH-1 but also with patient-derived viruses of all genotypes. One of the major drawbacks of this model is its immune deficiency, thus hampering the study of host-virus immune responses. Such chimeric mice have been useful for the study of basic aspects of the HCV life cycle, the assessment of novel antiviral therapies, and emergence of resistance-associated substitutions of HCV against DAAs.

Key Players in Immune Responses Against HCV

After HCV primarily infects the liver, viral replication continues and viral particles are continuously released into the circulation. The first lines of defense are provided by antivi-

ral type I IFN and subsequent IFN-inducible genes (ISGs). As for cellular components in innate immune system, natural killer cells (NK) and natural killer T cells (NKT) play major roles in liver immunology, the populations of which are relatively increased in the liver compared to the periphery. These cells are activated in the liver, where the expressions of IFN-α and ISGs are extremely high during the early phase of the hepatitis virus infection [24]. Activated NK and NKT cells secrete IFN-γ or TNF-α, which, in turn, inhibit the replication of the hepatitis virus mainly through non-cytolytic mechanisms (Fig. 17.2) [25].

Dendritic cells (DCs) or macrophages in the liver are capable of taking up viral antigens and processing and presenting them to other immune cells (see Fig. 17.2) [26]. As DCs and macrophages express distinct sets of Toll-like receptors (TLRs) and cytosolic pathogen sensors (RIG-I or MDA5) [27], it is likely that some viral components stimulate DCs and macrophages through ligation of these receptors. DCs develop a mature phenotype and migrate to lymphoid tissues (see Fig. 17.2), where they stimulate effectors, including T cells and B cells (see Fig. 17.2). Following the encounter of DCs with other cells, DCs secrete various cytokines instructing or regulating the functions of the adjacent cells [26]. In addition to these cytokines, DCs express various co-stimulatory molecules and ligands to enhance or limit the functions of immune and infected cells. The existence of functionally and ontogenetically distinct DC subsets has been reported, i.e., myeloid DC1 (mDC1), mDC2/BDCA3$^+$DCs, and plasmacytoid DCs (pDCs) [28]. Myeloid DCs predominantly produce interleukin (IL)-12 or TNF-α following pro-inflammatory stimuli, while PDCs release a considerable amount of IFN-α upon viral infection depending on the immune stimulus; both cytokines in actuality can be produced by both cells.

It is generally accepted that adaptive immunity performs a critical role during the clinical courses of hepatitis. The involvement of antigen-specific CD4$^+$ T cells in HCV eradication has been well described during both acute or chronic infections [29]. Helper CD4$^+$ T cells have an immunoregulatory function mediated by the secretion of cytokines that support either cytotoxic T lymphocyte (CTL) generation (Th1) or B cell function and antibody production (Th2) (see Fig. 17.2). In addition to Th1/Th2 paradigm, CD4$^+$ T cells secreting IL-17 (Th17) are induced with distinct cytokine conditioning and are involved in liver inflammation or autoimmunity. DC ontogeny and DC-derived cytokines are crucially associated with the differentiation or polarization of helper T cell subsets. There is little evidence that CD4$^+$ T cells mediate direct liver cell injury in viral infection. Thus, it is likely that CD4$^+$ T cells play a critical role in facilitating other antiviral immune mechanisms, such as enhancing CD8$^+$ effector function. The antigen-primed CTLs are recruited to the liver (see Fig. 17.2) and constitute the critical element in the eradication of virally infected cells (see Fig. 17.2). The increment of specialized immune suppressors such as regulatory T cells (Tregs) has been demonstrated in HCV infection [30, 31]. These cells are

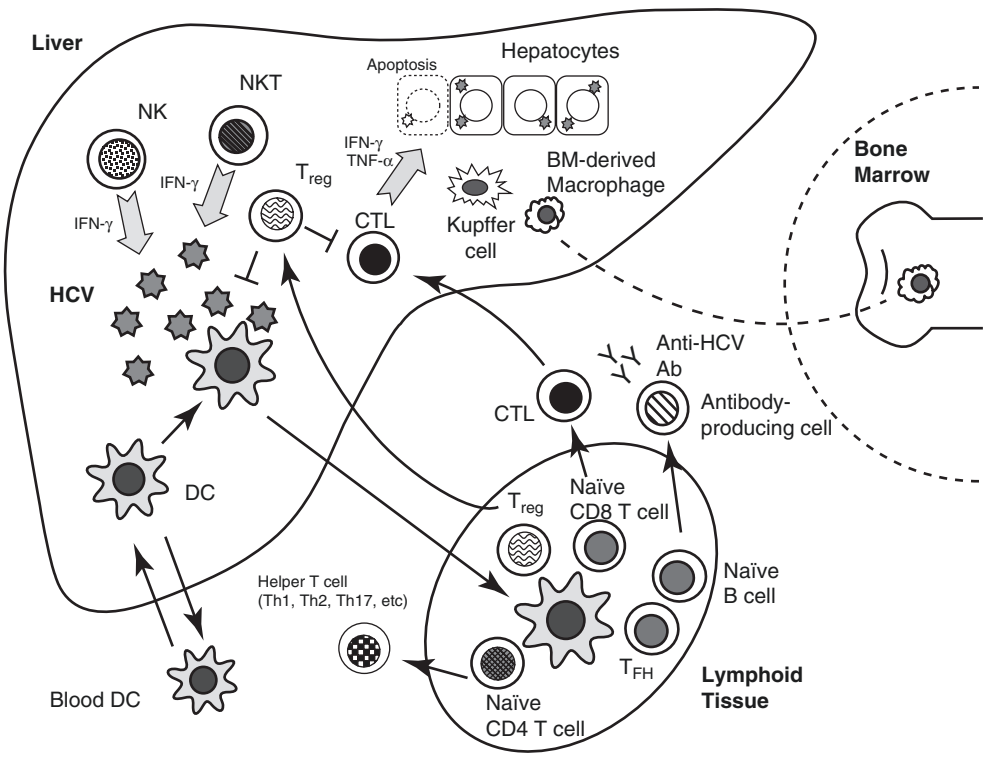

Fig. 17.2 Key players in the immune reaction associated with viral hepatitis. Immune cells involved in HCV infection are shown. The details are described in the text. CTL cytotoxic T lymphocyte, DC dendritic cell, HCV hepatitis C virus, NK natural killer cell, NKT natural killer T cell, TFH follicular helper T cell, Th helper T cell, Treg regulatory T cell

actively involved in the alleviation of Th1- or CTL-mediated liver inflammation, thus contributing to persistence of the hepatitis virus (see Fig. 17.2).

Clinical observation of patients who were repetitively infected with a single-source HCV strongly suggests that sterilizing immunity is barely induced against HCV even in patients with previous resolution. Coexistence of HCV with the anti-HCV antibody in the circulation of chronic hepatitis patients indicates that such an antibody lacks neutralization capacity. However, active differentiation from naïve B cells to antibody-producing cells is evident in patients with the HCV infection, because the anti-HCV antibody is abundant in serum spanning from the acute to chronic phase of infection. Escape mutations and impaired differentiation of B cell lineage might be involved. Myeloid-derived suppressor cells (MDSCs) constitute another type of regulatory cells that are induced from myeloid or granulocytic lineage, the inhibitory function of which is mediated by arginase-1 [32]. The increase of MDSCs is reported in patients with HCC, regardless of the etiology of liver disease [33]. Mucosal-associated invariant T (MAIT) cells are unique innate-like cells that link innate and adaptive immunity [34]. These cells are activated by bacterial products, which are derived from the synthesis of vitamin B6. MAIT cells are activated by not only the HCV infection but also by dengue or the influenza virus. Activation of MAIT cells is mediated by TCR-independent mechanisms and leads to suppression of the HCV replication [35]. The role of MAIT is still controversial in the course of the HCV infection.

Innate Immunity Against HCV: HLA Alleles and IL-28B/IFN-λ3

In primary HCV infection, HCV-RNA levels rapidly increase during the first few days of the HCV infection and continue to be high during the incubation periods [36], which lasts for up to 10–12 weeks following infection. The visualization of HCV-infected hepatocytes by in situ hybridization of the HCV genome has enabled to estimate that 20–30% of hepatocytes are infected and form clusters separated from each other [19]. Although HCV triggers expressions of type I IFN and ISGs in the liver during this phase [24], the HCV viral load does not decrease. This suggests that HCV impedes the execution of antiviral molecular mechanisms, including interferon regulatory factor (IRF)-3 [37], as well as NF-κB- and RNA-dependent protein kinase (PKR) [38]. Wieland et al. reported that, by using highly sensitive in situ hybridization system, HCV-infected hepatocytes and their adjacent uninfected hepatocytes exhibited positive signals of ISGs, suggesting that the stimulus driving ISG induction originates from HCV-infected cells [19]. In adults with primary HCV infection, approximately 20% of patients successfully clear HCV, while the remaining 80% of the cases fail to do so [4]. It has been acknowledged that some host factors, such as genetic backgrounds or immune responsiveness, are involved in influencing the distinct outcomes. Genome-wide association studies (GWAS) have disclosed that certain types of human leukocyte antigen (HLA) alleles are accumulated in patients who spontaneously cleared HCV, spanning from class I to class II including extended haplotypes, i.e., HLA-A03, HLA-B27, HLA-B57, HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*1101, and HLA-DQB1*0301 [39, 40]. Presumably, some HCV-derived peptides could specifically bind to relevant HLA alleles, thus tuning the directions and the tones of anti-HCV immunity. Further studies need to be performed to explore the functional impact of various HLA alleles or haplotypes on the pathogenesis of the disease.

Several groups reported that genetic polymorphisms (single-nucleotide polymorphisms, SNPs) upstream of the promoter region of the IL-28B/IFN-λ3 gene are critically involved in the efficacy of IFN-based treatment in patients with chronic hepatitis C in multiple ethnic cohorts [41–43]. Furthermore, the same IL-28B SNPs are involved in the successful spontaneous HCV eradication [44]. These reports clearly indicate that IL-28B or IFN-λ3 is essentially involved in HCV eradication, however, the mechanisms of which have been largely unknown. The interferon-lambda 4 (IFNL4) gene has been inactivated in a large part of the human population by a frameshift mutation (ss469415590, TT), and a dinucleotide variant (ss469415590, ΔG allele) is functional. Interestingly, the TT allele is positively associated with HCV clearance, suggesting that the disruption of the IFNL4 gene is beneficial for humans in the context of the HCV infection [45]. Recently, multi-ancestry GWAS of three studies on spontaneous HCV clearance identified important variants in the MHC locus, IFNL4-IFNL3, and G-protein-coupled receptor 158 gene (GPR158) [46].

IFN-λs, or type III IFNs, comprise a family of highly homologous molecules consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). In clear contrast with type I IFNs, they are released from relatively restricted types of cells, such as hepatocytes, intestinal epithelial cells, or DCs. In a case of successful HCV eradication, it is postulated that IFN-α/β and IFN-λ cooperatively induce antiviral ISGs in HCV-infected hepatocytes. It is of particular interest that, in primary human hepatocytes or chimpanzee liver, IFN-λs, but not type I IFNs, is primarily induced after HCV inoculation, the degree of which is closely correlated with the levels of ISGs [47]. These results suggest that hepatic IFN-λ could be a principal driver of ISG induction in response to HCV infection. Some investigators showed that the expression of IL-28 in PBMC was higher in subjects with

IL-28B major than those with IL-28B minor genotypes; however, the levels of IL-28 transcripts in liver tissue were comparable regardless of the IL-28B genotype [41, 48].

Innate Immunity Against HCV: DCS, Macrophages, and NK Cells

Dendritic cells, as immune sentinels, sense specific genomic and/or structural components of pathogens with various pattern recognition receptors and eventually release IFNs and inflammatory cytokines [49]. Limited reports have been published on the roles of blood DCs in acute HCV infection. Ulsenheimer et al. reported that, in acute hepatitis C, pDCs are reduced and functionally impaired; however, such a decrease is not specific to HCV infection and is due to liver inflammation [50]. DCs play a decisive role in shaping innate immunity by interacting with NK cells. It is tempting to speculate that the impairment of DCs in NK cell activation is partly responsible for the failure of HCV control in the early phase of the primary HCV infection. Further studies addressing the DC and NK cell functions in acute HCV infection are required for the successful design of immune therapies based on the induction of efficient antiviral responses.

In humans, the existence of phenotypically and functionally distinct DC subsets has been reported: myeloid DC1 (mDC1), mDC2/BDCA3$^+$DC, and plasmacytoid DC (pDC) [51]. Of particular interest is the report that mDC2/BDCA3$^+$DCs have a potent capacity of releasing IFN-λ in response to the TLR3 agonist [52]. We examined the frequency and functions of BDCA3$^+$DCs as well as other DC subsets in patients with HCV infection. We demonstrated that human BDCA3$^+$DCs are (1) present at an extremely low frequency in peripheral blood mononuclear cells (PBMC) but are accumulated in the liver; (2) capable of producing IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B/IFN-λ3 robustly in response to HCV; (3) able to recognize HCV by a CD81-dependent mechanism, endosome acidification, and TRIF-dependent mechanism; and (4) able to produce larger amounts of IFN-λs upon HCV stimulation in subjects with a IL-28B major genotype (rs8099917, TT) [53]. In contrast, pDCs are able to produce a large amount of IFN-α/β, instead of IFN-λ, upon HCV infection [53, 54].

Several investigators, including ourselves, reported that mDCs and pDCs in HCV-infected patients were reduced in number and impaired in their ability to promote Th1 polarization and IFN-α production in chronic HCV infection [54, 55]. One of the explanations for such a reduction is enhanced apoptosis of DCs, which is partly due to a diminished NF-κB activity [56]. Dysfunctional DCs are involved in the exhaustion of CD8$^+$ T cells, confirming that DCs play an important role in linking innate immunity to adaptive immunity [57]. Functional impairment of DCs diminished when HCV had been eradicated from patients, revealing the evidence of HCV-induced DC dysfunction [58]. NK cells from HCV-infected patients downregulate DC functions in the presence of hepatocytes by secreting suppressive cytokines, IL-10 and TGF-β1 [59].

Controversial reports have been published regarding the frequency and functions of DCs in patients with chronic hepatitis C [55, 58, 60–63]. By using cutting-edge mass cytometry, Doyle et al. reported that liver pDCs are polyfunctional for producing not only IFN-α but also multiple immune modulators in patients with chronic HCV infection [64]. The responsiveness of pDCs in HCV infection seems to be fair, because TLR7 or TLR9 agonists were effective for the enhancement of their IFN-α production and the suppression of HCV replication [65].

Macrophages are a major population of non-parenchymal cells in the liver. Ontogenetically, there are two cell subsets: the tissue-resident Kupffer cells and bone marrow-derived macrophages [66]. The concept of M1-/M2-polarized macrophages has been accepted for the understanding of macrophage functional diversity. Activation of macrophages in HCV infection is evident by several reports regarding the higher serum levels of macrophage-related factors, soluble CD163 [67], IL-34, and M-CSF [68]. These observations support the notion that macrophages play a critical role in the progression of liver fibrosis. HCV-E2 glycoprotein induced macrophages into the M2 phenotype by the activation of CD81, EGFR, to STAT1/STAT3 [69]. In various liver diseases, including the HCV infection, CD206$^+$ macrophages are reported to be involved in liver inflammation by releasing TNF-α or GM-CSF. However, single-cell transcriptomic analysis revealed that dichotomous plasticity may be a feature of macrophages in the liver. Saha B et al. reported that HCV-infected hepatocytes drive monocytes into M1/M2 mixed differentiation, thereby promoting hepatic stellate cell (HSC) activation by TGF-β [70]. For the regulation of macrophage differentiation or function, TLR2 [71], TLR3 [72], or TLR7/8 [73] agonist has been examined, potentially aiming at an anti-HCV vaccine.

NK cells are one of the key players that comprise the first line of antiviral immune responses. The precise mechanism of the NK cell activation in HCV infection has not yet been elucidated. Pollmann et al. reported that the interaction of monocytes and NK cells in the presence of HCV-infected hepatocytes is involved in NK activation, by way of the signals through an OX40 (NK)-OX40L axis [74]. NK cells express various functional receptors; one group transduces inhibitory signals (killer inhibitory receptors/KIRs, CD94, NKG2A) and the other transduces activating signals (NKG2D) [75]. The function of NK cells is dynamically regulated in vivo by the balance between expressions of counteracting receptors and their association with relevant ligands [76]. Large-scale cohort studies of the HCV infection have disclosed that certain combinations of HLA-C and KIR haplotypes (KIR2DL3) are closely associated with

spontaneous HCV clearance [77, 78]. Such epidemiological observations raise a possibility that NK cells play an active role in HCV eradication. Recently, it has been reported that CD94 plays an inhibitory role in NK cell function. Blockade of CD94 on NK cells could enhance not only NK cells but also bystander $CD8^+$ T cells, suggesting that CD94 is a checkpoint molecule on NK cells [79]. NK cells also play a regulatory role against other types of immune cells. Boelen L et al. reported that the ligation of inhibitory KIRs on NK cells enhanced $CD8^+$ T cell responses against viral infection, including HCV [80]. This finding is suggestive that NK cells could be a therapeutic target for enhancing not only innate but also adaptive immunity.

T Cell Responses in Acute or Chronic HCV Infection

HCV-RNA levels rapidly increase during the first few days of HCV infection and continue to be high during the incubation periods [36], which lasts for up to 10–12 weeks following infection. In parallel with the onset of acute hepatitis, activated HCV-specific T cells enter the liver [81]. HCV-specific $CD4^+$ and $CD8^+$ T cell responses and IFN-γ co-expression coincide with decreases in HCV quantity [81]. Vigorous, multi-epitope-specific, Th1-type, and sustained $CD4^+$ T cell responses are detected in resolved cases [36]. By contrast, in cases that progress to chronic hepatitis, $CD4^+$ T cell responses are weak, narrowly selected, and short-lived [82]. The frequency of HCV-specific $CD8^+$ T cells is high during the acute phase of infection (2–8% of peripheral $CD8^+$ T cells); however, the frequency decreases after HCV persistence develops (0.01–1.2%) [83]. Despite the high numbers of CTLs, some of these cells are "stunned" in the acute phase, as demonstrated by an inability to produce IFN-γ and to proliferate in response to HCV antigens [83, 84].

One of the crucial mechanisms of attaining HCV persistence during the primary infection is the rapid and frequent occurrence of escape mutations in the HCV genome. It has been acknowledged that selection pressure on epitopes by immune cells, relevant epitope-specific $CD4^+$ [85] and $CD8^+$ T cells, is the cause of such a phenomenon. Novel mechanisms on the induction of HCV escape mutations in patients with acute and chronic HCV infections have been reported. Interferon-induced transmembrane proteins (IFITMs), one of the IFN-inducible host factors that restricts HCV entry, drive HCV evasion in cooperation with the anti-HCV neutralizing antibody [86].

In chronic hepatitis C patients, HCV-specific $CD4^+$ T cells were functionally impaired, and their activity was not sustained [87], which was in clear contrast to the resolved cases. Inoculation studies of infectious HCV to recovered chimpanzees demonstrated that $CD4^+$ T cell help was indispensable for the development of effective $CD8^+$ T cell responses to protect from HCV persistence [88]. In patients who spontaneously cleared HCV, an increase of $CD161^+CCR6^+CD26^+$ Th17 cells capable of producing IL-21 was reported [89]. Follicular helper T cells (Tfh) are a subset of $CD4^+$ T cells that contribute to pathogen-specific antibody responses by supporting B cell maturation into antibody-producing cells. IL-21 is a signature cytokine released from Tfh or Th17. In patients with acute HCV infection, ICOS expressions on HCV-specific Tfh are correlated with an anti-HCV antibody response [90]. The decreased frequency of Tfh and lowered serum levels of IL-21 in patients with chronic hepatitis C may be involved in impaired B cell responses [91]. The functional relevance of Tfh and IL-21 in HCV infection needs to be further explored.

The mechanism of the decision fate of patients at the primary HCV infection, whether they become resolvers or non-resolvers, has been one of critical questions that need to be answered. By using an integrative system immunology approach, Wolski D et al. reported that metabolic dysregulation of $CD8^+$ T cells during early infection alters gene expression regarding nucleosome regulation, T cell differentiation, and inflammatory responses. Such responses were found to be correlated with age, sex, and HCV-specific $CD4^+$ T cell responses, thus associating with the state of T cell exhaustion [92].

With regard to HCV-specific $CD8^+$ T cells, observed during the chronic stages of disease, conflicting results have been reported for their roles in HCV replication and liver inflammation. HCV-specific $CD8^+$ T cells in chronic hepatitis C patients possess a lowered capacity to proliferate and produce less IFN-γ in response to HCV antigens [93]. As $CD8^+$ T cells are reported to be involved in HCV-induced liver inflammation [94], inefficient $CD8^+$ T cells may evoke only mild hepatocyte injury, a level which is not sufficient for HCV eradication [95].

T cell exhaustion is a mechanism that is involved in persistency of hepatitis virus. Under continuous exposure to a large amount of viral proteins, antigen-specific T cells become hyporesponsive to repetitive antigen stimulation in proliferation and cytokine production. With regard to an inducer of exhausted T cells, extensive studies have been performed on PD-1 expression on HCV-specific $CD8^+$ T cells [96]. In the transition from acute hepatitis to the chronic phase, it is reported that $CD8^+$ T cells expressing PD-1 are increased in the patients [97]. However, inconsistent observations regarding PD-1 have been reported by other investigators [98]. Of particular importance, HCV-specific $CD8^+$ T cell responses were restored in vitro in the presence of masking antibodies against PD-L1, suggesting that PD-1/PD-L1 pathway could serve as a therapeutic target [99, 100]. Pilot studies were performed in chimpanzees to verify the efficacy of the blockade of PD-1 using an anti-PD-1 antibody against HCV infection. In one out of three chimpanzees, the reduction of HCVRNA

was observed during the administration of the anti-PD-1 antibody but relapsed after the cessation of treatment [101]. In the clinical trial of anti-PD-1 antibody for patients with chronic HCV infection, 6 out of 47 patients who underwent low-dose anti-PD-1 antibody treatment showed a reduction in HCVRNA titers. However, such a viral response was not durable, and some of them exhibited immune-related adverse events such as severe liver injury or hyperthyroidism consistent with autoimmune thyroiditis [102]. A combination of the blockade of PD-1 with other inhibitory receptors or the addition of direct antiviral agents may be warranted to gain satisfactory results. Myers LM et al. reported that SIRPα is a marker of $CD8^+$ T cells that retained functionality even with the co-expression of co-inhibitory receptors [103]. Wieland D et al. reported that $TCF1^+CD127^+PD1^+$ HCV-specific $CD8^+$ T cells are exhausted and memory phenotype [104]. Interestingly, such T cells remained in patients even after HCV elimination for a long time and easily expanded in the case of HCV reinfection [104].

Several investigators have reported that the frequency of Tregs increases in chronic hepatitis C patients, either in the liver or in the periphery [30, 31]. Tregs are endowed with a suppressing capacity for NK cells, DCs, and HCV-specific T cells, thus leading to alleviation of collateral liver damage or impeding virus elimination. The question as to how Tregs are generated in HCV infection is still unknown. T follicular regulatory (Tfr) cells are a subset of regulatory T cells that suppress Tfh and the generation of antibody-producing B cells. Cobb et al. reported that Tfr cells are abundant in the HCV-infected liver, the induction of which was mediated by exosomes derived from HCV-infected hepatoma cells [105]. Myeloid-derived suppressor cells (MDSC) are induced and are capable of NK suppression not only in cancer patients but also in those with HCV infection. Although the precise mechanisms of MDSC induction are undisclosed, HCV-infected hepatocytes are involved [106]. An extensive sequential study of intravenous drug users or healthcare workers, who had higher chances of HCV infection, showed that repetitive exposure of minimal dose of HCV tended to induce Tregs, instead of raising effector T cells [107]. Such regulatory mechanisms need to be avoided in order to give sufficient immunity by awaiting protection via the vaccine against HCV.

Immune Response During and After Antiviral Therapy

From the time of the discovery of HCV, its impact on the progression of chronic liver disease has since been well acknowledged. In order to prevent the development of fatal liver disease, HCV clearance is definitely needed for patients with chronic HCV infection. Anti-HCV treatment has significantly improved from IFN monotherapy, a combination of pegylated (PEG) IFN-α and ribavirin to IFN-sparing DAA treatment [108]. Currently, standard care for chronic HCV infection, including liver cirrhosis, comprises DAAs regardless of HCV genotypes. In the clinical setting, SVR in patients who underwent DAA therapy is >95% when properly treated.

In the era of IFN-based treatment as a standard, the immunological research has been focused on the association of HCV-specific $CD4^+$ and $CD8^+$ T cell responses with successful HCV clearance. However, the results were not convincing enough, because some investigators failed to show the positive correlation between treatment-induced T cell responses and HCV clearance. GWAS revealed that the single-nucleotide polymorphisms (SNPs) neighboring IFN-λ3/IL28B gene are strongly associated with successful or unsuccessful viral responses in PEG-IFNα and ribavirin therapy for chronic hepatitis C [41, 109]. Of particular interest, it is reported that major alleles in such SNPs give a significant and positive impact on spontaneous HCV clearance [109].

The reduction of HCV quantity is quite rapid and reaches a lower limit of detection within 24 hours in patients who respond well to DAAs. Such viral dynamics in patients is considered to be influential on intrahepatic as well as the systemic immune system. Under potent DAAs, the percentage of patients who failed to clear HCV, breakthrough or relapse, is quite low, thus providing difficulty in the comparative analysis between SVR and relapsers. By comparing patients with SVR with those who experienced viral breakthroughs, it is reported that higher hepatic ISG and activated pSTAT1 and TRAIL-expressing activated NK cells at the baseline are involved in DAA-mediated HCV clearance [110].

One of the important questions that need to be answered is whether the function of immune cells was altered/restored after HCV clearance by DAAs. As NK cells are critical players of liver inflammation and HCV clearance, several investigators reported the changes of phenotypes, functions, and gene signatures of this cell population [111]. The expression of functional receptors, NKp30, NKp46, NKG2A, and TRAIL, on blood NK cells was reduced in accordance with the decrease of serum CXCL10, IL-12p40, and IL-18 levels after 24 weeks of DAA treatment [112]. The NK cell response to IFN-α improved after DAA treatment [113]. These reports suggest that HCV clearance by DAA results in not only the correction of the altered NK cell phenotype but also their function observed in patients with chronic HCV infection.

After HCV clearance by DAA, HCV-specific $CD8^+$ T cells in patients restored their proliferative capacity [114]. Of clinical importance, the recovery of $CD8^+$ T cell function seems to be dependent on the stages of liver disease. It is reported that $CD8^+$ T cells are hyperactivated in patients with liver cirrhosis, the status of which is not reversed by HCV clearance [115]. The frequencies of $PD\text{-}1^+CD8^+$ T cells co-

expressing inhibitory receptors Tim-3, CD160, 2B4, KLRG1, and Blimp-1 are increased at baseline and at the end of treatment in the patients who achieve SVR after 4 weeks of DAA treatment [116]. The frequency and the memory potential of TCF-1-expressing PD-1$^+$CD127$^+$ HCV-specific CD8$^+$ T cells are maintained after DAA-mediated HCV clearance [104, 117]. The blockade of the PD-1/PD-L1 pathway can preferentially increase the proliferative capacity of HCV-specific CD8$^+$ T cells. The proliferative capacity could not be restored following HCV clearance, suggesting that the mechanisms of T cell exhaustion are not simple. Because CD8$^+$ T cell exhaustion is a multifaceted dysfunction, which affects phenotype, capacity of cytokine production or proliferation, and metabolic alteration. Such dysfunctions were not completely reversed but sustained even after HCV clearance [117].

In chronic hepatitis C, Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells (Tregs) increase in both blood and liver tissue. It is reported that Tregs persisted both in blood and in the liver of patients who had been successfully treated with conventional IFN-based treatment [118, 119]. IFN-free DAA and IFN-based regimens resulted in only a small reduction in frequency of Foxp3$^+$CD25$^+$CD4$^+$ T cells co-expressing inhibitory molecules, such as GARP, OX-40, CTLA-4, GITR, Tim-3, and galectin-9, even 4 years after DAA-mediated HCV clearance [120]. These observations suggest that complete restoration of immune responses seems to be a tall order in patients with chronic HCV infection.

MAIT cells are a unique population of T cells that respond to bacterial products, possibly involved in gut-liver interaction of liver inflammation [121]. Circulating MAIT cells in patients remain at a low frequency while displaying an altered phenotype, and their impaired function does not change after HCV clearance by DAA therapy [122, 123], suggesting that HCV utilizes some cell-type-specific mechanisms of functional alteration.

The reports regarding the impact of DAAs on B cell lineage are limited. Lymphoproliferative malignancy or cryoglobulinemia is one of the extrahepatic manifestations of chronic HCV infection [124]. In patients with lymphoproliferative disorders, B cell frequency decreased, and serum gamma-globulins, but not total proteins, were significantly reduced after attaining SVR [125]. Similar changes were observed in patients with cryoglobulinemia vasculitis. HCV clearance by DAA therapy decreased percentages of IgM$^+$CD21$^-$/low memory B cells and decreased numbers of Tfh cells in HCV-infected patients [126]. Therefore, HCV clearance gives a significant benefit not only to the outcomes of liver-related diseases but also to extrahepatic organs.

Long-lived T cell memory, both HCV-specific CD4$^+$ and CD8$^+$ T cells, is reported to be maintained for more than 20 years after spontaneous HCV clearance [127]. The anti-HCV antibody was barely detectable at this point, suggesting that humoral immunity is not sustainable without the continuous existence of antigen. Actually, the mechanisms of T cell memory are yet to be clarified. Cytokines that support homeostatic T cell proliferation, such as IL-7, IL-15, and IL-21, are necessary. In addition, periodic stimulation of T cells with minor quantities of HCV-RNA might be involved [128]. However, questions still remain whether such sustainable T cell immunity is obtainable or not after treatment-induced HCV clearance. Upon re-exposure of HCV, protective immunity, but not sterilizing immunity, is invigorated, thus protecting infected hosts from severe ALT elevation. In the settings of HCV reinfection in individuals of high-risk behavior, PWID or MSM, protection upon re-exposure may be associated with the maintenance of polyfunctional HCV-specific memory CD4$^+$ and CD8$^+$ T cells. Additional conditions that are required are the frequency and breadth of epitope coverage of antigen-specific T cells. Based on the analysis of the TCR repertoire on HCV-specific CD8$^+$ T cells, it is reported that patients who successively cleared HCV spontaneously twice possessed TCR with higher functional avidity and polyfunctionality, which is more than patients who once succeeded but failed to be protected by HCV reinfection [129]. Further studies are urgently needed to focus on the mechanisms of DAA-induced immune memory status, shedding light on the strategy of protection of high-risk populations from HCV reinfection.

Anti-HCV Vaccine

Boosting virus-specific immune responses may be beneficial to HCV-infected patients; however, critical concerns remain unaddressed regarding potential risks of evoking severe types of hepatitis. In cases of excessive immunity such as fulminant viral hepatitis, meticulous attention needs to be paid for the application of immune intervention in order to avoid progression of liver failure. Clinical trials have been underway in order to assess efficacy or safety of anti-HCV vaccines. The development of effective vaccines against HCV has been challenging. One of the reasons is that HCV is a highly diverse virus with seven major genotypes and 67 characterized subtypes [130–132]. HCV displays greater sequence diversity than even the human immunodeficiency virus (HIV), and genotypes can differ by up to 30% in nucleotide sequences and subtypes by up to 15% [133]. This large sequence variation in HCV has been an ongoing challenge in the development of vaccines.

There have been three major approaches for designing vaccines against HCV. The first approach is the use of the recombinant envelope proteins that induce neutralizing antibodies. There are reports regarding the involvement of anti-HCV neutralizing antibody in the HCV clearance at its primary and re-exposure [134–136]. A vaccine consisting of recombinant E1/E2 viral envelope glycoprotein provides

partial protection against chronic HCV infection [137] and induces virus-neutralizing antibodies in healthy volunteers [138]. An E1/E2 glycoprotein vaccine has been tested in chimpanzees, and the elicited immune response was able to prevent infection by a homologous virus challenge, but this vaccine was unable to prevent acute infection of a heterologous strain [137]. While the recombinant 1a E1/E2 vaccine-induced antisera from a vaccinated goat exhibited low neutralization against the genotype 2a J6 virus, the antisera did efficiently neutralize JFH-1, a closely related genotype 2a virus [139]. The existence of broadly cross-neutralizing anti-HCV antibody that can block HCV infection of various genotypes has been reported in HCV chronically infected patients or immunized animals [140, 141]. Analysis on the structure of epitope-binding site of such neutralizing antibody could provide important information on vaccine design. Alternative approach was the usage of recombinant HCV E1/E2 proteins combined with adjuvants. In phase I trial in 60 healthy volunteers, immunization of HCV E1E2 (genotype 1a) with MF59C.1 adjuvant induced HCV-specific $CD4^+$ T cell response and humoral response [142]. The important finding from this trial was the sera from this cohort showed in vitro neutralizing capacity against heterologous strain, 1a, 1b, and 2a [138].

The second approach is the utilization of virus-like particles (VLPs) that express HCV structural proteins to induce humoral and cellular immunity. In this trial on chimpanzees, immunization elicited T cell response with cytokine production but failed to induce humoral response. Upon rechallenge, all four chimpanzees became infected, but three out of them cleared HCV later [143].

The third approach is aiming for inducing potent T cell responses. The selection of more immunogenic HCV antigen may be necessary for boosting cellular and humoral immune response. Nanoparticles consisting of adjuvant and HCV p7 protein, forming ion channels for viral assembly and release, induced $CD4^+$ and $CD8^+$ T cell response in HCV-infected chimpanzees and humans. In vaccinated mice with such nanoparticles, transgene-expressing cells were cleared [144]. Replication-defective recombinant adenoviral (Ad), vaccinia virus (VV), and modified vaccinia Ankara (MVA) vectors were used to deliver antigens to prime T cell response. A combination of viral vector prime and DNA or recombinant protein boost is a promising approach. In a phase I clinical trial in 40 healthy volunteers, heterologous prime/boost regimens with chimpanzee adenovirus Ad3Ch3 and a rare strain of human adenovirus (Ad6) expressing NS region of genotype 1b were used. Both vectors primed broad T cell responses capable of responding to heterologous strains of HCV genotypes 1a and 3a. HCV-specific T cells were sustained for a year after the boost [145]. This vaccine has been moved to phase II trial in a high-risk population, PWID. The other prime/boost vaccine candidate is priming with Ad-encoding NS5B genotype 1b and boosting NS5B-encoding plasmid DNA. Although $CD8^+$ T cell responses were observed in chimpanzees challenged with this vaccine, three out of five of them were not protected [146].

Perspectives

Pervasive and mild dysfunction of immune cells is a hallmark of HCV-induced status in chronically infected patients. However, fundamental principles of immune interventions taken by HCV have been still unclear. Potent DAAs that specifically suppress HCV replication are now used as standard of care in clinics. In addition to an inhibitory effect on viral replication, such compounds are expected to restore immunity indirectly by reducing the viral burden. In patients with chronic hepatitis C, decline of viral load and alleviation of liver inflammation may be beneficial for the restoration of the once-impaired immune cell function. The advancement of cutting-edge technologies, such as mass cytometry or single-cell sequencing analysis, has now enabled us to obtain sizable data from a limited human sample size and to get deeper insight on disease pathogenesis. Extensive studies are thus needed to elucidate how the therapeutic HCV elimination reinvigorates memory T cell responses and maintains sustainable protective immunity against HCV re-exposure. Newly/repetitive HCV-infected patients are on the rise especially in middle- to high-income countries because of the expansion of PWID due to social irritability. In order to solve such socioeconomic and healthcare problems, while trying to achieve the WHO HCV elimination target by 2030, the development of protective/therapeutic vaccines could be one of the remedies. Deciphering key elements of the survival strategy of hepatitis virus could shed light on the way to disarm such fatal and intractable pathogens.

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Hepatitis D

18

Olympia E. Anastasiou and Heiner Wedemeyer

Key Points

- The hepatitis delta virus (HDV) is a noncytopathic circular, single-stranded RNA virus; it is estimated that 15–25 million people are infected worldwide.
- HDV causes the most severe form of viral hepatitis; the development of fibrosis and the progression toward cirrhosis are faster than in HBV-monoinfected patients.
- The only currently available treatment option is peg-interferon-alpha, leading to HDV suppression in 25–40% of patients and HBsAg loss in less than 10%. Addition of nucleoside or nucleotide analogues used to treat hepatitis B may only be useful in patients with HBV DNA.
- New substances blocking different steps in the HDV life cycle show promising results in clinical studies; phase 3 trials are ongoing.
- Innate immunity in hepatitis delta is not well studied. HDV can interfere with IFNα signaling. NK and MAIT cells have been implicated in pathogenesis and as a predictor of treatment response.
- Adaptive cellular immune responses against HDV are detectible but weak. Few epitopes targeted by HDV-specific T cells have been described being associated with immune escape.

Introduction

Hepatitis D virus (HDV) is the cause of the most severe form of viral hepatitis, frequently leading to liver fibrosis, cirrhosis, hepatic decompensation, and development of hepatocellular carcinoma [1]. HDV infection can only be established in the presence of a hepatitis B virus (HBV) infection, although recent in vitro data challenge this dogma [2]. The virus needs the HBV envelope proteins for assembly and transmission. HDV infection can occur as simultaneous HDV/HBV coinfection or, more frequently, as superinfection of HBsAg-positive individuals. HDV infection is a public health issue causing considerable morbidity and mortality [1]. The interplay of HDV and the immune system has been extensively studied in the last years.

Epidemiology

HDV is mostly transmitted through percutaneous or mucosal contact with infectious blood, while HDV mother-to-child transmission seems to be a very rare event [3]. It has been previously reported that 15–20 million individuals are infected with HDV worldwide [4]. Recent data indicate that this estimation may be conservative and estimate the number of HDV-infected individuals to be up to 70 million [5], even though these data have been criticized [6].

Due to its dependence on HBV coinfection or superinfection, HDV infection follows the pattern of HBV infection from an epidemiological point of view. Implementation of vaccination programs against HBV has been shown to lead to a decrease in HDV incidence [5, 7]. Its overall prevalence has been estimated to be up to 1% and its prevalence in HBsAg-positive populations without other risk factors such as intravenous drug abuse 11%, twice as high as estimated before [5]. Interestingly, the prevalence of HDV infection in HBsAg carriers without other risk factors differs greatly from region to region, ranging from 0% in Canada to 66% in some regions of Peru and 83% in Mongolia. The prevalence

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of HDV in intravenous drug users and in people who have high-risk sexual behavior is higher than in the general population, which could be attributed to the parenteral transmission route of the infection [5]. Country of origin is an important risk factor for HDV infection. HDV is endemic in some parts of the world, such as Central Africa, Eastern Turkey, Central Asia, some Eastern European countries, and the Amazonian region of Brazil. Its prevalence is also high in immigrant populations originating from these regions, which accounts for many of the cases detected in European and North American countries [1].

To date, eight HDV genotypes have been described [8]. Each of them has a distinct geographical distribution (Fig. 18.1). Genotype 1 can be found all over the world, while the distribution of other genotypes tends to be more regional. HDV genotypes 3, 4, and 8 remain confined in South America, Southeast Asia, and West Africa, respectively. Genotypes 5, 6, and 7 had been previously only reported in Africa, but in recent years, they can be found in European countries (UK, France, and Switzerland) as well [5]. The HDV genotype plays a role in the severity of the disease (Table 18.1).

New challenges in HDV prevention, diagnosis, and therapy include its undiminished global burden, which seems to be higher than estimated before, and the emergence of previously undetected genotypes in Europe, probably due to great population shifts.

Natural Course

The clinical course of the infection varies according to the preinfection status of the host. HDV infection has only been described in the presence of a hepatitis B virus (HBV) infection, although recent in vitro and in vivo experimental data challenge this dogma [2]. Each natural course depends on the time point of HDV and HBV infection. In the case of simultaneous infection, only 2% of the patients become chronically infected. The infection resembles an acute HBV monoinfection, although the risk for acute liver failure is greater. On the other hand, HDV superinfection leads to chronic courses in 70–90% of cases [22, 23].

Chronic HDV infection frequently leads to liver fibrosis and potentially cirrhosis. The time needed for progression to cirrhosis can vary. Earlier studies reported that 23% of the patients develop cirrhosis 10 years after infection, while the percentage rises to 41% and 77% after 20 and 30 years [24]. In a minority of patients (10–15%), progression to cirrhosis occurs within merely 2 years after infection [23, 25]. More recent studies confirmed the severity of liver disease [9, 26].

Importantly, HDV replication has been associated with long-term outcome as HDV RNA-positive patients have a higher risk to develop liver-related complications as compared to anti-HDV-positive/HDV RNA-negative individuals [27]. Hepatic decompensation and hepatocellular carcinoma follow the development of cirrhosis at an annual rate of 4% and 2.8%, respectively [28]. The risk of HCC development in chronic HDV infection seems to be higher compared to cases with HBV monoinfection, although not all studies confirm this observation [29–31].

One particular feature of HDV is the association with autoimmune hepatitis-like features. LKM-3 antibodies are frequently present in HDV infection [32]. Worsening of autoimmune hepatitis during PEG-IFNa therapy of HDV infection has been described [33, 34].

The interplay between HBV and HDV is complex and not fully understood. Various patterns of reciprocal inhibition of

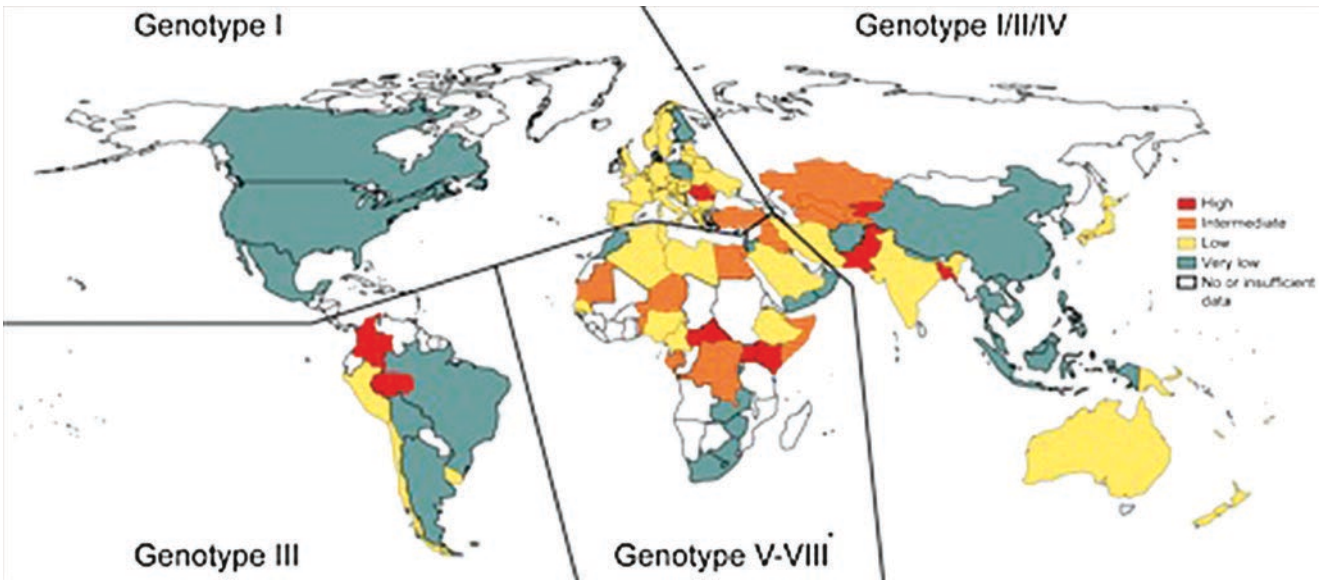

Fig. 18.1 Global prevalence of hepatitis delta infection and distribution of HDV genotypes

Table 18.1 Overview of the most important diagnostic parameters in HDV infection

Parameter	Clinical significance
Biochemical parameters	BEA score predicts with a very high accuracy the development of liver-related complications in chronic HDV-infected patients [9]
HBsAg	HBsAg levels correlate with HDV viremia and liver histological grading [10]
HBeAg	HBeAg positivity (10–20% of HDV-infected patients) is associated with higher biochemical activity, but similar clinical outcome to the absence of HBeAg, 60% of HBeAg-positive HDV-coinfected patients present with HBV DNA levels below 2000 IU/ml [11]
Anti-HDV IgM	Predictor of disease activity and treatment response [12]
Anti-HDV IgG	Screening parameter for HDV diagnosis, may persist for years after HDV clearance [13]
HDV RNA	Detection is a marker of active HDV infection, important for therapy monitoring; high levels of HDV RNA in non-cirrhotic patients are associated with progression to cirrhosis and HCC [14]; RNA extraction method and PCR assay influence significantly the measured viral load [15, 16]
HDV genotype	Epidemiological significance – genotypes 2 and 4 have been associated with milder forms of hepatitis, genotype 3 has been associated with outbreaks of fulminant hepatitis [17], genotype 5 is associated with favorable disease outcome and better response to PEG-IFNa treatment [18], and a combination of HBV genotype F with HDV genotype 3 was associated with more severe hepatitis [19]
Histology	Gold standard for staging and grading of liver disease; additional value in detecting autoimmune features in HDV
Noninvasive fibrosis markers	Noninvasive fibrosis scores have lower performance accuracy in chronic HDV-infected patients compared to HBV and HCV patients [20], delta fibrosis score (sensitivity 85% and PPV 93%) [21]

viral replication can be observed in HBV/HDV coinfection, an effect demonstrated both in vivo and in vitro. In vitro experiments demonstrate that HBV replication markers including HBeAg, total HBV-DNA, and pregenomic RNA significantly decrease upon HDV superinfection of HBV-infected cells, confirming the interference of HDV on HBV, while the levels of circular covalently closed HBV DNA (cccDNA) and HBsAg remained unchanged [35]. In patients, HDV dominance over HBV has been associated with reduced response to PEG-IFNa [36]. HDV replication is often associated with HBV suppression, sometimes to the point of undetectability of HBV DNA in patient serum [1, 37], an effect at least in part mediated through hepatitis delta antigen (HDAg). HDAg can inhibit HBV replication by trans-repressing its enhancers and by trans-activating the IFN-alpha-inducible MxA gene [38].

While HDV needs HBV to establish a productive infection, in vivo experiments indicate that HDV monoinfection can persist intrahepatically for at least 6 weeks and can subsequently be converted by HBV to a productive coinfection [39]. Clinical data support this, as HDAg-positive cells have been observed up to 19 months after liver transplantation without detected evidence of HBV replication [40]. Furthermore, in vivo and in vitro experiments suggest that, in contrast to HBV, HDV persists during liver regeneration and is amplified through hepatocyte division [41].

HDV Diagnostic

An overview of important diagnostic markers for HDV infection is presented in Table 18.1.

Antiviral Therapy: PEG-Interferon and New Treatments in Development (Table 18.2)

Treatment with interferon-a against HDV infection was first introduced more than 30 years ago, and it remains the only recommended therapy for HDV infection according to current international guidelines, in the form of pegylated interferon-a (PEG-IFNa) [48]. The treatment is moderately effective with 25–40% of the patients becoming HDV RNA undetectable during and after therapy [49]. Late HDV RNA relapses beyond 24 weeks after the end of therapy are frequently observed [50], even after prolonged treatment of up to 96 weeks [37]. HBV functional cure is attained even more rarely with only 10% achieving HBsAg loss [50, 51]. Few host and virological factors being associated with treatment failure have been described. In some but not all studies, cirrhotic patients had an unfavorable response to PEG-IFNa therapy compared to non-cirrhotic patients [52]. Undetectability of HDV RNA within 6 months of the therapy is associated with a favorable outcome, while baseline virological parameters are not good predictors of response to PEG-IFNa therapy [53, 54].

Despite all its limitations, therapy with PEG-IFNa has been shown to improve long-term outcome [51]. HDV RNA loss, even when combined with HBsAg persistence, has been shown to reduce the risk of hepatic decompensation and HDV-associated death [55]. In an effort to improve the treatment success rate, combination of interferon with other approved antiviral substances has been evaluated in clinical studies. Addition of ribavirin, lamivudine, adefovir, entecavir, or tenofovir to a PEG-IFNa could not show a significant improvement in the clinical outcome of the patients compared to an interferon monotherapy [34, 37, 42, 56, 57]. Nevertheless, treatment with nucleoside reverse transcriptase inhibitors still has its place in HDV treatment and is recommended for patients with HBV DNA levels persistently greater than 2000 IU/ml and for patients with advanced liver disease [48].

Table 18.2 Overview of selected treatment studies performed in HDV infection

Reference, number of patients	Substance	Summary
Wedemeyer et al. [34], *n* = 90	PEG-IFNa plus adefovir	Comparison of PEG-IFNa plus adefovir versus either drug alone. No effect on HDV replication of adefovir alone, no additional effect in combination with interferon
Abbas et al. [42], *n* = 40	PEG-IFNa plus entecavir	Comparison of PEG-IFNa plus entecavir versus PEG-IFNa alone. Addition of entecavir did not improve the overall response
Wedemeyer et al. [37], *n* = 120	PEG-IFNa plus tenofovir	Comparison of PEG-IFNa plus tenofovir versus PEG-IFNa alone prolonged PEG-IFNa treatment did not prevent posttreatment HDV RNA relapse; no significant improvement with TDF combination
Etzion et al., [43], *n* = 33	PEG-IFN lambda	PEG-IFN lambda monotherapy, comparable antiviral activity, and better tolerability compared to historical PEG-IFNa data
Wedemeyer et al. [44], *n* = 120	Myrcludex B plus tenofovir	Comparison of different myrcludex doses plus tenofovir versus tenofovir monotherapy. Myrcludex-B showed a dose-dependent antiviral efficacy with improvement of biochemical activity and liver stiffness
Bogomolov et al. [45], *n* = 24	Myrcludex B plus PEG-IFNa	Comparison of PEG-IFNa plus myrcludex versus either drug alone. Comparable effect of monotherapy with either substance, better chances for SVR under the combination
Bazinet et al. [46], *n* = 12	REP 2139 plus PEG-IFNa	Combination therapy, seven patients with SVR, four patients with HBsAg loss, therapy well tolerated
Yurdaydin et al. [47], *n* = 15	Lonafarnib plus ritonavir plus PEG-IFNa	Comparison of different lonafarnib doses vs. combination of lonafarnib with either ritonavir or PEG-IFNa; combination with ritonavir led to better tolerability, and combination with PEG-IFNa led to a greater HDV reduction

Available treatment options for HDV-infected patients are limited. Treatment with PEG-IFNa is not a solution for all patients, as it is not devoid of side effects and contraindications, one of them being decompensated liver disease. Liver transplantation remains an option for patients with HDV-mediated acute liver failure or end-stage liver disease [58], but this option has limitations due to the unsuitability of some patients for liver transplantation and the lack of available organs.

The high global health burden of HDV infection and the limited response to PEG-IFNa treatment underline the need to find alternative therapies. New substances are currently being tested in clinical studies. The first is an immunomodulatory agent, pegylated IFN lambda. Experiments on HBV/HDV-infected humanized mice demonstrate that administration of pegylated IFN lambda reduces all intrahepatic HDV infection markers [59]. A phase II randomized clinical trial is evaluating the safety and efficacy in HDV-infected patients. Interim results for 33 patients indicate that pegylated IFN lambda has antiviral activity against HDV in a similar range to PEG-IFN-alpha and is well tolerated [43].

Three other substances, impeding different steps in the life cycle of the virus, have been tested in clinical trials: myrcludex B, lonafarnib, and REP 2139-Ca. The first one, myrcludex B, is a myristoylated lipopeptide, corresponding to the amino acids 2–48 of the N-terminal domain of the L-HBsAg, and acts as a NTCP receptor inhibitor. Subsequently, it was shown that myrcludex blocks HDV and HBV entry into the hepatocyte without interfering in a relevant manner with the bile acid transport at therapeutic doses [60]. The substance has been tested in clinical trials as monotherapy and in combination with other antiviral substances. Myrcludex B administration in monotherapy and in combination with tenofovir for 24 weeks leads to reduction of HDV RNA and biochemical improvement, but longer therapies will be required as most patients relapsed after this short-term treatment [44]. The results of a small study evaluating the combination of myrcludex B and PEG-IFNa, though, indicate a significant benefit of dual therapy which may induce sustained HDV RNA suppression [45]. Importantly, treatment with myrcludex B is reported to lead to a decrease of intrahepatic HDV RNA levels [61].

The nucleic acid polymer REP 2139 has been tested in a small trial performed in Moldavia. Its precise mechanism of action remains unclear to date, but its effect on HDV/HBV coinfection seems to be mediated through interaction with HDAg and inhibition of HBsAg release from the hepatocytes [62, 63]. Its primary effect is a potent reduction of serum HBsAg levels [46]. Combination of REP 2139 and PEG-IFNa showed sustained HDV RNA suppression in more than half of the patients [46], while a considerable proportion of the patients has undetectable HBsAg 18 months after end of treatment [64]. Some patients developed significant ALT increases during treatment; however, no hepatic decompensation occurred.

Finally, lonafarnib, a farnesyltransferase inhibitor, has also been tested in clinical studies with HDV-infected subjects. Farnesylation (a type of prenylation) of L-HDAg is an important posttranslational modification for HDV. In vitro and in vivo experiments demonstrate that lonafarnib can prevent the production of infectious HDV particles, disrupting the interaction of HDV nucleocapsid-like ribonucleoprotein with the HBV protein envelope [65, 66]. This effect has also been observed in a small group of patients. Lonafarnib monotherapy for 4 weeks led to a significant reduction of HDV RNA levels in serum, accompanied, however, by mild to moderate gastrointestinal side effects [67]. In an effort to

improve the tolerability of lonafarnib, the substance was combined with ritonavir, a booster better known from HIV and HCV treatment. The combination has been shown to improve the tolerability as gastrointestinal side effects are frequent at higher doses [68]. Addition of PEG-IFNa to lonafarnib leads to a greater and faster HDV RNA reduction compared to historical responses with PEG-IFNα alone [47], while the combination of a relative low dose of lonafarnib with ritonavir and PEG-IFNa led to significant decrease of HDV viral load and biochemical improvement [69].

HDV Virology

HDV is unusual from a phylogenetic point of view, being unlike other hepatitis viruses. Its origin is unclear. Its sequence and mode of replication bear resemblance to certain plant pathogens (plant viroids), while other data support the evolution of the virus from a host mRNA precursor or from a virusoid/retrovirusoid [70]. In fact, an HDV-like sequence, the cytoplasmic polyadenylation element-binding protein 3 (CPEB3), has been discovered in the human genome [71].

It is the only member of the genus *Deltavirus* and the smallest virus infecting humans. The virus is a 36-nm particle and contains a single-stranded circular RNA genome of negative polarity, of approximately 1700 nucleotides [22]. Inside the virion is a ribonucleoprotein complex, consisting of the RNA genome intertwined with a structural protein, HDAg. This complex is coated by the HBV envelope glycoproteins, consisting of L-HBsAg, M-HBsAg, and S-HBsAg, encoded in the HBV genome. The ribonucleoprotein complex formation does not depend on HBV, but HDV cannot exit the cell and enter other hepatocytes without the coating of the HBV envelope proteins.

The HDV genome does not code any other proteins apart from the two HDAg variants, the large HDAg (L-HDAg) and the small HDAg (S-HDAg). HDV uses instead the cell's own enzymes to proliferate [72], limiting the potential targets of antiviral therapy. The large variant of HDAg is 214 amino acids long with a molecular weight of 27 kDa, and the small variant is 195 amino acids long with a molecular weight of 24 kDa. Both proteins are translated from the same open reading frame: the stop codon at the end of the sequence encoding the S-HDAg is altered by posttranscriptional modification to produce the L-HDAg [73]. This modification is performed by adenosine deaminase acting on RNA (ADAR1), a cellular and not viral enzyme [74].

The two variants of HDAg serve different purposes in HDV life cycle. The S-HDAg after translation relocates to the nucleus and inhibits transcription of host templates via the RNA polymerase, promoting viral replication instead [75]. This effect is mediated by replacing the cellular negative elongation factor (NELF), a negative regulator of RNA polymerase activity [76]. L-HDAg inhibits genomic replication but not antigenomic RNA synthesis, thus enabling initiation of HDV replication [77], and is essential for virion assembly [78]. The C-terminal domain of L-HDAg interacts with HBsAg, promoting the formation of the HDV coating [79, 80]. A balance between viral replication and virion assembly is achieved through the changing relative quantity of the two HDAg isoforms [81]. This is achieved through the editing of the antigenomic RNA by ADAR1, which promotes the transition from S-HDAg mRNA to L-HDAg mRNA transcription [82].

HDV enters the hepatocytes via the NTCP, as HBV does, using its HBV envelope glycoprotein coating [83]. The N-terminal region of the pre-S1 domain of L-HBsAg is essential for HDV entry into the hepatocyte. Mutations/deletions in a highly conserved pre-S1 region and treatment with myristoylated HBV preS peptides have been found to inhibit HDV entry into the hepatocytes [84]. A heparan sulfate proteoglycan, glypican-5, has been shown to act as an entry factor through its interactions with the HBV envelope proteins during viral attachment [85]. After cell entry, the viral particles lose their HBsAg mantle, and HDAg translocates the viral genome into the nucleus for replication [86].

Replication is performed in a rolling circle mechanism, similar to the replication of bacterial plasmids, and starts with the transcription of the antigenome using the viral genome as template and producing an antigenomic RNA of more than one-unit length [81]. The circular genome is replicated into a linear, multimeric molecule which is later self-cleaved by autocatalytic activity through the formation of so-called ribozymes [87, 88], a procedure found widespread in nature [89]. Then it is ligated to form a circular antigenome using cellular ligases [81]. HDV replication is highly unusual, representing a hybrid of the conventional DNA-dependent transcription and the unique RNA-dependent RNA synthesis in the absence of an RNA-dependent RNA polymerase. RNA-dependent RNA replication is used exclusively by RNA viruses for replication and not by cellular RNAs. HDV does not encode an RNA-dependent RNA polymerase but still manages to replicate successfully in the cell, hijacking the cellular mechanism for genome replication and using the host RNA polymerase II for this purpose [88].

The next steps of the HDV life cycle include the transcription of HDV mRNA and its translation to S- and L-HDAg and the association of HDAg particles and viral RNA. After nuclear export, final viral morphogenesis is completed at the Golgi apparatus, where the complexes are coated with hepatitis B virus surface antigen to form infectious particles, which are finally secreted from the cell via the trans-Golgi network [90].

A schematic overview of the HDV life cycle is presented in Fig. 18.2.

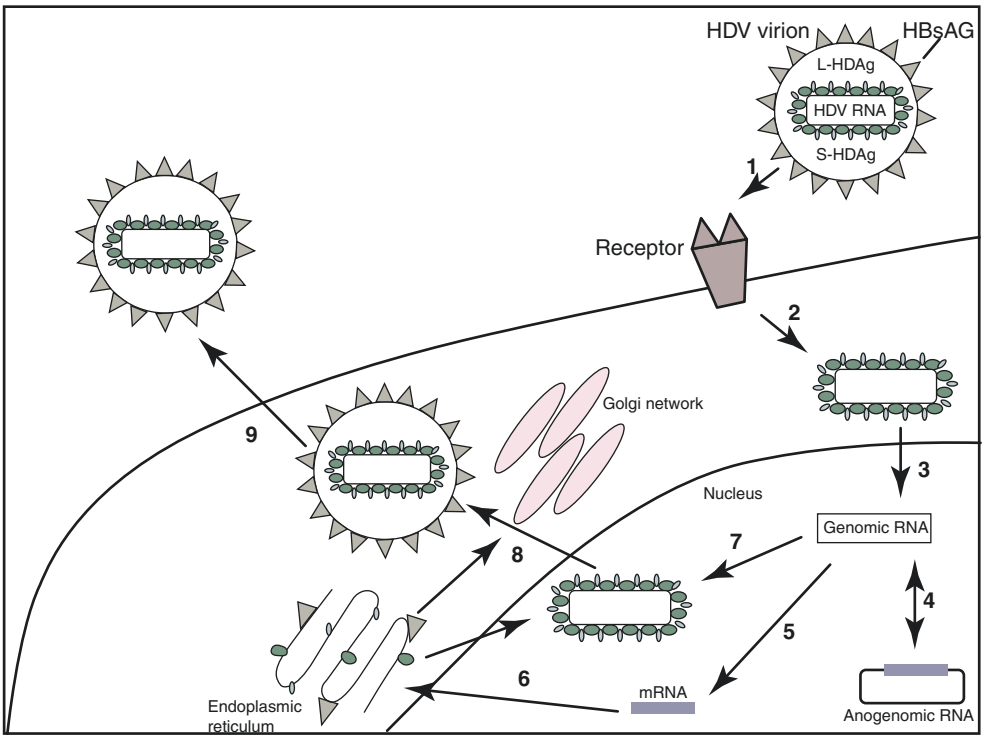

Fig. 18.2 Replication and morphogenesis of hepatitis D virus. (1) Cell entry and uncoating. (2) Nuclear import mediated by the nuclear localization signal. (3) Replication of the HDV genome. (4) Transcription of the mRNA. (5) Translation of the mRNA to small and large HDAg particles at the endoplasmatic reticulum (ER). (6) Association of HDAg particles and RNA. (7) Final viral morphogenesis in the Golgi apparatus. (8) Secretion of new viral particles

Host elements play a key role in HDV life cycle. A recent study identified two key host factors for the HDV life cycle: (a) carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD) enzyme and (b) estrogen receptor alpha (encoded by ESR1). CAD is a multifunctional enzyme exhibiting carbamoyl-phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase activities catalyzing the three first steps of the pyrimidine biosynthesis pathway [91]. CAD can limit viral replication through uridine starvation, affecting both genomic and antigenomic HDV RNAs, while ESR1 inhibition leads to a decrease in CAD protein expression [92].

HDV and Innate Immunity

The immunology of HDV infection is a subject of great academic interest and of even greater potential clinical significance. Although less studied than that of other hepatotropic viruses, recent studies have managed to illuminate some aspects of it. One area of interest is the interplay of HDV and innate immunity. A long-standing issue is the (non)cytopathic nature of HDV. To date, it remains inconclusive if HDV is indeed cytopathic or if the liver damage in HDV infection is exclusively immune-mediated. Some experimental data support the concept of an immune-mediated hepatitis through a noncytopathic virus [93]; other in vitro and in vivo studies support the notion of a direct cytopathic effect of HDV on hepatocytes [94–96]. However, irrespective of the presence of HDV-mediated cytopathic effects, the role of the immune system in inducing liver damage during HDV infection is well documented [97–99].

HDV infection induces an innate immune response. In vitro data demonstrate that HDV superinfection of HBV-infected hepatocytes leads to a strong type I IFN response with significant induction of interferon-stimulated genes, RSAD2 (Viperin) and IFI78 (MxA) [35]. The IFN-β/λ response to HDV replication is predominantly mediated by MDA5 [100]. In a human liver chimeric mouse model, HDV coinfection led to a more pronounced induction of innate immune responses compared to HBV monoinfection. A significant induction of interferon-stimulated genes and cytokines occurred in HDV-coinfected mice compared to uninfected and HBV-monoinfected ones. Moreover, hepatocytes displaying very high HDAg levels demonstrated weaker signal transducers and activators of transcription (STAT) nuclear accumulation, suggesting that HDAg may in part limit STAT signaling [101].

As noted above, HDV replication induces a sustained type I interferon response. This effect is, however, significantly reduced in immunodeficient mice and almost absent in mitochondrial antiviral signaling protein (MAVS)-deficient ones [102]. An experimental model with humanized mice lacking adaptive immunity and thus depending only on innate immunity underlines the role of interferon alpha but also lambda in controlling HDV infection. Administration of PEG-IFNα and PEG-IFNλ reduced HDV viremia and intrahepatic levels of genomic and antigenomic HDV RNA and increased the number of HDAg- and antigenomic RNA-negative hepatocytes [59].

HDV not only induces but also interferes with innate immunity. HDV can inhibit IFNa signaling by blocking the activation of the molecule Tyk2, a part of the Janus kinase (JAK)-STAT pathway [103]. Furthermore, in vitro data suggest that the IFN response to HDV infection and exogenous IFN treatment have only a moderate effect on HDV replication [100] possibly explaining the moderate success of interferon-based treatment of HDV infection.

HDV and Cellular Innate Immunity

The role of cellular innate immunity in HDV pathogenicity and outcome is not fully understood. Previous studies have evaluated the role of natural killer (NK) cells in HDV infection. Boosted NK function has been associated with decreased HDV RNA levels in HDV-infected subjects receiving IFNa treatment; this effect of the treatment on NK function varies greatly between different individuals [104]. A more recent study examined in more detail the role of NK cells in chronic HDV infection. Untreated HDV-infected patients had a higher frequency of NK cells in the peripheral blood with unaltered phenotypic NK cell differentiation status compared to healthy controls. Thus, chronic HDV infection is associated with elevated levels of peripheral blood NK cells; however, these cells show reduced functional capacity to respond to IFNα [105]. Long-term IFNα treatment influences the differentiation status, the function, and the IFN signaling of NK cells. It leads to a selective loss of terminally differentiated NK cells, a relative enrichment in immature NK cell subsets, and a marked functional impairment of the NK cells, which is independent on the changes in NK cell differentiation status. Interestingly, a high frequency of CD56(dim) NK cells at baseline was a positive predictive factor for IFNα treatment outcome [106].

A recent study evaluated the role of another group of cells, the mucosa-associated invariant T (MAIT) cells. MAIT builds a group of innate-like T cells which are highly enriched in the human liver. HDV-infected patients demonstrate a decreased number of MAIT cells in peripheral blood and liver compared to healthy controls and HBV-monoinfected patients while exhibiting monocyte activation signs and elevated levels of proinflammatory cytokines IL-12 and IL-18 in peripheral blood. Furthermore, the functional response of MAIT in HDV infection is impaired, indicating that chronic HDV infection interacts with MAIT cells causing functional impairment and subsequent progressive loss of MAIT cells as the HDV-associated liver disease progresses. This effect may be mediated via IL-12 and IL-18. In vitro experiments showed that IL-12 and IL-18 promote MAIT cell death and induce an activated MAIT cell phenotype similar to the one observed ex vivo in HDV-infected patients [107].

HDV and Adaptive Immunity (Table 18.3)

Only recent studies provide insights on how HDV adapts to the immune system and evades elimination. However, HDV does not seem to elicit strong adaptive immune responses in general. Antibodies against HDV are produced and can be detected in serum during both acute and chronic HDV infections [114]. They are useful for diagnostic purposes, but they

Table 18.3 Overview of selected studies focusing on immune responses in HDV infection

Study	Cell	Summary
Alfaiate et al. [35]	Hepatocyte	HDV superinfection of HBV-infected hepatocytes leads to a strong type I IFN response with significant induction of interferon-stimulated genes
Lunemann et al. [105]	NK	Chronic HDV infection is associated with elevated levels of peripheral blood NK cells with reduced functional capacity to respond to IFNα
Lunemann et al. [106]	NK	Long-term IFNα treatment in HDV infection leads to functional impairment and selective loss of terminally differentiated NK cells
Dias et al. [107]	MAIT	HDV infection is associated with functional impairment and severe loss of MAIT cells
Fiedler et al. [108]	T and B lymphocytes	Antibodies induced by HDAg in woodchucks induce both T cell and humoral immune responses but fail to protect from hepatitis delta virus infection
Aslan et al. [109]	CD4+ T cells	Perforin-positive cytotoxic CD4+ T cells accumulate in HDV chronically infected patients with more advanced liver disease
Karimzadeh et al. [110]	CD8+ T cell	Identification of two HLA-B*27-restricted CD8+ T cell epitopes within the L-HDAg in patients with resolved HDV infection vs. escape mutations within these identified epitopes in HLA-B*27-positive patients with chronic HDV infection
Karimzadeh et al. [111]	CD8+ T cell	Association of certain HDV polymorphisms with HLA class I alleles, presence of uncommon HLA class I alleles linked to HDV immune escape mutations, indicating HDV evolution at population level
Landahl et al. [112]	CD4+ and CD8+ T cell	Detection of low-level MHC I- and MHC II-restricted HDV-specific T cell responses in patients, response not associated with HDV RNA detectability
Kefalakes et al. [113]	CD8+ T cell	Some activated HDV-specific CD8+ T cells target conserved epitopes; about half of them have a memory-like phenotype and fail to clear HDV due to the presence of escape variants

do not provide protective immunity, an effect that has been replicated in in vivo experiments in the past. Antibodies induced by an expression vector encoding HDAg in woodchucks induced both T cell and humoral immune responses but failed to protect the animals from hepatitis delta virus infection [108]. Similarly, the presence of antibodies did not prevent HDV reinfection of chimpanzees that had previously cleared HDV infection [115].

Cellular immunity is implicated in the pathogenesis and control of HDV infection. Vaccination studies have shown that inoculation with a DNA plasmid encoding for the sequence of the hepatitis delta antigen of mice leads to the development of both CD4+ [116] and CD8+ T cell responses [117]. A study focusing on cytotoxic CD4+ lymphocytes in viral hepatitis demonstrated that perforin-positive cytotoxic CD4+ T cells accumulate with advanced age in chronically infected patients with more advanced liver disease, possibly contributing to the more severe course of HDV-associated liver diseases [109]. Four different MHC class II-restricted epitopes were discovered in a screening of T helper cells from eight HDV-infected patients [118]. A study by the same group suggested that the generation of immunogenic epitopes directly by serum proteases could play a role in the immune response against hepatitis delta virus during infection [119].

CD8+ T cell responses have also been documented in in vivo experiments and in the context of human HDV infection. CD8+ T cells specific for two HLA-A*0201-restricted HDV epitopes could be detected in HLA-A2-transgenic mice after DNA vaccination with a plasmid encoding the HDAg. Cytotoxic T-lymphocyte responses against these two epitopes could also be detected in HDV-infected patients without active disease [120]. A following study by the same group showed that the previously described native HDV epitope spanning the amino acids 43–51 of HDAg produced limited cytotoxic immune response. Modifying the epitopes enhanced the immunogenicity [121].

Previous studies have identified a limited number of HLA-restricted cytotoxic T-ligand epitopes in HDV-infected patients. Three HLA-A*0201-restricted epitopes were previously identified (amino acids 43–51, 50–58, and 114–122) in patient samples [122]. A more recent study evaluated the impact of substitutions within L-HDAg epitopes on the CD8+ T cell response and HDV outcome experimentally and by in silico studies. The authors could identify only two HLA-B*27-restricted CD8+ T cell epitopes within the L-HDAg in patients with resolved HDV infection, while escape mutations within these identified epitopes could be observed in HLA-B*27-positive patients with chronic HDV infection [110]. Certain HDV polymorphisms have been associated with HLA class I alleles, while the presence of uncommon HLA class I alleles has been linked to HDV immune escape mutations. This would be indicative of HDV evolution at population level to evade recognition by common HLA class I alleles [111]. The low number of described epitopes would be in line with the hypothesis of HDV evolution from a host mRNA precursor [70, 123] coding a host protein.

HDV-specific T cell responses against HLA class II-restricted epitopes have been observed as well. A recent study described two such epitopes (amino acids 11–30 and 41–60), as well as one major histocompatibility complex class I-restricted epitope (amino acids 191–210). Interestingly, the breadth of the T cell response was not associated with HDV RNA detectability [112].

A common conclusion of several recent studies is that CD8+ T cell response in HDV infection is ineffective but may still play a role in controlling HDV infection. On the one hand, chronic HDV infection has been linked to a progressed immune senescence with weak HDV-specific proliferative and functional responses, an effect that could be attenuated by the addition of IL-12 [124]. HDV-specific CD8+ T cells recognize HDV epitopes. While some activated HDV-specific CD8+ T cells do target conserved epitopes, about half of them have a memory-like phenotype and fail to clear HDV due to the presence of escape variants [113].

On the other hand, the results of a study evaluating HDV-specific cytokine responses of T cells under IFN-a treatment suggest a different conclusion. In this study, PBMC from HDV-infected patients before and under PEG-IFNa therapy were stimulated with overlapping HDV peptides covering the entire HDV proteins. HDV-specific interferon-gamma and IL-2 responses were more pronounced in patients with lower HDV viral load suggesting a contribution of virus-specific T cell responses to control of HDV replication [125].

Conclusion

Many questions about the HDV infection, in a clinical, virological, and immunological context, remain unanswered. The enigma of HDV origin remains unsolved, aspects of the viral life cycle are not fully elucidated, and the knowledge about the interaction of HDV with the immune system is limited. HDV continues to cause considerable morbidity and mortality.

Still, significant progress has been made in the last years. Clinical and experimental evidence emerged that immune control of HDV infection is possible, at least in a subgroup of patients. This may become even more important when new antiviral treatments such as HBV entry inhibitors are used in clinical practice. A better understanding of which patients may achieve immune control could allow to personalize these novel treatment approaches. Development of HDV-

specific immunotherapies seems possible and may be needed as current and new treatment options are not optimal and will not lead to cure of all patients.

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Hepatitis E

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Key Points

- Hepatitis E virus (HEV) infection was previously thought to be limited to certain developing countries by waterborne transmission. Now it is known that HEV infection can also occur in developed countries by a zoonotic infection and foodborne transmission.
- HEV infection mainly causes a self-limiting acute hepatitis, but can also lead to acute liver failure (ALF) in pregnant women or elderly people. Chronic hepatitis E has been described in immunocompromised individuals such as those with solid organ transplant or human immunodeficiency virus infection.
- The innate immune responses via pathogen-recognition receptors (PRRs) and NK and NKT cells play a key role in preventing and eliminating acute HEV infection.
- Adaptive immunity including both humoral and cell immunity play a key role in determining the clinical course and manifestation of HEV infection.
- Most patients with acute hepatitis E do not require a special treatment, whereas those who developed ALF need an intensive liver and systemic support. For chronic hepatitis E, weaning the immunosuppressive agents and mounting antiviral therapy with ribavirin may help to clear the virus and improve the clinical outcomes.
- Improvement in water and food hygiene is the cornerstone to prevent HEV infection. Vaccine against HEV genotype 1 has been shown highly efficacious and approved in China.

Introduction

Hepatitis E virus (HEV), identified over 30 years ago, remains a serious threat to health and productivity in developing countries where a safe water supply is limited [1]. Recently, it has been recognized that HEV is also transmitted as a zoonotic and foodborne pathogen in developed countries, where it clinically manifests differently [1, 2]. HEV infection is usually an acute self-limiting disease; however, it could causes acute liver failure (ALF) in pregnant women and chronic infection in immunocompromised subjects with rapid progression to cirrhosis and extrahepatic manifestations [2].

Both clinical and animal studies have shown that the immune response to, rather than direct cytopathic effect of, HEV may drive different clinical manifestations of the disease, ranging from self-limiting acute viral hepatitis (AVH) and ALF to chronic HEV infection; furthermore, it could cause extrahepatic symptoms, which typically coincides with a rise in anti-HEV antibodies, pro-inflammatory cytokines, and cellular immune responses and a decline in viral load [3].

A specific therapy for hepatitis E is not required in a self-limiting acute infection. The management of chronic HEV infection in immunocompromised subjects mainly includes reduction of immunosuppressive therapy and appropriate antiviral therapy. The best way to avoid HEV infection is to improve the drinking water and food hygiene as well as to immunize with HEV vaccine.

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In this chapter, we summarize the current knowledge on the epidemiology, virology, immunology, diagnosis, treatment, and prevention of hepatitis E.

Virology

The HEV is a small (with a size of 27–34 nm) non-enveloped virus with a positive-sense single-stranded RNA genome [4]. It belongs to genus *Hepevirus* in the *Hepeviridae* family [5]. The HEV strains affecting humans are divided into four species [4] and fall under the species *Orthohepevirus* A, which consists of eight genotypes [6].

The HEV genome has approximately 7200 bases with three open reading frames (ORFs) 1–3 and contains three short untranslated regions [5]. Besides, a fourth ORF4 has been found only in HEV gt 1, which can be translated into a protein that increases the activity of the RNA-dependent RNA polymerase (RdRp) [1].

ORF1 is the largest viral gene product of HEV. Translated as a large polyprotein, it subsequently undergoes posttranslational cleavage into its component proteins including a methyltransferase, a putative protease, an RNA helicase, and an RdRp [7].

As the second largest HEV gene, ORF2 is located downstream of ORF1 and codes structural capsid proteins for virion assembly [2]. Neutralizing antibodies can also be raised against this domain which is a potential target for vaccine development [4].

ORF3, almost entirely overlapping with ORF2, is 360 bp in length. It encodes a functional ion channel for the release of infectious viral particles [8]. Additionally, pORF3 has been identified to interact with a variety of host proteins, including tumor susceptibility gene 101 protein (TSG101). As a key component of the endosomal sorting complexes required for transport pathway, TSG101 is used by a number of viruses including human immunodeficiency virus (HIV) for budding of progeny virions [9].

Epidemiology

Hepatitis E is a global burden, and there are estimated 20 million events of HEV infection every year [1]. In the late 1970s, HEV was first recognized during a large-scale, waterborne epidemic of unexplained hepatitis through 200 villages in the Kashmir Valley of India, causing 1700 deaths [10]. From April 2014 to January 2015, there was an outbreak of hepatitis E among refugees from South Sudan – Gambella, Ethiopia [11].

Most of the eight HEV genotypes have regional distribution preference [6]. Genotype (gt) 1 and 2 infections are endemic in Mexico, Africa, and South Asia and accounting for the major waterborne HEV infection in these regions [12]. The gt 3-associsted zoonotic infections have been reported in Japan, North America, and Europe [5]. In recent years, it has been found that farmed rabbits are important animal hosts of gt 3 [4]. Other data also shows that HEV gt 3 has found in dolphins in Cuba [13]. The gt 4 has also been associated with HEV in East Asia [14]. Thus far, gt 5 and 6 have only been isolated from wild boar in Japan [1]. Gt 7 and 8 infect dromedary and Bactrian camels, respectively [1]. Recently, HEV gt 7 was found to be prevalent in camels in several countries and identified in a patient who regularly consumed camel meat and milk [15].

HEV can spread by four different transmission modes: fecal-oral, foodborne, blood-borne, and vertical transmission [4].

In developing countries, the most popular circulating way of HEV is fecal-oral route usually through consumption of contaminated drinking water. This is also the most common mode of transmission of HEV globally and is responsible for the majority of HEV infection outbreaks [16].

In developed countries, the primary routes of HEV transmission are zoonotic, through consumption of either raw pork or deer meat of the infected animals [5]. HEV can be inactivated by heating at 71 °C for 20 min; therefore, zoonotic transmission primarily occurs through the intake of uncooked or undercooked products [17]. Studies showed the presence of HEV RNA in commercial pork-based food products in 47% of pork pâtés (Canada), 22% of pork liver sausages (Germany), and 30% of figatelli (French-Corsican liver sausage) (France) samples [18–20]. Another interesting phenomenon is that HEV has consequently been found in contaminated seafood and in soft fruits and salads irrigated with infected water [2, 4].

The high rate of HEV infections has raised extensive concern of blood transfusions or organ transplants from donors infected with HEV [5]. Indeed, cases of HEV transmission by blood transfusion have been recorded in several European countries, such as Great Britain, Germany, and France [21], as well as in Canada [22] and Japan [23].

Vertical transmission from mother to child has also been reported in developing countries [4], but it is very rare.

In addition, in some countries, the epidemiology of HEV is changing. China is the best example, where previously HEV gt 1 was the dominant transmission genotype, especially in Eastern China; however, nowadays, gt 1 has become much less common, and gt 4 is the most widespread genotype discovered in patients [5, 24]. For instance, HEV gt 4 has been found in cattle in Yunnan province of China through consumption of their milk [25]. Moreover, an observational study described a low-level endemicity of HEV driven by foodborne transmission from seafood or pork products in Shenzhen, a southern city [26], and the east coastal areas of Shandong province of China [27].

Immunopathogenesis

The HEV usually causes benign and spontaneously resolving acute hepatitis in immunocompetent individuals. However, in immunocompromised patients, HEV infection may cause ALF or chronic hepatitis. Therefore, the clinical outcomes may be determined by the interplay between host antiviral immunity and immunopathology during HEV infection. Upon infection, pathogen-recognition receptors recognize the virus genome, leading to the rapid activation of intracellular signaling cascades, which trigger antiviral immune responses in HEV target cells, as well as recruitment of immune cells to mobilize various immune activities [28].

Innate Immune Response to HEV Infection

As the first-line defense, the host innate immune system plays an important role in protection against infection. However, its dysregulation may partially contribute to severe pathogenesis.

HEV infection induced innate immune mainly focuses on the recognition of HEV by pathogen-recognition receptors (PRRs), including toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). They could activate various signaling molecules such as mitochondrial antiviral-signaling protein (MAVS), TANK-binding kinase 1 (TBK1), interferon regulatory factor (IRF)3, IRF7, and nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) [29], which eventually trigger antiviral activities through the production of interferons (IFNs) and activation of multiple IFN-stimulated genes (ISGs) [28, 29].

TLRs, the cell membrane-associated PRRs, on the cell surface or in the endosomes are found remarkably expressed in target cells of HEV. HEV-infected patients expressing a high level of TLR3, a sensor of dsRNA, can inhibit HEV replication and achieve uneventful recovery [30]. Mechanically, on recognition of HEV, TLR3/7 will induce NF-κB activation through tumor necrosis factor receptor-associated death domain protein, which further facilitates the production of IFNs. Of note, ORF3 of HEV leads to more poly(I:C)-induced IFN-β-expression by enhancing TLR3-mediated TBK1 pathway [31]. In HEV-infected pregnant women, the expression levels of TLR2, TLR3, TLR4, and IFN-γ in those who recovered from acute infection are significantly higher than those who progressed into ALF [30].

RLR family is another important system to detect viral nucleic acid existing in the cytoplasm, with RIG-I and melanoma differentiation-associated protein 5 (MDA5) being two typical ambassadors. Theoretically, 50-triphosphate (50-ppp) RNA of the HEV RNA can bind RIG-I and MDA5, activate the downstream-signaling cascades, and subsequently lead to the production of type 1 and 3 IFNs and ISG expression [32]. Moreover, both MDA5 and RIG-I consist of two caspase activation recruitment domains, which, upon binding to foreign RNA, recruits mitochondrial antiviral MAVS signaling to activate IRF3/7 and NF-κB and leads to a massive release of IFNs and other cytokines [28, 33]. In a noncanonical pathway, RIG-1 is independent of IFN production but partially through the activation of the JAK-STAT cascade to inhibit HEV replication [34]. This may shed light on novel therapy by using RIG-I agonists like ImOl-100 and Rigontec, which may theoretically avoid the severe side effects associated with excessive exposure to IFN treatment.

Multiple cells have been speculated to play significant roles in the pathogenesis and recovery of HEV-infected liver diseases like acute hepatitis and ALF. NK and NKT cells are important modulators of antiviral response through secretion of IFN-γ and tumor necrosis factor (TNF)-α. It has been reported that, compared with health controls, in AHE patients, the proportions of NK and NKT cells in peripheral blood mononuclear cells (PBMCs) are much lower whereas the percentages of activated NK and NKT cells are higher, implying a role of these cells in the pathogenesis of this disease [28].

Vδ2 cells, similar to the pattern of distribution and level of activation of NK and NKT cells, are strong producers of IFN-γ and TNF reported in patients with ALF [35]. In addition, it has been known that infiltration of activated and TNF-α- and reactive oxygen species (ROS)-secreting mononuclear macrophages can cause hepatocyte injury in ALF resulting from other etiologies. In HEV-related ALF, however, a significant functional impairment and deactivation of macrophages were found, which may cause reduced ROS production and deficient phagocytosis. In Mongolian gerbils, massive mast cells were assembled in the liver and the small intestine of animals infected by HEV, where a high level of the tryptase and 5-hydroxytryptamine was detected. The activation of mast cells can increase the local vascular permeability and lead to portal inflammation [36].

Adaptive Immune Response to HEV Infection

Acquired immunity, mainly consists of humoral immunity and cellular immunity, prevents primary infection and alleviates severity of the disease caused by the residual infection that had evaded host immune surveillance.

In humoral immunity, HEV infection provokes modest antibody response and cytokine mediation. Anti-HEV antibodies and cytokines generated in humans by infection or vaccination are related to protection for HEV; however, mechanisms thereby mediated protection against HEV infection and HEV-induced liver disease are still not fully understood.

Anti-HEV IgM is first detected during the early phase of infection and declines sharply after convalescence. Approximately coincident with the onset of the IgM response, anti-HEV IgA response is also commonly identified in patients with acute primary HEV infection [37]. Serum anti-HEV IgG antibodies against the ORF2 capsid are detected at the later stage of acute hepatitis E (AHE) as the patients recover [37]. Recent studies from India have demonstrated that, compared with those who recover spontaneously, significantly higher levels of anti-HEV IgM and IgG antibodies and Th1 (IFN-γ, interleukin (IL)-2, and TNF) and Th2 (IL-10) cytokines were recorded in patients who develop ALF [38]. This may imply that persistent inflammatory response may promote progressive liver damage and result in ALF.

A single nucleotide polymorphism analysis was also conducted to reveal the association of TNF-α-308AA genotype with susceptibility to HEV and that of TNF-α-1031CC and IFN-γ+874TT and TA with relevant clinical liver disease. Higher 308A allele frequency was associated with susceptibility to HEV and the prognosis to ALF [39].

In chronic hepatitis E, it is a controversial concept of durable and perhaps lifelong humoral immunity [3]. The anti-HEV IgM and IgG responses vary greatly among patients with chronic hepatitis E, perhaps reflecting different immune status in underlying diseases and immunosuppressive regimens. Differing from individuals with typical acute self-resolving infections, the IgM response can persist throughout chronic infection [40]. A similar co-circulation of everlasting virus with anti-HEV IgG was also described in macaques experimentally infected with HEV isolate during treatment with immunosuppressive drugs [41].

A more recent analysis of sporadic and epidemic HEV outbreaks was reported in small numbers of subjects with serologic reinfection evidence. In these individuals, IgM response is of absence, but increasing titer of high-avidity anti-HEV IgG antibodies was considered a significant sign of reinfection, representing virus replication and that hepatitis were too transient or attenuated [42]. Reinfection was also observed in the setting of solid organ transplantation (SOT) [37]. A retrospective cohort study described one of the four SOT recipients who were previously anti-HEV IgG positive became reinfected with HEV 8 years later, and this reinfection became chronic [40]. A phase 3 HEV vaccine study found that lower anti-HEV IgG concentration at baseline was associated with reinfection in individuals [43].

Hepatitis E may be attributed to cell immune-mediated damage or protection [44]. Replication of the virus is rapid during the prodromal stage of infection, as well before the onset of hepatitis. Thus, a better understanding of adaptive cellular immunity is necessary to resolute acute infection and prevent chronic HEV disease.

There is a remarkable deficit in knowledge about the contribution of CD4+ and/or CD8+ T-cell immunity to the pathogenesis of HEV infection. Using the IFN-γ ELISpot assay as a readout in most early studies of T-cell immunity against HEV gt 1 viruses, an elevated frequency of circulating ORF2-specific T cells was detected in patients with AHE and resolved HEV infections, compared with uninfected controls [45]. However, the relative contribution of CD4+ versus CD8+ T cells to IFN-γ production was not provided by ELISpot assay. In order to facilitate the detection of CD4+ and CD8+ T-cell responses, some studies analyzed T-cell responses through PBMC stimulation, with overlap peptide sets spanning the ORF1, ORF2, and ORF3 domains. They concluded that significantly lower proportions of CD3/CD69/IFN-γ and CD3/CD69/TNF-α staining PBMCs and higher proportions of CD4 cells, but similar levels of CD3/CD69/IL-4 staining PBMCs and CD8 cells, were detected in HEV-infected patients, compared with healthy controls [46–48]. Moreover, in vitro study showed that IFN-γ was upregulated in the supernatants of cultures cultivated with ORF2-stimulated PBMCs from HEV-infected patients compared with controls [49].

Analyses of T-cell immunity were undertaken to identify the antigenicity in ORF proteins in patients with acute, convalescent, and chronic HEV gt 3 infections. CD4+ and CD8+ T cells in the peripheral blood target all three HEV open reading frames and produce cytokines like macrophage inflammatory protein-1β (MIP-1β), IFN-γ, and TNF-α [46–48].

Broad HEV ORF1-specific CD4+ and CD8+ T-cell responses were assessed in patients with acute, resolved, and chronic hepatitis E without distinct dominant regions. It was shown that the magnitude and frequency of which were similar to T-cell responses against HEV ORF2/3 [48]. Brown et al. analyzed CD4+/CD8+ T-cell responses and polyfunctionality in 41/44 immunocompetent HEV-exposed volunteers and concluded that powerful HEV-specific T-cell responses generated during HEV infection acute phase predominantly target ORF2, but decline in magnitude and polyfunctionality over time [47]. In the Aggarwal study, CD4 T-cell epitopes in ORF2 and ORF3 proteins of HEV were mapped by using lymphocyte proliferation assays and overlapping peptide libraries. They showed that HEV ORF2 proteins were associated with significant proliferation while HEV ORF3 peptide pools did not induce proliferative responses [50].

Chronic HEV infection mainly occurs when immunity is persistently impaired and the number of circulating CD8+ and CD4+ T cells is distinctly reduced [46]. When compared with simple AHE, virus-specific CD4+ and CD8+ T cells are present at very low frequency in patients with chronic HEV hepatitis [46, 47]. Suneetha et al. analyzed T-cell responses against HEV in 38 different HEV infection individuals and found that the strongest and multi-specific HEV-targeted

T-cell responses are existing in healthy controls, and to a lesser extent also present in recovered AHE patients after transplantation, and absent in patients with chronic hepatitis E, but detectable in subjects after their viral clearance. They further observed that HEV-specific T-cell responses could be reversed in vitro by blocking the programmed death gene 1 (PD-1) or cytotoxic T-lymphocyte antigen 4 pathways [46]. It is noteworthy that inhibitory PD-1 signaling is a major driver of CD8+ T cells from chronically infected subjects to regain antiviral effector functions [46].

An interesting study displayed that the introduction of HEV-specific T cell receptors (TCRs) into lymphocytes of immunocompetent donors and patients with chronic hepatitis E enabled the lymphocytes to bind HEV dextramers, secrete multiple cytokines, and exert cytotoxicity in a target-specific manner [51].

Furthermore, Cao et al. successfully constructed a pig model with chronic HEV infection and observed serum levels of cytokines and cell-mediated immune responses. They found reduced serum levels of Th1 cytokines IL-2 and IL-12 and Th2 cytokines IL-4 and IL-10, as well as IFN-γ-specific CD4+ T-cell responses in HEV infection immunocompromised pigs, particularly during the acute phase of infection. However, TNF-α-specific CD8+ T-cell responses are increased during the chronic phase of infection. Therefore, active suppression of cell-mediated immune responses under immunocompromised conditions may facilitate the establishment of chronic HEV infection [52].

Clinical Aspects

Hepatitis E most commonly manifests as a self-limiting acute hepatitis; only a minority (probably less than 5%) develop acute hepatitis with the symptoms of elevated liver enzymes, jaundice, and nonspecific manifestations such as fatigue, pruritus, and nausea [53]. Immunocompetent patients with hepatitis E infection can clear the HEV spontaneously. On the contrary, immunocompromised patients could fail to clear HEV infection. Therefore, hepatitis E is a concern in pregnant women and patients with underlying immunosuppression, chronic liver disease, and HIV. Acute HEV infection with ALF is mainly seen in pregnant women, especially those at the third trimester, whereas the clinical presentation of chronic HEV infection has mainly been described in organ transplant recipients, patients with hematological malignancy requiring chemotherapy, and individuals with HIV [2, 54, 55], which could lead to cirrhosis and even, in some cases, decompensation or death. Fortunately, liver fibrosis can regress after HEV clearance [56]. In addition, extrahepatic HEV-associated manifestations, including neurological and renal injuries, have been observed in different periods of HEV infection [57, 58].

Pregnant Women

The mortality rate is high and can be up to 25% in pregnant women, particularly in Indian/Asian pregnant women at the third trimester [1]. Fulminant hepatic failure and high virus level appear to be more common in HEV RNA-positive pregnant women [59]. A recent study on 220 consecutive pregnant women presenting with jaundice found that patients with AHE had a higher maternal mortality rate and worse obstetric and fetal outcomes than pregnant women with the same symptoms caused by other types of viral hepatitis [60]. So far, the mechanisms underlying the increased HEV virulence in pregnant women are unclear but could be related to the potential role of changes of immunity status and hormone secretion during pregnancy [3].

Immune profiles among pregnant women with AHE were noticeably different from those in nonpregnant patients with hepatitis E, healthy pregnant women, or nonpregnant women. Pal et al. reported a lower lymphocyte proliferation response to phytohemagglutinin and a T-cell subpopulation tilting toward T-helper cell (Th2) polarization in pregnant women with AHE [61]. Functionality of monocyte-macrophage is impaired in pregnant women with acute liver failure-hepatitis E viral infection (ALF-E) compared to HEV-infected patients. Probably, reduced expression of TLR3, TLR7, and TLR downstream-signaling molecules and further inadequate triggers for the immune responses contribute to the progression and severity of ALF-E [62].

The alteration in hormone secretion during pregnancy may also be associated with the clinical manifestation of hepatitis E infection. A high level of steroid hormones and diminished cellular immunity (lowered CD4/CD8 cell ratio), which influence viral replication and virulence during pregnancy, appear to be a plausible explanation for more severe clinical manifestation and worse prognosis of the pregnant women with HEV infection [63].

Progesterone inhibits Th1 cell and promotes Th2 cell development and impairs the transition of pro T-cells to early pre T-cells in mice models. Furthermore, studies have reported that increased progesterone leads to a decrease in bone marrow B-cell production [64]. In addition, human chorionic gonadotropin has been shown to inhibit cell-mediated immunity in guinea pigs [64].

Recent data demonstrated that estrogen level was positively correlated with viral load in feces and cells, suggesting that estrogen may be able to promote HEV replication in vivo and in vitro [65, 66]. Immunohistochemistry in the liver, ovary, and placenta showed that the proportion of CD8+ T cells was higher than that of CD4+ T cells in pregnant rabbits infected with HEV [66]. Yang et al. also found that estradiol increased the HEV infection in a dose-dependent manner in an in vitro model [65].

Elderly People

One observation in England demonstrated that older males are more likely to develop clinically serious acute hepatitis. As reported previously, in developed countries HEV gt 3 group is the major cause of acute infections, and older men are at higher risk for this disease [67]. Indeed, a study demonstrated that patients with anti-HEV IgM-positive results among those with suspected drug-induced liver injury were mainly from older men (89%; mean age, 67 years) [68].

Patients with Hematological Diseases

Both severe acute and chronic HEV infections have been described in association with hematological diseases. For example, the cases of thrombocytopenia and aplastic anemia have been reported in association with acute HEV infection [69, 70]. In the contrary, a small number of patients with untreated hairy cell leukemia, idiopathic CD4 T lymphopenia, chronic myelomonocytic leukemia, and non-Hodgkin lymphoma taking rituximab have been detected to have chronic HEV infection [2, 71]. Furthermore, a study assessed HEV RNA and HEV serology in 328 pre- and post-stem cell transplant patients and found 8 patients (2.4%) had HEV infection, with 5 of them developed chronic HEV infection [72].

Immunosuppressed Patients

Chronicity is very rare but has been described in immunocompromised patients, such as organ transplant recipients and individuals infected with HIV [2].

Chronic hepatitis caused by HEV infection is observed in more than 60% of recipients of solid organ transplants. An observational study performed in solid organ transplant recipients showed few spontaneous HEV clearances during 3 and 6 months after infection [73], indicating HEV-induced chronic hepatitis.

Evidence showed that immunosuppressive agents can increase the risk of developing chronic HEV in SOT recipients. It may be due to a lower lymphocyte count and weaker immune response caused by immunosuppressive therapy. In line with this notion, reductions of immunosuppressive therapy result in viral clearance in more than 30% of patients [55].

In 14 cases of HEV acute infection with positive serum HEV RNA in patients receiving different organ transplants, Kamar et al. found significantly lower total counts of lymphocytes and of CD2, CD3, and CD4 T cells in patients who developed chronic disease [74].

It is newly found that phosphoinositol-3-kinase-protein kinase B-mammalian target of rapamycin signaling pathway could inhibit HEV infection by acting as a key goalkeeper in human HEV target cells. This discovery provides a potential strategy to safely maintain immunosuppression in HEV-infected organ transplantation recipients [75].

Chronic HEV infection has also been described in immunocompromised individuals with HIV infection. The seroprevalence of anti-HEV IgG in HIV-positive groups ranges from 1.5% to 11.2%, while the positive rate of serum HEV RNA is low, only ranging from 0% to 1.3% [54]. Around 20 cases of HEV-HIV coinfection have been documented worldwide through the detection of HEV RNA, including 5 cases of HEV-HIV chronic coinfection and 2 cases of HEV-related cirrhosis [76]. Notably, all of the individuals with HEV infections have a common feature of low CD4 counts (<250/mm) [2].

Intravenous drug use may also be a risk factor for HEV infection [77]. The possibility of HEV sexual transmission has always been controversial in HIV-positive patients. In a recent study, it was shown that men having sex with men might be at risk for HEV acquisition [78]. In contrast, another recent study analyzed 3293 cases of HIV-positive patients in Taiwan and found that neither sexual orientation nor acquisition of sexual transmission of HIV was associated with prevalent or sporadic of HEV infection and no patients had prolonged HEV viremia or related clinical symptoms, except a slight elevation of serum aminotransferase [79].

Diagnosis

Clinically, cases of hepatitis E are not so distinguishable from other types of AVH. However, appropriate epidemiologic settings or settings with risk of contaminated drinking water may be of help. A definitive diagnosis of HEV is not so easy to be established, and the proprietary assays have different sensitivity, specificity, and reproducibility. Usually, the diagnosis of hepatitis E depends on serologic assessment of antibodies directed against HEV (IgM or IgG) as well as the real-time polymerase chain reaction assays for HEV RNA detection. Moreover, HEV antigen that derived from both blood and stool can be detected with a double-antibody sandwich method.

Anti-HEV IgM

During HEV infection, IgM antibodies appear first. The IgM antibodies are relatively short-lived, which usually last no longer than 3–4 months, but may persist for up to a year. The reappearance of anti-HEV IgM represents new infection. At present, this method is mainly applied in clinical diagnosis of hepatitis E [80].

Anti-HEV IgG

After IgM antibody appearance, IgG antibodies to HEV follow. Compared with IgM, the IgG response is long-lasting with increasing antibody avidity over time [81]. As an indicator of past infection of HEV, it is often used in epidemiological investigation. Clinically, if the level of anti-HEV IgG increases more than four times, it can also be used as a diagnostic criterion for acute HEV infection.

HEV Antigen

As a capsid protein of virus, HEV ORF2 protein exists in the window phase and acute stage of infection. It is of significance in early diagnosis and treatment monitoring of hepatitis E by detecting HEV antigen with a double-antibody sandwich method. Because of the specificity of each assay, it is critical to interpret the results in the context of epidemiological and clinical information [82].

HEV RNA

The acute phase of hepatitis E is usually accompanied by viremia and fecal excretion of the virus. At the same time, HEV RNA can be detected from serum, feces, and even urine of patients. As detection of HEV RNA is a direct indicator of acute HEV infection, it is of great significance in immunosuppressed patients (such as organ transplant recipients) in whom anti-HEV IgM and anti-HEV IgG are likely to be negative after infection with HEV. So far, HEV RNA can be detected by both nested PCR and real-time PCR techniques [83].

Therapy

Treatment of Acute HEV Infection

Acute HEV infection does not usually require antiviral therapy because it is self-limiting and the virus could be spontaneously cleared. General support and symptomatic treatment will be enough. However, some patients may develop liver failure. Therefore, in such circumstance the patients need to be treated with ribavirin monotherapy, aiming at a rapid clearance of HEV and avoidance of liver transplantation [2]. Besides, corticosteroid therapy has been reported effective in HEV-associated acute hepatic failure by averting the need for liver transplantation [84]. However, there is insufficient evidence to support the routine use of corticosteroids in patients with ALF due to HEV infection.

Treatment of HEV Infection in SOT Patients

In an immunosuppressed patient with chronic HEV, the treatment options for chronic HEV are as follows. Firstly, reducing the intensity of immunosuppression, especially drugs targeting T cells such as calcineurin inhibitors, could lead to viral clearance in nearly one-third of patients [55, 85]. Studies had reported HEV clearance rates of up to 25% after reducing immunosuppressive medication [85]. Indeed, studies showed that patients with chronic HEV infection who had significantly lower tacrolimus pre-dose concentrations cleared the virus while others remained viremic [55, 86]. Administration of pegylated interferon-alpha (PEG IFN-α) is the second option to treat chronic HEV. But the concern of possible augmentation of organ/tissue rejection limits the widespread use of this therapy in the setting of SOT [85]. Thirdly, the antiviral drugs such as ribavirin could be applied [7], which induce a change in viral nucleotides and impede RNA virus replication [87]. Recently, sofosbuvir, a NS5b inhibitor for HCV, has shown some activity against HEV RNA replication in vitro, and the antiviral effect is additive with ribavirin [88].

However, IFN-α and ribavirin belong to off-label treatment options, and their applications are limited by the side effects. On the other hand, immunotherapy, more specifically T-cell-based therapy, may be an alternative and encouraging approach. One example is the recently identified TCRs targeting HEV-specific CD8+ T-cell epitopes which shows a potential clinical value in future T-cell-based therapy [51].

Treatment of Chronic HEV Infection in Other Immunosuppressed Patients

Chronic HEV infection has also been described in non-transplant immunocompromised individuals, such as patients with hematological disorders or HIV infection. The clinical and biological presentations of hematological disorders or HIV infections are quite similar to that observed in SOT patients. Therefore, PEG IFN-α, ribavirin, or their combination was effective for treating HEV infection in patients with hematological disorders and those with HIV [89, 90].

Prevention

The prevention of HEV infection depends on avoidance of viral exposure or active vaccination. HEV gt 1 infection can be prevented by providing clean drinking water and improving the health infrastructure in developing countries. HEV

gt 3 infection may be prevented by avoiding the consumption of undercooked or uncooked meat, especially pork products [91]. Two vaccine candidates had shown good tolerability, high immunogenicity, and strong efficacy against HEV infection, with one of them already licensed in China [92, 93].

The first vaccine is a 56-kDa protein, which is encoded by ORF2 of a HEV1 strain and expressed in insect cells. This vaccine was tested in Nepal where 2000 participants with seronegativity or low-titer seropositivity were randomly divided into two groups, receiving 3 doses of 20 ug of the 56-kDa vaccine (n = 898) or placebo (n = 896). The cohorts were followed for a median time of 804 days, and the overall efficacy was 95.5% (95% CI = 85.6–98.6%) [92].

The second vaccine, HEV 239 expressed in *Escherichia coli*, is a 26-kD a protein encoded by ORF2 of HEV gt 1 [94]. In China, a clinical trial on this vaccine performed in more than 11,000 subjects demonstrated a 100% efficacy rate for the prevention of HEV following three doses of vaccine [93], and a 4.5-year long-term efficacy assay revealed an overall protective rate of 86.8% (95% CI = 71–95%) [95]. This vaccine has been licensed for use in the People's Republic of China where the most prevalent HEV genotype is gt 1.

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Alcohol-Associated Liver Disease

20

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Key Points

- Alcohol-associated liver disease is the liver manifestation of the end-organ effects of chronic excessive alcohol intake.
- Alcohol and its metabolites directly damage hepatocytes through generation of oxidative stress and other mechanisms.
- The effects of alcohol on gut integrity and the adipose tissue contribute to the development of ALD.
- Activation of the innate immune system is a major component in the development and progression of ALD.
- Gut-derived and endogenous danger signals contribute to innate immune activation in ALD.
- Understanding specific molecular mechanisms involved in ALD may guide development of new therapeutic interventions.

Introduction

This chapter focuses on the immune-mediated aspects of the pathogenesis of alcohol-associated liver disease (ALD). Alcohol not only directly injuries the liver but also exerts profound effects on other organs, which indirectly contribute to liver damage. Here, we will discuss the cellular effects of alcohol and its metabolites, innate and adaptive immune responses, and intracellular signaling pathways involved in ALD. Finally, an overview of current and emerging therapeutics will be reviewed, with a focus on immune-targeted approaches. Many of these therapies aim to translate new insights in pathogenesis of ALD into clinical treatment.

Clinical Characteristics of Alcohol-Associated Liver Disease

Epidemiology and Natural History of ALD

In 2017, an estimated 14.1 million adults in the USA had alcohol use disorder [1], and 88,000 deaths per year are attributable to alcohol use, making alcohol the third leading preventable cause of death in the USA [2]. The clinical-histologic spectrum of ALD includes liver steatosis, steatohepatitis, steatohepatitis with fibrosis, and cirrhosis, which increases the risk for hepatocellular carcinoma (HCC) [3]. Heavy alcohol consumption, including binge drinking, leads to liver steatosis in over 90% of individuals [4], although fat deposition usually resolves after cessation of alcohol use in the absence of advanced liver disease (Fig. 20.1). Persistent

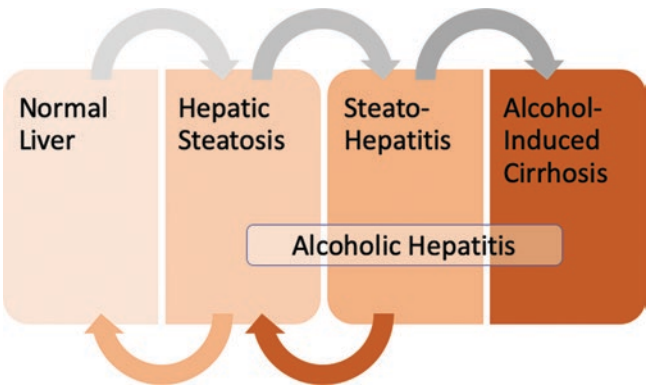

Fig. 20.1 Progression of alcohol-associated liver disease (ALD). Alcoholic hepatitis may develop from a histologic background of hepatic steatosis, steatohepatitis, or cirrhosis

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heavy alcohol use leads to liver steatosis with inflammation and sets the stage for progressive liver disease. Inflammation triggers fibrosis, which is the deposition of extracellular matrix and collagen that over time leads to irreversible cirrhosis [3, 5]. Continued alcohol intake is the most important risk factor for progression of ALD [3, 5, 6]. Cirrhosis, decompensated liver disease, and hepatocellular carcinoma (HCC) are life-threatening complications of ALD, and a major cause of morbidity and mortality in individuals with alcohol use disorder.

Clinical Diagnosis of ALD

The diagnosis of ALD differs at various stages of the disease, and these diagnoses are predicated on a history of heavy and chronic alcohol consumption. The exact amount of alcohol required to cause liver injury differs among individuals and is affected by a variety of cofactors, some genetic and others environmental [3, 7–9]. In general, excess alcohol use has been defined clinically as greater than 28 g of alcohol daily in men and greater than 14 g of alcohol daily in women [3, 6]. Clinical manifestations of ALD also vary greatly. Alcohol-induced liver steatosis is often asymptomatic but may garner medical attention with elevated liver enzymes and fat deposition in the liver on imaging. In fact, aside from a history of heavy alcohol use, alcohol-induced liver steatosis may be difficult to distinguish from nonalcoholic fatty liver disease (NAFLD), even with the help of liver biopsy [4]. However, elevated transaminase level with aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio >1.5 is more typical of ALD than NAFLD and may suggest alcohol as the likely etiology [10]. Chronic alcohol can also lead to liver inflammation, or alcoholic steatohepatitis (ASH); typical features found on liver histology include steatosis, hepatocyte ballooning, neutrophil infiltration, Mallory-Denk bodies, and fibrosis [4]. While alcoholic steatohepatitis is a histologic diagnosis, a clinical syndrome of alcoholic hepatitis characterized by severe liver inflammation and dysfunction may arise from alcoholic steatohepatitis and is associated with high mortality (discussed in further details below). Chronic and persistent inflammation from alcohol abuse can lead to alcoholic cirrhosis, with findings of fibrosis and steatosis on liver biopsies. Hepatocellular carcinoma is more common in patients with cirrhosis and may be seen arising from a background of liver fibrosis.

Alcoholic Hepatitis

Alcoholic hepatitis (AH) is a clinical syndrome defined by rapid onset of jaundice with liver injury in a patient with chronic heavy alcohol use. Impaired liver synthetic function, portal hypertension, and elevated inflammatory markers are common features. A pattern of alcoholic steatohepatitis would be seen on liver biopsy though biopsy is usually not required for diagnosis of alcoholic hepatitis. Inflammation is a hallmark of alcoholic hepatitis. Many inflammatory markers may be elevated, including peripheral white blood cell count. This is caused by the recruitment of immune cells from the bone marrow to the liver in response to the massive proinflammatory cytokine activation [11]. Molecular mechanisms and biomarkers that trigger the development of AH from stable ALD are yet to be delineated. Previous studies identified tumor necrosis factor (TNF) as a central mediator of ALD. TNF-α was increased both in the serum and liver of patients with alcoholic hepatitis [12–15]. Patients with severe AH have a high mortality and often develop jaundice, portal hypertension, and other signs of hepatic decompensation. While many cases of AH manifest as acute on chronic liver injury, portal hypertension develops even in the absence of cirrhosis as a result of sinusoidal congestion in the inflamed liver [3]. The clinical course of AH may be complicated with upper GI bleeding, ascites, peripheral edema, systemic infections, and renal insufficiency. Alcohol withdrawal and its physical and behavioral symptoms provide additional challenges in the clinical management of these patients. Different scoring systems are used to establish the severity of AH. The Maddrey discriminant function ≥32 and Model of end-stage liver disease (MELD) score ≥21 are the most commonly used cutoffs for defining severe alcoholic hepatitis [3]. Mortality in severe AH may be greater than 30% [16, 17]. Although corticosteroids can be used in this setting, they have not been shown to improve long-term survival, and efforts in search for a more efficacious treatment are ongoing.

Pathogenesis of ALD

Alcohol affects virtually all organs in the body, and it is increasingly evident that alcohol-induced changes in one organ can influence the function of other organs. Multiple key elements have been identified in the pathogenesis of ALD, including direct toxicities of alcohol and its metabolites on liver cells, crosstalk between liver and gut, and crosstalk between liver and adipose tissue. Activation of both the innate and adaptive immune systems is crucial for pathogenesis of the disease and links interorgan crosstalks. The next section will highlight the pathogenesis of alcohol-associated liver disease with an emphasis on the inflammatory and immunological responses involved in the process.

The Effects of Alcohol, Metabolites, and Oxidative Stress

Alcohol Metabolism

Alcohol is metabolized by alcohol dehydrogenase (ADH) into acetaldehyde, which is further metabolized into acetate by aldehyde dehydrogenase (ALDH) [18]. Acetaldehyde and acetate are short-lived and toxic; thus, many direct tissue effects of alcohol have been attributed to these metabolites (Fig. 20.2). Both of ADH and ALDH enzymes have limited capacity to metabolize alcohol [18]. At high tissue concentrations, alcohol metabolism involves alternate enzymatic pathways including cytochrome P450 2E1 (CYP2E1) and microsomal enzymes, which are upregulated with chronic alcohol use [19]. Reactive oxygen species (ROS), such as the superoxide anion radical and hydrogen peroxide, are by-products of CYP2E1-dependent ethanol metabolism, and ROS directly induces cellular oxidative stress in hepatocytes [19, 20]. The role of CYP2E1 in hepatocyte damage in ALD has been established by multiple studies both in vitro and in vivo [19, 21]. Alcohol metabolism also results in increased NADH/NAD+ and NADPH/NADP+ ratios in the cytoplasm and mitochondria of hepatocytes [19, 22]. Increased NADP inhibits mitochondrial β oxidation and accumulation of lipids in hepatocytes, contributing to the development of steatosis [19]. Ethanol metabolism also inhibits histone deacetylation via suppression of sirtuin deacetylases, thereby compromising epigenetic regulation of fat and glucose metabolism [23].

Reactive Oxygen Species and Mitochondrial Stress in ALD

In addition to ROS generated from direct alcohol metabolism, alcohol also increases mitochondrial oxidative stress [22, 24]. Alcohol leads to alteration in mitochondrial membrane permeability and transition potential and activates apoptosis pathways through release of cytochrome c and caspase-3 activation [25–27]. ROS also damages mitochondrial DNA and ribosomes. Mitochondrial dysfunction that ensues further contributes to ROS production [22, 24]. In mice models, a single dose of ethanol was sufficient to cause mitochondrial DNA damage, which could be rescued by administration of antioxidants or by mitochondrial biogenesis [28]. However, replication of damaged mitochondrial DNA may result in mitochondrial DNA mutation, which is seen infrequently in healthy controls but present in up to 85% of patients with severe alcohol-induced steatosis [29]. Apart from the mitochondria, NADPH oxidase complex is an alternative source of ROS production in both immune and parenchymal cells in the liver and becomes activated in ALD [30, 31]. NADPH p47phox was shown to contribute to Kupffer cell (KC) activation in ALD [32], providing another mechanism for ROS generation.

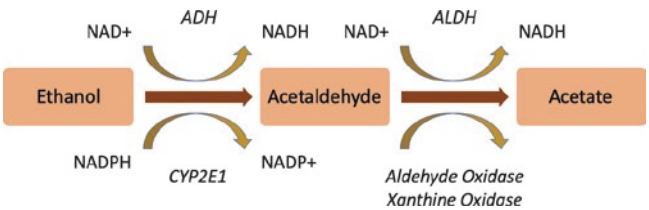

Fig. 20.2 Ethanol metabolism. The enzymes and intermediates of alcohol metabolism

Endoplasmic Reticulum (ER) Stress

The unfolded protein response also referred to as ER stress is a protective cellular mechanism that can be deregulated by alcohol [33–35]. Alcohol consumption results in increased expression of key components of the unfolded protein response including glucose regulatory proteins (GRP78, GRP94, CHOP, and caspase-12) [36]. C/EBP Homologous Protein, or CHOP, was found to be a key factor in this process, and mice deficient in CHOP were protected from alcohol-induced apoptosis in the liver [37]. ER stress also results in upregulation of transcription factors SREBP-1c and SREBP-2, which are key regulators of lipid accumulation in the liver [38]. Furthermore, ER stress contributes to increased homocysteine levels [39], which can cause undesirable protein modifications.

Decreased Antioxidants

While alcohol increases ROS, it also reduces the availability of most antioxidant systems, thereby promoting oxidative stress and ROS-induced liver damage [20]. Alcohol-fed mice had decreased expression of superoxide dismutase (SOD), an important antioxidant [40]. The activities of other antioxidants, particularly glutathione sulfhydryl (GSH) and glutathione-S-transferase (GST), are also decreased in ALD [41, 42].

Gut-Liver Axis in ALD

Increasing evidence suggests that interactions between the liver and gut contribute to the development of ALD (Fig. 20.3). In normal homeostasis, a balance is maintained among the gut microbiome, gut permeability, and translocation of gut-derived substances that reach the liver via the portal circulation [5, 11, 43–45]. The liver, as an immune organ, expresses sensitive receptor systems on all of its cell types, which can trigger responses to pathogen-derived signals from the gut. Lipopolysaccharide (LPS), a component of Gram-negative bacteria, is present at increased levels in the portal and systemic circulation in humans and in animals

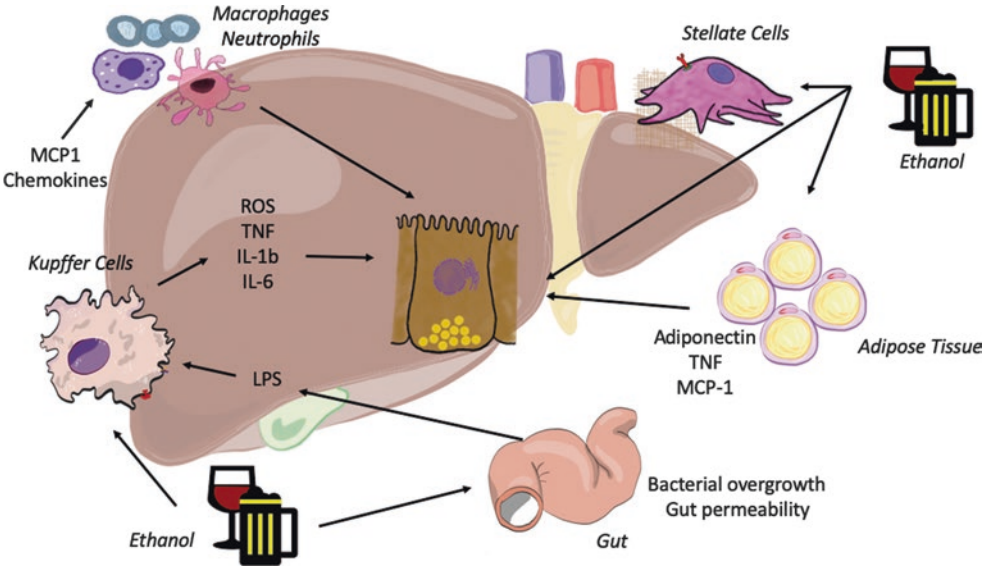

Fig. 20.3 Pathogenesis of ALD. Interorgan crosstalks between gut-liver and adipose-liver play important roles in pathogenesis. Both hepatic and immune-derived cells are activated in ALD under a variety of stimuli, including cytokines, chemokines, and reactive oxygen species

after excessive alcohol intake [46–48]. The central role of LPS has been demonstrated by multiple studies [48–50]. Increased serum levels of peptidoglycan were found in mice after chronic alcohol administration, suggesting that components of Gram-positive microbes may also increase in the serum after prolonged alcohol use [51]. Increased translocation of bacterial products has been attributed to changes in intestinal permeability. Indeed, chronic alcohol exposure increases gut permeability by compromising epithelial cell barrier functions through multiple mechanisms [47, 52]. Activation of inducible nitric oxide synthase by ethanol metabolites has been implicated [53]. In vitro alcohol treatment of colonic epithelial cells decreases the expression of tight junction proteins such as zona occludin-1 (ZO-1), possibly via ROS-induced microRNA-221 expression that in turn downregulates ZO-1 protein levels [54].

In addition to the direct effects of alcohol on gut epithelium, alcohol consumption results in changes in the gut microbiome [55]. Animal studies have revealed quantitative and qualitative changes in the gut microbiome after prolonged alcohol feeding [56, 57]. Small bowel bacterial overgrowth is seen in patients with ALD [58]. The expression of the antimicrobial peptides, Reg3b and Reg3g, decreases from colonic epithelium [59] and, along with increased gut permeability, contributes to increased bacterial translocation. Furthermore, the composition of the bacterial species changes after alcohol treatment. ALD is associated with decreased intestinal production of long-chain fatty acids that support the growth of commensal *Lactobacillus* [55], and the relative proportions of *Firmicutes* increases at the expense of *Bifidobacteria* in alcohol fed mice [56]. Changes in bacterial flora have also been linked to increased level of unconjugated bile acids, which causes suppression of farnesoid X receptor activity in enterocytes that leads to upregulation of hepatic CYP7A1 expression and bile acid synthesis [55]. Alcohol-induced fungal dysbiosis has also been recognized recently [60]. The specific role of gut microbiome changes in the pathogenesis of ALD remains unclear; however, sterilization of the gut with nonabsorbable antibiotics has a significant protective effect on alcohol-induced steatosis and inflammation in animal models of ALD [48].

Adipose-Liver Axis in ALD

The role of adipose-liver crosstalk has been increasingly recognized (see Fig. 20.3). Adipose tissue is not only important for storage of excess fat but also acts as an important endocrine organ that engages in extensive interorgan crosstalk, including with the liver. Adipose tissue is inflamed under chronic alcohol exposure and releases a wide range of proinflammatory cytokines, also called adipokines [61]. These adipokines, including adiponectin, also have important roles in pathogenesis of ALD [62]. Adiponectin is a cytokine with anti-inflammatory properties and is decreased with chronic alcohol use [63]. Decreased secretion of adiponectin and adiponectin resistance hinder lipid metabolism in the liver, which contributes to hepatic steatosis [61]. Fat metabolism is also regulated by osteopontin, which is increased in the adipose tissue, liver, and serum of patients with alcohol-related liver fibrosis [64]. Osteopontin has been suggested as a marker of liver disease progression [65–67].

Innate and Adaptive Immune Responses

The liver is a major immune organ that contains all cell types of the immune system. In ALD, there is recruitment of a variety of immune cells to the liver including neutrophil, monocytes, T cells, and B cells [68, 69]. Liver parenchymal cells

also engage in the inflammatory process through crosstalk with immune cells and cytokine production. The immunotolerant state of the healthy liver is profoundly changed in ALD where a proinflammatory state prevails and disturbs parenchymal cell functions [11]. The pathogenesis of ALD involves complex interactions between the effects of alcohol and its toxic metabolites on cells in the liver and gut, induction of ROS, and propagation of the inflammatory cascade.

Role of Innate Immunity

The innate immune system is the first line of defense in recognition and response to danger signals in the liver. Innate immunity comprises of chemical-physical barriers (gut epithelium) as well as cellular defenses. The impairment of gut barrier function discussed above can be considered as a breach of innate defense mechanisms. Cellular components of the innate immune system of the liver include resident liver macrophages or Kupffer cells (KCs), hepatic dendritic cells (DCs), and bone-marrow-derived immune cells that circulate through the liver [70, 71]. Although other liver parenchymal cells such as hepatocytes and liver sinusoidal endothelial cells are not formally considered to be immune cells, they take on important immune functions in liver injury [72]. Innate immune cells and signaling pathways recognize exogenous danger signals such as pathogen-derived molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) that are released from stressed, injured, or dying cells [70, 71]. The slow blood flow in the liver sinusoids and the proximity of liver parenchymal cells and immune cell in the liver sinusoids allow ample interactions among danger signals, immune cells, and parenchymal cells during the different states of ALD. Both soluble mediators and innate immune cells contribute to liver and systemic inflammation that is characteristic of ALD and particularly AH [70, 71]. Overexpression of proinflammatory cytokines and chemokines (TNF-α, interleukin (IL)-1α, IL-1β, MCP-1, IL-8) and decreased levels of anti-inflammatory mediators (IL-10) in AH are reflective of innate immunity dysregulation [50, 68, 73, 74]. Despite this immune cell activation, AH patients are actually predisposed to infection, reflecting immune dysregulation.

Soluble Mediators

Cytokines and Chemokines

Cytokines and chemokines are secreted mediators of the immune system and are critical in coordination of inflammatory crosstalks between various cell types and organ systems. The importance of pro-inflammatory cytokines has been well recognized in pathogenesis of ALD, particularly in alcoholic hepatitis, the most inflammatory condition within ALD [74, 75]. Patients with alcoholic hepatitis have increased circulating and liver levels of TNF-α, IL-6, CXCL8, and IL-1 [14, 15, 76–78]. Several studies have proposed using cytokine/chemokine levels as prognostic indicators in alcoholic hepatitis as increased serum level of CXCL5 and CXCL8 correlated with higher mortality [79]. CXCL8 is highly upregulated in alcoholic hepatitis, while it is only moderately increased in alcoholic cirrhosis [80]. CXCL chemokines are involved in neutrophil recruitment and activation, and thought to be responsible for the intense neutrophilic liver infiltration seen in alcoholic hepatitis. Monocyte chemoattractant protein (MCP)-1, also called CCL2, is a strong recruiter of monocytes and macrophages to the liver in ALD [81]. The pathologic role of these pro-inflammatory cytokines was suggested by animal studies involving knockout mice where deficiency in TNF receptor 1 (TNFR1), MCP-1, or IL-1 receptor (IL-1R) ameliorated ALD [82, 83]. Furthermore, administration of recombinant IL-1R antagonist, that prevents the biological effects of IL-1β and IL-1α on the IL-1R, attenuated the development of ALD in a mouse model [82]. Among these cytokines, TNF-α has been identified as a central mediator of ALD. RNA sequencing analysis identifies TNF-α as an upstream regulator to many differentially expressed genes in ALD, including many chemokines and cytokines [12, 13]. The sources of cytokines and chemokines in the liver likely originate from multiple cell types. While resident liver KCs are thought to be the major source of pro-inflammatory cytokine production, particularly TNF-α [50], circulating immune cells such as monocytes, neutrophils, and lymphocytes as well as resident liver cells such as endothelial cells, hepatic stellate cells, and hepatocytes have all been implicated to produce cytokines [11, 84]. In alcohol-induced liver injury, an inciting injury may cause the activation of KCs, leading to the release of pro-inflammatory cytokines such as TNF-α. These cytokines kick off a positive feedback loop leading to the release of more cytokines and chemokines, which attracts circulating immune cells to propagate this process. In this way, inflammation begets more inflammation.

In addition to fueling inflammation, many cytokines have direct effects on hepatocytes that contribute to the pathogenesis of ALD, and sometimes, these cytokines concurrently promote both liver injury and regeneration [74, 85, 86]. TNF-α is a good example of this. While TNF-α binding on healthy hepatocytes does not have deleterious effects, TNF-α binding on injured hepatocytes potently triggers apoptosis [87, 88]. At the same time, TNF-α is also involved in liver regeneration, and blockade of TNF-α signaling may have the unintended side effect of impairing organ healing [86]. IL-1β is an endogenous pyrogen, an inducer of other proinflammatory mediators [89]. It also has direct effects on hepatocytes by inducing steatosis [82]. Furthermore, IL-1β sensitizes hepatocytes to the cytotoxic effect of TNF-α, further potentiating hepatocyte injury caused by inflammatory cells [82]. IL-6 causes fat accumulation in hepatocytes and, like TNF-α,

promotes liver regeneration in ALD [90]. IL-22, a member of the IL-10 family, was shown to have hepatoprotective effects in ALD. IL-22 is produced by Th17 T and natural killer (NK) cells, and its levels were diminished in the liver after chronic alcohol feeding [91]. IL-22 was shown to promote liver regeneration, decrease apoptosis, and reduce steatosis. Consistent with this, administration of recombinant IL-22 was protective in an alcohol binge drinking mouse model and currently being tested in clinical trials for treatment of AH [74, 91–93]. Monocyte production of MCP-1 is increased in AH [94], and MCP-1 appears to induce liver steatosis [95], possibly through the action of hypoxia-inducible factor-1 (HIF-1) and PAPR-γ in hepatocytes [83, 96, 97]. Given the complexities involved in the actions of cytokines and chemokines in ALD, clinical use of cytokine inhibitors needs to proceed with caution, mindful of the potential impact on not only antimicrobial defense but also liver regeneration [98].

Complement

The complement system is an integral part of the innate immunity that carries important functions in the activation of both the innate and adaptive immunities. Complement is activated in hepatic inflammation, and can be activated by ethanol exposure [11, 71, 99]. Receptors of classical complement pathway factors C3a and C5a were found to be involved in cytokine activation in mouse model of ALD [100]. C1q, the recognition subunit of the first complement component, was shown to bind to apoptotic cells, leading to exacerbation of ethanol-induced injury [84, 99]. On the other hand, other reports suggested that factor D, a component of the alternative complement pathway, assists in clearance of apoptotic cells and thus helps to ameliorate alcohol-induced liver injury [100, 101]. As is the case with cytokines, the complement system appears to have dichotomy of functions, playing important roles in both liver inflammation and healing.

Immune Cells

Neutrophil

In acute AH, a hallmark histopathological finding of alcohol-induced liver injury is infiltration of neutrophils into liver parenchyma [67, 102]. This is in contrast with the predominately macrophage-driven pattern of injury in chronic alcoholic steatohepatitis [4]. In animal models, binge drinking in alcohol-fed mice induced liver neutrophil infiltration [103–105]. As discussed previously, AH is associated with much higher morbidity and liver injury compared to chronic alcoholic steatohepatitis, and it is unclear if this may be due to increased neutrophil presence in the liver. Neutrophils are an important source of ROS production, exacerbating oxidative injury in hepatocytes that are already damaged by alcohol exposure. In patients with AH, there is remarkably increased expression of neutrophil recruiting chemokines, including CXCL1, CXCL5, CXCL6, and CXCL8, and the degree of elevation of these chemokines correlates with disease outcome [12, 79]. On the other hand, it has been hypothesized that neutrophilic inflammation may have beneficial effects as well, as one clinical studied found increased neutrophil infiltration to be associated with improved disease outcome [106]. Neutrophils have important functions in removing debris, secreting growth factors, and fighting bacterial infections. Due to increased gut-bacterial translocation seen in AH, increased neutrophilic presence may be necessary. The phagocytic and bactericidal abilities of neutrophils in patients with AH are often impaired, predisposing patients to infection [107, 108]. The persistence of both host and pathogen inflammatory factors may drive continued neutrophil recruitment into the liver [108]. Further research in this area is needed to better delineate the role of neutrophils in ALD.

Kupffer Cells, Macrophages, and Monocytes

A central role has been suggested for Kupffer cells (KCs) in ALD [109]. KCs are liver-resident macrophages that arise from the liver and are enriched in the livers of chronic alcohol users and alcohol-fed mice [50, 109–111]. In ALD, there is both activation of resident KCs and recruitment of bone-marrow-derived monocytes, which collectively contribute to an augmented macrophage immune response, though the increased number of macrophages is thought to be predominately bone-marrow derived [112–114]. These macrophages are powerhouses of cytokines production, and orchestrate the inflammatory cascade in the liver by modulating the recruitment and activation of other inflammatory cells [113]. The tremendous plasticity in the phenotype of macrophages has been increasing recognized in recent years. Depending on the tissue environment, danger signals, and cytokine milieu, monocytes differentiate into M1 or M2 macrophages. M1 macrophages promote inflammation, whereas M2 macrophages suppress inflammation and promote tissue healing [113]. Although this nomenclature has limitations, it still provides a construct to understand dual macrophage function in AH. M1 macrophages are classically activated by LPS, IFN-γ, or pro-inflammatory cytokines and have high phagocytic activity. These macrophages produce high levels of proinflammatory cytokines such as TNF-α, thus driving further Th1 response and Th17 cell activity [113]. M2 macrophage differentiation is triggered by IL-4, IL-10, TGF-β, or adiponectin and is typically involved in Th2 responses, such as allergy, granuloma formation, and wound healing [113, 115, 116]. M2 macrophages produce IL-10, IL-1R antagonist and TGF-β, which have anti-inflammatory effects and promote tissue repair [116].

KCs can adapt a similarly immune-activating (M1) or immune-suppressive (M2) phenotype. Isolated KCs in animals exposed to ethanol chronically become more activated [50, 113, 117, 118]. This has been linked to increased

expression of NF-κB, ERK, and MAPK pathways [119–121]. In vivo studies elegantly demonstrated that elimination of KC by gadolinium chloride in rats or clodronate in mice attenuated alcohol-induced liver injury [109, 122], establishing the significant contribution of these cells in pathogenesis of ALD. Bone marrow transplantation experiments in mice demonstrated that bone-marrow-derived inflammatory cells are also important in liver injury independent of KCs. For example, while mice deficient in caspase-1 or IRF3, molecules that mediate IL-1β and TNF-α, respectively, are protected from ALD [82, 123], alcohol feeding after transplantation of these mice with wild-type bone marrow resulted in steatosis, liver damage, and inflammation [123]. Human studies from patients with ALD demonstrated increased production of IL-1β, TNF-α, and IL-6 in monocytes [15, 75, 124]. Therefore, it is clear that both KCs and bone-marrow-derived monocytes play important and non-interchangeable functions in promoting liver injury in ALD.

Dendritic Cells

Alcohol exposure leads to dendritic cells (DCs) dysfunctions, manifesting as impaired antigen presentation capacity in inducing antigen-specific T cell activation, reduced immunomodulatory cytokines (IL-12) production, and altered expression of costimulatory molecules [125–127]. There is a decrease in the number of circulating DCs in alcoholic cirrhosis patients, and the composition of the dendritic cell population changes in the liver of alcohol-fed mice as well, resulting in an increased immature DC phenotype with reduced antigen presentation capacity [125, 128].

Adaptive Immunity

It has been shown that T-cell, NK-cell, and B-cell functions are altered by chronic alcohol use [11, 69]. In the liver, there is enrichment of T lymphocytes and increased activation of circulating T cells. T-cell receptor sequencing analysis demonstrated that there is enrichment of oligoclonal T cells in the livers of patients with ALD, which indicates that there is clonal expansion of antigen-specific T cells in the ALD livers and not simply a nonspecific recruitment of bystander inflammatory cells [129]. Besides translocation of pathogen-derived antigen, there is also neoantigen formation in the liver in ALD due to the formation of adducts [130]. Abundance of ROS in alcohol-induced injury as a result of reactive metabolites such as acetaldehyde, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) can bind to proteins, DNA, and RNA to form adducts [131]. These adducts are recognized by resident liver cells via the scavenger receptor and induce cytokine production [131]. In addition, protein adducts may be presented to T cells and B cells to elicit antigen-specific responses [131, 132]. Being a subtype of T helper cells, the Th17 cells have been shown to be important in ALD [133–135]. Th17 cells promote liver inflammation and fibrosis, in part by the release of IL17 as well as other chemokines, such as CXCL1 and CXCL8, which are potent neutrophil chemoattractants [135]. A recent study demonstrated a correlation between neutrophil recruitment and the presence of IL-17-producing T-helper cells within the inflammatory liver infiltrates in patients after alcohol intoxication [133]. However, Th17 cells also release IL22, which, as discussed previously, has regenerative effects. These studies illustrated not only the cytotoxic and proinflammatory effect of T cells in ALD, but also highlighted the potentially regenerative effect of these cells, illustrating the complexities of immune regulation in this disease.

Signaling Pathways

Pattern Recognition Receptors

Innate immune responses are triggered by danger signals from pathogens or injured cells through recognition by pattern recognition receptors (PRRs) (Table 20.1). The major families of PRRs in the liver are Toll-like receptors (TLRs), RIG-I-like RNA helicase receptors (RLHs), and NOD-like receptors (NLRs) [136–138]. Ample evidence demonstrates that activation of TLRs and NLRs is a pivotal element in the pathogenesis of ALD (Fig. 20.4). While most studies focus on the role of LPS as a trigger of innate immune activation, the role of other pathogen-derived or endogenous danger signals remains to be evaluated.

TLRs

TLRs play central roles in pathogenesis of ALD. Of the 13 TLRs, TLRs 1–6 are expressed on the cell surface and recognize extracellular PAMPs, while intracellularly localized TLRs (TLR3, 7, 8, 9) sense nucleic acid sequences [136, 138–140]. The cytoplasmic TIR domain of TLRs interacts with the TIR domain of adapter molecules such as the My88, the common adapter utilized by all TLRs except for TLR3,

Table 20.1 Potential danger signals activating innate immune responses in alcoholic liver disease

Danger signal	Sensor/receptor	Mediators
Exogenous danger signals		
LPS	TLR4	Inflammatory cytokine
	TLR2	Inflammatory cytokine
Endogenous danger signals		
Saturated fatty acids	TLR4, inflammasome	IL-1, inflammatory cytokine
Unsaturated fatty acids		
ROS		NF-κB, SIRT1
Apoptotic cells	Inflammasome	CIg
Necrotic cells (ATP?)	Inflammasome	
Hypoxia		HIF1α

Reprinted from Szabo [184]

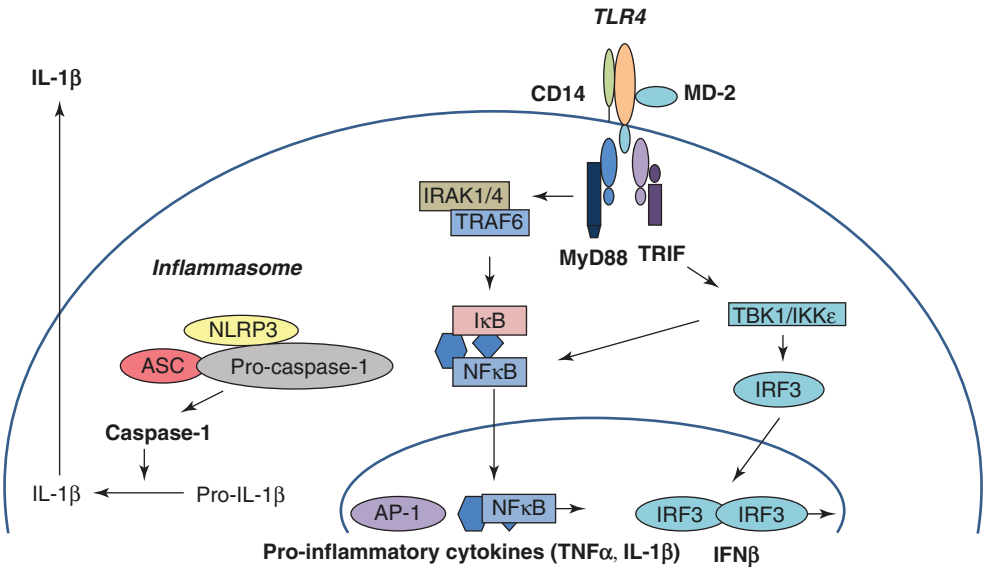

Fig. 20.4 Activation of TLR4 and inflammasome in ALD. Pattern recognition receptors (PRRs) are activated by danger signals, resulting in activation of proinflammatory genes and the production of inflammatory cytokines

or TRIF that is involved in TLR3 and TLR4 signaling. MyD88 recruitment triggers downstream signaling via IRAK1/4 kinases and leads to NF-κB activation and induction of proinflammatory cytokine genes [141, 142]. The TRIF adapter activates IKKε/TBK, leading to IRF3 or IRF7 phosphorylation and Type I Interferon (IFN) induction. TLR4 recognizes endotoxin derived from Gram-negative bacteria, TLR2 senses microbial lipopeptides, while TLR1 and TLR6 combined with TLR2 distinguish between triacyl- and diacyl-lipopeptides. TLR3 recognizes viral double-stranded RNA, and the bacterial flagellin stimulates TLR5. TLR7 and TLR8 are sensors of single-stranded RNA and TLR9 recognizes CpG-rich DNA. All TLRs are broadly expressed in the liver in different cell populations across immune and parenchymal cells [138]. TLR4, the receptor that senses LPS, plays a central role in ALD. TLR4 recognition of LPS is facilitated by the coreceptors CD14 and MD-2. CD14, a GPI-anchored protein, facilitates the transfer of LPS to the TLR4/MD-2 receptor complex that modulates LPS recognition [143]. MD-2 associates with TLR4 and binds LPS directly to form a complex with LPS in the absence of TLRs. The association between LPS and CD14 can be further facilitated by LPS-binding protein (LBP) [143]. Studies in animal models demonstrated that mutation in TLR4 or deficiency of TLR4 attenuated alcohol-induced liver steatosis, inflammation, and injury [49, 144]. Ligand engagement of TLR4 triggers rapid downstream signaling by recruitment of the adaptor molecules, MyD88 or TRIF. MyD88 recruitment leads to IRAK-1/4 activation and phosphorylation that triggers downstream activation of the inhibitory kinase (IKK) complex and NF-κB activation [140]. NF-κB has complex roles in ALD, including protecting hepatocytes from apoptosis and activating proinflammatory cytokine in immune and parenchymal cells [11, 73]. Nuclear translocation of the NF-κB p65/p50 dimer in immune cells correlates with proinflammatory cytokine induction in ALD [13, 73]. Recruitment of the TRIF adapter to TLR4 triggers downstream activation of the TBK/IKKε complex that phosphorylates IRF3, leading to IRF3 nuclear translocation and induction of Type I IFNs. Mice model demonstrated that TLR4 and IRF3 were critical in the development of liver steatosis, inflammation, and liver damage after chronic alcohol feeding [49, 123, 145]. Bone marrow chimera experiments revealed a cell-specific role for IRF3. Whereas the absence of IRF3 in bone-marrow-derived cells resulted in protection from alcohol-induced steatosis, inflammation, and liver damage, IRF3 deficiency in the liver parenchymal cells promoted alcohol-induced liver injury [123].

NOD-Like Receptors and the Inflammasome

Inflammasomes are multiprotein complexes that include NLR sensors, adapter molecules, and procaspase-1 that cleave procaspase-1 into active caspase-1 upon ligand engagement [146]. Caspase-1 activation results in cleavage of pro-IL-1β, pro-IL-18, or IL-33 into a biologically active IL-1β (17 kD), IL-18, or cleaved IL-33 [147]. The family of NLR is characterized by the presence of a central nucleotide binding and oligomerization (NACHT) domain, which is flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment domain (CARD) or Pyrin (PYR) domains [146, 147]. NLRs function as receptors with ligand sensing in the LRRs region, whereas the CARD and PYR domains provide protein–protein interactions for downstream signaling. Based on their domain structures, the NLR family consists of subfamilies including NODs (NOD1-9), NLRPs (NLRP1-14, also called NALPs), IPAF (IPAF or

NLRC4 and NAIP), and AIM2. The AIM2 inflammasome is not a formal member of the NLRs but like NLRs is composed of ASC and caspase-1, leading to IL-1β activation [148]. These NLRs all lead to caspase-1 activation and IL-1β cleavage, while their ligand activation is unique. Previous reports document increased serum IL-1β as a feature of human ALD [89]. Indeed, Il-1β levels are also increased in a mouse model of ALD, while IL-1α, which is mostly cell associated, is not elevated. The importance of the inflammasome was demonstrated in mouse model deficient in caspase-1, which had significantly attenuated alcoholic liver steatosis, inflammation, and liver damage [82].

Nuclear Receptors

Most nuclear receptors that have received attention in ALD are involved in regulation of both lipid metabolism and inflammation [149]. Hypoxia has been shown to play a role in the pathogenesis of ALD. Hypoxia-inducible factor-1 (HIF-1α) messenger RNA was increased in livers of chronic alcoholics and in mice after chronic alcohol administration [96]. Alcohol-induced steatosis was mediated by HIF-1α, and involvement of HIF-1α activation was found in both hepatocytes and liver immune cells [96]. Retinoid X receptor (RXR) was found to modulate alcohol metabolism by affecting ADH expression. Blood ethanol levels in hepatocyte-specific RXRα-KO mice were significantly lower than in wild-type controls, and the same mice had significantly increased liver damage and more pronounced liver steatosis [150–152]. PPAR-α is responsible for regulation of lipid metabolism. Decrease in PPAR-α was linked to liver steatosis after alcohol feeding and PPAR-α agonist treatment ameliorated ALD in mice [153]. Likewise, PPAR-γ is also regulated in chronic alcohol exposure in KCs and hepatocytes. Treatment with the PPAR-γ agonist pioglitazone prevented the development of alcohol-induced steatosis and inflammation [154]. Another transcription factor, SREBP1, contributes to lipophilic pathway in ALD as well, and deficiency of SREBP1 worsened steatosis [155]. Recently, the importance of hepatocyte nuclear factor 4 α (HNF4α) was reported in patients with different phenotypes of ALD. HNF4α activity is mediated by TGFβ1 and deranged in alcoholic liver injury, resulting in downregulation of HNF4α and other liver-enriched transcription factors [12]. Interestingly, while genetic polymorphisms in these transcription factors were not related to the development of AH, epigenetic changes, such as DNA methylation and histone modifications, were highly related to development and severity of AH [12]. This illustrates an example of epigenetic transcriptional regulation in ALD. Increasing number of studies have demonstrated the importance of the roles of various epigenetic mechanisms, including DNA methylation, histone modifications, chromatin remodeling, in gene regulation in ALD [9]. These findings open up new avenues of investigation and may introduce novel therapeutic target for treatment development.

MicroRNAs and EVs

MicroRNAs (miRNAs) are a class of evolutionarily conserved, single-stranded, noncoding RNAs of 19–24 nucleotides that control gene expression at the posttranscriptional level [156]. MicroRNAs contribute to the regulation of liver parenchymal and immune cells [157]. The expression and potentially the function of many miRNAs are changed in ALD in mice [157, 158]. MicroRNAs also regulate stem cell differentiation, regeneration, and cell death [159]. Innate immune responses are fine-tuned by miR-155, miR-125b, and miR-146a as these miRNAs positively or negatively regulate target genes/proteins in the family of TLR signaling, NF-κB, ERK, and MAPK inflammatory intracellular signaling pathways [160, 161]. MiR-155 is enriched in KCs and positively regulates TNF-α through enhancing its translation [157, 162]. One of the important effects of alcohol is sensitization of KCs to LPS-induced TNF-α production. It has recently been shown that miR-155 levels are increased in the liver after chronic alcohol feeding and that alcohol-induced upregulation of miR-155 is a major molecular mechanism for LPS sensitization in mice [163]. Alcohol-induced liver steatosis has also been linked to alterations in miRNA expression. For example, miR-122, which regulates many targets in lipid metabolism, is decreased in the liver in ALD, while miRNA-217 was shown to promote ethanol-induced fat accumulation in hepatocytes [164]. Epigenetic regulation of miR-34 has been linked to fibrosis progression in ALD [165]. MicroRNAs are important in the cells where they are produced; they can be packaged into extracellular vesicles (EVs) and transferred to other cells, playing an important role in cell-cell communication [166]. Many miRNAs are increased in EVs of alcohol-fed mice compared to controls and have been studied as biomarkers of ALD [167].

Aside from miRNAs, EVs can carry many other cargos, including protein, DNA and RNA, and lipids, and exert transcriptional control over their target cells [168]. For example, adipose-tissue-derived EVs can mediate adipose-liver axis interactions by varying adipokine cargo carried in these EVs [100]. Circulating hepatocyte derived-EVs are increased in cirrhosis, but are increased most markedly in acute AH [169]. Certain hepatocyte-EV sphingolipids are also increased in AH, although the functional roles of these EV cargos remain largely unknown [169]. These EVs show potential as biomarkers for ALD, as EV number and EV sphingolipids correlates well with disease severity and mortality in small clinical studies.

Treatment for Alcohol-Associated Liver Disease

Abstinence

Cessation of alcohol consumption and treatment of alcohol use disorder (AUD) is considered as the mainstay of ALD treatment [170, 171]. This becomes even more important in the context of liver transplantation eligibility given increased concern for alcohol relapse in these patients. Alcohol relapse is associated with higher mortality in alcoholic hepatitis patients, and alcohol rehabilitation was shown to decrease readmission rate, relapse, and mortality [172].

Current Medical Treatment

Treatment of alcoholic cirrhosis focuses largely on management of complications, as liver transplantation remains the only definitive treatment for liver cirrhosis [173, 174]. Symptomatic treatment is provided to tackle complications and stigmata of cirrhosis. Rifaximin and lactulose are first-line therapies for treatment of hepatic encephalopathy. Esophageal variceal bleeding is another major cause of morbidity and mortality in these patients and can be corrected by many approaches including endoscopic banding. Many patients present with ascites and other complications such as acute kidney injury [170, 171]. Despite intense immune activation in the liver, there is an "immunological paralysis" associated with the disease. Infections, including spontaneous bacteremia, spontaneous as well as secondary bacterial peritonitis, procedural, and nosocomial infections, are very common in this group of patients [170, 171, 175]. Alcoholic hepatitis (AH) is underlined by a severe inflammatory response and is associated with high morbidity and mortality. Corticosteroid treatment with prednisolone 40 mg daily for 28 days is considered to be the first-line medical therapy, which demonstrated trend for improved short-term (28 days) survival, but was not shown to improve long-term survival [17]. Corticosteroids are also contraindicated in many AH patients with or suspected to have infections. Therapeutic futility is assessed at day 7 by calculating the Lille score. With Lille score <0.45 at day 7, therapy is considered futile and corticosteroids are discontinued to prevent risk of infections [170, 171]. Pentoxifylline, a weak phosphodiesterase inhibitor, has been evaluated as an alternate to steroid treatment in AH; however, most studies found it inferior compared to steroids [17]. A large clinical trial investigated the combination of steroids and pentoxifylline and found no benefits over single therapy except for a small population of patients with hepatorenal syndrome [176]. It is generally not included as a therapy in guidelines at the present time.

Liver Transplantation in ALD

In the USA, patients with ALD that report active or recent alcohol abuse are not considered ideal candidates for liver transplantation. Many transplant centers in the USA require at least 6 months of abstinence and participation in support groups for eligibility of listing for liver transplantation. These rules are especially ominous for severe AH patients that have a remarkably high 6-month mortality rate. In a multicenter study in the European Union, liver transplantation was found to be effective as a treatment in patients with AH [177]. While all transplant recipients used alcohol heavily pretransplant, <10% had heavy relapse in alcohol use after liver transplantation for AH [177]. Similar results have been observed in US studies albeit at a smaller scale and in a retrospective manner. Ideal selection criteria for transplantation of patients with AH are evolving and beyond the scope of this chapter.

Liver transplantation for alcoholic liver cirrhosis is highly successful and part of standard of care in the USA and other parts of the world. Transplanted organ survival is excellent both in 1 and 5 years, and recipient survival is also high compared to transplantations for many other etiologies, particularly viral hepatitis [178]. The necessity of an arbitrary 6-month absence period has come into question, and some experts have argued to revise the requirement to expand the eligibility pool for transplantation.

Novel Therapeutics

Advances in the understanding of the cellular and molecular mechanism of ALD in the last decades provide multiple attractive therapeutic targets in ALD, and many of these therapeutics target immune activation. Accompanying table lists the most actively studied immune targeting therapy currently investigated in clinical trials (Table 20.2). For example, given the essential role of TNF-α in AH pathogenesis, multiple TNFα inhibitors have been trialed in AH. However, despite the clinical success of anti-TNF-α agents in treatment of autoimmune diseases, TNF-α inhibitors Infliximab and Etanercept failed in clinical trials for treatment of alcoholic hepatitis [98, 179]. The use of TNF-α inhibitors was associated with increased risk of infection and poor clinical outcomes. Similarly, IL-1β signaling has been studied as a potential target. Pro-IL-1β is activated and secreted upon cleavage with caspase 1, which gains function via recruitment to a multiprotein complex the inflammasome. Anakinra, an IL-1 receptor antagonist, has been studied in a clinical trial in combination with zinc sulfate and pentoxifylline compared to corticosteroids in patients with severe alcoholic hepatitis [180]. The study has shown preliminary positive trend toward improved 6-month mortality. Emricasan, another drug that act on caspases and thus potentially has

Table 20.2 Emerging immune targeted therapies in alcohol-associated liver disease

Therapy	Mechanism
Endotoxemia	
IMM 124-E	Decreases LPS and potentially reduces downstream inflammatory response
IL-1 inhibitor	
Canakinumab	IL-1 inhibition
Anakinra	IL-1 inhibition, in clinical trial in combination with other agents
TNF-α inhibitor	
Infliximab	TNF-α inhibition, no clinical benefit
Etanercept	TNF-α inhibition, no clinical benefit
Liver regeneration	
IL-22 (F-652)	Liver regeneration, anti-inflammatory, antisteatosis effects. Beneficial effects in a Phase IIa trial without any severe adverse events.
GCSF	Liver regeneration, immune reconstitution. Mixed results on efficacy in multiple clinical trials
Chemokines	
Cenicriviroc	Dual CCR2/CCR5 antagonist with improvement in NASH
Apoptosis	
Emricasan (IDN-6556)	Pan-caspase inhibitor, not shown to be beneficial in clinical trial

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benefit in inhibiting IL-1 signaling has been tested in trials [181]. Unfortunately, no clinical benefit was seen in a Phase II trial focused on alcohol-induced acute on chronic liver disease with Emricasan [182]. Chemokines, including C-C chemokine ligand 2 (CCL2) and C-X-C motif ligand 1 (CXCL1), are upregulated in ALD, leading to the recruitment of macrophages and neutrophils in propagation of liver inflammatory responses. More narrowly targeted and selective anti-inflammatory agents such as chemokine receptor antagonists have been used in preclinical setting with some success. Cenicriviroc, a CCR2/CCR5 receptor antagonist, was recently shown to reduce steatosis and fibrosis in a Phase II cohort with nonalcoholic steatohepatitis (NASH) [183]. Clinical trial for treatment of alcoholic hepatitis with Cenicriviroc is needed to explore its applicability to alcoholic hepatitis.

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2. SAMHSA. National Survey on Drug Use and Health (NSDUH). Table 5.5A—Alcohol use disorder in past year among persons aged 12 or older, by Age Group and Demographic Characteristics: Numbers in Thousands, 2016 and 2017. 2017 [cited 2019/10/1]. Available from: https://www.samhsa.gov/data/sites/default/files/cbhsq-reports/NSDUHDetailedTabs2017/NSDUHDetailedTabs2017.htm#tab5-5A.
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Nonalcoholic Fatty Liver Disease

21

William Alazawi and Gideon Hirschfield

Key Points

- Nonalcoholic fatty liver disease is the most prevalent of liver diseases.
- A substantial proportion of patients worldwide progress beyond simple steatosis to steatohepatitis.
- The presence of steatohepatitis is a marker of risk of developing progressive liver fibrosis and hepatocellular carcinoma.
- While not an organ-specific clinical syndrome, for many patients, the development of liver fibrosis/cirrhosis portends a substantial risk of ultimate liver failure and liver cancer, alongside raised cardiometabolic risks associated with the closely aligned obesity and metabolic syndrome/insulin resistance.
- Within the liver microenvironment, there are immunologic consequences directly and indirectly associated with the development of steatosis and steatohepatitis.
- These include cellular responses involving adaptive and innate immune responses, as well as pathophysiologic responses modulated by host genetics and microbiome variations.
- New therapies for patients, among lifestyle/metabolic interventions more generally, are needed, and immunoregulation is one potential target based on our evolving and better understanding of these inflammation and immune responses.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide. It affects 13–32% of the general population and is closely associated with the metabolic syndrome, affecting up to 68% of people living with type 2 diabetes and up to 75% of people with obesity [1, 2]. The disease is defined by the deposition of fat in more than 5% of hepatocytes in the absence of secondary causes such as excess alcohol consumption, drugs, and other causes of liver injury. Most people with NAFLD have uncomplicated or "simple" steatosis, also called nonalcoholic fatty liver or NAFL, and the risk of significant liver disease for such individuals is low. However, 10–19% develop nonalcoholic steatohepatitis (NASH) [3], characterized by liver cell injury, inflammation, and, in some cases, fibrosis (Fig. 21.1). NASH, and fibrosis can lead to cirrhosis, liver failure, and hepatocellular carcinoma which have high rates of morbidity and mortality. Closely associated with the obesity epidemic and metabolic syndrome, the rise of NASH has led to it becoming the leading indication for liver transplantation in women (and soon to be in men too) in the United States [4].

The definition of stage and grade of NAFLD has been based upon histological assessment of liver tissue. Composite disease scores assess the degree of steatosis, inflammation, and hepatocyte ballooning, as well as fibrosis. Ballooning degeneration of hepatocytes is the sine qua non of steatohepatitis. This form of hepatocyte degeneration is defined morphologically as swelling, enlargement, rounding, and characteristic reticulated cytoplasm. The absence of other, more objective, markers of steatohepatitis has resulted in overreliance upon liver biopsy in epidemiological and mechanistic studies of NASH. While noninvasive markers of fibrosis have been developed and are widely used in clinical practice and in research, similar tools to detect inflammation and ballooning degeneration are not well established.

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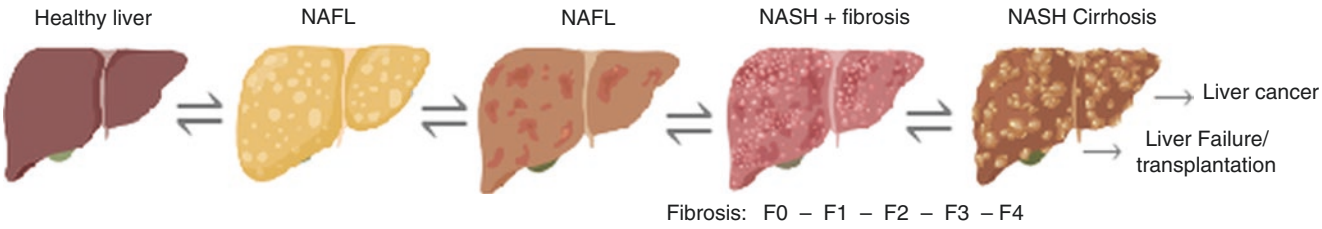

Fig. 21.1 The spectrum of disease in NAFLD. Uncomplicated or "simple" steatosis, also called nonalcoholic fatty liver (NAFL), can be complicated by liver cell injury and inflammation resulting in nonalcoholic steatohepatitis (NASH) which can be associated with fibrosis (staged F0-F1). NASH and fibrosis can lead to cirrhosis, liver failure, and hepatocellular carcinoma

Compared with patients with simple steatosis, life-threatening liver outcomes were more five times and ten times more common in patients with NASH and fibrosis, respectively, in a series of patients followed over 30 years [8]. Patients with more advanced stage of fibrosis are at particularly increased risk of mortality although the grade of NASH was not predictive of mortality [5–7]. Notwithstanding the risk of lead time bias, studies such as this have informed clinical practice, meaning that fibrosis has become a major focus of research including drug development. However, it is important not to overlook the fact that fibrosis is a pathological process that occurs because of an injury that is persistent or because of an ineffective inflammatory and immune responses that fail to resolve.

NAFLD is described as the hepatic manifestation of metabolic syndrome. More accurately, patients with NAFLD spectrum are likely to have elements of metabolic syndrome such as diabetes and obesity. Mortality rates in NAFLD are twice those in the general population [8]. Cardiovascular disease and malignancy are the most common causes of death in these patients although it remains unclear whether the increased cardiovascular risk is accounted for by the existence of other risk factors [9–12]. Cohorts followed over many years have shown that patients with histological NASH and fibrosis, rather than simple steatosis, are at greater risk of end-stage liver disease and liver-related mortality [5]. Paired biopsy studies have shown that progression through stages of fibrosis is particularly seen in patients with type II diabetes [13], and this is borne out in large-scale population-level studies [14]. These epidemiological data point to shared mechanisms of disease that, at least in part, explain the close association of obesity, diabetes, and the NAFLD spectrum.

An emerging body of evidence points to innate and adaptive immune responses shaping the natural history of these diseases. Our current understanding of the NAFLD spectrum is that hepatic fat content can increase in the context of, although not exclusively, insulin resistance. In some patients, this steatosis is accompanied by hepatotoxicity, believed to be driven in part by the directly toxic effects of lipids on the hepatocyte. This drives an inflammatory response that includes innate and adaptive immunity. Hepatic steatosis, insulin resistance, and obesity-associated metabolic inflammation can exist in patients without histological features of NASH. Setting aside the challenges of defining NASH, the low risk of progression seen in such individuals suggests further insult is required for the development of progressive NASH.

However, a major challenge in understanding the literature in NASH has been the heterogeneity in mouse models and in human studies. High-fat, amino-acid deficient or defined diets result in different patterns of liver injury in mice with varying degrees of obesity and insulin resistance. Human studies suffer inconsistent case definitions, sampling methods, and tools to assess immune involvement. In this chapter, we will explore the role that lipid accumulation and other "hits" play in triggering innate and adaptive immune responses and how these responses can lead to progressive fibrosis and eventually advanced liver disease.

Hepatic Steatosis, Insulin Resistance, and Inflammation

The hepatocyte can become lipid-laden as a result of a number of mechanisms. These include increased dietary fat content, impaired fat transport out of the liver, lipolysis in adipose tissue that increases free fatty acid flow to the liver, and de novo lipogenesis. Insulin resistance, a feature of metabolic syndrome, contributes to these biochemical processes.

NAFLD and obesity are closely linked epidemiologically and mechanistically. Overweight and obesity in adults are defined by body mass indices greater than 25 kg/m^2 and 30 kg/m^2, adjusted to 23 kg/m^2 and 27.5 kg/m^2 in people of South Asian ethnicity, respectively. Obesity is associated with an increased risk of abnormal liver biochemistry in adults as well as children and young people [15] and with risk of NAFLD in White and South Asian ethnicities [16]. Patients with overweight and obesity are more likely to have visceral adiposity and hepatic steatosis. Dietary intake contributes to this, but an important source of free fatty acids is lipolysis in adipose tissue. This is regulated by insulin. In

insulin resistance, peripheral tissues do not respond to insulin as sensitively as healthy tissues do, and in the adipose tissue, this results in a failure to switch off lipolysis. Insulin resistance also results in raised plasma insulin as well as glucose levels. High levels of glucose activate the transcription factor ChREBP and insulin activates SREBP1, both of which facilitate de novo lipogenesis, working via fatty acid synthetase and acetyl CoA-carboxylase. FOXO-1-mediated gluconeogenesis is inappropriately active in the insulin-resistant liver as is Akt2-mediated impairment of glycogen synthesis. Taken together, obesity and insulin resistance can account for the features of hepatic steatosis seen in NAFLD.

These liver-specific inflammatory events happen on a background of so-called *metabolic inflammation*, a pro-inflammatory shift in innate and adaptive immune cells in adipose tissue, skeletal muscle, and the liver, the numbers of which correlate with body mass index [17]. Metabolic inflammation is both a cause and a consequence of insulin resistance [18], and there is now clear evidence of a reciprocal relationship between products of metabolism (including diabetes and overnutrition) on innate immunity and inflammation [19]. Immunologically, therefore, the innate and adaptive changes associated with metabolic syndrome are likely to be present in these patients. Adipocyte hypertrophy in obese adipose tissue can result in hypoxia and contributes to adipocyte necrosis, triggering a local inflammatory response that recruits macrophages and T cells. Adipocytes and adipose-resident macrophages express major histocompatibility complex II and co-stimulatory molecules that can activate T cells and induce proliferation leading to the formation of crown-like structures. These pro-inflammatory "M1" adipose tissue macrophages secrete adipokines, cytokines, and chemokines including TNFα, MCP-1, adiponectin, and IL-6. TNFα in adipose tissue activates NF-kB signalling directly through its receptor or indirectly via TLR4 activated by free fatty acids released by TNFα-mediated lipolysis. Adipose tissue-derived inflammatory mediators called *adipokines* play an important role in development of metabolic inflammation, influencing inflammatory signalling pathways and affecting insulin sensitivity. Levels of pro-inflammatory adipokines such as IL-6 and visfatin are increased in the serum of patients with NASH compared to controls, while anti-inflammatory adipokines such as adiponectin are reduced. Pro-inflammatory signalling can promote insulin resistance by phosphorylating regulatory serine/threonine sites on the insulin receptor substrate (IRS) downstream of the insulin receptor (see below), worsening metabolic syndrome [20]. These changes seen in metabolic tissues are mirrored in the liver. The insulin-resistant and lipid-laden hepatocyte can, as a result of lipotoxicity, release inflammatory cytokines including type I interferons that attract and activate T cells.

Oxidative Stress and Damage Pathways

Lipidomic studies have shown an abundance of diacyl- and triacylglycerol among other lipid species in NAFLD. Mitochondrial β-oxidation of long-chain fatty acids produces acetyl CoA; however, this process is impaired in NAFLD, possibly due to regulators such as the sirtuin protein SIRT1 which can indirectly upregulate antioxidant genes [21]. SIRT3 can increase beta-oxidation and is decreased in animal models of NAFLD [22].

Loading of free fatty acids (FFAs) into the mitochondrion from the cytoplasm can disrupt the mitochondrial membrane electron chain, possibly through reduced NAD+, and impair ATP synthesis related to cytochrome C activity. This promotes the formation of reactive oxygen species rather than water and leads to oxidization of phospholipids and protein modification by the products of lipid peroxidation, oxidative stress. Modified proteins and phospholipids together with reactive aldehydes can act as damage-associated molecular patterns (DAMPs, see below) that can trigger inflammatory responses. In addition, the structurally modified macromolecules are potentially antigenic and are known as oxidative stress epitopes which may trigger adaptive immune responses as observed in other disease states [23–25]. Indeed, it has been suggested that esterification of free fatty acids to glycerol acts to protect the cell from free peroxidation of saturated fatty acids and subsequent oxidative stress [26].

Ligand-activated nuclear receptors include the peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), and thyroid hormone receptors (THR) families, acting in partnership with the retinoid X receptor (RXR) and other binding partners including NF-kB. These receptors regulate gene expression affecting a wide range of cellular processes including metabolic function including free fatty acid transport and mitochondrial β-oxidation. PPAR-α is a widely expressed protein that serves as a master regulator of hepatic lipid metabolism. PPAR-δ activation switches energy production toward fatty oxidation rather than glycolysis and can promote pancreatic insulin secretion and protect against insulin resistance in the liver. PPAR-γ is a crucial regulator of adipocyte differentiation and function and also of hepatic steatosis. Thyroid hormone receptor β is mostly expressed in the liver where it modulates hepatic cholesterol synthesis [27] and the FXR regulates lipid, as well as glucose and bile acid metabolism. The main endogenous ligands for the FXR are bile acids that are produced in the liver and secreted in the bile (under FXR control) and reabsorbed in the terminal ileum before recirculation to the liver. Acting directly as FXR agonists or through FGF19, bile acids can therefore influence hepatocyte metabolic function. Taken together, these pleiotropic metabolic effects of nuclear receptors affect hepatic steatosis and mitochondrial β-oxidation.

Experimental treatment of hepatocytes with long-chain saturated fatty acids, such as palmitate, results in lipid peroxidation and the generation of mitochondrial beta-oxidation-derived free radicals, endoplasmic reticulum stress, and activation of inflammasome and c-Jun N-terminal kinase (JNK) pathways and apoptosis [28]. The JNK pathway is also activated by free cholesterol in hepatocytes which depletes glutathione. Hepatotoxicity liberates damage-associated molecular patterns, which, together with abundant gut-derived pathogens and their metabolites, trigger an inflammatory response through the pathways outlined above and the inflammasome.

The NLRP3 inflammasome is a large intracellular complex comprised of multiple proteins. This complex includes a sensor component, such as an NOD-like receptor (NLR), and adaptor proteins, such as apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and the precursor procaspase-1. Inflammasome activation is a two-stage process that involves sensing of pathogen or damage patterns via PRRs that upregulates inflammasome components and procytokines (pro-IL1b and pro-IL-18) followed by assembly of the constituents of the inflammasome complex. This leads to the maturation of caspase 1 that cleaves pro-IL1b and pro-IL-18 into the active cytokines that are then released by the cell [29, 30].

Murine and human data point to a role for the NLRP3 inflammasome in the pathogenesis of NASH. Gene expression for NLRP3 inflammasome components, caspase-1 activity, and serum IL-1b levels are increased methionine-choline deficient and long-term high-fat diet-fed mice [31, 32]. Indeed, oral administration of the selective inhibitor of NLRP3, MCC950, resulted in improved liver histology in methionine-choline-deficient and choline-deficient diet-fed mice [33].

Inflammatory Triggers in NASH

The intestinal microbiome plays vital roles in digestion, metabolism, and homeostasis. The microflora breaks down dietary complex macromolecules, metabolizes bile acids, and releases metabolites that maintain intestinal health. Obesity and metabolic disorders have been associated with alterations in the intestinal microbiome. It is a matter for debate whether an obesogenic diet favors dysbiosis or whether dysbiosis leads to obesity and metabolic dysfunction; however, a large body of evidence now points toward an association between reduced diversity in obesity and diabetes with emergence of phyla such as Proteobacteria in NAFLD [34]. Many of these studies focus on the bacterial DNA harvested from stool. However, bacteria colonize the length of the orogastrointestinal tract, and therefore, focus is shifting onto the oral and small intestinal populations [35]. Bacterial structural and metabolic products such as lipopolysaccharide (LPS), flagellin, and DNA can move across the intestinal mucosa and are transported in the portal vasculature to the liver. In health, there is a degree of hepatic tolerance to these potentially inflammatory and immunogenic molecules. Emerging evidence points to impaired gut barrier function in obesity and NAFLD with translocation of bacterial products such as metabolites as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). These molecules can trigger innate and inflammatory responses as well as act as "danger signals" in the generation of adaptive responses (see below). In advanced liver disease, portal hypertension, impaired gut barrier integrity, and hepatic dysfunction reduce that tolerance, and this is believed to be an important driver of advanced liver disease.

PAMPs and DAMPs are sensed by a family of proteins called pattern recognition receptors (PRRs) that include the Toll-like receptors (TLR). The paradigm for TLR function is the interaction between TLR4 and LPS. LPS (together with the acute-phase protein, LPS-binding protein, LBP) binds to a complex of cell surface proteins that comprises CD14, MD-2, and TLR4 on the surface of many cell types, including myeloid, lymphoid, and endothelial cells. The interaction activates signalling cascades that involve TIR domain containing adaptor protein (TIRAP) and the major adaptor protein, myeloid differentiation primary response protein 88 (MyD88). Activated MyD88 recruits and phosphorylates cytosolic kinases including the interleukin-1 receptor (IL-1R)-associated kinases (IRAKs) which associate with TNF receptor associated factor 6 (TRAF6). This is subsequently ubiquitinated and recognized by the transforming growth factor beta (TGFβ)-activated kinase 1 (TAK1) complex which in turn activates the mitogen-activated protein kinase (MAPK) cascade. K63-linked polyubiquitin also binds to nuclear factor kappa B essential modulator (NEMO), an important regulatory subunit of the inhibitor of kappa B (IκB) kinase (IKK) complex, and this phosphorylates IκB (inhibitor of κ light-chain gene enhance in B cells) which leads to degradation of IκB proteins. In the resting state, IκB proteins prevent dimerization and activation of nuclear factor kappa B (NFkB) family members. Therefore, degradation of IκB releases this repression and allows transcription of cytokines such as tumor necrosis factor alpha (TNF-a), interleukin 6 (IL-6), and chemokines such as C-C motif chemokine ligand 2 (CCL2). TLR4 is also capable of signalling independently of MYD88, through a TIR-domain-containing adaptor inducing interferon-β (TRIF)-dependent pathway, RIP-1-mediated pathway. NFκB is activated by IκB degradation, and RIP-1 activation also leads to p38 MAPK and subsequent AP-1 transcription factor activation. The other key transcription factor activated in this MyD88-independent pathway is interferon regulatory factor 3 (IRF3) which transcribes, among other cytokines, type I IFNα/β.

Innate and Adaptive Immune Cells in NAFLD and NASH

NASH and fibrosis are characterized by the presence of immune cells within the liver lobule. B and T lymphocytes, neutrophils, and liver-resident and monocyte-derived myeloid cells, as well as NKT cells and γδ T cells, have all been shown to play a role in the pathogenesis of NASH. Rag1$^{-/-}$ mice that lack mature B cells, T cells, and NKT cells are protected from choline-deficient high-fat, diet-induced steatosis, hepatocyte ballooning, and liver fibrosis related to secreted TNFSF14 and lymphotoxin. However, in this model, CCR2$^{-/-}$ mice, which have reduced numbers of myeloid cells, were not protected [36].

The pathogenic role of adaptive responses in obesity is seen in high-fat diet-fed MHCII$^{-/-}$ mice which develop less adipose inflammation and insulin resistance compared to wild type. Patients with obesity with or without type 2 diabetes have increased numbers of circulating pro-inflammatory Th1 and Th17 cells, Th1-promoting IFNγ cytokine with a reciprocal reduction in Th2, and regulatory T cells. In keeping with this, mounting evidence points toward the involvement of adaptive immune system in the pathogenesis of NASH. Circulating autoantibodies and increased levels of IgA are associated with liver fibrosis in NASH (although these may reflect a priori diagnostic uncertainty and hence recourse to liver biopsy). The inflammatory infiltrate in NASH contains B and T lymphocytes, and the size and number of B- and T-lymphocyte aggregates are correlated with the degree of inflammation and fibrosis [37].

T Helper Cells

Murine and human data indicate a role for CD4+ T cells in steatohepatitis. The lineage-specific T-cell protein tyrosine phosphatase deletion mouse (*AlbCre;Ptpn2$^{fl/fl}$*) is unable to dephosphorylate signal transducer and activator of transcription (STAT)1 and STAT3 in hepatocytes. When fed a high-fat diet, *AlbCre;Ptpn2$^{fl/fl}$* mice develop hepatic steatosis, ballooning hepatocytes, fibrosis, and lymphocyte infiltration in a STAT1 and CXCL9 but not STAT3-mediated manner. The dominant cell types identified were effector memory (CD44^{hi}CD62L^{lo}) and activated (CD25^{hi}CD69^{hi}) CD4$^+$ T cells and cytotoxic (IFNγ^{hi}TNFα^{hi}) CD8$^+$ T cells. However, this study did not show differences in NK or NKT cells, monocytes, macrophages myeloid-derived suppressor cells Th17 cells, or immunosuppressive T cells. This is at variance to other, including human, studies that have shown a role for each of these cell types in NASH [36].

Th1 cell numbers in visceral and subcutaneous adipose tissue are higher in high-fat diet compared to control diet-fed mice. Impairing Th1 cell function in IFNγ or Tbet knockout mice attenuates adipose inflammation and glucose tolerance [38, 39]. The precise role for Th1 cells in metabolic disease remains to be fully elucidated with Th1 involvement in patients with type 2 DM but not obese patients without type 2 DM. In human NAFLD, Th1 cells were increased in the peripheral blood of 81 patients with NAFL and NASH compared to controls [40]. Although greater levels of IFNγ RNA expression in CD4$^+$ T cells were observed in $n = 51$ patients with biopsy-proven NASH [40], there were no differences in Th1 cell numbers between NAFL and NASH in this study. However, other studies have shown an immunological distinction between NAFL and NASH. Numbers of naïve IFNγ-expressing CD4$^+$ T cells were increased in the peripheral blood of adults and children with NASH [41, 42], and genes associated with Th1 differentiation were upregulated in in $n = 6$ patients with NASH compared to $n = 6$ with simple steatosis [41].

Th2 cells have an anti-inflammatory effect in obesity and metabolic disease. In high-fat diet-fed mice, Th2 cell numbers are reduced in visceral adipose tissue, and adoptive transfer of CD4+ T cells from wild-type mice but not STAT6-deficient mice (that lack Th2 cells) into high-fat diet-fed RAG knockout mice that are deficient in lymphocytes led to a reduction in body weight and insulin resistance [43]. In humans with NAFLD, Th2 cells are increased in peripheral blood, and in particular, there is an increase in the ratio of Th2: Treg cells which reverses 12 months after bariatric surgery. Beyond these studies, little is known of the role of Th2 cells in NAFLD.

Th17 Cells

Pro-inflammatory Th17 cells are characterized by expression of retinoic acid-related orphan receptor γt and production of IL-17, IL-22, and IL-23. In mice, data describing the role of Th17 cells in hepatic steatosis and steatohepatitis are somewhat conflicting. There is no significant change in IL17+CD4+ T cells in the high-fat diet-fed *AlbCre;Ptpn2$^{fl/fl}$* mice with steatohepatitis. On the other hand, methionine-choline-deficient diet-fed IL17$^{-/-}$ mice have lower levels of activated JNK1 and JNK2 and milder steatohepatitis compared to wild-type mice, suggesting a role for the cytokine in liver injury [44]. There are increased numbers of Th22 cells in the livers of protected IL17$^{-/-}$ mouse, and the abundance of Th22 and Th17 mirrors each other as they fluctuate during the course of the disease. In patients with NASH, RORγt, IL-21, IL-23, and IL-17 expressions are increased in liver tissue compared to controls [45]. Numbers of IL17$^+$CD4$^+$ cells in the liver are increased in NASH compared to steatosis patients, and in the peripheral blood, the ratio of Th17/Treg cells was increased in patients with NASH, which normalized 1 year after bariatric surgery [40]. Production of

short-chain fatty acids such as acetate, propionate, and butyrate from carbohydrate fermentation in the gut is increased in patients with NAFLD [45]. Increased fecal propionate and acetate are associated with lower numbers of Treg and higher Th17:Treg ratio in patients with NAFLD.

CD8 Cytotoxic T Cells

CD8+ cytotoxic T cells infiltrate visceral adipose tissue and are a major part of the type I immune response in obesity. CD8 T cells precede and are required for adipose infiltration by macrophages via the production of chemokines [46]. CD8+ T-cell-derived inflammatory cytokines inhibit insulin signalling mediators, compounding insulin resistance, and recruit further Th1 cells and $CD8^+$ T cells. A potential pathogenic role for CD8+ cytotoxic T cells in NASH is indicated by studying mice that lack CD8+ T cells – either through germline deletion of B2 macroglobulin [36] or by intraperitoneal injection with anti-CD8 antibodies [47] that deplete this cell type. When such mice are fed with a choline-deficient high-fat or high-fat high-carbohydrate diet, respectively, they are protected from the development of NASH compared to WT mice. A synergistic role of NKT cells (see below) was also implicated with additional protection observed in $CD1d^{-/-}$ mice. Studies in human tissue have also reported increased numbers of CD8+ T cells, particularly in portal inflammatory infiltrates [48] with numbers of portal CD8+ T cells per high-power field correlating with the NAS score [48]. In a study of $n = 9$ patients with NASH, CD8 staining positively correlated with a smooth muscle actin, a marker of activated hepatic stellate cells, but they did not observe any differences in CD8 staining between patients with NASH and those with steatosis or controls [49].

Innate T Cells

Innate T cells include natural killer T cells, gd T cells, and mucosa-associated invariant T cells. NKT cells are a subset of lymphocytes that are characterized by the co-expression of the TCR and NK cell surface markers. In response to lipid antigens presented by CD1d-expressing cells, NKT cells secrete a wide repertoire of cytokines recognizing that it can drive Th1, Th2, and Treg responses. Data on the role of NKT cells in adiposity and NAFLD in both murine and human studies are conflicting with studies supporting both increases and decreases in NKT cell numbers.

γδ T cells may represent another source of IL-17 in NASH, in addition to Th17 cells, described above. γδT cells are innate T cells that comprise 3–5% of intrahepatic lymphocytes. They do not require MHC-dependent peptide presentation to be activated and can be activated by non-peptide ligands such as phosphoantigens from bacteria to produce IL-17 and IFN-γ. Increased numbers of IL-17-producing γδT cells, but not Th17 cells, have been reported in adipose and hepatic tissue of high-fat diet-fed mice. Moreover, markers of liver injury and insulin resistance were reduced in $tcrd^{-/-}$ mice that lack γδT cells. Similar results were obtained when mice were treated with antibiotics to deplete commensal bacteria and could be reversed by administration of exogenous IL-17, suggesting a role for this axis in metabolic dysfunction and fatty liver [50]. Intrahepatic IL-17-producing γ4+, PD1+, and Ly6C+ CD44+ γδ T cells are increased and present in greater numbers in methionine-choline-deficient diet-fed mice with recruitment mediated by NOD2, CCR2, and CCR5 [51].

Mucosal associated invariant T (MAIT) cells are a group of innate T lymphocytes characterized by the presence of CD161 and a semi-invariant T-cell receptor composed of an invariant Va7.2Ja33 chain and predominantly Vβ6 and Vβ20 chains. MAIT cells are activated through this TCR by cells presenting bacteria-derived vitamin B metabolites on the MHC-like molecule MR1 [52]. Activated MAIT cells release pro-inflammatory cytokines TNFα, IFNγ, IL-17, and cytotoxins granzyme B and perforin [53, 54]. Large numbers of MAIT cells are found in the liver where they comprise up to 45% of intrahepatic lymphocytes. They are predominantly located within portal tracts reflecting their role in detecting bacteria-derived metabolites [55], although they are also present in peripheral blood and gastrointestinal mucosa, albeit at lower levels [53].

MAIT cell numbers are reduced in patients with type 2 diabetes and obesity, although the residual cells exhibit markers of activation such as CD25 and upregulated IL-17, IFNγ, and granzyme B compared to controls [56]. In NAFLD, peripheral MAIT cell numbers are also reduced compared to controls, but unlike data from patients with diabetes and obesity, markers of inflammation IFNγ and TNFα are decreased following stimulation while the frequency of IL-4-producing MAIT cells was increased. Conversely, intrahepatic MAIT cell numbers increase and correlate with NAS score [57], but these cells are able to induce a Th2 cytokine profile. MAIT cell-deficient mice ($MR1^{-/-}$) developed greater hepatic steatosis and inflammation that WT, further suggesting, in this study at least, that MAIT cells can regulate the immune response in NAFLD.

B Cells and Humoral Immunity

The inflammatory infiltrate in NASH also comprises B lymphocytes [58], and selected depletion of the B2 subset of B cells (which require T-cell help to proliferate and undergo class switching) results in mild NASH and less fibrosis [37]. The role of B cells in fibrosis is believed to relate to the acti-

vation of hepatic stellate cells by B-cell-derived inflammatory mediators and the reciprocal production of retinoic acid by stellate cells that promote B-cell maturation into plasma cells. As described above, increased levels of serum IgA have been described in NASH, but the origin and antigen specificity of these IgA molecules remain to be determined. One hypothesis is that oxidized phospholipids and reactive aldehydes such as malondialdehyde form antigenic adducts called oxidized stress epitopes (OSE). Anti-OSE antibodies can be detected in approximately 40% of patients with NAFLD or NASH [59], particularly targeting malondialdehyde-acetaldehyde adducts. Antibodies to such adducts are detected in mouse models of NASH and accompany the maturation of B cells. Intriguingly, preimmunization with malondialdehyde-acetaldehyde adducts leads to enhanced lymphocyte infiltration into the liver and more severe NASH-like liver injury in methionine-choline-deficient diet-fed mice [60]. As above, these can act as DAMPs and trigger innate immune responses.

Myeloid Cells

Large numbers of myeloid cells reside in and patrol the liver. Kupffer cells are tissue-resident macrophages and are responsible for homeostasis in the liver, clearing gut-derived injurious agents and inducing a degree of tolerance in other leukocyte populations. Blood monocytes patrol the hepatic vasculature and can leave the circulation to differentiate into blood-derived macrophages and dendritic cells. Recent single-cell techniques have shown 14 subtypes of myeloid cells in advanced liver disease, including NASH [61], highlighting the rather simplistic dichotomous classification of M1 pro-inflammatory and M2 anti-inflammatory phenotypes currently described. Nevertheless, this binary classification has been helpful in identifying an M1 profile in liver injury in NASH and in insulin resistance and visceral metabolic inflammation. In patients with NASH, expanded populations of CD11c+ CD206+ CCR2+ macrophages have been reported in adipose tissue [62], and the degree of hepatic histological injury correlates with activation of these pro-inflammatory pathways, leading to the interest in chemokine receptors as therapeutic targets in NASH. In Kupffer cells, inflammatory responses such as IL-6-induced and STAT3 activation and insulin resistance are controlled by the nuclear receptor PPAR-δ described above [63], and PPAR-γ can control the alternative (anti-inflammatory) activation of liver-resident macrophages [64].

Dendritic cells (DCs) play an important role in orchestrating adaptive immune responses, and expansion of lipid-containing myeloid DCs is associated with NASH. The expanded DCs are pro-inflammatory and express the fractalkine receptor CX3CR1 and monocyte markers, suggesting that these are monocyte-derived. A major role for these DCs is to present antigen to and activate T cells. OX40 is a co-stimulatory molecule in this activation and is upregulated, along with its ligand OX40L, in liver tissue taken from mouse models of NASH. OX40 deficiency reduces the total number of T cells in the liver, differentiation toward Th1 and Th17, serum transaminases, and the abundance of M1 macrophages, suggesting that this molecule and adaptive immune responses can influence the pathogenesis of NASH [65].

Therapeutic Implications

There are currently no drugs licensed for the treatment of NASH with or without fibrosis. Early randomized controlled trials of behavioral and pharmacological interventions have shown that it is possible to reverse NASH and fibrosis. However, even in the small numbers of patients who achieve weight loss more than 10% of body weight, or in the trials of drugs that affect weight and insulin resistance (such as liraglutide), disease reversal is not universal [66, 67]. Over 30 drugs with varied mechanisms of action are currently in phase I–III clinical trials in NASH, but there is no clear mechanism of action, candidate target, or drug that is leading the field. It is interesting that among the strategies being employed or explored are drugs that target aspects of the innate as well as adaptive immune response in NASH. Cenicriviroc is an inhibitor of CCR2 and CCR5 that results in reduced trafficking of immune, mostly myeloid, cells to the liver. The role of nuclear receptors and their ligands including bile acids has become a major focus for drug development, not just because of the effects on metabolism but also the effect of the PPARs and bile acids/FXR pathways on innate and adaptive immune cell function [68]. Trials to determine the safety and efficacy in NASH of drugs that target nuclear receptors either singly (e.g., pioglitazone, resmetirom, or obeticholic acid) or in combination (e.g., elafibranor or lanifibranor) are currently underway. Data from these trials will be informative as to the role of immunity in NASH, but further data are needed to understand the mechanisms by which immunity contributes to NAFLD spectrum and how targeted intervention may benefit patients.

Conclusion

Our current understanding of the NAFLD spectrum highlights hepatic steatosis, lipotoxicity, inflammation, and fibrosis as hallmarks of NASH and progressive disease. In patients with NAFL, there is, by definition, no evidence of inflammatory or immune cells in liver biopsy specimens. Although patients with NAFL follow a benign hepatological course, with low risk of developing significant liver outcomes, many patients with NAFL also have features of the metabolic syn-

drome including obesity and diabetes. Emerging evidence indicates that metabolic syndrome is an inflammatory-immune state, and therefore despite the absence of immune activity in the liver, the immune response is a feature in all patients with NAFLD spectrum. Multiple potential sources of inflammation and immune activation have been suggested, including dying adipose and hepatic cells, oxidative stress, and immunogenic epitomes associated with this and the gut microbiome, its component macromolecules, and metabolites. Inflammatory cytokines produce in the adipose tissue and liver can impact insulin sensitivity, worsening metabolic function, while innate immune cells, particularly myeloid cells, are key determinants of NASH resolution or progression to fibrosis. The role of T cells, in particular Th1 and Th17 cells, has emerged in recent years, raising the possibility of an adaptive immune response in NASH. Innate T cells, such as NKT, γδ T cells, and MAIT cells, are also likely to contribute.

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Primary Biliary Cholangitis

22

Atsushi Tanaka, Patrick S. C. Leung, Christopher L. Bowlus, and M. Eric Gershwin

Abbreviations

2-OADC	2-Oxo-acid dehydrogenase complex
AIH	Autoimmune hepatitis
ALP	Alkaline phosphatase
AMA's	Antimitochondrial antibodies
ANA	Antinuclear antibodies
APASL	Asian Pacific Association for the Study of Liver
BCOADC-E2	Branched chain 2-oxoacid dehydrogenase complex E2 subunit
BECs	Biliary epithelial cells
CA	Cholangitis activity
CNSDC	Chronic nonsuppurartive destructive cholangitis
E3BP	Dihydrolipoamide dehydrogenase-binding protein
EASL	European Association for the Study of the Liver
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FXR	Farnesoid X receptor
GWAS	Genome-wide association study
HA	Hepatitis activity
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
IBAT	Ileal bile acid transporter
IFN-γ	Interferon γ
LA	Lipoic acid
LPA	Lysophosphatidic acid
LT	Liver transplantation
MHC	Major histocompatibility complex
OCA	Obeticholic acid
OGDC-E2	2-Oxo-glutamic acid dehydrogenase complex
PBC	Primary biliary cirrhosis
PDC-E2	Pyruvate dehydrogenase complex E2 subunit
POISE	PBC OCA international study of efficacy
RANKL	Receptor activator of nuclear factor-kappa B ligand
RR	Relative risk ratio
UDCA	Ursodeoxycholic acid

Key Points

- PBC is a multifactorial and enigmatic disease, and a combination of genetic predisposition and environmental triggering factors plays a crucial role in tolerance breakdown.
- The progression of PBC pathogenesis is considered to occur in three steps: autoimmune reactions against biliary epithelial cells, intrahepatic cholestasis, and progression of fibrosis.
- Epidemiological studies indicate an increasing trend in the prevalence of PBC over time. Female predominance is still clearly observed today; however, it is less pronounced.
- A diagnosis of PBC is made when two or three following items are met: (1) consistent elevation of cholestatic enzymes, (2) detection of AMA, and (3) typical liver histology.

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- UDCA improves serum biochemical abnormalities, delays the histological progression and development of varices, and prolongs transplant-free survival, even in those with incomplete response.
- Fatigue and pruritus are dominant clinical symptoms in PBC and can significantly deteriorate the quality of life.
- Stratification of the risk for progression at diagnosis and at 12 months of UDCA treatment is important to improve long-term outcome.

Introduction

Primary biliary cholangitis (PBC), formally named primary biliary cirrhosis until 2016, is a chronic cholestatic liver disease that can potentially progress to cirrhosis and liver failure. PBC mainly develops in middle-aged women, but it can also occur in young women and men. Although the etiology of PBC has not been fully elucidated, robust evidence indicates that autoimmune reactions targeted to intrahepatic biliary epithelial cells (BECs) play a critical role in the pathogenesis of the disease. Indeed, PBC is considered a model autoimmune disease because of the presence of disease-specific antimitochondrial autoantibodies (AMAs), an intense infiltration of mononuclear cells damaging bile ducts, and a high prevalence of coincident autoimmune. As with other autoimmune diseases, a genetic predisposition and environmental triggers have been implicated in the development of PBC.

Patients with PBC frequently develop various symptoms including fatigue, pruritus, dryness of the eyes and mouth, and abdominal pain, but the disease is incidentally detected in a substantial portion of asymptomatic patients with cholestatic liver enzyme elevations. Histologically, PBC is characterized as degeneration and necrosis of intrahepatic BECs surrounded by a dense infiltration of mononuclear cells, coined as chronic nonsuppurative destructive cholangitis (CNSDC), which leads to destructive changes and disappearance of small- or medium-sized bile ducts. Other autoimmune diseases frequently coexist, such as chronic thyroiditis, Sjögren's syndrome, and rheumatoid arthritis.

Until 1990, the long-term outcome of PBC was very poor, with patients inevitably developing complications of cirrhosis including jaundice, ascites, and esophagogastric varices, resulting in liver transplantation (LT) or liver-related death. Earlier diagnosis with detection of AMAs and introduction of ursodeoxycholic acid (UDCA) as first-line treatment has greatly altered the clinical course of PBC, and LT-free survival of patients with PBC is now comparable to that of the general population. For those with an incomplete response to UDCA, which comprises 30–40% of PBC patients, several drugs have been approved or are anticipated to be approved as second-line treatment. Nevertheless, LT is the only option for survival in patients with decompensated cirrhosis. The history, pathogenesis, epidemiology, diagnosis, treatment, management of symptoms, disorders, and risk of progression of PBC are discussed in this chapter.

Historical Background

In 1851, the first patient presenting with symptoms resembling PBC was described in the literature [1]. The term "primary biliary cirrhosis" initially appeared in the title of an article published in 1949 [2] by Dauphinee et al., which referred to patients with "marked enlargement of the liver and spleen, and the occurrence of a persistent jaundice." Most early descriptions of PBC involved patients at the cirrhotic stage, with jaundice, ascites, and variceal bleeding; therefore, the nomenclature "primary biliary cirrhosis" was correct at that time. However, Sherlock had already noted in 1959 that this terminology should be changed because many patients were free of cirrhosis [3]. In 1965, Hans Popper and colleagues also suggested that the term "primary biliary cirrhosis" was a misnomer as neither septa nor nodules are present, and they suggested the use of "chronic nonsuppurative destructive cholangitis" instead [4], which is still used as a description of the typical histopathological finding of PBC.

The use of biochemical and immunological tests in the clinical settings has enabled the diagnosis of PBC at earlier stages. Further, the establishment of UDCA as a first-line treatment drug remarkably reduced disease progression to cirrhosis. The serious gap between the disease manifestation and its misnomer became wider, and the term "cirrhosis" became not merely an inaccuracy but an active stigma for patients. In 2014, during the second European Association for the Study of the Liver (EASL) monothematic conference on primary biliary cirrhosis, experts gathering from different parts of the world agreed that (i) the name "primary biliary cirrhosis" should be changed and that (ii) the alternative should be "primary biliary cholangitis," keeping the acronym "PBC." The EASL and American Association for the Study of Liver Diseases governing boards approved this agreement in 2014 and 2015, respectively [5–12]. The Asian Pacific Association for the Study of Liver (APASL) also officially approved this decision, and the new nomenclature "primary biliary cholangitis" is currently used in the official journal of the APASL [13].

Pathogenesis

PBC is a multifactorial and enigmatic disease; it remains unknown how and why PBC develops. PBC is a cholestatic disease with an autoimmune etiology that leads to intensive

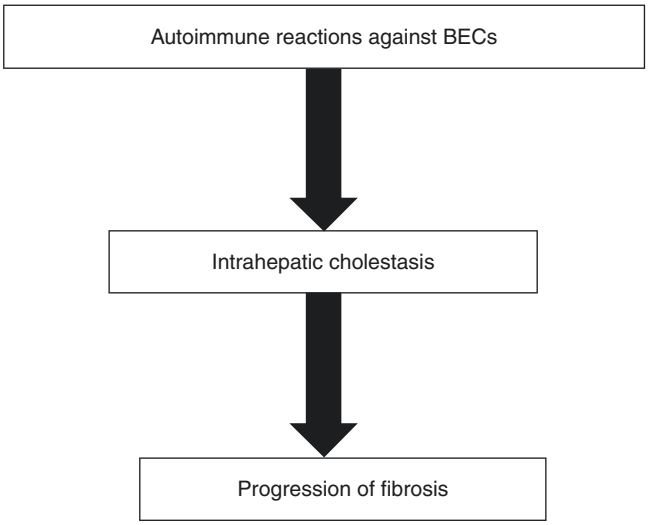


Fig. 22.1 (**a**–**c**) Three steps in the pathogenesis of primary biliary cholangitis. BECs biliary epithelial cells

fibrosis and cirrhosis. The progression of PBC pathogenesis is considered to occur in three steps (Fig. 22.1). Autoimmune attack targeted at BECs through tolerance breakdown triggers the disease onset. Accumulating evidence also suggests that a combination of genetic predisposition and environmental triggering factors plays a crucial role in tolerance breakdown.

Autoimmune Responses Against Mitochondrial Antigens

AMAs, detected in 90–95% of patients with PBC [14, 15], are the most disease-specific autoantibodies in human immunopathology. The high specificity of AMAs for PBC suggests that AMAs are not simply serological markers for diagnosis but are important in the immunopathology of PBC. In addition, the histological signature of PBC includes dense infiltration of mononuclear cells in the portal tracts near small-sized or medium-sized bile ducts. Immunohistochemical examination of these lymphocytes reveals a predominance of CD4+ and CD8+ T cells with B cells and natural killer (NK) cells [16, 17]. A major finding in PBC was the molecular identification of mitochondrial autoantigens and their B-cell and T-cell autoepitopes.

AMAs recognize a family of enzymes located in the inner membrane of the mitochondria, named the 2-oxo-acid dehydrogenase complex (2-OADC), which mainly includes the pyruvate dehydrogenase complex E2 subunit (PDC-E2), the branched-chain 2-OADC E2 subunit (BCOADC-E2), the 2-oxo-glutaric acid dehydrogenase complex E2 subunit (OGDC-E2), and the dihydrolipoamide dehydrogenase-binding protein (E3BP) [18]. Among them, PDC-E2 and

Table 22.1 AMAs and ANAs and their corresponding frequencies

AMA targets	
PDC-E2	95%
BCOADC-E2	53–55%
OGDC-E2	39–88%
PDC-E1α	41–66%
E3BP	95%
ANA targets	
Sp100	9–27%
gp210	16–26%

AMA antimitochondrial autoantibody, *ANA* antinuclear autoantibody, *2-OADC* 2-oxo-acid dehydrogenase complex, *PDC-E1α* pyruvate dehydrogenase complex E1α subunit, *BCOADC-E2* branched-chain 2-OADC E2 subunit, *OGDC-E2* 2-oxo-glutaric acid dehydrogenase complex E2 subunit, *E3BP* dihydrolipoamide dehydrogenase binding protein

E3BP are the most frequently detected autoantigens (Table 22.1). All these E2 enzymes have a common structure consisting of an N-terminal domain with a single attachment site or multiple attachment sites to a lysine (^{173}K in mammalian PDC-E2) of lipoic acid (LA) (Table 22.2). The dominant epitope sites recognized by AMAs are in contiguity with the LA attachment site(s) at the lipoyl domains of these target antigens [19–21]. The amino acid residues critical to maintaining the structural integrity of AMA epitope of the PDC-E2 lipoyl domain have been revealed by site-directed mutagenesis [22]. Furthermore, the immunodominant CD4+ T-cell epitopes of PDC-E2 peptide 163–176 (GDLLAEIETDKATI) also overlaps with the B-cell epitope of human PDC-E2 (see Table 22.2) [23]. Importantly, the frequency of PDC-E2-specific CD4+ T cells was 100- to 150-fold higher in the liver and hilar lymph nodes than in peripheral blood [24]. The CD8+ T-cell epitopes were also characterized as PDC-E2 peptide 159–167 (KLSEGDLLA), which again was mapped to the same region of PDC-E2 (see Table 22.2) [25]. Taken together, AMAs and autoreactive helper and cytotoxic T-cells epitopes are confined within a shared peptide sequence of the inner lipoyl domain of human PDC-E2.

Interaction of BECs and Autoimmunity

BECs and hepatocytes of patients with PBC express large amounts of human leukocyte antigen (HLA) classes I and II molecules [26, 27]. In patients with PBC, BECs act as non-professional antigen-presenting cells, and the interplay of BECs and T cells may to some extent account for bile duct loss. Indeed, BECs express adhesion molecules, cytokines, and chemokines and recruit mononuclear cells in the biliary tract of the liver. One example is fractalkine (CX3CL1), a chemokine with both chemoattractant and cell-adhesive functions [28]. Th1-cytokine predominance and lipopolysac-

Table 22.2 Molecular mimicry and immunodominant epitopes of human PDC-E2 155-185[a]

Human PDC-E2	KVGEKLSEGDLLAEIETD**K**ATIGFEVQEEGY
B cell	KVGEKLSEGDLLAEIETD**K**ATIGFEVQEEGY
CD4+ T cell	KVGEKLSEGDLLAEIETD**K**ATIGFEVQEEGY
CD8+ T cell	KVGEKLSEGDLLAEIETD**K**ATIGFEVQEEGY
E. coli PDC-E2[b]	---D-VEAEQS–ITV-G---SMEVPSPQA-I

E. coli Escherichia coli
[a]**K** denotes [173]lysine, which is the attachment site of lipoic acid
[b]Identical amino acids to human PDC-E2 are denoted as "-"

charide in the microenvironment of injured bile ducts induce the upregulation of fractalkine expression in BECs, followed by the chemoattraction of mononuclear cells expressing its receptor (CX3CR1), including CD4(+) and CD8(+) T cells [29, 30]. Serum fractalkine levels in PBC are high in patients with marked cholangitis activity (CA) at early stages, and they decreased in response to treatment [31].

PDC-E2 is a ubiquitous protein located in nearly all nucleated cells in the human body, and it remains unclear why autoreactive T cells specific for PDC-E2 elicit cytotoxicity against only BECs in the liver. In this regard, it should be noted that PBC recurs even after LT, indicating that the immunopathological susceptibility of BECs in PBC is not major histocompatibility complex (MHC)-specific but a general feature shared with autologous BECs. The hypothesis to solve this enigma is that human intrahepatic BECs could maintain PDC-E2 immunologically intact within apoptotic blebs (apotopes) during apoptosis [32]. Interestingly, a unique triad that consists of BEC apotopes, macrophages from patients with PBC, and AMAs could lead to rigorous production of inflammatory cytokine production [33].

Genetic Predisposition

Genetic predisposition is believed to be a major contributing factor in the development of PBC [34]. The phenomenon of familial clustering is evidently supported by epidemiological data of increased disease prevalence of PBC among first-degree relatives and siblings of an index patient with PBC [35, 36]. Moreover, the concordance rate of PBC is 63% in monozygotic twins, which is the highest rate among several autoimmune diseases [37].

A recent study in Iceland, which took advantage of the unique local genealogical database, demonstrated that the familial risk of PBC was present not only in first-degree relatives but in first-, second-, and third-degree relatives of patients with PBC, with increased relative risk ratios (RRs) of 9.13 (95% confidence interval, 4.17–16.76), 3.61 (1.48–8.92), and 2.59 (1.35–4.67), respectively [38]. Furthermore, the increased risk of PBC trended toward significance even in fourth- and fifth-degree relatives with RRs of 1.66 (1.00–3.02) and 1.42 (0.99–2.20), respectively. These findings clearly emphasize the importance of genetic risk in the pathogenesis of PBC.

In pre-genome-wide association studies (GWAS) era, case-control studies were the main tool to uncover genetic predisposing factors in human diseases. These studies collectively demonstrated the association of HLA class II alleles with the development of PBC. In particular, the *DRB1*08* allele family, with *DRB1*0801*, *DRB1*0803*, *DRB1*14*, and *DPB1*0301*, were described as susceptible alleles and *DRB1*11* and *DRB1*13* as protective alleles [39–42]. A recent study from Japan identified HLA-DQ alleles, *DQB1*06:04* and *DQB1*03:01*, as disease-protective alleles [43]. A high prevalence of *HLA DRB1**0301–*DQB1**0201 haplotype among patients with PBC in Sardinia was also reported [44]. It has remained unclear how HLA alleles affect the susceptibility of PBC, which is also confounded by the variation of susceptible HLA alleles in different populations. Nevertheless, a recent fine mapping study of the MHC region in Han Chinese, identifying major independent variants different from those in European populations, indicated that the predicted effects in antigen binding are likely to be very similar or even identical among ethnicities [45].

In addition to these HLA alleles, GWAS analyses from North America, European countries, Japan, and China identified other HLA alleles that are strongly associated with susceptibility to PBC and revealed more than 40 non-HLA alleles contributing to PBC susceptibility (Table 22.3) [46–57]. Although risk alleles differ among studies and populations, they primarily belong to genes and pathways involved in antigen presentation and production of interleukin (IL)-12 (*IRF5, SOCS1, TNFAIP3, NF-κB,* and *IL-12A*), activation of T cells, and interferon γ (IFN-γ) production (*TNFSF15, IL12R, TYK2, STAT4, SOCS1, NF-κB*, and *TNFAIP3*), as well as activation of B cells and production of immunoglobulins (*POU2AF1, SPIB, PRKCB, IKZF3,* and *ARID3A*). Logically, these immune pathways could be important in the pathogenesis of PBC.

As for other autoimmune diseases, GWASs were unable to clearly define alleles that are specific in PBC, i.e., most of the identified non-HLA loci were also found to be susceptible genes in other autoimmune diseases. Therefore, these common risk alleles of autoimmunity by itself cannot fully account for the specificity of autoimmune attack that leads to chronic cholangitis and specific BEC destructions exclusively found in PBC. GWAS identified significant differences for methylation profiles, copy number variation, and gene expression in three monozygotic twins and eight sibling pairs discordant for PBC [58]. Moreover, aberrant demethylation on the CXCR3 promoter of the X chromosome was noted in patients with PBC [59]. These findings are still only descriptive, and further studies are needed to elucidate the etiological implications of epigenetics.

Table 22.3 Major gene loci associated with susceptibility of PBC, PSC, and other autoimmune diseases[a]

Chromosome no.	Gene loci	PBC (Europe/ North America)	PBC (Japan/ China)	PSC	RA	IBD	MS	SLE
1	*CD58*		Yes		✔		✔	
1	*MMEL1, TNFRSF14*	Yes		Yes	✔	✔	✔	
1	*IL12RB2*	Yes						
1	*DENND1B*	Yes				✔		
2	*IL1RL2/IL1RL1*	Yes				✔		
2	*STAT4*	Yes	Yes		✔			✔
2	*CD28/CTLA4/ICOS*		Yes	Yes	✔		✔	
2	*CCL20 (LARC)*	Yes		Yes		✔		
2	*BCL2L11*			Yes				
2	*GPR35*			Yes		✔		
3	*PLCL2*	Yes					✔	
3	*CD80*	Yes	Yes				✔	
3	*IL12A, SCHIP1*	Yes	Yes				✔	
3	*FOXP1*			Yes				
3	*MST1*			Yes		✔		
4	*DGK Q*	Yes				✔	✔	
4	*NF-kB1*	Yes	Yes	Yes		✔		
4	*IL21*		Yes	Yes		✔		
5	*IL7R*	Yes	Yes			✔	✔	
5	*PAM/C5orf30*	Yes			✔			
5	*LOC285626/IL12B*	Yes				✔	✔	
6	*TNFAIP3*	Yes			✔	✔	✔	✔
6	*BACH2*			Yes		✔		
7	*ELMO1*	Yes				✔	✔	✔
7	*IRF5*	Yes			✔	✔		✔
9	*TNFSF15*		Yes			✔		
10	*IL2RA*			Yes	✔	✔	✔	
11	*RPS6KA4*	Yes				✔	✔	
11	*CXCR5*	Yes	Yes				✔	✔
11	*POU2AF1*		Yes					
11	*CCDC88B*	Yes		Yes		✔		
11	*SIK2*			Yes				
12	*TNFRSF1A*	Yes	Yes				✔	
12	*SH2B3*	Yes		Yes	✔			
12	*HDAC7*			Yes		✔		
12	*RFX4, RIC8B*			Yes				
13	*TNFSF11 (RANKL)*	Yes				✔		
14	*RAD51L1*	Yes						
14	*TNFAIP2*	Yes						
15	*IL16*		Yes					
16	*IL21R*		Yes					
16	*PRKCB*		Yes					✔
16	*CLEC16A, SOCS1*	Yes		Yes		✔	✔	✔
16	*CSNK2A2, CCDC113*		Yes					
16	*IRF8*	Yes			✔	✔	✔	
17	*IKZF3-ORMDL3*	Yes	Yes		✔	✔		
17	*MAPT, CRHR1*	Yes						
18	*TYK2*	Yes			✔	✔	✔	
18	*ARID3A*		Yes					
18	*SPIB*	Yes						
18	*TCF4*			Yes				
18	*CD226*			Yes	✔	✔		
19	*PRKD2, STRN4*			Yes				
21	*PSMG1*			Yes		✔		

(continued)

Table 22.3 (continued)

Chromosome no.	Gene loci	PBC (Europe/North America)	PBC (Japan/China)	PSC	RA	IBD	MS	SLE
21	*UBASH3A*			Yes	✔			
22	*MAP3K7IP1/RPl3, SYNGR1*	Yes	Yes			✔		

[a]Summary from eight GWAS/iCHIP analyses from European countries and North America [46–50, 52–54] and three GWAS analyses from Japan and China [51, 55, 56] in PBC and eight GWAS analyses from European countries and North America [203–210]. No., number; *PBC* primary biliary cholangitis, *PSC* primary sclerosing cholangitis, *RA* rheumatoid arthritis, *IBD* irritable bowel disease, *MS* multiple sclerosis, *SLE* systemic lupus erythematosus, *RANKL* receptor activator of nuclear factor-kappa B ligand, *GWAS* genome-wide association study

Environmental Triggering Factors

Although a study of monozygotic twins demonstrated a high concordance rate, recent epidemiological studies revealed a relatively low risk of developing PBC in first-degree relatives of the indicated patient during 8 years of follow-up, suggesting that genetic predisposition does not define the risk of PBC [60]. Large-scale case-control studies have consistently found an association of urinary tract infections and cigarette smoking with PBC [61–64]. Bacterial infection may have an impact on the etiology of PBC because PDC-E2, which is an immunodominant target of AMA, has a molecular mimic between human PDC-E2 and *Escherichia coli* PDC-E2 (see Table 22.2), and thus, *E. coli* infection may trigger the breaking of immunological tolerance against human PDC-E2. Another candidate bacterium that may be involved in the etiology of PBC through cross-reactivity is *Novosphingobium aromaticivorans*, a ubiquitous xenobiotic-metabolizing bacterium [65].

A case-control study also suggested that the frequent use of nail polish is associated with an increased susceptibility to PBC [63]. Furthermore, a geographically uneven distribution of patients with PBC in a particular region is reported, especially near toxic waste sites [66–68]. A detailed, quantitative structure-activity relationship analysis with 107 potential xenobiotic mimics coupled to the lysine residue of the immunodominant 15-amino acid peptide of the PDC-E2 inner lipoyl domain revealed that 2-octynamide, the conjugate derived from 2-octynoic acid present in cosmetics, lipsticks, and some chewing gums, was unique in both its quantitative structure-activity relationship analysis and reactivity with PBC sera [69]. Moreover, another xenobiotic, 2-nonyamide, provided an optimal chemical structure of the xenobiotics-modified epitope, which demonstrated enhanced recognition by AMA-positive PBC sera [70]. Remarkable molecular mimicry between lipoamide and 2-nonynamide was observed. These findings illustrate that xenobiotic modification of PDC-E2 with chemicals abundantly found in daily life has a role in generating immunogenic neoantigens and breaking tolerance in PBC. Finally, dysbiosis of the gut microbiota was found in patients with PBC, and interestingly, it was partially resolved with UDCA treatment [71].

Epidemiology

Since both genetic background and environment are involved in the development of PBC, logically the prevalence and incidence of PBC vary considerably worldwide. A systemic review in 2012 and the following epidemiological studies reported that the incidence and point prevalence of PBC ranged from 0.39 to 5.8 per 100,000 populations and from 1.91 to 58.2 per 100,000 populations, respectively [72, 73]. Both the incidence and point prevalence greatly vary depending on the study (Fig. 22.2a, b); this discrepancy can be attributed to the true epidemiological difference between regions or study periods, the variation in study designs for case finding or ascertainment, or the difference in the diagnosis of PBC among physicians. Notably, awareness of PBC may still not be satisfactory in some Asian and African countries where epidemiological studies are scarce, and the sample size in some studies are very low. PBC was believed to be a rare disease in the Asia-Pacific region, and both prevalence and incidence seemed to be lower in the Asia-Pacific region as indicated by recent epidemiological studies in South Korea or Hong Kong [74, 75] (see Fig. 22.2a, b). On the contrary, a 2016 study in Japan reported that point prevalence of PBC was 33.8 per 100,000 in the Japanese population [73], which was comparable to those in European countries, the United States, and Canada.

Other studies also indicated an increasing trend in the prevalence of PBC over time [72] (see Fig. 22.2a). Longitudinal studies in the identical regions consistently showed an increase in prevalence of PBC [73, 76, 77]. Since sequential studies demonstrated the increasing incidence of PBC (1.67 in 2009 and 5.31 in 2015, in Italy [78, 79]) or a relatively stable incidence (2.6 in Sweden [77]), it is unclear whether a true increase or improved overall survival has contributed to the increasing prevalence of PBC.

One of the signatures of PBC is its female preponderance. Retrospective analysis indicated that the female:male ratio was 9:1 [80, 81] in 1990s and early 2000s, and this overt female predisposition has provided researchers with a clue in clarifying the etiology of PBC. Notably, female predominance is still clearly observed today; however, it is less pronounced. As shown in Fig. 22.2c, the female:male ratio was less than 5:1 in most recent epidemiological studies and even 2.1:1 [78]. The reason for the relative increase of male patients with PBC remains unclear, but better recognition and a true increase in the incidence of PBC are likely responsible.

Diagnosis

A diagnosis of PBC is made when two or three of the following items are met: (1) consistent elevation of cholestatic enzymes, (2) detection of AMA, and (3) typical liver histology [82–84]. In Fig. 22.3, a diagnostic flowchart is shown. Because of the very high sensitivity and specificity of AMA in the diagnosis of PBC, detectable AMA and elevation of the alkaline phosphatase (ALP) level are adequate for the diagnosis of PBC, and liver biopsy is not mandatory in many cases. Nevertheless, fibrosis stage at baseline may be an

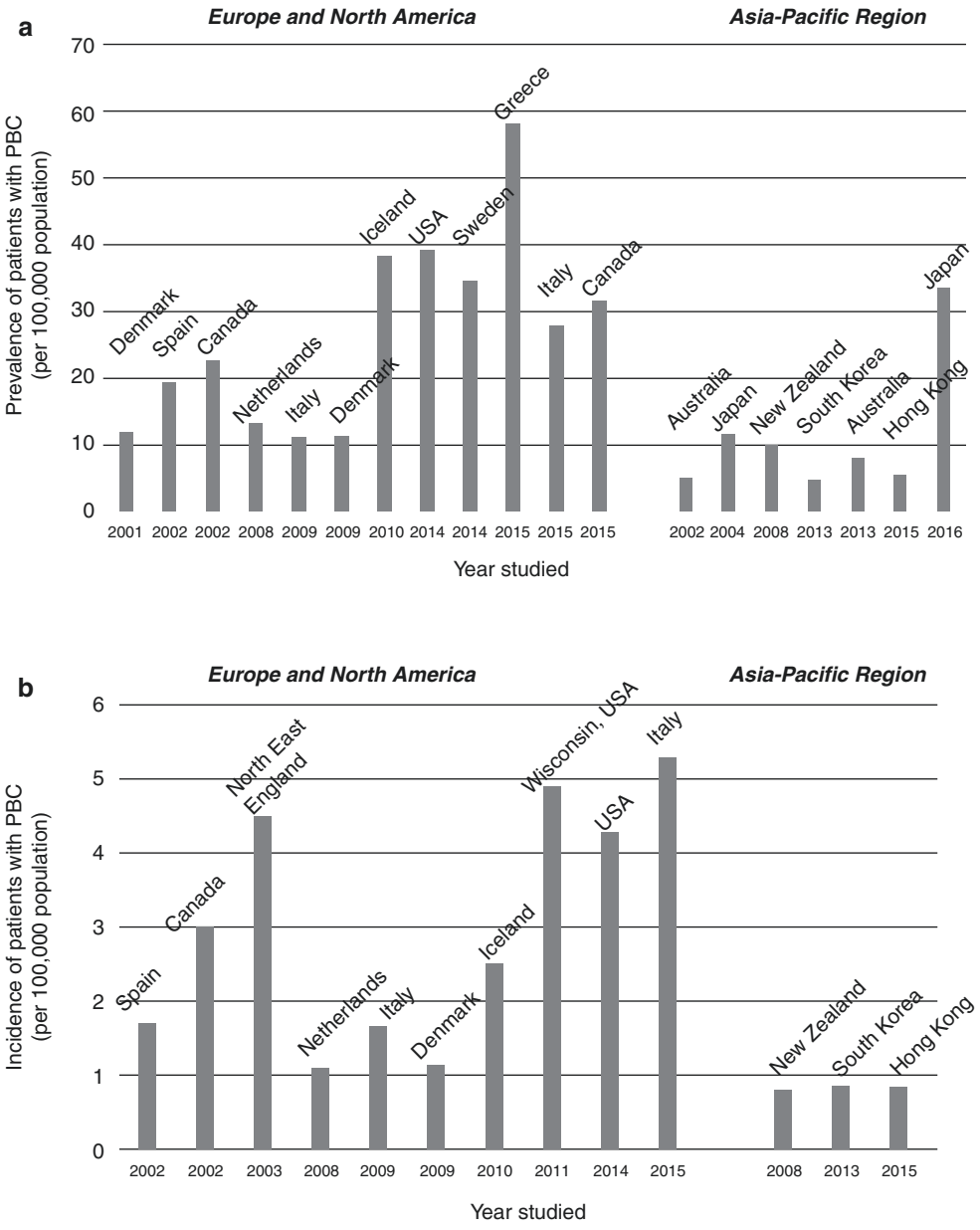
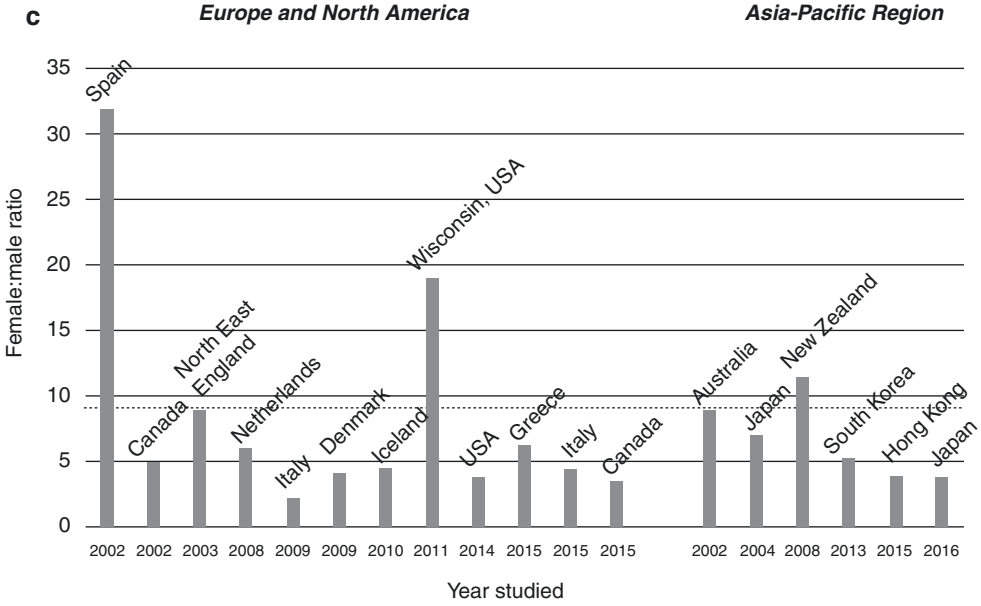


Fig. 22.2 Epidemiological data of PBC over time and in different geographical regions. (**a**) Prevalence (/100,000 population), (**b**) incidence (/100,000 population), and (**c**) female:male ratio of PBC. The dotted line indicates that the female:male ratio is 9, and please note that most recent studies showed that the female:male ratio is less than 9. PBC primary biliary cholangitis, USA United States

Fig. 22.2 (continued)



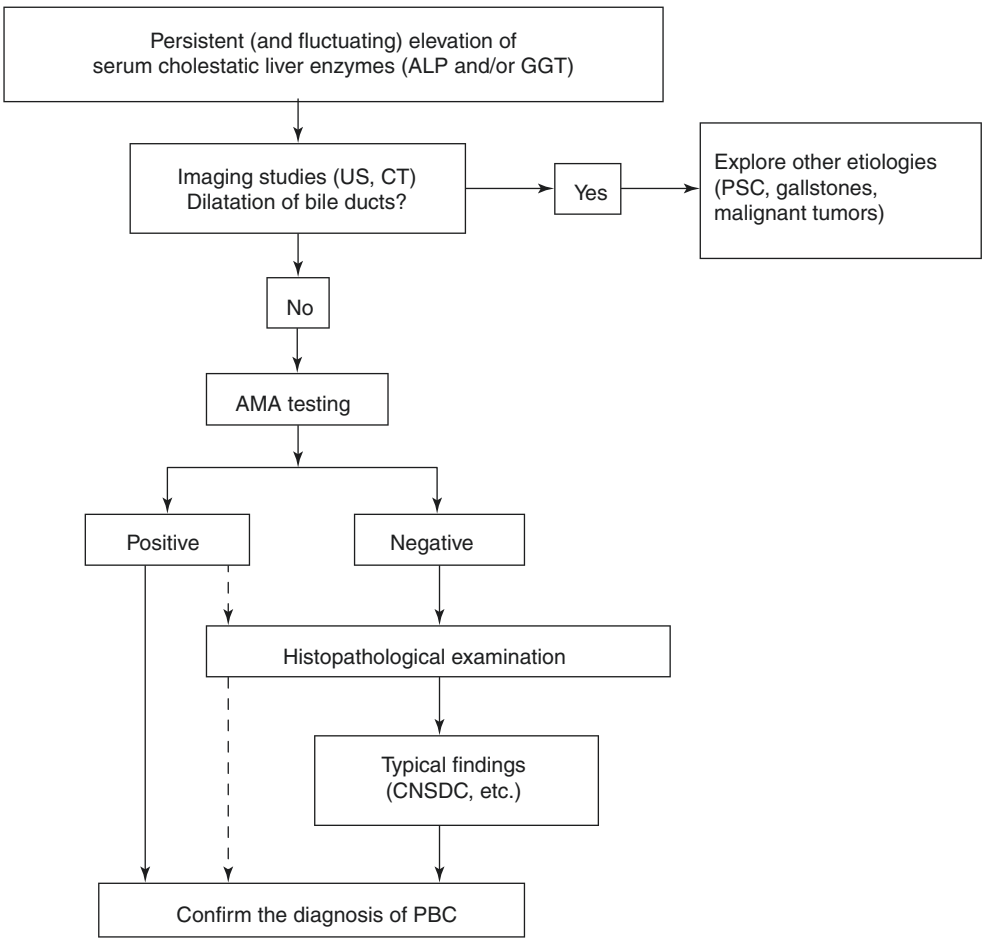


Fig. 22.3 Diagnostic flowchart of patients with PBC. PBC primary biliary cholangitis, ALP alkaline phosphatase, GGT gamma-glutamyl transferase, US ultrasonography, CT computed tomography, PSC primary sclerosing cholangitis, AMA antimitochondrial autoantibody, CNSDC chronic nonsuppurative destructive cholangitis, etc.

independent prognostic marker of survival as demonstrated by a recent large-scale retrospective study [85]. Thus, assessment of fibrosis stage by liver biopsy or noninvasive means, such as vibration-controlled transient elastography, may assist in predicting long-term outcome. A histological examination is required in atypical cases, including suspicious AMA-negative PBC and autoimmune hepatitis (AIH)/PBC overlap.

Liver Enzymes: Consistent Elevation of Cholestatic Enzymes

Patients with PBC complain of various subjective symptoms, such as pruritus, fatigue, dryness of the mouth or eyes, and body pain; yet, none of these symptoms is specific for PBC. Hence, an incidentally abnormal blood chemistry result is the first clue leading to the diagnosis of PBC in most cases. Similar to intrahepatic cholestasis, serum levels of ALP and/or gamma-glutamyl transferase are elevated in PBC. The levels of serum bilirubin can increase in patients with advanced PBC and jaundice, but this is relatively rare. Since PBC is a chronic disease that progresses very insidiously, liver enzymes fluctuate during the natural disease course. Imaging studies, such as abdominal ultrasonography or computed tomography, must be performed to exclude dilatation of intra- and extra-bile ducts, which is not observed in PBC.

Serological Tests: Detection of AMA

AMA is a disease-specific serological marker almost exclusively found in patients with PBC. In the clinical settings, AMA is often determined by enzyme-linked immunosorbent assay (EIA) or indirect immunofluorescence (Fig. 22.4). In EIA, a combination of three recombinant mitochondrial proteins (PDC-E2, BCOADC-E2, and OGDC-E2) or a purified mitochondrial fraction (M2) is used as the antigens. The titer of AMAs is not associated with disease progression or the patient's clinical course. AMAs are occasionally detected in less than 1% of healthy individuals with normal liver test results [86, 87]. Individuals who are AMA-positive are at higher risk for developing PBC and require close follow-up, although the risk does not appear to be high as previously believed. A large-scale cohort study in France demonstrated that the prevalence of AMA-positive patients without evidence of PBC was 16.1 per 100,000 population, and 1 in 6 patients with AMA positivity and a normal ALP level developed PBC within 5 years [88]. Conversely, a recent study from China demonstrated that more than 80% of patients with AMA without elevation of serum ALP levels also developed histological characteristics of PBC, suggesting the presence of undiagnosed PBC patients among those with normal ALP levels and AMA positivity [89]. It remains unclear whether these individuals will progress to advanced disease as in typical PBC and how they should be clinically treated.

Among several antinuclear antibodies (ANA), sp100 and gp210 are frequently found in the sera of patients with PBC (see Table 22.1) and, thus, aid in diagnosing patients with probable PBC but undetectable AMA positivity. A combination of AMA, sp100, and gp210 ("PBC screen") had a sensitivity of 83.8% and specificity of 94.7% for diagnosing PBC and is considered appropriate as the first-line screening test [90]. Molecular mimicry between mitochondrial antigens and sp100/gp210 was reported [91]. Detection of gp210 may be associated with progression of the disease in UDCA-treated patients [92], but this observation needs further validation.

Liver Histology: CNSDC

Histopathologically, PBC is exclusively located in intrahepatic small- or middle-sized bile ducts. Dense infiltration of mononuclear cells around the intrahepatic small bile ducts (interlobular bile ducts), coined as CNSDC, and granuloma formation are characteristic findings; eventually, intrahepatic small bile ducts disappear from the liver, and chronic cholestatic features gradually develop. Hepatitis activity (HA) and chronic CA contribute to progressive hepatocellular damage and fibrosis, resulting in liver cirrhosis and hepatic failure.

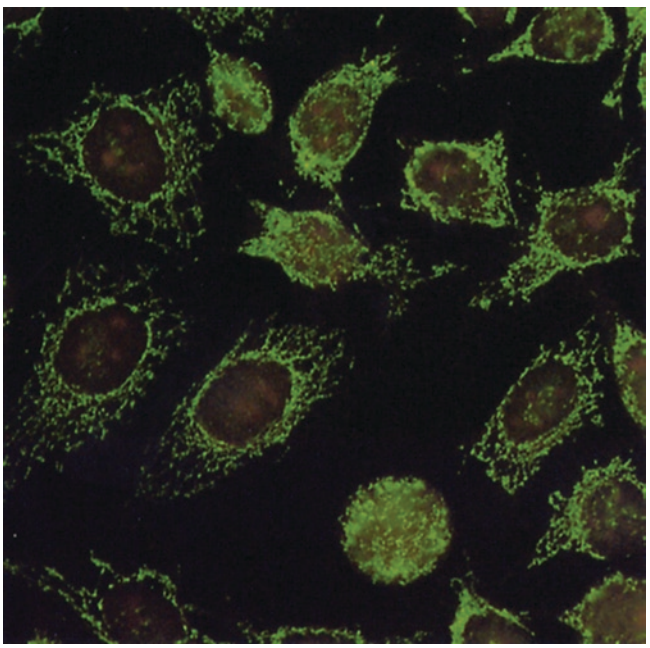


Fig. 22.4 Detection of the antimitochondrial autoantibody using the indirect immunofluorescence method with rat stomach-kidney cells. Note that the reticulated granules are stained in the whole cytoplasm

Table 22.4 Classic histopathological staging system of PBC

a. *Scheuer's classification* [93]
1. Florid duct lesion
2. Ductular proliferation
3. Scarring
4. Nodular cirrhosis
b. *Ludwig's classification* [94]
1. Portal hepatitis
2. Periportal hepatitis
3. Septal (bridging) fibrosis
4. Biliary cirrhosis

PBC primary biliary cholangitis

The Scheuer's [93] or Ludwig's classification [94] was developed as the classification system for staging of PBC pathology (Table 22.4). In the Scheuer's classification, florid duct lesions (CNSDC, Fig. 22.5a), ductular proliferation, scarring, and nodular cirrhosis are representative findings of stages 1, 2, 3, and 4, respectively. However, as described in the original report by Scheuer, there is considerable overlap of findings between these stages; CNSDC can be observed even in the liver with nodular cirrhosis. Additionally, the pathology of PBC is not always distributed evenly in the liver; hence, sampling error can occur when determining the stages with these systems.

In order to overcome these limitations, Nakanuma et al. proposed a new histological staging and grading system for PBC (Table 22.5) [95]. In Nakanuma's classification, the scores for fibrosis (Fig. 22.5b), bile duct loss (Fig. 22.5c), and deposition of orcein-positive granules (Fig. 22.5d) are used for staging (Table 22.5a), whereas CA and HA are used for grading (Table 22.5b). CA is determined by the presence of chronic cholangitis (Fig. 22.5e) or CNSDC, and HA is defined by the presence of interface hepatitis (Fig. 22.5c) or lobular hepatitis (Fig. 22.5f). Overall survival was stratified better with Nakanuma's classification than with the classic system [96].

Atypical Cases

AMA-Negative PBC

Serum AMAs are undetectable in approximately 5% of patients with PBC, i.e., "AMA-negative PBC." Although this category of PBC was formally named "autoimmune cholangitis" and considered as a distinct phenotype with different clinical features, the current presentation, histopathology, natural course, and treatment response of AMA-negative PBC are similar to those of AMA-positive PBC. AMAs are detectable even in most patients with AMA-negative PBC with a highly sensitive method such as the bead assay [15], and T-cell responses against mitochondrial antigens are evident in AMA-negative PBC [97].

PBC with AIH Features (PBC/AIH Overlap)

Although PBC typically presents as an elevation of cholestatic liver enzymes and detectable AMA, variant forms of PBC lacking one or more typical characteristics are occasionally encountered. Particularly, some patients simultaneously or consecutively present with features of AIH (i.e., elevation of transaminases, serum immunoglobulin G levels, and positive ANAs). This variant type is alternatively referred to as PBC/AIH overlap. It should be noted that this atypical disorder is neither a single clinical entity nor a combination of PBC and AIH but rather a variant form of classic PBC [98]. The Paris criteria [99] (Table 22.6) is most commonly used to define this variant of PBC, and patients who meet these criteria benefit from corticosteroid treatment in addition to UDCA [82]. Although patients with PBC may occasionally have ANA positivity (except for sp100 or gp210) or mild elevation of transaminases, such patients should be considered to have classic PBC and not the "overlap" variant.

Treatment

UDCA

UDCA is a naturally occurring hydrophilic bile acid that, when orally administered, becomes the dominant bile acid in the enterohepatic circulation, exerting a protective effect on bile duct cells and hepatocytes through its choleretic and bicarbonate-secreting effects [100]. Since the first report demonstrating its efficacy for PBC [101], UDCA has dramatically altered the natural course of PBC and has been approved as a first-line therapy for PBC worldwide [82, 84, 102]. UDCA is used at a dose of 13 to 15 mg/kg/day and is recommended for all patients with PBC with elevated liver biochemistry levels.

UDCA improves serum biochemical abnormalities, delays the histological progression and development of varices, and prolongs transplant-free survival [103–112]. Studies indicate that PBC patients who completely respond to UDCA treatment have a comparable survival as in the general population [113–117]. Interestingly, a retrospective study on a large cohort demonstrated that the LT-free survival of UDCA-treated PBC patients was significantly improved compared to those that received no treatment and also in a population with incomplete biochemical response to UDCA [118]. Although the safety profile of UDCA is generally excellent, side effects such as abdominal fullness, diarrhea, and constipation may infrequently occur, and a small fraction of PBC patients are intolerant to UDCA. As the discontinuation of UDCA frequently leads to elevation of serum liver enzymes, treatment with UDCA should be continued throughout the patient's life.

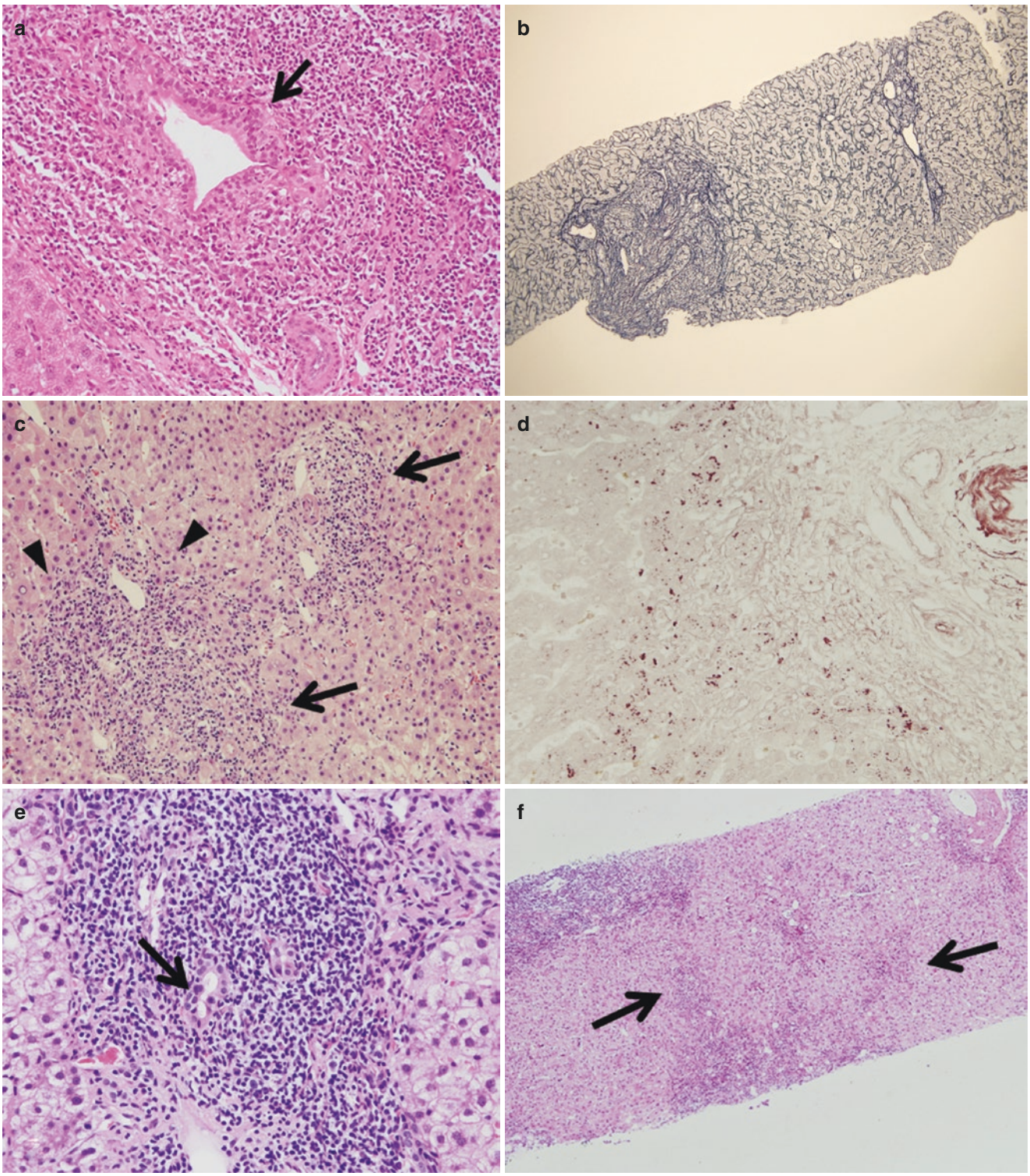


Fig. 22.5 Liver histology of PBC. (**a**) Chronic nonsuppurative destructive cholangitis (arrow, hematoxylin and eosin staining), (**b**) fibrous enlargement of the portal tract, (**c**) bile duct loss (arrow) and interface hepatitis (arrowhead), (**d**) orcein-positive granules, (**e**) chronic cholangitis, (**f**) lobular inflammation. (All these histological figures were kindly provided by Prof. Kenichi Harada (Kanazawa, Japan). PBC, primary biliary cholangitis)

Table 22.5 Nakanuma's classification [95]

a) *Staging of PBC*			
Stage 1 (no progression): score 0[a]			
Stage 2 (mild progression): score 1–3			
Stage 3 (moderate progression): score 4–6			
Stage 4 (advanced progression): score 7–9			
	Fibrosis	*Bile duct loss*	*Deposition of orcein-positive granules*
Score 0	No or limited portal fibrosis	No	No deposition
Score 1	Portal fibrosis	Yes, in <1/3 of the portal tracts	Deposition in several periportal hepatocytes in <1/3 of the portal tracts
Score 2	Bridging fibrosis	Yes, in 1/3–2/3 of the portal tracts	Deposition in variable periportal hepatocytes in 1/3–2/3 of the portal tracts
Score 3	Cirrhosis	Yes, in >2/3 of the portal tracts	Deposition in many periportal hepatocytes in >2/3 of the portal tracts
b) *Grading of PBC*			
	Cholangitis activity		
CA0 (no activity)	No cholangitis but mild duct epithelial damage may be present		
CA1 (mild activity)	One bile duct with evident chronic cholangitis		
CA2 (moderate activity)	≥2 bile ducts with evident chronic cholangitis		
CA3 (marked activity)	≥1 bile duct with CNSDC		
	Hepatitis activity		
HA0 (no activity)	No interface hepatitis and no or minimal lobular hepatitis		
HA1 (mild activity)	Interface hepatitis affecting ≥10 continuous hepatocytes in one portal tract or fibrous septum and mild to moderate lobular hepatitis		
HA2 (moderate activity)	Interface hepatitis affecting ≥10 continuous hepatocytes in ≥2 portal tracts or fibrous septa and mild to moderate lobular hepatitis		
HA3 (marked activity)	Interface hepatitis affecting ≥20 continuous hepatocytes in ≥1/2 of the portal tracts and moderate lobular hepatitis or bridging or zonal necrosis		

PBC primary biliary cholangitis, *CNSDC* chronic nonsuppurative destructive cholangitis
[a]The score for staging is the sum of the scores for fibrosis, bile duct loss, and deposition of orcein-positive granules, as shown above

Approximately 20–30% of patients with PBC exhibit incomplete biochemical responses to UDCA. The outcomes of these patients were significantly worse than those with complete responses to UDCA [118]. It is strongly recommended that patients with incomplete responses to UDCA commence a second-line treatment in addition to UDCA. For this purpose, various criteria employing combinations of biochemical markers at 1 year after commencement of UDCA treatment have been proposed, which will be discussed in the section "Stratification During Treatment." Furthermore, the "UDCA response score," which predicts treatment response before starting UDCA treatment with baseline clinical variables, has been proposed [119] but still needs to be validated.

Table 22.6 The Paris criteria for PBC with features of AIH [99]

PBC criteria	Features of AIH criteria
1) ALP level >2 × ULN or GGT level >5 × ULN	1) ALT level >5 × ULN
2) Positive AMA	2) IgG level >2 × ULN or positive SMA
3) Florid bile duct lesion on histology	3) Moderate or severe periportal or periseptal lymphocytic piecemeal necrosis

PBC primary biliary cholangitis, *AIH* autoimmune hepatitis, *ALP* alkaline phosphatase, *ULN* upper limit of normal, *GGT* gamma-glutamyl transferase, *AMA* antimitochondrial autoantibody, *SMA* smooth muscle antibody, *IgG* immunoglobulin G
The presence of at least two of three for each condition was required

Obeticholic Acid

Obeticholic acid (OCA) is a selective ligand of the farnesoid X receptor (FXR). Bile acid toxicity against BECs and hepatocytes is decreased by FXR signaling through impairment of bile acid synthesis and stimulation of choleresis. Compared with chenodeoxycholic acid, which is a primary bile acid and an endogenous FXR ligand, OCA is approximately 100 times more potent in activating FXR [120]. In the international, prospective, randomized, placebo-controlled trial (POISE trial), 217 patients with PBC who showed an incomplete response (serum ALP level >1.67 × upper limit of normal [ULN]) or an abnormal total bilirubin level (<2 × ULN) or were intolerant to UDCA were enrolled and received 5–10 mg of OCA daily, 10 mg of OCA daily, or placebo daily for 1 year. The primary end point was an ALP level <1.67 × ULN with >15% reduction from the baseline and normal bilirubin level. Among patients receiving OCA, 46–47% achieved the primary end point, compared to 10% of those receiving placebo [121]. Consequently, OCA received accelerated approval from the United States Food and Drug Administration (FDA) approval on May 27, 2016. Following the 1-year double-blind phase of the POISE study, all patients were offered enrollment in an open-label safety extension study. Interim results after 3 years of treatment suggested continued biochemical efficacy and safety [123].

Although OCA has become the long-awaited second-line drug approved for PBC, it is still unsatisfactory for several reasons. First, the response rate to OCA was less than 50%. Second, pruritus, a symptom frequently experienced by patients with PBC, is a frequent adverse effect of OCA. Third, given the high cost of OCA, the cost-effectiveness of

OCA for the treatment of PBC has yet to be demonstrated [122]. Fourth, it has not yet been confirmed whether the primary end points (ALP level <1.67 × ULN with >15% reduction from the baseline and normal bilirubin level) are associated with improvement of long-term outcomes. Although there is evidence of histologic improvement in a small group of patients who underwent paired liver biopsies [124], follow-up studies of the POISE trial were required by the FDA to demonstrate efficacy in clinical outcomes, which is being studied in an ongoing phase 3 trial (COBALT, NCT02308111). Finally, there are safety concerns of OCA, particularly in PBC patients with decompensated cirrhosis which has led to the FDA releasing a warning in September 2017 stating that the use of OCA in PBC patients with decompensated cirrhosis (Child-Pugh-Turcotte grades B and C) was associated with clinical worsening or even death, often when OCA was not appropriately dose reduced.

Fibrates and Other PPAR Agonists

Peroxisome proliferator-activated receptors (PPAR) are nuclear hormone receptors that bind fatty acids and fatty acid-derived molecules to regulate many metabolic pathways. Three PPAR isotypes, α, β/δ, and γ, are found in humans and differ in distribution, ligand activation, and metabolic regulatory pathways. PPARα is the primary receptor expressed in hepatocytes and enhances fatty acid and triglyceride metabolism, whereas PPARγ is essential for adipocyte differentiation and is the target of insulin-sensitizing thiazolidinediones. PPARβ/δ and PPARβ/γ are involved in energy use. These receptors are targeted by numerous drugs, including fenofibrate (α), bezafibrate (α, β/δ, γ), pemafibrate (α), elafibranor (α, β/δ), and seladelpar (β/δ).

In addition to the effects of fatty acid and triglyceride metabolism, activation of PPARs and pregnane X receptor results in reduction of de novo bile acid synthesis and upregulation of bile acid transporters [125]. Bezafibrate was first reported as potentially effective for patients with PBC who were refractory to UDCA in 1999 [126]. A French prospective, randomized, placebo-controlled study of bezafibrate in PBC patients with incomplete responses to UDCA demonstrated that an add-on of bezafibrate to UDCA for 2 years significantly improved liver biochemistry levels and liver stiffness [127]. In another study, additional evidence from Japan showed that the observed LT-free survival of patients treated with combination therapy of UDCA and bezafibrate was significantly superior to the expected LT-free survival of those treated with UDCA monotherapy according to the Globe and UK-PBC score [128]. Bezafibrate may also improve pruritus of PBC [129], and a prospective clinical trial for the treatment of cholestatic itch is ongoing (FITCH trial, NCT02701166).

Fenofibrate was reported to decrease serum ALP levels in studies from Japan and China [130, 131], whereas the adjunct use of fenofibrate with UDCA showed no association with decreased serum ALP levels in a United Kingdom study [132]. Participants are being recruited for a prospective randomized study in China (clinical trial ID: NCT02965911). However, these two prospective clinical trials showed notable improvements in liver enzyme levels at 12 or 24 months as the primary end point.

Novel PPAR agonists being developed for use in PBC include elafibranor, a PPARα/δ agonist, and seladelpar, a PPARδ agonist. In a phase 2b, 12-week trial of PBC patients with inadequate response to UDCA treated with placebo, 80 mg of elafibranor daily, or 120 mg of elafibranor daily, there was a significant decrease in ALP levels for patients receiving either dose of the drug. ALP levels decreased by 48% and 41% in patients treated with 80 mg and 120 mg, respectively, and increased by 3% in patients given placebo. In an initial phase 2 study of seladelpar testing 50 mg and 200 mg daily—doses previously shown to be well tolerated for other indications—seladelpar reduced ALP levels; however, three patients treated with seladelpar experienced ALT increases greater than five times the ULN, which resolved 2 to 4 weeks after drug discontinuation. In a subsequent phase 2 study of seladelpar at daily doses of 5 mg, 10 mg, or 5 mg followed by 10 mg for 12 weeks, ALT elevations were not observed and ALP levels decreased by 47% and 46% in the 5 mg to 10 mg and 10 mg groups, respectively. Despite the evidence of biochemical improvement, further development of seladelpar was terminated due to atypical histologic findings, including interface hepatitis with or without biliary injury, in a clinical trial of doses ranging from 10 to 50 mg daily for nonalcoholic steatohepatitis. Importantly, there was no biochemical evidence of hepatotoxicity in these patients, and it remains unclear if this is a unique property of seladelpar or a class effect.

The safety concerns related to hepatoxicity and PPAR agonists is not unique to seladelpar. There is a long history of hepatotoxicity with PPAR agonists including liver failure due to troglitazone as well as drug-induced liver injury with fibrates. Other safety concerns related to myositis and kidney toxicity exist. In the 50 patients treated with bezafibrate in a study by Corpechot and colleagues, one bezafibrate-treated patient developed stage 3 chronic kidney disease and 20% of patients in the treatment group experienced myalgias compared to 10% in the placebo group, with one patient in the bezafibrate group developing rhabdomyolysis. Importantly, four patients in the study (three receiving bezafibrate and one receiving placebo) developed ALT elevations five times the upper limit of normal. Levels returned to normal within 3 months of drug discontinuation, with two patients requiring glucocorticoids.

Further studies to determine the safety of these drugs and whether long-term outcomes are improved are needed.

LT

Despite improvements in medical treatment for PBC, LT is the only treatment option for patients with decompensating events or intolerable pruritus. A recent study utilizing the European Liver Transplant Registry demonstrated a significant decrease of LT in PBC over the last 30 years, after the introduction of UDCA in clinical settings [133]. The proportion of LT for PBC decreased from 20% of all LT cases in 1986 to 4% in 2015 ($p < 0.001$). The absolute number of transplants was the highest in 1994 ($n = 279$), which decreased to an average of 200 in the last decade. This decrease is striking in contrast to the substantial increase of prevalence of PBC at the same time [72]. Overall, the long-term outcome after LT for PBC is excellent (Table 22.7) [134–136].

Recurrence of PBC after LT is not uncommon. The reported incidence of recurrent PBC widely differs between 11% and 42% [137–152]. Although several studies have reported risk factors associated with recurrence of PBC, most of them consistently demonstrated that the use of tacrolimus is associated with an increased risk of recurrence [138, 139, 146, 149–151]. For example, a recent study of 785 patients with PBC from North America and Europe who underwent LT from February 1983 to June 2016 indicated that tacrolimus was linked to recurrence of PBC. Although the use of cyclosporine was protective, the 5-year probabilities of recurrence of PBC were reported to be 28% and 11% in patients receiving tacrolimus and cyclosporine, respectively ($p < 0.001$) [148]. On the other hand, the role of tacrolimus as an increasing agent of recurrent PBC does not seem to be the case in other ethnicities. Recently, two cohort studies from Japan demonstrated that the increased frequency of recurrent PBC is associated with initial treatment with cyclosporine after LT [140, 145].

Although it is believed that recurrence of PBC does not have a significant impact on long-term outcomes, such as overall survival [146, 153], a recent study of 785 PBC patients from 13 centers in North America and Europe who received LT with a median follow-up of 6.9 years (interquartile range 6.1–7.9) reported opposite and unexpected results that disease recurrence was found in 240 patients (31%), and importantly, graft and patient survival rates were significantly impaired in those with recurrent PBC ($p = 0.004$ and 0.001, respectively) [148]. It is imperative to determine whether recurrent PBC really has a clinically significant impact on patient and graft survival. Furthermore, preemptive UDCA treatment after liver transplantation seems to be effective in reducing the risk of recurrence of PBC after LT [153].

Table 22.7 Patient and graft survival at 5 and 10 years after LT[a]

		Patient survival		Graft survival	
Region	*N*	5 y	10 y	5 y	10 y
Europe	4515	80	71	75	66
USA	3052	84	79	78	72
Japan[b]	710	79	74	NA	NA

LT liver transplantation, *USA* United States, *NA* not applicable
[a]Registry data from Europe [134], USA [135], and Japan [136]
[b]Living-related LT in all

Management of Symptoms and Extrahepatic Manifestations

Patients with PBC frequently suffer from numerous symptoms. The most dominant clinical symptoms in the early stage of PBC are fatigue and pruritus. Since these symptoms can significantly deteriorate the quality of life of patients with PBC, it is strongly recommended to carefully monitor these symptoms with objective and reproducible measures such as the PBC-40 questionnaire [154].

Fatigue

Fatigue is the most common and debilitating symptom in PBC, experienced by approximately 50% of patients (ranging from 20% to 80% depending on each study) [155–157]. Although it is difficult to define the cutoff clearly according to the presence of fatigue, it has been repeatedly shown that fatigue impairs the quality of life of patients with PBC [156, 158]. Fatigue is not associated with disease severity or staging but may be related to age at onset and gender [159].

The cause of fatigue remains unknown but seems to be complex in origin, probably multifactorial in most patients and associated with depression, autonomic dysfunction, and sleep disturbance [158]. Recent studies with magnetic resonance imaging revealed neuroimaging changes in the brain can be detected even in early stages of PBC [160]. Fatigue is not affected by UDCA treatment, and a recent systemic review failed to define any established treatment for fatigue in PBC [161]. Fatigue may be improved by LT, but it can also persist in a substantial portion of patients even after LT, making the role of LT as a therapeutic option for severe fatigue questionable [162]. Modafinil, which is officially approved by the FDA for wakefulness disorders, has been used, but a randomized, placebo-controlled clinical trial failed to identify any beneficial effects of this drug in reducing fatigue in patients with PBC [163].

Pruritus

Pruritus is another common symptom in PBC, affecting 20–80% of patients. Pruritus can occur locally or diffusely, and its presence and severity change throughout the clinical

course of PBC. It tends to become more pronounced with the progression of PBC but can be present even in the very early stage. Pruritus can be highly bothersome and intolerable to patients, causing sleep disturbance and, in rare cases, can be an indication for LT. The severity of pruritus can be objectively assessable with the PBC-40 questionnaire as other patient reported outcome measures such as a numeric rating scale or 5-D Itch survey [154]. The cause of pruritus remains unknown, although several substances are hypothesized to be related to pruritus in cholestatic liver diseases [164]. Most notably, lysophosphatidic acid (LPA) may be a potential candidate for initiating pruritus [165], and the activity of serum autotaxin, which converts lysophosphatidylcholine into LPA, is related to the severity of pruritus and responds to therapeutic interventions [166, 167]. Hence, LPA-autotaxin is an important candidate as a therapeutic target, yet clinically unavailable. An ileal bile acid transporter (IBAT) inhibitor compound (Linerixibat) that inhibits reabsorption of bile acids in the ileum effectively decreased pruritus in patients with PBC in a phase 2a study [168], and a global phase 2b study (clinical trial ID: NCT02966834) investigating the efficacy of this compound is almost completed. A phase 2 trial of another IBAT inhibitor, Maralixibat, failed to demonstrate a significant antipruritic effect against placebo, presumably because of a significant placebo effect [169]. As described earlier, bezafibrate is now being investigated in a clinical trial for the treatment of cholestatic pruritus (clinical trial ID: NCT02701166) [129]. In Japan, nalfurafine hydrochloride, a selective κ-opioid receptor agonist, was recently approved in Japan for refractory pruritus in patients with PBC and exhibited a substantial antipruritic effect [170]. Human sensory neuron-expressed Mas-related G protein-coupled receptor X4 (MRGPRX4) is a bile acid receptor, and a recent experiment suggests that targeting MRGPRX4 may be a promising strategy for alleviating cholestatic itch [171].

Disorders Associated with PBC

Sicca Syndrome

Sicca complex is frequently present in patients with PBC, manifesting as dry eyes and/or dry mouth. External glands including the lachrymal or salivary glands are also affected in PBC. A current retrospective study revealed the prevalence of Sjögren's syndrome in up to 56% of patients with PBC [172]; however, the sicca complex affects patients with PBC who do not meet the criteria of Sjögren's syndrome. Patients with sicca syndrome may experience many symptoms including burning, itching, or irritated eyes, blepharitis, dysphagia, stomatitis, dental caries, and dry cough, resulting in severe impairment in their quality of life. Early recognition of sicca symptoms and consultations with ophthalmologists or dentists are suggested.

Osteopenia and Osteoporosis

Osteopenic bone disease, including osteopenia and osteoporosis, is a common disorder in PBC that mainly affects middle-aged women and is associated with an increased risk for fragility fracture. The decrease in bone mineral density found in PBC is multifactorial. Chronic cholestasis leads to malabsorption and deficiency of vitamin D, which is essential to bone metabolism. Other factors associated with bone diseases include age, gender, low body mass index, history of fragility fracture, and advanced stage of PBC [173, 174]. Intervention with bisphosphonate for patients with osteoporosis and those with a history of fragility fracture is safe and improves bone mineral density [175]; nonetheless, it remains unclear whether bisphosphonate use is associated with a decrease in fragility fractures. Recently, denosumab, a fully human monoclonal antibody against the receptor activator of nuclear factor-kappa B ligand (RANKL), was demonstrated to be effective for treating osteoporosis in patients with PBC [176]. Since the RANK-RANKL signaling might be implicated in the pathogenesis of PBC [177], treatment with denosumab could be used to target both osteoporosis and PBC.

Hyperlipidemia and Metabolic Syndrome

Chronic cholestasis is a main feature of PBC, and consequently, hyperlipidemia is common and affects up to 80% of patients [178]. Several prospective studies indicated that an increase in serum lipid levels is not associated with a higher risk for cardiovascular diseases related to atherosclerosis, and treatment for hyperlipidemia per se is not necessary. Notably, these studies were conducted in the 1990s when metabolic syndrome was relatively rare in patients with PBC. A recent study from Italy demonstrated that cardiovascular events developed more frequently in patients with metabolic syndrome [179], indicating the importance of treatment intervention for patients with hyperlipidemia if metabolic syndrome exists.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is occasionally encountered in patients with PBC. The life expectancy of these patients with UDCA treatment is comparable to the general

Table 22.8 Incidence and risk factors for HCC in patients with PBC

Country/region	Number		Incidence[a]				Risk factors
	Total	HCC	All	Male patients	female patients	Female	
Barcelona, Spain [180]	389	13	3.6	NA	NA		Advanced histological stage
Padova, Italy [180]	327	11	3.7	NA	NA		Advanced histological stage (all), male sex
Japan [211]	2946	71	3.6	9.5	2.9	2.9	Male sex, advanced histological stage (in female patients)
International [182]	4565	123	3.4	6.7	2.6		Advanced age, male sex, thrombocytopenia at 12 months, biochemical nonresponse
Beijing, China [183]	1865	70	6.6	NA	NA		Advanced age, male sex, coexistence of diabetes, Hx of HBV infection

HCC hepatocellular carcinoma, *PBC* primary biliary cholangitis, *Hx* history, *HBV* hepatitis B virus, *NA* not applicable
[a]Cases per 1000 patient-years

population. The reported incidences and risk factors for developing HCC from several large-scale retrospective cohorts are summarized in Table 22.8. Surprisingly, the incidence rates (cases per 1000 patient-years) of HCC in all patients with PBC are similar across different regions: 3.6 in Barcelona, Spain; 3.7 in Padova, Italy [180]; 3.6 in a nationwide study in Japan [181]; and 3.4 in an international cohort [182]. The incidence rate was exceptionally high, 6.6, in a cohort from Beijing, China [183], presumably because of the high rate of individuals with previous hepatitis B virus (HBV) infection. A history of HBV infection was identified as an independent risk factor for HCC in this study. The incidence rate was higher in men than in women. In those studies, male sex and advanced histological stage independently contributed to the development of HCC [180–183]. Treatment response was included among possible risk factors only in the international cohort study, and biochemical nonresponse at 1 year of UDCA treatment (Paris II not fulfilled) significantly increased the future risk of HCC (adjusted hazard ratio, 3.44) [182]. Taken together, close monitoring of HCC is strongly recommended for high-risk patients with PBC such as male patients, those with advanced-stage disease, and nonresponders to UDCA. The mean survival of patients who developed HCC was 36 months after diagnosis, and another cohort indicated 5- and 10-year survival rates of 49.5% and 31.7%, respectively [184].

Stratification of the Risk for Progression

Since the introduction of UDCA as the first-line drug, the prognosis of patients with PBC has been dramatically improved and is now comparable to those of the general population if the response to UDCA is complete. However, a substantial proportion of patients who are already in the cirrhotic stage at presentation or are refractory to UDCA unavoidably progress to liver failure and require LT. Approximately half of patients at early stages progressed to a moderate stage at 5 years [185]. Therefore, clinicians must stratify individual patients who are diagnosed as having PBC and estimate the risk of the patient for progression, both at presentation and at any time during treatment, especially at 1 year after the commencement of UDCA treatment [186].

Stratification at Baseline

Gender and age at diagnosis have been suggested to be associated with response to UDCA treatment and symptom development, and women who are younger than 50 years of age exhibited the lowest response rate to UDCA and the highest levels of symptoms [159]. However, a recent retrospective cohort study revealed that patient age, not sex, was associated with response to UDCA treatment and LT-free survival [187]. The AST/platelet ratio index at baseline is also a predictor of outcomes independent of the UDCA response [188]. The presence of ANAs, especially anti-gp210, at baseline may be associated with a more severe clinical course [92, 189, 190]. A current retrospective study from China also highlighted the significance of anti-gp210 as a biomarker of worse outcomes [191]. Advanced histological stages at presentation are obviously associated with poor prognosis, and a large-scale retrospective cohort study confirmed the association of fibrosis stage at baseline with long-term outcome despite biochemical treatment response [85]. However, assessment of liver histology requires liver biopsy as an invasive procedure, and sampling errors can be additional problems. In this regard, various noninvasive techniques for evaluating liver fibrosis have been developed, including the liver stiffness measurement by means of vibration-controlled transient elastography, magnetic resonance elastography, and serum biomarker measurements [192]. Recent studies indicated that serum levels of *Wisteria floribunda* agglutinin-positive mac-2 binding protein, solu-

ble CD-14, IL-8, and IFN-γ-inducible protein-10 are other candidate serum biomarkers for predicting liver fibrosis and the prognosis of PBC [193–195]. As an alternative attempt, the predictive scores of patients' UDCA response (the UDCA response score), based on pretreatment variables, were proposed [119], and the validity of the UDCA response score was comfirmed in another population [196].

Stratification During Treatment

It is important to stratify patients with PBC depending on their responses to treatment. Different criteria for defining biochemical responsiveness to UDCA have been proposed (Table 22.9). A global consensus has been established to judge responsiveness to UDCA at 1 year after the commencement of UDCA treatment with liver biochemistry tests, which are easy to conduct. Furthermore, although several simple definitions of unresponsiveness (responder or nonresponder) have been established in a nationwide scale in earlier studies [113–115, 117, 197–199] and later in an international consortium (Global PBC Group) to take into account a very large-scale cohort [200], these dichotomous definitions did not serve as predictors that could precisely and quantitatively assess the risk for progression in a given patient. Recently, two large-scale multicenter studies developed continuous predictive models (the GLOBE score and UK-PBC score) with age and liver biochemistries either at baseline or at 1 year after UDCA treatment [116, 201]. These scores are easy to use with computer-aided calculations and allow physicians to continuously quantify the risk for progression over time in a single patient. These scores have been validated in another cohort of 1746 UDCA-treated patients [202]. It is important to note that these scores were solely developed for a patient cohort treated with UDCA monotherapy, and further studies are needed to validate whether these scores based on biochemical responses at 1-year treatment are also applicable to patients treated with additional drugs, such as OCA or bezafibrate. In fact, the observed LT-free survival of patients was significantly improved by treatment with a combination of UDCA and bezafibrate compared to the expected survival by the GLOBE score and UK-PBC score [128].

Table 22.9 Criteria defining biochemical responses to UDCA

Criteria	Number of patients	Duration	Definition
Qualitative definition			
Barcelona [117]	192	1 y	Normal ALP level or reduction in the ALP level by >40%
Paris-I [113]	292	1 y	ALP level <3 × ULN, AST level <2 × ULN, normal bilirubin level
Rotterdam [114]	375	1 y	Normal bilirubin level, normal albumin level
Toronto [115]	69	2 y	ALP level ≤1.67 × ULN
Ehime [197]	83	6 m	Normal GGT level or reduction in the GGT level by ≥70%
Paris-II [198]	165	1 y	ALP level <1.5 × ULN, AST level <1.5 × ULN, normal bilirubin level
Rochester [199]	73	1 y	ALP level ≤1.67 × ULN, bilirubin level ≤1 mg/dL
International (Global PBC) [200]	4845	1 y	ALP level <2 × ULN, normal bilirubin level
Quantitative scores			
GLOBE score [116]	4119	1 y	Bilirubin level, ALP level, albumin level, and platelet count at 1 year, age at baseline
UK-PBC score [201]	3165	1 y	ALP level, AST/ALT level, and bilirubin level at 1 year, albumin level and platelet count at baseline

UDCA ursodeoxycholic acid, *ALP* alkaline phosphatase, *ULN* upper limit of normal, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transferase

Future Perspective

Despite recent magnificent progresses, many mysteries remain unresolved in PBC. Although PBC is considered to be an autoimmune liver disease, what trigger(s) for the loss of tolerance against BECs leading to the specific destruction of small-sized intrahepatic bile ducts remains unclear. While a diagnostic role of AMAs in PBC is remarkable, a pathogenic role of AMA is still undefined. The clinical presentations and disease progression vary among PBC patients, and their responses to treatment are not always predictable. While UDCA (and OCA or bezafibrate, hopefully) has dramatically improved the natural course of PBC, there is no therapeutic medical approach for advanced PBC or annoying subjective symptoms such as fatigue and pruritus.

Hence, it is necessary for clinicians to stratify patients depending on the risk for progression and subjective symptoms and individualize the treatment with choices of available therapeutic agents including an option of no treatment. Rigorous effort should be directed at improving our understanding on the environmental etiology and genetic basis of PBC, molecular mechanisms of disease progressions, and gender bias to identify critical pathways for therapeutic interventions. Relevant animal models that recapitulate human PBC should be established for preclinical studies with designer drugs guided by this new knowledge. Our goal is achieving the "cure" for PBC.

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PSC-AIH Overlap

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Abbreviations

AASLD	American Association for the Study of Liver Diseases
AIH	Autoimmune hepatitis
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANAs	Antinuclear antibodies
ANCAs	Antineutrophil cytoplasmic antibodies
ANNAs	Antineutrophil nuclear antibodies
ASC	Autoimmune sclerosing cholangitis
AST	Aspartate aminotransferase
CCA	Cholangiocarcinoma
CD	Crohn disease
EASL	European Association for the Study of the Liver
ERCP	Endoscopic retrograde cholangiopancreatography
GWASs	Genome-wide association studies
HLA	Human leukocyte antigen
IAIHG	International AIH Group
IBD	Inflammatory bowel disease
IgG	Immunoglobulin G
IPSCG	International PSC Study Group
MMF	Mycophenolate mofetil
MRCP	Magnetic resonance cholangiopancreatography
PBC	Primary biliary cholangitis
PSC	Primary sclerosing cholangitis
SMA	Smooth muscle antibodies
UC	Ulcerative colitis
UDCA	Ursodeoxycholic acid
γ-GT	γ-Glutamyl transpeptidase

Key Points

- Patients with overlapping characteristics between autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC) are classified as having a PSC-AIH "variant syndrome," although controversies exist whether this is a distinct nosological entity or a variant form of a dominant autoimmune liver disease (AIH or PSC).
- The International Autoimmune Hepatitis Group (IAIHG) and the European Association for the Study of the Liver (EASL) recommend the classification of these patients according to the predominant disease entity (AIH or PSC) and replacement of term "overlap" with "variant syndrome."
- There is no single diagnostic characteristic for the diagnosis or exclusion of this enigmatic syndrome and therefore, each patient should be viewed individually, over time, focusing particularly on the dominant histologic process.
- The true prevalence of PSC-AIH "variant syndrome" is unknown, as specific diagnostic criteria are missing, while the application of the IAIHG criteria for AIH diagnosis is not recommended in these patients.
- In routine clinical practice, the diagnosis of PSC-AIH "variant syndrome" is based on the typical cholangiographic or histological characteristics of PSC, along with AIH features. The presence of interface hepatitis at the histological level is a fundamental characteristic and therefore, histology is of major importance in evaluating patients with overlap presentation.
- In children and adolescents, the hepatitic feature can be dominant, but up to 50% of these patients with AIH have also cholangiographic abnormalities, suggestive of PSC. Therefore, the term "autoimmune sclerosing cholangitis" is used for the pediatric population.

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- "Variant syndromes" should be considered when a patient with autoimmune liver disease deviates from the expected clinical course, typical biochemical and serologic findings, and the expected response to treatment.
- Patients with PSC-AIH variant have a high prevalence of inflammatory bowel disease (IBD), like patients with PSC alone.
- Regarding the therapeutic strategy, the leading disease component should be treated first, though validated therapeutic guidelines for "variant syndromes" are missing.
- Patients with PSC-AIH variant should be consider for treatment with immunosuppressants with or without ursodeoxycholic acid. Their outcome seems better than patients with classic PSC phenotype but rather poorer than AIH alone.
- Patients with variant forms of autoimmune liver diseases should be referred to centers with expertise in diagnosis and treatment of autoimmune liver diseases.

Case Presentation and Introduction

A 40-year-old male submitted to the hospital because of torturous pruritus and obstructive jaundice. His past medical history revealed a very long history of liver disease during the last 23 years. Actually, his liver disease started at the age of 17 years with an acute episode of icteric hepatitis. The patient denied ever consumption of herbal agents and/or dietary supplements, intravenous or nasal illicit drugs, or alcohol abuse. Viral serological testing for hepatitis A, B, and C markers was negative while the liver autoimmune serology during his first episode revealed hypergammaglobulinemia and reactivity against liver kidney microsomal type-1 (anti-LKM1). Liver biopsy at this age showed centrolobular necrosis, interface hepatitis with lympho/plasma cells infiltration, and lobular hepatitis but without biliary lesions, suggesting the diagnosis of acute autoimmune hepatitis (AIH) type 2 (AIH-2). Since then the patient was under immunosuppression from his physicians with corticosteroids and azathioprine with partial response. Magnetic resonance cholangiopancreatography (MRCP) studies had not been performed up to the age of 37 years. At this time, because of a moderate increase of cholestatic enzymes [gamma glutamyltranspeptidase (γ-GT): 102 U/L (ULN: 36 U/L) and alkaline phosphatase (ALP): 204 U/L (ULN: 120 U/L)], MRCP imaging was done, showing mild dilatation of the intrahepatic bile ducts and the common bile duct without, however, the typical findings of primary sclerosing cholangitis (PSC).

Two years later because of palpable spleen, triplex imaging of the portal and splenic veins was performed, showing dilatation of portal vein with low flow and competitive increase of hepatic artery blood flow accompanied by splenomegaly, suggesting the development of portal hypertension. A new MRCP was now typical of PSC affecting the intra- and extrahepatic bile ducts. Liver biochemistry at this time was as follows: aspartate aminotransferase (AST)/alanine aminotransferase (ALT)/γ-GT/ALP: 135/436/499/296 U/L and immunoglobulin G (IgG): 1800 (ULN: 1600 mg/dL) with normal bilirubin levels. A new liver biopsy was decided, which showed the presence of hepatocyte rosetting, lobular spotty necrosis, and moderate interface hepatitis without destruction of the bile ducts, indicating at the histological level, a moderate exacerbation of the already known underlying AIH-2. However, there was also extended expression of CK7 in periportal hepatocytes, periductular fibrosis, focal absence of intralobular bile ducts and bile plugs in dilated bile duct capillaries along with positive orcein staining in periportal hepatocytes, suggesting apart from AIH-2, the presence of chronic cholestasis.

A year ago, the fibroscan showed a liver stiffness of 12.6 KPa, while during the last 3 years, his liver biochemistry was consistently abnormal albeit normal bilirubin levels, suggesting that the patient was not responding to the conventional treatment with corticosteroids and azathioprine (2015: AST/ALT/γ-GT/ALP: 84/107/164/179 U/L, IgG: 1694 mg/dL; 2016: 46/115/195/225 U/L and 2017: 59/106/99/128 U/L, IgG: 1874 mg/dL). Taking into account the results of the recent liver biopsy, the nonresponse to the conventional immunosuppression and the steadily abnormal AST, ALT, and IgG, an induction trial with second-line therapies was offered. Actually, the patient started an individualized course of immunosuppression with prednisolone 1 mg/kg/day and mycophenolate mofetil (MMF) 2 g/day in an attempt to control at least the AIH-related part of his disease [1, 2] along with 15 mg/kg/day of ursodeoxycholic acid (UDCA). After 5 months of treatment, his liver biochemistry has improved substantially (AST/ALT/γ-GT/ALP: 42/82/155/122; IgG: 1146 mg/dL and normal bilirubin).

However, during corticosteroids tapering, a new flare was observed, characterized now by the development of jaundice. In fact, in his last admission, the liver biochemistry was as follows: AST/ALT/γ-GT/ALP: 150/251/445/339 U/L; total bilirubin: 11.4 mg/dL/direct bilirubin: 8.8 mg/dL, and IgG: 2080 mg/dL. Liver autoimmune serology showed the presence of smooth muscle antibodies (SMA): 1/320, atypical perinuclear antineutrophil antibodies (pANCA): 1/80 and LKM-1 positivity by immunoblot. An ultrasound of the upper abdomen revealed common bile duct dilatation, while a following endoscopic retrograde cholangiopancreatography (ERCP) showed dilatation and strictures of the intra- and extrahepatic bile ducts including a dominant stricture of approximately 4 cm in the peripheral portion of common bile duct. Accordingly, balloon dilatation was performed along with brush cytology and a baseline colonoscopy, which proved negative. In addition, immunosuppression was stopped as

now PSC was the prevailing form of his variant liver disease. However, because of further deterioration of the jaundice and pruritus, the patient was transplanted and he is now in good health 2 months after undergoing liver transplantation.

According to this long-term history, the most probable scenario was the initial development of AIH-2, which then gradually got "complicated" by the additional presence of PSC. In other words, this representative case may constitute the story of autoimmune sclerosing cholangitis (ASC) reported in children and young adolescents by Giorgina Mieli-Vergani's group several years ago [3]. In addition, this case indicates how difficult and complicated could be the diagnosis and long-term outcome of patients with this enigmatic and mixed condition of autoimmune liver diseases, highlighting the range of disease manifestations and severity.

The spectrum of autoimmune liver diseases includes AIH, primary biliary cholangitis (PBC), and PSC including its variant form of small duct PSC. Each of them has characteristic clinical, laboratory, serologic, histologic, and imaging findings. From the immunopathogenetic perspectives, however, these immune-mediated liver disorders have different main targets, namely, the hepatocytes and bile ducts and therefore, they could be divided into two broad categories: those with a predominance of hepatocellular injury (AIH) and those with a predominant cholestatic feature (PBC or PSC) [4–10].

Regarding AIH and PSC, although the vast majority of patients meet the diagnostic criteria for AIH or PSC alone, some have features of both conditions presenting either simultaneously or sequentially ("PSC-AIH overlap"). A controversy exists whether these overlap presentations reflect a distinct disease entity or they represent atypical presentations of the aforementioned autoimmune liver diseases [4, 9, 11–13]. The key clinical characteristics and serologic markers of AIH and PSC are summarized in Table 23.1.

Table 23.1 Main clinical, laboratory, and histologic characteristics of patients with AIH or PSC

Feature	AIH	PSC
Gender	Female > male (3–4:1)	Male > female (recent studies ~ same prevalence)
Age at diagnosis	All ages (bimodal distribution in puberty and 4th–6th decades; a substantial proportion of patients, however, are older >65 years)	All ages but usually <40 years
Clinical presentation	From asymptomatic to acute/severe or even fulminant hepatitis	Asymptomatic with biochemical cholestasis In symptomatic disease, right upper quadrant discomfort, pruritus, features of cholangitis with fever
Aminotransferases	Elevated (usually >3×) – may be normal or slightly elevated	Normal or moderately elevated
ALP	Normal or slightly elevated	Elevated (usually >2–3×)
IgG	Elevated in ~85%	Elevated in ~60%
Autoantibodies		
ANA	70–80%	8–77%
SMA	70–80%	0–83%
Anti-LKM1	3–5%	Very rare
Anti-SLA/LP	10–30%	Few
pANCA	Up to 92% (often atypical)	26–94% (atypical often with concurrent IBD)
Liver biopsy		
Interface hepatitis	Typical finding	Variably present
Portal inflammation	Infiltration by lympho/plasmatocytes	Infiltration by lymphocytes
Biliary changes	~10% present	Inflammatory bile duct damage, with or without "onion skin" periductular fibrosis
Cholangiography	Usually normal, although mild intrahepatic ductal changes in advanced cirrhosis (10%) Cholangiographic abnormalities up to 50% (pediatric AIH)	Multifocal strictures of intrahepatic or extrahepatic bile ducts or both Normal cholangiography in small duct PSC
Coexisting IBD	~3–10%	~70%
Associated conditions	Hashimoto thyroiditis, Grave's disease, vitiligo, alopecia, rheumatoid arthritis, diabetes mellitus type 1, systemic lupus erythematosus, psoriasis, celiac disease, Sjögren's syndrome, panniculitis, mononeuritis, urticaria pigmentosa, Sweet's syndrome, idiopathic thrombocytopenic purpura, polymyositis, hemolytic anemia, uveitis, autoimmune polyendocrinopathy syndrome type 1	Pancreatitis (up to 25%), idiopathic fibrosing diseases (retroperitoneal fibrosis)
Treatment	Immunosuppression	UDCA

Abbreviations: AIH autoimmune hepatitis, *PSC* primary sclerosing cholangitis, *IBD* inflammatory bowel disease, *ALP* alkaline phosphatase, *SMAs* smooth muscle antibodies, *UDCA* ursodeoxycholic acid, *ANAs* antinuclear antibodies, *SLA/LP* soluble liver antigen/liver pancreas, *pANCA* peripheral antineutrophil cytoplasmic antibody, *IgG* immunoglobulin G, *anti-LKM1* anti liver kidney microsomal type-1

Overview of PSC Characteristics

PSC is a rare but serious immune-mediated cholestatic liver disease of unknown etiology with no real effective therapy so far. Therefore, in most patients, liver transplantation is needed although the disease may recur after transplantation [14]. The disease is characterized by inflammation that affects both the intrahepatic and extrahepatic bile ducts, leading to severe cholestasis, multifocal strictures of the biliary tree, and hepatic fibrosis/cirrhosis [9, 14–16].

Of note, despite previous assumptions for a male predominance among PSC patients, recent studies from the International PSC Study Group (IPSCSG) including more than 7.000 patients have shown that the disease may occur as commonly in females as in males [17, 18]. PSC is associated with the presence of inflammatory bowel disease (IBD) in approximately two-third of PSC cases from northern European countries and the United States, while there is a high risk for the development of cholangiocarcinoma (CCA) and colorectal cancer [15, 16, 19–21]. Conversely, only 3–8% of patients with IBD develop PSC.

In the era of MRCP, liver biopsy is rarely needed to establish the diagnosis of PSC as the pathognomonic "onion-skin" periductular fibrotic lesions are found infrequently. MRCP is indeed the noninvasive imaging modality of choice for PSC diagnosis as it bears excellent sensitivity and specificity (0.86 and 0.94, respectively) [9, 13–16, 22].

Small duct PSC is a specific variant of the disease, originally described in adult patients, characterized by typical cholestatic liver biochemistry and morphologic features of PSC on liver biopsy but with normal appearance of the bile ducts even after high-quality MRCP [13–16, 23]. Small duct PSC is found in 3–5% of patients with PSC, but the precise risk of progression to classic large duct PSC is unknown [13–16, 18, 24]. In a recent large retrospective study from the IPSCSG, it was shown that small duct PSC was associated with better prognosis in terms of the risk of liver transplantation or death and development of CCA in both sexes compared to the typical phenotype of PSC [18].

Overview of AIH Characteristics

AIH is a relatively rare, acute or chronic liver disease of unknown etiology characterized by large epidemiologic, clinical, genetic, serologic, and histologic heterogeneity [5, 7, 8, 25–34]. Its distribution is global, covering all ages, both sexes, and all ethnic groups [5, 7, 8, 32–34]. AIH diagnosis is based on clinicopathological characteristics such as polyclonal hypergammaglobulinemia with a distinct increase of serum IgG, circulating non-organ-specific autoantibodies, interface hepatitis on liver histology, absence of viral hepatitis, and a favorable response to immunosuppression [5, 7, 8, 25, 26, 33, 34]. The presence of interface hepatitis with dense plasma cell-rich lymphoplasmacytic infiltrates at the histological level and the presence of autoantibodies are the two diagnostic hallmarks of the disease [5, 7, 8, 25–28, 33, 35]. AIH patients can be considered for screening for other concurrent autoimmune diseases, especially autoimmune thyroiditis, as AIH is associated with a reduced quality of life and a broad variety of other autoimmune-mediated conditions [5, 7, 8, 25, 26, 31, 33, 36].

A useful tool for the diagnosis and study of AIH was the diagnostic scores established by the International AIH Group (IAIHG). The first score was developed in 1993 and subsequently revised in 1999 including descriptive criteria for the diagnosis and classification of AIH either as "definite" or "probable" based on a numeric scoring system [37, 38]. In 2008, this score was simplified further in an attempt to be friendly in everyday clinical practice [39]. It should be noted, however, that there is no single gold standard for AIH, and these diagnostic scores were established in order to conform the diagnostic criteria between the different centers and to give the opportunity to compare the different experiences, mainly for research purposes [40–42].

Patients with Overlapping Features Between PSC and AIH

Definitions and Terminology Issues

In the vast majority of PSC or AIH patients, the diagnosis is set on clinical grounds by using clinical, biochemical, histological, liver autoimmune serology, and imaging studies. However, several variants or atypical forms with overlapping characteristics among patients with autoimmune liver diseases are recognized, making sometimes the diagnosis difficult [4, 9, 11–13, 43–45]. Features of both PSC and AIH in the same patient were first described about three decades ago, including a small case series [46, 47]. Since these early reports, there has been an increasing awareness for patients with features of both conditions either simultaneously or consecutively, often called "PSC-AIH overlap" [48]. Compared to the previous 20 years, these "overlap syndromes" are now more frequently diagnosed and have been the subject of several reviews. However, a clear classification and definition of pathogenesis, prevalence, serologic profile, treatment options, and prognosis in these patients are still lacking. Besides definitions of "overlap syndromes" are variable and have changed over time as our understanding of autoimmune liver disease has been improved [12].

From the clinical points of view, "PSC-AIH overlap" should be considered in the differential diagnosis when a patient with a first diagnosis of PSC or AIH deviates from the usual clinical course, classical biochemical and serologic

findings, and the expected response to therapy. However, at the same time, it is important not to overdiagnose or consider "overlap syndrome" at presentation of a predominant disease process either PSC or AIH as a means of justifying the initiation of nonstandard therapies [49, 50].

Unfortunately, the lack of a universal agreement on what exactly constitutes an "overlap syndrome" and the lack of standardization of diagnostic criteria have generated confusion in the literature and as a result, "overlap syndrome" is one of the most misused descriptive terms currently used in every-day clinical practice in hepatology [11–13, 48–51]. Indeed, much debate exists whether these enigmatic syndromes represent manifestations within the spectrum of autoimmune liver diseases, variants of the classical autoimmune liver diseases, or distinct clinical entities [7, 8, 11–13]. The presence of interface hepatitis – although not specific – is a fundamental component of AIH and therefore, histology has been considered vital in evaluating patients with "overlap syndromes" [26, 28, 35]. In addition, the degree of interface hepatitis seems important as a measure of AIH-like disease activity irrespective of the coexistence or underlying cholestatic liver disease [11–13]. The European Association for the Study of the Liver (EASL) and the IAIHG suggest that although patients may have overlapping features across the spectrum of autoimmune liver diseases, individual cases should be categorized according to the predominant disease entity as AIH, PBC, PSC, or small-duct PSC and to be considered as parts of the respective "classical" disease with the addition of the presence of features of another autoimmune liver disease [7, 11]. As a consequence, it is now generally believed that the term "overlap syndromes" should be changed, as "overlap" suggests by definition the concurrence of two different liver diseases at the same time, which could be a misnomer, because it is not the case for all patients [7, 8, 11, 50, 53]. In this context, the use of "variant syndromes" has been proposed as much more appropriate, assuming that there is a continuous spectrum of autoimmune liver disease affecting hepatic parenchyma and bile ducts to varying degrees on the basis of an impaired immune homeostasis in the liver [11–13, 52, 54]. However, it should be kept in mind that "variant forms" of AIH should not be overdiagnosed in order not to expose PBC or PSC patients unnecessarily to the risk of immunosuppression side effects [7, 8, 11].

Pathogenesis

The "variant syndromes" are rather clinical descriptions than valid pathological entities, and their nature is uncertain [55, 56]. Several clinical presentations and pathophysiologic mechanisms have been suggested: (1) a pure coincidence of two independent autoimmune diseases; (2) a distinct disease entity sharing clinical, biochemical, and histologic features of both AIH and PSC; (3) the appearance of the middle of a continuous spectrum of two autoimmune liver diseases; and (4) the hypothesis of sequential transition in genetically susceptible individuals from PSC or AIH to the other clinical entity, resulting in either the "hepatitic form of PSC" (PSC-AIH variant) or the "cholestatic form of AIH" (AIH-PSC variant), with the latter being the most common in clinical practice [13, 49, 57] (Fig. 23.1).

In general, similar pathogenetic ways of injury have been proposed for PSC and AIH including environmental triggers, genetic predisposition, and failure of immune tolerance mechanisms [16, 26–28, 30, 53, 58–63]. These mechanisms may collaborate to induce an antibody- and T-cell-mediated immune attack against liver-specific targets (hepatocytes, intrahepatic bile ducts, and the extrahepatic biliary tree), leading to a progressive inflammatory and fibrotic process in the liver [53, 63]. Various patterns of injury within the liver starting from a nonspecific response either against hepatocytes (AIH) or intrahepatic/extrahepatic bile ducts (PSC) could subsequently lead to collateral damages, developing finally a phenotype with mixed features [56] (Fig. 23.2).

Moreover, AIH and PSC might share similar genetic predispositions to autoimmune reactions. Indeed, studies on human leukocyte antigen (HLA) haplotypes in PSC and AIH suggest a common genetic basis for the immunologic mechanisms attributed to these diseases [64, 65]. A high prevalence of HLA-B8 and DR3 has been reported in both diseases, whereas DR4 appears to predispose to AIH but not to PSC and DR52 to associate with PSC [64, 65]. The few data available regarding "overlap patients" show that patients with PSC-AIH "variant syndrome" have similar frequency of DR3 compared to patients with either AIH or PSC [44, 47, 66]. On the other hand, pediatric patients with AIH alone have increased frequency of DR3 compared with patients with ASC [3]. The apparently common genetic background of both AIH and PSC could be the basis for the overlapping features of both diseases.

Over the last few years, a series of genome-wide association studies (GWASs) have identified several HLA and non-HLA risk loci for autoimmune liver diseases and have greatly increased the understanding of the genetic signature of AIH, PBC, PSC, and autoimmunity in general [67, 68]. In regard to findings supporting the mixed genetic basis for "variant syndromes," GWAS in AIH found a strong inflation of single-nucleotide polymorphisms in AIH patients associated specifically with PSC and PBC, suggesting the involvement of pleiotropic loci in AIH and other autoimmune liver diseases [69]. On the other hand, a large GWAS including several subphenotypes of PSC patients showed that the alleles DQA1*05:01 and DQB1*02:01 were most significantly associated with PSC-AIH "variant syndrome" [70]. However, in an older study of genetic risk factors in autoimmune liver diseases, Czaja et al. found that patients

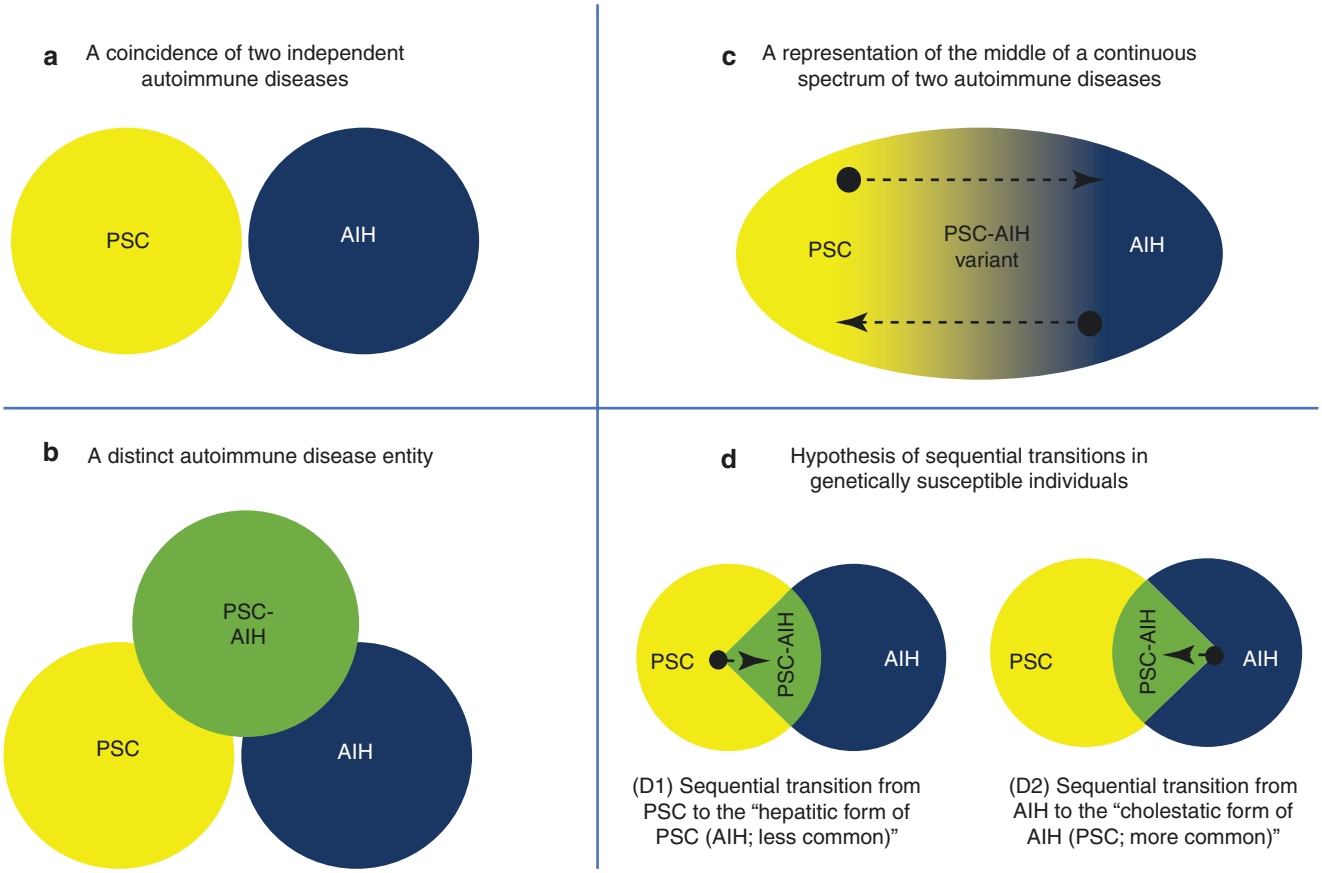

Fig. 23.1 Several clinical presentations and pathophysiologic mechanisms have been suggested for PSC-AIH variant syndrome: (**a**) A pure coincidence of two independent autoimmune diseases. (**b**) A distinct entity of autoimmune liver disease with clinical, biochemical, and histologic appearance of both AIH and PSC. (**c**) A representation of the middle of a continuous spectrum of two autoimmune diseases initiating from the one (PSC) or the other autoimmune liver disease (AIH). (**d**) A hypothesis of sequential transition from PSC or AIH to the other entity in genetically susceptible individuals, resulting in either the "hepatitic form of PSC" (**d**1; less common in clinical practice) or the "cholestatic form of AIH" (**d**2; more common), respectively

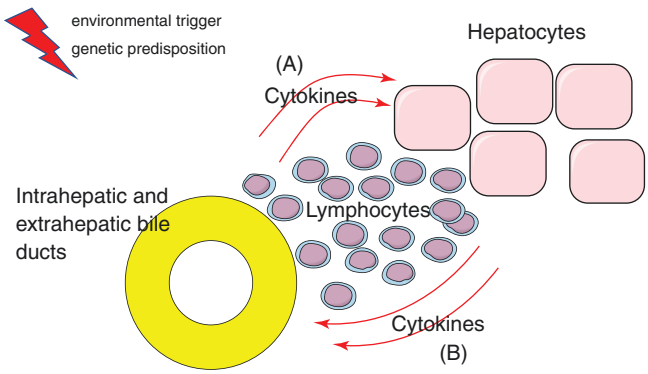

Fig. 23.2 Similar pathogenic ways of injury have been postulated for AIH and PSC including environmental triggers, genetic predisposition, and failure of immune tolerance mechanisms, which, in turn, may collaborate to induce an antibody- and T-cell-mediated immune attack against liver-specific targets (hepatocytes, intrahepatic bile ducts, and the extrahepatic biliary tree). The net result is a progressive inflammatory and fibrotic process in the liver. Various patterns of injury within the liver could reflect this unfocused immune response and create a phenotype with mixed features, depending on the first main attack ((**a**): Transition from PSC to AIH; (**b**): Transition from AIH to PSC)

with mixed genetic risk factors did not have distinctive features or manifestations of hybrid conditions, indicating that "overlap syndromes" rather are not due to the presence of mixed genetic factors and may have a different distinctive genetic predisposition than each primary disease or no genetic basis [65].

A "leaky gut" hypothesis has also been postulated in PSC due to its association with IBD [71–74]. Translocation of gastrointestinal flora from the inflamed gastrointestinal tract to the portal venous system causes a systemic inflammatory response, which may disrupt the tight junctions in biliary epithelial cells [75]. This alteration exposes cholangiocytes to bile acids that could promote injury and inflammation [76]. Another indirect indication for the role of intestinal microbiota is the association of atypical pANCA with both PSC and AIH, which could be marker of a pathogenetic link between these diseases in "variant syndromes" [77]. Particularly, environmental factors and genetic predisposition are thought to contribute to the development of atypical pANCA [78] as studies have shown that the B-tubulin antigen, which is a tar-

get antigen of atypical pANCA, cross-reacts with a bacterial antigen, namely, the FtsZ [79]. FtsZ is an evolutionary precursor protein of B-tubulin present in almost all bacteria of the intestinal microbiota. This suggests that molecular mimicry to bacterial products of enteric microbiome may also play a role in the development of "PSC-AIH variant."

In the same context, as both AIH and PSC can be associated with IBD, there is a study suggesting that liver disease may be driven by the recruitment of effector lymphocytes that were activated in the gut [80]. Twenty percent of the T cells infiltrating the liver in AIH or PSC complicating IBD were a4b7 + CCR9 +, suggesting their gut origin, whereas these cells are found at very low frequencies in other liver diseases [81, 82]. However, this hypothesis cannot explain the presence of liver disease in the absence of IBD.

PSC-AIH Variant

Epidemiology and Diagnosis

The prevalence of overlap characteristics between PSC and AIH is hard to ascertain because of the absence of well-validated diagnostic criteria, publication bias, and limitations in the interpretation of laboratory, histologic, and imaging studies [53]. Indeed, data regarding the PSC-AIH variant are derived not only from case reports and small series [47, 66] but also from larger groups of PSC patients [18, 44, 83–87]. According to these studies, the presence of PSC-AIH variant is observed in approximately 7–14% of mainly young male patients with PSC [7, 8, 13, 18]. In the most recent large multicenter study from the IPSCSG including 7121 patients with PSC, a prevalence of 6.6% was found [18]. Of interest, patients with PSC-AIH "variant syndrome" seem to have less common concurrent ulcerative colitis (UC) compared to patients with the classic PSC phenotype [18], although older studies have reported a comparable IBD prevalence [44, 52, 84, 87]. However, it should be emphasized that the diagnosis of the "variant syndromes" in most of the abovementioned studies was based on the application of the IAIHG diagnostic scoring systems, although these scoring systems have been developed rather to diagnose AIH and increase the discrimination of AIH from other liver diseases than to look for common features or the possible development/transition of one disease to another, making, therefore, their performance on the diagnosis of "variant syndromes" very problematic and suboptimal [7–9, 11, 13, 40, 41].

In everyday clinical practice, the diagnosis of PSC-AIH "variant syndrome" is based on the typical cholangiographic or histologic characteristics of PSC in combination with AIH features [7–9, 11, 13–16, 53, 88, 89]. However, the spectrum of PSC-AIH variant is diverse, because it can be presented as serologic/biochemical variant [high titers of antinuclear antibodies (ANAs)/SMA or LKM1, high IgG, high aminotransferases – usually ALT> 5×ULN – in PSC patients or ALP> 3×ULN (with or without elevated γ-GT; e.g., >5×ULN in children) in AIH patients], imaging variant (cholangiographic abnormalities suggestive of PSC in patients with clinical and laboratory features of AIH), or histologic variant (moderate or severe periportal or interface hepatitis on liver biopsy in patients with bile duct lesions, suggestive of PSC presence) (Table 23.2).

The situation is much more complex as up to 25% of patients with AIH have incidental histologic features of bile duct injury [90]. Therefore, bile duct changes at the histological level alone cannot justify the diagnosis of PSC-AIH "variant syndrome" in patients with AIH, but they should prompt a further cholangiographic work-up. Even then, imaging studies should be interpreted cautiously [22] as mild MRCP abnormalities have been found in up to a quarter of AIH patients that were rather attributed to secondary architecture distortion from hepatic fibrosis and nodular regeneration or from the development of cirrhosis, than from a concurrent development of PSC [91]. Indeed, it should be kept in mind that an intrahepatic biliary tree, which simulates a sclerosing pattern, can be observed in any liver disease after

Table 23.2 Diagnostic criteria and features of AIH and PSC-AIH variant

AIH (simplified IAIHG criteria)	ANA or SMA positivity ≥1/40 = 1 point[a] ANA or SMA positivity ≥1/80 = 2 points[a] or LKM positivity ≥1/40 = 2 points[a] or SLA/LP positivity at any titer = 2 points[a] IgG or γ-globulins levels > upper limit of normal = 1 point IgG or γ-globulins levels >1.1 upper limit of normal = 2 points Liver histology (evidence of hepatitis is a prerequisite) Typical with AIH = 2 points Compatible with AIH = 1 point Atypical = 0 points Absence of viral hepatitis B or C = 2 points
PSC-AIH variant syndrome	Probable or definite for AIH by the IAIHG criteria (interface hepatitis should be present) Cholangiography with multifocal bile duct strictures

Adapted from [7, 39]

Abbreviations: ANA antinuclear antibodies, *SMA* smooth muscle autoantibodies, *LKM* liver/kidney microsomal, *SLA/LP* soluble liver antigen/liver pancreas, *AIH* autoimmune hepatitis, *IAIHG* International Autoimmune Hepatitis Group, *PSC* primary sclerosing cholangitis

[a]Maximum 2 points from the sum of points achieved for all autoantibodies. Definite autoimmune hepatitis: ≥7; Probable autoimmune hepatitis: ≥6. Typical liver histology for autoimmune hepatitis includes all of the following features: interface hepatitis, lymphocytic/lymphoplasmocytic infiltrates in portal tracts and extending into the lobule, emperipolesis (active penetration by one cell into and through a larger cell), and hepatic rosette formation. Compatible liver histology for autoimmune hepatitis includes the presence of hepatitis with lymphocytic infiltration without all the features considered typical. Atypical liver biopsy includes signs of another diagnosis, such as steatohepatitis, granulomas, etc

extensive fibrosis [91]. Nevertheless, real cholangiographic abnormalities suggestive of the presence of PSC-AIH variant are found in AIH patients at a various prevalence depending on the age of patients: 2–10% in adults [91, 92] and up to 50% in children [3].

As in the clinical case presented in the introduction, in most of the reported cases of PSC-AIH "variant syndrome", the development of PSC and AIH usually occurs sequentially, with the diagnosis of AIH often proceeded that of PSC by several years [3, 66, 84, 93]. Therefore, in patients with a diagnosis of AIH, the development of biochemical cholestasis, coexisting IBD, or inadequate response to immunosuppressive therapy should prompt searching for PSC presence [92]. Sudden deterioration of liver function tests or suboptimal response to treatment of a previously well-controlled autoimmune liver disease should also raise the suspicion of "variant syndrome." In particular, an old study showed that if IBD is present, an abnormal cholangiogram can be found in up to 41% of patients [94]. However, due to the small number of patients in each report, the identification of a unique IBD-related phenotype specific to the PSC-AIH variant is difficult, although a relatively recent study has shown that intestinal disease had similar behavior compared to that observed in IBD-related PSC as most of the PSC-AIH patients with IBD had well-controlled colitis [83].

Less frequently, PSC and AIH may occur simultaneously [46, 47] or sequentially in patients with previously established PSC [84, 95, 96]. For this reason, in patients with PSC, an unusual elevation of serum aminotransferases, high ANA or SMA titers, and increased serum levels of IgG should raise the suspicion of AIH development. However, in the vast majority of cases with PSC-AIH variant, the exact time course of the two components of the syndrome cannot be defined precisely [47, 66, 93, 97]. A challenge for the diagnosis of the development of AIH in patients with previously established PSC is that liver biopsies are rarely required at the time of the diagnosis of PSC, leading to a paucity of data regarding the possible overall characteristics of AIH between PSC-AIH variant and AIH alone [98].

Apart from the classic PSC phenotype, patients with the small duct PSC variant (normal cholangiogram) have also been reported in association with AIH (small duct PSC-AIH variant). Of interest, the majority of these patients have also IBD (50–88%) [99]. However, because of the lack of a specific marker, it seems difficult to diagnose a "variant syndrome" of AIH and small-duct PSC, unless the typical periductular fibrosis is observed on liver biopsy in a patient with AIH and normal cholangiogram [24, 100].

Finally, the exclusion of IgG4-associated sclerosing cholangitis in patients with possible PSC-AIH variant is also important, as IgG4-related disease may cause both sclerosing cholangitis and hepatitis, which, in turn, may be mistakenly considered as PSC-AIH "variant syndrome" [4, 9, 101, 102]. The possibility for IgG4-associated sclerosing cholangitis should be raised in suspected PSC-AIH patients with older age, male gender, absence of IBD, presenting with jaundice, biliary strictures predominantly in the distal common bile duct, abnormal pancreatic imaging, multiorgan involvement, and substantial response after corticosteroid therapy [49]. Notably, the American Association for the Study of Liver Diseases (AASLD) guidelines for PSC diagnosis and management recommend measuring at least serum IgG4 levels in all patients with suspected PSC to exclude this condition [6].

Treatment and Outcome

The low prevalence of PSC-AIH "variant syndrome" has made it impractical to perform randomized controlled trials and to extrapolate standard guidelines for its treatment. Treatment of these patients is largely empiric and based on experience derived from the dominant autoimmune liver disease. This has led to high variability and conflicting results regarding treatment responses and outcomes [103]. Indeed, previous small case-studies showed better outcomes of patients suffering from the PSC-AIH variant compared to PSC alone probably because of the administration of combined treatments (immunosuppression along with UDCA) [83, 104], although other investigators have shown opposite results [88, 105]. Recently, the large study from the IPSCSG showed that the patients with PSC-AIH variant bear a similar risk for the progression of liver disease compared to the classic PSC albeit a significantly lower prevalence of hepatobiliary malignancy [18]. By contrast, the outcome of patients with PSC-AIH variant appears worse compared to AIH alone as attested by the higher rates of treatment failures, progression to cirrhosis and liver transplantation, resulting in a significantly reduced survival despite a good initial biochemical response to immunosuppression [47, 83, 88, 89, 97, 105]. However, it should be emphasized that many of these early reports did not use the currently accepted definition of AIH response defined as the complete normalization of all inflammatory parameters including AST, ALT, IgG, and liver histology [7, 8].

In general, it is now widely accepted that treatment should initially focus on the disease component that appears predominant and the other component of the variant should only be treated in the event of incomplete response. This approach of combined treatment with immunosuppressive agents and UDCA is advocated by international guidelines [6–8], although it is emphasized that because of lack of randomized controlled trials, treatment should be individualized based on clinical, laboratory, and histological findings. Indeed, the EASL clinical practice guidelines suggest the combination of UDCA and immunosuppressive therapy, although it is emphasized that this is not evidence based [7]. In parallel, the AASLD guidelines on PSC also recommend the use of corticosteroids and other immunosuppressants in patients with PSC-AIH "variant syndrome" [6], while the

IAIHG position paper suggests the consideration of immunosuppression with or without UDCA [11].

In this context, patients with PSC-AIH "variant syndrome" could be treated with combination of UDCA and immunosuppressive agents on an individual basis (Fig. 23.3). Indeed, administration of prednisone or prednisolone and azathioprine in combination with UDCA, at a daily dose not exceeding 15–20 mg/kg in order to avoid unexpected outcomes [106], has been associated in some studies with a better survival in adults with the "variant syndrome" than those suffering from classic PSC alone [83, 104, 107]. Of interest, in one of the latter studies, the use of immunosuppression resulted in better response in patients having the large-duct PSC component of the syndrome compared to those with the small-duct PSC component [107]. MMF has been ineffective in children with AIH and sclerosing cholangitis [108] and adults with classic PSC [109], while the reported experience with the calcineurin inhibitors in the treatment of the AIH-PSC "variant syndrome" has been scarce [110]. However, it should be kept in mind that in "variant syndromes," the response to therapy might be dependent on the predominance of AIH or PSC and at least in adults, the response rates to immunosuppressants, in particular the AIH component of the syndrome, can be excellent and occasionally may lead to the complete remission of disease activity (see Fig. 23.3) [85].

For patients with end-stage liver disease, liver transplantation is the only treatment option. Of note, in a retrospective study, the diagnosis of "variant syndrome" was associated with a three-fold increased risk of disease recurrence after liver transplantation compared to patients with one autoimmune liver disease, although the graft and patient survivals were not affected [111]. In addition, recurrence seems to present earlier in patients with "variant syndromes," suggesting a more aggressive disease or an additive effect of the combined autoimmune liver disorders [111]. Interestingly, all patients with PSC-AIH variant in this study had similar risk factors for recurrence compared to those that have been associated with PSC recurrence after liver transplantation in patients with PSC alone such as IBD [93, 112, 113] and recurrent acute cellular rejection [114]. The majority of patients (70%) had recurrence of the one component of the syndrome; however, it is not known if the features of the second disease will reappear in the long-term.

Autoimmune Sclerosing Cholangitis (ASC)

Epidemiology and Diagnosis

Pediatric autoimmune liver disease comprises AIH, ASC, PSC alone (infrequently) and de novo AIH after liver transplantation for other hepatic diseases [115]. Among them, ASC is a specific and unique variant that has been reported almost 20 years ago in approximately half of children and adolescents with an initial diagnosis of AIH, characterized by both AIH and features of sclerosing cholangitis [3, 89, 115–117]. In that large prospective case-series study of ASC in children and adolescents published in 2001 [3], 55 pediatric patients with clinical, biochemical, and histologic signs of AIH were followed for 16 years (1984–1997). Cholangiographic studies with invasive techniques (ERCP or percutaneous transhepatic cholangiography) were included in the initial work-up irrespective of clinical, biochemical, or histologic evidence of cholestasis. Additionally, patients underwent sigmoidoscopy with rectal biopsy, regardless of the presence of gastrointestinal symptoms. In about half the patients (27/55), cholangiographic findings were typical of sclerosing cholangitis at baseline, establishing the diagnosis of ASC. Of note, 50% of the patients with ASC were male. Abdominal pain, weight loss, and intermittent jaundice were frequently present in both ASC and AIH-1, while IBD

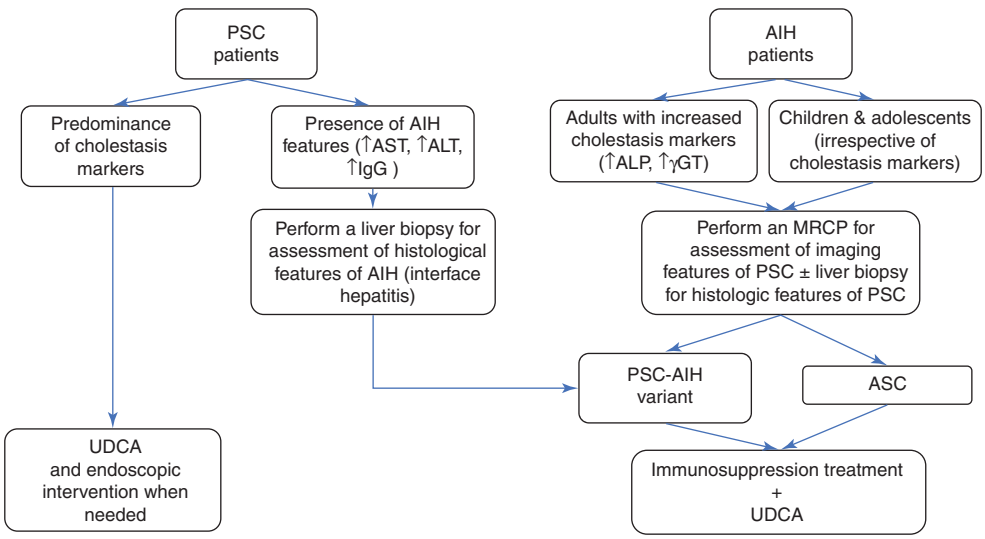

Fig. 23.3 Diagnostic algorithm and treatment of adult patients with PSC-AIH variant and children with ASC

affected more often patients with ASC than AIH (45% vs. 20%, respectively). Biochemical cholestasis markers did not help in discriminating between AIH and ASC at presentation, taking into account that γ-GT and ALP levels were within normal limits in 26% of patients with ASC, whereas only two had typical "onion skin" periductular fibrosis on liver biopsy [3]. Nearly all ASC patients in the original study had raised IgG serum levels, while ANA and SMA positivity was found equally between ASC patients and patients with AIH only in most of them. By contrast, anti-LKM was rarely reported in this "variant syndrome," as only 1 out of 27 patients with ASC had LKM reactivity. Finally, pANCAs were present in 75% of patients with ASC compared to 45% of patients with AIH-1 and 10% of those with AIH-2 [3]. According to the original authors, the term ASC was selected for this specific PSC-AIH "variant syndrome" in pediatric patients in an attempt to underscore the favorable response to immunosuppression compared to the classic PSC phenotype [3].

The reason why ASC is described more commonly in children (reported prevalence: 20–49%) [3, 89, 118] than PSC-AIH variant in adults is obscure, although it might reflect the lack of burnout of autoimmune hepatitic inflammation in children [119]. However, as it is shown in the case presentation, it is unclear whether PSC-AIH "variant syndrome" in adults represents an evolution of pediatric ASC in the same patient. From the genetic points of view, HLA studies have suggested that HLA-DR13 is associated more frequently with ASC, while DR3 confers susceptibility to AIH-1 and that of DR7 to AIH-2 [116]. Unlike AIH, male and female patients are equally affected by ASC. Approximately half of the children with ASC have concurrent extrahepatic autoimmune diseases, which is comparable to that observed in pediatric patients with AIH alone [120]. IBD, mostly asymptomatic or with minimal symptoms, represents the most common associated extrahepatic disease in pediatric patients with ASC [3, 89, 120].

In this regard, it has been proposed that the chronic IBD associated with ASC may represent a distinct entity, different from the classic phenotypes of UC and Crohn disease (CD), as attested by the presence of right-sided colitis with frequent rectal sparing and small bowel mucosal breaks on capsule enteroscopy [121]. Indeed, Bjarnason et al. [121] have given new insights on ASC, PSC, and IBD, implicating a potential interaction of the gut–liver axis. In particular, they studied the inflammation associated with ASC and compared the findings with those seen in patients with PSC-associated colitis and patients with IBD alone. The microscopic findings of the colonic mucosa were identical between patients with ASC and PSC. The inflammation was almost invariably more pronounced in the right colon, and there were no features suggestive of CD. However, the findings in the small bowel on capsule endoscopy were different between these entities with over a third of patients with ASC showing mucosal breaks similar to that seen in CD. By contrast, all capsule endoscopy studies in the PSC patients were normal apart from one patient who had features of backwash ileitis. Conclusively, this study clearly showed that patients with ASC and PSC had identical forms of colitis; however, 39% of ASC patients also had small bowel erosions, a pattern more representative of that of CD-like lesions, highlighting the contrasting pattern of liver–gut cross talk in PSC and ASC [121, 122]. These observations may suggest that IBD associated with liver diseases such as PSC or ASC rather need to be classified as separate disease entity, namely, chronic IBD associated with chronic liver disease, than being clustered as classic UC or CD [121–124]. Concerning the time course of this entity, a large retrospective study showed that IBD can precede the diagnosis of liver disease by many years, be diagnosed at the same time, or develop during follow-up [54].

Regarding diagnosis, a new scoring system has recently been proposed by the European Society of Paediatric Gastroenterology Hepatology and Nutrition Hepatology Committee, although a validation is required [115] (Table 23.3). Neither the revised nor the simplified diagnostic scoring systems

Table 23.3 Proposed scoring criteria for the diagnosis of juvenile autoimmune liver disease

Variable	Cut-off	Points	
		AIH	ASC
ANA and/or SMA[a]	≥1:20[b]	1	1
	≥1:80	2	2
Anti-LKM-1[a]	≥1:10[b]	1	1
	≥1:80	2	1
Anti-LC1	Positive[b]	2	1
Anti-SLA/LP	Positive[b]	2	2
pANCA/ANNA	Positive	1	2
IgG	>ULN	1	1
	>1.2 ULN	2	2
Liver Histology	Compatible with AIH	1	1
	Typical of AIH	2	2
Absence of viral hepatitis (A, B, E, EBV), NASH, Wilson disease	Yes	2	2
Presence of extrahepatic autoimmunity	Yes	1	1
Family history of autoimmune disease	Yes	1	1
Cholangiography	Normal	2	−2
	Abnormal	−2	2

Adapted from [115]

Abbreviations: AIH autoimmune hepatitis, *ASC* autoimmune sclerosing cholangitis, *ANA* antinuclear antibody, *SMA* smooth muscle antibody, *anti-LC1* antiliver cytosol type 1 antibody, *anti-LKM-1* antiliver kidney microsomal antibody type 1, *anti-SLA/LP* antisoluble liver antigen/liver pancreas, *pANCA/ANNA* peripheral antineutrophil cytoplasmic antibody/antinuclear neutrophil antibody, *IgG* immunoglobulin G, *ULN* upper limit of normal, *EBV* Epstein–Barr virus, *NASH* nonalcoholic steatohepatitis

[a]Antibodies measured by indirect immunofluorescence on a composite rodent substrate (kidney, liver, stomach)

[b]Sum of points achieved for ANA, SMA, anti-LKM-1, anti-LC-1, and anti-SLA/LP autoantibodies cannot exceed a maximum of 2 points. Score ≥7: probable AIH; ≥8: definite AIH. Score ≥7: probable ASC; ≥8: definite ASC

[37, 38] are suitable to discriminate between AIH and ASC, as they do not include cholangiographic studies at disease onset [115, 125–127]. Indeed, all 55 patients included in the original prospective study from UK [3] had a definite or probable AIH diagnosis according to the revised IAIHG diagnostic score indicating its insufficiency to differentiate between AIH and ASC. Therefore, current guidelines recommend active screening for ASC by using MRCP in every child or adolescent with an initial diagnosis of AIH irrespective of the presence of elevated cholestatic enzymes [5, 6, 8, 9, 115]. In children, the reported sensitivity and specificity of MRCP for the diagnosis of sclerosing cholangitis range are 81–89% and 84–100%, respectively [128, 129]. Accordingly, the increasing use and better performance of MRCP imaging has led to recategorize many pediatric patients previously characterized as AIH to ASC [130].

Contrary to AIH where liver biopsy and the presence of hepatitis is a prerequisite for AIH diagnosis [5, 7, 8, 25, 26], liver histology in childhood sclerosing cholangitis may or may not show bile duct damage with some features being more typical for other specific diseases [3, 131]. In this context, despite abnormal cholangiograms, one-quarter of the children with ASC in the original study had no histologic features suggestive of bile duct involvement [3]. Of note, the classic histologic picture of adult PSC with periductular concentric fibrosis ("onion-skin"-like) is rarely seen in pediatric sclerosing cholangitis [117], suggesting that it is the result of long-standing biliary inflammation. Therefore, the high-quality cholangiogram remains the only effective tool to differentiate patients with AIH alone from those with ASC, because ASC accounts for the majority of sclerosing cholangitis cases in childhood and it is as prevalent as AIH in children [22, 130, 132].

Treatment and Outcome

In ASC, the recommended immunosuppressive therapy is similar to that used in AIH (steroids with or without azathioprine) with the addition of UDCA (~15 mg/kg/day) [115; Fig. 23.3]. The King's College prospective study showed that if treatment started early, the hepatocellular damage responds adequately in terms of normalization of biochemical parameters with good medium- to long-term survival [3]. However, it is not clear whether the biliary changes are reversible with this combination therapy, because despite the improvement at the biochemical and histological levels, the biliary lesions progressed in about half of the patients, particularly in those with resistant IBD. This led to a poorer long-term outcome for patients with ASC compared to AIH patients, as 27% of the ASC patients ultimately required liver transplantation [3]. Later, other series reported similar outcome after immunosuppression [133], while some reported worse outcome with up to one-third of the patients being listed for liver transplantation over a median follow-up of 3.8 years [134, 135]. Postliver transplant recurrence rate in ASC was as high as 71%, with data suggesting that uncontrolled IBD is a risk factor for ASC recurrence after liver transplantation [3, 133, 136]. Recently, a retrospective study in patients with pediatric-onset PSC showed that the phenotype of PSC-AIH variant was an independent prognostic factor associated with poorer long-term outcome [137].

Management of Other Complications

Management of other complications of PSC should also be emphasized in patients with PSC-AIH "variant syndrome." There are few data for the treatment of pruritus in PSC, and most recommendations are extrapolated from trials in PBC [138]. The current guidelines for the management of pruritus recommend cholestyramine as first-line therapy, and rifampicin, naltrexone, and sertraline, as second-, third-, and fourth-line treatments, respectively [9, 139, 140]. However, pruritus associated with advanced liver disease is difficult to be managed medically, and therefore, treatable biliary obstructions should be sought. Accordingly, clinically significant dominant strictures should be managed by balloon dilatation with or without stenting [6, 141]. Surveillance for CCA including gall-bladder cancer and colorectal cancer in patients with IBD should also be performed as suggested for PSC [4, 6, 9, 10], although the development of these malignancies in PSC-AIH "variant syndrome" has not been reported so far [83, 88]. Unique indications for liver transplantation as intractable pruritus, recurrent bacterial cholangitis, and CCA, which have been suggested in patients with PSC, may also be applied for patients with PSC-AIH "variant syndrome" [4, 6, 9, 10].

Conclusion

Conclusively, MRCP screening is advised for all children and adolescents with an initial AIH diagnosis, while in adults with established AIH, cholangiography imaging studies should be considered only to those with marked cholestasis despite adherence and adequate immunosuppressive treatment. Similarly, it is reasonable to recommend further testing for the presence of AIH including liver histology among PSC patients with high aminotransferases (usually >5×ULN) and/or high IgG levels [7, 8, 115].

However, the establishment of precise and reliable criteria for the diagnosis of PSC-AIH variant is still an unmet need in both children and adults. Also, there is an urgent need to clarify which PSC patient will benefit from the addition of immunosuppression and therefore, multicenter randomized controlled trials for the management of PSC-AIH variants are needed.

In general, however, it should be kept in mind that PSC-AIH "variant syndromes" should not be overdiagnosed in order not to expose PSC patients unnecessarily to the long-term risk of steroid side effects. On the other hand, catastrophic consequences of a missed opportunity to initiate immunosuppression in overlap patients have occasionally been reported [7].

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Primary Biliary Cholangitis: Autoimmune Hepatitis Overlap Syndrome

24

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Key Points

- Some patients present with features of both primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH) either simultaneously or consecutively.
- The term overlap syndrome (OS) is used to describe these settings, but lack of universal agreement on what precisely constitutes an OS has generated considerable confusion.
- The low prevalence of OS (roughly 10% of PBC) has made it impracticable to perform randomized controlled trials.
- It remains unclear whether this syndrome forms a distinct entity or, more likely, a variant of PBC, or AIH.
- Moderate-to-severe interface hepatitis is a fundamental component and histology is vital in evaluating patients with overlap presentation. Use of the International Autoimmune Hepatitis Group criteria for the diagnosis of OS is not recommended.
- For PBC-AIH OS, EASL has provided diagnostic criteria and, in most cases, it is possible to define one primary disorder ("dominant" disease), usually PBC.
- Patients with OS seem to have a more severe disease compared to conventional PBC.
- Treatment of OS is empiric and includes ursodeoxycholic acid (UDCA) for the cholestatic component and immunosuppressive agents for the hepatitic component, either simultaneously or sequentially. Immunosuppressive treatment in addition to UDCA is recommended in patients with severe interface hepatitis and deserves consideration in those with moderate interface hepatitis.
- The dominant clinical feature should be treated first and therapy adjusted according to the response.
- OS is not uncommon but should not be over-diagnosed in order not to expose unnecessarily PBC patients to the risk of steroid side effects. Therapy has to be individualized and not be static.

Introduction

Three well-defined rare autoimmune diseases, namely, autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC), may affect the liver. AIH targets hepatocytes and is characterized by a predominant hepatocellular injury, whereas PBC and PSC target bile ducts and are characterized by predominant cholestatic features. These three diseases are generally differentiated easily on the basis of clinical, biochemical, serological, radiological, and histological findings. However, patients presenting with features of PBC on one hand and AIH on the other hand, either simultaneously or consecutively, have been repeatedly recognized. The term overlap syndrome is often used to describe these variant forms. Unfortunately, lack of universal agreement on what precisely constitutes an overlap syndrome has generated considerable confusion in the literature and the clinical phenotypes of patients with the same overlap syndrome designation exhibit considerable heterogeneity [1]. As a result, "overlap syndrome" is one of the most abused descriptive terms currently used in hepatology [2].

Broadly similar pathogenic themes of injury have been postulated for AIH and PBC and include environmental triggers, genetic predisposition, and failure of immune tolerance mechanisms, whereby liver disease represents the result of a cell- and antibody-mediated immunological attack against liver-specific targets.

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The overlap syndrome pathogenesis is highly debated, and it remains unclear whether two distinct diseases co-exist in one patient; whether these forms are an own entity or whether they represent a variant form of either disease (PBC or AIH). The latter seems to be the most appropriate, since a predominant phenotype can be identified in most cases. For example, in PBC-AIH overlap, it has been proposed that overlap represents a "hepatitic" form of PBC in genetically susceptible individuals (HLA-B8, DR3, or DR4 positive) [3]. This would fit with the hypothesis that immune-mediated disease can develop ("secondary" AIH) in any susceptible host if, for some reason, the local milieu becomes pro-inflammatory. In this regard, the name overlap that strongly suggests the presence of 2 distinct diseases could be a misnomer. As a result, according to the EASL AIH and PBC guidelines, the preferred terminology to describe these conditions is now "variants forms", primarily variants forms of the cholestatic autoimmune liver disease with autoimmune features [4, 5]. By contrast, recent British and US PBC guidelines still use the term "overlap" [6, 7].

The aim of this chapter is to describe the overlap syndrome (OS) between primary biliary cholangitis and autoimmune hepatitis (PBC-AIH), especially focusing on the clinical presentation of this syndrome compared to the individual diseases, the diagnostic criteria including the histological features, the therapy and the natural history of OS, and finally, the association with extra-hepatic autoimmune disorders.

The issue of PBC-AIH OS is still controversial because, since its first description [8–11], a number of studies, using different criteria to define the OS, have been published. Moreover, most studies are single-centre retrospective cohort studies including as control group PBC or AIH patients or both of the diseases. Because of the absence of well-validated diagnostic criteria and publication bias, the prevalence of overlap syndromes is difficult to ascertain and diagnosis remains a challenge. It should be kept in mind that overlap syndrome should not be over-diagnosed in order not to expose PBC patients unnecessarily to the risk of steroid side effects. On the other hand, tragic consequences of a missed opportunity of instituting immunosuppressive therapy in overlap patients have occasionally been reported [12]. The low prevalence of overlap syndromes has made it impracticable to perform randomized controlled trials. As a consequence, treatment of overlap syndrome is largely empiric.

Clinical Presentation of Overlap Syndrome

The frequency of PBC-AIH OS in PBC and AIH patients varies between 1% and 28% [13–35] and 1.2% and 27% [17, 18, 22, 24, 27, 30, 34, 36, 37], respectively, depending on the diagnostic criteria used to define the OS and the sample size of the control group.

PBC-AIH OS may present *simultaneously* or *consecutively*. The simultaneous presentation of the two diseases is more frequent than the sequential. However, some patients may present with features of the second disease during the follow-up. The *simultaneous occurrence* of PBC and AIH is characterized by a hepatitic and cholestatic profile at the same time, an elevation of both serum immunoglobulin G (IgG) and immunoglobulin M (IgM), the positivity of autoantibodies characterizing the two diseases, and the presence of histological features of both PBC and AIH [20, 30]. The *sequential development* of OS may present in two different modalities. In most cases, *PBC is the first diagnosis* and AIH occurs later during the follow-up, in a variable interval of time from the first diagnosis that ranges from 6 months to 14 years [13, 17, 27, 29, 38]. More rarely, patients with AIH may develop PBC within 1–20 years after the initial diagnosis of AIH [17, 26, 27, 29, 36, 39]. The clinical presentation of sequential AIH in previous PBC was described as the occurrence of increased hepatitis activity, hypergammaglobulinaemia, and, eventually, jaundice during the course of PBC [25, 38]. The development of AIH in a patient is unpredictable; indeed, similar baseline characteristics at the time of PBC diagnosis were observed in patients with "pure" PBC and patients who later developed AIH [29]. Differently, the *development of PBC in AIH patients* has been less characterized and again, no differences in baseline features between AIH patients who later developed PBC-AIH OS or patients with typical AIH were reported [27]. Dinani et al. reported three cases of AIH with anti-mitochondrial antibodies (AMAs) positivity, which later developed PBC [39].

The sequential development of overlap should be suspected when a hepatitic or a cholestatic flare appears during the course of the disease, or when an incomplete response to standard treatment is observed. In these cases, a diagnostic workup, including liver biopsy, to exclude or confirm the presence of OS is recommended [5, 40].

Symptoms of OS are usually fatigue in 67–83% of patients [20, 31, 38] and pruritus in 20–57% of patients [14, 18, 20, 23, 31, 34, 36, 38]. The latter seems to be less frequent in patients with OS compared to patients with pure PBC [28, 33]. Other reported symptoms are malaise, abdominal pain, weight loss, and general symptoms of chronic liver diseases [41].

Age at diagnosis is variable (just as in pure PBC or AIH), but, in some studies, patients with OS were reported to be younger at diagnosis than those with pure PBC [18, 28, 34].

Diagnosis of Overlap Syndrome

Diagnostic Criteria

The diagnosis of OS is based on the concomitant presence or sequential development of biochemical, serological, and histologic features of the two diseases. Three studies published at

the end of 1990s systematically described three series of PBC-AIH and proposed different diagnostic criteria [3, 20, 30].

Chazouillères et al. reported about 12 patients with PBC-AIH overlap syndrome, which were identified among PBC patients by the presence of PBC and AIH either simultaneously or consecutively [20]. For the diagnosis of each disease, the presence of at least two of the three accepted criteria was required. PBC criteria were (1) serum alkaline phosphatase (AP) levels at least two times the upper limit of normal values or serum gamma-glutamyl transpeptidase (GGT) levels at least five times the upper limit of normal values; (2) a positive test for anti-mitochondrial antibodies (AMAs); and (3) a liver biopsy specimen showing florid bile duct lesions. AIH criteria were the following: (1) serum alanine transaminase (ALT) levels at least five times the upper limit of normal values; (2) serum immunoglobulin G (IgG) levels at least two times the upper limit of normal values or a positive test for anti-smooth muscle antibodies (ASMAs); and (3) a liver biopsy showing moderate or severe periportal or periseptal lymphocytic piecemeal necrosis [20]. These criteria are known as *Paris criteria* (Table 24.1).

In the same year, Lohse et al. described a series of 14 OS identified among AIH and PBC patients by selecting those displaying histological and clinical features of both diseases but with less strict criteria [3].

Finally, Czaja reported a series of variant syndromes of autoimmune liver diseases and identified 15 cases of PBC-AIH which were AIH-1 and PBC, all displayed an aggregate score for AIH, according to the original score proposed by the International Autoimmune Hepatitis Group (IAHG) [42] (Table 24.2), of 10 or greater and seropositivity for AMA [30].

Paris criteria were applied in several studies (Table 24.3) [13, 16, 19, 21, 23–25, 27–29, 33–35, 41, 43] and were shown to be effective in diagnosis of OS with a sensitivity of up to 92% and a specificity of 97% [43], keeping in mind that it is difficult to assess a diagnostic performance in the

Table 24.1 Paris criteria for the diagnosis of overlap PBC-AIH [20, 46]

Autoimmune hepatitis
1. Alanine aminotransferase (ALT) ≥ 5 X upper normal limit 2. Immunoglobulin G (IgG) ≥ 2 X ULN or presence of anti-smooth muscle antibodies 3. Liver biopsy with moderate or severe periportal or periseptal lymphocytic piecemeal necrosis
Primary biliary cholangitis
1. Alkaline phosphatase (ALP) ≥ 2 X ULN or gamma-glutamyl transferase ≥5 X ULN 2. Presence of AMA 3. Liver biopsy with florid bile duct lesions
At least two of three accepted criteria for PBC and AIH, respectively, should be present. Histologic evidence of moderate-to-severe lymphocytic piecemeal necrosis (interface hepatitis) is mandatory for the diagnosis

Table 24.2 Original IAIHG scoring system for diagnosis of AIH [42]

Category	Factor	Score
Gender	Female	+2
ALP:AST ratio	≥3 <3	−2 +2
γ-globulin or IgG levels above the normal	>2.0 1.5–2.0 1.0–1.5 <1.0	+3 +2 +1 0
ANA, SMA, or anti-LKM1 titres	>1:80 1:80 1:40 <1:40	+3 +2 +1 0
AMA	Positive	−2
Viral markers	HBsAg IgM anti-HAV HCV RNA Other viruses Anti-HCV All negative	−3 −3 −3 −3 −2 +3
HLA	DR3 or DR4	+1
Alcohol	<25 g/day >40 g/day	0 −2
Immune disease	Patient or relative	+1
Histological features	Interface and acinar hepatitis with bridging Interface hepatitis Rosettes Plasma cells Biliary changes Other features	+3 +2 +1 +1 −1 −1
Blood transfusion or drugs	Yes No	−2 +1
Pre-treatment score	Definite diagnosis Probable diagnosis Non-diagnostic	>15 10–15 <10

Table 24.3 Summary of studies regarding PBC-AIH overlap syndrome and diagnostic criteria used to define OS

Authors	Year	Patients' country	Population	Diagnostic criteria
Chazouillères et al. [20]	1998	France	PBC ($N = 130$)	*Paris criteria*
Czaja [30]	1998	USA	AIH ($N = 162$), PBC ($N = 37$), PSC ($N = 26$)	Type I AIH or PBC with revised score ≥ 10 and AMA+
Lohse et al. [3]	1999	Germany	OS ($N = 20$), PBC ($N = 20$), AIH ($N = 20$)	*Histology*: bile duct damage and hepatitis *Serology*: AMA+ and other autoantibodies + *Biochemistry*: transaminases >2 x ULN and cholestatic enzymes elevation

(continued)

Table 24.3 (continued)

Authors	Year	Patients' country	Population	Diagnostic criteria
Talwalkar et al. [31]	2002	USA	PBC ($N = 137$)	Revised IAHG vs. original IAHG
Joshi et al. [16]	2002	USA, Canada	PBC ($N = 331$)	Paris criteria
Muratori et al. [15]	2002	Italy	PBC ($N = 142$), AIH ($N = 70$)	Revised score
Suzuki et al. [22]	2004	Japan	AILD ($N = 227$)	Diagnostic criteria for both PBC and AIH at presentation or during follow-up
Gheorghe et al. [37]	2004	Romania	AIH ($N = 82$)	Revised score for AIH and 2 out of 3: cholestatic profile AMA >1:40 florid bile duct lesions
Chazouillères et al. [21]	2006	France	PBC ($N = 190$)	Paris criteria
Poupon et al. [29]	2006	France	PBC ($N = 282$)	Paris criteria
Saito et al. [23]	2006	Japan	PBC ($N = 103$)	Paris criteria
Amarapurkar et al. [38]	2006	India	AILD ($N = 102$)	Simultaneous or sequential diagnosis of PBC and AIH
Heurgué et al. [34]	2007	France	AIH, PBC, OS ($N = 115$)	Paris criteria
Alric et al. [35]	2007	France	AIH, PBC, OS ($N = 102$)	Paris criteria (AMA+ and AMA− PBC)
Silveira et al. [32]	2007	USA	PBC ($n = 135$)	Revised IAHG score in AMA+ PBC
Gossard and Lindor [14]	2007	USA	PBC ($n = 1476$)	Transaminases >5 × ULN and liver biopsy confirming AIH in AMA+ PBC
Al-Chalabi et al. [36]	2008	UK	AIH, AIH-PBC, AIH-PSC ($N = 238$)	Definite AIH (original score) and both histological features of PBC and AIH
Lindgren et al. [17]	2009	Sweden	AIH, PBC, variant forms	Concurrence of PBC and AIH (revised score)
Muratori et al. [24]	2009	Italy	PBC ($N = 120$), AIH ($N = 120$) OS = 15	Paris criteria
Yokokawa et al. [25] (extension of Saito 2006)	2010	Japan	PBC ($n = 144$), AIH ($N = 73$)	Paris criteria

Table 24.3 (continued)

Authors	Year	Patients' country	Population	Diagnostic criteria
Bonder et al. [13]	2011	USA	PBC ($N = 609$)	Paris criteria 2 Paris criteria + response to IS therapy 1 Paris criteria + response to IS therapy
Tanaka et al. [18]	2011	Japan	PBC ($N = 1081$), AIH ($N = 597$)	Simultaneous biochemical, serological, and histopathological features of both PBC and AIH at presentation
Ozaslan et al. [51]	2013	Turkey, France, Italy, USA, Sweden	–	Paris criteria
Liu et al. [19]	2014	China	PBC ($N = 65$)	Paris criteria; revised and simplified IAHG score
Efe et al. [27]	2014	Turkey, France, USA, Italy, Sweden	PBC ($N = 483$), AIH ($N = 582$)	Paris criteria
Yoshioka et al. [26]	2014	Japan	PBC ($N = 280$)	PBC with AIH features: high transaminases, ANA or SMA positivity, and/or histological features of moderate-to-severe IH or lobular hepatitis
Levy et al. [33]	2014	USA	PBC ($N = 204$)	Paris criteria or PBC + simplified score > 6
Yang et al. [28]	2016	China	PBC ($N = 323$)	Paris criteria in AMA+ and AMA- PBC
Fan et al. [41]	2018	China	–	Paris criteria

absence of gold standard. In other studies, both the *revised AIH score* [44] (Table 24.4) and the *simplified AIH score* [45] (Table 24.5) were applied to PBC patients to retrospectively identify patients treated with corticosteroids. However, these AIH scores were not originally developed to diagnose cholestatic variants of AIH or to diagnose AIH in patients with PBC. Indeed, the presence of PBC features (i.e. ALP/AST ratio > 3, AMA positivity and biliary changes at liver biopsy) is scored negatively and this accounts for a lower diagnostic performance in patients with OS compared to Paris criteria

Table 24.4 Revised IAIHG scoring system for diagnosis of AIH [44]

Category	Factor	Score
Gender	Female	+2
ALP:AST ratio	<1.5 1.5–3.0 >3.0	−2 0 −2
γ-globulin or IgG levels above the normal	>2.0 1.5–2.0 1.0–1.5 <1.0	+3 +2 +1 0
ANA, SMA, or anti-LKM1 titres	>1:80 1:80 1:40 <1:40	+3 +2 +1 0
AMA	Positive	−4
Hepatitis viral markers	Positive Negative	−3 +3
Drug history	Positive Negative	−4 +1
Alcohol	<25 g/day >60 g/day	+2 −2
Histological features	Interface hepatitis Predominantly lymphoplasmacytic infiltrate Rosettes None of the above Biliary changes Other changes	+3 +1 +1 −5 −3 −3
Other autoimmune disease(s)	Patients or first-degree relatives	+2
Optional additional parameters (for patients seronegative to ANA, SMA, anti-LKM-1) Seropositivity for other autoantibodies	pANCA, anti-LC1, anti-SLA, anti-ASGPR, anti-LP, and anti-sulfatide	+2
HLA	DR3 or DR4	+1
Response to therapy	Complete relapse	+2 +3
Interpretation of aggregate scores		
Pre-treatment	*Definite AIH* *Probable AIH*	*>15* *10–15*
Post-treatment	*Definite AIH* *Probable AIH*	*>17* *12–17*

Table 24.5 Simplified diagnostic criteria for AIH [45]

Component	Result	Score
ANA or SMA	≥1:40 ≥1:80	1
Or LKM1 antibody Or SLA	≥1:40 Positive	2
IgG	≥Upper limit of normal ≥1.1 times upper normal limit	1 2
Liver histology (evidence of hepatitis is a necessary condition)	Compatible with AIH Typical of AIH	1 2
Absence of viral hepatitis	Yes	2
Interpretation of scores	*Definite AIH* *Probable AIH*	≥7 ≥6

Table 24.6 Diagnostic performance of different diagnostic criteria of OS [43]

	Sensitivity	Specificity
Paris criteria	92%	97%
Revised AIH score (definite AIH)	0%	79%
Revised AIH score (probable AIH)	60%	83%
Simplified AIH score (definite AIH)	40%	80%
Simplified AIH score (probable AIH)	73%	78%

(Table 24.6) [43]. For these reasons, the use of revised and simplified AIH scores is not recommended in clinical practice for the diagnosis of overlap syndrome PBC-AIH [40, 46]. Most authors agree that the Paris criteria provide a diagnostic template that can be consistently applied and the 2009 European Association of the Study of the Liver guidelines on the management of cholestatic liver diseases endorsed the Paris criteria for the diagnosis of OS and specified that histologic evidence of moderate-to-severe lymphocytic piecemeal necrosis (interface hepatitis) was mandatory for the diagnosis of PBC-AIH OS. Moreover, the same guidelines stated that OS should always be considered once PBC has been diagnosed and in case of poor response to UDCA because of potential therapeutic implications [46]. Nevertheless, there are still several areas of uncertainty including the cut-offs for IgG/gamma-globulins and transaminases levels to indicate liver biopsy and the grade of hepatitis activity to indicate immunosuppression [5]. Indeed, the recent EASL guidelines on AIH recommends treatment for patients with AIH at lower cut-offs for transaminase or IgG levels and an histological HAI score as low as 4 [4].

Otherwise, Paris criteria may not identify patients with less severe forms of OS, which did not fulfil the biochemical criteria or serological criteria despite the presence of histologic features of both PBC and AIH. To overcome these limitations, *a new scoring classification for OS* was recently proposed (Table 24.7) [47]. However, this score is potentially associated with an over-estimation of diagnosis of OS and thus over-treatment of these patients. An external validation is mandatory before its dissemination.

Biochemical Features

Biochemical features of patients with OS are typically characterized by hepatitic and cholestatic profile and an elevation of both immunoglobulin G and immunoglobulin M. In comparison with patients with pure PBC, patients with OS showed, as expected, higher transaminases [3, 18, 22–25, 28, 34, 35], higher gamma-globulin [3, 31, 34], and higher IgG [16, 18, 23–25, 28]. Moreover, even if less frequently reported, patients with OS may show higher alkaline phosphatase [23], higher GGT [25], and higher total bilirubin [28] compared to pure PBC patients. Otherwise, compared

Table 24.7 New scoring classification for OS [47]

Component	Result	Score
Biochemical category		
AST or ALT above ULN	>2	+3
	1.5–2	+2
	1–1.5	+1
	<1	0
ALP above ULN	>1	+2
	0.75–1	+1
	<0.75	0
Serum globulin above ULN	>1.5	+2
	1–1.5	+1
	<1	0
Immunological category		
ANA, ASMA, or LKM1	>1:80	+3
	1:80	+2
	1:40	+1
	<1:40	0
or		
Anti-SLA, pANCA	Positive	+2
AMA	Positive	+3
Histologic category		
	Interface hepatitis	+3
	Lymphoplasmacytic	+1
	Hepatic rosettes	+1
	Biliary damage	
	Granulomas	+3
	Florid ductal lesions	+1
	Ductular proliferation	+1
	Bile duct loss	+1
Other categories		
Viral marker	Positive	−3
	Negative	+3
Drugs	Yes	−4
	No	+1
Alcohol	<25 g/day	+2
	>60 g/day	−2
Interpretation of scores	*Definitive* *Probable* *Rejected*	*>21* *19 or 20* *<19*

to patients with pure AIH, OS patients show higher alkaline phosphatase [3, 18, 24, 25, 34, 35, 48] both at baseline and also during remission [3], higher GGT [3, 25, 34], and IgM [3, 23, 25, 34], and lower transaminases [35, 36] and bilirubin [36].

Serology

Serum autoantibodies are frequently described in autoimmune liver disease, especially PBC and AIH, and their serological pattern of reactivity is used to sub-classify disease. PBC-AIH OS may present serological pattern of both PBC and AIH; however, the concomitant presence of autoantibodies of the two diseases is not sufficient for the diagnosis of OS and, moreover, is not predictive of the sequential development of OS in a patient with a previous diagnosis of PBC or AIH. Indeed, O'Brien et al. reported about 15 AIH patients with AMA positivity but without clinical, biochemical, and histological features of PBC, neither at the time of AIH diagnosis, nor during a median follow-up of 8 years [49].

Type-I AIH is typically characterized by anti-nuclear antibodies (ANAs) and/or anti-smooth muscle antibodies (ASMAs), while type-II AIH is characterized by anti-liver kidney microsomal type-I (anti-LKM-1) antibodies which are mostly directed toward the human cytochrome P450IID6, or rarely anti-liver cytosol (anti-LC) antibodies. Anti soluble liver antigen/liver-pancreas (anti-SLA/LP) antibodies were originally thought to identify a third group of AIH but more than 75% of anti-SLA/LP positive patients are also ANA and/or SMA positive. PBC is characterized by anti-mitochondrial autoantibodies (AMAs) positivity in up to 95% of patients. ANA positivity is also reported in 30–50% of patients, but, in PBC, some ANAs are directed against specific antigens, namely, gp210 and sp100. The presence of anti-gp210 and/or anti-sp100 antibodies in PBC patients is more often observed in AMA-negative patients and their identification supports the diagnosis of PBC in these patients with biochemical features of cholestasis. The serological pattern of reactivity of PBC-AIH OS has been largely reported (Table 24.8) and is characterized by *AMA* positivity in 60–100% of patients, with a few studies reporting a lower frequency [15, 35, 36]. Serological reactivity to *SMA* was reported in up to 75% of PBC-AIH OS and, clearly, a lower frequency of SMA reactivity was reported in studies not adopting Paris criteria for the diagnosis of OS [14, 15] and in the Eastern population where the frequency ranges between 4% and 20% [19, 26, 28, 41, 50]. Patients with PBC-AIH OS showed *ANA* positivity in 33–100% of cases, and PBC-specific ANA (i.e. anti-gp210 and anti-sp100) positivity was found in up to 55% of ANA-positive cases [51]. Among ANA-positive OS, several immunofluorescence patterns of ANA in OS are possible: homogeneous in 28–33% of cases, speckled pattern in 33–43%, nuclear rim in 14–33%, and anti-centromere in 7–14% [20, 24]. *Anti-SLA* was reported in 7–33% of PBC-AIH OS and since these antibodies had the highest specificity for AIH among AIH-related autoantibodies [52], some authors suggest that the presence of anti-SLA/LP antibodies could be helpful in the diagnosis of a "variant" syndrome of PBC with AIH features and that immunosuppressive treatment should be offered to these patients when a relevant inflammatory activity is suspected [3, 53]. The presence of *anti-LKM-1* has been poorly reported in adult patients with OS and varies between 1% and 7% in different studies. *Anti-double-strand DNA* (anti-dsDNA) positivity was reported in 38–60% of patients with PBC-AIH OS diagnosed according to Paris criteria [24, 51, 54], and this frequency was significantly higher than in patients with pure PBC (3%) and pure AIH (26%) [24]. Anti-dsDNA was not associated, in these cases, with clinical, serological, or radiological signs of sys-

Table 24.8 Serological pattern of reactivity in studies describing natural history of PBC-AIH OS patients

	Autoantibodies positivity in PBC-AIH OS						
Study	AMA	ANA	SMA	Anti-SLA	Anti-LKM	Anti-dsDNA	Other
Chazouillères et al. [20]	75%	58%	75%	–	–	–	
Czaja [30]	100%	67%	67%	–	–	–	
Lohse et al. [3]	80%	70%	50%	20%	1%	–	ASGPR 55%
Talwalkar et al. [31]	100%	27%	27%	–	–	–	–
Joshi et al. [16]	100%	63%	69%	–	–	–	
Muratori et al. [15]	33%	67%	33%	33%	0%	–	pANCA 0%
Suzuki et al. [22]	100%	100%	0%	–	–	–	
Chazouillères et al. [21]	94%	47%	65%	–	–	–	
Poupon et al. [29]	100%	67%	67%	–	–	–	
Saito et al. [23]	100%	100%	70%	–	–	–	
Amarapurkar et al. [38]	100%	100%	17%	–	0%	–	pANCA 0%
Heurgué et al. [34]	60%	73%	67%	–	–	–	
Alric et al. [35]	43%	76%	52%	–	–	–	pANCA19%
Gossard and Lindor [14]	100%	38%	0%	–	–	–	
Silveira [32]	100%	–	–	–	–	–	ANA, SMA, LKM-1 > 1:80 23%
Al-Chalabi et al. [36]	33%	90%	50%	–	0%	–	
Lindgren et al. [17]	100%	48%	32%	–	–	–	
Muratori et al. [24]	87%	93%	33%	7%	0%	60%	AMA + anti-dsDNA 47%
Yokokawa et al. [25]	100%	100%	57%	–	–	–	
Kuiper et al. [43]	60%	67%	27%	–	–	–	
Bonder et al. [13]	100%	–	–	–	–	–	
Tanaka et al. [18]	88%	88% (ANA or SMA)	88%(ANA or SMA)	–	–	–	
Ozaslan et al. [51]	81%	33%	18%	13%	2%	53%	ANA+SMA41%
Liu et al. [19]	100%	100%	100%, 8%, 20% Paris; revised and simplified criteria	–	–	–	
Efe et al. [27]	100%	29–42%	17–29%	–	–	–	ANA+SMA 8–42%
Yoshioka et al. [26]	86%	100%, 21% Gp210	43%	–	–	–	
Levy et al. [33]	85%	80%	–	–	–	–	
Yang et al. [28]	94%	98%,51% Gp210,26% Sp100	4% SMA or SLA	4% SMA or SLA	–	–	
Fan et al. [41]	64%	89%	–	0%	0%	–	LC-1 0%
Nguyen et al. [54]	–	40% Gp210, 40% Sp100	–	13%	7%	–	Anti-dsDNA 38%, anti-p53, 31%

Note: *AMA* anti-mitochondrial antibody, *ANA* anti-nuclear antibody, *SMA* anti-smooth muscle antibody, *SLA* anti-soluble liver antigen antibody, *LKM* anti-liver-kidney microsomal antibody, *ASGPR* asialo-glycoprotein receptor, *pANCA* perinuclear anti-neutrophil cytoplasmic antibodies, *LC-1* anti-liver cytosol type 1 antigen antibody, *dsDNA* anti-double-strand DNA

temic lupus erythematosus at diagnosis or during follow-up. Moreover, the double positivity for anti-dsDNA and AMA was observed in 47% of patients with OS compared to 3% and 1% of pure PBC and pure AIH, respectively. The overall specificity of the concomitant AMA and anti-dsDNA for the diagnosis of PBC-AIH OS was 98%, with a reported likelihood ratio for a positive and a negative test of 28 and 0.5, respectively [24]. The specificity of anti-dsDNA antibodies for PBC-AIH OS was recently confirmed in a study in which an enlarged panel of novel and classical autoantibodies was analysed in patients with PBC and with PBC-AIH OS. The serum positivity of anti-dsDNA by CLIFT assay was the only autoantibody associated with PBC-AIH OS, and in a multivariate model, the presence of anti-dsDNA, ALT, and IgG was independent predictor of PBC-AIH OS with an area under the receiver operator curve of 0.84 [54]. Autoantibodies directed against p53 protein (*anti-p53*) have been also reported in 8 (57%) PBC-AIH OS diagnosed according to Paris criteria and the presence of anti-p53 protein was significantly higher in patients with PBC-AIH and pure AIH compared to patients with pure PBC with a specificity for the diagnosis of PBC-AIH and AIH equal to 97.8%. Anti-p53-positive PBC-AIH patients tended to be younger at diagnosis compared to anti-p53 negative; however, no other differences in clinical, biochemical features or response to therapy were reported between the two groups. A signifi-

cant positive correlation between the titre of anti-dsDNA and anti-p53 was observed. Serum reactivity to anti-p53 was independent from apoptosis in the liver of these patients. Indeed, p53 was not identified in the liver biopsy specimens and caspase-3 was detected in liver tissue independently of the serum positivity [55]. Unfortunately, the specificity of anti-p53 for the diagnosis of AIH was not confirmed in another study which found a similar frequency of anti-p53 antibodies in patients with PBC and PBC-AIH OS [54].

Compared to pure AIH, patients with PBC-AIH OS were more frequently AMA and ANA positive [3, 24, 34, 35], had higher level of AMA and ANA titres [36], and were less frequently SMA positive [20, 24] . The latter differ in Japanese OS where SMA positivity was more frequent than in pure AIH patients [23, 25].

Compared to pure PBC patients, PBC-AIH OS is more frequently characterized by SMA positivity [3, 16, 18, 23, 28, 29, 31, 32, 34], ANA positivity [16, 18, 24, 31, 32, 34], with more often a diffuse pattern [24], and a double positivity for AMA and anti-dsDNA positivity [24].

Additionally, overlap of AMA-negative PBC with AIH has also been reported [20], but the diagnosis of overlap is highly challenging in this context because of the histological biliary injury that may be observed in "pure" AIH, probably representing collateral damage in the context of a marked inflammation (see below). As a consequence, a diagnosis of overlap in these patients lacking "specific" PBC autoantibodies can be reasonably made only if marked biochemical cholestasis and/or granulomatous (not purely lymphocytic) cholangitis are present.

Liver Biopsy

Liver biopsy is considered a prerequisite for the diagnosis of AIH [4, 56], and it is mandatory in clinical practice when PBC-AIH OS is suspected [5, 40]. Histological features of OS were extensively reported (Table 24.9) and include in most cases the concomitant presence of typical findings of both diseases (Fig. 24.1).

The most frequent histological finding in *AIH* is the presence of lymphocytic interface hepatitis, which is characterized by the presence of lymphocytic, often lymphoplasmacytic, inflammatory infiltrates invading the limiting

Table 24.9 Histological features of OS patients

Authors	Lymphocytic cholangitis	Ductopenia	Portal inflammation	Interface hepatitis	Parenchymal inflammation	Fibrosis
Chazouillères et al. [20]	80%	20%	–	–	Moderate 50% Severe 50%	Mild/moderate 90% Advanced 10%
Lohse et al. [3]	100% Granulomas in 45%	–	–	Severe IH, PMN 5.8	Lobular inflammation between 2 and 3in 15%	–
Muratori et al. [15]	–	–	–	100%	–	–
Suzuki et al. [22]	–	–	Portal inflammation more severe than lobular inflammation 21%	–	Acidophilic bodies in high or moderate grade in 79%	–
Chazouillères et al. [21]	–	–	–	–	Mild 6% Moderate 56% Severe 38%	Mild/moderate 59% Advanced/ cirrhosis 41%
Poupon et al. [29]	75–82%	Ductopenia >50% in 18–25% OS vs. 31% PBC alone		Severe in 25–45% of OS vs. 4% of PBC alone	Severe 83%	25–41% of OS vs. 29% of PBC alone
Saito et al. [23]	100%	–	–	Moderate or severe in 100%	Severe in 50%	Advanced fibrosis or cirrhosis 50%
Joshi et al. [16]	–	–	–	–	43% lobular inflammation >2	–
Amarapurkar et al. [38]	–	–	–	–	–	Cirrhosis 100%
Heurgué et al. [34]	93%	–	–	Moderate or severe 86%	Moderate 36% Severe 64%	Advanced 43% Cirrhosis 14%
Alric et al. [35]	24%	24%	86%	Mild 29% Moderate to severe 24%	1.52 ± 0.2 Metavir activity	Advanced 38%
Gossard and Lindor [14]	71%	75%	88%	86%	100%	Advanced 75%

Table 24.9 (continued)

Authors	Lymphocytic cholangitis	Ductopenia	Portal inflammation	Interface hepatitis	Parenchymal inflammation	Fibrosis
Al-Chalabi et al. [36]	–	–	–	–	–	Cirrhosis 20%
Lindgren et al. [17]	–	–	–	–	–	–
Muratori et al. [24]	21%	79%	–	Moderate or severe 100%	Severe centrilobular necrosis 64%	Advanced/ cirrhosis 36%
Yokokawa et al. [25]	100%	–	–	–	Severe 38%	Advanced/cirrhosis 44%
Bonder et al. [13]	–	–	–	–	–	–
Tanaka et al. [18]	82% *Cholangitis activity CA score* *1.27 ± 0.98*	56%		Moderate and severe 76%	91% *HA score* *2.27 ± 0.67*	
Ozaslan et al. [51]						Absent to moderate 55% Advanced/ cirrhosis 45%
Liu et al. [19]	80%	–	–	Moderate 50% Severe 50%	–	–
Efe et al. [27]	75% of PBC to AIH at baseline and at the time of OS 80% AIH-PBC at the time of OS	25% PBC to AIH at baseline and 50% at the time of OS 40% AIH-PBC at the time of OS	–	Moderate in 8% and severe in 92% PBC to AIH	–	Advanced/ cirrhosis in 42% PBC to AIH, 40% in AIH to PBC at the time of OS
Yoshioka et al. [26]	43–55%	40%	–	Moderate to severe 71–85%	moderate-severe lobular hepatitis 86–92%	Advanced/ cirrhosis 68%
Levy et al. [33]	–	–	–	At least moderate in 100%	–	Advanced 59%
Yang et al. [28]	No difference compared to PBC alone	No difference compared to PBC alone	–	Severe in 95% OS vs. 17% PBC($p < 0.001$)	High grade of inflammation more frequent in OS than PBC alone	Less cirrhosis in OS vs. PBC alone
Fan et al. [41]	82%	54%	–	Moderate in 93% or severe in 7%	Moderate or severe in 86%	100%

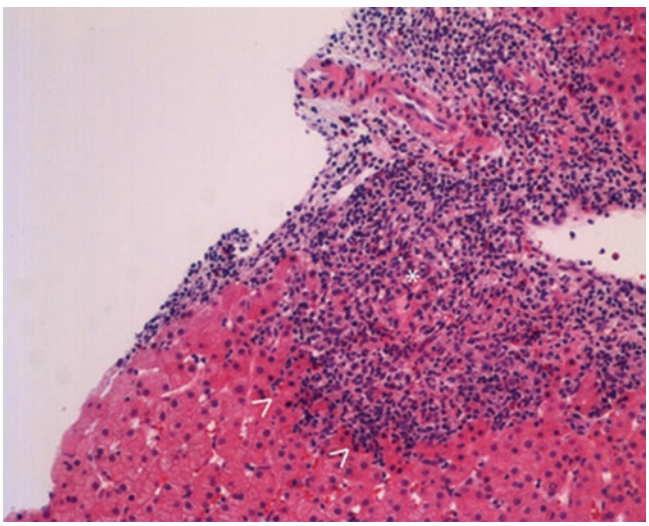

Fig. 24.1 Histological features of PBC-AIH overlap syndrome. Lymphocytic cholangitis (*) and lymphocytic interface hepatitis (^) (HE staining, original magnification ×100). (Courtesy of Prof. Dominique Wendum)

plate and extending from portal tracts into acinar tissue with hepatocyte injury [57, 58]. Interface hepatitis differs from biliary interface modifications (previously described as "biliary interface activity") that is the consequence of major cholestasis and associated ductular reaction, neutrophilic inflammation, and cholate stasis of periportal hepatocytes [59] (Fig. 24.2). Nevertheless, lymphocytic interface hepatitis is not pathognomonic of AIH, since it can be also seen in approximately 25% of PBC and PSC patients [40], in drug-related liver injury, and also in viral hepatitis. *PBC* histological hallmarks are chronic non-suppurative destructive cholangitis, which is characterized by lymphocytic infiltration of the biliary epithelium, biliary epithelial cells' senescence, and bile duct loss, with areas of macrophage-rich fibrosis replacing bile ducts in portal tracts. However, interface hepatitis develops to some degree in untreated pure PBC and is associated with disease progression [60, 61].

A Japanese study compared 41 PBC with interface hepatitis and 43 AIH treatment-naïve patients [62]. The degree of interface hepatitis did not differ between the two groups,

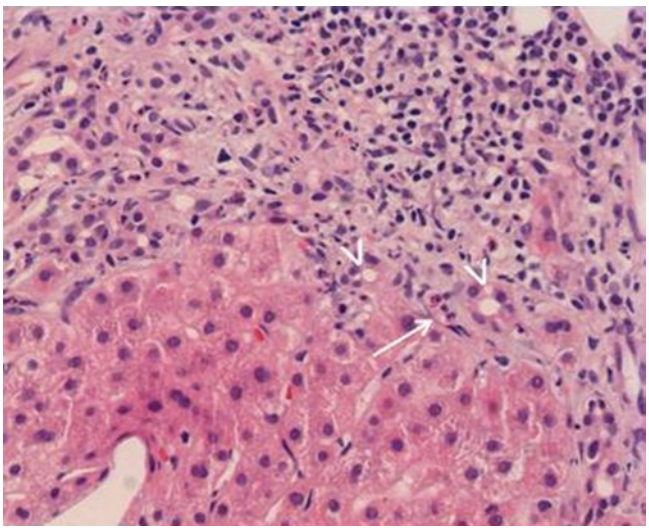

Fig. 24.2 Biliary interface modifications. Ductular reaction (^) and neutrophils (→). (HE staining, original magnification ×200). (Courtesy of Prof. Dominique Wendum)

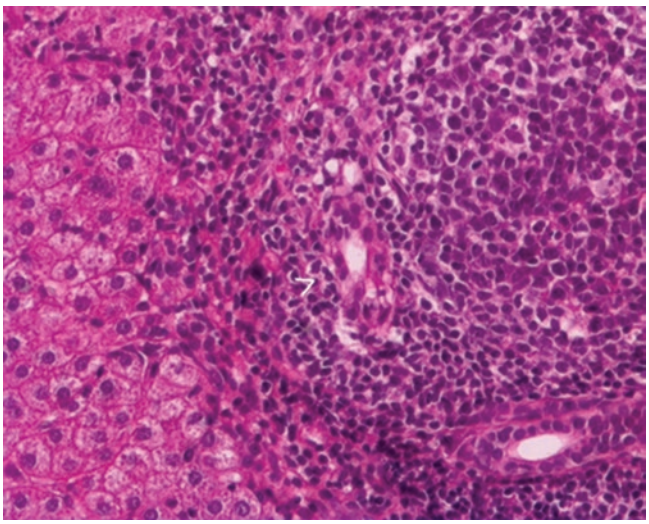

Fig. 24.3 Autoimmune hepatitis with cholangitis (^). (HE staining, original magnification ×200). (Courtesy of Prof. Dominique Wendum)

but AIH showed higher scores of lobular hepatitis with zonal or even bridging necrosis and focal hepatocellular necrosis, higher scores of hepatitic rosette formation, and emperipolesis compared to PBC. Despite the presence of a similar degree of interface hepatitis, immunophenotypes of infiltrating inflammatory cells were different between the two diseases. Indeed, higher scores of CD3+ (T cells), CD4+, (helper T cells), CD8+ (cytotoxic T cells) cells at the interface and within the hepatic lobules were observed in AIH compared to PBC. Hepatocyte necroinflammation and CD38+ cells infiltration were correlated to elevated AST in PBC patients, thus suggesting that these mononuclear cells play a role in immune-mediated hepatocellular injuries at the interfaces and within the hepatic lobules in PBC [62]. Analysis of infiltrating plasma cells with respect to immunoglobulin classes showed that IgG+ plasma cells were frequently present at the interfaces in PBC and AIH and their scores were significantly higher in AIH. These authors concluded that the hepatocellular injuries associated with interface and lobular hepatitis in AIH and PBC with interface hepatitis may not be identical [61]. Other authors reported that IgM/IgG ratio was significantly higher in PBC than in AIH or OS [63], but OS is not characterized by a predominant immunostaining of lymphocytes and plasma cells with either IgG and IgM predominance [64]. On the other hand, pure *AIH* may be characterized in one-quarter of patients by *bile duct injury*, namely, non-destructive, destructive cholangitis and ductopenia [65] (Fig. 24.3). Non-destructive cholangitis is identified by the presence of a mononuclear inflammatory infiltrate surrounding and infiltrating but not damaging the bile duct epithelium or destroying the bile duct basement. Otherwise, destructive cholangitis is defined as the presence of mononuclear inflammatory infiltrate surrounding and penetrating the bile duct epithelium with associated epithelial damage and/or destruction of the basement membrane. Finally, ductopenia is characterized by the absence of a bile duct adjacent to an arteriole within the portal tract. For example, in a study focusing on histologic features of bile duct injury and including 84 patients with classic AIH at presentation, 12% of patients showed destructive cholangitis, 12% non-destructive cholangitis, and 4% ductopenia. Patients with features of bile duct injury were indistinguishable from patients without bile duct injury by clinical, biochemical, histological activity and fibrosis score, frequency of cirrhosis at baseline, response to therapy, and natural history except for a younger age at diagnosis in patients with destructive cholangitis compared to patients with non-destructive cholangitis and a higher serum bilirubin in patients with ductopenia compared to patients without bile duct changes [65].

However, other groups reported much higher prevalence of biliary damage in AIH. Verdonk et al. analyzed the presence of bile duct injury and ductular reaction in a group of 35 treatment-naive patient with AIH fulfilling the simplified score. Ductular reaction was defined as a proliferation of bile ductules at the periphery of portal tracts accompanied by inflammatory cells and stromal reaction. Bile duct injury was assessed as injury of the interlobular bile duct, centrally located in the portal tract adjacent to the hepatic artery, and was sub-grouped in two different patterns of injury including a PBC-like and a PSC-like pattern. The first pattern was characterized by a dense lymphocytic or lymphoplasmacytic periductal inflammation, epithelial infiltration by inflammatory cells, epithelial damage and malformed, tortuous or irregularly shaped bile ducts. Differently, PSC-like pattern was defined by less relevant periductal inflammation with epithelial atrophy, disruption of basement membrane, and/or

concentric periductal fibrosis. Bile duct injury was present in 83% of patients, 50% of them showing a PBC-like pattern, 14% a PSC-like pattern, and 12% a mixed type. No associations between the presence of bile duct injury and any clinical, biochemical, or other histological features were noticed except for the presence of ductular reaction and hepatic rosettes. In the follow-up biopsies, 43% of patients still showed bile duct injury, and in all cases, a degree of inflammation was still present. Emperipolesis and hepatocyte rosettes were present in 89% and 83% of patients, respectively [66]. Similar to these observations, bile duct damage was reported in another series of 63 AIH patients which demonstrated in 70% of cases the presence of bile duct damage [67].

The general opinion is that bile duct injury in AIH is reliably a collateral injury associated with an exuberant inflammatory process due to a possible promiscuous nature of the immune-mediated response targeting, not only hepatocytes, but also cholangiocytes [65, 68] and the presence of bile duct injury and ductular reaction in AIH do not necessary imply a change in therapeutic management in such cases [65, 69].

In clinical practice, it appears that good-quality liver biopsy interpretation is key and a specialist review of liver biopsies has a major added value [70].

Finally, it should be kept in mind that no autoimmune liver disease has an absolute diagnostic test (the possible exception being PBC) as summarized in Table 24.10. Their diagnosis is based on the presence and relative absence of various clinical, biochemical, serological, and histological markers, with some being less categorical and objective than others [1]. As a result, there is intrinsic scope for individuals to present with overlapping features of more than one of these conditions, although, in most cases, it is possible to define one primary disorder ("dominant" disease). In a landmark review, Woodward and Neuberger emphasized that "true overlaps" should be differentiated from simple "crossover" or "outlier" syndromes (one clear diagnosis while having one feature associated with another) [71]. Overlapping presentations include: biochemical overlap (AST or ALT > 5 ULN in patients with PBC; or ALP > 3ULN in patients with AIH), serological overlap (positive ASMA in AMA-positive PBC; or positive AMA in AIH), histological overlap: interface hepatitis on liver biopsy with biliary lesions indicative of PBC, and finally varying combinations of the above. However, these overlap features have various significance, the weaker being probably immunoserology. Indeed, autoantibody profile should never be used in isolation but rather interpreted in conjunction with biochemical and histological features. For example patients with histological AIH and AMA positivity generally behave like typical AIH [49] and the same holds true for AMA-negative but ANA and/or ASMA-positive PBC (sometimes described as autoimmune cholangitis) when compared with typical PBC [72]. Laboratory features lack sensitivity considering that cholestasis in itself can cause raised ALT levels in the absence of marked inflammation and that cirrhosis can lead to high IgG levels in the absence of histological hepatitis. By contrast, a good-quality liver biopsy interpretation is the strongest means to diagnose overlap. Lastly, the diagnosis of AIH is, at least in part, a diagnosis of exclusion and that other causes of liver damage have to be ruled out, including intercurrent drug-induced liver injury and hepatitis E occasionally.

Table 24.10 Key features of PBC and AIH

	AIH	PBC
Gender	Female > male (4:1)	Female > male (9:1)
Co-existing IBD	3–10% (PSC should be excluded)	Not characteristic
ANA	70–80%	30–50% (some specific)
ASMA	*70–80%*	May be present: <10%
AMA	5–10%	*95%*
Immunoglobulins	IgG elevated	IgM elevated in most
Cholangiography	Usually normal	Normal
Interface hepatitis	*Characteristic*	Variably present
Biliary changes	10–20%	*Inflammatory duct lesion*
Response to immunosuppression	Yes	Mild

Course of the Disease and Therapy

Patients with PBC-AIH overlap syndrome seem to have a more severe disease compared to conventional PBC as illustrated by a higher frequency of extensive fibrosis at presentation, despite a younger age in some reports [34].

It is well recognized that in patients with PBC, *ursodeoxycholic acid* (*UDCA)* (15 mg/kg/day) leads to slowed progression of fibrosis and liver failure, particularly in patients who demonstrate an adequate biochemical response to therapy [73, 74]. Several models have been developed to evaluate UDCA response in PBC, including qualitative binary definitions such as the Barcelona criteria [75], Paris I and II criteria [73, 76], Toronto criteria [77], Rotterdam criteria [74], and continuous scores, namely, the Globe score [78] and the UK-PBC score [79]. Independently of the definition used to define response, patients who respond to UDCA have a significantly better transplant-free survival than non-responders. However, PBC patients presenting with significant interface hepatitis at liver biopsy may show a rapid progression of fibrosis and thus, in this case, the institution of immunosuppression may be considered [18, 21, 30]. Moreover, patients non-responders to UDCA, with persistent cholestatic enzyme elevation, showed a clear benefit after

starting second-line therapy with obeticholic [80, 81] acid or fibrates [82]. On the other hand, once the diagnosis of AIH is achieved, the institution of *immunosuppressive therapy*, based on the use of steroids (usually prednisone/prednisolone) monotherapy or in combination with azathioprine, is mandatory [4, 56]. The goal of therapy in AIH is the achievement of biochemical remission, defined as normalization of transaminases and IgG, and histological remission, defined as score of inflammatory activity below 4/18 according to the modified HAI grading [83].

Patients with overlapping features of PBC and AIH showed, in most of cases, a positive response to the *immunosuppressive and UDCA combination therapy* [3, 13, 14, 17, 20, 21, 23–30, 33–36, 38, 51], but the criteria of response for the single diseases have not yet been validated in OS and thus, the evaluation of response in OS patients remains a challenge.

Chazouillères et al. retrospectively reported about 17 patients with OS, identified according Paris criteria, and followed up for a mean interval time of 7.5 years. Among them, 11 patients were initially treated with UDCA alone and the remaining 6 with UDCA and immunosuppressive drugs, initially prednisone/prednisolone 0.5 mg/kg/day in monotherapy, and progressively tapered according to ALT decrease with subsequent addition of azathioprine or mycophenolate mofetil as corticosteroids-sparing agents. Three patients treated with UDCA alone were responders and a subsequent liver biopsy showed a decrease or stable inflammatory activity and no increase in fibrosis after a median time of 4.5 years. Non-responders to UDCA alone showed, in subsequent liver biopsy, an increase of activity in 38% of cases and of fibrosis in 89% of patients without cirrhosis at baseline. On the other hand, all patients initially treated with immunosuppressive and UDCA in combination were responders and subsequent liver biopsies showed a decreased or stable activity in 67% and 17% of cases, respectively, and a stability of fibrosis in all non-cirrhotic patients. Non-responders to UDCA monotherapy were then treated with immunosuppressants and after 4 years, liver biopsy available in half of them, they showed decreased or stable fibrosis in two and one cases, respectively. Finally, one patient, non-responder to UDCA monotherapy, showed an increase of fibrosis during follow-up biopsy. Similar to these results, Czaja reported that biochemical remission was achieved in 75% of patients treated with immunosuppressive treatment but not with UDCA alone and an alkaline phosphatase higher than 2 times the upper limit of normal was predictive of non-response to corticosteroids treatment [30]. Lohse et al. also reported biochemical response in most of patients treated with immunosuppressive agents and UDCA [3]. A similar efficacy of immunosuppressive therapy was also reported in patients with sequential development of OS [27, 29].

Other data suggested that OS patients less likely have a complete response to immunosuppressive agents compared to AIH alone but, in these studies, UDCA therapy was not given in combination from the beginning but subsequently added during the follow-up [36, 37]. Differently from the previous evidence, Joshi et al. reported on 16 patients retrospectively identified with OS, which were included in the Canadian trial of UDCA, a similar percentage of improvement in serum bilirubin, alkaline phosphatase, cholesterol, and IgM between patients with OS and PBC patients treated with UDCA. Unfortunately, histological fibrosis course was not assessed in these patients and no firm conclusions can be drawn from this study [16].

The more recent results of a large retrospective multicentre study (88 patients defined according to Paris criteria) have underlined the predictive role of the interface hepatitis degree: as first-line therapy, 30 patients received UDCA alone and 58 patients a combination of UDCA and immunosuppression (prednisone +/− azathioprine); in patients with moderate interface hepatitis, UDCA alone and combination therapy had similar efficacy (80%) in terms of biochemical response, whereas in patients with severe hepatitis, efficacy of UDCA alone was much lower (14 vs. 71%, respectively). Second-line immunosuppressive agents (cyclosporine, tacrolimus, and mycophenolate) led to biochemical remission in half of the patients who were non-responders to initial immunosuppression and UDCA combination [51]. Several studies confirmed the efficacy of the combination of UDCA and immunosuppression to achieve biochemical remission [13, 25, 26, 28, 33], to improve interface and lobular hepatitis and to avoid fibrosis progression [26, 29]. Moreover, the efficacy of combination therapy with UDCA and immunosuppressive was also confirmed in OS with cirrhosis decompensation at baseline [38, 41]; vice versa, UDCA monotherapy in this setting was associated to a lower remission rate and a lower transplant-free survival compared to patients treated with combination therapy with UDCA and immunosuppressive agents.

Other Therapies

Anecdotical use of several different agents in association with UDCA or as third-line therapy in non-responders to standard combination therapy was reported in OS patients. Budesonide in association with azathioprine was reported in five OS patients and was ineffective in the majority of them [14, 84] and treatment failure was found to be associated with the presence of advanced fibrosis at the initial liver biopsy [84]. Another study, including five OS patients treated with budesonide as first- or second-line therapy, showed a significant reduction of liver enzymes after budesonide introduction [85]. Finally, a recent meta-analysis supported the effective-

ness of budesonide in combination with UDCA compared to UDCA alone in PBC-AIH OS and moreover, budesonide was associated with fewer side effects compared to prednisone [86]. Cyclophosphamide was used in combination with prednisone in one patient [3], and cyclosporine showed to be effective in addition to UDCA in five of six non-responders to standard combination therapy [34, 51]. Tacrolimus was used in four OS patients: it was effective to induce remission in one of them, was associated with partial response in two cases, and one patient developed cirrhosis decompensation and received a transplant [51]. Mycophenolate mofetil was reported in three cases and was associated with complete response in two of them, and one patient showed partial response which was then achieved with the addition of cyclosporine [51]. Methotrexate was reported in two OS patients, but its effectiveness has not been reported [32].

Recently, obeticholic acid (OCA) has been approved as a second-line therapy for PBC patients with an inadequate response to UDCA monotherapy [80]. Impressive results of fibrates have also been reported in these patients [82]. It is important to differentiate patients with "classical" PBC and non-response to UDCA from those with overlap who are also non-responsive to UDCA. Whether the pleiotropic effects of fibrates or farnesoid X receptor agonists like OCA have sufficient immunosuppressive capacities and could be beneficial for overlap syndromes is currently unknown, but bezafibrate in association to UDCA was reported to be effective in 1 patient with OS [18]. *Relapse after immunosuppressive agents' withdrawal* was variably reported in different studies. Czaja observed 100% of relapse, but these patients were not treated with UDCA [30]. Similarly, Al Chalabi et al. documented relapse in all patients after drug withdrawal [36]. Chazouillères reported that one-third of patients successfully stopped immunosuppressive agents after a median interval time of 2.7 years and maintained persistent normal transaminases and no progression of fibrosis at subsequent biopsy was reported [21]. Heurgué et al. noted that one-fourth of patients relapse after drug withdrawal [34]; however, all of the patients responded well to reintroduction of immunosuppressive agents [26, 34]. *Corticosteroid therapy* in OS is generally safe despite the fact that mild bone loss was described in 38% and vertebral fractures in 15% of patients [21] but was not increased compared to patients treated with UDCA alone [25]. Moreover, diabetes mellitus was reported in 15% of patients, cosmetic effects in 15%, and obesity in 8% of patients. Finally, the combination therapy with UDCA and immunosuppressive agents was safe also in the rare patients with decompensated cirrhosis [41]. The natural course of OS is aggressive if an adequate therapy is not established, due to the persistence of inflammatory activity and the progression of fibrosis. On the other hand, patients with OS, responders to appropriate therapy, showed a comparable liver transplant-free survival to patients with PBC [32] and AIH [36]. However, a higher rate of portal hypertension, oesophageal varices, gastrointestinal bleeding, and adverse outcomes (death for all causes and/or orthotopic liver transplantation) was documented in the whole group of patients with OS compared to patients with PBC [32]. Similarly, a higher frequency of adverse outcome and a lower adverse outcome-free survival was recently reported in 46 patients with OS compared to patients with PBC [28]. However, in this study, the prognosis was better in patients with OS who were treated with combination therapy with UDCA and immunosuppressive agents compared to patients treated with UDCA alone. Total bilirubin was an independent prognostic factor in OS and also in PBC patients [28]. In decompensated cirrhosis, prognosis was strongly related to the efficacy of the combination therapy with UDCA and immunosuppressive agents [26, 38, 41]. Finally, Hispanics with OS were shown to have more relevant biochemical abnormalities, more frequently were non-responders, and developed more complications of portal hypertensions than non-Hispanics [33].

Liver transplantation (LT) for end-stage liver disease in 12 patients with OS (both PBC-AIH and PSC-AIH) was analysed in comparison with patients having a single autoimmune liver disease [87]. Patients with OS showed a shorter duration from diagnosis to LT, had a higher probability of recurrence of at least one disease (5-years: 53% vs. 17%; 10-years: 69% vs. 29%, $p < 0.001$), and showed a shorter median time to recurrence compared to patients with a single autoimmune liver disease. The diagnosis of OS and mycophenolate mofetil use, as part of immunosuppression, were independent predictive factors of recurrence. However, no differences in graft loss and patients' survival between patients with OS and patients with single autoimmune liver disease were reported. The type of recurrence in patients with OS was variable; indeed, two of them showed a recurrence of OS, while others developed a single disease's recurrence. On the other hand, no recurrent OS was described in patients transplanted for single autoimmune liver diseases. Moreover, patients with OS with a recurrent OS showed a significantly lower graft survival compared to patient with a recurrence of single disease [87].

In conclusion, the combination therapy of UDCA and immunosuppressive agents appears to be effective in patients with OS to achieve biochemical remission, to reduce hepatic inflammation, and to prevent fibrosis progression. To date, it is recommended in patients with severe interface hepatitis at initial biopsy. Differently, patients with mild or moderate interface hepatitis and no advanced fibrosis may benefit from UDCA monotherapy and, in these patients, immunosuppressive agents may be added in case of persistent biochemical activity, as suggested by EASL guidelines. Otherwise, there are no criteria to evaluate response to therapy in OS, neither the optimal time to perform a second biopsy to assess histological remission and thus eventually support the

decision regarding immunosuppressive drug withdrawal. Normalization of transaminases, IgG, and alkaline phosphatase in these patients seems a reasonable target, but whether biochemical remission is indicative of absence or minimal histological activity in patients with OS is still unknown.

Associated Extra-hepatic Autoimmune Diseases

Different concurrent autoimmune diseases may occur in the same patient and this association has been described both in patients with multi-systemic autoimmune diseases (e.g. rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus) and also in patients with organ-specific autoimmune diseases (e.g. Grave's disease, myasthenia gravis, polymyositis) [88]. Similarly, PBC and AIH have been also reported to occur in association with systemic and organ-specific extra-hepatic autoimmune diseases (EHADs) [89–104]. Finally, patients with PBC-AIH OS may also present with one or more associated EHAD; however, data regarding this association are scarce (Table 24.11).

Chazouillères et al. reported in the first series of PBC-AIH OS, diagnosed using Paris criteria, that extra-hepatic disorders including Sjogren's syndrome, Raynaud's phenomenon, and arthropathies occurred in one-third of patients [20]. Other different studies, using Paris criteria or revised and/or simplified IAIHG scoring system to diagnose the presence of OS, reported a variable frequency of the association of EHAD and PBC-AIH OS that ranges between 27% and 91% [18, 26, 31–34, 38, 41, 105, 106]. The largest series of PBC-AIH OS, diagnosed according Paris criteria, showed that 44% of 71 patients with OS had an associated EHAD [106]. Similarly, in a large study including AIH patients seen in two reference centres in the UK, 42% of 562 patients had at least one concomitant EHAD [107] and in PBC, the presence of concomitant EHAD varies between 32% and 61% [99, 108].

The *frequency* of different types of EHAD in OS varies among different studies and is summarized in Table 24.12. *Autoimmune thyroid diseases*, namely, Hashimoto's thyroiditis and Grave's disease, were reported in 9–36% of patients with PBC-AIH OS diagnosed according to Paris criteria [18, 33, 106] and in up to 59% of OS diagnosed according to the revised IAIHG scoring system. Levy et al. observed that hypothyroidism in Hispanics was significantly more frequent in PBC-AIH OS compared to pure PBC (35% vs. 6.4%), but this difference was not confirmed in non-Hispanics. On the other hand, autoimmune thyroid diseases were reported in 18% of 562 patients with pure AIH [107] and 12% of 921 patients with pure PBC [109]. The cross-reactivity of anti-thyroid autoantibodies or the presence of

Table 24.11 Frequency of extrahepatic autoimmune diseases (EHAD) in patients with PBC-AIH overlap

Study	PBC-AIH OS Total number	Extrahepatic autoimmune disease, *n* (%)	Time of diagnosis of EHAD	Comparison vs. pure PBC or pure AIH	Clinical impact of EHAD on course of PBC-AIH
Chazouillères et al. [20]	12	4 (33%)	Previous	–	–
Talwalkar et al. [31]	26	19 (69%)	Previous or concurrent	69% vs. 34% in pure PBC	–
Gheorghe et al. [37]	11	–	–	No differences compared to AIH	–
Amarapurkar et al. [38]	6	2 (33%)	–	–	–
Poupon et al. [29]	39	7 (18%)	–	–	–
Heurgué et al. [34]	15	7 (47%)	–	No differences compared to both PBC and AIH	–
Silveira et al. [32]	Extension of Talwalkar et al.: same frequencies of EHAD				
Tanaka et al. [18]	33	9 (27%)	–	No differences compared to both PBC and AIH	–
Yoshioka et al. [26]	28	21(75%)	–	No differences	No impact of EHAD on response to treatment
Levy et al. [33]	39	15 (38%)	–	Hypothyroidism significantly more frequent among Hispanics with OS compared to Hispanics with pure PBC (35% vs. 6.4%)	–
Fan et al. [41]	28	10 (36%)	–	–	–
Neuhauser et al. [105]	43	39(91%)	–	EHAD more frequent in OS than pure PBC (91% vs. 60%)	–
Efe et al. [106]	71	31 (44%)	–	–	No impact of EHAD on progression of liver disease or response to therapy

Table 24.12 Frequency of different extra-hepatic autoimmune diseases in patients with PBC-AIH overlap

Study	Sjogren's syndrome	Raynaud's phenomenon	Arthropathies including rheumatoid arthritis	Autoimmune thyroid diseases	Psoriasis	Celiac disease	SLE	Vitiligo	Others
Chazouillères et al. [20]	1(8%)	1(8%)	2/17%)	–	–				
Amarapurkar et al. [38]	1 (16%)	–	–	–	1(16%)				
Tanaka et al. [18]	6 (18%)	–	–	3 (9%)	–				
Levy et al. [33]	1 (3%)	–	–	14 (36%)	–				
Neuhauser et al. [105]	28 (65%) or 11 (48%) according to revised or simplified criteria for OS diagnosis	2 (5%), 2(9%)	21 (49%), 7(30%)	25 (59%), 9 (39%)	–	–			
Efe et al. [106]	6 (8%)		3 (4%)	13 (18%)	3 (4%)	3 (4%)	2 (3%)	2(3%)	7(22%)[a]

Notes: *SLE* systemic lupus erythematosus
[a]Other EHADs included one case each of the following diseases: autoimmune haemolytic anaemia, anti-phospholipid syndrome, multiple sclerosis, membranous glomerulonephritis, sarcoidosis, systemic sclerosis, and temporal arteritis

autoreactive T cells or similar epithelial antigens with other tissue and organs has been suggested as possible pathophysiological mechanism that may explain the association of overlap autoimmune thyroid diseases and other autoimmune diseases [110]. Moreover, a genetic variant of protein tyrosine phosphatase non-receptor 22 (PTPN22) has been found to be significantly associated to the risk of developing PBC and autoimmune thyroid diseases and several other polymorphisms were shown to be associated with shared susceptibility to autoimmune thyroid disease and PBC [111]. The presence of *Sjogren's syndrome* in patients with PBC-AIH OS varies between 3% and 18% [18, 20, 33, 38, 106], and these frequencies are intermediates between the reported frequency of Sjogren's syndrome observed in 3% of AIH patients [107] and in up to 34% of PBC patients [108]. PBC and Sjogren's syndrome share a common immunopathogenesis in which genetics and environmental factors interact to determine the disease onset inducing salivary or biliary epithelial cell apoptosis and contributing to the breakdown of tolerance to self-antigen exposed to the apoptotic blebs [112]. *Raynaud's phenomenon* was reported in 8–9% of patients with OS [20, 105], in 2% of patients with AIH [107], and in 18% of patients with PBC [108]. Autoimmune arthropathies, including *rheumatoid arthritis*, were reported in 4–17% of patients with OS [20, 106], in 5% of patients with AIH [107], and in up to 10% of patients with PBC [108, 113, 114]. *Systemic lupus erythematosus* (SLE) was reported in 4% of 71 patients with PBC-AIH OS, in 3% of patients with AIH, and 2% of patients with PBC. Moreover, when analysing the causes of liver enzymes abnormalities in 147 patients with SLE, the presence of PBC-AIH OS was found to be responsible for these alterations in 3% of patients, AIH in 11%, and finally PBC in 6% of patients [103]. A common genetic predisposition between SLE and AIH may justify this association, since the two diseases shared the HLA-DR3 susceptibility allele. Among autoimmune cutaneous diseases, *psoriasis* was reported in 4% of PBC-AIH OS patients [106], whereas it is rarely reported in AIH and PBC patients. *Vitiligo* was reported in 3% of patients with PBC-AIH OS, 1–2% of patients with AIH [89, 107] and together with other cutaneous autoimmune diseases in 5% of patients with PBC [108]. *Celiac disease* was described in 4% of PBC-AIH OS and in 1.4% of AIH and PBC patients [107, 108]. In the largest series including 71 PBC-AIH OS, other EHADs were reported, each one accounting for one case: autoimmune haemolytic anaemia, antiphospholipid syndrome, multiple sclerosis, membranous glomerulonephritis, sarcoidosis, systemic sclerosis, and temporal arteritis [106].

The *temporal presentation* of EHAD compared to the diagnosis of PBC-AIH OS has been poorly described and the available data comes from case-reports studies. Efe et al. reported about three cases of development of PBC-AIH OS during the course of connective tissue diseases [104]. The reported association and the sequential development of different autoimmune hepatic and/or extra-hepatic disease supports the concept that clinical expression of autoimmune diseases may be affected by multiple factors contributing to the development of additional autoimmune manifestations. Indeed, it is commonly believed that autoimmune conditions develop after an environmental trigger which upsets the immune system equilibrium in a genetically predisposed host. These alterations of the immune system may lead to the development of one autoimmune disease in some patients or several different clinical manifestations affecting different organs in other patients. This concept has been referred as the *mosaic* of autoimmunity

by Shoenfeld and colleagues and implies that the integration of genetic, environmental, and hormonal factors into the aetiology of autoimmune responses may emerge as different overlapping conditions [88, 115, 116]. Regarding the genetic predisposition, GWAS studies, published in the last 15 years, showed that the same genetic variants, which are associated with an increased risk of autoimmune and immune-mediated conditions, are common in more than one disease. This phenomenon, genetically denominated "pleiotropy", refers to the fact that a collection of different risk genes can predispose individuals to a variety of different autoimmune conditions [117]. For example *IRF5* variants were found to be involved in PBC, ulcerative colitis, rheumatoid arthritis, SLE, and systemic sclerosis. Notably, genetic variants overlap between PBC, PSC, and AIH were described with a *IRF5, STAT4, IL12A,* and *IL12RB* associations occurring in AIH and PBC, *BACH2* and *CTLA4/CD28* associations occurring in AIH and PSC, and *SH2B3* and *TNFRSF14* occurring in all three diseases [118]. It remains to be defined to what extent this genetic overlap contributes to the clinical overlap between PBC and AIH and PSC and AIH. HLA associations in the three diseases have been also clearly documented with some shared HLA alleles (e.g. the DR4 association in PBC and AIH) and some distinguishing one (e.g. DR8 in PBC), but what is the causal HLA class I and II gene for each condition remains to be determined [117]. Regarding environmental triggers, Floreani et al. recently reviewed the environmental basis of autoimmunity, underlining that a number of infections and environmental agents have been identified as possible triggers in PBC, AIH, and EHAD including virus, bacteria, drugs, cosmetics, and chemical agents [119]. Some of these agents are common to different diseases, thus supporting the idea that a single trigger may contribute to the pathogenesis of different autoimmune diseases. For example Epstein–Barr virus (EBV) has been suggested as a possible trigger factor for AIH in genetically predisposed individuals [120]. Moreover, EBV was also documented in peripheral blood mononuclear cells, liver tissues, and saliva of PBC patients [121] and finally, in patients with rheumatoid arthritis, anti-citrullinated protein antibodies were observed to react with a viral deaminated protein of EBV [122]. Similarly, a previous exposition to nail polish was suggested as a possible environmental trigger for both PBC and SLE [99, 123]. Lastly, an association between cadmium-rich areas with PBC and high levels of arsenic with PSC was recently reported. This valuable research confirmed a possible role of environmental factors in the pathogenesis of autoimmune liver diseases and it will be of notable interest to know whether in the same region, a cluster association with other autoimmune diseases is also present.

PBC-PSC Overlap Syndrome

Primary biliary cholangitis overlapping with primary sclerosing cholangitis has been reported only in a few case-reports of variable quality and does not represent a real issue. Indeed, in most of these cases, the diagnosis of PBC-PSC was controversial due to lack of clear manifestation of both diseases including the absence of associated inflammatory bowel disease [124–129]. As a consequence, the overlap between PBC and PSC still remains a controversial issue in the field of autoimmune liver diseases due to the small number of reported cases and the lack of properly defined diagnostic criteria.

Conclusion

Liver overlap syndromes do exist but are rare. Whatever be the name used (e.g. variant PBC with autoimmune hepatitis features or variant autoimmune hepatitis with PBC features), recognition of autoimmune overlap syndromes is of interest not only from a classification standpoint but also, and more importantly, because of therapeutic implications. Overlap syndromes should be diagnosed conservatively by using as strict criteria as possible. Appraisal has to be performed longitudinally rather than at a single point in time. Treatment decisions should be tailored to the individual and not be static. In most cases, it is possible to define one primary (dominant) disorder. As a rule, the dominant clinical feature should be treated first and therapy should be individualized and adjusted according to the response. In difficult cases, referral to a specialist centre with a high volume of caseload with autoimmune liver diseases is recommended.

International effort for collection of a large database and discovery of more specific molecular signatures with the ability to identify sub-groups within the spectrum of autoimmune liver disease should be encouraged.

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Primary Sclerosing Cholangitis

25

Christopher L. Bowlus

Key Points

- Primary sclerosing cholangitis (PSC) is a cholestatic liver disease strongly associated with inflammatory bowel disease (IBD) and is characterized by fibrotic strictures of medium and large size bile ducts.
- The precise mechanisms that lead to PSC have not been established, but current evidence suggests that impaired mucosal barrier functions, intestinal microbes, and gut-derived lymphocytes play key roles in genetically susceptible individuals.
- PSC typically progresses to biliary cirrhosis and also predisposes to cholangiocarcinoma and, in those with IBD, colon cancer.
- Immunomodulators and therapies targeting the bile acid pool have failed to prove effective leaving liver transplantation as the only treatment option once end-stage liver disease is reached.

Introduction

Sclerosing cholangitis was first described by Delbet in 1924 as an "obliterative cholangitis" of the extrahepatic biliary tree with diffuse thickening of the wall and narrowing of the lumen [1]. The term now refers to a spectrum of cholestatic conditions that are defined by the cholangiographic appearance of diffuse stricturing and segmental dilatation of the intrahepatic and/or extrahepatic bile ducts, with primary sclerosing cholangitis (PSC) denoting a specific form of sclerosing cholangitis typified by its strong association with inflammatory bowel disease (IBD) involving the colon, in the form of either ulcerative colitis (UC) or Crohn's colitis. The IBD associated with PSC is typically one of a pancolitis and frequently involves the ileum but spares the rectum. Often, the IBD is mild, asymptomatic, and limited to the right colon which can lead to the diagnosis of Crohn's colitis. The association between PSC and IBD appears to be greater in Northern latitudes, although even there, the frequency of non-IBD PSC is increasing. Within PSC, several subtypes can be distinguished. The most common is *large-duct PSC*, which refers to the classic form with cholangiographic evidence of sclerosis of the larger bile ducts. *Small-duct PSC* accounts for 5% to 20% of PSC patients who have clinical, biochemical, and histological features of PSC, but a normal cholangiogram [2–5]. Some small-duct PSC cases may in fact be patients with AMA-negative primary biliary cholangitis (PBC) or who carry variants of the *ABCB4* gene [6]. Overall, 12% to 17% of patients with small-duct PSC will progress to classic large-duct PSC with long-term follow-up. The third PSC subtype referred to as *PSC-AIH overlap* makes up from 1% to 53.8% of patients with PSC, though when a standardized scoring system is used for the diagnosis of autoimmune hepatitis, only 7.5% of patients with PSC can be characterized as PSC-AIH overlap [7–9]. In contrast, *secondary sclerosing cholangitis* (SSC) refers to a syndrome that results from any of a number of underlying diseases and may be indistinguishable from PSC (Table 25.1).

PSC in children has many of the same features as PSC in adults, namely, a male predominance and strong association with IBD [10–12]. However, unlike PSC in adults, childhood PSC appears to be responsive to immunosuppressive therapies and has a higher frequency of AIH overlap, sometimes referred to as *autoimmune sclerosing cholangitis* [13]. Although most studies of PSC have been performed in populations of Northern ancestry, the incidence and prevalence rates of PSC among African Americans appear to be similar to Caucasians [14–16], though African Americans appear to have a less striking male predominance and lower rate of IBD.

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Table 25.1 Causes of secondary sclerosing cholangitis

Pediatric	Benign	Malignant
Cystic fibrosis Primary and secondary immunodeficiency Histiocytosis X Neonatal sclerosing cholangitis Biliary atresia Ichthyosis with sclerosing cholangitis Congenital bile duct abnormalities Sickle cell disease Progressive familial intrahepatic cholestasis type 3	Auto-inflammatory Sarcoidosis, eosinophilic cholangitis, mast cell cholangitis Iatrogenic bile duct injury Cholecystectomy Liver transplantation Anastomotic stricture Non-anastomotic stricture Cholelithiasis (Mirizzi syndrome) Chronic pancreatitis Vascular Ischemic cholangiopathy Vasculitis Intra-arterial chemotherapy Portal hypertensive biliopathy Infectious AIDS cholangiopathy Recurrent pyogenic cholangitis Biliary inflammatory pseudotumor	Cholangiocarcinoma Pancreatic adenocarcinoma Metastatic cancer Gall bladder cancer Ampullary adenocarcinoma Hepatocellular carcinoma Lymphoma

Epidemiology

Estimations of incidence and prevalence of PSC are complicated by multiple factors including barriers to diagnosis, inconsistent diagnostic criteria, and referral bias. Data from large cohorts of patients suggest that the incidence of PSC in North America and Northern Europe is approximately 1 to 1.5 cases per 100,000 person-years, with a prevalence of 6 to16 cases per 100,000 [17–19]. Estimates of the prevalence of PSC in other parts of the world are limited but suggest a lower prevalence [20]. Data collected prior to widespread use of MRI/MRCP, lack of population-based cohorts, and until 2018, the absence of a specific International Classification of Diseases code defining PSC have likely led to an underestimation of the true incidence and prevalence of PSC.

Peak incidence of PSC is between the ages of 25 and 45 years, with a median age of diagnosis ranging from 36 to 39 years, but PSC has been diagnosed in neonates and as late as the eighth decade of life [5, 21, 22]. Overall, men account for approximately two-thirds of patients with PSC, but among PSC patients without IBD, the male predominance is much lower [5]. Women with PSC are generally older at diagnosis. Similar to UC, PSC is associated with nonsmoking, but whether this effect is independent of smoking's effect on IBD remains controversial [23–25].

Natural History

The natural history of PSC is complicated and remains an important topic of debate. Identifying the start of PSC is problematic due to delays in diagnosis by clinicians and the fact that the diagnosis itself requires the presence of fibrotic strictures that are likely the sequelae of a preceding injury. In addition, the outcomes of PSC include not only the progression of liver fibrosis to cirrhosis and liver failure but also cholangiocarcinoma, gallbladder cancer, colon cancer, and sepsis which may occur independent of the degree of liver fibrosis. Clearly, some patients are diagnosed late in the disease process or progress rapidly, in contrast to others who have an indolent course. The latter group may appear to be growing due to increased awareness of and testing for PSC leading to the diagnosis of these less severe cases. In fact, PSC may be identified as an incidental finding on MRCP or through MRCP screening studies of individuals with long-standing IBD even in the setting of normal serum liver biochemical values and no PSC symptoms [26, 27]. In most cases, follow-up MRI evaluation has suggested that the subclinical disease progresses very slowly, if at all. Thus, the large range in reported mean transplant-free survival from 12 to more than 20 years is not surprising [5, 21, 22, 28] .

A large proportion (15–44%) of PSC patients have only biochemical abnormalities, typically elevation of serum alkaline phosphatase levels with variable elevations of serum bilirubin and aminotransferase levels and no symptoms related to liver disease or cholestasis [29, 30]. This group of patients appears to have a better prognosis. For example, in a study of 305 Swedish patients with PSC of whom 44% were asymptomatic, median survival was significantly better for the asymptomatic patients compared to those with symptoms at presentation [30]. Nevertheless, even among asymptomatic PSC patients, the median survival is significantly lower than an age-, sex-, and race-matched control population [31]. Symptoms of cholestasis typically include pruritus, cholangitis, and jaundice and may occur concomitantly. When present, these patients have more severe biochemical derangements, more abnormalities on cholangiography, and a higher histologic stage on liver biopsy specimens than asymptomatic patients. Like all chronic liver diseases, the final stage of PSC is characterized by decompensated cirrhosis and its complications of ascites, hepatic encephalopathy, and variceal bleeding.

Next to cirrhosis, the most common outcome in PSC is malignancy with cholangiocarcinoma being the most common and most feared. The greatest incidence of cholangio-

carcinoma is within the first year of the diagnosis of PSC followed by a 0.5–1.5% annual incidence and culminating in a lifetime risk between 5% and 20% [5, 21, 32, 33]. Risk factors for the development of cholangiocarcinoma in patients with PSC include older age, male sex, large-duct PSC, and UC, whereas small-duct PSC and Crohn's disease and absence of IBD appear to be protective [5, 32, 34–36]. The median survival of cholangiocarcinoma in PSC is only 5 months after diagnosis [35, 37]. Due to the high rate of recurrence of cholangiocarcinoma after liver transplantation, few patients with cholangiocarcinoma are considered candidates to liver transplantation.

In addition to cholangiocarcinoma, patients with PSC are at increased risk for the development of gallbladder cancer and, in patients with cirrhosis, hepatocellular carcinoma. Although prior estimates of gallbladder cancer in PSC have been 3–14% [38], a recent analysis of over 7000 PSC patients from an international consortium found the prevalence of gallbladder cancer and hepatocellular carcinoma in patients with PSC to be 0.8% for each [5].

Patients with concomitant PSC and UC are at significantly increased risk for developing colonic dysplasia or adenocarcinoma and, in fact, are at greater risk than patients with UC alone [21, 39]. Of 590 cases of PSC in the Netherlands, the cumulative risk of high-grade dysplasia or colorectal cancer was 3%, 7%, and 13% after 10, 20, and 30 years of PSC diagnosis, respectively, a risk 9-fold greater compared to an age- and gender-matched population and 10-fold greater compared to patients with UC without PSC [21]. In a large longitudinal collaborative study of 1911 patients with IBD, the 293 patients with IBD and PSC had a 2-fold greater risk of advanced colorectal neoplasia and a 2.5-fold greater rate of development of advanced neoplasia following a diagnosis of low-grade dysplasia compared to IBD patients without PSC [39]. In addition, patients with PSC and UC are also more likely than patients with UC alone to have synchronous sites of dysplasia in the colon [40]. Notably, PSC patients without IBD do not appear to be at increased risk of colorectal cancer [21].

In general, reduced transplant-free survival from PSC has been associated with older age at the time of diagnosis, male gender, large-duct PSC (as opposed to small-duct disease), and coexisting ulcerative colitis (in contrast to Crohn's disease or no IBD) [5].

Patients with small-duct PSC generally do well with the largest cohort of 83 patients finding that only 22% progressed to large-duct disease over a median follow-up of 7.4 years, and compared to 157 age- and gender-matched patients with large-duct PSC, the median transplant-free survival was significantly better [4]. Children with PSC appear to have a similar natural history to adults based upon a large cohort of 781 pediatric patients with PSC in which the transplant-free survival was 88% at 5 years and 70% at 10 years [11].

Table 25.2 Common signs and symptoms at diagnosis of PSC

Symptoms	Frequency (%)
Fatigue	65–75
Abdominal pain	24–72
Pruritus	15–69
Fever/night sweats	13–45
None	15–44
Weight loss	10–34
Signs	
Jaundice	30–73
Hepatomegaly	34–62
Splenomegaly	32–34
Hyperpigmentation	14–25
Ascites	4–7

Clinical Features

The most common symptoms if present at the time of presentation are generally nonspecific and include jaundice, fatigue, pruritus, and abdominal pain [29, 30, 41–43]. Frequently, symptoms are intermittent and coincide with biliary obstruction and worsening cholestasis. Physical examination may be normal in patients with early-stage PSC, while hepatomegaly, jaundice, and splenomegaly indicate advanced disease (Table 25.2). Skin findings seen in other chronic cholestasis conditions can also be seen in PSC and include cutaneous hyperpigmentation, excoriations from pruritus, and xanthomata. Other signs of advanced liver disease appear as fibrosis progresses, including spider telangiectasias, muscle atrophy, peripheral edema, and ascites.

Like other cholestatic liver diseases, the main indication of a biliary disease is elevation of the serum alkaline phosphatase levels, frequently three to five times the normal. However, normal alkaline phosphatase levels are present in up to 6% of patients with PSC [27]. Serum alanine and aspartate aminotransferase levels are typically elevated but only mildly. Elevations of transaminases more than four or five times the normal range are seen in episodes of acute cholangitis or in patients with an overlap syndrome with autoimmune hepatitis [7, 11, 44]. Serum bilirubin levels often fluctuate and may reflect biliary disease or hepatic dysfunction, the latter associated with advanced liver disease and concurrent hypoalbuminemia and coagulopathy.

Imaging Findings

Multifocal stricturing and ectasia of the biliary tract typify the PSC cholangiogram. Segmental strictures with normal or proximal dilatation result are the classic "beaded" appearance of the biliary tree (Fig. 25.1). The strictures are usually short though longer confluent strictures may be seen along

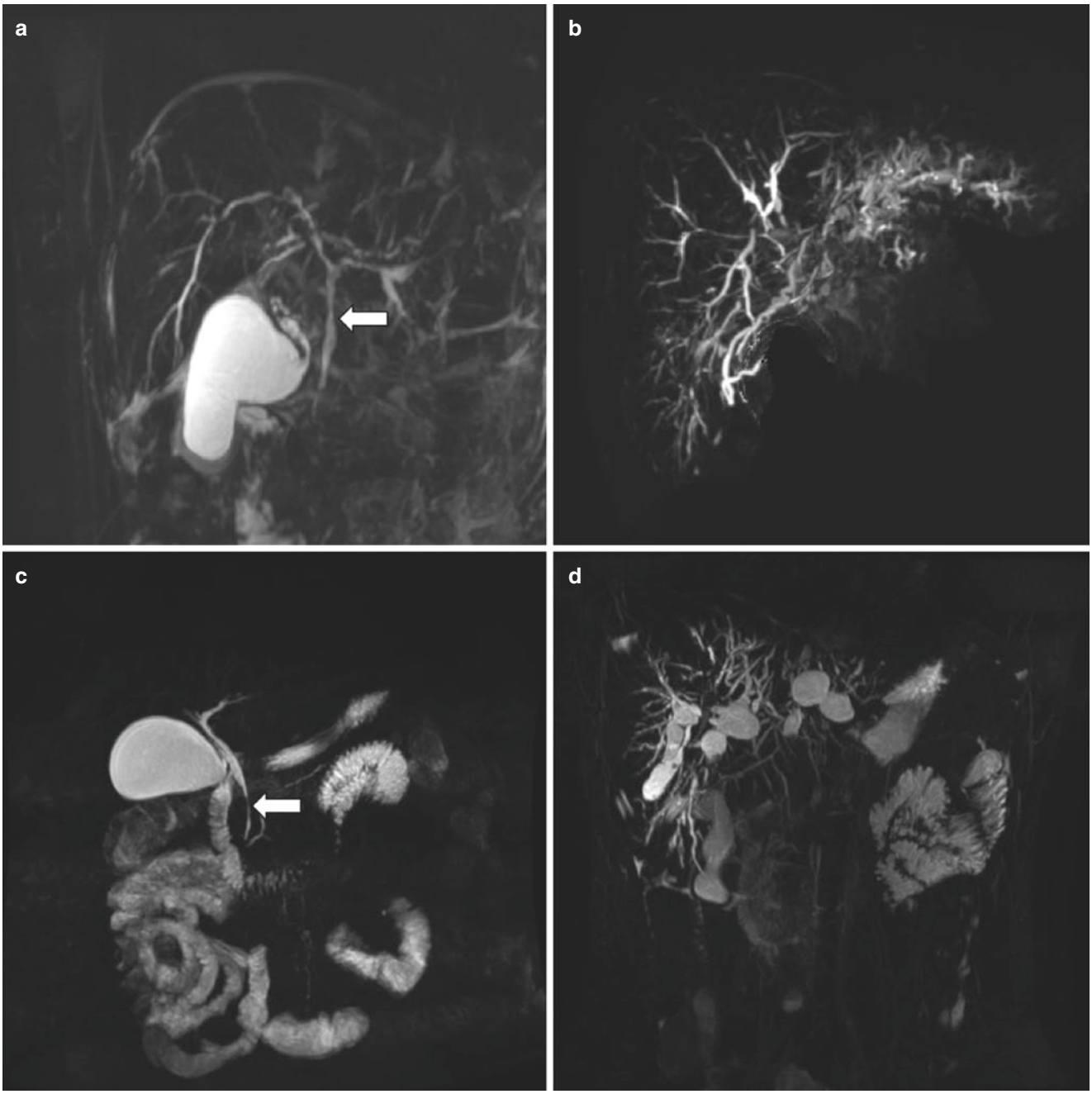

Fig. 25.1 (**a**–**d**) MRCP images in patients with PSC. (**a**) Typical beaded appearance of intrahepatic ducts. In (**b**), there is a dominant stricture at the level of the proximal common bile duct (*arrow*). (**c**) A dominant stricture in the distal common bile duct with proximal dilation. (**d**) Sacculations of intrahepatic bile ducts is a rare finding in PSC

with saccular or diverticular structures. The term *dominant stricture* has been used to refer to focal strictures of the main bile ducts leading to severe cholestasis [45]. The extent of biliary involvement is frequently classified by the involvement of the extrahepatic bile ducts, the intrahepatic bile ducts, or both which is present in approximately 75% of cases. Involvement of only the intrahepatic ducts has been observed in 15–20% of cases with disease isolated to the extrahepatic ducts reported in fewer than 6% of patients [27, 31, 46]. Periportal lymphadenopathy is very common, detected in up to 77% of patients, but nonspecific and not indicative of malignancy [47, 48].

Histology

The gross and histologic appearance of PSC reflects the cholangiographic findings which include diffusely thickened and fibrotic extrahepatic bile ducts. The fibrosis may be accompanied by various degrees of a mixed inflammatory infiltrate involving the biliary epithelium and biliary glands [49, 50]. Histologic findings on liver biopsy are wide ranging and are not typically diagnostic for PSC. The classic "onionskin" of concentric fibrosis surrounding medium-sized bile ducts is neither unique to PSC nor common and in fact is present in a minority of cases (Fig. 25.2). Fibro-obliterative cholangitis refers to the loss of the smaller interlobular and septal bile duct branches and is also uncommon, being present in only 5–10% of biopsy specimens [51]. Bile duct proliferation, periductal inflammation, and ductopenia or other findings are observed in PSC. Although a portal-based infiltrate of lymphocytes, plasma cells, and neutrophils is frequently observed, the severity of inflammation varies considerably. Occasionally, lymphoid aggregates may be seen [51, 52]. The presence of lymphocytic interface hepatitis and/or lobular infiltrates may indicate coexistent AIH [53, 54].

Histologic staging of PSC has typically used the system described by Ludwig and colleagues in 1981 [55] and is similar to the same authors' system for staging PBC. The portal stage (I) designates changes including portal inflammation, connective tissue expansion, and cholangitis that are limited to the portal tracts. The periportal stage (II) is illustrated by the spillage of the inflammatory infiltrate and fibrosis beyond the limiting plate, specifically interface hepatitis, also referred to as piecemeal necrosis, and periportal fibrosis. The septal stage (III) is characterized by bridging fibrosis consisting of septa that form bridges between portals. The final cirrhotic stage (IV) depicts nodules formed by bands of fibrosis which is a pattern of biliary cirrhosis. The rate of histological progression in PSC is not well documented. A retrospective study of 307 liver biopsies from 107 PSC patients with a median time between biopsies of 11 months estimated that 93% of PSC patients with stage II disease would progress over 5 years and that 14% would progress to cirrhosis in 1 year [56]. However, in clinical trial involving paired liver biopsies, significant changes in histologic stage over 1–5 years of the study have not been demonstrated [57–66]. Even with the use of morphometric measures of hepatic collagen content, no significant change was found in a clinical trial of 225 patients over the course of 2 years regardless of treatment assignment [67]. Other staging systems such as the Ishak and METAVIR systems have been used infrequently [63, 67–70]. More recently, the Nakanuma system for staging PBC [71] has been applied to PSC [68, 69]. The advantage of the Nakanuma staging system is the additional features of bile duct loss and cholestasis measured by orcein-positive granules.

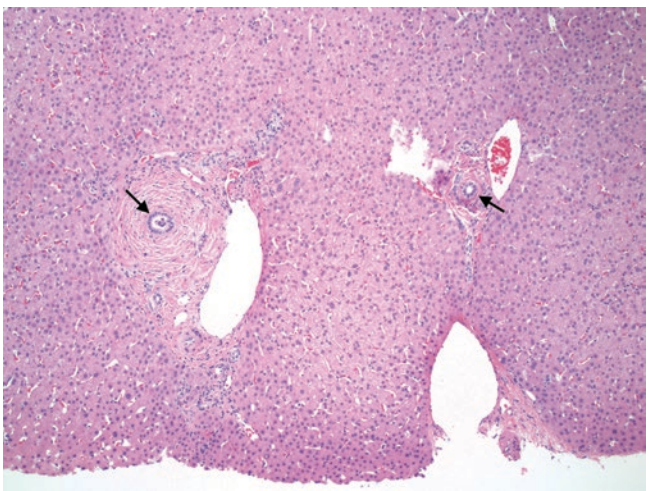

Fig. 25.2 Liver histopathology in PSC. Two interlobular ducts (*arrows*) surrounded by concentric fibrosis with an onionskin appearance. Note the variable degree of fibrosis between the ducts and the paucity of inflammatory cells. Although considered to be classic features of PSC, these changes are present in the minority of patients with a liver biopsy and can be seen in other causes of chronic biliary disease. (Courtesy of Karen Matsukuma, MD)

Etiology and Pathogenesis

The strong clinical association of PSC with IBD and other autoimmune diseases along with their shared genetic risks lends strong support to an underlying immune-mediated mechanism of disease. However, the precise mechanisms underlying PSC remain poorly understood. The initial insult is thought to be derived from the intestine in the form of intestinal lymphocytes, microbes, or microbial products in genetically susceptible persons. This initial insult leads to a fibrotic reaction and biliary strictures that can be recognized as PSC. Progression of PSC may be due to the same ongoing immunologic mechanisms of initiation, chemical injury from bile acids retained secondary to the cholestasis that develops as a result of the biliary strictures, or a combination of both.

Genetic Factors

The risk of PSC has been estimated to be 9- to 39-fold greater among siblings suggesting a strong heritable risk

[84]. Strong associations with HLA-B8 and HLA-DR3 were identified decades ago, and HLA remains the most impactful risk loci. Interestingly, HLA-DR3, which is strongly associated with PSC in European populations, is rare among African Americans and is not associated with PSC in African American PSC patients listed for liver transplantation, though the HLA-B8 association is shared between both Caucasian and African American PSC patients [15]. Although there is an association between both UC and Crohn's disease with HLA, the HLA risk alleles of IBD are distinct and are not of the same magnitude as those associated with PSC. In addition, HLA-B8 and HLA-DR3 are not overrepresented in patients with IBD without PSC.

Molecular genotyping of HLA has further refined the extended HLA haplotypes that are most strongly associated with PSC including [15, 85–87]:

- B*08:01
- DRB1*03:01-DQA1*05:01-DQB1*02:01
- DRB1*13:01-DQA1*01:03-DQB1*06:03
- DRB1*15:01-DQA1*01:02-DQB1*06:02
- DRB1*01:01-DQA1*01:01

Haplotypes that have been associated with protection from PSC include:

- DRB4*01:03-DRB1*04:01-DQA1*03-DQB1*03:02
- DRB4*01:03-DRB1*07:01-DQA1*02:01-DQB1*03:03
- DRB4*02:02-DRB1*11:01-DQA1*05:01-DQB1*03:01

Interestingly, small-duct PSC in the presence of IBD is also associated with HLA-B*08 and DRB1*13:01, but small-duct PSC without IBD is only weakly associated with HLA-DRB1*13:01 suggesting that small-duct PSC without IBD is a separate clinical entity [10]. Among PSC patients with elevated IgG4 levels, associations with HLA-B*07 and HLA-DRB1*15 have been found [88].

Identifying the causative gene or genes responsible for the HLA associations in PSC remains challenging. Fine mapping of the region and modeling of the effects of variants on the HLA-DR peptide binding groove have implicated changes in residues 37 and 86 in the HLA-DRβ chain which affect the binding of peptide antigens to be presented by class II molecules [86]. These findings have led to speculation of a PSC-specific antigen responsible for ongoing immune activation. Other studies have implicated HLA-C and HLA-B variants that have been associated with PSC and can act as inhibitory ligands for killer Ig receptors (KIRs) on natural killer cells [89, 90]. Further, an HLA-independent association with the *NOTCH4* gene in the class III region has been reported [85].

Subsequent genome-wide association studies (GWAS) have identified additional non-HLA risk alleles in cohorts of several thousand patients and controls [91–97]. Sufficient validation is available for 22 risk loci (Table 25.3), which account for only a fraction of the estimated PSC susceptibility. The majority of identified loci have been associated with other immune-mediated diseases and are near genes with roles in the function of innate and adaptive immune responses, particularly T cell responses [95, 98]. An additional 33 genes have been implicated at a lower level of significance based upon an a priori assumption that there is overlap in the genetic associations between PSC and other immune-mediated disease such as rheumatoid arthritis and type 1 diabetes, so called pleiotropy.

In addition to providing insights into the potential aberrancies leading to PSC, these genes give important insights into the genetic architecture of PSC [95]. Only half of the PSC-associated genes are also associated with UC, Crohn's disease, or both. Although the genetic correlation between PSC and IBD generically is strong ($r = 0.56$), the correlation is weak ($r = 0.29$) with UC specifically and was not found to be statistically significant ($r = 0.04$) with Crohn's disease [97]. In addition, network analysis has failed to identify common functional pathways that predispose to both IBD and PSC.

Genetic modifiers of disease progression in PSC are even less well documented. Several studies have investigated HLA haplotypes and clinical outcomes with inconsistent findings likely due to the small cohort size of these early studies. A more recent study of 635 PSC patients from the United Kingdom did find that HLA-DR*03:01 copy number was associated with younger age at diagnosis and increased risk of death or liver transplantation [99]. However, analysis of GWAS data from 3402 patients on time to clinical events did not find an association with the HLA region, though there was a significant association with rs853974 on chromosome 6 with the AA genotype being protective compared to the GG and AG genotypes (hazard ratios of 0.46 and 0.55, respectively) [100]. The rs853974 polymorphism is located near the R-spondin 3 (*RSPO3*) gene which is highly expressed in cholangiocytes suggesting a potential mechanistic role in PSC disease progression.

Table 25.3 Primary sclerosing cholangitis risk alleles

Chromosome	Polymorphism	Risk allele	OR	P-value	Candidate gene(s)	Reference
Genome-wide significance						
1	rs3748816	A	1.21	7.41E-12	*TNFRSF14*, *MMEL1*	[95]
2	rs6720394	G	1.6	4.10E-08	*BCL2L11*	[92]
2	rs7426056	A	1.3	1.89E-20	*CD28*, *CTLA4*	[95]
2	rs7556897	T	0.85	4.73E-9	*CCL20*	[238]
2	rs4676410	A	1.38	2.43E-09	*GPR35*	[96]
3	rs3197999	A	1.33	2.45E-26	*USP4*, *MST1*	[95]
3	rs80060485	C	1.44	2.62E-15	*FOXP1*	[97]
4	rs17032705	G	1.18	3.8E-10	*NFKB1*	[238]
4	rs13140464	C	1.3	8.87E-13	*IL2*, *IL21*	[95]
6	rs3099844 rs2844559		4.8 4.7	2.60E-26 4.2E-26	*HLA-B*	[91]
6	rs56258221	G	1.23	8.36E-12	*BACH2*	[95]
10	rs4147359	A	1.24	8.19E-17	*IL2RA*	[95]
11	rs7937682	G	1.17	3.17E-09	*SIK2*	[95]
11	rs663743	G	1.20	2.24E-13	*CCDC88B*	[97]
12	rs11168249	G	1.15	5.49E-09	*HDAC7*	[95]
12	rs12369214	G	0.85	1.3E-9	*RFX4*, *RIC8B*	[238]
12	rs3184504	A	1.18	5.91E-11	*SH2B3*, *ATXN2*	[95]
16	rs725613	T	1.20	3.59E-13	*CLEC16A*	[97]
16	rs11649613	T	1.19	1.00E-11	*CLEC16A*	[238]
18	rs1452787	G	0.75	2.61E-08	*TCF4*	[96]
18	rs1788097	A	1.15	3.06E-08	*CD226*	[95]
19	rs60652743	A	1.25	6.51E-10	*PRKD2, STRN4*	[95]
21	rs1893592	A	1.22	2.19E-12	*UBASH3A*	[97]
21	rs2836883	G	1.28	3.19E-17	*PSMG1*	[95]
Suggestive but not reaching genome-wide significance						
Chromosome	Polymorphism	Risk allele	OR	P-value	Candidate gene(s)	Reference
2	rs7608910	A	1.12	7.21E-6	*PUS10*	[97]
2	rs11676348	C	1.14	6.48E-7	*TGR5*, *CXCR2*	[97]
8	rs2042011	G	0.92	0.001	*RN7SKP226*	[97]
10	rs2497318	C	0.01	0.0001	*EIF2S2P3*	[97]
10	rs10748781	A	1.13	4.37E-6	*NKX2-3*	[97]
11	rs559928	C	0.88	0.0001	–	[97]
16	rs7404095	C	0.91	0.0003	*PRKCB*	[97]
18	rs12968719	G	1.12	0.003	*PTPN2*	[97]
19	rs679574	C	1.10	1.78E-4	*FUT2*	[97]

Diagnosis

The diagnosis of PSC is based on typical cholangiographic findings along with clinical, biochemical, serologic, and histologic features of cholestasis along with the exclusion of secondary causes of sclerosing cholangitis (Fig. 25.3). In the majority of patients, both intrahepatic and extrahepatic bile ducts are affected, though isolated strictures of the intrahepatic bile ducts can occur in 20% to 28% of cases. Dominant strictures, defined as strictures with a diameter of less than 1.5 mm of the common bile duct or less than 1.0 mm of a hepatic duct within 2 cm of the bifurcation, develop at a cumulative incidence of 36–57%.

With improvements in image quality and standardized protocols [72, 73]. MR cholangiopancreatography (MRCP) has largely replaced ERCP for diagnosis [45, 74]. MRI also has the advantage of identifying hepatic dysmorphy, features of portal hypertension, and evidence of hepatic malignancies [46]. Increasingly, MR elastography is also performed with the MRCP to provide disease staging, which can also be done by vibration-controlled transient elastography [70]. ERCP is still performed in select patients in whom there is a high index of suspicion and MRCP is negative or equivocal [45] or if therapeutic interventions are anticipated. Percutaneous transhepatic cholangiography (PTC) may also yield diagnostic images and allow therapeutic intervention but requires

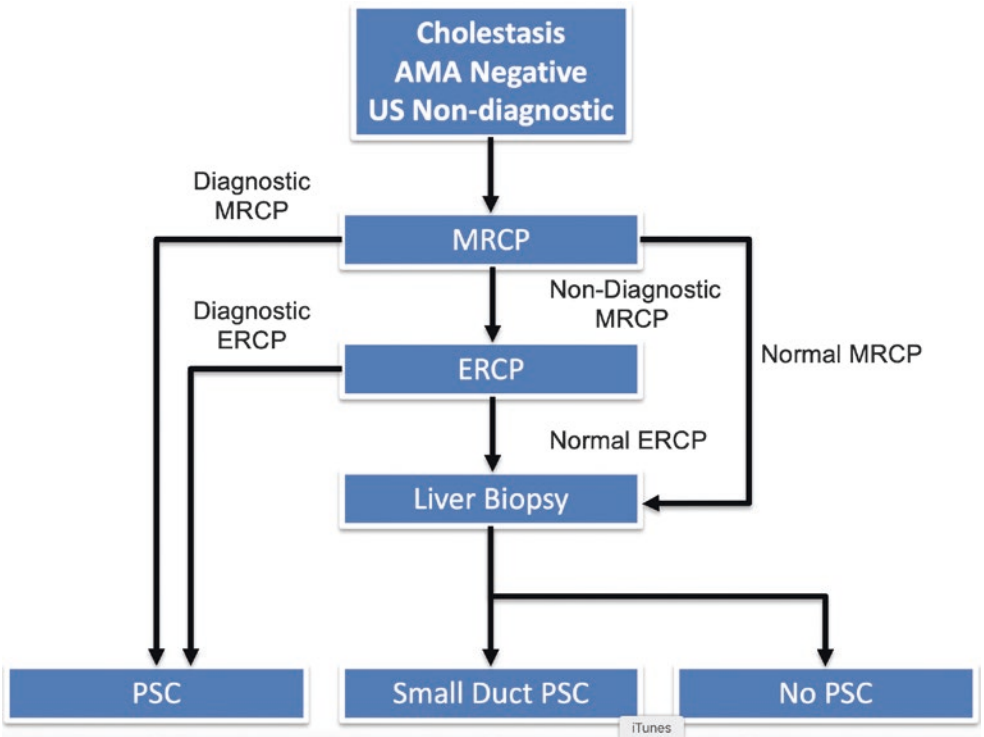

Fig. 25.3 Diagnostic algorithm for PSC. Patients typically present with cholestasis evaluated initially with an ultrasound (US). Antimitochondrial antibodies (AMA) should also be tested to exclude primary biliary cholangitis (PBC). In the absence of secondary causes of sclerosing cholangitis, an MRCP with typical chances of PSC (see Fig. 25.1) is diagnostic of PSC. If the MRCP is nondiagnostic and suspicion of PSC is high, then an ERCP should be considered. A liver biopsy with typical PSC histology and in the absence of other causes of cholestasis such as AMA-negative PBC may lead to the diagnosis of small-duct PSC, particularly in the presence of IBD

percutaneous puncture and may be technically difficult if the intrahepatic bile ducts are not sufficiently dilated.

In a patient with a characteristic cholangiogram of sclerosing cholangitis and a diagnosis of IBD, the diagnosis of PSC can be made confidently, while in the absence of IBD, secondary causes must be excluded. Differences in the cholangiographic appearance of primary and secondary sclerosing cholangitis have been described but are not sufficiently specific to distinguish the two entities [45]. In particular, excluding IgG4-sclerosing cholangitis, also known as immunoglobulin (Ig) G4-associated cholangitis, can be difficult because of serum IgG4 levels in PSC as well [75].

Choledocholithiasis and cholangiocarcinoma may mimic PSC but are also found in PSC leading to difficulty in diagnosis. Other challenges in diagnosis occur in patients with cirrhosis of other causes who may have nonspecific cholangiographic changes and patients with autoimmune hepatitis who may demonstrate features of PSC on MRCP [76, 77].

Serologic testing to establish the diagnosis of PSC is of limited value due the lack of sensitivity and specificity of currently available markers. Hyperglobulinemia and elevations in serum IgM levels are common [42], and serum autoantibodies are present in the majority of patients with PSC [11, 78]. Up to 53% of PSC patients have antinuclear antibodies (ANA), and smooth muscle antibodies are present in 13% to 20%, whereas antimitochondrial antibodies (AMA) are rarely, if ever, found [79]. Anticardiolipin antibodies are also commonly detected in patients with PSC with the titer correlating with disease severity in at least one case series [79, 80]. Perhaps the most investigated autoantibodies are the perinuclear antineutrophil cytoplasmic antibodies (pANCA) which are detected in 65% to 88% of PSC patients. These antibodies in PSC, which have an atypical pANCA pattern, have also been described as "antineutrophil nuclear antibodies" (ANNA) [81]. However, these antibodies are also commonly found in patients with IBD or autoimmune hepatitis. Although titers of pANCA do not correlate with disease activity or severity [82], pANCA positivity has been associated with a younger age at the diagnosis, a lower frequency of cholangiocarcinoma, and a higher prevalence of HLA-B*08 and DDRB1*03 [80]. Measurement of autoantibodies is therefore of limited clinical value in patients with PSC.

Liver biopsy is rarely required to establish a diagnosis of PSC except in cases of small-duct PSC or overlap with autoimmune hepatitis. If an MRCP is of good quality and nondiagnostic, then liver biopsy to diagnose small-duct PSC of other liver disease should be considered, particularly in cases with underlying IBD. Small-duct PSC should not be confused with AMA-negative PBC which would typically demonstrate granulomas and florid duct lesions [83].

PSC and IBD

Most series have found IBD in 70–80% of PSC patients [17–19, 21], and the typical IBD has been classified as ulcerative colitis in 80% of cases with the remaining classified as either Crohn's colitis or indeterminate colitis [17–19, 21].

Fibrostenosing Crohn's disease is rare if ever found [101], and in some cases, only subtle histologic changes in the colonic mucosa can be found, or overt colitis may develop at a later date [102]. In contrast, fewer than 5% of all patients with either UC or Crohn's disease develop PSC [24, 103]. In addition, the colitis of PSC often has an unusual clinical phenotype that makes classification problematic. UC typically is defined as starting in the rectum with involvement extending proximally and occasionally into the terminal ileum. However, the IBD of PSC often has rectal sparing and may only involve the proximal colon and ileum (Fig. 25.4) [104, 105]. Interestingly, PSC patients who have undergone proctocolectomy and ileal pouch-anal anastomosis have a higher incidence of pouchitis [106–108]. This may be due to the inherent propensity to ileal inflammation or secondary effects of altered fecal bile acids and their impacts on intestinal epithelium or microbes. In addition, there is a lack of PSC seen in patients with Crohn's disease isolated to the small intestine. Whether this IBD phenotype directly predisposes to PSC or is simply an epiphenomenon of shared genetic and environmental factors remains to be determined. Racial and gender differences in the association between PSC and IBD also exist. Concomitant IBD is present in only 58.8–60.5% in African Americans with PSC, while in Asian PSC patients, wide ranges of IBD have been reported from as low as 21% up to 54% [15, 16, 109–112]. IBD is also less frequent in women being found in 48% to 51% [5, 113].

Despite the strong link between PSC and IBD, two key issues suggest that other factors are at play in the development and/or progression of PSC. First, unlike uveitis and erythema nodosum which wax and wane with the activity of the IBD, PSC typically progresses independently of IBD activity [102], although colectomy has been associated with improved clinical outcomes [114]. In addition, the colitis of PSC is often clinically mild or quiescent suggesting that the severity of the intestinal inflammation is not a factor in developing PSC [105, 115–117]. Second, some cases of PSC have been diagnosed years after total colectomy for UC [118, 119]. Whether the PSC was already present but undiagnosed at the time of colectomy or truly developed after colectomy cannot be determined. The former is consistent with the concept of progression due to cholestas, while the latter supports a theory of aberrant trafficking of intestinal memory cells to the liver for the initiation and progression of PSC.

Early studies comparing PSC between patients with or without IBD could not identify any histologic [55] or cholangiographic [120] differences. However, recent studies have found that the presence and type of IBD impacts the outcomes of PSC and that patients with concurrent UC have an earlier age of diagnosis and higher rates of hepatobiliary cancer, liver transplantation, and death [5, 121].

Despite the improved understanding of the clinical features IBD in PSC, their functional relationship remains an enigma. Anatomically and functionally, the intestine and liver are directly linked with the movement of nutrients, bile acids, and immune cells from the intestine and associated lymphoid tissue to the liver. Any theory of the pathogenesis of PSC must address this unique relationship. Generally, these theories can be grouped into three categories. First are those of a "leaky gut" in which the intestinal epithelial barrier is impaired allowing the entry of metabolites, microbes, and/or antigens into the portal circulation of the liver. However, the evidence of impaired gut barrier function in PSC is limited [122–124]. Second are theories of aberrant trafficking of intestinal lymphocytes activated by intestinal inflammation that are recruited to the liver resulting in subsequent biliary

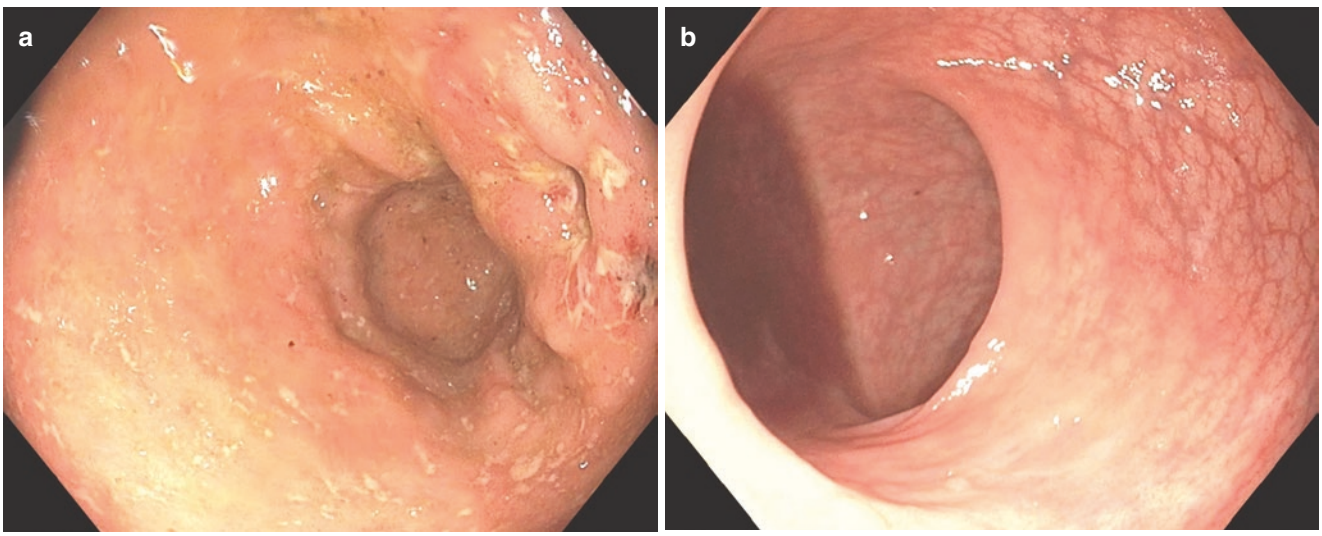

Fig. 25.4 Atypical inflammatory bowel disease of PSC. (**a**) Proximal colon and ileocecal valve with erythema, edema, and ulceration. (**b**) Normal-appearing distal colon of same patient

inflammation. Both of these groups of theories would suggest that more severe colitis would lead to a more pronounced impaired intestinal barrier and greater intestinal lymphocyte burden and therefore would be associated with a greater risk of PSC, which is not the case. The third group of theories posit that intestinal dysbiosis contributes to both an inflammatory colitis and subsequent biliary inflammation.

Immune Responses

A popular hypothesis linking the IBD-PSC connection is that PSC is triggered by an innate immune response to bacteria or pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) that enter the portal circulation through an inflamed, permeable intestine ("leaky gut"). PAMPs activate macrophages, dendritic cells, and NK cells through pattern recognition receptors, including TLRs and CD14, leading to the secretion of cytokines. NK cells are activated in turn by IL-12 and promote recruitment and activation of lymphocytes via TNF-α, IL-1β, and CXCL8. NK cells may be activated by MHC class I chain-related gene products MICA and MICB, stress-induced proteins that can promote the cytotoxic function of NK, NKT, and γδT cells through the NKG2D receptor.

Macrophages, key cells in the transition from innate to adaptive immune responses, appear to play a key role in PSC. Tissue resident macrophages, such as Kupffer cells, are derived from the yolk sac, while other macrophages may be recruited from bone marrow-derived monocytes and regulate the initial inflammatory response and late healing response. Macrophage phenotypes have frequently been divided into M1-like or pro-inflammatory macrophages and M2-like or restorative, reparative macrophages. In PSC, macrophages are a predominant cell type in comparison with PBC or hepatitis C [125, 126]. Not only accumulating in the sinusoidal and perisinusoidal spaces, macrophages accumulate in the portal areas [126] with peribiliary macrophages consisting of recruited macrophages of both M1 (pro-inflammatory) and restorative (M2) phenotypes, though the M1 phenotype cells are more pronounced in advanced disease [125]. Peripheral blood mononuclear cells from PSC patients also support an important role for innate immune response in PSC, particularly in pathways involved in macrophage differentiation by M-CSF [127]. In addition, associations have been identified with *MST1* and *GPBAR1*, genes involved in macrophage function, and PSC.(96:Hov, 2010 #47) *MST1* encodes macrophage stimulating protein 1, a circulating preprotein that is activated by various inflammatory stimuli and exerts negative feedback on macrophages to prevent excessive inflammation. *GPBAR1* encodes a member of the G protein-coupled receptor superfamily (TGR5) and functions as a cell surface receptor for bile acids on macrophages, BEC, and intestinal epithelial cells [128–130]. On macrophages, GPBAR1 activation leads to the suppression of macrophage functions. Notably, sequencing of the GPBAR1 gene in 267 Norwegian PSC patients and 274 healthy controls identified 6 nonsynonymous variants, 4 of which were only found in PSC patients, though only 1 patient each [131]. However, functional analysis revealed that five of the six variants had reduced or abolished function.

Eosinophils are associated with not only sclerosing cholangitis caused by the hypereosinophilic syndrome and IgG4-related diseases but also with PSC. Patients with UC who have eosinophilia are at tenfold increased risk of having PSC [132], and the eosinophil-specific chemokine eotaxin-1 (CCL11) has been shown to be elevated in the serum of PSC patients compared to PBC and autoimmune hepatitis [133]. Further studies found that serum levels of CCL11 are elevated only in PSC-UC patients with active colitis but that the intestinal CCL11 levels and other markers of eosinophil activation are not elevated in patients with PSC and UC in contrast to UC [134].

Despite the strong clinical and genetic evidence of an adaptive immune response as a basis for PSC, a specific antigen or immune response that leads to PSC has yet to be delineated. Also unknown is whether the targeted destruction of bile ducts is directed at specific self-antigens, antigens of the intestinal flora, or dietary antigens. High-throughput sequencing of the complementarity-determining region 3 (CDR3) of the B cell receptor immunoglobulin heavy chain in paired gut and liver tissue from PSC patients with IBD and normal tissue from cancer patients demonstrated a significantly greater frequency of overlapping clonotypes in paired gut and liver samples in PSC patients compared to the controls. In addition, these overlapping gut and liver clonotypes had shorter CDR3 lengths and higher rates of somatic hypermutation consistent with antigen-driven activation suggesting that B cell antigens are shared across the gut-liver axis in PSC. As noted previously, autoantibodies are frequent in PSC patients with pANCA being the most common autoantibody found. However, despite extensive studies, a specific autoantigen for the pANCA of PSC has not been validated [82, 135, 136]. Interestingly, the presence of pANCA in PSC patients with or without IBD and UC patients without PSC is associated with HLA-DRB1*03 [80]. In comparison with primary biliary cholangitis (PBC) in which there is a clear autoantibody response to the pyruvate dehydrogenase complex E2 subunit (PDC-E2), PSC livers have far fewer antibody secreting B cells and lack a specific autoantigen target [137]. An intriguing autoantibody to biliary epithelial cells (BEC) has been reported to have a high prevalence in PSC (63%) compared to healthy controls (8%), PBC (37%), or autoimmune hepatitis (16%) [138]. Sera from PSC and PBC patients with this antibody also induced IL-6 expression from BEC. Interestingly, IgG

from PSC patients with anti-BEC antibodies induced the expression of Toll-like receptor (TLR) 4 and TLR9 on BEC in culture along with the secretion of GM-CSF, IL-1β, and IL-8 [139]. This might in turn lead to the recruitment of neutrophils, macrophages, and T cells. However, the target(s) of these anti-BEC antibodies remains unknown.

Immunohistochemical studies have defined the lymphoplasmacytic infiltrate in PSC liver to consist primarily of non-activated memory $CD8^+$ T cells and appear to be oligoclonal [140–142]. Despite this observation, the functional roles of conventional CD4+ and CD8+ T cells in PSC remain poorly understood. Interestingly, in the IL-2 receptor-α (*IL-2AR*)-deficient mouse which develops both colitis and cholangitis, deletion of CD4 ameliorates the colitis but not the colitis with the opposite effect when CD8 is deleted suggesting that CD8+ T cells are the primary drivers of liver injury in this model [143]. Unconventional T cells including mucosal-associated invariant T (MAIT) and γδ T cells along with innate immune cells are also suspected to play key roles. Importantly, the distribution of immune cells is not uniform within the PSC liver with T cells and MAIT cells localizing to areas of fibrosis, whereas NK cells and Kupffer cells were being evenly distributed [142]. In addition to MAIT cells and γδ T cells, innate lymphoid cells, which have been implicated in the pathogenesis of IBD and have also been suggested to play a role in PSC [144], are particularly important for recognition of bacterial pathogens. All of these cell types have been implicated in chronic liver injury models, including the *Mdr2−/−* mouse in which IL-17 production by γδ T cells has been implicated in the development of cholestatic fibrosis and inflammation [145]. Notably, the frequency of circulating CCR6 + CCR4 + CXCR3- Th17 cells has been shown to be higher in patients with PSC-UC compared to healthy controls, though a similar finding is found in those with UC alone [144]. In addition, peripheral blood mononuclear cells from PSC patients have a significantly greater IL-17 response after stimulation with *E. faecalis* or *C. albicans*, and IL-17A producing cells are prominent in PSC livers [146].

Peripheral regulatory T cells (Tregs), important mediators for resolution of immune activation, are reduced in PSC compared to healthy controls and patients with PBC or UC with the most pronounced reduction in patients homozygous for the PSC risk allele in the *IL2RA* gene [147, 148]. The function of peripheral Tregs has also been found to be impaired in PSC compared to controls. In livers, Tregs in PSC are reduced compared to PBC livers [147] though a greater frequency of $CD4^+CD25^+$ T cells in the periphery of UC patients with PSC compared to UC patients without PSC has been found [149, 150]. In the dextran sodium sulfate (DSS) colitis model, both Tregs and Th17 cells infiltrate the liver with no apparent liver pathology, but when Th17 cells are transferred into mice without Tregs, significant liver injury is induced [151]. However, it should be noted that prior reports have documented a reduced frequency of peripheral and tissue Tregs in both PBC and UC relative to healthy and disease controls suggesting that changes in Tregs may be a generic feature of inflammatory diseases and not specific to PSC [152, 153].

Among peripheral T cells from PSC patients, CD4+ T cells, but not CD8+ T cells, have reduced apoptosis in response to repeated TCR stimulation or cytokine withdrawal [154]. Although resistance to apoptosis was associated with reduced upregulation of proapoptotic Bim in T cells, a polymorphism in the *BCL2L11* gene which encodes Bim and has been associated with PSC did not influence resistance to apoptosis, and T cell activation, indicated by expression of CD69, CD25, and CD28, was similar in PSC and controls. However, in the liver, $CD4^+CD28^-$ T cells are enriched compared to the periphery in PSC and are more frequent in PSC livers compared to PBC, nonalcoholic steatohepatitis (NASH), and normal livers [150]. These $CD28^-$ T cells have been defined as activated memory cells with intracellular stores of cytotoxic molecules, adhesion molecules, and chemokine receptors that can promote tissue infiltration and localization to bile ducts and are able to activate BEC in vitro. Intriguingly, the gene encoding CD28, a co-stimulatory molecule for T cell activation, survival, and proliferation, has been genetically associated with PSC.

Lymphocyte Trafficking

An important step in developing an understanding of the link between PSC and IBD came with the investigation of specific adhesion molecules, chemokines, and chemokine receptors which were initially thought to be intestinal specific but later revealed to also be expressed in the inflamed liver leading to the recruitment of lymphocytes of intestinal origin [155–158]. Tissue-specific recruitment of lymphocytes to inflammation involves the coordinated recognition of "addressins" expressed by vascular endothelial cells by homing receptors on the lymphocyte along with interactions of chemokines and chemokine receptors. In addition to tissue specificity, chemokines and chemokine receptors also impart lymphocyte lineage specificity [159]. Activation of lymphocytes by dendritic cells in gut-associated lymphatic tissue results in the expression of the α4β7 integrin and the CCR9 chemokine receptor. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is the ligand for α4β7 and is specifically expressed on the intestinal endothelium and during inflammation on intestinal mucosa along with the CCR9 ligand, CCL25, which is also preferentially expressed in the intestine.

However, MAdCAM-1 is not confined to gut endothelium but is also expressed in the portal vein and sinusoidal endothelium in autoimmune-mediated liver diseases, includ-

ing PSC [160]. The expression of MAdCAM-1 in the liver appears to be mediated by deamination of methylamine by vascular adhesion protein 1 (VAP-1), a semicarbazide-sensitive amine oxidase expressed in the human liver [161]. In the presence of tumor necrosis factor-α, methylamine induces the expression of functional MAdCAM-1 in hepatic endothelial cells as well as CCL25 which is specifically upregulated in PSC liver [157]. In addition, CCR9+ liver lymphocytes preferentially migrate to CCL25 rather than to CXCL12 or CCL5 and are triggered by CCL25 to bind immobilized MAdCAM-1 via α4β7.

Interestingly, the frequency of α4β7+ lymphocytes in the liver does not appear to be increased relative to peripheral blood in PSC, though lymphocytes expressing $\alpha_E\beta7$ are [157]. In contrast, CCR9+ liver lymphocytes have been found to be increased in PSC compared to PBC with approximately 20% of liver lymphocytes from PSC livers expressing CCR9 compared to <2% in livers from controls or patients with PBC. The CCR9+ liver lymphocytes included both CD8+ and CD4+ T cells, the former demonstrating a memory phenotype. Although this reflects an enrichment of these cells, it is far less significant compared to Crohn's disease in which nearly 100% of lamina propria lymphocytes express CCR9 + .

Direct evidence of intestinal lymphocyte homing to the liver has been difficult to achieve. In mouse models, using fluorescently labeled cells and inducing colitis have allowed direct imaging to demonstrate migration directly from the inflamed intestine into the liver [151]. In addition, hepatic CD4+ T cells from the SAMP1/YitFc mouse, a model of Crohn's disease which also develops liver inflammation, have the ability to induce not only liver but also ileal inflammation upon transfer to SCID mice [162]. Supporting evidence that PSC liver α4β7+ CCR9+ lymphocytes originate in the intestine comes from findings that only gut-derived dendritic cells and not liver-derived dendritic or stellate cells were able to imprint these homing markers on CD8+ T cells [155]. In addition, CD8+ T cells primed in the gut in vitro can migrate to both the gut and the liver, while liver-primed CD8+ T cells only migrate to the liver [163]. However, CD4+ T cells primed by liver sinusoidal endothelial cells (LSEC) were able to induce α4β7 and CCR9 expression in vitro with subsequent migration into gut and gut-associated lymphoid tissue [164].

PSC Dysbiosis

The study of intestinal microbiota and its association with a host of extraintestinal disorders has exploded in recent years. Perhaps in no other situation is the potential importance of a direct link between gut microbiota and extraintestinal disease greater than it is in PSC. However, studies in PSC are confounded by the presence of IBD and liver disease, both of which have impacts on the intestinal microbiome. In UC and Crohn's disease, the most common finding is a decrease in the diversity in the microbial population along with some specific alterations in the members of the population, findings reminiscent of the "hygiene hypothesis" of autoimmunity. Dysbiosis is also frequently found in patients with chronic liver diseases, especially in those with advanced cirrhosis. These two important caveats can confound microbiome studies of PSC which typically includes patient with and without IBD and of varying degrees of liver disease. In addition, bile acids can significantly impact the microbiome in PSC and IBD [165]. However, the studies to date suggest that the intestinal microbiome of patients with PSC is distinct from IBD [165–173]. In fact, patients with PSC with IBD tend to have a microbiome more closely related to PSC without IBD compared to IBD alone. In addition, the biliary microbiome may also be altered in PSC. The fucosyltransferase-2 gene (*FUT2*) is involved in protein glycosylation, and genetic variants leading to truncated FUT2 proteins, so-called nonsecretors, have been linked to PSC and Crohn's disease. Interestingly, biliary microbes in PSC varied by *FUT2* genotypes with *Firmicutes* being significantly elevated and *Proteobacteria* significantly decreased among nonsecretors.

Experimental evidence that this dysbiosis is a cause of PSC rather than a consequence is supported by studies in the *Mdr2*-null mouse model of sclerosing cholangitis in which germ-free mice had higher alkaline phosphatase, aspartate aminotransferase, and bilirubin compared to conventionally housed *Mdr2*-null mice [174]. In addition, fibrosis, ductular reaction, and ductopenia were significantly more severe in the germ-free environment. No differences in primary bile acids were detected, and not surprisingly, secondary bile acids were absent in the germ-free environment. In a second study, the frequency of *Lactobacillus* in fecal samples from *Mdr2*-null mice was increased compared with control mice [145]. In addition, *Lactobacillus gasseri* was enriched in *Mdr2*-null livers and when heat-killed could still induce γδ TCR+ cells from *Mdr2*−/− livers to produce IL-17. Further, intraperitoneal injection of *L. gasseri* into control mice increased serum levels of IL-17 and resulted in liver inflammation, which was blocked by injection with anti-γδ TCR. Interestingly, γδTCR+ cells from livers of patients with primary sclerosing cholangitis, but not those from patients with hepatitis C virus infection, produced IL-17.

More recently, studies in gnotobiotic mice have identified *Klebsiella pneumonia* and others as candidate organisms involved in the development of PSC. Mice inoculated with stool from patients with PSC, but not from UC or healthy controls, developed increased IL-17-expressing T cells (TH17) in the liver and were more susceptible to hepatobili-

ary injury by diethoxycarbonyl-1,4-dihydrocollidine (DDC). Mesenteric lymph nodes from control mice showed no viable organisms, but in mice inoculated with PSC stool, *K. pneumoniae*, *Proteus mirabilis*, and *Enterococcus gallinarum* were isolated from mesenteric lymph nodes. Interestingly, translocation of *E. gallinarum* has also been linked to systemic lupus erythematosus and autoimmune hepatitis [175]. Further, the *K. pneumoniae* isolated from PSC patients could induce epithelial damage in a bacterial-organoid coculture system. Importantly, these organisms were also found to be prevalent in stool from patients with PSC.

Toxic Bile Theory

Although genetic evidence does not support a role of bile as an initiator of PSC, several lines of evidence suggest that bile is important in the progression of PSC. Bile is a complex mixture of bile acids, bilirubin, cholesterol, phospholipids, and proteins for which several protective mechanisms have evolved. Changes in the composition of bile, decreased bile flow, and increased biliary pressure in PSC may all disrupt the normal homeostasis and lead to toxic bile formation. BEC are protected from bile by dilution and alkalization, the so-called bicarbonate umbrella. In addition, mixed micelles with phosphatidylcholine and cholesterol prevent bile acid toxicity. However, these mechanisms can be compromised by impairment of transporters responsible for maintaining the bile acid/phospholipid ratio (MDR3 or BSEP) or bicarbonate excretion and hydration of bile (CFTR or AE2). Alternatively, bile stasis, a frequent phenomenon in PSC, may lead to toxic bile formation and exacerbation of bile duct injury.

Support for the toxic bile acid theory comes primarily from the multidrug resistance gene *Mdr2*-null mouse [176–178]. Targeted disruption of *Mdr2* leads to sclerosing of the biliary tree with extra- and intrahepatic biliary strictures and dilations, onionskin-type periductal fibrosis, and focal obliteration of bile ducts similar to that seen with primary and secondary sclerosing cholangitis in humans [176]. In humans, variants of the human orthologue of *Mdr2* (*MDR3* or *ABCB4*) are associated with intrahepatic cholestasis of pregnancy and gallbladder disease in an autosomal dominant fashion and progressive familial intrahepatic cholestasis type 3 (PFIC3) in a rare autosomal dominant condition. In addition, some rare variants have been associated with sclerosing cholangitis [179, 180], but genetic studies have not found any association of genetic variants in *ABCB4* with PSC susceptibility [181]. In addition, PSC patients with normal serum bilirubin levels have been shown to have normal biliary excretion of bile acids and lipids suggesting that the toxic bile theory may only play a role in the later stages of PSC [181, 182].

Biliary Epithelial Cells

The role of BEC in the pathogenesis of PSC remains unclear, but the understanding of the function of BEC in the recruitment and activation of immune cells has grown recently and suggests that BEC are active participants rather than innocent bystanders. Biliary epithelial cells when activated express a host of receptors, cytokines, and chemokines that can orchestrate a number of immunological processes. In addition to MHC class II antigens, BEC express CD1d and can present lipid antigens to NK T cells [183]. Interestingly CD1d is downregulated in PSC. Toll-like receptors (TLR) which are also expressed on BEC and IgG found in the sera of some PSC patients directed against BEC induced the expression of TLR4 and TLR9 on BEC in culture [139]. In fact treatment of BEC with PSC sera-containing anti-BEC antibodies induced secretion of GM-CSF, IL-1β, and IL-8. However, the target(s) of these anti-BEC antibodies remains unknown. BEC expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) could also play a role in the recruitment of T lymphocytes [184].

Infectious and Antigenic Factors

Attempts to identify infectious or other antigenic factors in the gut which may enter the liver via the portal venous system via a "leaky gut" and induce PSC have so far been unfruitful. Bile cultures are positive in a majority of patients with PSC but appear to be linked primarily to endoscopic intervention [185]. In addition, bacterial endotoxin has been shown to accumulate in biliary epithelial cells in patients with PSC and PBC [186].

Treatment

Except for liver transplantation, no specific therapy has proven to be effective for treating PSC. The objectives of management prior to liver decompensation should be the treatment of complications, such as bacterial cholangitis and pruritus, prevention of osteoporosis and nutritional deficiencies, and early diagnosis of malignancies including cholangiocarcinoma, gallbladder cancer, and colon cancer. Once the liver disease is advanced, then evaluation for liver transplantation should be initiated.

Medical Treatment of Underlying Disease

A wide variety of medications have been studied in patients with PSC with only a few randomized, placebo-controlled trials of significant size (Table 25.4). In addition, the defined

Table 25.4 Randomized controlled trials in PSC with more than 20 subjects

Drug	Year	N	Treatment	Time of treatment	Outcome	Reference
Colchicine	1995	84	Colchicine (1 mg) vs. placebo	36 months	No benefit in histology, liver biochemistry, or clinical outcomes	[239]
UDCA	1997	105	UDCA (13–15 mg/kg/day) vs. placebo	24 months	No benefit in time to treatment failure (composite of death, transplant, cirrhosis, histologic progression >2 stages, decompensated cirrhosis, liver biochemistry, or symptomatic progression) Improved liver biochemistry	[61]
UDCA	2001	26	UDCA (20 mg/kg/day) vs. placebo	24 months	Improved liver biochemistry, reduced histologic and cholangiographic progression	[63]
UDCA Metronidazole	2004	80	UDCA/metronidazole (600–800 mg/day) vs. UDCA/placebo	36 months	Improved liver biochemistry and Mayo Risk Score and histology, not cholangiography	[64]
UDCA	2005	219	UDCA (17–23 mg/kg/day) vs. placebo	60 months	No benefit in transplant-free survival, liver biochemistry, quality of life	[190]
UDCA	2008	31	UDCA (10 vs. 20 vs. 30 mg/kg/day)	24 months	Improved liver biochemistry, improved Mayo Risk Score (high dose)	[65]
UDCA	2009	150	UDCA (28–30 mg/kg/day) vs. placebo	60 months	No benefit, increased adverse events	[59]
Vancomycin Metronidazole	2013	35	Vancomycin (125 mg or 250 mg q.i.d.) vs. metronidazole (250 mg vs. 500 mg t.i.d.)	3 months	Improved liver biochemistry, Mayo Risk Score in low-dose metronidazole and vancomycin	[206]
nor-UDCA	2017	161	*nor*-UDCA (500 mg/d, 1000 mg/d, or,1500 mg/d) vs. placebo	12 weeks	*nor*-UDCA reduced ALP levels (12.3% to 26.0% reduction compared to 1.2% increase with placebo)	[198]

study endpoints, whether clinical, biochemical, histologic, or a mathematical risk score, have varied greatly among published studies. Recent consensus on the surrogate endpoints that should be included in clinical trials has been established which should improve the likelihood of successful drug development [187, 188]. However, no current medical treatment has been shown to alter the natural course of PSC.

Ursodeoxycholic acid (UDCA) has been the most extensively studied drug in patients with PSC through several controlled clinical trials with varying doses [59, 61, 63, 189, 190]. The mechanisms by which UDCA is thought to exert a beneficial effect in cholestatic conditions include protection of cholangiocytes against cytotoxic hydrophobic bile acids, stimulation of hepatobiliary secretion, protection of hepatocytes against bile acid-induced apoptosis, and induction of antioxidants [191]. Although the majority of clinical trials have demonstrated improvement in serum liver biochemical test levels, none has demonstrated a survival benefit or delay in the requirement for liver transplantation. In addition, there were no benefits to UDCA with regard to fatigue, pruritus, or development of cholangiocarcinoma.

Because of the disappointing results with standard-dose UDCA, several groups studied the use of UDCA up to 30 mg/kg daily, twice the dose recommended for primary biliary cholangitis [59, 63, 190, 192]. A large study of 219 Scandinavian patients randomized to 17 mg/kg/d to 23 mg/kg/d of UDCA (n = 110) or placebo (n = 109) for 5 years failed to show any difference in transplant-free survival [190]. However, this study was unable to recruit the number needed to adequately power the study, and only 18 of 219 patients

reached the endpoint over 5 years reflecting the inherent difficulty with PSC clinical trials. The results of a prospective, placebo-controlled randomized trial of 25–30 mg/kg/d of UDCA for 6 years, however, demonstrated a higher risk of death, need for liver transplantation, and development of varices in patients on high-dose UDCA compared to placebo [59]. Nevertheless, post hoc analyses of these studies suggest that patients that improve liver biochemistries may obtain some clinical benefit [193, 194] and withdrawal of UDCA has been associated with deterioration in serum liver tests and Mayo Risk Score and increased pruritus [195].

Several newer bile acid modulating agents have shown promising early-stage results in improving liver biochemistries. These have included nor-ursodeoxycholic acid (*nor*-UDCA), a C_{23} homolog of UDCA with potent choleretic activity [196] that in preclinical studies showed significant anti-cholestatic, anti-inflammatory, and antiproliferative properties [197] with less toxicity than UDCA. In a multicenter, phase II clinical trial in Europe, *nor*-UDCA improved serum alkaline phosphatase regardless of concomitant UDCA use [198]. Other therapies in development include obeticholic acid (OCA), approved for the treatment of PBC, an epimer of UDCA and a farnesoid X receptor agonist that regulates bile acid homeostasis and many other metabolic processes as well as a peptidomimetic FGF19 agonist that downregulates bile acid synthesis by CYP7A.

The clear immunologic basis of PSC would appear to make immunosuppressive therapy a reasonable treatment option. Glucocorticoids, administered both orally and via nasobiliary lavage, have not shown a clear benefit in uncontrolled studies [199, 200]. Oral budesonide has been evaluated in an uncontrolled pilot study in 21 patients with PSC but was not effective and resulted in significant loss of bone mass [57]. In a small prospective, controlled trial of methotrexate, no biochemical, histologic, or cholangiographic differences from therapy with placebo were seen after 2 years of treatment [201]. A study of tacrolimus demonstrated significant biochemical improvement after 1 year, but no change in cholangiographic or histologic severity [202]. Neither infliximab nor etanercept, TNF-α inhibitors, showed a benefit in patients with PSC [66, 203].

Antibiotics have been used with no clear benefit but remain under study. In pediatric PSC patients treated with oral vancomycin, all 14 had improvement in liver biochemistries, especially those without cirrhosis [204]. The same investigators subsequently found that oral vancomycin improved liver histology and imaging while increasing plasma levels of transforming growth factor-β (TGF-β) and peripheral Tregs suggesting an immunomodulatory mechanism [205]. In adults, oral vancomycin demonstrated a modest reduction of serum alkaline phosphatase over 12 weeks of treatment [206]. Despite these promising results, the potential harm from indiscriminate removal of gut flora as illustrated by the *Mdr2*-null mouse raised in a germ-free environment should temper their widespread use [174].

Other approaches under study include anti-fibrotic medications but to date have also not shown significant benefits. Combination therapy targeting several pathways may be needed for an effective therapy in PSC. Historically, combinations of various agents such as azathioprine, glucocorticoids, UDCA, and antibiotics have been studied in a limited fashion [64, 207, 208]. The results of these studies have been mixed, with some showing no benefit and others demonstrating histologic improvement in small numbers of patients. Of note, with combination therapy comes an increased risk of adverse drug reactions.

Endoscopic Management

In select patients, endoscopic therapy for PSC carries the potential to relieve jaundice, pruritus, and abdominal pain; improve biochemical cholestasis; decrease the frequency of episodes of bacterial cholangitis; and improve bile flow. In theory, improved long-term biliary patency could slow the progression of the disease and prevent or delay biliary cirrhosis, but studies of endoscopic intervention in patients with PSC have been small, retrospective series and uncontrolled trials. Thus, routine endoscopic therapy in PSC is not recommended.

Patients most likely to benefit from endoscopic intervention are those with a known or suspected dominant stricture defined as a stenotic area with diameter ≤1.5 mm in the common bile duct or ≤1 mm in the hepatic duct [209], particularly if they present with worsening jaundice or pruritus, cholangitis, or abdominal pain. Dominant strictures are associated with reduced transplant-free survival [33], and multiple studies have reported significant improvements in clinical, biochemical, and cholangiographic endpoints in patients with a dominant stricture treated with endoscopic therapy [210–214], usually with balloon dilation, with or without temporary stent placement. Sphincterotomy is controversial since it can result in further sclerosis of the distal biliary tree and increase the risk of bacterial cholangitis. Despite an increased risk of periprocedural bleeding especially in cirrhotic patients, sphincterotomy may protect against post-ERCP pancreatitis in those who are likely to undergo multiple ERCPs with complex cannulation.

Choledocholithiasis should be considered in patients with worsening cholestasis. In as many as 30% of the cases, small stones may be missed by ERCP and regarded as wall irregularities, consistent with PSC [215]. The use of direct chol-

angioscopy allows for detection of these stones and use of lithotripsy is needed. Direct visualization with cholangioscopy is also useful for evaluation of dominant strictures as it allows for targeted biopsies which improve overall diagnostic accuracy compared to ERCP [216].

Placement of a biliary stent after balloon dilatation appears to increase the risk of complications compared with balloon dilation alone [217, 218]. Professional society guidelines recommend avoiding routine stenting of dominant biliary strictures in PSC, although short-term stenting (<2 weeks) may be required for severe strictures [45, 74]. Importantly, patients with PSC should receive antibiotic prophylaxis prior to undergoing ERCP, with most groups recommending continuing treatment for 3–5 days after the ERCP.

Three studies have suggested that progression of the underlying disease process may be slowed by endoscopic therapy of a dominant stricture. Baluyut and colleagues [219] performed graduated and balloon dilation, with or without stent placement, in 63 patients with PSC, with a median follow-up of 34 months, and observed a 5-year survival that was significantly better than the survival predicted by the revised Mayo model score. Stiehl and colleagues [209] performed endoscopic balloon dilation and occasional stent placement in 52 patients with PSC in whom a dominant stricture developed while the patients were on therapy with UDCA. Actuarial survival free of liver transplantation at 3, 5, and 7 years was significantly better than that predicted from the multicenter model score. An extension of this study including 96 patients suggested that there was an improvement in liver transplantation-free survival with dilation [34]. Finally, a retrospective study by Gluck and colleagues [220] reported that patients who had endoscopic therapy performed had a significantly higher survival rate than predicted by the revised Mayo model score at 3 and 4 years.

Endoscopic therapy in PSC also has important limitations, including increased risks of complications of ERCP, such as pancreatitis, cholangitis, worsening cholestasis, and perforation, at an overall rate of 7.3–10% [220]. Patients with diffuse intrahepatic biliary stricturing and no dominant stricture are less likely to derive benefit from endoscopic intervention and may be at higher risk for post-ERCP cholangitis [209]. In light of the limitations of the studies suggesting a benefit and the risks of biliary manipulation in PSC, routine endoscopic intervention for stricture management remains controversial.

Percutaneous Management

Percutaneous transhepatic cholangiogram with balloon dilation, stenting, or both can also be undertaken to treat biliary strictures in patients with PSC. This approach is typically recommended only when endoscopic intervention is contraindicated or unsuccessful because of the added risks of bleeding and bile peritonitis, as well as increased patient discomfort, associated with percutaneous intervention.

Biliary Surgery

With improvements in endoscopic therapy and liver transplantation, biliary surgery for PSC is rarely indicated. Occasionally, dominant strictures at or near the hepatic bifurcation are resected with hepaticojejunostomy or choledochojejunostomy, but due to the high mortality in cirrhosis and risk of undiagnosed cholangiocarcinoma, caution should be taken when considering this procedure [221]. In addition, the viability of future liver transplantation may be impacted by this surgery.

Liver Transplantation

Liver transplantation remains the only therapy that improves the natural history of PSC as well as quality of life [222, 223]. The most common indication for liver transplantation for patients with PSC is decompensated cirrhosis and complications of portal hypertension. Less frequently, recurrent cholangitis, intractable pruritus, and early-stage perihilar cholangiocarcinoma are indications for liver transplantation. Overall, adult liver transplants for PSC in the United States are an uncommon indication accounting for between less than 5% of all transplants performed [224].

Outcomes from liver transplantation for PSC in terms of both patient and graft survival are excellent and generally are significantly better than those for any other indication with the exception of PBC [225]. Recipient factors that have been associated with a worse prognosis after liver transplantation for PSC are older age, decreased serum albumin level, renal failure, Child-Pugh class C cirrhosis, and advanced United Network for Organ Sharing status [226].

Cholangiocarcinoma even when discovered incidentally in the explant portends a poor prognosis with 1- and 5-year survival rates of 65% to 82% and 35% to 42% [227, 228]. Even with external radiation, brachytherapy, radiosensitizing therapy, and/or chemotherapy prior to liver transplantation, 5-year recurrence-free survival is only 65% [229].

Recurrent PSC following transplantation is common, though the incidence rates vary widely between studies, and is associated with a reduced rate of survival [230]. To definitively diagnose recurrent PSC, other causes of posttransplant biliary strictures including ABO blood group incompatibility, hepatic artery occlusion, chronic ductopenic graft rejection, Roux-en-Y-related cholangitis, and preservation-related ischemia must be ruled out. When a strict diagnosis of recurrent PSC is applied including a cholangiographic pattern consistent with PSC and compatible liver histology as well as a lack other risk factors for biliary strictures or

non-anastomotic strictures within 90 days of transplantation [231], the prevalence of recurrent PSC after liver transplantation ranges from 5.7% to 59.1% after 2.6 to 9.1 years [232–236]. Multiple risk factors implicated in recurrent PSC include active IBD prior to transplant and use of tacrolimus-based immunosuppression. Colectomy before liver transplantation is associated with reduced rates of recurrent PSC [230, 233, 237].

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Autoimmune Hepatitis

26

Rodrigo Liberal, Giorgina Mieli-Vergani, and Diego Vergani

Key Points

- Autoimmune hepatitis (AIH) is characterized by a histological lesion called interface hepatitis in which mononuclear cells infiltrate the portal tracts and invade the parenchyma, disrupting the limiting plate.
- A set of inclusion and exclusion criteria for the diagnosis of AIH have been established by the International Autoimmune Hepatitis Group.
- There are two main types of AIH: type 1, positive for anti-nuclear (ANA) and/or anti-smooth muscle (SMAs) antibodies and type 2, positive for anti-liver kidney microsomal antibody type 1 (LKM-1) and/or anti-liver cytosol type 1 (LC-1) antibody.
- Autoantibodies should be tested by indirect immunofluorescence at an initial dilution of 1/40 in adults and 1/10 in children on a rodent substrate that includes kidney, liver and stomach to allow simultaneous detection of all reactivities relevant to AIH.
- Anti-LKM-1 antibody is often confused with anti-mitochondrial antibody (AMA) if only rodent kidney is used as substrate in indirect immunofluorescence.
- The identification of the molecular targets of anti-LKM-1 and AMA has led to the establishment of immuno-assays based on the use of the recombinant or purified autoantigens.
- Perinuclear anti-nuclear neutrophil antibody (p-ANNA) is an additional marker of AIH-1; anti-soluble liver antigen (SLA) antibodies are specific for autoimmune liver disease, can be present in AIH-1 and AIH-2 and are associated to a more severe clinical course. Anti-SLA are detectable by ELISA or radio-immuno-assays, but not by immunofluorescence.
- Predisposition to AIH-1 is conferred by the possession of HLA-DR3 in young patients, and HLA-DR3 and HLA-DR4 in older patients, whilst susceptibility to AIH-2 is conferred by possession of HLA-DR7 and HLA-DR3.
- Patients with AIH respond well to immunosuppressive treatment, even in the presence of cirrhosis, and have an excellent long-term prognosis.
- In AIH-2, the autoantigen targeted by anti-LKM-1 is cytochrome P4502D6 (CYP2D6) and that targeted by anti-LC1 is formiminotransferase cyclodeaminase (FTCD).
- All arms of the immune system, including CD4, CD8 and B lymphocytes, are involved in the liver autoimmune attack.
- Impairment in number and function of regulatory T cells (Tregs) plays a permissive role in the development of AIH.
- Adoptive transfer of in vitro expanded antigen-specific Tregs is an attractive treatment prospect, which is currently under investigation.

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Introduction

The first account of autoimmune hepatitis (AIH) dates back to the 1950s, when Jan Waldenström described a group of young women affected by severe and fluctuating persistent hepatitis associated with acneiform rashes, spider angiomas,

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anovulatory amenorrhea and profoundly elevated serum immunoglobulins [1]. The presence of lupus erythematosus cells and the detection of antinuclear antibody (ANA) seropositivity subsequently led to the adoption of the term "lupoid hepatitis" and the idea that the condition stems from a loss of immunological tolerance [2]. The positive impact of steroid therapy, initially recognized in the early 1960s, resulted in the publication of three controlled clinical trials which incontrovertibly showed the life-saving value of corticosteroids in the treatment of what was then referred to as "hepatitis B surface antigen (HBsAg)-negative hepatitis" [3–5]. The recognition that "chronic active autoimmune hepatitis," as it was later termed, constituted a distinct clinical entity followed the systematic evaluation of its clinical symptoms, laboratory features and molecular immunopathology. During two working meetings held in the early 1990s, the International Autoimmune Hepatitis Group (IAIHG) introduced the term "autoimmune hepatitis," as originally suggested by Ian Mackay in 1965 [6], since the disease frequently presents acutely and often has a fluctuating course, characterized by spontaneous remission, being therefore occasionally inactive. The IAIHG continues to monitor developments in the field regularly and was responsible for the development of an initial scoring system for the diagnosis of AIH [7], that was subsequently revised [8]. Later, a simplified system, designed for use in clinical practice, has been proposed by the group [9].

Two types of AIH are recognized, based on the serological autoantibody profile: AIH type 1 (AIH-1) is defined by positivity for ANA and/or anti-smooth muscle antibody (SMA), whereas AIH type 2 (AIH-2) is characterized by the presence of anti-liver kidney microsomal type 1 antibody (anti-LKM-1) or anti-liver cytosol type 1 antibody (anti-LC-1). Besides the presence of autoantibodies, AIH is associated biochemically to elevated transaminase levels, histologically to interface hepatitis and serologically to increased levels of immunoglobulin G (IgG).

AIH is the first liver disease for which medical therapy was shown to improve survival [10]. Immunosuppressive therapy with steroids alone or in combination with azathioprine, which remains the standard of care, should be instituted as soon as the diagnosis is made. Normalization of serum transaminase and immunoglobulin levels is generally accepted as an endpoint for the treatment of AIH and used to define complete remission [11, 12]. Patients not achieving complete remission usually experience histological progression [13]. Patients who do not respond or who do not tolerate standard therapy are challenging, and their therapeutic needs remain unmet.

The aetiology of autoimmune hepatitis is unknown, though both genetic and environmental factors are likely to be involved. An immune response targeting liver autoantigens, unrestrained because of the failure of immunoregulatory mechanisms, is thought to initiate and perpetuate the liver damage [14].

This chapter will review recent breakthroughs in our understanding of the pathogenesis of AIH, linking them to advances in clinical practice.

Epidemiology

AIH most commonly affects females, with a male:female ratio of 1:4 [12]. Although the peak incidences of the disease are in adolescence and at 30–45 years of age, AIH can affect children and adults of all ages [12].

The exact incidence and prevalence of AIH are unknown, because most studies were conducted before the introduction of standardized criteria developed by the IAIHG [7]. Moreover, early studies are marred by the possible inclusion of patients with chronic hepatitis C. The mean annual incidence and prevalence of AIH in one Norwegian study were 1.9 cases per 100,000 people per year and 16.9 cases per 100,000 people, respectively [15]. In a Spanish population, the mean annual incidence in the population over 14 years of age was 0.83 cases per 100,000, with a prevalence of 11.6 cases per 100,000 inhabitants [16], but these figures are biased by the fact that the study was hospital-based in a tertiary referral centre. Notably, the first study to utilize the IAIHG scoring system reports a much higher prevalence of definite AIH; 35.9 cases per 100,000 within the native Alaskan population [17]. Another study using the IAIHG standardized criteria reported an annual incidence of 2.0 cases of AIH per 100,000 and a point prevalence of 24.5 cases per 100,000 in New Zealand [18]. A study from the United Kingdom reported an annual incidence of 3 cases per 100,000 inhabitants [19]. A large study conducted in the Netherlands showed an AIH-1 prevalence of 18.3 cases per 100,000, with an annual incidence of 1.1 per 100,000 per year in adults, the peak incidence being in women aged 40–60 years [20]. A nationwide registry-based cohort study from Denmark reported an incidence rate of 1.68 cases per 100,000 people and demonstrated that the incidence of the disease increased during 1994–2012 [21]. AIH cases are thought to be less frequent in Asia. In Japan, the incidence is estimated to fall between 0.08 and 0.15 cases per 100,000 people per year [22]. In China, where autoimmune liver disease has historically been considered very rare, AIH is being reported with increasing frequency after the adoption of a more refined diagnostic work-up [23]. Epidemiological studies are detailed in Table 26.1.

The diagnosis of AIH-2, which affects mainly children and young adults, is often overlooked; hence, the prevalence remains unknown. The King's College Hospital tertiary paediatric hepatology referral centre has seen a seven-fold increase in the incidence of both AIH-1 and AIH-2 over the last decade. AIH represents approximately 10% of some 400 new referrals per year, with two-thirds of cases diagnosed

Table 26.1 Epidemiology of autoimmune hepatitis

Location	Year	Incidence (per 100.000 inhabitants)	Prevalence (per 100.000 inhabitants)	References
Japan	1997	0.08–0.15	n/a	[22]
Norway	1998	1.9	16.9	[15]
Alaska	2002	n/a	35.9	[17]
Spain	2004	0.83[b]	11.6[b]	[16]
United Kingdom	2007	3.0	n/a	[19]
New Zealand	2010	2.0	24.5	[18]
Denmark	2014	1.68	23.9	[21]
Canada	2015	0.23[a]	n/a	[24]

n/a not applicable
[a]Paediatric population only
[b]In individuals over 14 years of age

with AIH-1 and one-third with AIH-2. In addition, a study from Canada which included 159 children and adolescents with AIH, the annual incidence was 0.23 cases per 100,000 children; AIH-1 was diagnosed 5.5-times more frequently than AIH-2 [24].

Aetiology and Pathogenesis

Genetics

AIH is a complex genetic disorder as the susceptibility to the disease is influenced by several genes. The strongest predisposition to AIH is linked to Major Histocompatibility Complex (MHC) class II genes (Table 26.2), more specifically to the Human Leukocyte Antigen (HLA)-DR locus, located on the short arm of chromosome 6 – which are involved in the presentation of antigenic peptides to T cells, and are therefore implicated in the initiation of an adaptive immune response [25, 26].

In Europe and North America, the alleles conferring susceptibility to AIH-1 in adults are HLA-DR3 (*DRB1∗0301*) and HLA-DR4 (*DRB1∗0401*): both are heterodimers containing a lysine residue at position 71 of the DRB1 polypeptide and the hexameric amino acid sequence LLEQKR at positions 67–72 [25]. The first genome-wide association study (GWAS) in AIH performed in Dutch AIH-1 patients and replicated in a cohort of German patients, confirming the HLA association, identified *DRB1*0301* and *DRB1*0401* as primary and secondary susceptibility genotypes, respectively [27]. In Japan, Argentina and Mexico, susceptibility is linked to *DRB1*0405* and *DRB1*0404* alleles encoding arginine rather than lysine at position 71 but sharing the motif LLEQ-R with *DRB1*0401* and *DRB1*0301* [25]. Thus, the two basic amino acids lysine and arginine at position 71 in

Table 26.2 HLA associations in autoimmune hepatitis

HLA Locus	Allele Association	AIH-1	AIH-2
HLA-B	B8	LD with DRB1*0301 Severe disease course Relapse after drug withdrawal More frequent requirement for LT	
HLA-C	Cw7	Susceptibility in United Kingdom (LD with DRB1*0301)	
HLA-DRB1	DRB1*0301	Susceptibility in Europe and North America Younger age at onset, higher rate of treatment failure, relapse after drug withdrawal and requirement for LT than DR4 More expression of SLA/LP	Second most frequent susceptibility allele in children Associated with seropositivity for both anti-LKM-1 and anti-LC-1
	DRB1*0401	Susceptibility in Europe and North America Later age at onset than DR3 Higher frequency in women Low frequency of progression to hepatic failure and death Higher frequency of ANA positivity Protection in juvenile AIH	
	DRB1*0404	Susceptibility in Mexico	
	DRB1*0405	Susceptibility in Japan and Argentina	
	DRB1*0701		Susceptibility in Europe and in Brazil Predominant amongst patients positive for only anti-LKM-1 Aggressive disease course and worse prognosis
	DRB1*1301	Susceptibility in South America and in DR3/DR4-negative North American patients	Early age at onset in Brazil
	DRB1*1501	Protection in United Kingdom	

(continued)

Table 26.2 (continued)

HLA Locus	Allele Association	AIH-1	AIH-2
HLA-DQ	DQB1*0201	Susceptibility in United Kingdom and South America	Susceptibility in Europe and in North America (LD with DRB1*0301 and DRB1*0701)
	DQB1*0301	Protection in South America	
	DQB1*0601	Susceptibility in Brazil (LD with DRB1*1301)	

Abbreviations: HLA human leukocyte antigen, *AIH* autoimmune hepatitis, *LD* linkage disequilibrium, *LT* liver transplantation, *SLA/LP* soluble liver antigen/liver pancreas, *anti-LKM-1* anti-liver microsomal antibody type 1 antibodies, *anti-LC-1* anti-liver cytosol type 1 antibodies, *ANA* anti-nuclear antibodies

the context of LLEQ-R may be critical for susceptibility to AIH, favouring the binding of autoantigenic peptides complementary to this hexameric sequence [28].

In northern Europe, paediatric AIH-1 is also associated with *DRB1*03*, whereas *DRB1*04* confers protection [25, 29]. In Brazil and Egypt, the primary susceptibility allele for paediatric AIH-1 is *DRB1*1301*, but a secondary association with *DRB1*0301* has also been identified [30].

Susceptibility to AIH-2 is conferred by the possession of *DRB1*0701* and *DRB1*0301* [31], and those patients who are positive for *DRB1*0701* have a more aggressive form of the disease with worse overall prognosis [32].

A number of genes outside the MHC have also been linked to susceptibility to AIH. For example, a substitution from A (adenine) to G (guanine) in exon 1 of the *CTLA-4* gene confers susceptibility to AIH-1 in Caucasians from North America [33]. Additionally, a polymorphism at position 308 in the tumour necrosis factor α (*TNFA*) gene promoter is particularly frequent in patients with AIH-1 from Europe and North America and is associated with a poorer response to steroids [34]. A *FAS* gene promoter polymorphism at position 670 also enhances susceptibility to AIH and influences progression to a more aggressive form characterized by the early development of cirrhosis [35]. Polymorphisms in the vitamin D receptor can also be predisposing factors to the development of autoimmune liver disease [36]. The AIH GWAS reported that AIH-1 is associated with variants of *SH2B3* locus, a gene which is a negative regulator of T-cell activation, tumour necrosis factor and Janus kinase 2 and 3 signalling, and plays an essential role in normal haematopoiesis [27].

The occurrence of an AIH-like picture in patients with rare monogenic disorders, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndromes – caused by mutations in *AIRE-1* and *FOXP3* genes, respectively – as well as in patients with *CTLA-4* or *GATA-2* mutations, further supports the role of non-HLA genes in the pathogenesis of AIH [35]. Interestingly, in all these conditions, patients have an impairment of regulatory T cells, a feature that characterizes AIH and is likely to be involved in its pathogenesis.

Potential Triggers

In patients with increased genetic susceptibility, a potential trigger for AIH development is an immune response to exogenous pathogens that cross-reacts with structurally similar liver autoantigens, a phenomenon known as molecular mimicry. The strongest support for this model is in the context of viral hepatitis, where autoimmunity is a common feature during chronic infection. Indeed, 50% of patients with chronic hepatitis B or C eventually develop autoantibody seropositivity [37, 38]. In chronic HCV, some 10% of patients are anti-LKM-1 positive, the autoantibody titre correlating with disease severity and being associated with adverse reactions to interferon treatment [39]. The target antigen of anti-LKM-1 is cytochrome P4502D6 (CYP2D6) in both AIH-2 and HCV infection. Within anti-LKM-1-positive chronic HCV patients, reactivity against a key autoantigenic target of anti-LKM-1, the epitope $CYP2D6_{193\text{-}212}$, can be seen in 50% of patients. There is direct evidence of cross-reactivity between anti-LKM-1 and antibodies directed against homologous regions of HCV (NS5B $HCV_{2985\text{-}2990}$) and cytomegalovirus (exon $CMV_{130\text{-}135}$) [40]. There is also sequence homology between $CYP2D6_{254\text{-}271}$ and amino acids present in the E1 HCV and the IE1 75 regions of the Herpes Simplex Virus 1 (HSV-1). As anti-LKM-1 antibodies cross-react with homologous regions of CYP2D6, HCV, CMV and HSV, a "multi-hit" mechanism for the generation of autoantibody seropositivity and possibly for the development AIH-2 can be envisaged. In this model, on a background of genetic susceptibility, sequential exposure to common viral pathogens favours the development of cross-reactive T cells. It is therefore conceivable that as yet, unidentified single or repeated viral infections could contribute to the initiation of the autoimmune attack in AIH [14]. One case-report describes a 10-year-old girl who acquired HCV infection following liver transplant for end-stage liver disease caused by α1-anti-trypsin deficiency. Two weeks after HCV infection, IgM anti-LKM-1 antibodies appeared, followed by IgG anti-LKM-1 antibodies. This finding is suggestive of HCV as a trigger of a primary anti-LKM-1/anti-CYP2D6 autoimmune response [41]. Interestingly, 10 years later, the patient developed florid AIH type 2, which responded satisfactorily to immunosuppressive treatment; by this time, there was no trace of the previous HCV infection (unpublished data). Moreover, in a recent report, up to 8.7% of patients with autoimmune disease, including cryoglobulinaemia, Hashimoto

thyroiditis and inflammatory bowel disease (IBD) [42], had serum HCV antibody-positivity, linking HCV infection with a breakdown of immune tolerance.

The antibiotics nitrofurantoin and minocycline [43], as well as the statins and the anti-TNF agents adalimumab and infliximab, have been reported as non-viral environmental triggers of AIH. However, because drug-induced liver injury with features of AIH does not usually require long-term immunosuppressive treatment, these triggers should be considered independently [43].

Though the evidence for molecular mimicry is convincing, a universal trigger has not yet been discovered. Moreover, other non-mutually exclusive mechanisms that may contribute to the initiation and perpetuation of AIH, such as epitope spreading or exposure to previously hidden autoantigens during hepatocellular injury, should also be explored.

Mechanisms of Liver Damage

The dense infiltrate of lymphocytes, plasma cells and macrophages characteristic of the histological picture of AIH suggests that an auto-aggressive cellular immune attack is the basis of this condition. Over the past four decades, intense investigations have begun to uncover the mechanisms by which this inflammatory infiltration mediates liver damage.

The predominant population within the cellular infiltrate is composed of α/β T cells [44]. Amongst these cells, the majority are $CD4^{pos}$ T helper (Th) cells, with a sizable minority of cytotoxic $CD8^{pos}$ T cells. Immunohistochemically, lymphocytes of a non-T-cell lineage are seen relatively rarely, and include natural killer (NK) cells, macrophages, B cells and plasma cells [44].

Whatever the trigger, the pathogenic mechanisms leading to liver damage in AIH are complex, involving the intervention of both innate and adaptive arms of the immune system. They can be summarized as follows (Fig. 26.1): an autoantigenic peptide is presented to an uncommitted T helper (Th0) lymphocyte within the HLA class II molecule of an antigen-presenting cell. Th0 cells become activated and, according to the cytokines present in the microenvironment, differentiate into Th1, Th2, or Th17 cells, initiating a series of immune reactions determined by the cytokines they produce.

Th1 cells secrete IL-2 and IFN-γ, which stimulate CD8 T cells, enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and

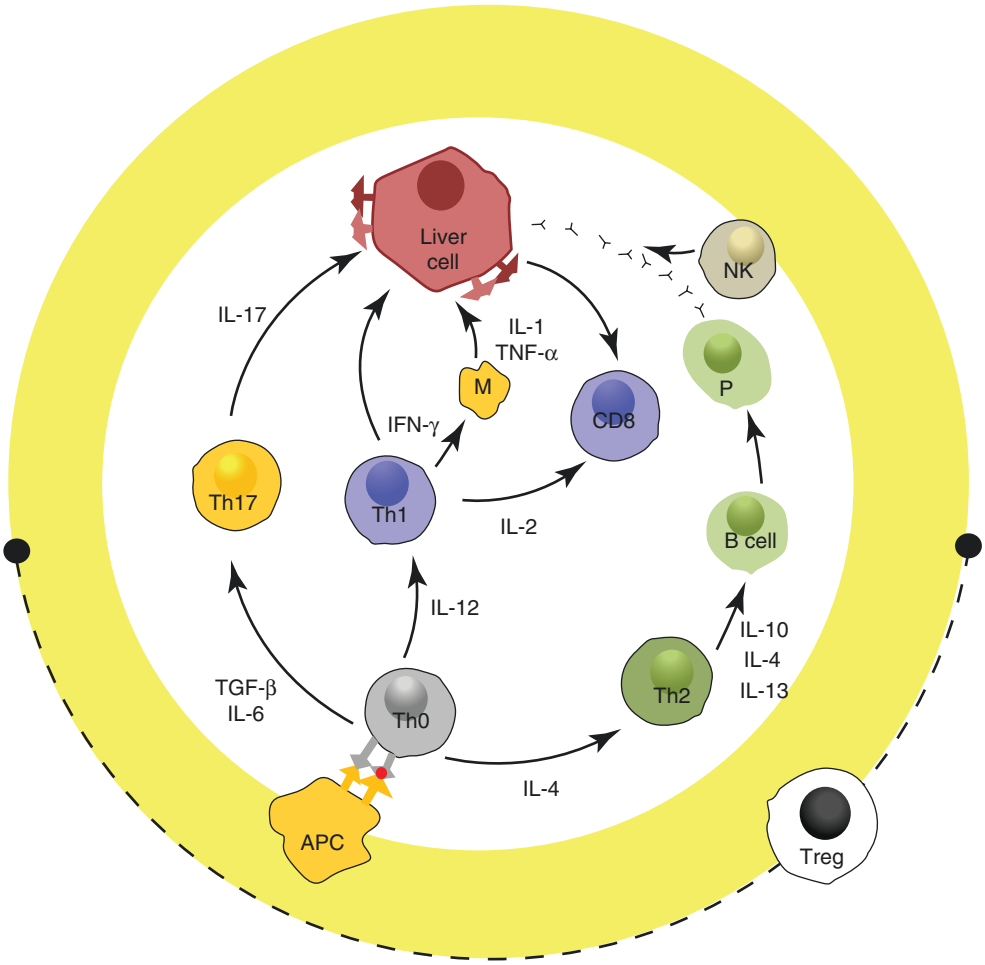

Fig. 26.1 Mechanisms of liver damage. Liver damage is initiated by the presentation of a self-antigenic peptide within a major histocompatibility molecule (MHC) by professional antigen-presenting cells (APCs). The presence of appropriate co-stimulation alongside exposure to various cytokines drives the differentiation of uncommitted CD4 helper T cells (Th0). Exposure to IL-12 leads to the differentiation of Th1 cells secreting IFN-γ, which induces monocyte differentiation, activates cytotoxic CD8 T cells and promotes NK-cell killing. IFN-γ also increases MHC class I and induces class II expression by hepatocytes, further exacerbating inflammation. Exposure to IL-4 leads to Th2 differentiation. Th2 cells secrete IL-4, IL10 and IL-13, cytokines that enable B-cell maturation into plasma cells with the consequent production of autoantibodies. Autoantibodies are in turn involved in antibody-mediated cellular cytotoxicity and complement activation. The autoimmune attack takes place due to impairment of regulatory T cells (Tregs)

activate macrophages [45, 46]. In mouse models, IFN-γ drives hepatocyte apoptosis and cell cycle arrest [47], whilst enhancing chemokine- and adhesion-molecule-driven leukocyte infiltration of the liver [48]. The key role of IFN-γ in the initiation and perpetuation of liver injury has been demonstrated in a mouse model of acute hepatitis. IFN-γ deficiency [49] or the administration of anti-IFN-γ monoclonal antibodies [50, 51] protects mice from developing concanavalin-A (Con-A)-induced hepatitis. There is a greater proportion of circulating Th1 cells in AIH patients [52] and a higher secretion of IFN-γ in AIH patients compared to healthy subjects [53].

Th17 cells produce IL-17A and IL-17F [54]; both inducing the production of pro-inflammatory cytokines (IL-6, IL-1 and TNF), pro-inflammatory chemokines (CXCL-1, CXCL-6 and IL-8) and metalloproteinase, ultimately leading to the recruitment of neutrophils to the site of inflammation [55]. Besides IL-17A and IL-17F, Th17 cells also produce IL-21 and IL-22, neither of which are Th17-exclusive cytokines [54, 56, 57]. The role of Th17 cells in AIH is incompletely understood. There is, however, robust evidence that they contribute to the cholangiopathy, characteristic of primary biliary cholangitis [58], implying that Th17 cells contribute to cholestatic forms of liver injury. Th17 cells are also elevated in the circulation and the liver of patients with AIH [59]. Moreover, IL-17 production by Th17 cells has been shown to induce hepatocytes to produce IL-6, which further enhances Th17-cell activation [59]. The Con-A model provides some support for a pathogenic role of Th17 cells in liver injury, since both IL-17- [60, 61] and IL-17-receptor-deficient [61] mice have reduced hepatic injury compared to wild-type controls.

B cells are also involved in the pathogenesis of AIH. The fluctuating course of AIH is mirrored by the activation of B cells, which results in hypergammaglobulinaemia and production of autoantibodies, whose levels reflect disease activity [14]. Autoantibodies have been reported to contribute to liver damage in AIH: titres of anti-liver-specific membrane lipoprotein, for example, correlate with biochemical and histological indices of disease severity [62, 63]. The role of B cells in the autoimmune attack has been recently shown in an AIH mouse model, in which the administration of an anti-CD20-depleting antibody resulted in significant biochemical and histological improvements [64].

Loss of Self-Tolerance

The development of autoimmune diseases is favoured by the breakdown of self-tolerance mechanisms that, in health, prevents the majority of auto-reactive T-cell clones from entering the periphery. As circulating auto-reactive T cells are, however, present in health, there are both intrinsic and extrinsic peripheral tolerance mechanisms to limit autoimmune tissue damage. Key to this homeostatic process is the control exerted by regulatory T cells (Tregs), which are specialized suppressive cells central to immune tolerance maintenance [65]. The archetypal Treg is characterized by high constitutive expression of CD25 [66], the alpha chain of IL-2 receptor. IL-2 is required for the maintenance of tolerance to self. IL-2 neutralization [67] and deficiency [68] lead to reduced Treg numbers and are therefore associated with a variety of autoimmune manifestations [69]. The $CD25^{high}$ population is enriched in what is the most widely recognized marker of Tregs, the forkhead winged helix transcription factor (Foxp3) [70]. Tregs can exert their suppressive function either through a contact-dependent mechanism – by directly targeting the function of effector T cells as well as modulating the maturation and/or function of dendritic cells (DCs), which in turn are required for the activation of the former – and/or through contact-independent mechanisms [71].

In the context of AIH, seminal studies conducted during the 1980s demonstrated that cells with "suppressor" function were impaired, and that this defect could be minimized by their exposure to therapeutic doses of steroids in vitro [72]. Such cells, constituting a subpopulation of T lymphocytes, were able to control immune responses against a liver-specific membrane autoantigen [72]. These early experiments paved the way for a series of studies, performed almost 20 years later, demonstrating that regulatory T cell impairments are pivotal to loss of immune tolerance in AIH [73–78]. These studies have shown that in both children and adults with AIH, there is a reduced frequency of $CD4^{pos}CD25^{high}$ Tregs, which express lower levels of FOXP3 compared to healthy controls [73, 74, 77]. Tregs isolated from AIH patients are also less able to restrain the proliferation and IFN-γ production of CD4 and CD8 effector T cells compared to those from the healthy control population [73, 74]. These defects relate to the stage of liver disease, being more pronounced at presentation compared to drug-induced remission. Interestingly, the frequency of Tregs is inversely correlated with autoantibody titre; therefore, Treg reduction potentially favours the serological manifestations of AIH [73]. Moreover, in AIH, Tregs enhance the activation of monocytes, cells of the innate immune system abundantly present in the portal-periportal inflammatory infiltrate [79], and fail to create a regulatory milieu that would support and enhance their own function [75]. Subsequent reports have challenged these observations and showed that the number of $CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ cells was similar in adult AIH patients and controls and higher in patients with active disease compared to those at remission [49, 50].

More recently, we have re-examined the phenotypic and functional Treg properties of patients with juvenile-onset AIH and found that circulating $CD4^{pos}CD25^{pos}CD127^{neg}$

Tregs are decreased in AIH compared to health, their frequency being inversely correlated with indices of disease activity and not affected by the immunosuppressive treatment [51]. These "bona fide" Tregs produce less IL-10 and are impaired in their ability to suppress CD4 target cells, a feature that in healthy subjects, but not in patients, is dependent on IL-10 secretion. Notably, decreased IL-10 production by Tregs in AIH is linked to defective responsiveness to IL-2 and pSTAT-5 up-regulation [51].

The reasons for Treg impairment in AIH remain unclear. There is evidence showing that Tregs in AIH are defective in the expression of CD39, an ectonucleotidase that initiates an ATP/ADP hydrolysis cascade, culminating with the generation of immunosuppressive adenosine [52]. $CD39^{pos}$ Tregs from AIH patients are therefore defective in their ectoenzymatic activity and inhibition of Th17-cell function. Of note, in AIH but not in healthy individuals, $CD39^{pos}$ Tregs undergo a marked increase in the production of IFN-γ and IL-17 upon challenge with pro-inflammatory stimuli. This suggests that in AIH, Tregs are more prone to be skewed into effector cells, therefore contributing to the maintenance of the effector lymphocyte pool and to the perpetuation of autoimmune liver damage [52].

In addition to the dominant form of suppression performed by Tregs, effector cell intrinsic peripheral tolerance mechanisms have been described. For example, in healthy people, autoantigen-specific T cells express inhibitory receptors such as CD5, CTLA-4 and programmed cell death-1 (PD-1). Interestingly, in AIH, $CD4^{pos}$ T cells are, to some extent, resistant to Treg suppression. This defect is accounted for by the reduced expression of the inhibitory receptor T-cell-immunoglobulin-and-mucindomain-containing-molecule-3 (Tim-3), which upon ligation of galectin-9 expressed by Tregs, induces effector cell apoptosis [53]. The mechanisms that account for the impaired function of Tregs in AIH are depicted in Fig. 26.2.

Treg cell therapy, aimed at reconstituting self-tolerance, is a highly promising candidate for alternative and effective immune intervention in AIH. To date, this approach has been hindered by the limited ability of Tregs to expand and by their propensity to apoptose. However, because corticosteroid therapy can partially restore the potency of the Treg population, Tregs in AIH do have the potential to expand and regain their function [73, 74]. Using a polyclonal T cell stimulation strategy (that engages the T cell receptor via CD3 and the co-stimulatory molecule CD28, whilst providing exogenous IL-2, a key cytokine for Treg survival and growth), Tregs can be expanded from circulating $CD4^{pos}CD25^{pos}$ Tregs, and also generated de novo from non-regulatory $CD4^{pos}CD25^{neg}$ T cells in both healthy subjects and patients with AIH [76]. Interestingly, expanded Tregs express higher levels of FOXP3 and are more effective suppressors compared to freshly isolated Tregs [76].

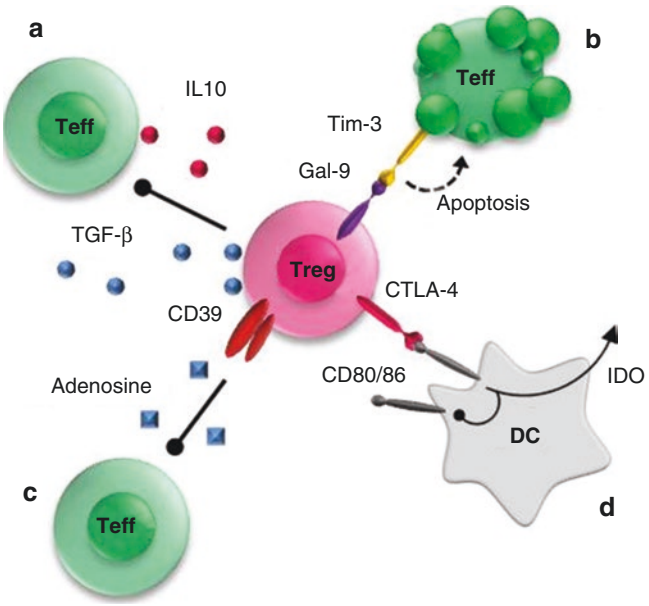

Fig. 26.2 Regulatory T cells in autoimmune hepatitis. Several mechanisms may determine the defective suppressive ability of regulatory T cells (Tregs) in autoimmune hepatitis (AIH): (**a**) low Treg number and impaired ability to suppress proliferation of effector cells and to secrete the anti-inflammatory cytokines transforming growth factor-β (TGF-β) and IL-10; (**b**) impaired apoptosis of activated effector T cells due to reduced Treg expression of galectin-9, which physiologically binds the T-cell-immunoglobulin-and-mucin domain-containing-molecule-3 (Tim-3) on effector cells leading to their apoptosis; (**c**) reduced Treg expression of the ectoenzyme ectonucleoside triphosphate diphosphohydrolase 1 (CD39), leading to impaired production of the inhibitory molecule adenosine (mechanism under investigation); (**d**) low Treg expression of the inhibitory molecule cytotoxic T lymphocyte antigen-4 (CTLA-4), leading to down-regulation of CD80/86 on dendritic cells (DCs) with consequent reduction in the production of immunosuppressive indoleamine 2,3-dioxygenase (IDO)

Although FOXP3 is the most specific marker of human Tregs, its intracellular location limits its use in the laboratory setting. In addition to the lack of specific cell-surface markers for Tregs, the human $CD4^{pos}$ $CD25^{high}$ population contains a proportion of activated effector T cells. Furthermore, Tregs and Th17 cells share a common progenitor, though their developmental pathways diverge. Since de novo generation of Tregs relies on strong T cell receptor (TCR) signalling, there is a risk of effector Th17 cell expansion and contamination, which needs to be addressed when considering Treg therapy for AIH [14]. The physical removal of $IL17^{pos}$ cells, or the use of small interfering RNAs specific for the Th17-associated transcription factor RORC, leads to elevated FOXP3 expression and increased suppressive function by expanded Tregs from AIH patients [80].

The potential for successful Treg therapy is particularly strong in AIH-2, given that the antigenic regions ($CYP2D6_{217\text{-}260}$ and $CYP2D6_{305\text{-}348}$), targeted by B, CD4 and CD8 T cells, are well characterised [81]. Several lines of evidence demonstrate that autoantigen-specific Tregs suppress

more efficiently than their non-antigen-specific counterparts. In this regard, antigen-specific Tregs generated from AIH-2 patients are able to suppress CD4 and CD8 T cell responses more potently than polyclonally expanded Tregs. The most efficient suppression of auto-reactive T cells has been achieved by Treg co-culture with semi-mature dendritic cells loaded with the CYP2D6 peptides [78].

Natural killer T (NKT) cells are another population with suppressive potential. This population, well represented within the liver, has been implicated in the regulation of immune responses in autoimmune liver disease. Indeed, NKT cells are reduced in frequency in the peripheral blood of AIH patients, particularly during the active phases of the disease and their number is partially restored during drug-induced remission. The behaviour of NKT cells, therefore, mirrors that of $CD4^{pos}CD25^{high}$ regulatory T cells [77]. In addition, NKT cells from AIH patients produce lower quantities of the regulatory cytokine IL-4 compared to healthy controls [77].

Animal Models

The Con-A-induced hepatitis model has been a useful tool to identify key cell populations and cytokines involved in hepatocellular injury. However, it is merely a model of acute injury mediated by cytokine storm, and it does not accurately reflect the chronic disease seen in human AIH [82]. In fact, animal models faithfully reflecting all the characteristics of AIH – which should include a well-defined initiating event followed by chronic inflammation leading to fibrosis – are lacking, although there are several candidates [82–90] (Table 26.3).

Transgenic models which express antigen under the control of liver-specific promoters feature prominently. Tolerance to these antigens is generally broken by the adoptive transfer of adjuvant and/or antigen-specific T cells. The TF-OVA transgenic mouse, in which ovalbumin (OVA) expression is driven by the hepatocyte transferrin promoter, is an example. In this model, OVA-specific OT-1 cells are administered to produce acute, transient hepatitis [84]. Although transgenic models have benefits – the initiating antigen is well defined and confined to the liver – liver injury is usually transient. One exception to this is the human CYP2D6 model, in which human CYP2D6 is delivered to the liver via an Adenovirus construct. Both wild-type and humanized CYP2D6 mice have been used to produce chronic persistent hepatitis [85, 86]. More recently, Yuksel et al. developed a model based on the HLA-DR3 transgenic mouse on the non-obese diabetic background by immunization with a DNA plasmid coding for human CYP2D6/formiminotransferase cyclodeaminase (FTCD) fusion protein (the target antigen of anti-LC1) [87]. Immunization with CYP2D6/FTCD fusion protein leads to increased transaminase levels, development of autoantibodies, interface hepatitis and fibrosis [87].

Table 26.3 Selected mouse models of autoimmune hepatitis

Model	Strategy	Characteristics	References
Experimental autoimmune hepatitis	Repeated immunization with liver homogenate and adjuvant	Persistent liver damage Perivascular liver infiltration	[83]
Con-A induced hepatitis	Administration of Con-A	Non-specific T-cell activation Cytokine storm Acute liver damage	[82]
TF-OVA transgenic	OVA expression under control of hepatocyte TF promoter OT-1 cell administration	Acute liver injury Transient hepatitis	[84]
Human CYP2D6	Human CYP2D6 delivered by adenovirus construct targeted to the liver of WT or humanized mice	Persistent cellular infiltration and fibrosis High titre autoantibodies	[85]
CYP2D6 DNA vaccine	Injection of plasmids encoding CYP2D6 and IL-12	CD4-mediated liver damage Transient autoantibodies Variable transaminase levels	[88, 89]
PD-1 deficient	Neonatal thymectomy in PD-1-deficient mice	CD4 and CD8 infiltrate Fatal hepatitis	[90]
CYP2D6/ FTCD vaccine	Injection of plasmids encoding CYP2D6/FTCD	Autoantibodies Interface hepatitis and fibrosis	[87]

Abbreviations: *Con-A* concanavalin-A, *CYP2D6* cytochrome P4502D6, *FTCD* formiminotransferase cyclodeaminase, *OVA* ovalbumin, *PD-1* programmed death-1, *TF* transferrin, *WT* wild-type

Murine studies have been used to investigate the role of Tregs in AIH; collectively, these investigations suggest that Tregs are able to protect against experimental liver injury. In one study – using a mouse model generated by DNA vaccination against CYP2D6 – the immunoregulatory defect associated with AIH was attributed to defects in the peripheral tolerance compartment, low thymic expression of the autoantigen being necessary, but not sufficient, to induce the disease [88, 89]. Diseased mice had a lower frequency of $CD4^{pos}CD25^{pos}Foxp3^{pos}$ Tregs compared to other strains [89]. Importantly, the adoptive transfer of ex vivo expanded Tregs was able to alleviate disease symptoms, restoring peripheral tolerance to the autoantigen [89]. Another model was generated using mice deficient in the inhibitory molecule PD-1 [90]. Neonatal thymectomy was used to dramatically reduce the number of circulating Tregs in PD-1-deficient mice, leading to fatal AIH characterized by pronounced $CD4^{pos}$ and $CD8^{pos}$ T cell infiltration, massive lobular necrosis and elevated titres of ANA. Importantly, adoptive transfer of Tregs

could prevent fatal hepatitis in this model, confirming the proposed roles of pathogenic auto-reactive T cells and protective Tregs in this condition [90].

Clinical Presentation and Natural History

AIH can present with diverse clinical manifestations [91]. There are basically three patterns of disease presentation: an acute onset; characterized by non-specific symptoms such as malaise, nausea/vomiting, anorexia and abdominal pain; followed by jaundice, dark urine and pale stools; an insidious onset, with an illness characterized by progressive fatigue, relapsing jaundice, headache, anorexia, amenorrhea and weight loss and finally a presentation with complications of portal hypertension [26]. The mode of presentation of AIH is therefore variable, and the disease should be suspected and excluded in all patients complaining of symptoms and signs of prolonged or severe liver disease. Some patients, however, are completely asymptomatic and are diagnosed after incidental discovery of abnormal liver function tests.

Without treatment, the reported 5- and 10-year survival rates are 50% and 10%, respectively [3–5]. Because of the use of corticosteroid treatment, the 10-year survival rate has risen to approximately 90% [14].

The complications associated with AIH are similar to those of other progressive liver diseases. Chronic hepatitis can evolve to cirrhosis and ultimately to hepatocellular carcinoma (HCC) despite the use of immunosuppressive therapy.

Histological evidence of cirrhosis is described in at least 30% of patients, regardless of the mode of presentation, suggesting that subclinical disease has been present for some time [92]. Indeed, in a study comprising over 450 AIH patients, 30% had evidence of cirrhosis at diagnosis, with a further 10% developing cirrhosis during a median follow-up time of 7.2 years. The presence of cirrhosis at diagnosis correlated with negative outcome [liver transplantation (LT) or death] [93]. In another study, including 126 AIH patients, Feld et al. reported that 33% had histological evidence of cirrhosis at diagnosis. With the exception of platelet count, which was lower in patients with cirrhosis, laboratory parameters, patient demographics and AIH scores did not differ between cirrhotic and non-cirrhotic patients. A similar frequency of patients from each group was symptomatic at diagnosis and an equivalent proportion had good response to treatment [94]. Importantly, similar response to treatment has also been reported elsewhere [18]. Feld et al. also found, however, that the presence of cirrhosis significantly increased the risk of progression to LT or death [94]. Consistent with the above studies, Verma et al. reported that 28% of AIH patients were cirrhotic at diagnosis. In this study, a further 20% of patients developed cirrhosis during 52 months of follow-up. Again, cirrhosis was an independent predictor of poor outcome in this cohort [95]. On the other hand, studies in the adult [18, 96] and paediatric [97] settings, of comparable size and methodology to those described above, have not found associations between the presence of cirrhosis at diagnosis and the likelihood of poor outcome. In one study, patients diagnosed between 21 and 60 years of age were more likely to present with cirrhosis than those outside this range. Male patients were also more likely to have cirrhosis compared to their female counterparts. Low serum albumin concentrations, prolonged INR and low platelet count were all more frequently associated with the AIH cirrhotic group [98]. There are indications that cirrhosis is more common amongst AIH-1 patients compared to patients with AIH-2. In a paediatric study, 69% of ANA-/SMA-positive patients had evidence of "definite cirrhosis" on initial biopsy, whereas only 38% of patients positive for anti-LKM-1 were cirrhotic. On follow-up, these values increased to 74% and 44%, respectively [29].

HCC development is relatively rare in patients with AIH. In a meta-analysis of 25 studies, 93 out of 6528 AIH patients were found to have developed HCC during a median follow-up of 8 years [99]. In the face of an overall incidence of HCC of 3.1 cases per 1000 patients/year, the incidence of the tumour was more than threefold higher (10.1, range: 6.9–14.7 per 1000 patients/year) in those with cirrhosis, which was present in 92 out of 93 patients with HCC [99]. The strict association of HCC with the development of cirrhosis was chronologically proven in two retrospective cohort studies published in London and Rochester. In the first cohort, out of 243 patients with AIH, 169 of whom were receiving immunosuppressive therapy, HCC developed exclusively in 15 (6.1%) out of 122 cirrhotic patients after a follow-up of over 40 years [100]. HCC occurred in the same proportion of females and males and was more frequent in patients who had cirrhosis at presentation or signs of portal hypertension [100]. The same was true in the Mayo Clinic's cohort of 212 patients where HCC was detected in 3 patients (1.4%) with cirrhosis, after a median follow-up of 68 months, and independently of immune suppressive therapy uptake [101]. The strict association of HCC risk with cirrhosis led the American Association for the Study of Liver Disease (AASLD) to recommend 6 monthly screening with abdominal ultrasonography in cirrhotic AIH patients [12].

Diagnosis and Scoring Systems

There is no single diagnostic test for AIH; thus, diagnosis is based upon several indicative clinical, serological, biochemical and histological findings. The presence of other causes of liver disease must also be excluded [12].

The IAIHG has established and revised a set of diagnostic criteria for AIH [7, 8] to be used mainly for research purposes (Table 26.4). This score comprises clinical, laboratory and histological parameters, including response to treatment

Table 26.4 International autoimmune hepatitis group's revised diagnostic scoring system

Parameter	Feature	Score
Sex	Female	+2
ALP: AST (or ALT) ratio	>3	–2
	1.5–3	0
	<1.5	+2
Serum globulins or IgG (times above normal)	>2.0	+3
	1.5–2.0	+2
	1.0–1.5	+1
	<1.0	0
ANA, SMA or anti-LKM-1 titres	>1:80	+3
	1:80	+2
	1:40	+1
	<1:40	0
AMA	Positive	–4
Viral markers of active infection	Positive	–3
	Negative	+3
Hepatotoxic drug history	Yes	–4
	No	+2
Average alcohol	<25 g/day	+2
	>60 g/day	–2
Histological features	Interface hepatitis	+3
	Plasma cells	+1
	Rosettes	+1
	None of the above	–5
	Biliary changes[a]	–3
	Atypical changes[b]	–3
Immune diseases	Thyroiditis, colitis, other	+2
HLA	DR3 or DR4	+1
Seropositivity for other autoantibodies	Anti-SLA/LP, actin, ASGPR, p-ANNA	+2
Response to therapy	Remission	+2
	Relapse	+3

Pre-treatment score >15: definite AIH; 10-15: probable AIH; post-treatment score >17: definite AIH; 12-17: probable AIH. Adapted from [8]
Abbreviations: ALP alkaline phosphatase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *IgG* immunoglobulin G, *ANA* anti-nuclear antibody, *SMA* anti-smooth muscle antibody, *anti-LKM-1* anti-liver kidney microsomal type 1 antibodies, *AMA* anti-mitochondrial antibodies, *SLA/LP* soluble liver antigen/liver pancreas, *ASGPR* asialoglycoprotein receptor, *p-ANNA* peripheral anti-nuclear neutrophil antibody, *HLA* human leukocyte antigen
[a]Including granulomatous cholangitis, concentric periductal fibrosis, ductopenia, marginal bile duct proliferation and cholangiolitis
[b]Any other prominent feature suggesting a different aetiology

[8]. Although too cumbersome for general bedside use, the system is clinically useful when evaluating patients with few or atypical features of AIH [102]. The distinction between a definite and probable diagnosis of AIH predominantly relates to the extent of the increase in serum gamma-globulin/IgG or autoantibody titre, as well as exposure to alcohol, hepatotoxic medication or infection. Laboratory and histological features associated with cholestasis carry a negative score. In the rare instances where conventional autoantibodies are not detected, the presence of anti-asialoglycoprotein receptor (anti-ASGPR), anti-soluble liver antigen/liver pancreas (anti-SLA/LP) or atypical perinuclear anti-neutrophil cytoplasmic antibodies (atypical p-ANCA, currently better referred to as p-ANNA) weigh towards a probable diagnosis of AIH. The scoring system also incorporates response to corticosteroids, with a definite diagnosis before steroid treatment requiring a score higher than 15, and a definite diagnosis after treatment institution requiring a score greater than 17 [8].

Table 26.5 Simplified criteria for the diagnosis of autoimmune hepatitis

Variable	Cut-off	Points
ANA or SMA	≥1:40	1
ANA or SMA	≥1:80	2[a]
or anti-LKM-1	≥1:40	
or SLA	Positive	
IgG	>upper limit of normal	1
	>1.10 times upper limit of normal	2
Liver histology	Compatible with AIH	1
	Typical of AIH	2
Absence of viral hepatitis	Yes	2

Abbreviations: ANA anti-nuclear antibody, *SMA* anti-smooth muscle antibody, *anti-LKM-1* anti-liver kidney microsomal antibody type 1, *SLA* soluble liver antigen, *IgG* immunoglobulin G, *AIH* autoimmune hepatitis
Score ≥ 6: probable AIH; ≥7: definite AIH. Adapted from [9]
[a]Addition of points achieved for all autoantibodies cannot exceed a maximum of 2 points

In an attempt to devise a less complicated and more practical process, the IAIHG has proposed in 2008 a simplified scoring system (Table 26.5) to be used in clinical practice [9]. The system, which only uses four parameters (hypergammaglobulinaemia, autoantibodies, histology and exclusion of viral hepatitis) [9], has since received external validation [102].

Neither the original nor the simplified IAIHG scoring systems are suitable for the diagnosis of AIH in children and adolescents. Thus, the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has recently proposed a dedicated scoring system for juvenile autoimmune liver disease (Table 26.6) [103].

Laboratory

The typical laboratory abnormalities in AIH include elevations of aspartate transaminase, alanine transaminase and γ-glutamyltransferase levels with either normal or slightly elevated alkaline phosphatase levels [11, 12]. Spontaneous fluctuations of aspartate transaminase and alanine transaminase levels, even dropping into the normal range, should not dissuade diagnostic testing [11]. Levels of total and direct bilirubin vary from normal to significantly abnormal.

AIH is also often associated with a generalized elevation of serum globulins, particularly gamma globulins, mainly due to an increase in IgG, which is present at diagnosis in 85% of patients [11, 12].

Table 26.6 Proposed scoring criteria for the diagnosis of juvenile autoimmune liver disease

Variable	Cut-off	Points	
		AIH	ASC
ANA and/or SMA[a]	≥1:20[b] ≥1:80	1 2	1 2
Anti-LKM-1[a] or Anti-LC-1	≥1:10[b] ≥1:80 Positive[b]	1 2 2	1 1 1
Anti-SLA	Positive[b]	2	2
p-ANNA	Positive	1	2
IgG	>ULN >1.20 ULN	1 2	1 2
Liver histology	Compatible with AIH Typical of AIH	1 2	1 2
Absence of viral hepatitis (A,B,E,EBV), NAFLD, NASH, Wilson disease & drug exposure	Yes	2	2
Presence of extrahepatic autoimmunity	Yes	1	1
Family history of autoimmune disease	Yes	1	1
Cholangiography	Normal Abnormal	2 −2	−2 2

Abbreviations: AIH autoimmune hepatitis, *ASC* autoimmune sclerosing cholangitis, *ANA* anti-nuclear antibody, *SMA* anti-smooth muscle antibody, *anti-LKM-1* anti-liver kidney microsomal antibody type 1, *anti-LC-1* anti-liver cytosol type 1, *anti-SLA* anti-soluble liver antigen, *IgG* immunoglobulin G, *EBV* Epstein–Barr virus, *NAFLD* non-alcoholic liver disease, *NASH* non-alcoholic steatohepatitis, *ULN* upper limit of normal
Score ≥7: probable AIH; ≥8: definite AIH. Score ≥7: probable ASC; ≥8: definite ASC. Adapted from [103]
[a]Antibodies measured by indirect immunofluorescence on a composite rodent substrate (kidney, liver, stomach)
[b]Addition of points achieved for ANA, SMA, anti-LKM-1, anti-LC-1 and anti-SLA autoantibodies cannot exceed a maximum of 2 points

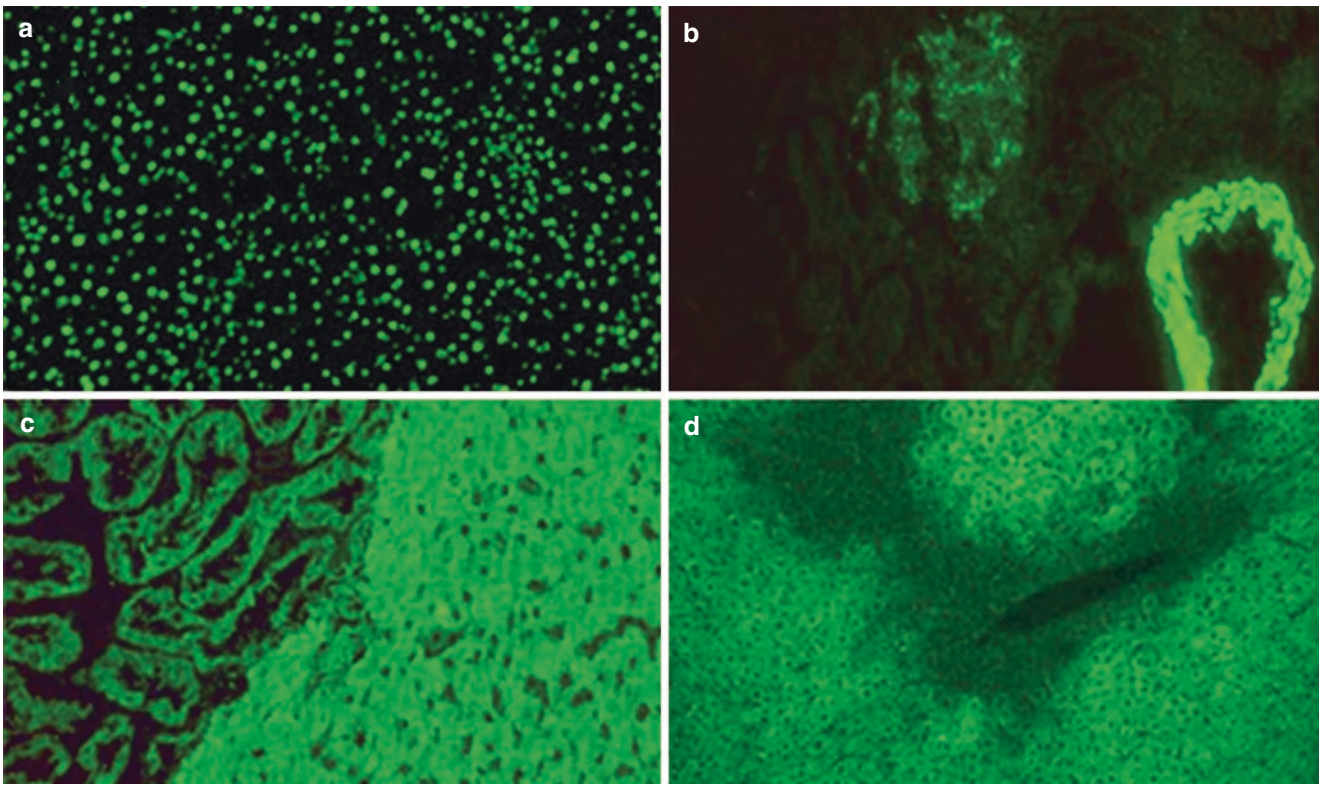

Fig. 26.3 Indirect immunofluorescence pattern of anti-nuclear (**a**), anti-smooth muscle (**b**), anti-liver-kidney microsome type 1 (**c**) and anti-liver cytosol type 1 (**d**) antibodies

Autoantibodies

The detection of antibodies against components of the nuclei, smooth muscle and liver kidney microsome is a crucial step in the diagnostic work-up of patients with suspected AIH (Fig. 26.3), and for that reason, it has been incorporated in all scoring systems [8, 9, 103].

Besides aiding the diagnosis, the autoantibody profile is used to define two types of AIH: AIH-1, characterized by positivity for ANA and/or SMA, and AIH-2, characterized by the presence of anti-LKM-1 and/or anti-LC-1 [12].

The IAIHG has published a consensus statement detailing the methods for autoantibody detection, acknowledging the central role played by autoantibodies in the diagnosis of AIH [104]. The document recommends that first-line screening should consist of indirect immunofluorescence on fresh, multi-organ rodent sections (usually rat liver, kidney and stomach) to enable the simultaneous screening of a range of autoantibodies relevant to liver disease: ANA, SMA, anti-LKM-1, anti-LC-1 and anti-mitochondrial antibodies (AMAs) [104]. If only rodent kidney tissue is used, AMA may be confused with anti-LKM-1. The identification of the molecular targets of anti-LKM-1 and AMA (Table 26.7) has led to the establishment of immuno-assays based on the use of the recombinant or purified autoantigens [105].

Anti-Nuclear Antibodies

The target antigens of ANA in AIH are heterogeneous and incompletely defined, although ANAs have been shown to react with single- and double-stranded deoxyribonucleic acid (DNA), small nuclear ribonucleoproteins (sn-RNPs), centromeres, histones, chromatin and cyclin A. A better definition of these target antigens will follow the development of new techniques using recombinant nuclear antigens and immuno-assays [106]. In terms of IFL, ANA gives a readily detectable nuclear staining of kidney, stomach and liver sections. In AIH, a homogenous pattern of staining is commonly observed, particularly in the liver, with coarsely or finely speckled patterns visualised less frequently [104]. A clearer definition of the nuclear pattern should be sought using human epithelial type 2 (HEp2) cells which are characterized by prominent nuclei. These, however, should not be used for screening purposes due to a high positivity rate in healthy subjects. A clinically relevant titre of ANA in AIH is considered 1/40 in adults and 1/20 in children, in whom titres correlate with disease activity. ANA can also be identified in up to 52% of patients with PBC. However, in contrast to AIH, in which no disease-specific ANA has been reported, the PBC-specific ANAs showing multiple nuclear dot or rim-like membranous patterns are highly diagnostic for this condition. They are recognised by IFL when HEp-2 or HeLa cells are used as substrate. ANAs are also present in other autoimmune disorders, such as SLE, Sjögren syndrome and systemic sclerosis, as well as non-autoimmune conditions, like viral hepatitis, drug-induced hepatitis and alcoholic and non-alcoholic fatty liver disease [107].

Table 26.7 Autoantibodies and their antigens in autoimmune liver disease

Autoantibody	Target antigen(S)	Liver disease	Value in AIH	Conventional method of detection	Molecular-based assays
ANA	Chromatin, histones, centromeres, Cyclin A, ribonucleoproteins	AIH PBC PSC Drug induced Chronic hepatitis C Chronic hepatitis B NAFLD	Diagnostic of AIH-1	IIF	N/A
SMA	Microfilaments (filamentous actin) Intermediate filaments (vimentin, desmin)	Same as ANA	Diagnostic of AIH-1	IIF	N/A
Anti-LKM-1	Cytochrome P4502D6	AIH-2 Chronic hepatitis C	Diagnostic of AIH-2	IIF	ELISA, IB, RIA
Anti-LC-1	Formiminotransferase cyclodeaminase	AIH-2 Chronic hepatitis C	Diagnostic of AIH-2 Prognosis of severe disease	IIF, DID, CIE	ELISA, RIA
SLA/LP	O-Phosphoseryl-tRNA:selenocysteinyl-tRNA synthase (SepSecS)	AIH Chronic hepatitis C	Diagnostic of AIH Prognostic of severe disease, relapse and treatment dependence	Inhibition ELISA	ELISA, IB, RIA
p-ANNAs	Nuclear lamina proteins	AIH PSC/ASC	Point towards diagnosis of AIH	IIF	N/A
AMA	E2 subunits of 2-oxoacid dehydrogenase complexes, particularly PDC-E2	PBC	Against diagnosis of AIH	IIF	ELISA, IB, RIA

Abbreviations: ANAs anti-nuclear antibodies, *SMAs* anti-smooth muscle antibodies, *anti-LKM-1* anti-liver kidney microsomal antibody type 1, *anti-LC-1* anti-liver cytosol antibody type 1, *SLA/LP* soluble liver antigen/liver pancreas, *p-ANNAs* peripheral anti-nuclear neutrophil antibodies, *AMAs* anti-mitochondrial antibodies, *AIH* autoimmune hepatitis, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis, *NAFLD* non-alcoholic fatty liver disease, *IIF* indirect immunofluorescence, *DID* double-dimension immunodiffusion, *CIE* counter-immune-electrophoresis, *ELISA* enzyme-linked immunosorbent assay, *IB* immunoblot, *LIA* line-immuno-assay, *RIA* radio-immuno-precipitation assay, *N/A* not applicable

Anti-Smooth Muscle Antibodies

The first targets of AIH-specific SMA to be recognized – following pre-incubation of serum with thrombosthenin (platelet actomyosin) or purified thrombosthenin-A (the actin fraction of thrombosthenin) – were constituents of actin. Later, SMAs were also shown to be directed against other components of the cytoskeleton such as tubulin, vimentin, desmin and skeletin [108]. SMA IFL patterns can be visualized on kidney, stomach and liver sections, where they stain the artery walls. In the stomach substrate, they also bind the muscularis mucosa and the lamina propria. In the kidney, the SMA typical of AIH stains the smooth muscle of the vessels, glomeruli and tubules (VGT pattern). The VG and VGT IFL patterns are much more specific for AIH than the isolated V pattern [104]. The AIH-1-specific target of SMA responsible for the VGT pattern remains elusive. However, when vinblastine-arrested cultured fibroblasts were used as a substrate, AIH-1-specific SMA VGT-positive sera predominantly stained the microfilaments. In contrast, non-AIH-1 SMA V-positive sera reacted with non-actin-containing intermediate filaments. Several studies point to actin in its filamentous form as the target of the SMA, giving the VGT pattern. However, whilst this pattern is highly specific for AIH-1, some 20% of SMA-positive AIH patients do not have it. Moreover, when molecular assays using purified F-actin are employed, some AIH VGT positive cases are negative, whilst anti-F-actin positivity is reported in diseases distinct from AIH-1 [104, 108]. SMA titres by IFL are usually equal or above 1/80 in AIH, although very young patients may have titres as low as 1/20. SMAs giving the V pattern have been reported in advanced liver disease of other aetiologies, infectious diseases and rheumatic disorders; hence, like ANA, these are not specific for AIH.

Anti-Liver-Kidney-Microsomal Type 1 Antibodies

The molecular target of anti-LKM-1, the hallmark of AIH-2, is CYP2D6. This autoantibody stains the hepatocellular cytoplasm and the P3 portion of the renal tubules. Some confusion can occur between the IFL patterns of anti-LKM-1 and AMA, because both autoantibodies stain the liver as well as the kidney. However, AMAs stain the liver more faintly than anti-LKM-1, and mark the renal tubules more diffusely, whilst accentuating the distal tubules. Importantly, AMAs stain gastric parietal cells, whilst anti-LKM-1 do not [104, 108]. Since the molecular targets of anti-LKM-1 – CYP2D6 – and of AMA – enzymes of the 2-oxo-acid dehydrogenase complexes – are known, immune-assays based on the use of recombinant or purified antigens have been developed. Commercially available enzyme-linked immunosorbent assays (ELISAs) accurately detect anti-LKM-1, at least in the context of AIH-2, and detect AMA reasonably accurately. These assays can therefore be utilized when there is doubt about IFL patterns [104]. A clinically relevant anti-LKM-1 titre is considered equal or above 1/40 in adults and 1/10 in patients under 18 years of age; the titre of this autoantibody is associated with disease activity [104]. Interestingly, anti-LKM-1 are also detected in some 5–10% of patients with chronic hepatitis C virus infection, as alluded to above.

Anti-Liver Cytosol Type 1 Antibodies

Anti-LC-1 react with the folate-metabolizing enzyme formiminotransferase cyclodeaminase (FTCD), which is found at high levels within the liver. This autoantibody stains the liver cell cytoplasm with relative sparing of the centrilobular area. Importantly, anti-LC-1 frequently occurs together with anti-LKM-1, which obscure the anti-LC-1 staining. Thus, in the presence of anti-LKM-1, anti-LC-1 can be detected by the use of liver cytosol in double-dimension immunodiffusion or counterimmunoelectrophoresis, with the use of a positive reference serum, or by ELISA detecting reactivity to its target FTCD [104]. This autoantibody was originally described alone or in combination with anti-LKM-1 to define a clinical entity indistinguishable from AIH-2. Although anti-LC-1 was subsequently detected also in patients positive for serological markers associated with AIH-1, and in patients with chronic HCV infection, anti-LC-1 in isolation scores positively towards a diagnosis of AIH-2, allowing prompt initiation of treatment [8]. The presence and titre of anti-LC-1 antibodies correlate with disease activity, and represent a potentially useful marker of residual hepatocellular inflammation in AIH [108].

Anti-Soluble Liver Antigen/Liver-Pancreas Antibodies

Anti-SLA and anti-LP were originally believed to be distinct antibodies, but they were subsequently shown to bind the same target, an UGA tRNA suppressor-associated antigenic protein ($tRNP^{(ser)sec}$), more precisely O-Phosphoseryl-tRNA:selenocysteinyl-tRNA synthase (SepSecS) [109]. They can, therefore, be considered one and the same. Since anti-SLA/LP cannot be detected by IFL, this antibody is detected by radio-immuno-assay and ELISA [104]. As anti-SLA/LP have been reported in the absence of seropositivity for conventional autoantibodies, the existence of a third group of AIH patients was suggested. However, these early reports used a particularly high cut-off point for conventional autoantibody detection – higher than those currently used for the diagnosis of AIH – therefore, the nosological entity of AIH-3 has not been accepted by the IAIHG. Though anti-SLAs have been reported in occasional HCV-infected anti-LKM-1-positive patients, their presence is highly specific for the diagnosis of AIH, and its detection at the time of diagnosis identifies patients with a more severe disease and a worse prognosis [32].

Anti-Neutrophil Cytoplasmic Antibodies

ANCAs react to constituents of the cytoplasm of neutrophils to give a perinuclear (p-ANCA) or cytoplasmic (c-ANCA) IFL pattern. The predominant target of c-ANCA is proteinase 3 and this autoantibody is frequently detected in Wegener granulomatosis. p-ANCA binds myeloperoxidase and is most commonly found in microscopic polyangiitis. In addition to primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD), p-ANCAs are frequently detected in AIH-1, although the IFL pattern is somewhat atypical. Staining is associated with peripheral nuclear membrane components, hence the name of peripheral anti-nuclear neutrophil antibody (p-ANNA).The proposed target of p-ANNA is a 50 kDa neutrophil-specific nuclear protein belonging to the nuclear pore complex, potentially the tubulin β chain 5 [108]. Positivity for p-ANNA is very rare in AIH-2. In AIH-1, however, its detection can aid in the diagnosis, particularly when other autoantibodies are absent [8].

Anti-Asialoglycoprotein Receptor Antibodies

In an attempt to identify putative auto-antigens specifically expressed on hepatocytes in AIH, a crude liver extract preparation, known as the liver-specific protein (LSP), was obtained. A key constituent of LSP is a type II transmembrane glycoprotein, the asialoglycoprotein receptor (ASGPR) [110], also known as hepatic lectin. ASGPR is the only known liver-specific auto-antigen, and is constitutively expressed on the hepatocellular membrane. Antibodies to ASGPR are found in 88% of AIH patients, co-existing with ANA, SMA and anti-LKM-1 [111], and their titre correlates with inflammatory disease activity, providing an additional marker to monitor treatment efficacy [112]. Anti-ASGPR is, however, also found in PBC and viral- and drug-induced hepatitis [113]. Moreover, commercial assays for the detection of anti-ASGPR await validation.

Histology

Liver histology is essential to confirm the diagnosis of AIH, as highlighted in all diagnostic scoring systems [8, 9, 103]. Hepatitis at the portal-parenchymal interface, known as interface hepatitis (Fig. 26.4), is characteristic, but not exclusive, of AIH [114]. In addition, there are other non-specific features that may point to the diagnosis of AIH, such as multilobular collapse in cases presenting acutely, and emperipolesis and hepatocyte rosetting. Interestingly, a recent study suggests that the latter two characteristics are stronger indicators of AIH than interface hepatitis or plasma-cell rich infiltrate [115]. Histology is also the gold standard for evaluating the extent of fibrosis, and it helps in identifying overlap syndromes as well as the possible presence of concomitant diseases, such as alcoholic and non-alcoholic fatty liver disease [116]. Moreover, the information provided by histology is important in terms of management, as the presence of certain features, such as bridging necrosis or multiacinar necrosis, indicates that treatment is necessary, whilst in the presence of inactive cirrhosis or mild portal inflammation, treatment may not be necessary [12].

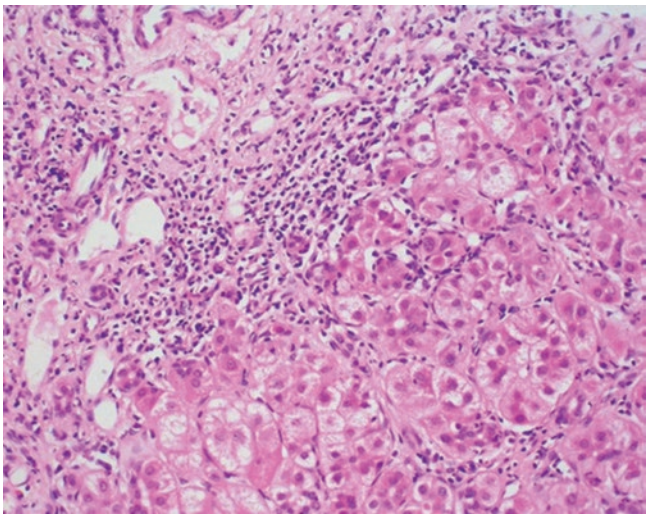

Fig. 26.4 Interface hepatitis. Liver biopsy histology specimens of autoimmune hepatitis typically reveal a dense portal and periportal mononuclear cell infiltrate

Treatment

Standard Treatment

The goal of AIH treatment is to induce and maintain complete suppression of the inflammatory activity, thus preventing progression to cirrhosis and liver decompensation [11, 12]. In contrast to previous guidelines [117] – where remission was defined by achievement of transaminase levels below twice the upper limit of normal – current guidelines require normal levels of transaminases, bilirubin and IgG [11, 12]. A retrospective single-centre analysis shows that when the old definition was used, over 70% of patients achieved remission, whereas when the new definition was used, only 26% did so [13]. In this study, 54% of patients fulfilling the old criteria for remission had histologically progressive disease, whilst when the new definition was applied, only 4% showed histological deterioration, underscoring the importance of achieving normal biochemical and serological indices in order to prevent progression of disease [13].

The induction regimen usually consists of high-dose predniso(lo)ne with or without azathioprine (Table 26.8). When used as monotherapy, the starting dose of steroids is 60 mg/day in adults and 1– 2 mg/kg/day (up to 60 mg/day) in children [12]. Regarding combination therapy, there are

Table 26.8 Therapeutic options for induction of remission in patients with autoimmune hepatitis according to AASLD and EASL guidelines

Week	Monotherapy (AASLD)	Combination therapy (AASLD)		Combination therapy (EASL)	
	Prednisone (mg/day)	Prednisone (mg/day)	Azathioprine (mg/day)	Prednisolone[a] (mg/day)	Azathioprine[a] (mg/day)
1	60	30	50	60	0
2	40	20	50	50	0
3	30	15	50	40	50
4	30	15	50	30	50
5	20	10	50	25	100
6	20	10	50	20	100
7+8	20	10	50	15	100
8+9	20	10	50	12.5	100
From week 10	20 and below	10	50	10 and below	100

Adapted from [11, 12]

[a]Considering an adult patient weighting 60 kg; initial prednisolone dose of 1 mg/kg body weight; azathioprine dose of 1–2 mg/kg body weight

differences between EASL and AASLD guidelines: whilst AASLD recommends a fixed dose of 50 mg/day of azathioprine to be started at the same time as steroids [12], EASL recommends 1–2 mg/kg/day of azathioprine to be started only 2 weeks after the introduction of steroids [11]. In addition, budesonide having been approved in some European and non-European countries [102], EASL guidelines suggest that remission can also be induced by replacing prednisolone with budesonide (starting dose of 9 mg/day), particularly in patients in whom the occurrence of steroid-specific side effects is expected [11]. Data supporting the use of budesonide come from a relatively recent multicentre, randomized controlled trial conducted in a large cohort of non-cirrhotic AIH patients, in which treatment with azathioprine plus budesonide 9 mg/day was compared with azathioprine plus prednisolone 40 mg/day (tapered to 10 mg/day) [118]. Steroid-specific side effects were less frequent in patients on budesonide compared to those on prednisone (28% vs. 53%). Remission, the definition of which included absence of steroid side effects, was achieved in 60% of the budesonide arm versus only 39% of those in the prednisolone arm [118]. However, it should be stressed that this rate of remission is lower than that observed when a higher starting dose of prednisolone is used. The trial also shows that budesonide at the dose employed offers no benefit over prednisone, apart from less weight gain, in children and adolescents [119, 120], a group of patients in whom higher remission rates have been reported with standard prednisolone and azathioprine treatment [121]. In addition, budesonide cannot be used in the presence of cirrhosis, excluding at least one-third of AIH patients who have cirrhosis at diagnosis [122].

Once remission is achieved, it can be maintained with azathioprine monotherapy or a combination of steroids with azathioprine. A systematic review of randomized controlled trials in adult patients showed that maintenance therapy with prednisolone monotherapy was inferior to azathioprine alone or in combination with prednisolone [123]. The European budesonide trial mentioned above, in which patients on the prednisolone arm were switched at 6 months to open-label budesonide, shows that budesonide (plus azathioprine) not only maintained remission but also reduced the frequency of steroid-specific side effects, suggesting that, more than a first-line induction-agent, budesonide may play a role as a maintenance drug in non-cirrhotic patients who experience steroid side effects [118].

Although maintenance of remission after treatment withdrawal is possible in some patients, a recent multicentre retrospective study including 131 patients in whom treatment was discontinued after achieving biochemical remission shows that over 80% relapsed within 3 years, reinforcing the notion that the majority of patients require long-term, if not life-long, maintenance therapy [124]. It is cautious not to attempt immunosuppression withdrawal within 2 years of diagnosis [12]. During withdrawal attempts, it is essential to monitor liver function tests closely, as relapse may be severe and even fatal. Patients who have successfully stopped immunosuppression should undergo long-term follow-up, as relapse can occur as long as 10 years later [125].

Alternative and New Treatments

Despite a lack of comparative or randomized studies, there are several novel treatment strategies in use in AIH. Most of these are pan-immunosuppressive agents, used as second-line therapy when standard therapy with prednisolone and azathioprine fails due to non-response or intolerance [11, 12, 126, 127].

6-Mecaptopurine

6-Mercaptopurine (6-MP) derives from non-enzymatic cleavage of azathioprine nitroimidazole group [142]. Although less frequently used, 6-MP is a potential drug for patients intolerant to azathioprine, since it has fewer side

effects, as reported in inflammatory bowel disease [128]. A retrospective study evaluated the safety and efficacy of 6-MP in 22 AIH patients who were either intolerant of or have insufficient responsive to azathioprine. 6-MP was started at a dose of 25 mg/day and increased up to 100 mg/day if tolerated. Amongst the 20 patients intolerant to azathioprine, 15 responded to treatment with 6-MP, whilst the 2 patients with inadequate response to azathioprine did not respond also to 6-MP [129].

Mycophenolate Mofetil

MMF is the morpholinoethyl ester prodrug of mycophenolic acid (MPA) [130]. Following oral absorption, MMF is rapidly converted into MPA [130]. Akin to azathioprine, it is a purine antagonist, leading to inhibition of B- and T-cell proliferation [130, 131]. However, and in contrast to azathioprine, MMF's potent immunosuppressive properties are independent of thiopurine methyltranferase activity [132]. Since it is more potent and better tolerated than azathioprine, MMF has largely replaced azathioprine in many transplant centres due to its effectiveness in preventing allograft rejection [133, 134].

Several studies comprising variable numbers of patients have shown that MMF can be used in AIH patients intolerant to azathioprine, with a reported response rate of 60% to 80%. In these studies, therapy with MMF was safe, with only few patients having to withdraw from treatment owing to severe side effects [135–144]. In almost all studies, the efficacy of MMF as a second-line agent in patients with previous non-response to azathioprine was low [141, 143, 144]. More recently, a retrospective study from 19 expert centres in Europe, North America and Asia reported an overall complete response to MMF as second-line therapy of 69.4%, with 92% and 34% success rates amongst patients switched to MMF due to azathioprine intolerance or insufficient response respectively [145]. A recent report on a prospective non-controlled study found that MMF may also be an effective first-line alternative agent in maintaining remission after induction in AIH [146].

Caution should be exerted in the use of MMF in fertile women in view of its teratogenicity.

Cyclosporine/Tacrolimus

Calcineurin inhibitors, cyclosporine and tacrolimus, have been used as a rescue treatment for difficult-to-treat cases of AIH [147].

Cyclosporine is a calcineurin inhibitor extracted from the *Tolypocladium inflatum* and *Cylindrocarpum lucidum* fungi. It acts on calcium-dependent signalling and inhibits T-cell function, suppressing the expression of the interleukin 2 gene [148]. The experience in AIH is limited to two pilot studies including a small number of patients [149, 150]. The data are, however, encouraging; thus, cyclosporine might be considered an alternative therapy in patients who do not achieve a complete remission with steroids and azathioprine or MMF. However, side effects are a serious problem and include hypertension, renal failure, hyperlipidaemia, hirsutism, infection and malignancy.

Tacrolimus is a macrolide lactone antibiotic which acts as a potent immunosuppressive agent on CD4+ T-helper cells. There are no controlled trials on the use of tacrolimus in AIH. However, case-series have reported the efficacy of low-dose tacrolimus (usually with a 2–5 ng/ml trough level target) [147, 151]. A recent study reporting the combined experience of two large European centres, Birmingham and Hamburg, shows that out of 16 patients switched to tacrolimus due to non-response to standard therapy, the majority achieved improved biochemical and immunological profiles, though only 29% reached normal transaminase and 50% normal IgG levels within 1 year of therapy [152]. Despite the acknowledged risk of nephrotoxicity, all patients showed stable renal function. Of 9 patients on long-term tacrolimus treatment, only one progressed to end-stage liver disease requiring transplantation, the other 8 had significant biochemical improvement on a reduced dose of steroids [152]. Despite these encouraging results, the experience is limited: this coupled to the toxicity profile should limit the use of tacrolimus to carefully selected cases followed up in experienced centres.

Infliximab

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine implicated in AIH pathogenesis [153]. Additionally, genetic polymorphisms in the TNF promoter region have been identified in patients with AIH-1 and associated with a poorer response to corticosteroid therapy and a higher frequency of progression to cirrhosis [34, 154]. Infliximab, etanercept and adalimumab are all anti-TNF-α therapies which have been approved for use in immune-mediated diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel disease.

Over the last decade, some reports suggested that anti-TNF-α therapies are an alternative option in controlling difficult-to-treat cases of AIH [155, 156]. In 2012, a retrospective series reported the use of infliximab in 11 AIH patients who did not achieve remission with standard immunosuppression or other alternative treatments [157]. After 3 infusions of infliximab (at a dose of 5 mg/kg at weeks 0, 2 and 6), all patients showed biochemical improvement. Additionally, in 5 patients in whom a liver biopsy was performed after treatment, a histological improvement was observed [157]. However, 6 of the 11 patients developed an infection whilst on infliximab therapy [157], in line with experience in other autoimmune conditions [158]. Moreover, infliximab therapy has been associated with the induction of severe de novo AIH in some patients treated for other diseases [159]. Nevertheless, anti-TNF-α is an option for con-

trolling the disease in difficult-to-treat cases; whether the same applies for the induction of remission in newly diagnosed AIH cases remains to be elucidated.

Rituximab

CD20 is a surface marker expressed on B lymphocytes, from early pre-B to memory B cells [160]. Rituximab is a chimeric monoclonal antibody to CD20 [161]. Treatment with rituximab leads to B-cell depletion through both antibody-dependent cellular cytotoxicity and complement-mediated lysis [161]. Rituximab was initially developed for the treatment of B-cell lymphoma, but it has since proven effective for the treatment of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and autoimmune haemolytic anaemia [162]. A case report of a patient with AIH, who developed Epstein–Barr-virus-associated lymphoproliferative disease secondary to azathioprine, showed that a treatment regimen including rituximab resulted not only in the remission of the lymphoma, but also in the normalization of liver function tests [163]. A later report of a patient with the concurrent diagnoses of B-cell lymphoma and steroid-resistant AIH/PBC overlap syndrome showed that a total of a 12-week treatment with rituximab resulted in clinical, biochemical and histological remission of the liver disease [164]. Rituximab has also been reported as an effective treatment of AIH in patients with concomitant idiopathic thrombocytopenic purpura [165], cryoglobulinaemic glomerulonephritis [166] or Evans syndrome [167]. In a phase 1 study, 6 patients with isolated AIH refractory to standard treatment were treated with rituximab (1000 mg at days 1 and 15) [168]. All patients were maintained on stable doses of prednisolone plus azathioprine for at least 1 month before and 3 months after rituximab infusions, after which steroids were tapered. All patients achieved biochemical remission by week 12, and the treatment was well tolerated with no serious adverse events being reported during the 72-week follow-up [168]. Although these results are promising and the toxicity profile is favourable, controlled clinical trials are needed before rituximab can be recommended as an alternative treatment in AIH.

Liver Transplantation

The indications for liver transplantation (LT) in AIH are similar to those for other end-stage liver diseases, comprising end-stage chronic liver disease, HCC meeting transplant criteria and onset with acute liver failure (ALF) unresponsive to steroids [118, 169]. Overall, AIH accounts for some 3% and 5% of paediatric and adult LTs performed in Europe and the United States [12].

LT for AIH shows a very successful outcome, with reported 1- and 5-year graft survival rates of 84% and 75%, respectively, and 5- and 10-year patient survival rates of 80–90% and 75%, respectively [170].

Despite the overall good outcome and the use of immunosuppression to prevent rejection, AIH may recur post-LT. The reported recurrence rate is highly variable, ranging from 12% to 46%, depending on the diagnostic criteria used, the length of follow-up and the performance of per-protocol biopsies [169]. The severity of necroinflammatory activity in the native liver and high IgG levels at the time of LT, as well as the presence of inflammatory bowel disease, are the best predictors of recurrence [171, 172]. Recurrent AIH is responsive to the reintroduction (or to an increase in the dose) of corticosteroids and azathioprine. Only in rare cases, recurrent AIH leads to graft failure despite aggressive immunosuppression [169].

Interestingly, AIH can also arise de novo following LT for non-autoimmune liver diseases. This form of graft dysfunction, known as de novo AIH, is characterized by biochemical, serological and histological features identical to those of classical AIH [173]. Treatment with steroids with or without azathioprine or MMF is successful in most cases, leading to excellent graft and patient survival [174].

Special Presentations

Variant Syndromes

AIH, PBC and PSC are generally viewed as distinct autoimmune liver diseases. There are, however, patients presenting with clinical, biochemical, serological and/or histological features of both a cholestatic liver disease and AIH, either simultaneously or consecutively. These variant conditions are often designated as overlap syndromes, and comprise PBC with features of AIH (PBC/AIH overlap) and AIH with biliary features suggestive of PSC (AIH/PSC overlap). There is debate as to whether these syndromes represent distinct entities or are variants of the main autoimmune liver disease. The IAIHG advocates that patients with overlapping features should not be categorized as separate diagnostic entities, but instead considered to be part of the "classical" diseases [175].

Due to its low frequency and lack of standardized diagnostic criteria, the prevalence of PBC/AIH overlap syndrome is difficult to establish; it is estimated that it accounts for 2–20% of AIH patients and up to 10% of those with PBC [176, 177]. The diagnosis of this condition remains a challenge and there is no validated scoring system, but in most reports, PBC/AIH overlap syndrome has been defined using the "Paris criteria" proposed by Chazouillères et al., where the diagnosis of overlap requires the presence of at least two of the three key criteria for the diagnosis of each component of the overlap [176]. The PBC criteria comprise (1) alkaline phosphatase (ALP) ≥2 the upper limit of normal (ULN) or

gammaglutamyl transpeptidase (GGT) ≥5 ULN; (2) presence of AMA and (3) histological evidence of florid bile duct lesions. The AIH criteria include (1) alanine aminotransferase (ALT) ≥5 ULN, (2) IgG ≥2 ULN (or IgG ≥1.5 ULN, Chazouillères personal communication) or presence of SMA, and (3) liver biopsy with moderate or severe periportal or periseptal inflammation [176]. These criteria have been incorporated in the EASL guidelines for the management of cholestatic liver diseases published in 2009, where it is, however, stressed that histological evidence of interface hepatitis is essential for making the diagnosis of overlap [178]. Compared to PBC alone, patients with PBC/AIH overlap appear to have a more aggressive course, a worse response to ursodeoxycolic acid (UDCA) and a more rapid progression in terms of fibrosis [176, 179]. When compared to AIH, the outcome does not differ significantly. Since no controlled studies are available, treatment is largely empiric [175]. According to EASL guidelines, patients with PBC/AIH overlap should receive combined therapy with UDCA and immunosuppressants; alternatively, patients with dominant AIH phenotype should be started on immunosuppressants only, and have UDCA added in case of insufficient response [11].

Besides PBC/AIH overlap, it is now well recognized that a variable proportion of patients with cholangiographically confirmed PSC also have features of AIH [180]. AIH/PSC overlap is characterized by hypergammaglobulinaemia, autoantibody seropositivity, and interface hepatitis – all features typical of classical AIH – in conjunction with cholestatic biochemical alterations, histological bile duct injury, frequent concurrence of inflammatory bowel disease and poor response to therapy [175].

In children, overlapping features of AIH and PSC are much more common than in adults and the term autoimmune sclerosing cholangitis (ASC) has been coined [181]. Interestingly, some 50% of children with clinical and histological evidence of AIH-1 have cholangiographic changes characteristic of sclerosing cholangitis, including some 25% that despite abnormal cholangiograms have no histological features suggesting bile duct involvement. Compared to AIH, children with ASC more commonly have concurrent inflammatory bowel disease, and more often progress to end-stage liver disease requiring LT [181]. Whether childhood ASC and adult PSC belong to the same disease spectrum remains undefined, although a retrospective study has shown that a high proportion of adults initially diagnosed as having AIH were found to have sclerosing cholangitis on cholangiography at follow-up [182].

Acute Severe to Fulminant AIH

Although AIH typically manifests as a chronic liver disease, it is estimated that up to 20% of patients have an acute presentation, which can be associated with the development of acute liver failure (ALF). Diagnosis of AIH in this setting is difficult, as the classical autoimmune manifestations may be absent, the published scoring systems not being readily applicable to this cohort of patients [8, 9]. The management of patients with AIH presenting acutely with severe hepatitis or liver failure is challenging. Although some of these patients do respond to corticosteroids, for the majority of those with ALF, LT remains the only available rescue treatment. It is therefore of utmost importance to identify early those patients with a higher likelihood to respond to steroids. Although a study did not find differences in prognostic scores between steroid responders and failures [183], others have reported that a model for end-stage liver disease (MELD) score of ≤28 on admission, absence of massive necrosis on histology and initial stabilization or improvement of bilirubin levels and INR within 4 days of therapy were associated with a higher response rate to steroids [95, 184]. If no improvement is observed during the first few days of treatment, continuing corticosteroid therapy may be a futile exercise and may result in serious adverse events, such as sepsis. Even if therapy with corticosteroids is maintained, assessment for LT should occur simultaneously [11, 12].

AIH and Pregnancy

Since its first description, fertility and pregnancy issues have been a concern for a disease that affects mainly females [1]. Successful pregnancies have been reported in AIH patients. Worse pregnancy outcomes – that is high incidence of AIH exacerbations and serious maternal adverse events – have been associated with positivity for anti-SLA, absence of immunosuppressive drug therapy during pregnancy and the occurrence of a flare in the year before conception [185, 186], underscoring the need for stable immunosuppression before and throughout pregnancy. The use of azathioprine appears to be safe during pregnancy, whilst MMF is contra-indicated [11, 12]. Of note, most disease flares occur in the postpartum period, even in patients whose condition improved during pregnancy [186]; thus, it is recommended to increase pre-emptively the dose of immunosuppression shortly before the expected date of delivery, and to closely monitor disease activity in the weeks following delivery [187].

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IgG4-Related Disease: Current Concept, Diagnosis, and Pathogenesis

27

Kazuichi Okazaki, Takashi Tomiyama, Toshihiro Tanaka, Tsukasa Ikeura, and Kazushige Uchida

Abbreviations

ANA	Anti-nuclear antibody
AIH	Autoimmune hepatitis
AIP	Autoimmune pancreatitis
CA-II	Carbonic anhydrase-II
CBD	Common bile duct
CTLA-4	Cytotoxic T lymphocyte antigen-4
ERCP	Endoscopic retrograde cholangio-pancreatography
FCRL	Fc-receptor-like
IFN-γ	Interferon-γ
GWAS	Genome-wide association study
IgG4-RD	IgG4-related disease
IgG4-SC	IgG4-related sclerosing cholangitis
IL-4	Interleukin-4
LF	Lactoferrin
LPSP	Lymphoplasmacytic sclerosing pancreatitis
PSC	Primary sclerosing cholangitis

Key Points

- IgG4-related disease (IgG4-RD) is a fibroinflammatory disorder of unknown origin with either synchronous or metachronous multi-organ involvement.
- Clinico-pathological features are diffuse or focal organ enlargement and mass-forming or nodular/thickened lesions with abundant infiltration of IgG4-positive plasmacytes and fibrosis, and respond well to steroid treatment.
- Multi-factors, such as genetic factors, disease-related antigens, abnormal innate and adaptive immunity may be involved in the development of IgG4-RD.

Introduction

IgG4 related-disease (IgG4-RD) is a fibroinflammatory disorder of unknown origin and recognized as a novel clinical entity with either synchronous or metachronous multi-organ involvement [1–3]. The patients with IgG4-RD show increased serum levels of IgG4, diffuse or focal organ enlargement and mass-forming or nodular/thickened lesions, with abundant infiltration of IgG4-positive plasmacytes and fibrosis, and respond well to steroid treatment [1–3]. It should be differentiated from mimickers by a combination of serum IgG4 level, imaging features, and histopathological findings. In the hepatobiliary system, IgG4-related sclerosing cholangitis (IgG4-SC), IgG4-related lymphoplasmacytic inflammatory pseudotumor and IgG4-related hepatopathy are originally considered part of the spectrum of IgG4-RD [4]. In addition to the original concept of IgG4-related lesions in the liver, IgG4-related autoimmune hepatitis (IgG4-AIH) has been proposed as a novel concept of hepatic lesions in IgG4-RD [5, 6], although not established yet. In this chapter, the concept and diagnosis of IgG4-RD, including hepatic lesions, are discussed.

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Current Concepts of IgG4-RD

Patients with IgG4-RD, either synchronous or metachronous, show diffuse or focal organ enlargement and mass-forming or nodular/thickened lesions in various organs with abundant infiltration of IgG4-positive plasmacytes with fibrosis [1–3]. IgG4-RD includes a wide variety of diseases, including Mikulicz's disease (MD), autoimmune pancreatitis (AIP), hypophysitis, Riedel thyroiditis, interstitial pneumonitis, interstitial nephritis, prostatitis, lymphadenopathy, retroperitoneal fibrosis, inflammatory aortic aneurysm, and inflammatory pseudotumor [1–3] (Fig. 27.1). About 10–20% of the patients have a single organ involvement. Although it is unclear whether the pathogenic mechanism is the same among individual organs or not, recent studies have suggested possible multi-pathogenic factors in the development of IgG4-RD similar to other immunogenic diseases. Based on genetic factors, disease-specific or -related antigens, abnormal innate and adaptive immunity may be involved [7]. IgG4-RD mainly affects middle-aged to elderly men except for MD, in which previous epidemiological studies did not show gender difference [1–3]. Clinical symptoms vary, depending on involved individual organs and dramatically relieved by steroid therapy in many cases; however, the long-term prognosis still remains unclear. Some patients develop serious complications, such as obstructive jaundice due to hepatic, gallbladder, or pancreatic disease; hydronephrosis due to retroperitoneal fibrosis; or respiratory symptoms due to pulmonary disease [1–3]. The infiltration of IgG4-positive cells, increased serum levels of IgG4, storiform fibrosis, and obliterative phlebitis are characteristic in most organ involvements of IgG4-RD, including pancreatic, biliary tract, retroperitoneal, and renal lesions, but storiform fibrosis and obliterative phlebitis are rarely observed in lymph nodes, salivary, or lacrimal glands [1–3].

In the First International Symposium on IgG4-RD, the nomenclature of individual organ manifestations of IgG4-RD was proposed (Table 27.1) using "IgG4-related" as a modifier, except for the pancreatic manifestation [2]. Formerly called Mikulicz's disease, Riedel thyroiditis, or Küttner tumor, Ormand's disease is replaced by IgG4-related dacryoadenitis and sialoadenitis, IgG4-related thyroid disease, IgG4-related submandibular gland disease and IgG4-related retroperitoneal fibrosis, respectively. The pancreatic manifestation of IgG4-RD is termed "type 1 autoimmune pancreatitis (IgG4-related pancreatitis)", because it is widely accepted among gastroenterologists and pancreatic surgeons, and helps to discriminate between type 1 and type 2 AIP, which is not a part of the IgG4-RD spectrum. When the pathogenesis of type 2 AIP is clarified, the term "type 1 AIP" might be replaced by "IgG4-related pancreatitis" [1–3]. In the hepatobiliary system, IgG4-related sclerosing cholangitis (IgG4-SC), IgG4-related lymphoplasmacytic inflammatory pseudotumor and IgG4-related hepatopathy are originally considered part of the spectrum of IgG4-RD [1–3].

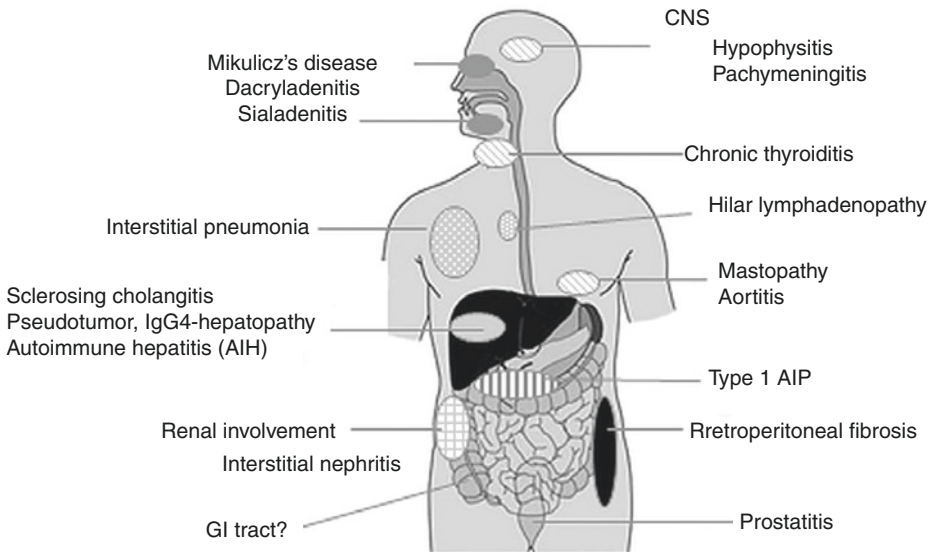

Fig. 27.1 Current concept of IgG4-related disease. IgG4-RD encompasses simultaneous or metachronous multi-organ involvement. (Reprinted with permission from Okazaki et al. [3])

Table 27.1 Preferred nomenclature for individual organ manifestations of IgG4-related disease

Organ system/tissue	Preferred name
Pancreas	Type 1 autoimmune pancreatitis (IgG4-related pancreatitis)
Eye	IgG4-related ophthalmic disease is the general term for the peri-ocular manifestations of this disease. There are several subsets, outlined below.
Lacrimal glands	IgG4-related dacryoadenitis
Orbital soft tissue (orbital inflammatory pseudotumor)	IgG4-related orbital inflammation
Extra-ocular muscle disease	IgG4-related orbital myositis
Orbit with involvement of multiple anatomic structures	IgG4-related pan-orbital inflammation (includes lacrimal gland disease, extra-ocular muscle involvement, and other potential intra-orbital complications)
Salivary glands (parotid and submandibular glands)	IgG4-related sialadenitis or, more specifically, IgG4-related parotitis or IgG4-related submandibular gland disease
Pachymeninges	IgG4-related pachymeningitis
Hypophysis	IgG4-related hypophysitis
Thyroid (Riedel's thyroiditis)	IgG4-related thyroid disease
Aorta	IgG4-related aortitis/peri-aortitis
Arteries	IgG4-related periarteritis
Mediastinum	IgG4-related mediastinitis
Retroperitoneum	IgG4-related retroperitoneal fibrosis
Mesentery	IgG4-related mesenteritis
Skin	IgG4-related skin disease
Lymph node	IgG4-related lymphadenopathy
Bile ducts	IgG4-related sclerosing cholangitis
Gallbladder	IgG4-related cholecystitis
Liver	IgG4-related hepatopathy (refers to liver involvement that is distinct from biliary tract involvement)
Lung	IgG4-related lung disease
Pleura	IgG4-related pleuritis
Pericardium	IgG4-related pericarditis
Kidney	IgG4-related kidney disease. The specific renal pattern should be termed IgG4-related tubulointerstitial nephritis and membranous glomerulonephritis secondary to IgG4-RD. Involvement of the renal pelvis should be termed IgG4-related renal pyelitis.
Breast	IgG4-related mastitis
Prostate	IgG4-related prostatitis

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Diagnosis of IgG4-Related Disease

Clinical symptoms of the patients with IGG4-RD vary, depending on the organ in which the lesions are located, which suggests that it is hard to establish criteria covering all patients with IgG4-RD. Therefore, some specific diagnostic criteria have been proposed for each involved organ, such as IgG4-related MD (IgG4-related dacryoadenitis/sialadenitis) [8], type 1 AIP (IgG4-related pancreatitis) [9], IgG4-SC [10], and IgG4-related kidney disease [11]. However, these organ-specific criteria do not cover other organs or are not familiar to general clinicians and specialists. Therefore, Japanese investigators have proposed the comprehensive diagnostic criteria (CDC) for IgG4-RD, containing three major criteria (clinical, laboratory, and histopathological examinations), have been proposed for practical use of general physicians and non-specialists [12] (Table 27.2).

Table 27.2 Comprehensive diagnostic criteria for IgG4-related disease, 2011

1. Clinical examination showing characteristic diffuse/localized swelling or masses in single or multiple organs
2. Hematological examination shows elevated serum IgG4 concentrations (135 mg/dl)
3. Histopathologic examination shows:
(1) Marked lymphocyte and plasmacyte infiltration and fibrosis.
(2) Infiltration of IgG4+ plasma cells: Ratio of IgG4+/IgG+ cells >40% and > 10 IgG4+ plasma cells/HPF
Definite: 1) + 2) + 3) Probable: 1) + 3) Possible: 1) + 2)
However, it is important to differentiate IgG4-RD from malignant tumors of each organ (e.g., cancer, lymphoma) and similar diseases (e.g., Sjögren's syndrome, primary sclerosing cholangitis, Castleman's disease, secondary retroperitoneal fibrosis, Wegener's granulomatosis, sarcoidosis, Churg–Strauss syndrome) by additional histopathological examination. Even when patients cannot be diagnosed using the CCD criteria, they may be diagnosed using organ-specific diagnostic criteria for IgG4-RD

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Clinical Examination

Physical examinations and imaging on US/CT/MRI can show the characteristic diffuse/localized swelling, masses, or thickness in single or multiple organs.

Laboratory Examination

The cutoff value for serum IgG4 concentration, 135 mg/dL, was based on receiver operating characteristic (ROC) curves [13], and its validity was confirmed. In patients with single-organ involvement and serum IgG4 concentration less than 135 mg/dL, the IgG4/IgG ratio may be helpful in making a diagnosis. However, elevated IgG4 may be also observed in other diseases (e.g., atopic dermatitis, pemphigus, asthma, and multicentric Castleman's disease), especially in about 10% of malignancy, which suggests that high serum IgG4 is not necessarily specific marker of IgG4-RD [14]. Therefore,

at present, the significance of elevated IgG4 in the pathogenesis/pathophysiology of IgG4-RD still remains unknown.

Histopathologic Examination

Although tissue biopsies are difficult to obtain from some organs, including the pancreas, retroperitoneum and ocular cavity, histopathological examination is important. Marked lymphocyte and plasmacyte infiltration with storiform fibrosis or obliterative phlebitis is characteristic of IgG4-RD. IgG4/IgG positive cells more than 40% have been reported in lymph nodes of the patients with IgG4-RD. On the other hand, more than 10 IgG4-positive plasma cells are recommended in diagnosis of type 1 AIP [9]. Based on these findings, the CDC for IgG4-RD recommend both the ratio of IgG4/IgG-positive cells >40% and infiltration of >10 IgG4-positive plasma cells/HPF for the definitive diagnosis [12]. Eosinophilic infiltration is often observed along with infiltration of IgG4-positive cells.

Although sensitivity of the CDC for definitive IgG4-RD is low in the organs to be difficult in taking biopsy specimens, it can detect possible cases of IgG4-RD. In the probable or possible cases, organ-specific criteria should be used concurrently.

Current Concept and Diagnosis OF IgG4-SC

IgG4-SC is a distinctive type of cholangitis of unknown origin, that is characterized by increased serum levels of IgG4 [1–3, 10], massive infiltration of IgG4-positive plasma cells with storiform fibrosis, and/or obliterative phlebitis in the bile duct wall, and responds well to steroid [10]. Patients with IgG4-SC are frequently associated with AIP [3], the concept of which was originally proposed by Yoshida et al. [15], and Hamano et al. reported increased serum levels of IgG4 in Japanese patients with AIP [13]. Now, it is recognized as a biliary manifestation of IgG4-related disease (IgG4-RD) [1–3]. Clinically, it is important to distinguish IgG4-SC from malignancy, such as cholangiocarcinoma, pancreas cancer, or a benign counterpart, PSC [10, 16].

Bile Duct Images of IgG4-SC

Cholangiogram

Four types of the characteristic cholangiographic features of IgG4-SC have been proposed based on the regions of stricture (Fig. 27.2) [10]. Type 1 IgG4-SC shows stenosis only in the distal CBD to differentiate it from chronic pancreatitis, pancreatic cancer, or cholangiocarcinoma. Type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic/proximal bile ducts, should be differentiated from PSC. Type 3 and type 4 of IgG4-SC show stenosis in the hilar hepatic bile duct, similar to hepatic hilar colangiocarcinoma.

Circular/Symmetric Thickening of the Bile Duct

Circular and symmetric thickening of the bile duct wall, smooth outer and inner margin, and homogenous internal echo demonstrated by abdominal ultrasonography (US), abdominal computed tomography (CT), abdominal magnetic resonance imaging (MRI), endoscopic ultrasonography (EUS), and intraductal ultrasonography (IDUS) are most characteristic images of the bile duct [10]. These characteristic features are recognized not only in the stenotic areas or occasionally in the gallbladder but also in areas without stenosis that appear normal in cholangiogram [10].

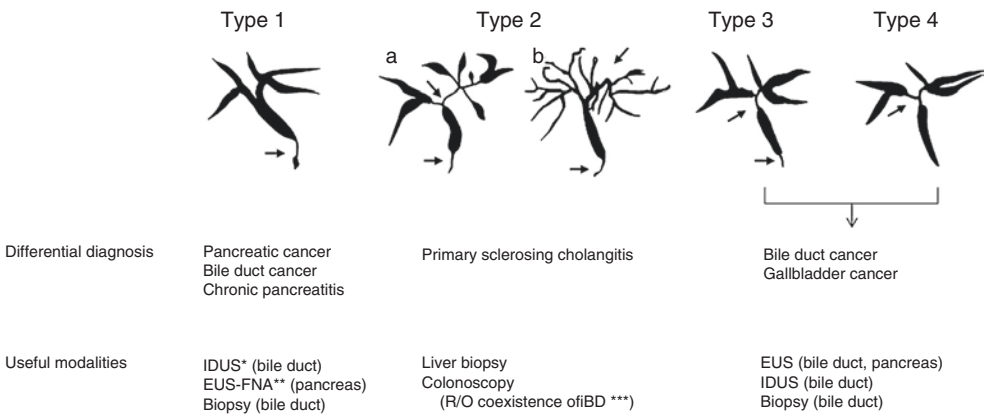

Fig. 27.2 The cholangiographic classification of IgG4-related sclerosing cholangitis and differential diagnosis. Stenosis is located only in the lower part of the common bile duct in type 1; stenosis is diffusely distributed in the intra- and extrahepatic bile ducts in type 2. Type 2 is further subdivided into 2 types: extended narrowing of the intrahepatic bile ducts with prestenotic dilation is widely distributed in type 2a; narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches are widely distributed in type 2b. Stenosis is detected in both the hilar hepatic lesions and the lower part of the common bile ducts in type 3; and strictures of the bile duct are detected only in the hilar hepatic lesions in type 4. *IDUS intraductal ultrasonography, **EUS-FNA endoscopic ultrasound-guided fine needle aspiration, ***IBD inflammatory bowel disease. (Reprinted with permission from Ohara et al. [10])

Characteristic Hematological Findings

More than 80% of the patients with IgG4-SC show hepatobiliary enzymes, total bilirubin in cases of obstructive jaundice, and elevation of serum IgG4 (upper limit of normal value (ULN) of 135 mg/dL or higher, nephelometric method), one of the diagnostic cardinal findings of IgG4-SC [1]. However, elevation of serum IgG4 levels is not necessarily specific to IgG4-SC; it is also observed in atopic dermatitis, pemphigus, asthma, and some malignant cholangio-pancreatic diseases [3].

Histopathological Findings of Bile Ducts

In IgG4-SC, massive infiltration of IgG4-positive plasma cells, storiform fibrosis, and/or obliterative phlebitis in the bile duct wall are characteristic and called as lymphoplasmacytic sclerosing cholangitis (LPSC) [10, 16]. Such fibroinflammatory involvement is mainly observed in the submucosa of the bile duct wall, whereas the epithelium of the bile duct is intact [10, 16]. Endoscopic transpapillary bile duct biopsy or cytological examinations are useful for differential diagnosis of cholangiocarcinoma, although it is difficult to take enough biopsy samples for characteristic histopathological findings of IgG4-SC [10, 16]. Liver biopsy is sometimes useful in the diagnosis of IgG4-SC in cases of intrahepatic bile duct involvement. [10, 16]

Diagnosis of IgG4-SC Using the Japanese Clinical Diagnostic Criteria

The Japanese study group for IgG4-SC proposed the clinical diagnostic criteria for IgG4-SC [10, 16] (Table 27.3) based on the combination of the following four criteria: (1) characteristic biliary duct images, (2) increased serum levels of IgG4, (3) coexistence of other organ involvements (OOIs), and (4) characteristic histopathological features. The effectiveness of steroid therapy is an optional diagnostic criterion to ensure accurate diagnosis of IgG4-SC like AIP only after negative workup of malignancy [10, 16].

Table 27.3 The Japanese clinical diagnostic criteria 2012 for IgG4-related sclerosing cholangitis

Diagnostic items
(1) Biliary tract imaging reveals diffuse or segmental narrowing of the intrahepatic and/or extrahepatic bile duct, associated with the thickening of bile duct wall
(2) Hematological examination shows elevated serum IgG4 concentrations (C135 mg/dl)
(3) Coexistence of autoimmune pancreatitis, IgG4-related dacryoadenitis/sialadenitis, or IgG4-related retroperitoneal fibrosis
(4) Histopathological examination shows:
a. Marked lymphocytic and plasmacyte infiltration and fibrosis
b. Infiltration of IgG4-positive plasma cells: >10 IgG4-positive plasma cells/HPF
c. Storiform fibrosis
d. Obliterative phlebitis
Option: Effectiveness of steroid therapy A specialized facility, in which detailed examinations, such as endoscopic biliary biopsy and endoscopic ultrasound-guided fine needle aspiration (EUS-FNA), can be administered, may include in its diagnosis the effectiveness of steroid therapy, once pancreatic or biliary cancers have been ruled out.
Diagnosis
Definite diagnosis
(1) + (3),
(1) + (2) + (4) a, b
(4) a, b, c
(4) a, b, d
Probable diagnosis
(1) + (2) + option
Possible diagnosis
(1) + (2)
It is necessary to exclude PSC, malignant diseases, such as pancreatic or biliary cancers, and secondary sclerosing cholangitis caused by the diseases with obvious pathogenesis. When it is difficult to differentiate from malignant conditions, a patient must not be treated with facile steroid therapy but should be referred to a specialized medical facility

Reprinted with permission from Ohara et al. [10]

Current Concept of IgG4-Related Hepatopathy and IgG4-Related Autoimmune Hepatitis

"IgG4-hepatopathy" is the comprehensive concept of various hepatic parenchymal lesions in the patients with type 1 AIP and IgG4-SC [2, 6]. Lesions of IgG4-hepatopathy are heterogeneously recognized as follows: (1) portal inflammation and sclerosis due to direct extension of IgG4-SC into the small portal tracts of bile ducts, or secondary to obstruction of large biliary ducts damage and cholestasis; (2) lobular hepatitis and portal inflammation [6]. Histopathological findings of the liver in patients with IgG4-SC occasionally resemble chronic active hepatitis, showing portal inflammation with periportal hepatitis and lobular hepatitis, but no parenchymal necroinflammation, such as zonal, bridging, or broad collapse. Therefore, at this moment, IgG4-hepatopathy is defined as primary and secondary hepatic lesions observed in livers of patients with IgG4-SC and type 1 AIP [6].

On the other hand, different from the concept of IgG4-hepatopathy, Umemura et al. have proposed a challenging novel concept of IgG4-AIH, which is histopathologically diagnosed as AIH and characterized by high serum IgG4 levels and abundant IgG4 positive plasma cell infiltration, and these cases of AIH may belong to a spectrum of IgG4-RD, although a few cases have been reported [5]. Based on these findings, Nakanuma et al. have proposed diagnostic criteria of IgG4-AIH (Table 27.4) [6].

Table 27.4 Proposed diagnostic criteria for IgG4-related autoimmune hepatitis (From reference [6] with permission)

Conditions
1. Serum IgG4 concentration _135 mg/dL
2. Increased IgG4-positive cells in liver tissue _10 per high power field
3. Chronic hepatitis with zonal and bridging necrosis or broad collapse
4. Metachronous or synchronous association of other IgG4-related disease(s)
Definite IgG4-related AIH criteria: 1 + 2 + 3 + 4
Probable IgG4-related AIH criteria: 1+ 2 + 3
Possible IgG4-related AIH criteria: Any 2 conditions

Reprinted with permission from Nakanuma et al. [6]
Abbreviations: *AIH* autoimmune hepatitis, *IgG4* immunoglobulin G4

Recent Advances in the Pathogenesis of IgG4-RD

Although the pathogenetic mechanism still remains unclear, recent studies suggest that abnormal conditions of innate and acquired immunity, regulatory T cells, and B cells based on genetic backgrounds, may be involved in the development of IgG4-RD (Fig. 27.3) [7].

Immunogenetic Backgrounds

Although genes susceptible to IgG4-RD remain unclear, the class II antigen haplotype of the major histocompatibility complex (HLA-DRB1*0405-DQB1*0401) [17], polymorphism of nuclear factor-κB and Fc-receptor-like (FCRL) 3 genes expressed on B cells [18] have been reported in the Japanese patients with AIP. An inhibitory molecule, cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152) [19], expressed on the activated memory T cells or CD4 + CD25+ regulatory T cells (Tregs), was independently reported as a susceptibility factor. Recently, a Japanese multicenter GWAS study, using more than 800 Japanese patients with IgG4-RD, confirmed a significant

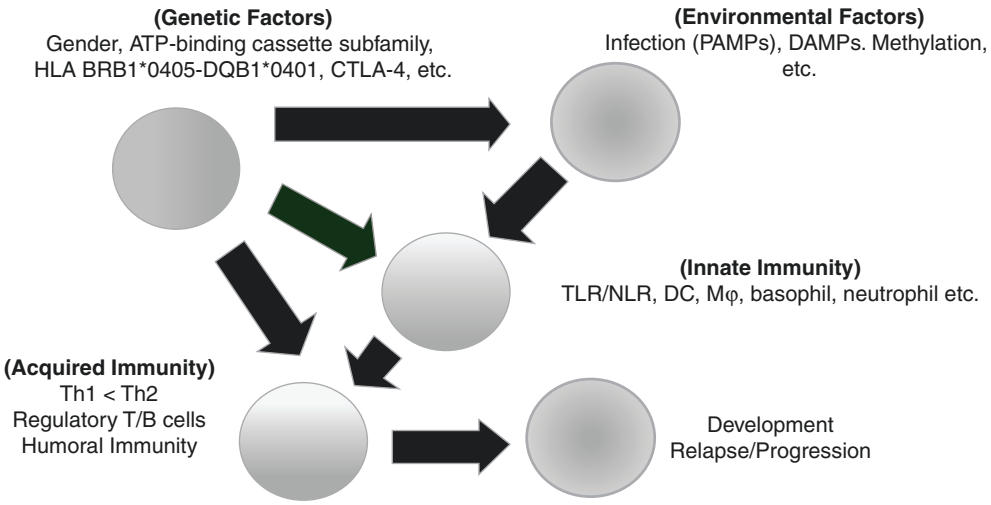

Fig. 27.3 Classification of cholangiography in IgG4-related sclerosing cholangitis. The characteristic features of IgG4-SC can be classified into four types, based on the regions of stricture as revealed by cholangiography and differential diagnosis. Type 1 IgG4-SC shows stenosis only in the lower part of the common bile duct, and it should be differentiated from chronic pancreatitis, pancreatic cancer, or cholangiocarcinoma. Type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic and extrahepatic bile ducts, should be differentiated from PSC. Type 2 is further subdivided into 2 types. Type 2a, with narrowing of the intrahepatic bile ducts with prestenotic dilation and Type 2b, with narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches, which is caused by marked lymphocytic and plasmacyte infiltration into the peripheral bile ducts. Type 3 IgG4-SC is characterized by stenosis in both the hilar hepatic lesions and the lower part of common bile duct. Type 4 IgG4-SC shows strictures of the bile duct only in the hilar hepatic lesions. Cholangiographic findings of type 3 and type 4 need to be discriminated from those of cholangiocarcinoma. (Reprinted with permission from Okazaki and Uchida [7])

association with HLA-DRB1*0405 and FCGRB2 (Fcγ receptor b2 gene) [20]. Tomiyama et al. reported the patients with IgG4-RD show significantly increased methylated MST1 gene, related with activation of integrin, and a possible candidate of disease resistant gene [21], because MST1 gene knockout mice show multi-organ lesions similar to those in IgG4-RD [22].

Innate Immunity

Recently, abnormal innate immunity has been demonstrated in patients with IgG4-RD. Activation of NOD-2 and TLR ligands on monocytes or basophils from patients with IgG4-related AIP enhances IgG4 responses via B cell activating factor (BAFF) and IL-13, although specific pathogens still remain unclear [23]. Moreover, abundant infiltration of TLR-7 positive M2-macrophages was observed in the pancreatic tissues from type 1 AIP cases [24]. Recently, possible roles of basophils, which are activated via TLR signaling, may be involved in the development of type 1 AIP [25].

In animal models, activation of TLR3 (polyinosinic:polycytidylic acid) or TLR4 (LPS) can induce immune-mediated cholangitis, pancreatitis, and sialadenitis similar to human IgG4-RD [26].

Possible Roles of IgG4 in IgG4-RD

Although the association of IgE-mediated allergy and IgG4 antibodies is well known, IgG4 characteristics are still poorly understood. IgG4 is involved in an immune process referred to as 'Fab-arm exchange', which is a swapping of a heavy chain and attached light chain (half-molecule) with a heavy-light chain pair from another molecule. This usually results in asymmetric antibodies with two different antigen-combining sites [27]. While these modified antibodies are hetero- bivalent, they behave as monovalent antibodies. Another aspect of IgG4 is that it mimics IgG rheumatoid factor (RF) activity by interacting with IgG, namely, through Fc-mediated aggregation [28]. IgG4 seems to be associated with a pathogenic effect in a few situations. In pemphigus, recognition of skin autoantigens (desmogleins) by IgG4 is at the origin of the disease process [29]. The most recent study of structural determinants of human IgG4-Fc, using crystallization, suggested that Fc-Fc interactions are compatible with intact IgG4 molecules and may provide a model for the formation of aggregates of IgG4 that can cause disease pathology in the absence of antigen [30].

Another recent study of the regulation of IgG4 showed that IgG4-related diseases may reflect an excessive production of anti-inflammatory cytokines such as IL-10, which triggers an overwhelming expansion of IgG4-producing plasma cells [4, 31–33]. Increased peripheral inducible-memory Tregs are positively correlated with serum levels of IgG4 [31]. In addition, prominent infiltration of Tregs upregulated IL-10 in livers of patients with IgG4-SC [32]. These findings suggest that IgG4 does not act as a pathogenic factor, nor is it an anti-inflammatory factor in IgG4-RD. Further studies are necessary to clarify the precise role of IgG4 in IgG4-RD.

The Complement System

Patients in active stages of AIP occasionally show decreased complement (C3, C4) with elevated circulating immune complex, as well as elevated serum levels of IgG4 and the IgG4 subclass of immune complexes [34]. However, a previous study showed that the classical pathway of complement activation through IgG1 may be involved in the development of AIP, as opposed to mannose-binding lectin or alternative pathways through IgG4 [34].

Autoantibodies and Candidate of Target Antigens

Although some patients with IgG4-RD have non-specific antibodies, such as an anti-nuclear antibody (ANA), this is rare. From the view of IgG4 function, it remains unclear whether IgG4-RD is an autoimmune or an allergic disease. Although disease-specific targets are unknown, the occasional coexistence of OOIs leads us to consider that there may be common target antigens in the involved organs, especially in the pancreas, which exhibit a high coincidence. Among candidate antigens previously reported, lactoferrin (LF) [35, 36], carbonic anhydrase (CA)-II [35–38], CA-IV [39], pancreatic secretory trypsin inhibitor (PSTI) [40] are distributed in the pancreas, salivary glands, biliary duct, lungs, and renal tubules, among others. Immunization with CA-II or LF-induced systemic lesions, such as pancreatitis, sialadenitis, cholangitis, or interstitial nephritis in mice models is similar to human IgG4-RD [41]. Amylase alpha-2A [42], heat shock 10 kDa protein 1 (HSP10) [43] and Helicobacter pylori [44, 45] are also disease-associated antigen candidates. Among the involved organs in IgG4-RD, recent studies suggest an extremely high association of pancreatic and biliary lesions. Peribiliary glands in the biliary tract and pancreatic duct glands associated with pancreatic ducts in humans are intermingled with small amounts of pancreatic exocrine acini [46], and biliary tree-derived stem cells constitute a pancreatic organogenesis in mice [47]. Thus, Nakanuma et al. have proposed a new concept of the "biliary diseases with pancreatic counterparts" [46], in which targets of type 1 AIP and IgG4-SC may be periductal glands around the bile and pancreatic ducts. Further studies of the biliary

tract's pathophysiology, based on its similarity to pancreatic counterparts, are warranted.

Recently, three novel candidates of target antigens related with connective tissues in some of the patients with type 1 AIP have been reported; annexinA11 [48], laminin 511 [49, 50], and galectin3 [51, 52]. Anti-annexin A11 IgG4-antibodies are positive in 9/50 patients, and IgG1-Abs in 7/97 patients, and anti-laminin 511 IgG4-and IgG1-antibodies are positive in a half of the patients.

Role of B Cells

In addition to steroid and immune-modulators, the B-cell depletion by rituximab, which reduces only IgG4, but not IgG1, IgG2, or IgG3, is useful in the therapeutic strategy in IgG4-RD [53–55]. A recent study showed expansion of IgG4+ B-cell receptor (BCR) clones in blood and tissue from patients with active IgG4-cholangiopathy, and disappearance of the clones with corticosteroid treatment [56]. A recent study showed that increased $CD19^{+}CD24^{high}CD38^{high}$ Bregs may suppress the disease activity of type 1 AIP, whereas the decreased $CD19^{+}CD24^{high}CD27^{+}$ Bregs might be involved in the development of type 1 AIP [57]. These findings suggest that specific B-cell responses may play a pivotal role in the pathogenesis of IgG4-RD.

Th1 and Th2 Immune Balance

The effector cells in IgG4-related diseases are poorly understood. CD4+ T-cells differentiate from naïve T-cells (Th0) into Th1, Th2, Th17, and regulatory T (Treg) cells. In the livers of IgG4-SC patients, a Th2-type immune reaction [4, 58] is induced in addition to the Th1 responses [36]. Th2 cytokines may be involved in the progression of the disease process, especially through the maturation and proliferation of local B-cells and plasmacytes.

Regulatory T Cells

Forkhead box P3 (FOXP3) is a member of the forkhead/winged-helix family of transcriptional regulators and functions as the master regulator in the development and function of CD4 + CD25+ regulatory T cells (Tregs) [59]. FOXP3 is classified as a naturally occurring CD4 + CD25+ Treg (nTregs) that originates in the thymus and while adaptive Tregs (aTregs) are induced in the periphery by different antigens [41, 59]. In type 1 AIP, circulatory naïve (CD45RA+) Tregs are significantly decreased in the peripheral blood, whereas memory (CD45RA-)-Tregs are significantly increased [59]. In addition, prominent infiltration of Tregs with upregulation of IL-10 is observed in the liver of type 1 AIP and IgG4-SC patients [31–33]. These findings suggest that increased memory-Tregs in the periphery and local tissues may be an inhibitory immune response against inflammation, although decreased naïve Tregs may be pathogenic.

Conclusion

Recent advances support the concept of IgG4-RD, a unique clinical entity, as a systemic disease. As hepatic lesions of IgG4-RD, concepts of IgG4-hepatopathy and IgG4-related autoimmune hepatitis (IgG4-AIH) have been proposed in addition to the concept of IgG4-sclerosing cholangitis (IgG4-SC) and inflammatory pseudotumor, although not established yet. In the pathogenesis of IgG4-RD, multi-pathogenic factors, including genetic backgrounds, disease-specific antigens, and the role of IgG4 must be clarified.

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Pediatric Liver Disease

28

Rishi Gupta and Nanda Kerkar

Key Points

- Biliary atresia, a progressive fibro-inflammatory process that damages the bile ducts, presents in infancy with cholestasis and pale stools, and is the most common indication for liver transplantation in childhood.
- Gene identification for cholestatic liver diseases, including progressive familial intrahepatic cholestasis, Alagille syndrome, and neonatal sclerosing cholangitis, have reduced the number of infants who were previously placed in the category of "idiopathic giant cell hepatitis." Alpha-1 antitrypsin deficiency is the most common genetic cause of liver disease in children.
- Nonalcoholic fatty liver disease is the most common cause of elevated serum aminotransferases in childhood.
- The availability of direct-acting agents for the treatment of hepatitis C in children in recent years has changed the lives of many children and families with chronic hepatitis C.
- The definition and etiologies of acute liver failure are different in children compared with adults with almost a third of indeterminate etiology.
- Metabolic diseases, such as methylmalonic acidemia, ornithine transcarbamylase (OTC) deficiency, and others, are indications of liver transplantation in children, where the liver may be structurally normal but lacks a specific enzyme secondary to a genetic mutation, causing progressive neurological and multisystemic damage.
- Autoimmune liver disease in children, including sclerosing cholangitis and *de novo* autoimmune hepatitis, has unique distinguishing features in childhood compared with adulthood.

Biliary Atresia

A progressive fibro-obliterative inflammatory process of unknown origin causes damage to the extrahepatic and intrahepatic biliary system, resulting in biliary atresia. Biliary atresia is the most common indication of liver transplantation in children in the USA and numerous parts of the world. The incidence of biliary atresia is estimated to be 1 in 8000–15,000 live births. Two forms of biliary atresia have been described – a fetal or embryonic form and a perinatal or "acquired" form. The King's group reported that 10% of their 548 patients with biliary atresia had associated splenic malformation (asplenia, polysplenia), were more likely to be female ($p < 0.04$), had antenatal diabetes ($p < 0.0001$) and extra-hepatic abnormalities, including pre-duodenal portal vein in 62%, cardiac anomalies in 45%, and situs inversus in 37% [1]. The North American Childhood Liver Disease Research and Education Network (ChiLDREN) prospectively analyzed data in 289 biliary atresia infants from 15 centers and classified biliary atresia into three groups: (1) non-syndromic, isolated BA (without major malformations in 84%), (2) biliary atresia with at least one major malformation but without laterality defects (6%), and (3) syndromic biliary atresia with laterality defects (10%) [2]. It was concluded that a new group (group 2 above) distinct from the historic syndromic or non-syndromic types had been identified and that careful phenotyping of biliary atre-

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sia may provide insight into the pathogenesis and outcomes. Biliary atresia is not believed to be an inherited disorder. The pathogenesis is unclear despite multiple studies that have investigated the role of infectious, toxic, metabolic insults in isolation or in combination with genetic or immunological factors in the development of biliary atresia [3].

Infants with biliary atresia classically present with prolonged jaundice, acholic stools and biochemically have a conjugated hyperbilirubinemia with elevated gamma-glutamyl transferase. Difficulty in accurate identification of acholic stools has led to the development of stool cards with pictures of normal pigmented stool and white/gray acholic stool, so that infants with pale stools can be brought to medical attention earlier by parents and pediatricians. On physical examination, apart from jaundice, there may be hepatomegaly and/or splenomegaly. If the disease is advanced, there may be history of gastrointestinal bleeding, and infants may present with failure to thrive as well as pruritus. Laboratory tests include fractionated bilirubin, serum aminotransferases, and gamma-glutamyl transferase; assessment of synthetic function with serum albumin and international normalized ratio (INR); serum bile acids and levels of fat-soluble vitamins (A, D, and E) as Vitamin K deficiency is reflected by the INR. It is necessary to check for other causes of cholestasis, including alpha-1 antitrypsin phenotype, thyroid hormone levels, metabolic screen with urine-reducing substances, serum/urine amino acids, and viral hepatitis screen. Much can be gleaned from results of newborn screening and maternal antenatal tests, so that the amount of blood drawn from the infant can be minimized. Ultrasonography is important, as it can rule out other causes of cholestatic jaundice, such as choledochal cyst, and is particularly helpful in the biliary atresia splenic malformation subgroup to allow the determination of situs inversus and other anatomical abnormalities before proceeding for liver biopsy. It may also reveal absence of the gall bladder, non-visualization of the bile duct, and a "triangular cord sign" (fibrous cone of tissue at the bifurcation of the portal vein) that has been reported in biliary atresia. Hepatobiliary scintigraphy using iminodiacetic acid analogues (HIDA) after phenobarbital priming has also been used. This allows biliary atresia to be ruled out if dye excretion is noted in the gut. Moreover, it is useful particularly in premature infants and

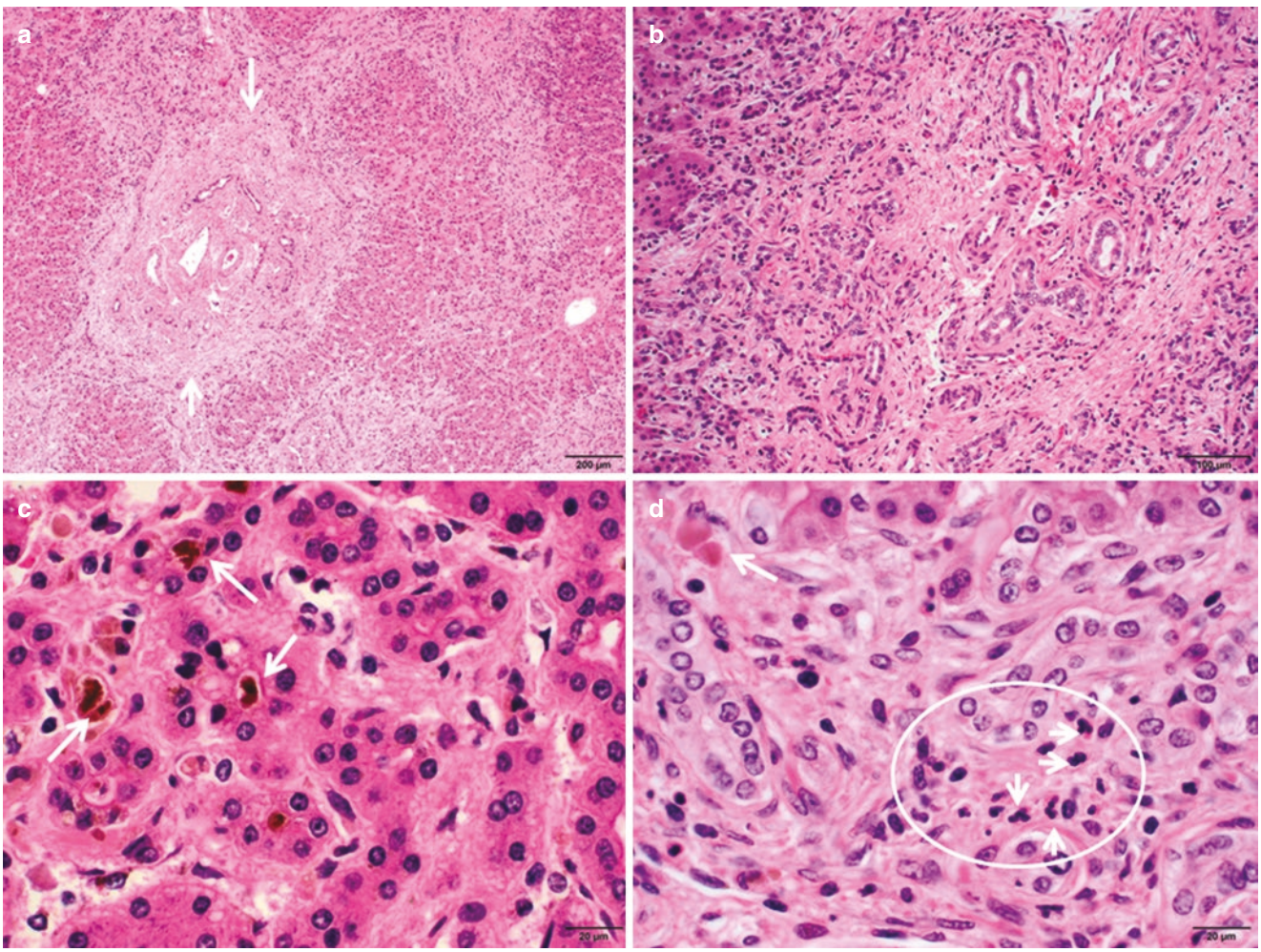

Fig. 28.1 Changes in biliary atresia include widening of the portal tract (between arrows in **a**) with marked bile ductular proliferation (**b**), cholestasis (long arrows in **c** and **d**), and mixed portal inflammation, including neutrophils (short arrows in circle) (original magnifications: **a** – 40×; **b** – 100×; **c**, **d** – 400×)

when liver biopsy is hazardous secondary to other medical/surgical issues.

Histology of liver tissue obtained by liver biopsy (Fig. 28.1) can reveal bile ductular proliferation, canalicular bile stasis, portal periportal fibrosis, and fibrosis with expanded portal tracts. About a third of infants may have portal inflammation with giant cells and extramedullary hematopoiesis. In very young infants, the changes may be early, and re-biopsy may be required in a few weeks. Some employ magnetic resonance cholangiography to reveal the bile duct. The gold standard for confirming the diagnosis of biliary atresia is laparotomy with intraoperative cholangiography and moving forward with Kasai portoenterostomy after establishing the diagnosis. This procedure was originally described by a Japanese surgeon Morio Kasai wherein biliary drainage is attempted by excision of the fibrosed extrahepatic biliary tree and then the resected surface of the porta hepatis is anastomosed to the bowel mucosa by using a loop of jejunum in a Roux-en-Y fashion. The success in establishing bile flow after the operation is variable and depends on the infant's age at surgery – the earlier the better, preferably at 8 weeks – disease severity, skill/experience of the surgeon, and microscopic patency of the bile ducts at the porta hepatis. If by 8 weeks or so post Kasai the stools remain acholic and cholestasis has not improved clinically/biochemically, then it is regarded as a "failed" kasai, as biliary drainage has not been achieved and the infant is referred for liver transplant evaluation. Several strategies have been employed to improve outcomes following the Kasai operation, including modifications to the original surgery, re-operation, use of steroids in the post-operative period [4], and use of intravenous immunoglobulin (IVIG) [5]; unfortunately, none have been successful. Biliary atresia remains the most common indication for liver transplantation in children in the USA and Europe. There is less than 10% survival with native liver into adult life. Some have proposed liver transplantation as the primary treatment for biliary atresia; however, in the current climate of organ shortage as well complications in smaller-size infants, the Kasai portoenterostomy remains the preferred modality of surgery, provided that infants are brought to medical attention before 10 weeks of life.

Alagille Syndrome

This syndrome was first described by Daniel Alagille in 1962 [6], but the mutation in Jagged 1 (JAG1) encoding a ligand in the Notch signaling pathway, which caused Alagille syndrome (AGS), was identified more than three decades later [7, 8]. This mutation is found in more than 90% of patients with AGS, but mutations in the NOTCH 2 receptor have been identified in a small percentage of patients with AGS [9]. JAG1 is a transmembrane cell-surface protein that interacts with Notch receptors to regulate cell fate during embryogenesis.

Table 28.1 Clinical manifestations of multisystemic involvement in Alagille syndrome

	Organ/system	Anomalies/manifestations
1.	Liver	Bile duct paucity is present in about 90%, hepatomegaly, cholestasis, pruritus, xanthomas
2	Cardiac	Seen in up to 94%, with right heart lesions being more common – typically peripheral pulmonary arterial stenosis, Tetralogy of Fallot with pulmonary atresia, ASD, VSD
3	Skeletal	Butterfly vertebrae (33–87%), osteoporosis, with recurrent fractures, short stature
4	Facies	Triangular with broad forehead, deep-set eyes, pointed chin, saddle nose with a bulbous tip
5	Ocular	Posterior embryotoxon- prominent Schwalbe's ring at the point where the corneal endothelium and uveal trabecular network join, seen in 56–88%, microcornea, keratoconus, cataracts, strabismus
6	Renal	In 40–73%, renal dysplasia, renal tubular acidosis, vesicoureteric reflux, and urinary obstruction
7	Vascular	Aneurysms of basilar, middle cerebral arteries, internal carotid artery, and Moyamoya disease (progressive intracranial arterial occlusive disease); aortic aneurysms and coarctation; intracranial bleeds in 15%, when it is fatal in 30–50%
8	Growth and development	Growth retardation in 50–87%; neurocognitive and developmental delay in 16–52%, intellectual impairment

There is a wide spectrum of clinical features in AGS ranging from completely asymptomatic to multi-systemic involvement (Table 28.1) with significant morbidity and mortality [10]. Children can present with severe cholestasis and hepatomegaly. The presence of a murmur in association with cholestasis in children alerts clinicians of the need to rule out AGS. The high levels of bile acids cause an intense pruritus that can be mutilating and disrupt the quality of life to such an extent that it has been an indication for liver transplantation. The high cholesterol levels exceeding 2 g/dL leads to the development of xanthomas, but they do not usually contribute to coronary disease.

The liver disease is generally severe for the first 5 years, after which it appears to spontaneously improve in up to 80%. The clinical diagnosis of AGS is made on the basis of the presence of cholestasis with at least three of the following characteristics: AGS facies, cardiac disease, renal disease, posterior embryotoxon, butterfly vertebrae, and a structural vascular anomaly. Cholestasis with associated pruritus is managed with ursodeoxycholic acid to promote bile flow, formula with high medium-chain triglycerides, and supplementation of fat-soluble vitamins. Anti-histaminic agents are used for relief from pruritus, as are rifampin, cholestyramine, and naltrexone. Apical bile salt uptake inhibitors are available through research study participation and in some patients have shown improvement, both in xanthomas and pruritus. Biliary diversion has been used

successfully to manage pruritus when medical therapy has failed. Liver transplantation is required in about a quarter of AGS patients with chronic liver disease.

Progressive Familial Intrahepatic Cholestasis (PFIC)

The group of progressive familial intrahepatic cholestasis (PFIC) encompasses an increasing number of discrete bile acid transport disorders with identified genetic mutations (Table 28.2). This along with other advances has led to the reduction in the number of children placed in the category of "idiopathic neonatal hepatitis" or "giant cell hepatitis."

Byler's disease (PFIC1), an autosomal recessive disease with progressive cholestasis, normal or low GGT, and pruritus and which progresses to cirrhosis in early childhood, was one of the first in this group of bile acid transport defects to be described [11]. The FIC1 protein encoded by the ATP8B1 gene is a P-type ATPase aminophospholipid translocase that flips phosphatidylserine and phospholipid ethanolamine from the outer to the inner layers of the canalicular membrane. Pruritus is debilitating, and infants often have severe cutaneous mutilation. The FIC1 gene is expressed in different tissues, such as the liver and intestines, leading to the development of extrahepatic manifestations, including sensorineural hearing loss, recurrent pancreatitis, diarrhea, pulmonary symptoms such as cough and wheezing, as well as growth impairment. Benign recurrent intrahepatic cholestasis (BRIC) is a similar but non-progressive form of low-GGT cholestasis characterized by intermittent jaundice and pruritus and is also caused by a mutation in the FIC1 gene [12]. Management is largely supportive, and biliary diversion has been successful in mitigating problems with cholestasis and pruritus in some children [13]. More recently, apical bile salt uptake inhibitors have been used successfully to ameliorate cholestasis and pruritus in the setting of clinical trials.

Table 28.2 Progressive familial intrahepatic cholestasis: mutations and salient clinical features

Deficiency	Gene mutation	GGT	Clinical features
Familial intrahepatic cholestasis 1 (PFIC1)	*ATP8B1*	Normal	Multisystemic disease, bland cholestasis, coarsely granular bile on electron microscopy Liver transplantation does not cure extrahepatic disease
Bile salt export pump (BSEP)	*ABCB11*	Normal	Higher incidence of hepatocellular carcinoma and gallstones. Can recur after liver transplantation
Multidrug resistance 3 (MDR3)	*ABCB4*	High	Progressive inflammation of the bile ducts
Tight junction protein 2 (TJP2)	*TJP2*	Normal	Bland cholestasis; progresses rapidly

Progressive familial intrahepatic cholestasis type 2 or bile salt export pump deficiency is clinically seen more frequently than the others in this group. The *ABCB11* gene encodes the bile salt export protein located in the canaliculus with an ATP-binding cassette and pumps bile acids through the canalicular domain against a negative gradient. The severe reduction in BSEP function causes jaundice with pruritus in the newborn period. There is no extra-hepatic involvement, but there is an unusually high incidence of hepatocellular carcinoma [14]. The disease recurrence following liver transplantation has been attributed to the development of antibodies against a component of the bile salt export protein [15]. The *ABCB4* gene encodes the canalicular protein MDR3, also with ATP-binding cassette, and works as a phospholipid flippase in the canalicular membrane. This enables the incorporation of phosphatidylcholine into bile micelles. In PFIC3, the bile becomes more detergent without phosphatidylcholine, thus causing injury to the cholangiocytes. The GGT is high, unlike types 1 and 2, and the disease progresses slower. Cholestasis is not common, and it is managed with ursodeoxycholic acid which improves the biochemical outcome, but the overall impact on prognosis in the long term is not clear. Tight junction protein belongs to the family of membrane-associated guanylate kinase homologs that are involved in the organization of epithelial and endothelial intracellular junction and regulate paracellular permeability.

Alpha-1 Antitrypsin Deficiency

Alpha-1 antitrypsin deficiency (A1ATD) is the most common genetic cause of liver disease in pediatric patients. It is an autosomal co-dominant condition, and the homozygous phenotype PiZZ (protease inhibitor) is observed in 1 in 3000 live births [16]. Sveger and colleagues conducted prospective screening studies in Sweden and revealed that only 8 to 10% of the PiZZ population developed clinically significant liver disease in the first 20 years of life [17]. Alpha-1 antitrypsin is a secretory glycoprotein that inhibits destructive neutrophil enzymes, including proteases and elastases. In A1ATD, a single nucleotide substitution (glutamine to lysine) results in an abnormally folded mutant protein that accumulates in the endoplasmic reticulum, triggering a cascade of events leading to hepatocellular injury. This is considered a "gain-of-function" mechanism of injury. Conversely, in the lungs, the pulmonary injury is said to occur through a "loss-of-function" mechanism, in that the uninhibited elastases and proteases attack the connective tissue matrix of the lung and produce emphysema. While liver

disease is observed in children, destructive lung disease and emphysema is observed in adults. The normal phenotype is PiMM, where normal levels of alpha-1 protein are present. The phenotypes PiZZ and PiSZ are considered abnormal and associated with sub-normal levels of alpha-1 protein. The mutant S protein polymerizes only when it is expressed with mutant Z protein, and this may explain the presence of liver disease in the compound heterozygote state PiSZ, but not in those with SS phenotype [18, 19]. Individuals who have PiMZ phenotype (one normal allele and one mutant Z allele) are "carriers" and are generally healthy from both the liver and lung disease perspective. However, if an individual with PiMZ phenotype has another cause of liver disease, prognosis is generally worse; thus, it is considered by many as a genetic modifier of liver disease.

The clinical manifestations of A1ATD are variable. In the newborn period, presentation may be with prolonged jaundice with/without hepatosplenomegaly on physical examination and a conjugated hyperbilirubinemia, with elevation of serum aminotransferases. If cholestasis is severe or prolonged, there may be associated features of poor growth and fat-soluble vitamin deficiency. The features of portal hypertension with splenomegaly and hypersplenism are observed with advanced fibrosis. It can also present later in childhood with isolated mild elevation of serum aminotransferases. Not surprisingly, the histological findings may vary from giant cell transformation, hepatitis, to microvesicular steatosis, bile ductular damage, and proliferation. The biliary changes may be similar to that observed in biliary atresia, and it is customary to ensure that A1AT phenotype is normal (PiMM) before proceeding for laparotomy with or without Kasai. The histologic hallmark is the presence of eosinophilic inclusions that represent dilated endoplasmic reticulum engorged with polymerized mutant protein (A1AT mutant Z), stain positive with periodic acid-Schiff (PAS) (Fig. 28.2), and resist digestion with diastase (in contrast to glycogen that is digested).

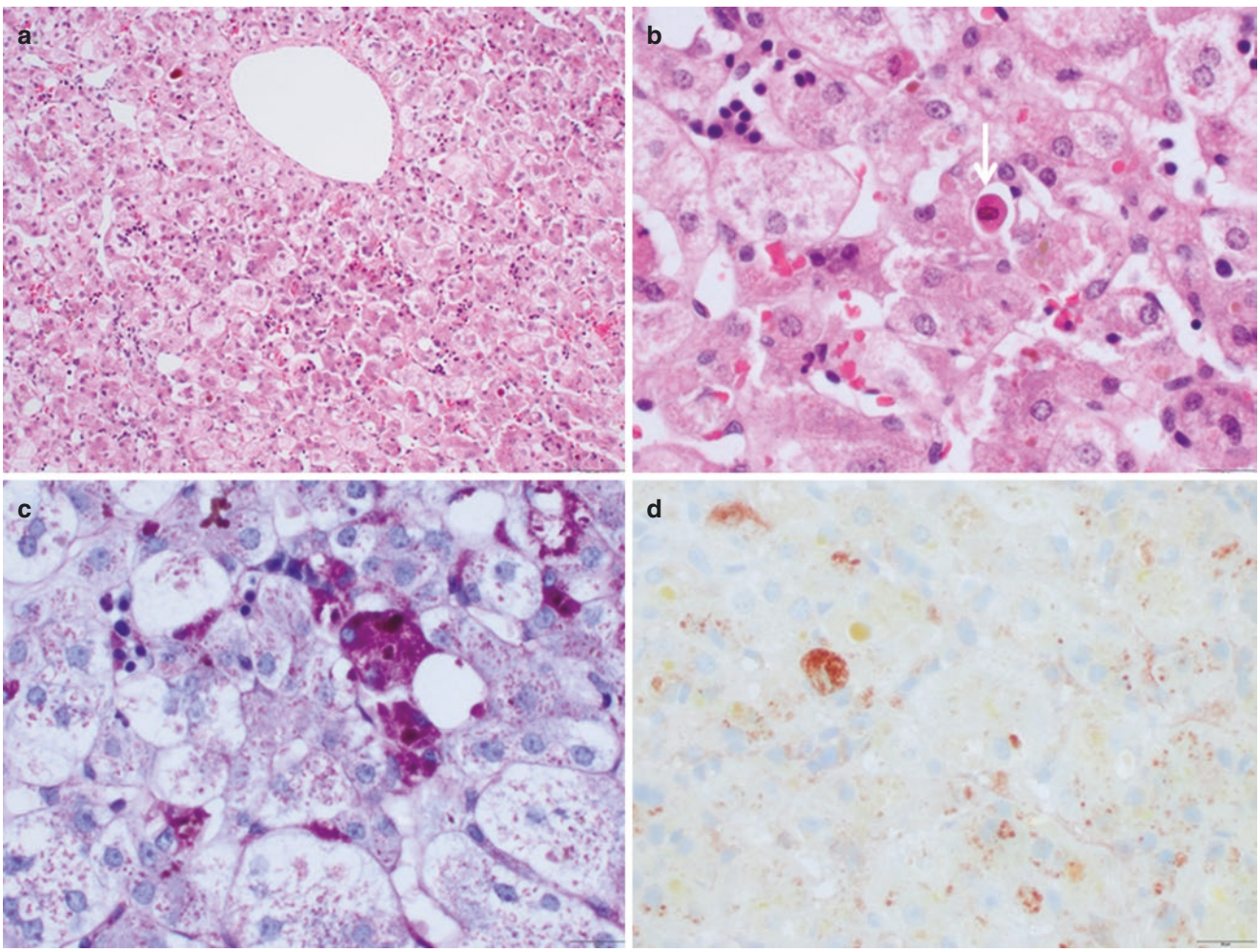

Fig. 28.2 (**a**) Liver in alpha-1-antitrypsin (A1AT) deficiency contains enlarged, eosinophilic cells that on higher power (**b**) contain a granular cytoplasm and scattered acidophilic bodies (degenerated hepatocytes, arrow). The granules are periodic acid-Schiff-positive and diastase-resistant (**c**, stain purple on PAS-D stain) and are positive on an A1AT immunostain (**d**). (Original magnifications: **a** – 100×; **b**–**d** – 400×)

The diagnosis of A1ATD is made on the basis of phenotype or analysis of the genotypic DNA, with the former being used more commonly [20]. While liver biopsy is not essential for diagnosis, histology is important in assessing the degree of liver damage, particularly the extent of fibrosis and the presence of cirrhosis. Moreover, it is an important tool for estimating disease prognosis. Treatment is supportive as there is no specific drug for the treatment of liver disease in A1ATD. Monitoring liver function with bilirubin, serum aminotransferases, and gamma-glutamyl transferase; synthetic function of the liver with serum albumin and international normalized ratio (INR); as well as screening for portal hypertension with complete blood count, assessment of spleen size on physical examination with imaging is standard care. Surveillance endoscopy may be considered in older children with portal hypertension. Using non-invasive methods, such as transient shear wave elastography (FibroScan), to annually monitor the state of fibrosis, can be a useful bedside tool for the clinician. Families are advised to keep children away from smoke as this may adversely impact the risk of developing emphysema in adult life. Children with PiZZ are often referred to a pulmonologist at 18 years of age for a baseline screening and earlier if any respiratory issues are clinically manifested. In those with cholestasis, special attention should be paid to growth with the use of high medium-chain triglycerides containing formula and supplementation of fat-soluble vitamins. In those with advanced liver disease, liver transplant evaluation needs to be undertaken in a center capable of transplantation. Research directed at gene therapy, gene repair, degradation of mutant protein by autophagy, and cell transplantation show promise. It is hoped that a specific treatment to manage liver disease in A1AT will be available in the near future.

Nonalcoholic Fatty Liver Disease (NAFLD)

Epidemiology

The increasing prevalence of pediatric NAFLD is linked to pediatric obesity but may also be attributed to increased awareness and acceptance of 'normal' serum aminotransferase values at lower levels than previous cutoffs. NAFLD is predominantly associated with obesity, though it has also been described in 5% of non-obese children [21]. While it is difficult to assess the true prevalence of pediatric NAFLD, based on a recent large meta-analysis, the prevalence of pediatric NAFLD is around 34.2% in obese children [22]. Before diagnosing a child with NAFLD, a comprehensive history, physical examination, and focused investigations are required to rule out other conditions that can present with fatty liver (Table 28.3).

Table 28.3 Differential diagnosis of pediatric hepatic steatosis

Genetic/metabolic diseases	Medications/ toxins	Systemic disease	Nutrition
Wilson disease	Glucocorticoids	Nephrotic syndrome	Severe protein energy malnutrition
Lysosomal acid lipase deficiency	Methotrexate	Hepatitis C infection	Anorexia nervosa
Fatty acid oxidation disorders	Valproic acid	Hypothyroidism	Parenteral nutrition
Fructosemia	Amiodarone	Inflammatory bowel disease	Rapid weight loss
Galactosemia	Alcohol	Hypopituitarism	
Abetalipoproteinemia	Carbon tetrachloride	Lipodystrophy	
Hemochromatosis	Organic phosphates		

Screening and Diagnosis

Screening for NAFLD is recommended in obese children and adolescents with or without other components of the metabolic syndrome, as well as in those with significant family history of fatty liver [23, 24]. An elevated serum ALT level, which is twice the upper limit of normal (normal being 22 U/L for girls and 26 U/L for boys) [25, 26], is the screening tool used widely for pediatric NAFLD. It is important to rule out the common causes of liver disease, including infectious hepatitis, autoimmune hepatitis, alpha-1 antitrypsin deficiency, Wilson disease, myopathies, and other metabolic diseases, before making a diagnosis of pediatric NAFLD (Table 28.4). Drug-induced liver injury and non-hepatotropic virus-induced liver dysfunction should be considered as a cause of elevated serum aminotransferases, and taking an appropriate history of drug/antibiotic use over the preceding weeks is advised.

Ultrasound is the most widely used imaging technique, although it is not very sensitive, especially if steatosis is present in less than 33% of the hepatocytes [27]. MR spectroscopy of the liver, though sensitive, is still considered a research tool. Small children require anesthesia for this modality. Transient hepatic elastography (Fibroscan) is increasingly utilized to screen and monitor NAFLD, particularly after FDA approval and the fact that cost will be covered by insurance. It is more sensitive than ultrasonography and also gives assessment of hepatic fibrosis, in addition to steatosis. Serum AST, GGT, and platelet counts are generally not used as screening tests for NAFLD, but may be used for the assessment of fibrosis scores [28] and prognostication of disease. Liver biopsy, though desirable for the histopathological diagnosis of NAFLD, is not commonly performed in pediatrics. The invasive nature of a liver biopsy, as well as sampling error and inability to repeat it regularly for monitoring, makes it a less attractive option in children. The indications of liver

Table 28.4 Screening laboratory tests for pediatric NAFLD

Liver function tests	Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, bilirubin, prothrombin time/international normalized ratio
Infectious work-up	Hepatitis B surface antigen, hepatitis B surface antibody, anti-hepatitis C virus antibody
Autoimmune hepatitis	Total serum immunoglobulin G, anti-nuclear antibody, anti-liver kidney microsomal antibody, anti-smooth muscle antibody
Endocrine testing	Thyroid-stimulating hormone, free thyroxine level
Metabolic diseases	Alpha-1 antitrypsin phenotype, serum ceruloplasmin, 24-h urinary copper, creatinine kinase level
Metabolic syndrome screening	Hemoglobin A1C, fasting serum insulin, lipid profile
Other baseline tests	Complete blood count, basic metabolic profile
Advanced metabolic testing (if required)	Lysosomal acid lipase deficiency testing, urine organic acid, plasma amino acid profile, serum carnitine level, urinary succinyl acetone level, plasma lactate, pyruvate, and ammonia level

biopsy, though variable, include the finding of steatosis in children below 5, where metabolic diseases need exclusion, if there is advanced disease evidenced by portal hypertension or poor synthetic function, and/or there is concern for a co-existing diagnosis, including autoimmune hepatitis and Wilson disease.

Management

Interpretation of long-term data can be challenging in determining the progression of pediatric NAFLD. First, there is a dearth of large-volume pediatric NAFLD studies using ALT as screening criteria. Secondly, puberty-induced hormonal changes can still be a confounding factor in interpreting the progression of pediatric NAFLD [29]. The mainstay of treatment remains life style changes. Vitamin E and metformin have not shown any benefit in disease resolution as compared to placebo in pediatric NAFLD [30]. In adult studies, weight loss ≥10% of baseline weight show promising results in NASH resolution [24]. Pediatric studies have revealed that even 5–10% body weight reduction and maintenance can improve complications associated with insulin resistance [31]. Metformin helps improve insulin resistance in children with metabolic syndrome, but its effect on NAFLD *per se* is not clear. Pediatric obesity, weight loss, and metabolic health centers are staffed by pediatric nutritionists, behavioral psychologists, exercise therapists, social workers, endocrinologists, hepatologists/gastroenterologists, pediatricians and use a family-based approach. The child's diet is quantified by using a 24-h or 72-h recall method, and appropriate suggestions are provided. In terms of macro composition of food, low glycemic index diet is preferred to a high monosaccharide-based diet. Portion control, mindful eating, and family meal times are reinforced. Aerobic exercise is recommended for cardiac and lung conditioning, whereas anaerobic exercise is taught to improve insulin resistance. Most of so-called pediatric obesity/nutrition centers hold individual/group sessions for 6 months or so, dictated by individual health insurance, and provide follow-up for 2 years. Bariatric surgery is considered for severely obese adolescents (BMI ≫ 95th centile) with NASH and other comorbidities of metabolic syndrome. There is paucity of studies examining the progression of NAFLD/NASH following bariatric surgery in adolescents, though limited results exhibit positive trends. Studies in adults have shown reversal in fibrosis [32].

Viral Hepatitis

Hepatitis A, B, C, and E can cause hepatitis in children. Hepatitis B and C more commonly cause chronic hepatitis in children and are discussed below. Their natural history depends on the age of contracting infection, mode of transmission – vertical or horizontal – and virus genotype.

Hepatitis B

After the implementation of universal hepatitis B vaccination of infants in 1991, the incidence of hepatitis B infection in the USA has decreased from 13.8/100,000 to 0.34 /100,000 children (0–19 years of age) [33]. The majority of pediatric hepatitis B in the USA are now represented by new infections diagnosed in immigrant children. The immune system of a child responds to hepatitis B virus (HBV) exposure differently than that of an adult. Around 90% of perinatally infected infants develop chronic HBV infection compared with only 5% of adults exposed to HBV, necessitating more rigorous surveillance and treatment of pregnant mothers and newborn infants infected with HBV. The HBeAg status of pregnant mothers and the genotype of the virus are both important determinants for perinatal transmission of HBV to the infant. The transmission risk varies from 70 to 100% with the mother's HBeAg positive status compared with 5–30% with HBeAg negative status [34]. Infants born in Africa have lower likelihood of acquiring perinatal HBV infection compared with those born in Asia, likely secondary to the prevalence of different genotypes. The administration of HBV vaccine at birth to neonates significantly reduces the rate of perinatal transmission. Decreasing maternal viral load antenatally has had a further impact on making neonatal vaccination more effective.

The natural progression of HBV is also different when infection is acquired in childhood than when acquired during adult life. Most children enter an "immunotolerant" phase, characterized by minimal liver inflammation and normal serum aminotransferases with high viral loads that can last for decades. Thereafter at a variable period that increases with time, children enter an immune clearance phase, marked by liver inflammation and elevated serum aminotransferases. The spontaneous HbeAg seroconversion rate to HBeAb in pubertal children can reach as high as 8–12% annually compared with only 2% in infants and toddlers, leading to inactive carrier state [35]. The diagnostic work-up for chronic HBV infection in children starts with the documentation of HBsAg positivity for more than 6 months and ruling out of other co-existent infections, including HDV, HCV, and HIV. In endemic areas, checking the status of hepatitis A virus immunization is also important. After establishing the diagnosis of chronic HBV, the child is placed in immune tolerant, immune clearance, chronic inactive carrier, and cirrhosis stage respectively with active monitoring for hepatocellular carcinoma (HCC) (Table 28.5).

Management

Knowing the exact phase of HBV infection in children is critical for giving antiviral treatment and for preventing drug resistance. Treatment is not recommended during the immune tolerant phase as spontaneous seroconversion can occur and chances of developing drug resistance are high. For younger infants, Interferon-alfa is the only available treatment. Entecavir and tenofovir are approved for children above 2 years of age. The treatment is continued until 1 year after seroconversion of HBeAg and appearance of HBeAb. Liver transplantation for chronic HBV infection is rare in children, except in fulminant HBV infection or with HCC. Interferon-alfa is not recommended during hepatic decompensation. An approach to pediatric chronic HBV treatment is suggested in Fig. 28.3.

Hepatitis C

The estimated worldwide prevalence of HCV in children is around 5 million, with 0.2–0.4% prevalence in the USA [36]. After mandatory screening for HCV in blood banks in 1992, the main mode for HVC transmission is vertical (60%). The mother-to-infant transmission rate is 2–7%, which is also influenced by maternal viral load and co-infection with HIV, both of which further increase the rate of perinatal viral transmission. The mode of delivery – cesarean section versus vaginal delivery – does not influence the vertical transmission rate, but complicated labor with prolonged rupture of amniotic membrane and episiotomy can increase the chances of viral transmission to the newborn. Up to 50% of vertically acquired HCV infection can be spontaneously cleared by 3 years of age, this being the rationale for withholding any hepatitis C treatment until 3 years of age in children. Older children can also have around 20–25% spontaneous clearance of the virus following acute infection, which can be manifested as fever, lethargy, and myalgia. The remainder go on to develop chronic HCV infection, and 1–2% may

Table 28.5 Phases of infection in chronic hepatitis B

Phase	HBsAg	Anti-HBs	HBeAg	Anti-HBe	HBV DNA	ALT	Histology/inflammation
Immune tolerant	Present	Absent	Present	Absent	≥200,000	Normal	Minimal
Immune clearance	Present	Absent	Absent	Present	2000–200,000	Increased	Moderate/severe
Chronic carrier	Present	Absent	Absent	Present	Undetectable	Normal	Minimal
Reactivation	Present	Absent	Absent	Present	>2000	Increased	Moderate

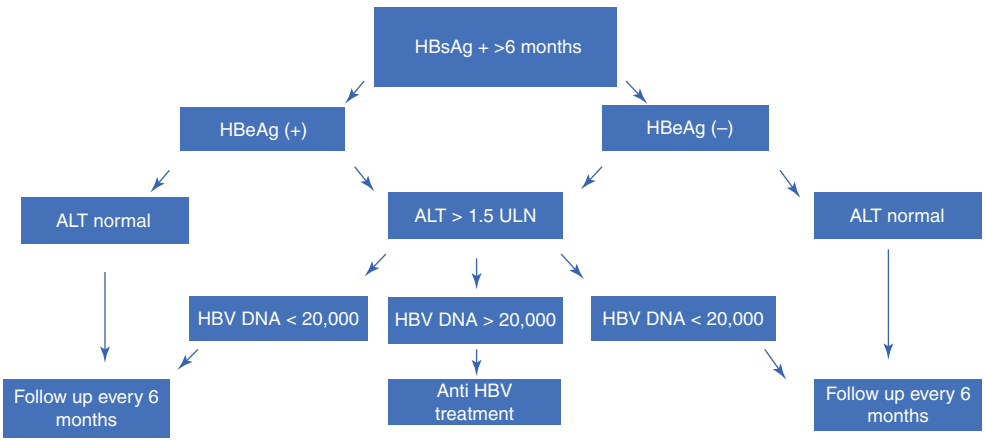

Fig. 28.3 Suggested treatment approach for children with chronic HBV infection

develop cirrhosis during childhood. Coinfection with HIV and obesity decreases the chances of spontaneous clearance in children [37].

Actively screening and treating all HCV+ pregnant women will help reduce vertical transmission to neonates. All newborns born to HCV+ mothers should be screened at or after 18 months of age for the presence of HCV antibodies, as before that age, interference with maternal antibodies can render the antibody test results unreliable [37]. If HCV antibodies are present at 18 months, further confirmation with HCV RNA and genotype testing should be performed. In noncompliant or high social risk families, newborns and infants can be tested earlier with RNA-based methods, but should also have the test repeated at 18 months of age.

Management

The US food and drug administration (FDA) approved the treatment of chronic hepatitis C in >12 years of age children with direct-acting antiviral (DAA) therapy (Ledipasvir and Sofosbuvir) in 2017 based on the results of a large multicenter open-label trial in children [38]. Treatment is being given for 12 weeks, with results as good as that in adults with cure rates reaching almost 100%. Recently, an open-label trial for 24 weeks with sofosbuvir and ribavirin combination in children (3–12 years old) infected with chronic hepatitis-C genotype 2 was successfully conducted with almost 100% elimination of virus [39]. In another open-label study, patients 3 to <6 years old chronically infected with HCV genotype 1 received a combination of ledipasvir-sofosbuvir for 12 weeks, with 97% sustained virological response (SVR) at the end of the treatment [40].

Acute Liver Failure (ALF)

Acute liver failure (ALF) accounts for 10–15% of pediatric liver transplants annually in the USA. Pediatric ALF is defined by the presence of biochemical evidence of acute liver injury along with coagulopathy that is not correctable by vitamin K administration in the absence of chronic liver dysfunction, and an INR of 2 or more does not require the presence of encephalopathy, whereas an INR of 1.5–1.9 requires its presence to fulfill the criteria of having pediatric ALF [4]. This definition was proposed by the pediatric acute liver failure (PALF) consortium in which 19 different pediatric liver transplant centers from the USA, Canada, and UK gathered data from 653 pediatric ALF patients between 1999 and 2014 [62, 63]. Though the etiological characterization of PALF has significantly improved with improving diagnostics, up to 30% of PALF are still considered indeterminate.

Table 28.6 Pediatric acute liver failure laboratory evaluation according to age of the patient

>3 months of age	Total serum immunoglobulin G, antinuclear antibody, anti-liver kidney microsomal antibody, anti-smooth muscle antibody (autoimmune hepatitis) Hepatitis B surface antigen, hepatitis B surface antibody, anti-hepatitis C virus antibody, hepatitis A-IgM antibody, hepatitis E IgM antibody, anti-Epstein-Barr virus IgM and IgG antibodies (viral hepatitis) Serum ceruloplasmin and 24-h urinary copper level (Wilson disease) Blood acetaminophen level (Tylenol overdose) Serum lactate, pyruvate, creatinine kinase levels (mitochondrial disease) Blood acylcarnitine and amino acid profile (fatty acid oxidation and urea cycle disorders) Soluble interleukin-2 receptor, triglyceride, and ferritin (hemophagocytic lymphohistiocytosis)
<3 months of age	Herpes simplex virus and enterovirus blood PCR (viral etiology) Serum ferritin (gestational alloimmune liver disease) Serum lactate, pyruvate, creatinine kinase levels (mitochondrial disease) Urine succinyl acetone (tyrosinemia) Review of newborn screening (additional testing for galactosemia) Blood acylcarnitine and amino acid profile (fatty acid oxidation and urea cycle disorders) Soluble interleukin-2 receptor, triglyceride, and ferritin (hemophagocytic lymphohistiocytosis)

Diagnostic Approach

A detailed history and examination along with age-appropriate investigations can lead to the etiology of pediatric ALF in more than 50% of cases (Table 28.6). In the newborn, perinatal and antenatal history is very important in terms of previous pregnancies, history of infant death, intrauterine infections, maternal-fetal blood group incompatibility, and known genetic conditions. In children, exposure to infection, medications including antibiotics in preceding weeks, history of blood transfusions, developmental delay, seizures, and family history of metabolic disease such as Wilson disease can guide investigations towards the right direction. Physical examination includes the assessment of jaundice and pallor, hepatomegaly, splenomegaly, ascites, petechiae, and altered mental status. Examples of disease-specific examination includes slit-lamp examination for Kayser-Fleischer rings in Wilson disease and chorioretinitis in CMV hepatitis. Extensive laboratory testing can be challenging in infants due to the need for large volumes of blood. The investigations have to be tailored to assess general hepatic function, metabolic and electrolyte status, as well as diagnostic tests prioritized to elucidate the etiology. The need for extensive laboratory testing as part of liver transplant evaluation

adds to the problem, and hence, laboratory testing in small infants requires a thoughtful approach.

Management

Ideally, pediatric ALF is managed in an intensive care setting with provision for pediatric liver transplantation, if necessary. Frequent vital and mental status assessments are mandatory along with strict fluid management. Baseline and follow-up laboratory monitoring include complete blood count, complete metabolic panel, serum aminotransferases, blood ammonia, and coagulation profile. In the early stages of hepatic encephalopathy (HE stages 0–II), venous ammonia level from a free-flowing venous sample is usually sufficient. Fluid restriction to two-thirds maintenance is standard, and more intensive management may be required based on the fluid electrolyte status, blood pressure, and serum ammonia levels. Other complications, including cerebral edema, acute kidney injury, secondary infections, coagulopathy, and hypoglycemia, are managed on an anticipatory basis and treated according to the local intensive care protocols.

Liver Transplant in the Setting of ALF

Liver transplant decisions in the setting of pediatric ALF can be challenging. Almost half of the patients do not have an identifiable cause of liver failure, and there are reasonable chances of spontaneous recovery. Fulminant hepatic failure in children is defined as the onset of hepatic encephalopathy within 8 weeks of the first symptom of liver disease in the absence of pre-existing liver disease. These patients also have to meet the following criteria per UNOS:

1. Ventilator dependence
2. Need for dialysis
3. INR>2

Children with ALF and after primary liver transplantation can be allocated United Network for Organ Sharing (UNOS) status 1A, if they meet the following criteria in an ICU setting. The status 1A designation is good for 1 week, unless the patient is re-listed by the attending physician as status 1A again the following week:

1. Presence of fulminant hepatic failure
2. Acute decompensated Wilson disease
3. Primary non-function following liver transplant
4. Hepatic artery thrombosis

Prognosis

Most predictive models including the King's College Hospital Criteria and Liver Injury Unit Scoring combine death and liver transplant in the outcome, making it difficult to follow the natural course of PALF.

Metabolic Liver Disorders

Inborn errors of metabolism (IEM) mainly present in two forms in children, although overlap can occur between them. The first group presents as bio-energetic failure**,** including glycogen storage, mitochondrial, and fatty acid oxidation disorders. The second group presents as disruption of synthesis or breakdown of complex molecules, leading to abnormal toxic intermediate metabolite accumulation and deficiency of the desired end products [39]. In certain IEMs, the liver is inherently healthy despite lacking a specific enzyme (e.g., urea cycle pathway), although this results in multisystemic injury. Hence "prophylactic" liver transplant is warranted in some disorders, such as ornithine transcarbamylase (OTC) deficiency, sometimes in the absence of biochemical evidence of liver dysfunction. The liver dysfunction in IEM can manifest as hydrops fetalis, acute liver failure, chronic cholestatic disorders, or hepatomegaly. Any history of acute fatty liver of pregnancy and hemolysis, elevated liver enzymes and low platelet count (HELLP syndrome) during pregnancy, parental consanguinity, and unexplained neonatal or childhood death in previous siblings should raise suspicion for IEM. Neurodevelopmental delays, dysmorphism, hepatosplenomegaly, or cardiomegaly can also be observed in several IEMs. The comprehensive metabolic testing can be very taxing, but baseline blood glucose, liver function tests, GGT, blood ammonia, lactate, pyruvate, carnitine, and acylcarnitine levels specially during times of metabolic stress (fever, infection, trauma, and hypoglycemia) are very helpful as a starting point.

Glycogen Storage Disorders (GSD)

Glycogen, the main storage form of carbohydrates in animals, is mainly stored in the liver and muscles and can provide substrate to maintain euglycemia for a few hours during fasting. GSDs occur due to specific enzyme deficiencies, leading to defective glycogen synthesis or breakdown. This leads to fasting hypoglycemia and organomegaly, specifically hepatomegaly, due to abnormal storage of glycogen. Other symptoms, such as muscle cramping, weakness, and cardiac involvement, depend on the subtype of GSD involving different enzymes in the glycogen metabolism. GSDs have been numbered classically based on the historical sequence in which they have been recognized, and the overall incidence of all GSDs is around 1 in 20,000–25,000 live births. The diagnosis is currently made by genetic test-

ing from peripheral blood or chorionic villus sampling by identifying the specific mutation compared to histopathological diagnosis in the past.

GSD I

In GSD1, glucose-6-phosphate cannot be catabolized due to either glucose-6-phosphatase deficiency (type 1a or von Gierke disease) or defective glucose-6-phosphate transporter (GSD 1b), leading to hypoglycemia and glycogen accumulation in the liver, kidney, and intestines. Clinical presentation is with hypoglycemia, hepatomegaly, and metabolic acidosis secondary to hyperlactacidemia. Older children can have doll-like facies, thin extremities, protuberant abdomen, and short stature. Serum aminotransferases can be mildly elevated with normal serum bilirubin and preserved hepatic synthetic function. Liver biopsy reveals increased glycogen content of hepatocytes along with prominent lipid vacuoles. Maintaining a constant glucose source is necessary to prevent hypoglycemia, often achieved by giving cornstarch to children several times a day and particularly overnight.

GSD Ib

Patients with GSD1b have features of GSD1a, along with neutropenia and altered neutrophil function. Children can present with recurrent aphthous ulceration and Crohn's disease-like presentation. A constant source of glucose helps mitigate the hypoglycemic symptoms without having any effect on neutropenia, and the latter may require granulocyte colony-stimulating factor (G-CSF) administration.

GSD II (Pompe Disease)

Deficiency of acid alpha-glucosidase (GAA), a lysosomal enzyme, leads to excessive accumulation of glycogen in the lysosomes and cytoplasm, leading to tissue destruction. The infantile form presents in initial months of life with cardiomyopathy and generalized muscular hypotonia. Hepatomegaly in infants is caused by heart failure and not secondary to metabolic liver disease. In juvenile and adult forms, the clinical presentation can vary from asymptomatic to progressive skeletal myopathy. Creatine kinase levels are elevated in both forms. GAA enzyme activity can be measured in white blood cells, and gene sequencing is another diagnostic tool which is commonly available these days. The primary treatment is enzyme replacement therapy with alglucosidase alfa.

GSD III

Deficiency of debrancher enzyme amylo-1,6-glucosidase results in the abnormal accumulation of glycogen that is partially broken down. Patients with GSD IIIb (less common, 20%) have debrancher deficiency confined to the liver, whereas in GSD IIIa (more common, 80%), the enzyme deficiency affects the liver, muscle, fibroblasts, cardiac muscle, and erythrocytes. The clinical presentation is just like GSD 1, but is milder. Symptom distribution depends on the localization of defect. Liver biopsy might reveal less steatosis and more fibrosis compared with GSD 1. Management of hypoglycemia is as given above, but the overall prognosis is better. Patients are at risk for developing hepatic adenoma and hepatocellular carcinoma.

GSD IV

In this condition, abnormal poorly soluble glycogen accumulates in various organs due to a defect in the enzyme required for normal branching of the glycogen molecule (alpha 1,4-glycan-6-glycosyltransferase). In contrast to other GSDs, hypoglycemia is not the presenting feature. Children can present with hepatomegaly which progresses to cirrhosis with failure to thrive. It is rare compared to other GSDs. These patients can also present with severe hypotonia and neurological involvement without hepatic dysfunction.

GSD VI

Patients with muscle phosphorylase deficiency present with muscle cramping and exercise intolerance. The liver is not affected in GSD VI.

GSD IX

Patients with liver phosphorylase kinase deficiency present with hepatomegaly, hypoglycemia, transaminitis, and growth retardation. Autosomal recessive inheritance has more severe hepatic progression compared with the more common X-linked variant of GSD IX. Treatment is symptomatic with more frequent feedings and nighttime cornstarch ingestion.

GSD XI (Fanconi- Bickel Syndrome)

This disease is metabolically manifested as fasting hypoglycemia, postprandial hyperglycemia, and hypergalactosemia. The primary defect is in the functioning of glucose transporter-2 (GLUT2) in hepatocytes along with pancreatic b-cells, enterocytes, and renal tubular cells. Other manifestations are mild metabolic acidosis, glycosuria, galactosuria, and other metabolic abnormalities secondary to renal tubular defects and mild elevation of serum aminotransferases. Diagnosis is made by DNA mutation analysis. Treatment is supportive, and overall, the prognosis is good.

Mitochondrial Respiratory Chain Disorders

Due to the ubiquitous presence of mitochondria, these disorders present as multiorgan dysfunction and develop protean manifestations. The age of onset ranges from the newborn period and infancy to early childhood. Organs

with high energy/metabolic needs, such as the liver and heart, are affected earlier than the other organs and present with liver failure, cardiomyopathy, seizures, neurodevelopmental delays, and hypoglycemia as prominent features. Due to bio-energetic failure, anaerobic metabolism increases, especially during a stressful episode, leading to hypoglycemia and lactic acidosis. Targeted exome or whole exome sequencing is the mainstay for the diagnosis of mitochondrial disorders, where available. Tissue-specific diagnosis involves liver and muscle biopsies and skin fibroblast culture. Liver biopsy may reveal steatosis, cholestasis, and necrosis on H&E staining and increased number of abnormal mitochondria on electron microscopy. The occurrence of liver failure makes the interpretation of respiratory chain enzyme complex analysis difficult based on liver biopsy alone. Even though muscle biopsies can sometimes be of higher diagnostic yield for these enzymes, the results might or might not reflect liver involvement due to the non-uniform effect of mitochondrial diseases on the different organs. Chorionic villus sampling is diagnostic if the mutation has already been identified from the affected family.

Currently, none of the treatment regimens, including free radical scavengers (e.g., coenzyme Q10, vitamin E, carnitine, or cofactor therapy such as riboflavin and vitamin C), have been found to be useful in symptom reversal or inhibition of progression of mitochondrial disorders. Still, most of the affected children get some sort of "mitochondrial cocktail" in the absence of another effective therapy. Most of the patients have progressive multisystemic disease, which makes them unsuitable candidates for liver/heart transplantation. A few cases of isolated mitochondrial disease with successful liver transplantation with excellent long-term outcomes have been reported in the literature. In sudden-onset acute liver failure of unknown etiology in children, assessing candidacy for liver transplantation can pose an ethical dilemma. As the heart and other organs may not exhibit signs of dysfunction early in the course of disease and results of genetic testing for mitochondrial diseases can still take days to weeks to become available, the presence of underlying mitochondrial disorders becomes difficult to assess. Several pediatric transplant centers, have had a case in which a child gets a liver transplant as a life saving procedure urgently and later presents with cardiac failure or progressive neurological involvement, leading to a subsequent diagnosis of mitochondrial disorder. Better and rapid diagnostic genetic testing for mitochondrial respiratory chain disorders in the future might help in preventing these situations. In the meanwhile, hepatocyte infusion is an interesting option to stabilize liver function in the setting of acute liver failure, although it is still experimental, and sometimes used while waiting for the results of genetic testing result to become available.

Urea Cycle Disorders

The urea cycle, consisting of five different enzymes, is the primary mechanism to eliminate nitrogen waste and is primarily located in the human liver. The symptoms of untreated disease are primarily neurological, secondary to cerebral edema. In terms of prevalence, ornithine transcarbamylase deficiency (55%) is the most common urea cycle disorder, followed by argininosuccinic acid synthase deficiency and carbamoyl phosphate synthetase deficiency [41]. In general, patients self-learn to avoid high-protein food. Hyperammonemia is the key feature in these disorders, along with elevated metabolite levels, depending on the precise enzyme deficiency. The ammonia level in neonates and young children can be difficult to interpret as newborns can have normal ammonia levels up to 100 μM/L. Moreover, poor sampling, including poorly flowing venous blood, can add to the confusion. Hyperammonemia with normal anion gap and normal blood glucose levels is characteristic of urea cycle disorders. The ability to perform DNA sequencing has replaced enzyme and metabolite analyses for the diagnosis of urea cycle disorders. The management of all suspected urea cycle disorders consists of immediate cessation of all protein intake and provision of high dextrose-containing solution to suppress protein catabolism. Removal of excessive ammonia and nitrogen as needed *via* dialysis is also helpful. Sodium phenylacetate and sodium benzoate are the nitrogen scavengers and are used in metabolic crisis in urea cycle disorders. Other measures to reduce cerebral edema, including hyperosmolar agents, are employed based on individual center experience. Every effort should be made to avoid dehydration, hypercatabolic state, high protein diet, and intake of valproic acid and alcohol in these children. Despite urea cycle disorders being a recognized indication for liver transplantation in the absence of liver failure, neurocognitive function can still be affected secondary to pretransplant hyperammonemia episodes.

Fatty Acid Oxidation Disorders (FAO Disorders)

Fatty acids are next in line to maintain fasting euglycemia after the depletion of glycogen stores. In the mitochondria, fatty acids are transported inside through carnitine transporter and then further oxidized to acetyl Co-A. Acetyl Co-A is further catabolized to ketone bodies, and ATP is produced through citric acid cycle. During acute metabolic decompensation, FAO disorders present as nonketotic hypoglycemia. FAO disorders can present with hepatomegaly, elevated serum aminotransferases, hyperammonemia, cholestasis, and sometimes acute liver failure. The blood acylcarnitine profile can be very helpful diagnostically during metabolic decompensation. FAO disorders affecting the

fetus, especially long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD), are an important cause for acute fatty liver of pregnancy in heterozygous mothers. The treatment consists of preventing lipolysis by providing a constant glucose source and avoiding hypoglycemia. Cornstarch intake before sleep also helps. Long-chain triglycerides should be avoided in LCHAD. In medium acyl-coenzyme A dehydrogenase defect, medium-chain triglyceride (MCT) should be avoided. Carnitine supplementation in general does not help, except when treating carnitine transporter deficiency.

Wilson Disease

Wilson disease is caused by a mutation in the ATP7B gene that results in excessive copper deposition in several organs, leading to impaired function [31]. The ATP7B protein is a P-type ATPase that acts as a copper export pump. Traditionally, it has been important to include Wilson disease in the differential diagnosis of liver disease in adolescents, but more recently, after the identification of the gene and increased access to genetic testing, the age at diagnosis is much earlier in childhood.

The clinical presentations include hepatic, neurologic, psychiatric, and "silent." The "hepatic" and "silent" forms are more common in the pediatric age group [42]. "Silent" WD refers to children who may have genetic and/or biochemical manifestations of Wilson disease but are asymptomatic and have an unremarkable physical examination. Liver disease can vary from acute hepatitis, acute liver failure, to cirrhosis and portal hypertension. Acute liver failure associated with WD (Wilsonian acute liver failure) has several characteristic features, including cholestasis, extremely low alkaline phosphatase, low ceruloplasmin, Coombs-negative hemolytic anemia, renal failure, and encephalopathy. The massive amount of copper released from the necrotic liver contributes to the severe intravascular hemolysis, hemoglobinuria, and renal failure. Neurological disease is uncommon in the pediatric population, and the changes are predominantly extrapyramidal with "wing beating" or flapping tremor and "risus sardonicus" being classic, but late. More commonly, subtle features, such as changes in behavior and handwriting or learning disabilities, are reported. Drooling, dysarthria, and gait abnormalities may also be observed. Kayser–Fleischer rings are often observed in children with neurological manifestations of WD. The other ocular manifestation of WD is Wilson disease. Psychiatric involvement is observed more commonly in those with neurological disease, and symptoms may often precede the development of neurological or liver disease, leading to delay in diagnosis and treatment. Worsening performance at work or school may also be secondary to WD.

The combination of high serum bilirubin levels with low alkaline phosphatase is highly suggestive of WD. Demonstration of Coomb's positivity, low serum ceruloplasmin, and increased 24-h urinary copper excretion are important diagnostic parameters, but the gold standard for establishing diagnosis of WD is demonstration of liver copper >250 μg/g dry weight of liver tissue. Genetic analysis of the ATP7B gene can allow the diagnosis of WD, but one must be aware that there are several mutations observed in WD. It is necessary to screen family members for WD following diagnosis of the index case. This can be done with liver chemistries, ceruloplasmin, and 24-h urine copper excretion. In those with an identified mutation, genetic testing of first-degree relatives is an efficient mode of screening. Magnetic resonance imaging (MRI) is considered the most sensitive modality for the detection of neurological changes in the brain, and nuclear medicine investigations are helpful in assessing functional deficits in those with neurological symptoms.

In terms of medical management, zinc and oral chelators are the mainstay of therapy. High copper-containing foods, such as shellfish and chocolate, need to be avoided. D-penicillamine and trientine are the two most widely used chelators. For children below the age of five, zinc is the preferred form of therapy. Zinc interferes with the absorption of copper in the intestine by inducing the enterocyte metallothionein to bind copper more avidly than zinc, and the latter is lost when the enterocytes are shed routinely as part of physiologic turnover. Zinc is also preferred in those with neurological disease and is often used alone as a maintenance therapy in WD. Monitoring patients on therapy can be done with routine chemistries, but measuring 24-h urinary copper and non-ceruloplasmin bound copper can be very useful.

In Wilsonian acute liver failure, albumin dialysis and other forms of liver assist devices may act as a bridge to liver transplantation. Scoring systems are valuable in the assessment of disease severity and in guiding clinical decision making with respect to need for liver transplantation.

Autoimmune Liver Disease

Childhood autoimmune liver diseases include autoimmune hepatitis, autoimmune overlap with sclerosing cholangitis, recurrence of autoimmune hepatitis post liver transplantation, and development of *de novo* AIH following liver transplantation in children not transplanted for AIH.

Autoimmune hepatitis (AIH) is a chronic inflammatory condition of the liver with elevation of serum aminotransferases, hypergammaglobulinemia, presence of autoantibodies, and lymphoplasmacytic interface hepatitis of unknown etiology. Children with AIH may be asymptomatic, have an acute hepatitis like picture including acute liver fail-

ure or present with complications of cirrhosis and portal hypertension. Type 1 AIH or ANA/SMA-positive AIH is more commonly seen in childhood than type 2 AIH. Type 1 AIH is often associated with a "chronic" presentation, and children are more likely to have cirrhosis as well as 'overlap' with sclerosing cholangitis. Children with type 2 AIH or liver kidney microsomal antibody-positive AIH are often younger at presentation, more likely to have an acute liver failure presentation and unlikely to have autoimmune overlap with sclerosing cholangitis. Diagnosis is made after ruling out other common causes of liver disease, including viral hepatitis, Wilson disease, alpha-1 antitrypsin deficiency, and drug ingestion. Liver biopsy is usually performed to secure diagnosis and also to allow better ability to prognosticate. Scoring systems are not commonly used in clinical practice but appear to be useful in complicated cases and research studies. If gamma-glutamyl transferase is high and biliary dilatation is seen on ultrasound and/or biliary changes noted on histology, then magnetic resonance cholangiography should be performed to assess for autoimmune overlap with sclerosing cholangitis.

The first line therapy of AIH in children is steroids (usually prednisone 2 mg/kg/day – max 40–60 mg daily) in combination with azathioprine. Azathioprine is started at variable intervals after starting prednisone, with the author (NK) preference being to start azathioprine after receiving the results of enzymatic status of thiopurine methyltransferase (TPMT). The azathioprine metabolites are helpful in optimizing the dose of azathioprine and also in monitoring adherence. Budesonide is a synthetic corticosteroid that undergoes 90% first-pass effect in the liver. Budesonide can also be used instead of prednisone, but should always be given with azathioprine during induction and should not be given when there is cirrhosis, as the steroid-sparing effects are lost. Budesonide is an attractive option when the BMI is high and in teenagers as they are focused on their appearance and want to avoid standard steroids whenever possible. Ursodeoxycholic acid (15–20 mg/kg/day in two divided doses) is added when there is evidence of overlap with sclerosing cholangitis. The second line therapy is mycophenolate mofetil or calcineurin inhibitors, such as tacrolimus or cyclosporin. Trough levels of the calcineurin inhibitor should be monitored. Response is higher when one switches to second line if the first drug has not been tolerated as opposed to the patient not responding to first line therapy. Salvage therapy with infliximab [43] and rituximab [44] has been successful in suppressing autoimmune disease. Liver transplantation for AIH accounts for about 2–3% of liver transplants. The indications of liver transplantation include (1) failure of medical treatment, (2) acute liver failure presentation with encephalopathy, and (3) hepatocellular carcinoma (rare). Management of AIH including children has recently been reviewed and is a useful resource [45].

Recurrent Autoimmune Hepatitis and De Novo Autoimmune Hepatitis

Recurrent autoimmune hepatitis has been described in adults and pediatric patients [46]. The incidence is variable (12–46%) and depends on the immunosuppressive regimens used, length of follow-up, and diagnostic criteria used [47, 48]. The diagnosis of recurrent AIH requires elevated serum aminotransferases, elevated serum immunoglobulin G, presence of serum autoantibodies, and interface hepatitis on histology in the absence of other known causes of graft dysfunction, including vascular, biliary, and infectious etiologies as well as classical rejection. Factors associated with the recurrence of AIH include (1) HLA DR3 or HLA DR4; (2) low levels of immunosuppression, either secondary to nonadherence or per protocol; and (3) severity of autoimmune inflammatory activity in the explant. Chronic AIH that fails medical therapy and requires liver transplantation is more likely to recur in the allograft than when the indication is an acute liver failure presentation of AIH. Once diagnosis is established, it is managed with bolus of steroids and addition of a third agent, e.g., azathioprine or mycophenolate mofetil. If patient is already on the third agent, substitution with a M-TOR inhibitor such as sirolimus can be effective [49]. Early diagnosis and prompt institution of appropriate therapy can save grafts and prevent re-transplantation.

The term de novo AIH was first used to describe a unique form of graft dysfunction that resembled AIH biochemically and histologically, but occurred in children transplanted for non-immune etiologies like biliary atresia and Alagille syndrome [50]. This form of graft dysfunction has since been described in multiple pediatric centers from around the world. A similar phenomenon has been described in adults but has been given different names, including "plasma cell hepatitis" [51] and "graft dysfunction resembling AIH" [52], as the majority of the adult liver transplants are performed for liver disease secondary to hepatitis C or other immune-mediated etiologies, such as primary sclerosing cholangitis and primary biliary cholangitis. Hepatitis C is linked with autoimmunity, and the International Autoimmune hepatitis Group developed a scoring system for the diagnosis of AIH following the identification of hepatitis C virus in 1989, as the hepatitis histologically appeared similar in both chronic hepatitis C and AIH. More recently, in an effort to establish guidelines for the management of graft dysfunction predominantly antibody-mediated rejection, the Banff group has suggested that "plasma cell hepatitis" and *de novo* AIH' should be referred to as "plasma cell rejection" [53]. While this nomenclature may be suitable for adult liver transplant recipients, the entity described histologically as plasma cell rejection has features of rejection with bile duct damage and sometimes histological features consistent with recurrent hepatitis C. These features exclude the diagnosis

of AIH. Given this, a case has been made to separate this entity of plasma cell hepatitis/rejection in adults from *de novo* AIH in children – an entity that fulfills all criteria for AIH and scores as AIH using the international AIH scoring system [54].

Sclerosing Cholangitis

Sclerosing cholangitis was first described in 1924 and is a progressive inflammatory condition affecting the biliary system leading to fibrosing strictures, beading, and dilation of the bile ducts. The incidence and prevalence of sclerosing cholangitis are estimated to be 0.2 and 1.5/100,000 children, respectively [55]. Sclerosing cholangitis is associated with IBD in almost 90% of cases and referred to as PSC, similar to adults. But, in pediatrics, other forms of sclerosing cholangitis have been described, including (1) neonatal sclerosing cholangitis; (2) overlap with AIH – autoimmune sclerosing cholangitis; (3) small duct primary sclerosing cholangitis; (4) immunodeficiencies such as Wiskott–Aldrich syndrome and x-linked agammaglobulinemia; (5) neoplasm like Langerhans cell histiocytosis and Hodgkin disease; (6) infection with *E. coli* and cryptosporidium; (7) cystic fibrosis; (8) sickle cell disease; and (9) MDR3 deficiency.

PSC with IBD children in this group are more likely to be male. No correlation has been observed between the severity of IBD and PSC. In a single-center study, IBD was diagnosed concurrently with PSC in 59%, before PSC in 26%, and after PSC in 15% [56]. Neonatal sclerosing cholangitis is a severe cholangiopathy which affects the intrahepatic bile ducts in the neonatal period and progresses to end-stage liver disease, thus requiring liver transplantation. This condition was first described in 1987. Since neonatal SC also presents with cholestatic jaundice with pale stools, it should be considered in the differential diagnosis of biliary atresia. Unlike the PFIC group of cholestatic disorders, GGT is high in this condition, but the histology is similar with ductular reaction, cholestasis, and varying degrees of fibrosis. Recently, using next-generation sequencing, mutations in the gene encoding two doublecortin domain (DCDC2), a signaling and structural protein located in the primary cilia of cholangiocytes, were identified in 7 of 24 children with neonatal sclerosing cholangitis in whom DNA was available [57]. The disease is thought to be inherited in an autosomal recessive fashion, as this condition has been observed in consanguineous kindred. Of the 29 children, extrahepatic diseases, including renal disease and posterior cerebral aneurysm, were noted. Almost half of the patients (16 of 29) underwent liver transplantation, and two died with end-stage liver disease waiting for a liver transplant. The recurrence of the disease post-transplant has not been described to date.

The overlap of AIH and sclerosing cholangitis has been termed autoimmune sclerosing cholangitis or autoimmune overlap in sclerosing cholangitis. This was first described by the King's group that reported 27 of 56 children with liver disease and positive autoantibodies had biliary changes on ERCP and histology [58]. About 44% had associated IBD, and the disease was equally present in males and females, unlike the female predominance of AIH. More recently, in a study involving 36 centers around the world, 33% of 781 children with SC were noted to have overlap with AIH [55]. Of these, 7% (52/781) had an additional immune-mediated disease, including thyroiditis, celiac disease, type 1 diabetes, and juvenile idiopathic arthritis. Given the high prevalence of AIH, it may be helpful for children with sclerosing cholangitis to be screened for IgG elevation and presence of autoantibodies – antinuclear antibody, smooth muscle antibody, and perinuclear staining anti-neutrophil antibody. Conversely, children with AIH should be screened for SC using MRCP/ERCP, particularly when GGT is high. The current scoring systems are not helpful in the diagnosis of autoimmune sclerosing cholangitis, and a new scoring system has been proposed, but not validated [59]. The term small duct primary SC is used when there is clinical and biochemical evidence of cholestasis with biliary changes on histology but no macroscopic evidence of biliary changes on imaging – magnetic resonance cholangiopancreatography or endoscopic retrograde cholangiopancreatography. The incidence in childhood has been found to be more common in children (13–36%) than adults (5%) [56, 60], and the prognosis appears to be better than that in other forms of PSC [61].

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Mechanisms of Acute Liver Failure

29

Christian Trautwein and Alexander Koch

Key Points

- Acute liver failure is characterized by the sudden onset of liver failure in a patient with no evidence of chronic liver disease.
- Four different mechanisms are mainly responsible for ALF: (1) infectious (mostly viral), (2) drugs/toxins/chemicals, (3) cardiovascular, and (4) metabolic.
- Suicidal acetaminophen ingestion is the most frequent cause of drug-induced liver failure.
- Three factors determine the prognosis of liver failure: (1) the metabolic consequences resulting from liver failure, (2) the release of mediators and toxic metabolites, and (3) the capacity of the remaining hepatocytes to restore liver mass.
- Cerebral edema, infections, and multiorgan failure are important clinical complications that limit patient survival.
- Ammonia levels can be used for risk stratification in patients with acute liver failure and subsequent hepatic encephalopathy.
- Intravenous administration of N-acetylcysteine improves transplant-free survival of patients with early-stage non-acetaminophen-related acute liver failure.
- Treatment with high-volume plasma exchange improves the outcome of patients with ALF by increasing liver transplant-free survival, potentially by attenuating innate immune activation and ameliorating multiorgan dysfunction.
- Cytokines are involved in the pathogenesis of acute liver failure and in controlling the balance between survival and hepatocyte proliferation.
- The mode of liver cell death that is predominantly induced in ALF (apoptosis or necrosis) is determined by the underlying etiology, the duration of the disease, and the extent of liver injury.
- Future characterization of the molecular cell death mechanisms might establish potential diagnostic and therapeutic targets in ALF.
- Intestinal dysbiosis has been recently identified as a driver of ALF severity. The understanding of gut-liver interaction during ALF might facilitate innovative therapeutic interventions.

Introduction

Acute liver failure (ALF) is characterized by the sudden onset of liver failure in a patient with no evidence of chronic liver disease. This definition is important as it differentiates patients with acute liver failure from patients who suffer from liver failure due to end-stage chronic liver disease (decompensated cirrhosis and acute-on-chronic liver failure, ACLF).

ALF is a rare condition and affects about 2000 persons per year in the USA. It is defined as severe hepatopathy with elevated transaminases twofold the upper limit of normal, liver dysfunction (icterus and coagulopathy with an international normalized ratio (INR) >1.5), and hepatic encephalopathy.

Chronic liver disease and the secondary causes of liver dysfunction, such as sepsis and cardiac shock, have to be ruled out. Nevertheless, acute decompensation of Wilson's disease, reactivation of chronic hepatitis B, and autoimmune

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hepatis – in fact, chronic liver diseases – are considered as cases of acute liver failure.

Other common clinical features of ALF are cardiovascular instability, susceptibility to infection, acute kidney injury, and cerebral edema. Owing to the affection of all organ systems, ALF is associated with an overall mortality of approximately 30%. ALF accounts for 6–8% of liver transplantations in the USA and Europe [1]. The data of the US ALF study group are presented in Fig. 29.1; spontaneous survival occurs in approximately 45%, liver transplantation in 25%, and death without transplantation in 30% of adults with ALF [1].

The time between the first symptoms and the manifestation of hepatic encephalopathy has been shown to be crucial for the prognosis of these patients. Therefore, several groups have included in their definition the time frame between the onset of symptoms and the start of encephalopathy.

The definition of the US ALF Study Group (ALFSG) uses the term acute liver failure as an umbrella and differentiates the three subgroups: hyperacute, acute, and subacute (Fig. 29.2). The time between the first symptoms and encephalopathy in hyperacute ALF is 7 days; in acute ALF, it is 8–28 days; and in subacute ALF, it is 5–26 weeks [2].

Hepatocyte injury can be caused by direct toxic necrosis, often related to hyperacute ALF, or by apoptosis and immune injury, which is a common feature of acute and subacute ALF.

Typically, in hyperacute ALF, very high aminotransferase concentrations and low bilirubin concentrations can be observed, whereas in acute and subacute ALF, lower aminotransferase levels and higher bilirubin levels are common. In general, patients with hyperacute liver injury have better short-term survival in comparison with patients with slowly progressing liver injury. Nevertheless, the cause of hepatic injury has superior prognostic potential as compared with time frame to evolve ALF.

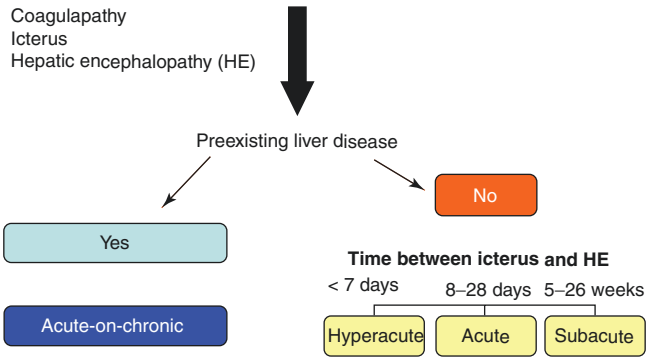

Fig. 29.1 Natural history of ALF. Liver regeneration with spontaneous survival occurs in approximately 45%, liver transplantation in 25%, and death without transplantation in 30% of adults with ALF. (Data from the United States); LTX liver transplantation [1]

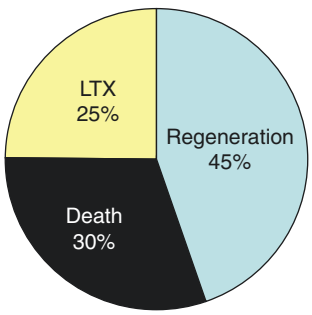

Fig. 29.2 Definition of ALF. ALF is defined as a severe liver injury, clinically characterized by coagulopathy and hepatic encephalopathy within 26 weeks of symptom onset in previously healthy subjects

Mechanisms of Disease

There are different causes of ALF. In principle, four different classes can be differentiated: (1) infectious (mostly viral), (2) drugs/toxins/chemicals, (3) cardiovascular, and (4) metabolic [3] (Table 29.1). In developed countries, acetaminophen toxicity, ischemia, drug-induced liver injury, hepatitis B, and autoimmunity account for nearly 80% of the cases [4].

There are obvious differences in the mechanisms that initially trigger liver failure. However, at the time of clinical presentation, in most cases, a common final stage has been reached in ALF patients. At this stage, three main factors seem to be important in determining the prognosis: (1) the

Table 29.1 Causes of acute liver failure

Infectious causes
Hepatitis A-E
Rare causes of infectious etiology
Herpes simplex virus types 1 and 2, Human herpes virus type 6, Varicella virus, Cytomegalovirus, Epstein-Barr virus, Parvovirus B19, Togavirus, Paramyxovirus, Parainfluenza virus
Drugs and toxins
Acetaminophen, halothane, isoniazid, valproate, tetracycline, nonsteroidal anti-inflammatory drugs (NSAIDs), pirprofen, ketoconazole *Amanita phalloides* (tuber toadstool poisoning) Illicit drugs: amphetamines, ecstasy, cocaine
Cardiovascular syndromes
Budd-Chiari syndrome, hypotension (circulatory shock, left ventricular failure), heart failure (e.g., right ventricular failure, valvular heart diseases), hyperthermia, malignant tumors, veno-occlusive disease, portal vein thrombosis, sepsis
Metabolic diseases
Wilson's disease, Reye's syndrome, acute fatty liver of pregnancy (AFLP), HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count), galactosemia, hereditary fructose intolerance, hereditary tyrosinemia

Table 29.2 Specific therapeutic options in ALF

Cause of ALF	Treatment	Dosage
Acetaminophen	N-acetyl cysteine	300 mg/kg
Amanita poisoning	Silibinin	20–50 mg/kg/day
Acute hepatitis B	Lamivudine Entecavir Tenofovir	100–300 mg/day 0.5–1 mg/day 245 mg/day
HELLP/AFLP	Termination of pregnancy	–
Autoimmune hepatitis	Prednisolone	1–2 mg/kg/day
Budd-Chiari syndrome	TIPSS/surgical shunt	–
Herpes simplex hepatitis	Aciclovir	3x10 mg/kg/day

Modified from Ref. [9]
TIPSS transjugular intrahepatic portosystemic stent shunt

metabolic consequences resulting from the loss of liver cell mass, (2) the release of mediators and toxic metabolites from the liver tissue, and (3) the capacity of the remaining vital hepatocytes to restore liver mass [5, 6].

Therefore, in terms of the mechanisms that are important during ALF, two different phases of ALF can be differentiated: the mechanisms that initially trigger liver failure and those that eventually determine the outcome.

The etiology of ALF and coma grade on admission are two prominent factors influencing prognosis. ALF caused by acetaminophen toxicity, hepatitis A, ischemia, and pregnancy are associated with at least 60% short-term transplant-free survival, whereas drug-induced liver injury, (reactivation of) hepatitis B, autoimmune hepatitis, and indeterminate causes are associated with a spontaneous recovery rate of only 30% [7]. Patients presenting with early grades of hepatic encephalopathy in ALF (independent of etiology) usually have a more favorable outcome than those with established stupor or coma [8]. Liver transplantation, intensive care medicine, and specific therapeutic options (Table 29.2) can improve prognosis [9].

Etiology

Infectious Causes

Viruses in particular are an essential cause of ALF and, depending on the geographical region can comprise between 30% and 70% of all forms of ALF [3, 5, 6]. In the developing world, infections with hepatitis A, B, and E viruses are accounting for most cases of ALF. In Europe, the data from the ELTR database reveal that liver transplantation for ALF due to HAV and HBV decreased significantly in the last 5 years (from 1% to 0.5% and from 17.9% to 13.2%, respectively) [10].

Hepatitis A Virus

Due to effective use of vaccination, infections with the hepatitis A virus (HAV) have declined over the last decade and accounted for 3% of the ALF cases in the USA [11]. The proportion of patients with ALF is higher in older than in younger patients. This is relevant, as in Western countries over the last decades, HAV infection has occurred more frequently in older patients, and thus, the risk of ALF increases in this population [12, 13]. Recent widespread outbreaks of hepatitis A infections among homeless people in the USA resulted in an increased rate of hospitalizations and demonstrated a lack of vaccination in the general population [2]. Furthermore, patients with underlying chronic liver disease, especially chronic hepatitis C, have an increased risk of ALF in the context of HAV infection [14].

The pathogenesis of HAV-related ALF is not completely understood. Current studies indicate that a combination of a direct cytopathic effect of the virus and immune-mediated mechanisms results in liver destruction. In comparison with other hepatotropic viruses, ALF caused by hepatitis A has a favorable prognosis with spontaneous or transplant-free survival of nearly 70% [11].

Hepatitis B Virus

The risk of acute liver failure of all patients who are hospitalized due to an acute hepatitis B virus (HBV) infection is around 1% [15]. Fulminant HBV is the most predominant viral cause of ALF in Western countries [7, 16] and accounts for 7–10% of ALF in Europe and 7% in the USA [1, 10]. Due to the implementation of routine vaccination, the incidence of fulminant HBV has decreased. In fulminant acute HBV infection, antiviral therapy with lamivudine, entecavir, or tenofovir has been proven efficient and safe, with a significant reduction in HBsAg concentrations (see Table 29.2) [17, 18]. Once ALF is established, the clinical benefit of antiviral therapy is not proven. Nevertheless, antiviral therapy should be given to transplantation candidates, since viral suppression prevents HBV recurrence after following transplantation [19].

Approximately two-thirds of the cases of ALF due to hepatitis B are caused by new infections, and the remainder are caused by reactivation of (unrecognized) chronic hepatitis B infection in the setting of chemotherapy or immunosuppression. Reactivation of HBV or infection with highly replicative HBV harboring precore and core-promoter gene mutations became a more important cause of ALF [20, 21].

Virus reactivation is associated with a much higher risk of ALF than is novel acute HBV infection, and antiviral prophylaxis should be administered to HBsAg-positive patients who are about to receive chemotherapy or immunosuppressive therapy [22, 23]. Clinical differentiation of ALF due to acute or chronic hepatitis B infection is often difficult if there is no previous history of hepatitis B infection. Quantitative measurements of immunoglobulin M (IgM) anti-hepatitis B

core antibody (anti-HBc) titers and HBV viral loads might allow etiological discrimination [24].

In general, the HBV itself is not cytopathic, but the immune response directed against the virus is essential [25]. Frequently at the time of hospitalization, the viral load is already decreasing, whereas transaminases are still increasing. This may reflect the possibility that different factors contribute to the elimination of the virus. The data indicate that cytokines – namely, interferon (IFN) – are operating through a noncytopathic mechanism to eliminate the HBV genome in hepatocytes, whereas at a later stage, the T cells infiltrate the liver and destroy the hepatocytes [26]. Therefore, the activation of HBV-specific T cells is important to determine the degree of hepatic injury during ALF.

In the case of HBV/hepatitis D virus (HDV) coinfection, the risk of ALF increases [27]. The exact mechanisms that lead to more pronounced liver failure are not defined.

Hepatitis C Virus

The risk of ALF through hepatitis C virus (HCV) is very low [5]. In Japan, in particular, cases of HCV-related ALF have been documented [28]. As there are only a few reports in the literature, the pathogenesis of HCV-related ALF is not completely understood. However, there is evidence that elimination of HCV-specific T cells is associated with chronic HCV infection [29]. This indicates that the HCV-specific immune response is involved during acute infection and thus is most likely the determining factor during ALF.

Hepatitis E Virus

Acute liver failure owing to hepatitis E virus (HEV) infection is seldom observed in Western countries. However, hepatitis E has a predilection for older men in whom it causes substantial morbidity and mortality [30]. Based on a poor prognosis in combination with preexisting liver disease, patients with unexplained hepatitis should be tested for HEV [31]. Epidemic outbreaks are known in developing countries, especially in patients with ALF. In India, Pakistan, China, and Southeast Asia, HEV infection is the most predominant cause of ALF [31]. Pregnant women, especially in the third trimester, have been regarded to have a high risk for ALF (up to 20%) [32].

However, recent data indicate that pregnancy does not affect the outcome of ALF resulting from HBE infection [33]. The mechanisms operating in patients with HBE infection-induced ALF have not yet been sufficiently studied. Therefore, there is no clear hypothesis in the literature, and it is only speculative to draw parallels with HAV.

Rare Cases of Viral Hepatitis

In rare cases, different systemic virus infections can present as ALF owing to a predominant manifestation in the liver. These are the herpes simplex virus types 1 and 2 (see Table 29.2), human herpes virus type 6, cytomegalovirus, varicella-zoster virus, Epstein–Barr virus, and parvovirus B19. A few cases of ALF related to an infection with the togavirus, paramyxovirus, and parainfluenza virus have also been described.

Drugs, Toxins, Chemicals

Drug toxicity is the predominant cause of ALF in Western countries. Several drugs, chemicals, and toxins can cause ALF (see Table 29.1) by either direct toxicity or idiosyncratic drug reaction. The most frequent examples are discussed in this review.

Acetaminophen

Acetaminophen (Paracetamol, Tylenol) is the most common cause of ALF. In adults, only higher doses (in general, more than 10–12 g) are dangerous, and in most cases, acetaminophen was taken in a suicide attempt. Patients who consume alcohol chronically and those with non-alcoholic fatty liver disease (NAFLD) may be more susceptible for acetaminophen toxicity, as cytochrome P450 has been induced in their liver [34].

Measurement of serum acetaminophen-protein adducts (toxic byproducts of cell injury: acetaminophen bound to cell proteins) can reliably identify acetaminophen toxicity in cases of ALF, in which no clinical or historic data are given that would reveal the cause up to 3 days following ingestion [35, 36]. At present, these analyses are only available in specialized laboratories. Acetaminophen toxicity causes 46% of the cases of ALF in the USA and 65% in the UK [37, 38].

The pathogenesis of acetaminophen injury is related to the formation of toxic metabolites through the cytochrome P450 enzymes, especially cytochrome P450 2E1 [39, 40]. These toxic metabolites are normally conjugated and inactivated through glutathione. However, when glutathione stores are depleted, these toxic metabolites accumulate, resulting in hepatocyte injury (Fig. 29.3).

The pattern of hepatic injury in acetaminophen toxicity is similar to ischemia, with a rapid-onset necrosis beginning 8–12 h following ingestion. The typical clinical features are very high levels of aminotransferase and elevated INR but normal or slightly increased bilirubin levels. Peak levels are expected at approximately 72 h. Necrosis has been shown to be the more prominent form of cell death in acetaminophen toxicity [41]; however, *in vitro* data and animal data suggest that apoptosis also contributes to acetaminophen-induced ALF [42–44].

In fact, it has been demonstrated that the course of disease in acetaminophen-induced liver failure is on the one hand influenced by the acetaminophen dose and the initial hepatocyte damage and on the other hand by the inflammatory

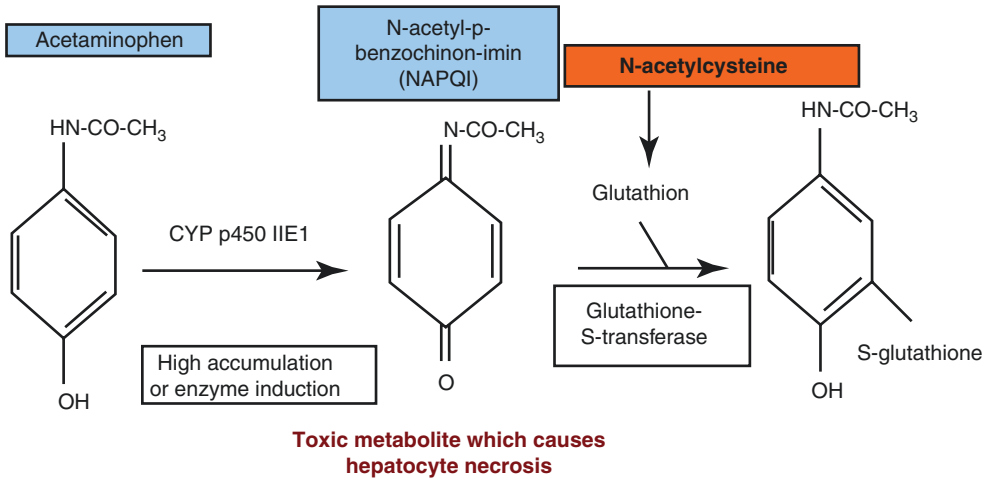

Fig. 29.3 Pathogenesis of acetaminophen injury. Formation of toxic metabolites (N-acetyl-p-benzoquinone imine, NAPQI) through the cytochrome P450 enzymes, especially cytochrome P450 2E1, as a result of acetaminophen accumulation and/or enzyme induction. Toxic metabolites are normally conjugated and inactivated through glutathione. Glutathione store depletion leads to the accumulation of these toxic metabolites and finally hepatocyte injury. Administration of N-acetylcysteine facilitates the refilling of glutathione stores and thereby supports detoxification

response following acetaminophen-induced liver failure. Necrotic hepatocytes release danger-associated molecular patterns (DAMPs) which are recognized by hepatic macrophages, Kupffer cells, and neutrophils and consecutively result in the activation of these cells. The detection of DAMPs and pathogen-associated patterns (PAMPs) is exerted by the inflammasome, a highly regulated signaling system in myeloid cells, which conclusively leads to the activation of monocytes and neutrophils by the release of pro-inflammatory cytokines, interleukin (IL)-1ß), and IL-18 through a proteolytic cleavage pathway mediated by the activation of caspase-1 [45]. Also, activated macrophages release pro-inflammatory cytokines (e.g., TNF-α, IL-1ß, and IL-18) as well as chemokines (e.g., CCL2), thereby enhancing hepatic inflammation. Additionally, monocytes which are mainly recruited by their receptor CCR2 further aggravate inflammation. Those liver-infiltrating monocytes can mature into monocyte-derived macrophages (MoMF), which are involved in the resolution of inflammation. Natural killer T cells are additional parts of the inflammatory response to acetaminophen toxicity and may maintain hepatic inflammation [46].

N-acetylcysteine (NAC), the standard antidote for acetaminophen overdose, exerts its therapeutic effects by restoring the depleted hepatic glutathione stores and is usually given at a cumulative dose of 300 mg/kg BW over 21 h (see Table 29.2) [47].

A recent multicenter study from Australia (NACSTOP) has shown that shortening of the NAC regimen for acetaminophen poisoning is possible in selected low-risk patients. Low risk was defined as normal ALT levels at baseline and after 12 h, and acetaminophen level <20 mg/l at 12 h. In this cohort, reduction of the NAC regimen to 12 h with a total NAC dose of 250 mg/kg BW was safe [48].

Moreover, intravenous NAC improves transplant-free survival in patients with early-stage non-acetaminophen-related ALF. However, patients with advanced coma grades do not benefit from NAC and typically require emergency liver transplantation [49].

Mushroom (Amanita) Poisoning

Mushroom poisoning, mainly through the species *Amanita phalloides* (tuber toadstool) frequently leads to ALF, especially in fall. The clinical spectrum of *Amanita* poisoning varies from acute gastroenteritis to the development of ALF.

After ingestion of the tuberous toadstools, there is initially a symptomless latency phase for 5–24 h, until vomiting, massive diarrhea, abdominal colic, and exsiccosis are in the foreground in the gastrointestinal phase over a period of 12–24 h. In this phase, tuber-toed mushroom intoxication is often misinterpreted as "food poisoning" or gastroenteritis. This is followed by the hepatorenal phase after 2–3 days, which is characterized by an increase in transaminases and evolving coagulopathy, icterus, and liver and kidney failure. A deleterious course can be prevented by liver transplantation or a spontaneous restitution of the liver function taking place within 2–3 weeks [50].

The toxic agent of tuber toadstool poisoning is the amanita toxin (amanitin). It mainly blocks RNA polymerase II and thereby inhibits transcription and protein biosynthesis and leads to cell death. The result is a multiorgan failure, especially the liver, kidney, and brain.

The foreground of the therapy is the primary elimination of toxins with repeated administration of activated carbon,

since the amanita toxin undergoes an enterohepatic cycle. As an antidote, silibinin can prevent the uptake of amanitin into liver cells and improve biliary excretion [51]. Although there no controlled trials proving its efficiency, silibinin is used in Europe owing to its cytoprotective effects against the amanita toxin and has been reported to be more effective than penicillin G in the amanita poisoning (silibinin is not available as a licensed drug in the USA) (see Table 29.2) [51, 52].

There are more than 1300 case reports on the clinical efficacy of silibinin as an antidote in tuber toadstool poisoning. Based on these case reports, an initial dose of silibinin of 5 mg/kg BW intravenously (I.V.) for 1 h, followed by a continuous application of 20 mg/kg BW/d I.V. until liver function has recovered, seems to be indicated. Concomitant therapy with NAC 300 mg/kg BW over 20 h I.V. potentially exerts additive positive effects [51].

Despite the advances in intensive care therapy, the morality rate in patients who develop ALF following amanita ingestion is high [52].

A recent animal study has investigated the effects of combined antidote therapy with polymyxin B and methylprednisolone in amanita intoxication [53]. The rationale for the use of these substances is, on the one hand, that polymyxin B can reverse the inhibition of RNA polymerase II caused by amanitin. On the other hand, methylprednisolone is an inhibitor of the Na^+ -taurocholate cotransporter polypeptide transporter (NTCP), which also mediates the toxic effects of amanitin and has anti-inflammatory effects.

The experimental animals received 0.33 mg/kg of amanitin intraperitoneally (I.P.) 4 h after the application of 2.5 mg/kg of polymyxin B and 10 mg/kg of methylprednisolone as antidotes I.P. Under this combination therapy, all animals survived the amanitin intoxication; without antidote, only 40% survived. The antidote combination of polymyxin B and methylprednisolone may be a new therapeutic option in tuber-toed mushroom poisoning.

It should be noted, however, that currently, there are only animal experimental data, and no dose information or suggestions for use in humans have been proposed. However, due to the expected therapeutic safety of polymyxin B and methylprednisolone, this approach seems promising.

Halothane

Halothane is the prototype of an idiosyncratic drug reaction that (less frequently) can also be found after anesthesia with other members of the same family. In general, halothane-related ALF is only found after the second exposure to the drug. Halothane hepatitis is a paradigm for immune-mediated adverse drug reactions. The mechanism appears to be related to the development of sensitization to both autoantigens, including CYP2D6, and halothane-altered liver cell determinants [54]. For the pathogenesis of the disease, specific antibodies are involved in hepatic injury. These antibodies can only be determined in specialized laboratories.

Cardiovascular Disorders

Cardiovascular diseases can lead to ALF, either by ischemia or by impaired blood flow leaving the liver. Examples of ischemic events are severe hypotension or heart failure. Aminotransferase concentrations ≥3000 U/l and bilirubin levels <5 mg/dl are strong indicators of either hepatic ischemia or acetaminophen toxicity. Ischemic hepatic injury rarely requires liver transplantation [55]. Hepatic injury due to severe heart failure can be promptly diagnosed *via* echocardiography by assessing the left-ventricular ejection fraction. Stasis of blood flow in the liver may occur owing to malignant tumors, veno-occlusive disease, or Budd-Chiari syndrome.

Budd-Chiari Syndrome

Classically, Budd-Chiari syndrome is characterized by a symptomatic occlusion of the hepatic veins and is more frequently observed in females [56]. Depending on the disease progression, Budd-Chiari syndrome may result in ALF when a sudden occlusion of at least two main liver veins occurs. Typically, acute Budd-Chiari syndrome presents with ascites, abdominal pain, jaundice, and hepatomegaly [57]. Budd-Chiari syndrome is frequently associated with primary myeloproliferative disorders, a factor V Leiden mutation, anticardiolipin antibodies, and protein C and S deficiency, which increase the risk of thrombotic complications [58]. In general, the course of disease in Budd-Chiari syndrome leads to liver transplantation. Transjugular intrahepatic portosystemic stent shunt (TIPSS) or percutaneous transjugular direct portocaval shunt, in patients with inaccessible hepatic veins, seems to be therapeutic options to decrease the portal pressure gradient, improve synthetic functions, reduce transaminase levels, and control ascites (see Table 29.2) [59, 60].

Metabolic Disorders

Different metabolic disorders may present as ALF, for example, Reye's syndrome, which is more common in children; its frequency has declined over the last decades. Also, during pregnancy, acute fatty liver of pregnancy (AFLP) or HELLP syndrome (hemolysis, elevated liver enzymes, and a low platelet count) may develop. Patients with HELLP syndrome typically present with LDH, ALT, and increased bilirubin level. Immediate termination of pregnancy and delivery usually reverse hepatopathy, but patients are at increased risk for complications in future pregnancies (see Table 29.2) [61].

Wilson's Disease

Wilson's disease is an autosomal recessive genetic disorder of copper metabolism and a rare cause of ALF. Wilson's disease protein (WND, ATP7B protein) is a copper-transporting P-type ATPase and is encoded by the ATP7B gene. The ATP7B protein is located in the trans-Golgi network of the liver and brain.

ATP7B protein regulates the copper concentration level in the body by excreting excess copper into the bile and blood. Genetic disorder of the ATP7B gene (by single base pair mutations, deletions, frameshifts, and splice errors in ATP7B gene located at chromosome 13, 13q14.3) may cause Wilson's disease, which is characterized by copper accumulation in the tissues. Hepatic disease occurs when the accumulation of copper in the liver causes mitochondrial damage and hepatocellular necrosis. The reduced excretion of copper into the bile results in increased urinary copper concentrations, leading to renal dysfunction.

The clinical appearance of Wilson's disease comprises hepatic, renal, ophthalmic, cardiac, neurologic, and psychiatric disorders. In general, patients with ALF due to Wilson's disease present with only moderately elevated aminotransferases and reduced levels of alkaline phosphatase but high bilirubin. Hemolytic anemia induced by copper ions leaking from necrotic hepatocytes into the circulation, causing lysis of erythrocytes, and acute kidney injury are further typical clinical features of Wilson's disease which allow appropriate diagnosis [62]. The patients already frequently have liver cirrhosis and are therefore not in accordance with the "real" definition of ALF. However, many of the patients were healthy before the onset of the disease and therefore are categorized as patients with ALF [63].

The pathogenesis of hepatocyte injury in the context of Wilson's disease is not completely understood. Both necrosis and apoptosis may be encountered. There is evidence that elevated copper levels are directly toxic for the cell and involve CD95-mediated apoptosis [64]. The current hypothesis postulates that excess copper generates free radicals that deplete the cellular stores of glutathione and oxidize lipids, enzymes, and cytoskeletal proteins [65].

Mechanisms of Organ Failure

As a consequence of ALF, multiorgan failure (MOV) develops rapidly. Different factors contribute to MOV (Fig. 29.4).

Frequent problems that occur during this process are cerebral edema and encephalopathy, an impairment of the immune response with an increased rate of infections, coagulation disorders, and cardiovascular and kidney failure; pulmonary and metabolic complications also develop. Figure 29.5 presents an overview of the common clinically relevant complications of ALF.

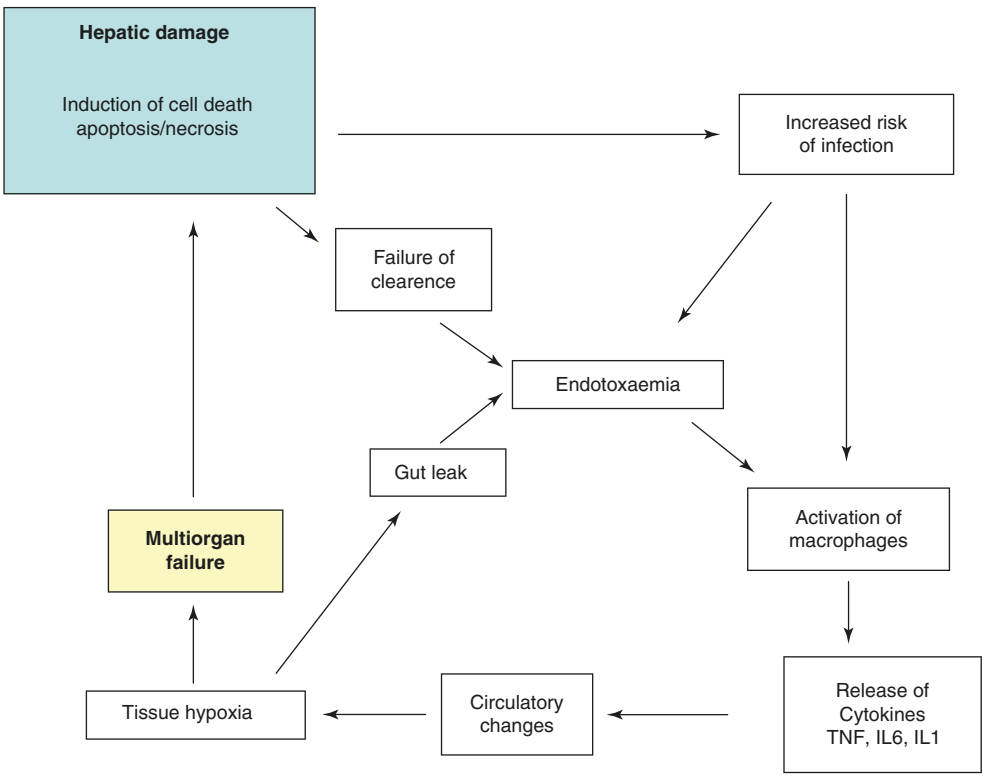

Fig. 29.4 Mechanisms that contribute to multiorgan failure during acute liver failure

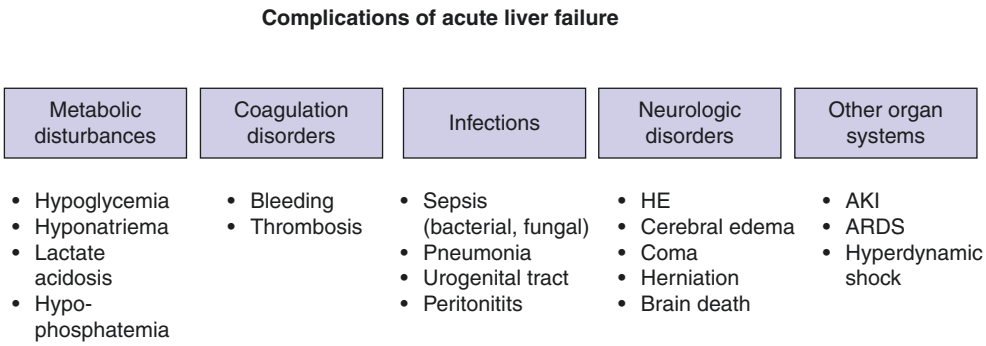

Fig. 29.5 Clinically relevant complications of acute liver failure

Table 29.3 Stages of acute hepatic encephalopathy

Stage	Mental state	EEG	GCS
I, prodrome	Mild confusion, slurred speech, slowness of mentation, disordered sleep rhythm, euphoria/depression	Usually normal	15
II, apathy, lethargy	Accentuation of stage I, drowsy but speaking, inappropriate behavior, incontinence	Generalized slowing	11–15
III, stupor	Sleeps most of the time but rousable, incoherent or no speech, marked confusion	Abnormal	8–11
IV, coma	Patient may (stage IVa) or may not (stage IVa) respond to painful stimuli	Abnormal	<8

Modified from Ref. [66]
EEG electroencephalogram, *GCS* Glasgow Coma Scale

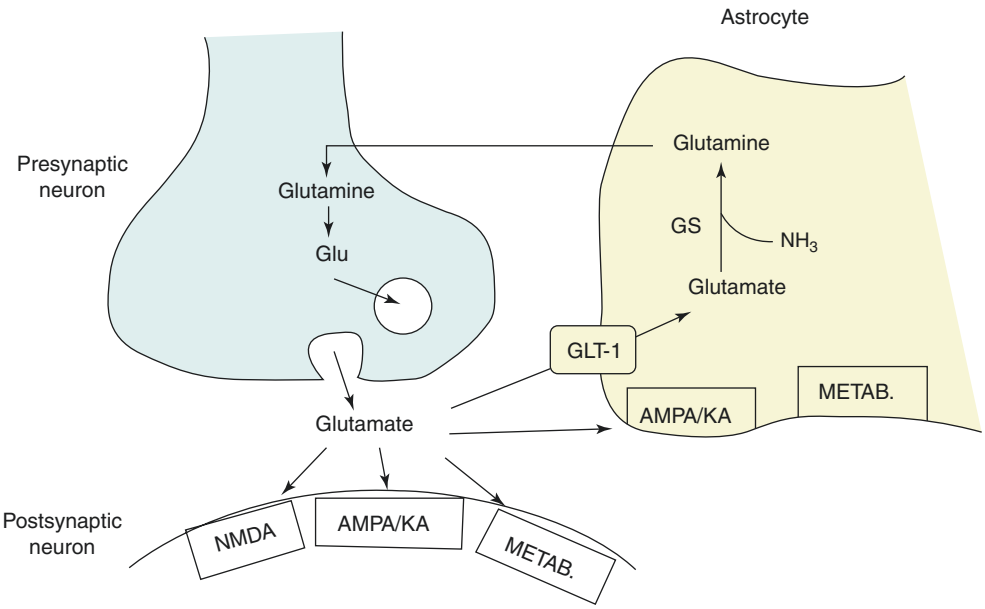

Fig. 29.6 The role of glutamate/glutamine in the brain. The localizations of the glutamate transporter (GLT-1) and glutamate receptor subtypes (NMDA, AMPA/KA, METAB) on astrocytes and neurons involved in glutamatergic neurotransmission are shown. Glu glutamate. (Modified from Ref. [70])

Encephalopathy and Cerebral Edema

Hepatic encephalopathy (HE) is essential for the diagnosis of ALF and is subdivided into four different grades: I–IV (Table 29.3).

In 75–80% of the patients in stage IV, cerebral edema develops independent of the cause of ALF. The treatment measures in hepatic encephalopathy comprise quiet environment, upper body elevation (30°), and, if necessary, intubation, analgesic sedation, and mechanical ventilation (at HE >3°) [67].

The precise pathophysiological mechanisms leading to hepatic encephalopathy are not completely understood [68]. However, laboratory studies indicate that the cause is an ammonia-induced deficit in neurotransmitter synthesis rather than a primary deficit in cerebral energy metabolism [69]. Most likely, the astrocytes and the pre- and postsynaptic neurons contribute to the clinical picture of hepatic encephalopathy (Fig. 29.6).

Astrocytic swelling during ALF determines the degree of cerebral edema and thus the degree of cerebral dysfunction [71].

In the literature, several factors are discussed that contribute to hepatic encephalopathy, but ammonia (with a consequent dysregulation of the glutamate neurotransmitter system) seems especially relevant for the development of hepatic encephalopathy and cerebral edema. Ammonia is primarily metabolized from glutamine in the small bowel and is converted to urea in healthy liver, but in ALF, concentrations increase, and ammonia is alternatively metabolized back to glutamine.

Arterial ammonia levels at presentation have been demonstrated to be predictive of outcome in patients with ALF. Patients with encephalopathy grade III and IV exhibited significantly higher serum ammonia levels than those with lower-grade encephalopathy. Possibly, patients with advanced cerebral dysfunction can be determined by a serum ammonia cutoff value of 124 µmol/l or more. Ammonia levels can be used for risk stratification [72]. Ammonia exerts effects on cerebral function by direct and indirect mechanisms (Table 29.4).

There is clear evidence that arterial ammonia concentrations directly correlate with cerebral edema and thus herniation [73]. Experimental evidence also demonstrates that physiological ammonia concentrations alone result in astrocyte swelling. Additionally, higher glutamine concentrations are a consequence during this process, and they accelerate cerebral edema [70, 74].

Higher ammonia concentrations have a direct effect on the glutamate neurotransmitter system. Glutamate is the major excitatory neurotransmitter in the mammalian brain (see Fig. 29.6). After the release at the presynaptic neuron, glutamate binds to glutamate receptors on the postsynaptic neuron (NMDA) or on both the postsynaptic neuron and astrocytes (AMPA/KA). Additionally, glutamate transporter on astrocytes (GLT-1 and GLAST) and neurons (EAAC1) limit the expression of glutamate in the neuronal cleft. After the uptake of glutamate in astrocytes *via* GLT-1, it is transformed into glutamine. Ammonia downregulates GLT-1 expression on astrocytes, and this results, in higher and prolonged extracellular glutamate concentrations in patients with ALF. Additionally, there is evidence that the glutamate receptors are differentially expressed during ALF, and thus, dysregulation of the glutamate system is one of the important determinants of hepatic encephalopathy during ALF [70, 74].

Table 29.4 Effects of ammonia on brain function

Electrophysiological effects of the ammonium ion
Effects on the inhibitory postsynaptic potential
Effects on glutamatergic neurotransmission
Effects on brain energy metabolism
Inhibition of α-ketoglutarate dehydrogenase
Effects on astrocyte function
Decreased expression of the glutamate transporter GLT-1
Increased expression of "peripheral-type" benzodiazepine receptors
Alzheimer type II astrocytosis
Effects on the glutamate neurotransmitter system
Direct postsynaptic effects
Impaired neuron-astrocytic trafficking of glutamate
Inhibition of glutamate uptake
Altered glutamate receptors
Effects mediated by the formation of glutamine in brain
Cytotoxic brain edema
Increased uptake of aromatic amino acids
Other effects
Stimulation of L-arginine uptake and neuronal nitric oxide synthase (nNOS)expression

Data from Ref. [70]

Other neurotransmitters that participate in hepatic encephalopathy are GABA, serotonin, and the opioid system. Systemic circulation of pro-inflammatory mediators during ALF might also contribute to hepatic encephalopathy, as they might modulate cerebral permeability to neurotoxins, initiate inflammatory responses, and impair cerebral blood flow [75]. Hyponatremia should be avoided and corrected, as it leads to water entry into astrocytes and further promotes astrocyte swelling. Targeting serum sodium levels at 145–155 mmol/l results in lower intracranial pressure, as compared with normal sodium values of 137–142 mmol/l [76].

A few uncontrolled studies [77–79] show a protective effect of mild hypothermia in ALF and cerebral edema. Hypothermia (32–35 °C) can be safely and easily applied. The risk of complications (arrhythmias, myocardial ischemia, infections, and coagulopathy) increases with the degree and duration of hypothermia, mainly with body temperatures below 32 °C. Hypothermia reduces intracranial pressure and reestablishes disturbed autoregulation of cerebral blood flow. Some studies suggest that hypothermia can reduce the extent of liver injury in ALF [80]; in contrast, hypothermia might also lead to impaired liver regeneration.

In a prospective multicenter study, 46 patients with ALF and high-grade hepatic encephalopathy were evaluated for a protective effect of hypothermia. There was no difference in the intracranial pressure or survival between patients who were cooled to 33–34 °C compared with those cooled to 36 °C body temperature [81]. The established measures for the treatment of high-grade hepatic encephalopathy in ALF are the application of mannitol 20% (150 ml) or hypertonic saline 2.7% (200 ml) or 30% (20 ml) I.V. over 20 min and short-term hyperventilation [67, 82]. Insertion of monitors for intracranial pressure does not improve outcome but might be significant in the identification of patients who should not undergo transplantation due to prolonged intracranial hypertension or low cerebral perfusion pressure [67]. Therefore intracranial probes for measuring intracranial pressure should not be routinely used and in fact the application rate in the US and Europe is very low.

Cardiovascular Dysfunction

Patients with ALF are characterized by hypotension and tachycardia. The basis for this observation is vasodilatation in the periphery that results in relative hypovolemia, hypotension, and high output failure. Factors that contribute to this dysregulation are capillary leakage, low osmotic pressure, and systemic inflammatory response syndrome (SIRS). Persistent hypotension (mean arterial pressure, MAP <60 mmHg) should prompt volume substitution (normal saline or balanced electrolyte solutions) and vasopressor therapy, primarily noradrenalin [83]. In refractory shock, vasopressin should be administered where necessary in combination with hydrocortisone 300 mg I.V. daily as adrenal insufficiency may occur in a substantial number of patients with ALF [84].

Some patients with ALF may suffer from hypertension. This problem may arise, especially in patients with hepatic encephalopathy grade IV, and typically occurs when cerebral edema is evolving.

Infection

Infection and thus sepsis are major problems in patients with ALF. Patients with a long stay in the ICU have a very high risk in particular, and this may actually be the ultimate reason for death [85]. Studies from the King's College Hospital group clearly indicated that monitoring by daily cultures (sputum, urine, and blood) identifies bacteria in up to 90% and fungal infections in around 30% of the patients [86, 87]. Frequently, the classical signs (fever, leukocytosis, and biochemical parameters, such as c-reactive protein and procalcitonin) in patients with ALF are not directly correlated with infection or are absent. The sites of the body with the most common infections are the lung, the urinary tract, and the blood (Fig. 29.7).

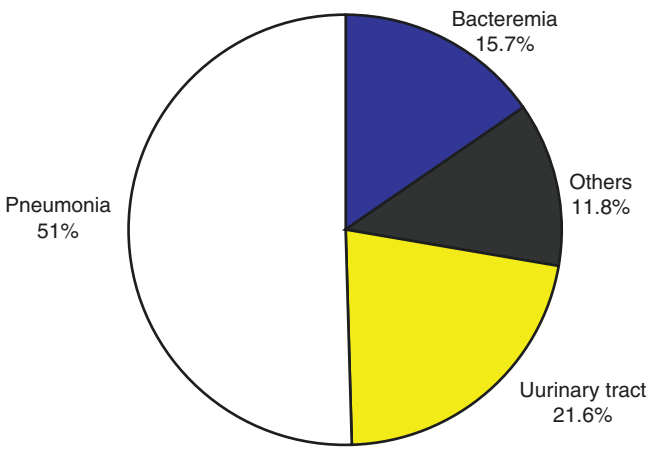

Fig. 29.7 Sites of infections during acute liver failure [86]

If antibiotic or antifungal treatment is necessary in these patients, the potential of further liver injury caused by antibiotic drugs should be considered. The basic principles are regular microbiological sampling and early antibiotic or antimycotic medication, but no prophylactic antibiosis.

Besides the increased risk of patients being managed in ICU, additional factors contribute to the higher risk of infections in patients with ALF, namely, defects in the immunological defense mechanisms (complement, Kupffer cell function, polymorphonuclear cell function, and cell-mediated immune response). The liver is the main source of complement (e.g., C3 and C5) production. As a consequence of lower complement levels, the activity of polymorphonuclear leukocytes and complement-mediated opsonization is reduced.

Therefore, phagocytosis and killing of polymorphonuclear cells are inhibited in patients with ALF. Through the portal circulation, bacterial toxins are regularly brought to the liver tissue that are cleared by the resident Kupffer cells of the liver. In ALF, there is a correlation between hepatic damage and Kupffer cell dysfunction. Additionally, Kupffer cells are a major source of cytokines, and their dysregulation also contributes to impaired immune response. Defective lymphocyte function has been attributed to impaired interleukin-2 (IL-2) production in these patients. Thus, the defect in immune response can be explained on different levels of the immune system [3, 86].

Pulmonary Complications

Pulmonary complications are frequent [88]. Different mechanisms contribute to this observation. Up to 50% of the patients have infections, especially following intubation and subsequent mechanical ventilation [89]. The possible consequent capillary leakage can result in an ARDS-like syndrome that is further augmented by the infusion of albumin, fresh frozen plasma, and coagulation factors.

Besides these local mechanisms, systemic causes, as a result of liver failure, also lead to intrapulmonary vasodilatation and pulmonary arteriovenous shunting, which further increase the risk of hypoxic complications and deteriorate peripheral tissue oxygenation [90].

Renal Failure

Renal failure with oligo- and anuria is observed in up to 70% of patients with ALF and requires renal replacement therapy in 30% of cases [91]. In acetaminophen and *amanita* poisoning, as well as halothane toxicity, direct toxic effects additionally contribute to kidney failure.

The association between liver failure and kidney failure is functional and known as hepatorenal syndrome.

Pathophysiologically, the syndrome is characterized by a contraction of the vessels with a distinctively reduced renal perfusion. At this stage, renal dysfunction is potentially reversible. In the further course of ALF, which is typically characterized by progressive shock, tubular necrosis can occur, which potentially results in terminal renal failure.

Additional severe complications in patients with hepatorenal syndrome, such as long periods of hypotension or sepsis, have a fatal effect on kidney function and significantly reduce the prognosis of patients with fulminant hepatic failure [92].

As systemic inflammatory response syndrome (SIRS) has been identified as an independent predictor of renal dysfunction in patients with non-acetaminophen-induced ALF, SIRS has been suggested to be functionally linked to the development of renal dysfunction in patients with non-acetaminophen-induced ALF, but not in patients with acetaminophen-induced ALF [93]. Renal replacement therapy, mostly in the form of continuous veno-venous hemofiltration (CVVH), should be initiated early in patients with oliguria, volume overload, or clinically significant hyperammonemia (NH_3 >150 µmol/l).

Aside from renal replacement therapy, plasmapheresis, which is another extracorporeal procedure, is also a promising measure in ALF. A prospective multicenter study has investigated the effects of high-volume plasma exchange in ALF. About 182 patients received either standard treatment or treatment with complete plasma exchange with FFP for 3 days. A beneficial effect for plasmapheresis-treated patients who did not receive or could not receive liver transplantation was demonstrated, whereas patients receiving (or being listed for) liver transplantation did not significantly benefit from plasmapheresis [94].

Artificial liver assist devices, such as the molecular absorbent and recirculating system (MARS®) and the Prometheus® system, can improve HE but have no mortality benefit in ALF [95]. Their use should currently be limited to clinical trials.

Metabolic Complications

The liver is essential for several metabolic functions. Two particular problems are frequently encountered in patients with ALF: hypoglycemia and acid-base disturbances.

Different mechanisms lead to hypoglycemia during ALF. The damaged liver loses its capacity to mobilize glycogen stores and to perform gluconeogenesis. Additionally, the liver is the major site of insulin metabolism, and the disintegration of insulin, which is consequently reduced, results in elevated insulin serum levels. All three mechanisms contribute to hypoglycemia, and this may also aggravate mental status. In terms of treatment, it might be important to differentiate hypoglycemia from hepatic encephalopathy as possible causes for disturbed mental status at certain stages. In ALF, glucose serum levels should be targeted at 140 mg/dl by glucose infusions [67].

Both acidosis and alkalosis might be present. Metabolic alkalosis is most frequent, as urea synthesis in the liver is impaired, which results in the accumulation of the two precursor substrates, bicarbonate and ammonium. Alkalosis is associated with hypokalemia, which is further aggravated by high sodium reabsorption in patients with ALF.

Acidosis, with a high anion gap, occurs in up to 30% of patients with acetaminopen poisoning and evolving acetaminophen-dependent ALF. In patients with a different etiology, acidosis is evident in only 5%. In those cases, acidosis is caused on the one hand by accumulation of lactate due to impaired microcirculation and resulting tissue hypoxia, and on the other hand by the inability of the injured liver to metabolize lactate. Early renal replacement therapy should be initiated in both, the event of severe metabolic acidosis and refractory hyperlactatemia.

Coagulation Disorders

Bleeding complications in patients with ALF are uncommon, occur in approximately 10% of cases, and are usually clinically not significant [96]. Patients with ALF exhibit increased INR and various degrees of thrombocytopenia, depending on the extent of inflammation (e.g., SIRS/sepsis).

In ALF, these deficits in hemostasis are counterbalanced by compensatory mechanisms, such as hypersecretion of clotting factor VIII and von Willebrand factor by endothelium. Conversely, factor VIII might compensate the deficit of liver-derived coagulation factors and von Willebrand factor thrombocytopenia [97]. Interestingly, the development of thrombocytopenia in ALF is associated with the development of multiorgan failure and poor outcome [98]. Furthermore, procoagulant microparticles, as a result of systemic inflammation, might compensate for deficiencies in hemostasis.

In fact, the use of blood products (packed red blood cells, platelet concentrates, fresh frozen plasma [FFP], 4-factor prothrombin complex concentrate [PCC], and single coagulation factors) has been decreased during the past decades in the USA, whereas bleeding complications remained stable in approximately 10% of cases with ALF.

Without evidence of relevant bleeding, blood products should not be administered routinely. For signs of bleeding or thromboembolism, differentiated coagulation diagnostics (e.g., thromboelastography) and on-demand substitution is indicated [67].

Pathophysiological Aspects of ALF

ALF occurs when the extent of hepatocyte death exceeds the regenerative capacity of the liver. Mainly two different mechanisms of liver cell death can be differentiated: (1) direct cellular damage and activation of cell signaling cascade pathways, resulting in the disturbance of intracellular homeostasis, and (2) innate and adaptive immune responses leading to immune-mediated liver injury.

Similar to sepsis, patients with ALF commonly exhibit immune paralysis with characteristic features of systemic inflammation and cellular immune depression contributing to severe extrahepatic complications, such as multiple organ failure [85, 99]. In this context, cytokines exert crucial pathophysiological functions in ALF, comprising hepatocellular death, extrahepatic complications, and hepatocellular regeneration.

Dysregulation of the Cytokine Network in ALF

In the last years, it has become obvious that there is a dysregulation of cytokine expression during ALF in humans. For example, it has been shown that mediators of the acute phase response – IL-6 and tumor necrosis factor (TNF) – are strongly elevated in the liver and serum of ALF patients. The meaning of this observation becomes more evident through the development of animal models whereby the role of each of the molecule can be more clearly defined. As there is evidence that several cytokines might be involved in the pathogenesis of ALF, all the different aspects cannot be covered in this review. Here, we focus on two cytokines, TNF and IL-6.

IL-6/gp130-Dependent Signals

IL-6 interacts on the cell surface with the IL-6 receptor (gp80). This complex associates with two gp130 molecules, resulting in the activation of Janus kinases and in turn in the phosphorylation of tyrosines at the intracellular part of gp130. After phosphorylation of tyrosines, the ras/map kinase pathways and transcription factors Stat1 and Stat3 become activated [100]. In hepatocytes, IL-6 is one of the main inducers of the acute phase response, and in recent years, it has become evident that IL-6 also contributes to the regulation of additional pathophysiological conditions in the liver [101, 102].

One of the simplest models to study the loss of liver tissue is the removal of two-thirds of the liver by surgical resection. This model has been applied mainly in rodents (e.g., rat and mouse), and after 1–2 weeks, the liver tissue has been restored by hepatocyte proliferation. In recent years, it has become obvious that IL-6 and TNF are involved in the restoration of liver mass [103], as it

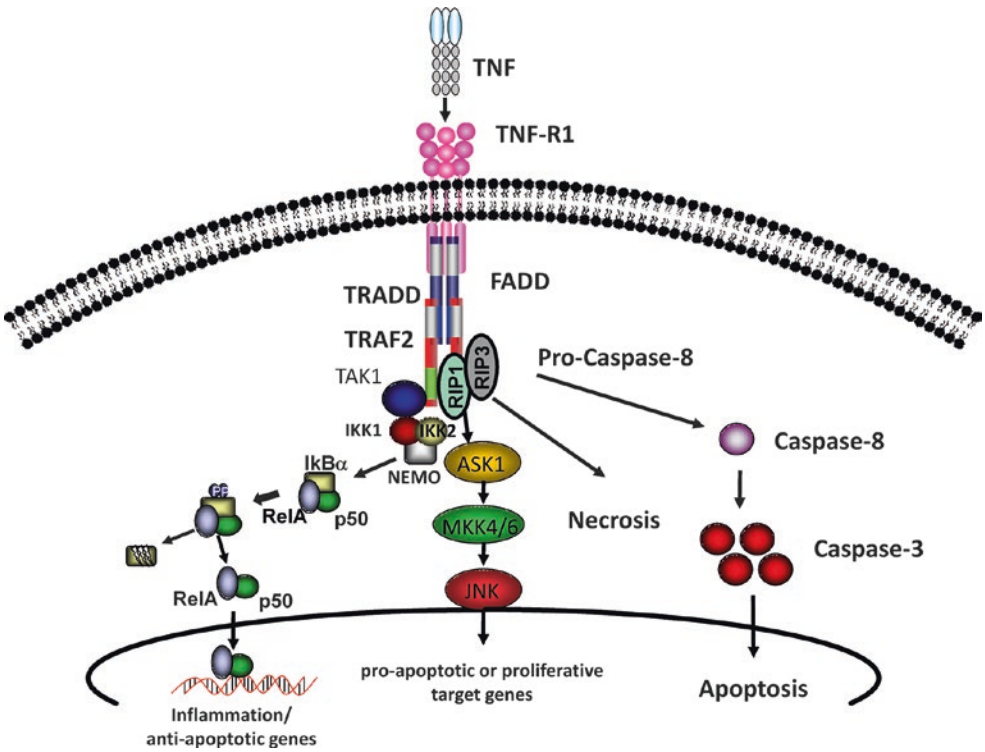

Fig. 29.8 TNF-dependent signaling pathways. The molecules and pathways that are involved in TNF/TNF-R1-dependent signaling are depicted. After TNF/TNF-R1 interaction, different adaptor proteins bind to the intracellular part of TNF-R1. As a consequence, at least four pathways (NF-kB, jun Kinase [JNK], apoptosis, and necrosis) can be activated. Recently, it has been demonstrated that downstream from FADD – dependent on the cellular context – programmed apoptosis or necrosis can be initiated

has been observed that liver regeneration was impaired in IL-6 and TNF receptor 1 (TNF-R1) knockout mice after two-thirds hepatectomy. The defect in regeneration in both knockout strains could be restored through IL-6 stimulation [104, 105]. The model of how IL-6 and TNF may work in concert during liver regeneration following partial hepatectomy is presented in Fig. 29.8.

In humans suffering from ALF, the IL-6 serum levels are high, and in the liver infiltrating cells express tremendous (10-fold higher compared with controls) amounts of IL-6 [101, 102, 106]. In animal models of ALF, the IL-6 serum levels are also greatly increased [107], and treatment with a hyper-IL-6 designer molecule reduces liver cell damage in several animal models [108, 109]. IL-6 plays a protective role for hepatocytes, not only during liver regeneration but also during ALF; cDNA arrays further demonstrate that IL-6 activates antiapoptotic pathways, e.g., Bcl-xl, in hepatocytes [110, 111].

Most IL-6 data in animal models show that gp130-dependent pathways in hepatocytes activate protective mechanisms [101, 102], and in humans, it is also likely that IL-6 renders hepatocytes more resistant. Therefore, it might be promising to modulate IL-6/gp130-dependent pathways in humans during ALF as a potential therapeutic approach.

TNF-Dependent Pathways

TNF belongs to a family of several known Fas (CD95) and TNF receptor apoptosis-inducing ligands (TRAIL). There is also evidence of an involvement in the pathogenesis of fulminant hepatic failure. At present, the role of TNF has been studied in more detail in both human and animal models.

TNF binds to two receptors, TNF-R1 and TNF-R2, on the cell surface. After ligand binding, the intracellular domains of the receptors interact with adapter molecules that activate different pathways (see Fig. 29.8). In the case of TNF-R1, first the molecule TNF-R-associated death domain (TRADD) and then additional molecules bind to activate the caspase cascade either *via* Fas-associated death domain (FADD) or *vi*a TNF-associated factor/receptor-interacting protein (TRAF/RIP) jun kinase (JNK) and nuclear factor-kB (NF-kB) (see Fig. 29.8) [112].

It has become evident that besides inducing apoptosis, TNF can also trigger necrosis. Therefore, TNF and its family members seem to be essential mediators of cell death during ALF. In humans, it has been shown that TNF serum levels correlate with the prognosis in ALF patients [106]. In animal models, blocking experiments using anti-TNF attenuates liver failure, and therefore, it is obvious that TNF plays a central role in the pathogenesis of ALF. However, further studies indicated that TNF has no uniform role in the different models. Depending on the model, the TNF-dependent effect might be related to a different cell in the liver or another intracellular pathway. Three models of ALF and the role of TNF will be discussed.

Endotoxin/Galactosamine Model

During LPS/galactosamine (GaIN)-induced liver injury, TNF induces the transcription of several pro-inflammatory genes, e.g., chemokines, nitric oxide, and adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin [113–115]. These changes in the liver are essential for triggering the extravasation of neutrophils into the liver parenchyma, which results in cytotoxic liver cell damage. During this scenario, a stepwise cascade has been described, which consists of three events: (1) sequestration of neutrophils in the liver vasculature, (2) transendothelial migration, and (3) adherence-dependent cytotoxicity against hepatocytes [116].

Therefore, in the LPS/GalN, model TNF obviously triggers an inflammatory mechanism mediated *via* NF-kB that results in liver cell damage. In this model, not only parenchymal but also non-parenchymal cells are involved in this process.

Galactosamine/TNF Model

The administration of GaIN and TNF triggers apoptosis of hepatocytes *in vivo and in vitro*. The essential role of TNF-R1 in this model has been demonstrated by TNF-R1 knockout mice that are resistant against GalN/TNF treatment [117]. GaIN directly inhibits transcription and thus synthesis of antiapoptotic signals. Therefore, in this model, the FADD-dependent pathway leading to apoptosis is the essential step in ultimately inducing liver cell damage. In contrast, the NF-kB and JNK pathways do not seem to be involved in the pathogenesis of liver damage, and also, nonparenchymal cells play no role. In this model, simple administration of an adenoviral construct expressing a dominant molecule blocking the FADD pathway is protective [106]. These data indicate that the caspase cascade activated by TNF might be a relevant target during ALF.

Concanavalin A Model

Concanavalin A (ConA) is a leptin with high affinity towards the hepatic sinus [118]. Accumulation of ConA in the hepatic sinus results in the activation of liver natural killer T (NKT) cells, i.e., NK 1.1 $CD4^+$ $CD8^-$ T cell receptor (TCR)$\alpha\beta^+$, and NK1.1. $CD4^-$ $CD8^-$ TCR $\alpha\beta^+$, which are essential for triggering the early phase of ConA-induced liver injury [119, 120]. Consecutively, CD4-positive and polymorphonuclear cells are attracted to the hepatic sinus and trigger an increase in cytokines, such as TNF, IL-2, IFN-γ, IL-6, granulocyte macrophage colony-stimulation factor (GM-CSF), and IL-1 [58]. TNF-α and IFN-γ have direct implications for the induction of liver cell injury, whereas anti-TNF-α and anti-IFN-γ antibodies have protective effects in ConA-induced liver injury [121, 122] and IFN−/− and TNF−/− mice are resistant to ConA-induced liver cell damage.

Until now, a stepwise process of liver damage, as shown for the endotoxin/LPS model, could not be defined for the ConA model. Adhesion molecules, such as ICAM-1 or VCAM-1, seem to play a minor role. Mice pretreated with antibodies against both adhesion molecules or ICAM-1 knockout mice still undergo liver cell injury [123].

Recently, it has been shown that hepatocyte-specific caspase-8 knockout mice are more susceptible to ConA-induced liver injury [124]. These results show that during ConA-induced liver injury, necrosis is the more prevalent form of cell death. Therefore, the ConA model is especially helpful to better define this form of hepatocyte injury *in vivo*.

Apoptosis and Necrosis in ALF

Apoptosis – the programmed form of cell death – is inevitable to maintain the balance of cell proliferation and elimination of injured cells. Caspase proteases are involved in the initiation, execution, and regulation of apoptotic pathways. Effector caspases (e.g., caspase-2, -6, -7) cleave various cellular proteins (e.g., cytokeratin-18) [125], and initiator caspases (e.g., caspase-8, -9, -10) exhibit regulatory functions by activating the downstream effector caspases [126]. The major signaling routes for caspase activation are the extrinsic death receptor and the intrinsic mitochondrial pathway [127] (see Fig. 29.6).

Death receptors are transmembrane proteins that consist of the following domains: (1) extracellular ligand-interacting domain, (2) transmembrane domain, and (3) intracellular death domain. Typically involved in ALF are death receptors CD95 (Fas), tumor necrosis factor receptor 1 (TNF-R1), and tumor necrosis factor-related apoptosis inducing ligand receptors 1 and 2 (TRAIL-R) and death receptors 3 and 6. Binding of death ligands such as TRAIL, CD95L, and tumor necrosis factor to their specific receptors leads to the recruitment of the adapter protein FADD and caspase-8 into death-inducing signaling complex (DISC), wherein caspase-8 is activated [128]. In most cells and hepatocytes, respectively, only low amounts of caspase-8 are activated in the DISC, which is not effectual for cell death. In order to exert cell death, the extrinsic receptor pathway has to be amplified by the intrinsic mitochondrial apoptotic pathway through the caspase-8-effected cleavage of Bid (a pro-apoptotic Bcl-2 family protein). Subsequently, together with the Bcl-2 family members Bak und Bax, the release of pro-apoptotic mediators from the mitochondrion is initiated [129].

ALF, induced by agonistic CD95 antibody, could be abolished by silencing of CD95- or caspase-8-protected mice [130, 131]. Conversely, CD95 and caspase-8 are involved in liver regeneration by inducing the differentiation of stellate cells and other non-parenchymal liver cells [132, 133]. TNF-α plays a key role in liver regeneration by activating NF-kB, which exerts antiapoptotic functions in the liver [134].

Necrosis is mediated by opening of the mitochondrial membrane permeability transition (MPT) pore, leading to the disruption of ATP formation and finally resulting in mitochondrial swelling and rupture of the outer mitochondrial membrane. Interestingly, it has been recently shown that TNF can also induce controlled necrosis. Therefore, necrosis is now also considered a programmed form of cell death, which is initiated by RIP1/RIP3 activation. Additionally, massive ATP depletion, formation of reactive oxygen species (ROS), activation of non-apoptotic proteases, and strongly increased intracellular calcium concentrations – aggravating ATP deficiency by loss of mitochondrial membrane potential – contribute to necrosis. As loss of ATP leads to necrosis and apoptosis is ATP-dependent, the intracellular amount of ATP itself might determine the way of cell death, either by apoptosis or by necrosis [135, 136]. Necrosis is associated with inflammation, as necrotic cell rupture induces an inflammatory response owing to the release of intracellular components, including the M65 form of cytokeratin-18 (CK-18), whereas apoptotic cells are rapidly cleared by phagocytic cells, thereby preventing the release of intracellular contents.

Cytokeratin-18 as a Prognostic Biomarker in ALF

CK-18 is a filament protein cleaved by caspases into specific fragments, which can be measured in the serum using the M30 ELISA (Fig. 29.9).

CK-18 levels at the time of admission have been demonstrated to be predictive of mortality in patients with ALF, with a prognostic impact comparable with the model for end-stage liver disease (MELD). Additionally, a modified MELD score where uncleaved necrotic CK-18 (M65 ELISA) substi-

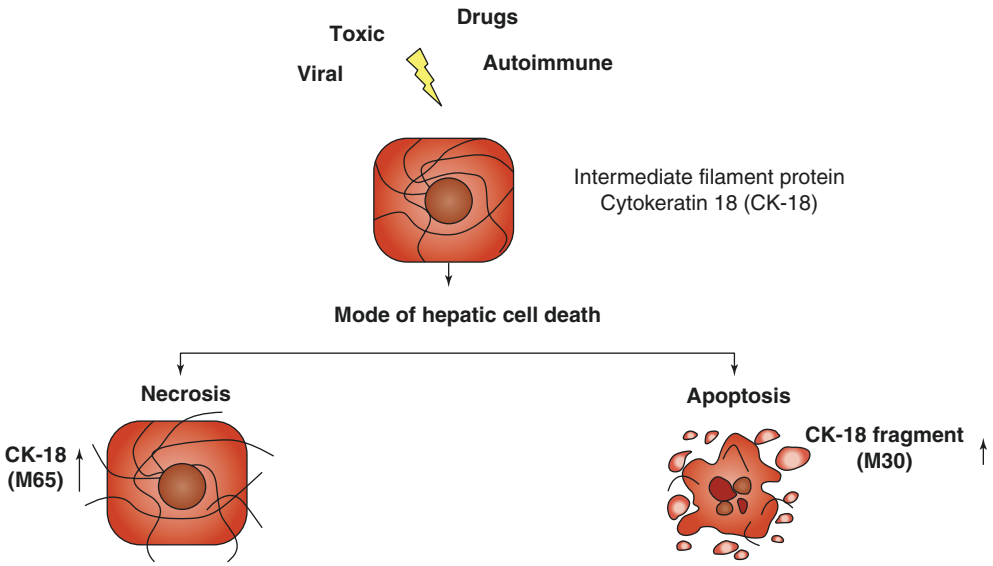

Fig. 29.9 Cytokeratin-18 is associated with the mode of hepatic cell death. In apoptotic cell death (induced by toxin, drugs, viruses, or autoimmune etiology), cytokeratin (CK)-18 is cleaved by caspases into specific fragments, which can be measured in serum by the M30 ELISA. Whereas cleaved CK-18 levels represent apoptosis, uncleaved CK-18 (M65 ELISA) reflects necrosis

tuted for bilirubin predicted significantly better prognosis of ALF patients compared with the current MELD score [137].

The observation that ALF patients who die or require transplantation exhibited increased serum levels of total CK-18, but the reduced levels of caspase cleaved fragments indicate that necrosis, not apoptosis, is the more prominent cell death mode in these most critically ill ALF patients [138]. In line with this, patients with acetaminophen-induced liver injury, where necrosis is the predominant cell death mode, exhibited higher levels of total CK-18 than caspase cleaved CK-18.

Intestinal Microbiome and Acute Liver Failure

The link between intestinal dysbiosis and chronic liver disease is well described by numerous studies [139]. Moreover, the intestinal-microbiota-liver axis has been proposed as a promising target to prevent the progression of chronic liver disease [140]. Nevertheless, little is known about a potential functional link of gut-liver interaction during ALF. Recently, interesting data of an animal model with wild-type (WT) and dysbiotic Nlrp6−/− mice and liver injury induced by acetaminophen (APAP) or lipopolysaccharide (LPS) have been presented [141]. Liver injury was studied based on liver functions tests, histology, flow cytometry immunophenotyping (FACS), and 16S rRNA-based microbiota profiling.

Interestingly, dysbiotic Nlrp6−/− mice exhibited significantly increased liver injury, as assessed by the extent of hepatic inflammation and necrosis compared with WT controls. Enhanced liver damage in Nlrp6−/− mice was associated with markedly increased infiltration of Ly6Chi monocyte-derived macrophages (MoMFs). As a potentially protective response to hepatic injury, WT mice exhibited a shift in microbiota composition and an expansion of colonic mucus layers, whereas this effect was absent in Nlrp6−/− mice. Fecal microbiota transfer (FMT) from Nlrp6−/− mice into WT mice aggravated liver injury upon APAP treatment in WT mice with a Ly6Chi inflammatory phenotype. These data reveal novel, so far unknown functions of intestinal microbiota during ALF and identify intestinal dysbiosis as a driver of ALF severity (Fig. 29.10). Future clinical studies should investigate the intestinal microbiome as a promising therapeutic target in ALF.

Translation of Experimental Data Into Therapeutic Approaches in Humans

The current data in animal models and humans indicate that TNF plays a significant role in the pathogenesis of ALF. However, as demonstrated for the three animal models discussed, depending on the pathogenesis, the intracellular pathways activated by TNF could have opposing effects.

The mode of liver cell death in ALF is still controversial. Induction of apoptosis or necrosis of hepatic cells potentially depends on the etiology and the duration and extent of liver injury. Severe liver damage causes oxidative stress and concomitant depletion of ATP, resulting in necrosis. Conversely, sufficient cellular ATP stores are essential for the execution of apoptosis. Necrosis as a consequence of severe hepatic injury is associated with an unfavorable prognosis.

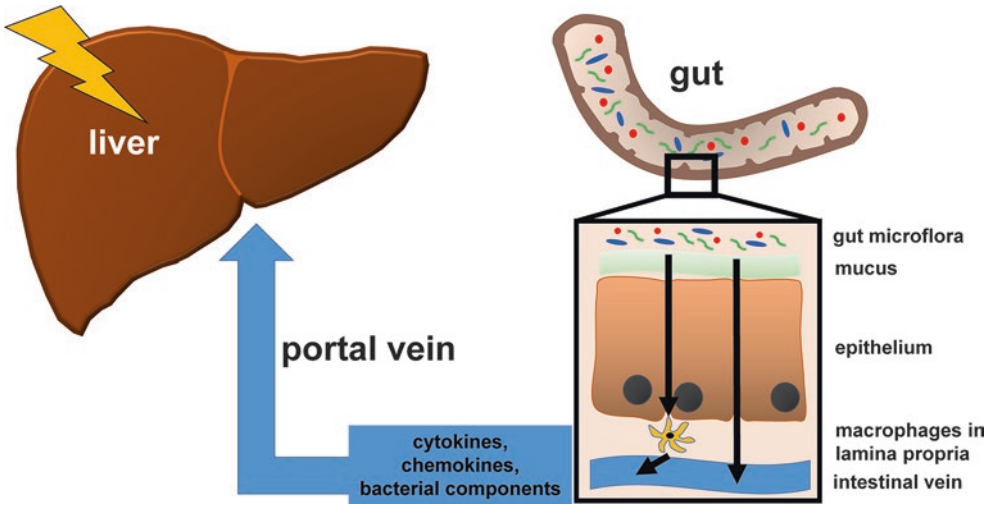

Fig. 29.10 Intestinal dysbiosis can trigger inflammatory reactions in acute liver injury. In the situation of intestinal dysbiosis, bacteria (or their components) of the intestinal flora can overcome the intestinal barrier of the mucous layer and epithelium and translocate into the portal blood stream. Additionally, these can be detected by macrophages, which then release cytokines and chemokines that can also enter the portal circulation. Both mechanisms can trigger inflammatory reactions in acute liver damage

Potentially, the differentiation of necrosis and apoptosis might enable the early identification of patients requiring transplantation. The identification of the molecular cell death mechanisms might offer new therapeutic perspectives for ALF. Reduction of cellular death without inhibition of the hepatic regenerative capacity seems to be the main goal for new therapeutic interventions. Whereas extreme liver injury results in necrosis, milder injury leads to apoptosis. Potentially, inhibition of apoptosis by caspase inhibitors can prevent liver cell death but can also possibly change only the cell death mode from apoptosis to necrosis. Considering the therapeutic use of caspase inhibitors to prevent apoptosis, the involvement of caspases in liver regeneration must not be ignored, as this might lead to potential severe adverse effects. Therefore, further studies are required to better understand the molecular mechanisms that determine the mode of cell death during ALF.

In mouse models, the administration of cyclooxygenase (COX) inhibitors resulted in decreased oxidative stress and reduced hepatic necrosis [142]. Therefore, COX inhibitors could be further investigated as potential agents to prevent ALF.

Another promising novel target in acetaminophen-induced ALF is cyclophilin A. Cyclophilin A is an intracellular protein that is pro-inflammatory when released by cells. In an animal model of acetaminophen-induced liver injury, it has been demonstrated that cyclophilin A acts as a DAMP to mediate acetaminophen toxicity and that experimental inhibition of cyclophilin A ameliorates acetaminophen-induced liver injury [143].

Recent data hint at so far unknown functions of intestinal microbiota during ALF. Intestinal dysbiosis of Nlrp6−/− mice was transferrable to healthy wild-type controls by fecal microbiota transfer which led to pro-inflammatory Ly6Chi macrophage polarization and finally resulted in the aggravation of hepatic injury.

Concluding Remarks and Open Questions

ALF is characterized by the sudden onset of liver failure in patients without evidence of chronic liver disease, by which ALF is differentiated from end-stage chronic liver disease. According to the time between the first symptoms and encephalopathy, ALF is divided into three subgroups: hyperacute, acute, and subacute. The prognosis of ALF patients is determined by the metabolic situation resulting from the loss of liver cell mass, the release of mediators and toxic metabolites from injured liver tissue, and the capacity of the remaining vital hepatocytes to restore functional liver mass.

Suicidal acetaminophen ingestion is the most frequent cause of drug-induced liver failure worldwide, with approximately 500 deaths a year in the USA. Other important mechanisms are viral hepatitis and cardiovascular and metabolic disorders.

ALF leads to multiorgan failure, especially to cerebral edema and encephalopathy. Owing to the diminished liver function, higher rates of infections and coagulation disorders are observed. Cerebral edema, infections, and renal failure are important clinical complications limiting survival. For risk stratification in patients with ALF and subsequent hepatic encephalopathy, serum ammonia levels can be used. Advanced cerebral dysfunction is expected at serum ammonia levels of 124 μmol/l or higher.

Cardiovascular dysfunction is characterized by peripheral vasodilatation that results in relative hypovolemia, hypotension, and high-output failure. Capillary leakage and high-volume therapy can lead to an ARDS-like syndrome and cause hypoxic complications. Prothrombin time is a useful parameter for assessing the extent of the remaining liver function.

Intensive care therapy is crucial for patients with ALF for managing multiorgan failure, and mild hypothermia to reduce cerebral edema should be considered. Further research and controlled clinical studies are required to evaluate the importance of hypothermia.

The mode of liver cell death which is predominantly induced in ALF (apoptosis or necrosis) is potentially determined by the underlying etiology, the duration of the disease, and the extent of liver injury. Severe liver injury leads to oxidative stress and depletion of ATP stores favoring necrosis, whereas sufficient cellular ATP resources are required for the execution of apoptosis. As necrosis is associated with an inferior outcome as compared with apoptotic cell death, the discrimination of the cell death mode in ALF might be a prognostic tool to instantly identify patients requiring transplantation. Moreover, the molecular cell death mechanisms in ALF are promising targets for future research aiming at reducing hepatocellular death without inhibiting liver regeneration.

The potential functional link of gut-liver interaction during ALF, where dysbiosis has been recently identified as potential driver of ALF severity, might be a promising novel therapeutic target and future studies should aim at further investigating the significance of the intestinal microbiome in ALF.

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Immune-Mediated Drug-Induced Liver Injury

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Key Points

- The traditional classification of DILI into immunologic or metabolic idiosyncracy is too simplistic; the development of *idiosyncratic* DILI is a multistep process involving both metabolic as well as immunologic factors.
- Hypersensivity or immunoallergic reactions are usually characterized by fever, rash, eosinophilia, and a rapid recurrence on rechallenge; the occurrence of eosinophilia in DILI implies a favorable prognosis in most cases
- Drug-induced AIH is a syndrome with clinical, biochemical, and histological features indistinguishable from idiopathic AIH; relapse rate after discontinuation of immunosuppressive therapy is much lower in drug-induced AIH than in *idiopathic* AIH.
- Biologics are commonly prescribed drugs for various chronic inflammatory conditions and some malignancies. Tumor necrosis factor alpha inhibitors and checkpoint inhibitors have more than other agents linked to immune-mediated hepatitis.
- Recent discovery of HLA alleles as risk factors for DILI due to the increasing number and variety of drugs has undoubtedly highlighted the role of adaptive immunity in the pathogenesis.
- Considering the low incidence of DILI in the cohort of patients undergoing therapy, genotyping would have a limited value in pretreatment screening; however, high negative predictive value of genotyping as a diagnostic test may still be useful and should be explored.

Introduction

Drug-induced liver injury (DILI) has previously been classified into immunologic or metabolic idiosyncrasy. Metabolic idiosyncrasy implies that a subject developing adverse reaction metabolizes the drug in a different way than most individuals or lacks adequate protective mechanisms to neutralize reactive metabolites formed. An immunologic idiosyncrasy implies that the susceptible individual has an immune system that would more readily recognize the formed neoantigens. Alternatively, immune system through cytokines and chemokines may modulate the degree of hepatic inflammation secondary to toxic injury. However, this classification derived from clinical observations, such as latent period, presence or absence of manifestations attributable to hypersensitivity, and pattern of response to rechallenge, is too simplistic to be accurate. Increasingly, it is evident that the development of idiosyncratic DILI is a multistep process involving both metabolic and immunologic factors.

Superimposition of drug-metabolizing enzymes and the immune system within the liver which may act both as a lymphoid organ and a target for toxicity create a setting suitable for the interaction between a variety of factors that influence the rate and extent of pathogenic process leading to liver injury. Liver is involved in 80% of the cases of drug rash with eosinophilia and systemic symptoms (DRESS) syndrome, a severe form of idiosyncratic reaction involving multiple organ systems [1, 2]. This syndrome has been associated with drugs, such as phenobarbital, carbamazepine,

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phenytoin, lamotrigine, minocycline, sulfonamides, allopurinol, modafinil, and dapsone. In patients with DRESS syndrome, drug-reactive T cells are in a pre-activated state and, therefore, may have a lower threshold for activation by drugs [3]. Evidence for involvement of immune system in the pathogenesis of idiosyncratic DILI have existed for decades; family studies performed over 30 years ago have revealed that the lymphocytes from first-degree relatives of patients with amineptine-induced liver injury demonstrated increased sensitivity to the drug metabolites [4]. Consistent with this, several candidate gene and genome-wide association studies (GWAS) involving well-characterized patient cohorts conducted in the past decade have indicated that immune mechanisms may underlie the pathogenesis of a range of clinically diverse DILI secondary to therapeutically and structurally unrelated compounds.

Immunoallergic DILI: Signs of Hypersensitivity

Concomitant eosinophilia in peripheral blood and in the liver in a patient with suspected DILI generally supports the role of drug etiology [5, 6]. These classical hypersensivity reactions are usually characterized by fever, rash, eosinophilia, and a rapid recurrence on rechallenge [7, 8]. Two prospective studies of DILI demonstrated that hypersensitivity features were present in 20–25% of cases [9, 10]. In a large meta-analysis of case reports of DILI, eosinophilia in peripheral blood was reported in approximately 30% of all cases in which the presence or absence of eosinophilia was documented, and overall 37% had infiltration of eosinophils in liver biopsies [11]. A study of patients with disulfiram-induced liver injury demonstrated that eosinophilic infiltration in liver biopsies was associated with favorable but hepatocyte dropout or hepatic necrosis with a poor outcome [12]. A prospective multicenter study from Spain over a 10-year period, in which mortality from DILI was observed in a substantial number of patients, found peripheral eosinophilia in only a single case among patients who died from suspected DILI [10]. In the meta-analysis mentioned above [11], the impact of eosinophilia on the prognosis of patients with DILI was also evident for other drugs than disulfiram. Thus, eosinophilia was associated with a favorable prognosis in DILI due to amoxicillin/clavulanic acid, carbamazepine, diclofenac, erythromycin, flucloxacillin, halothane, isoniazid, phenytoin, sulindac, and trimethoprim/sulfamethoxazole [11]. Peripheral eosinophilia was significantly more common in patients who recovered (37 vs. 15.6%) and also among those with hepatic eosinophilia (48 vs. 18.8%) than in those who died or underwent liver transplantation [11]. A recent study from India involving children with DILI due to antituberculous medications [13] was in agreement with these observations indicating that the occurrence of eosinophilia was associated with a favorable prognosis [10–12]. Children with features of hypersensitivity presented earlier (25 vs. 35 days; $P = 0.24$) had less severe disease (MELD, 16 vs. 29; $P = 0.01$) and no mortality (0/16 vs. 12/23; $P < 0.001$) compared with those without hypersensitivity [13]. The role of eosinophils in DILI is unclear. In patients with ulcerative colitis (UC), the activity of eosinophils was shown to be higher in patients with inactive phase of UC than in those with active intestinal inflammation [14], which might suggest that eosinophils are involved in the resolution of inflammation and repair of damaged intestinal tissues. The observation that eosinophilia can be associated with a favorable prognosis provides a hypothesis that could be tested in prospective studies. At the present time, no consensus exists on what constitutes eosinophilia in biopsies. A criterion for eosinophilia on a liver biopsy has been proposed as either many portal areas with occasional eosinophils or several portal areas with many eosinophils [15, 16].

Immune Mechanisms Underlying the Pathogenesis

Development of idiosyncratic DILI is an intricate process involving both concurrent and sequential events determining the direction of the pathways, degree of liver injury, and its outcome. Limited understanding of pathogenesis has led to the classification of DILI as metabolic or immunological idiosyncrasy based on their associated clinical features; this is not just simplistic and incomplete, but, more importantly, fails to reflect the key role that immune system plays in the pathogenesis (Fig. 30.1) even when the liver injury doesn't overtly manifest features of hypersensitivity.

The key upstream events include drug specific pathways triggered by particular drugs or their metabolites leading to an increased formation of reactive metabolites. The expression of these drug-metabolizing enzymes (phase I and II) and transporters involved in the excretion (phase III) and elimination of drug metabolites is regulated by transcription factors (nuclear hormone receptors), such as pregnane X receptor. Genetic and environmental factors that influence the expression and activities of proteins involved in phase I, II, and III of drug disposition or their regulation will determine the rate of formation and accumulation of reactive metabolite [17, 18]. In this chapter, we have focused on the downstream events involving the immune system leading to clinically significant DILI.

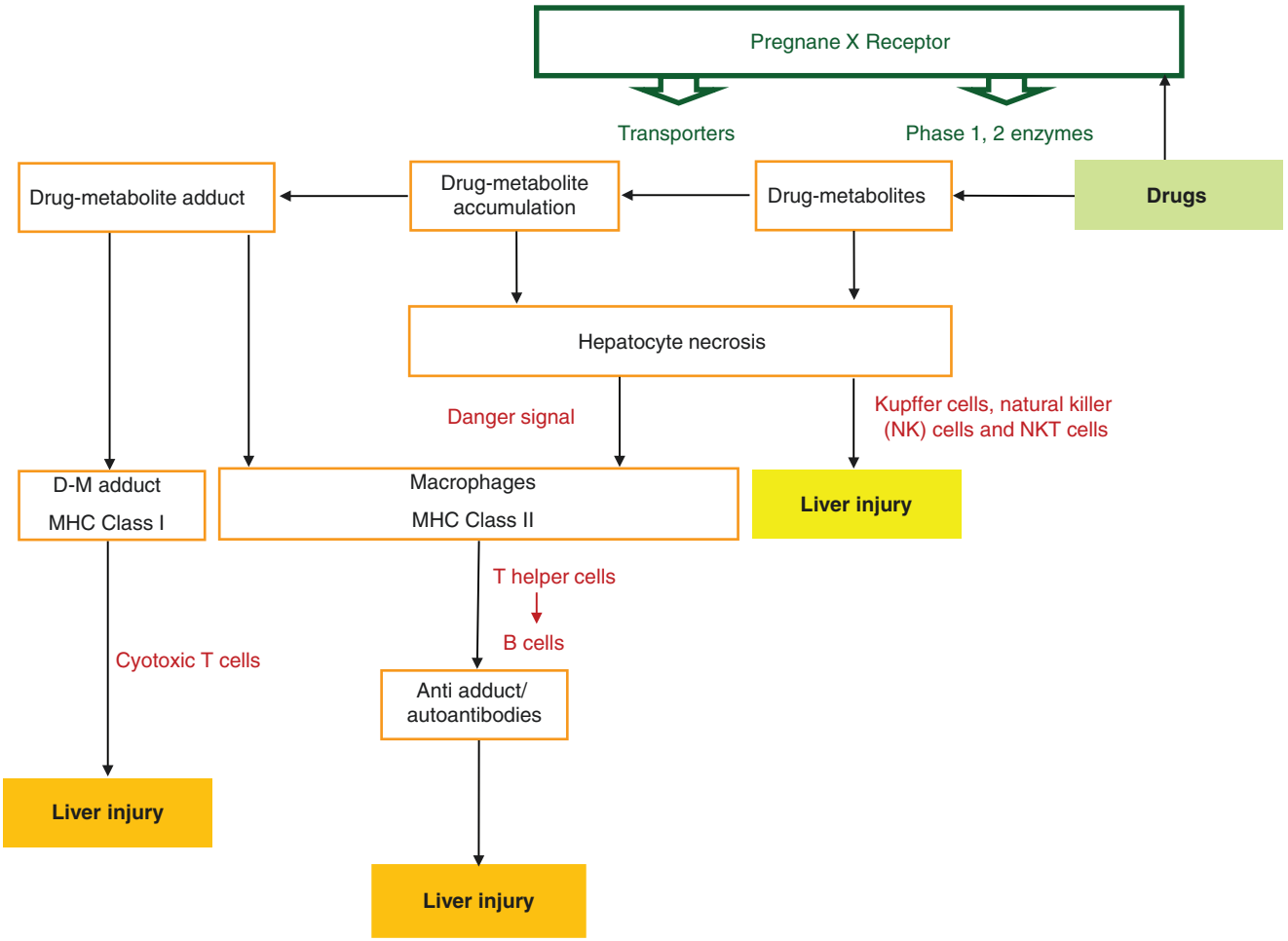

Fig. 30.1 Putative role of MHC class I or II molecules in the pathogenesis of DILI

Generation of Hapten

Drugs in general are too small (low molecular weight) to act as antigens and only gain immunogenic potential following conjugation with a protein carrier. For most drugs, metabolism is required to generate an electrophilic intermediate that can attack nucleophilic residues on proteins. Covalent binding of a reactive metabolite to a protein leads to the formation of adduct [19]. Inhalation anesthetic, such as halothane, is the best example of a drug causing what has been considered an immunoallergic DILI. Halothane is metabolized by cytochrome P450 2E1 to form a chemically reactive acyl halide. Acyl halide targets lysine residues of proteins; antibodies that recognize autoantigens and neoantigens created by trifluoroacetylation (TFA) of hepatic proteins have been demonstrated in patients with halothane-induced DILI [19]. However, there is no conclusive evidence that these antibodies are directly involved in causing liver injury.

Diclofenac is a commonly used nonsteroidal anti-inflammatory drug associated with idiosyncratic DILI that has been well investigated [20]. Diclofenac undergoes glucuronidation by UDP-Glucuronosyltransferase 2B7 forming an unstable acyl glucuronide which in turn can modify proteins covalently. Potential diclofenac adducts have been identified in the liver of a patient with diclofenac-induced liver failure, and antibodies to diclofenac metabolite-modified liver protein adducts have been found in the sera of all patients with DILI [21]. However, the observation that similar antibodies were also present in the sera from 60% of subjects who had not developed hepatotoxicity while on diclofenac therapy suggests that antibody production may be a prerequisite, yet, may not be sufficient on its own to cause clinically significant hepatotoxicity.

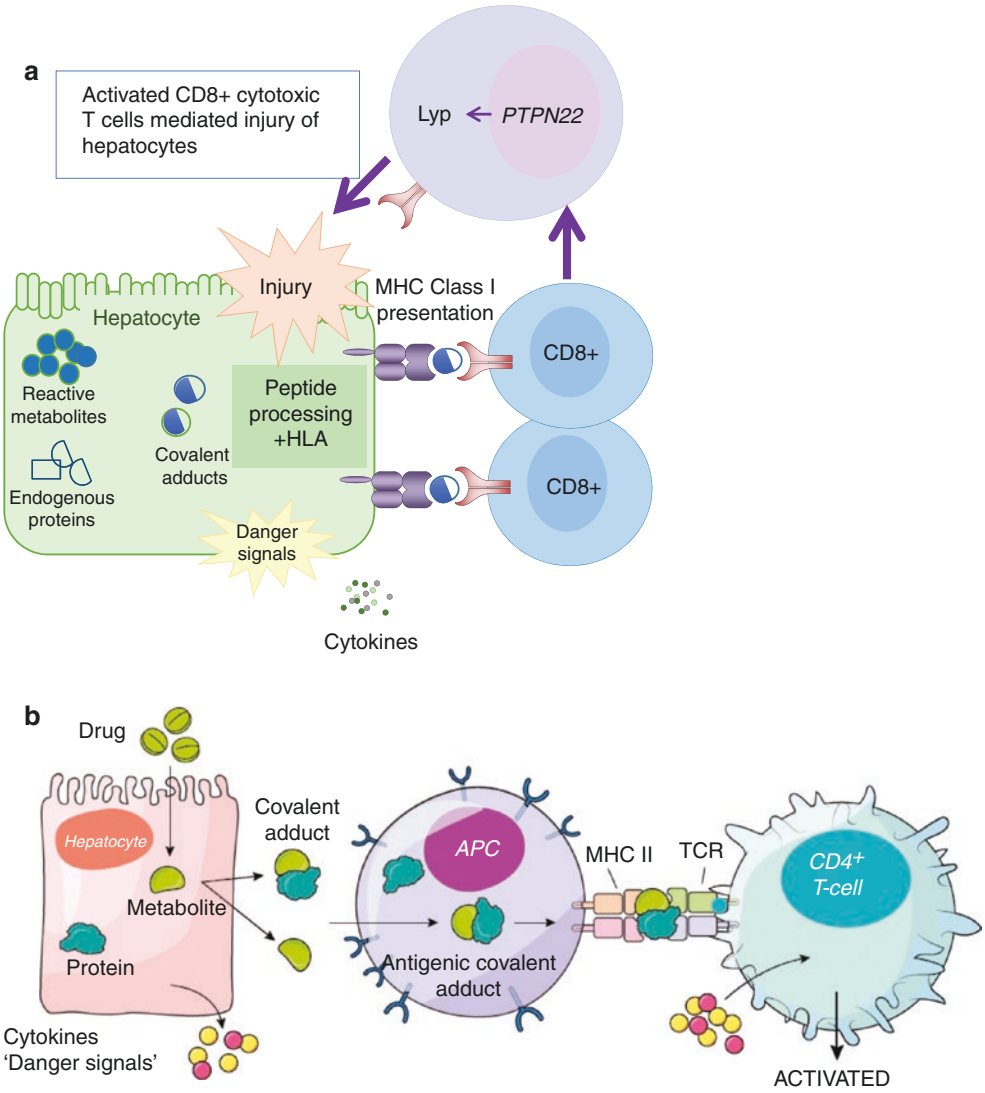

Fig. 30.2 (**a**, **b**) Unifying hypothesis of pathogenesis of DILI highlighting immune mechanisms involved

Role of Adaptive Immune System

To initiate an immune response, the hapten must be processed within the antigen presenting cells, cleaved into peptide fragments that can be presented to T cells *via* major histocompatibility complex (MHC) class I or II molecules in a microenvironment rich in costimulatory signaling and cytokines, which are necessary for sustained T-cell activation, proliferation, and expansion [22] (Fig. 30.2).

Several candidate gene and GWAS presented in Table 30.1 have demonstrated that the human MHC plays a major role in increasing or decreasing susceptibility to DILI. A seminal GWAS demonstrated that possession of *HLA-B*5701* allele was associated with 81-fold increased risk of DILI on exposure to flucloxacillin when compared with ancestry-matched controls [23]; this strong association has been replicated in a larger cohort recently [24]. Flucloxacillin binds covalently to selective lysine residues on albumin, and the level of protein binding determines the strength of the T-cell proliferative response [25]. Consistent with the role of adaptive immune system, drug-specific peripheral blood mononuclear cell (PBMC) responses can be detected in those who had suffered DILI. Flucloxacillin-responsive CD4+ and CD8+ T-cell clones have been isolated and characterized from patients who suffered DILI; the drug also activates naive CD8+ T cells from *HLA-B*5701*-positive volunteers [25]. In addition, a recent meta-analysis of two GWAS has identified *HLA-A*31:01* as the main genetic predisposing factor for both hypersensitivity skin reactions and DILI secondary to carbamazepine [26], further supporting the role of adaptive immune system in the pathogenesis of DILI.

HLA variants associated with toxicity determine the specificity of the peptide-binding groove for the drug or drug-peptide complex, hence enhancing the presentation of these molecules as antigens to T cells and leading ultimately to immunological destruction of hepatocytes [27]. A number of studies have confirmed association of co-amoxiclav DILI with the *DRB1*1501–DQB1*0602* haplotype; a protective association of *DRB1*07* family with co-amoxiclav DILI has also been demonstrated [28]. In contrast, with regard to

Table 30.1 HLA alleles associated with increased susceptibility to DILI secondary to medications that are currently in use

Allele	Drug	Odds Ratio	*P* Value
HLA Class I			
*A*02:01*	Amoxicillin-clavulanate (119–120)	2.3 (1.8–2.9)	1.8×10^{-10}
*B*57:02 & B*57:03*	Anti-HIV & anti-TB combination	30.1 (3.4–263.1)	0.002
*A*31:01*	Carbamazepine	7.3 (2.5–23.7)	0.0004
*A*33:01*	Enalapril	34.8 (3.9–302.9)	0.001
	Erythromycin	10.2 (2–51.7)	0.005
	Fenofibrate	58.7 (12.3–279.8)	3.2×10^{-7}
	Methyldopa	97.8 (12.8–743.8)	0.00001
	Sertraline	29 (4–207.2)	0.0008
	Terbinafine	40.5 (12.5–131.4)	6.7×10^{-10}
	Ticlopidine	163.1 (16.2–1642)	0.00002
*A*33:03*	Ticlopidine	13.0 (4.4–38.6)	1.2×10^{-5}
*B*14:01-C*08:02*	Trimethoprim-sulfamethoxazole	8.7 (3.2–19.5)	2.3×10^{-4}
*B*35:02*	Minocycline	29.6 (7.8–89.8)	2.57×10^{-8}
*B*57:01*	Flucloxacillin	36.6 (26.1–51.3)	2.6×10^{-97}
	Pazopanib	2.1 (1.3–3.6)	0.0058
*B*57:03*	Flucloxacillin	19.8 (3.37–116.1)	0.001
*C*03:02*	Methimazole	14.9 (2.4–182.9)	0.03
HLA Class II			
*DRB1*07:01*	Lapatinib	2.9 (1.3–6.6)	0.007
*DRB1*15:01*	Lumiracoxib	5.0 (3.6–7.0)	6.8×10^{-25}
	Amoxicillin-clavulanate	2.8 (2.1–3.8)	3.5×10^{-11}
*DRB1*16:01*	Flupirtine	18.7 (2.5–140.5)	0.002

flucloxacillin DILI, *DRB1*07* has been associated with an increased risk of disease and *DRB1*15* with a reduced risk. There are clear structural differences between the DR15 and DR7 antigens encoded by these alleles. These differences are concentrated in the peptide-binding groove of the MHC molecule and, hence, may determine the functional significance of these genetic associations [28]. As a corollary, peptide-binding groves of MHC molecules that have similar physicochemical properties function similarly with regard to antigen presentation. Therefore, *DRB1*15:02–DQB1*06:01* are predicted to have a similar association with amoxicillin/clavulanic acid DILI in Asian populations as described for *DRB1*15:01–DQB1*06:02* in Caucasians [29].

HLA molecules are central to the activation of T cells, which are responsible for initiating the inappropriate immune response that underlies DILI; both branches of the highly specific adaptive immune response rely on the selective presentation of antigens to T cells by HLAs, highly polymorphic proteins also known as MHC proteins. MHC class I molecules are expressed by almost all nucleated cells, including hepatocytes. MHC class I proteins usually associate with peptide antigens generated by the partial degradation of self-proteins which could include metabolite-cellular protein adducts generated by a compound. The MHC class I antigen is then expressed on the cell surface and elicits an immune response if a nonself-antigen is recognized, causing the activation of CD8+ T cells, which leads to the cell-mediated killing of the original cell (see Fig. 30.1a).

Evidence for the role of cytokine environment determining the evolution of the pathological process comes from a candidate gene study involving patients with diclofenac-induced hepatotoxicity, in which a combination of variant IL-10 and IL-4 alleles was associated with increased risk of hepatotoxicity [21]. Low IL-10-producing genotype could increase the antigen presentation of diclofenac-related neoantigens by monocytes and lead to the subsequent activation of T cells and immune-mediated liver injury. High IL-4-producing genotype, in addition, could promote a Th2-mediated immune response and induce B-cell differentiation. Both genetic polymorphisms in combination may increase susceptibility to hepatotoxicity by influencing the magnitude and pattern of immune reaction. In contrast, in nitrofurantoin-induced DILI, CD8+ cytotoxic T cells may play a pivotal role in the pathogenesis [30].

Loss of Immune Tolerance

Recently, rs2476601, a nonsynonymous polymorphism that encodes a substitution of tryptophan with arginine in the protein tyrosine phosphatase, non-receptor type 22 gene (*PTPN22*), has been associated with DILI caused by multiple drugs [31]. This variant has been associated with increased risk of type 1 diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus, vitiligo, and Graves' disease, as well as with decreased risk of Crohn's disease and Behçet disease [32]. In addition, rs2476601 appears to be associated with DILI regardless of which HLA alleles are associated with DILI risk. This effect is consistent with the fact that *PTPN22* controls events downstream from HLA presentation of neoantigen with switch in function associated with variant allele reducing immune tolerance of T cells, hence, promoting autoimmunity (see Fig. 30.1a) [33].

Danger Signals

According to the "danger hypothesis" [34], primary function of immune system does not rely upon the distinction of nonself from self, but the need to detect danger and protect against it. In the context of DILI, the induction of pathogenic immune responses may be dependent on the immune system receiving "danger" signals resulting from tissue damage, rather than tolerogenic stimuli associated with normal cell turnover. Consistent with this, macrophages that have taken up necrotic cell debris present antigens to T lymphocytes with greater efficiency, whereas those that have ingested apoptotic cells are ineffective in antigen presentation since

they secrete inhibitory cytokines [35]. In the context of DILI, additional "danger signals" may be provided by the drug-dependent events, such as oxidative stress induced by reactive drug metabolites or modifications of critical proteins through formation of drug adducts, leading to hepatocyte necrosis (see Fig. 30.1b), which generate subclinical liver injury manifested by transient and often self-resolving elevation of liver enzyme. Subclinical cellular toxicity may therefore be a prerequisite to the development of serious DILI [36]; indeed, a number of drugs, such as diclofenac and halothane, are associated with both asymptomatic elevations of liver enzymes in a substantial minority of recipients and rare, yet clinically significant, immune-mediated DILI.

In addition, concomitant nondrug-dependent factors such as disease-induced oxidative stress or bacterial and viral infections could also act as "danger signals" [37] and, hence, influence the immune equilibrium [38]. In rodent models, several drugs, such as trovafloxacin, ranitidine, sulindac, chlorpromazine, halothane, amiodarone, and diclofenac, cause hepatotoxicity when coupled with a nontoxic dose of an inflammogen [39]. *In vitro* studies have used bacterial endotoxins, such as lipopolysaccharide and staphylococcal enterotoxin B; flu viral proteins, cytokines, such as interleukin (IL)-1β, IL-6, and IL-10; tumor necrosis factor-α; interferon-γ; transforming growth factor-β; inflammatory molecules, such as prostaglandin E2, human serum complement, and activated protein C; and oxidants, such as buthionine sulfoximine and H_2O_2, and hyperthermia to mimic "danger signals" [40]. In the presence of these *in vitro* "danger signals" that mimic various pathological conditions encountered by patients treated with sulfamethoxazole, the metabolism of the drug in human antigen presenting cells can be markedly altered with increased formation of drug-protein adducts [40]. It is plausible that concomitant infection may contribute to susceptibility to DILI, and therefore, as a group, antimicrobials, including co-amoxiclav, flucloxacillin, and anti-tuberculous medications, are common among drugs associated with hepatotoxicity. Antituberculous DILI, in particular, has been shown to be more common in patients with chronic hepatitis B [41] and C [42], as well as those coinfected with human immunodeficiency virus [43].

Innate Immune System

Reactive drug metabolites, if not promptly cleared, induce the production of excessive reactive oxygen species leading to lipid peroxidation and cell death. Cellular environment can modulate the threshold for hepatocyte death secondary to oxidative stress. Activated cells of the hepatic innate immune system, such as Kuppfer cells, natural killer (NK) cells, and natural killer T (NKT) cells, can further produce a range of inflammatory mediators that contribute to the progression and cycle of liver injury. Animal model of halothane-induced liver injury in BALB/c mice is associated with increased mRNA levels of tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL6, and IL8 which in turn correlated with a higher number of neutrophils recruited into the liver [44]. Neutrophil recruitment was found to be dependent on NKT cells. Another animal model, CD1d−/− mice, which are deficient in NKT cells, are resistant to developing halothane-induced liver injury and exhibit a significantly lower number of hepatic infiltrating neutrophils upon halothane challenge [45].

Evidence that innate immune system may contribute to the pathogenesis of idiosyncratic DILI in humans comes from studies on genetic susceptibility to hepatotoxicity. In *HLA-B*5701* carrier cases of flucloxacillin DILI, an intronic single nucleotide polymorphism (SNP) in *ST6GAL1*, which encodes for Beta-galactoside alpha-2,6-sialyltransferase, an enzyme involved in transfer of sialic acid to cell surface and serum glycoproteins was associated with a four-fold risk of hepatotoxicity [23]. Increased hepatic expression of *ST6GAL1* has been demonstrated during acute inflammation. Another analysis involving a large number of hepatocellular DILI showed a trend association for a SNP, in the vicinity of signal transducer and activator of transcription 4 (*STAT4*); this association was replicated in an independent cohort [46]. Through the regulation of several cytokines, *STAT4* has been involved in inflammation and implicated in T-cell maturation. Association of SNP in this gene with DILI across a large number of implicated drugs supports a potential role of innate immunity in the pathogenesis of hepatocellular pattern of DILI [46].

Histology in Immunoallergic Hepatitis and Other Types of DILI

The prototype of the liver histology in immune-mediated DILI might be considered intensive infiltration of eosinophils [5]. Prototypical inducers of immune-mediated liver reactions are anticonvulsants [47]. Eosinophilia in peripheral blood was observed in 77%, and hepatic eosinophilia was present in 72% of liver biopsies of cases with phenytoin hepatotoxicity [11]. Focal changes on imaging of the liver, when biopsied, can reveal that drug can induce granulomatous eosinophilic hepatitis [48, 49]. Distinguishing DILI from autoimmune hepatitis (AIH) can be challenging. In some cases, it is very difficult to exclude potential drug involvement, and the differential diagnosis between DILI and AIH can be very problematic. Some cases of AIH are seronegative [50], at least in the beginning of their disease course, and drug etiology is often the most important differential diagnosis. This is of clinical importance as a prompt identification and cessation of drug therapy can prevent further liver injury,

but, if AIH is the likely diagnosis, steroid treatment is needed and discontinuation of the suspected drug unnecessary. The role of liver biopsy in differentiating between these two conditions is uncertain. In a recent study, a group of pathologist undertook a blinded systematic evaluation of liver biopsies from a clinically well-characterized DILI and AIH cases [51]. A model combining portal inflammation, portal plasma cells, intra-acinar lymphocytes and eosinophils, rosette formation, and canalicular cholestasis yielded an area under the curve of 0.90 in predicting hepatocellular type of DILI versus AIH [51]. The occurrence of prominent intra-acinar lymphocytes and canalicular cholestasis favored the diagnosis of DILI, whereas more severe portal inflammation, portal plasma cells, intra-acinar eosinophils, and rosette formation favored the diagnosis of AIH [51]. Thus, a considerable histologic overlap existed between these two conditions. As in AIH, chronic hepatitic pattern was more common than acute hepatitic pattern in both hepatocellular (HC) and cholestatic (CS) type of DILI. Similarly, histologic features often cited as "typical" of AIH were also observed in a significant proportion of DILI cases, such as interface hepatitis (89%), emperipolesis (34%), and rosette formation (40%) [5, 15, 52]. Prominent eosinophil infiltration which has been considered to be one of the histologic findings suggesting DILI [5] does not appear to be useful in distinguishing DILI and AIH. Interestingly, prominent eosinophilic infiltration in portal and intra-acinar areas was in fact higher among AIH than both HS and CS type of DILI cases [51]. Although, the differences in eosinophil counts were not significantly higher in univariate analysis, a prominent intra-acinar eosinophilic infiltrate was one of the predictors in the multivariate analysis that favored AIH over HC type of DILI [51]. It seems that different inflammatory cells may be enhanced in DILI vs. AIH as prominent portal neutrophil infiltrate was favoring CS type of DILI [51]. Table 30.2 demonstrates histologic features favoring AIH versus DILI. In Fig. 30.3, histology in a patient with disulfiram-induced liver injury with marked eosinophilia is presented in a liver biopsy.

Table 30.2 Histologic features favoring AIH vs. DILI

Histologic Features	FavoringAIH	FavoringDILI
Severe portal inflammation (≥grade 2)	*	
Prominent intra-acinar lymphocytes		*HC
Prominent intra-acinar eosinophils	*	
Cholestasis-canalicular		*HC, *CS
Prominent portal-plasma cells	*	
Rosette formation	*	
Any levels of fibrosis (≥grade 1)	*	
Prominent portal-neutrophils		*CS
Hepatocellular cholestasis		*CS
Severe focal necrosis (>grade 4)	*	

HC hepatocellular drug-induced liver injury, *CS* cholestatic drug-induced liver injury
*significance relates to significance differences between the two groups in reference 51.

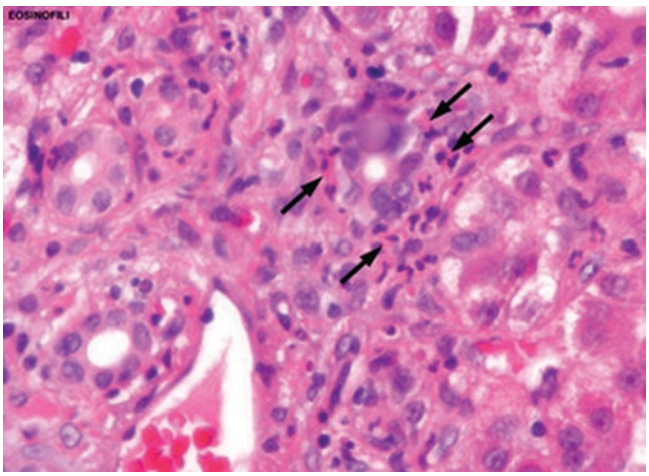

Fig. 30.3 Eosinophilic infiltration of the liver in disulfiram-induced liver injury

Drug-Induced Autoimmune Hepatitis (DIAIH)

Many drugs have been reported to have induced the syndrome of drug-induced autoimmune hepatitis (DIAIH) [53, 54]. Most of these drugs have appeared in case reports or small case series [53, 54]. The most common drugs previously found to provoke DIAIH were dihydralazine [55] and tielinic acid [56], both that have been removed from the market. Later on, accumulating reports have been published on the occurrence of DIAIH by nitrofurantoin [53] and minocycline [57]. More recently, increasing number of reports have been on statins [58–62] and antitumor necrosis α agents [63–65] inducing DIAH. Currently, it is unclear what proportion of patients with drug-induced liver injury developed DIAIH. Conversely, it is not clear what proportion of patients who fulfill the criteria for AIH have DIAIH. In large series on DILI [10, 66, 67], the occurrence of DIAH has not been reported. The only study at the current time describing the frequency of DIAIH, in a patient cohort with the diagnosis of AIH, 24/261 (9.2%) was considered to be induced by drugs [68]. Two drugs, nitrofurantoin (n = 11) and minocycline (n = 11), were the main causes in this series [68]. The proportion patients with DIAH might be higher, as the diagnosis of AIH is often made in the context of a patient on treatment with many drugs [69]. In the best documented drugs leading to AIH-like picture, the vast majority of patients consist of females [68]. The majority of patients with idiopathic AIH not induced by drugs are females, but the female preponderance is more pronounced in DIAIH [68], which is consistent with female propensity of autoimmune diseases. This has been confirmed in three other recent studies [70–72]. In a study of 88 patients with autoimmune features associated with nitrofurantoin, minocycline, methyldopa, or hydralazine from the DILIN network, 91%

were women [71]. In that study 72% had increased levels of ANA and 60% elevated SMA titers, whereas only 39% had elevated IgG, which is lower than in patients with genuine AIH [52, 68]. Autoimmune phenotype was more pronounced in patients with nitrofurantoin-induced hepatitis (82%) and minocycline (73%) than in methyldopa (55%) and hydralazine (43%) [71]. Interestingly, autoimmune scores and titers of autoantibodies were found to decrease during follow-up [71]. Similar proportion of the DILI–AIH induced had *HLA-DRB1*03:01* and *HLA-DRB1*04:01* as controls [71].

DIAH and idiopathic AIH have very similar biochemical, clinical, and histological picture. However, it seems that DIAIH is more likely to be of acute onset [68, 71], is rarely associated with the development of cirrhosis, and very rarely shows relapse after steroid discontinuation, when this has been tried [68, 70, 72, 73]. Two recent studies have not been able to identify any inflammatory features discriminating DIAIH and AIH [51, 68]. However, in the largest series comparing histological features of these two conditions, cirrhosis was observed in 21% of AIH cases, whereas no cirrhosis was present among DIAH cases at presentation [68]. The findings of another study are consistent with this, as advanced fibrosis was observed only in AIH, but not in DIAIH cases [51]. Consistent with these, none of the patients with AIH induced by antitumor necrosis α agents had histologically proven cirrhosis at presentation [65]. A small series of nitrofurantoin-induced AIH, precirrhosis or cirrhosis, was present in one case [74]. Thus, in general, fibrosis and cirrhosis are less frequently observed in DIAIH cases than in idiopathic AIH [51, 68, 70, 72].

Risk Factors for Drug-Induced Autoimmune Hepatitis

In a long-term follow-up of patients with DILI with concomitant jaundice leading to hospitalization, autoimmune hepatitis developed in 5/23 (22%) patients after the initial event over a mean period of 6 years [75]. Although causality is very difficult to assess in this context, it is conceivable that previous insult to the liver, such as DILI, might increase the risk for AIH in the future. Indeed, there are a few reports that support such a relationship. In a Japanese study, anti-nuclear antibody (ANA) was detected after DILI in six patients, and 5/6 (83%) were females [76]. All five patients who developed AIH after the initial DILI were females in a long-term follow-up study, which is in line with these results [75]. In the Spanish DILI registry, 9/742 (1.2%) patients had evidence of two DILI episodes caused by different drugs [61]. An interesting finding in that series was that four out of nine cases (44%) developed DIAIH in the second episode during follow-up [61]. This clearly exceeds the chance of association of this liver injury phenotype in the Spanish DILI registry's general patient cohort, as six out of nine cases in the series were AIH-like [61]. Although patients with past history of DILI in general seem to have a very low probability of hepatotoxicity in the future, the majority of these patients developed AIH-like type of liver injury in the second episode, which argues against preexisting or subclinical AIH [61]. Interestingly, Sugimoto et al. reported seven cases which were diagnosed as DILI but features of AIH became apparent later despite discontinuation of the drug, suggesting a different pattern of etiology [77]. Interestingly, ANA titers and immunoglobulin (Ig) G levels increased during the course [77].

The Role of Specific Drugs

Nitrofurantoin

Autoimmune hepatitis induced by nitrofurantoin was reported from the USA in a small series of five patients from the 1970s and six patients from the Netherlands from the 1980s [53, 74]. However, patients reported in these early series had a limited follow-up, and the need for immunosuppression as well as their long-term prognosis was uncertain. A number of case reports has been published on nitrofurantoin induced AIH before and after these series [71, 78]. Nitrofurantoin has also been associated with other types of DILI, such as acute liver failure and also liver cirrhosis [67, 71, 74, 78]. In a series from the Mayo Clinic of cases of DIAH among patients with AIH, nitrofurantoin was found in 11/24 (46%) of all cases [68]. Patients with nitrofurantoin-induced AIH have been reported to have radiologically "cirrhotic" liver with confluent fibrosis and massive fibrotic bands, but no cirrhosis was present on histology [68], which is similar to a report from the Netherlands showing no cases of nitrofurantoin induced cirrhosis in 52 cases [53]. The changes observed on imaging showing "cirrhotic" changes [68] might be explained by postnecrotic changes in the liver as seen in acute liver failure. Thus, radiological features of "cirrhosis" should not discourage clinician from using steroids in DIAH cases.

Minocycline

Minocycline-induced hepatitis is associated with the presence of ANA and SMA, as well as elevated IgG and histological picture identical of classical AIH [67, 79, 80]. In the previously mentioned series from the Mayo Clinic of cases of DIAH among patients with AIH, minocycline was found in 11/24 (46%) of all cases [64]. In general, DIAH induced by minocycline seems to have a favorable prognosis [57, 71, 79, 80], although other types of liver injury associated with the use of minocycline have in some cases induced acute

liver failure and need for liver transplantation [81–83]. In a patient with acute liver failure, requiring liver transplantation, anti-smooth muscle antibody, anti-double-stranded DNA antibody, anti-mitochondrial antibody, and antinuclear antibody were positive, indicating an autoimmune process rather than a necrotic and/or inflammatory process in the liver [83]. However, the explant of the liver showed severe necrotic changes, and the autoantibodies might have been secondary to the severe liver failure, that has been previously demonstrated in different types of acute liver failure [84].

Statins

Although rare statin-induced hepatotoxicity has been well documented [60, 85]. Many case reports [58–62, 86, 87] and some cases series [61, 62, 85] have been published describing DIAH with the use of statins. Most have been related to the use of atorvastatin which in general is the statin mostly associated with DILI [58, 62]. Most of patients with DIAIH due to statins were reported to have favorable prognosis. Cross-reactivity, with development of DILI after exposure of another statin, has been reported [60, 62], but it has also been observed that another type of statin could be tolerated and, hence, the "class effect" isn't universal [62], as with other types of DILI induced by statins [85]. It is possible that the drug might serve as a hapten in genetically susceptible host with a specific haplotype who might be reexposed to the same or another statin [88–90].

Anti-Tumor Necrosis Factor α (TNF α) Agents

More than 100 cases of DILI related to the use infliximab have been reported [91]. This has been in patients with all indications for these drugs, such as psoriasis, ankylosing spondylitis, inflammatory bowel disease, and rheumatoid arthritis [63–65, 92–94]. Taken together, TNF α agents are probably the most common cause of DIAIH among drugs in use nowadays. Many cases have been published [65, 70, 72, 92–94]. Most of these reports have been associated with infliximab, but DIAIH has also been associated with etanercept and adalimumab. Hepatic reactions due to these drugs seem to appear after a relatively short time of exposure, most commonly after the fourth infliximab infusion [70, 72]. Most cases have ALT levels >10 times the ULN, show generally good response to immunosuppressive therapy or can resolve without immunosuppression. Moreover, to our knowledge, advanced fibrosis or cirrhosis has not been reported, which is similar to reports on DIAIH due to other drugs, although classical AIH can be associated with advanced fibrosis in a significant proportion of cases [68]. After the resolution of liver injury, patients have been successfully switched to another TNF α agent without recurrence of liver injury [70, 72, 92–94]. As mentioned above, in most instances, the prognosis is favorable. However, a case has been reported where infliximab was considered a likely cause of vanishing bile duct syndrome leading to liver failure and need for liver transplantation [95].

Checkpoint Inhibitors

Immune-modulatory drugs are increasingly used to treat many types of malignancies [96]. By interfering with immune system, these novel therapies have been shown to result in several immune-associated adverse effects and can lead to many autoimmune conditions [97]. In the native state, expression of immune regulatory "checkpoint" receptors that downregulate immune functions lead to inhibition of activated T cells [97]. Monoclonal antibodies against these checkpoint receptors can therefore block the inhibition of T cells and unleash antitumor immunity [97]. Shortly after marketing of the first subgroup of checkpoint inhibitors, ipilimumab (anti-CTLA-4 inhibitor), hepatitis was observed which was not always severe and sometimes only required temporary interruption of therapy [98].

Results from liver histology demonstrated focal or confluent necrosis with prominent lymphocytic infiltrates of activated T cells, which was found to be consistent with an immune-mediated hepatic injury [99]. With more experience in post-marketing, not only mild elevations in liver tests, but also liver related death from acute liver failure were observed [99, 100]. If both mild and severe hepatotoxicity were taken together, this was found to effect 4–9% of anti-CTLA-4 mAbs and up to 18% of patients treated with combination of anti-CTLA-4 mAbs andanti-PD-1 mAbs [100, 101]. Thus, other checkpoint inhibitors, including nivolumab, pembrolizumab, and cemiplimab, are PD-1 antagonists that aim to modulate T cell immune reactivity have all been linked to clinically apparent liver injury [102–105].

In a landmark paper in this context, De Martin et al. compared anti-programmed cell death protein 1(PD-1/PD ligand 1 (PD-L1) and anticytotoxic T lymphocyte antigen 4 (CTLA-4) monoclonal antibodies [106]. Among 16 patients (3.5% of the total cohort treated with these drugs) DILI, the time between start of therapy and hepatitis was 5 weeks, and the median number of immunotherapy injections was two, indicating that most patients develop these adverse reaction early after therapy initiation, although not all [106]. Liver histology among these two types of mAbs was quite different, showing granulomatous hepatitis, including central vein endotheliitis and fibrin ring granulomas associated with anti-CTLA-4 mAbs and lobular hepatitis due to anti-PD-1/PD-L1 mAbs [106]. Overall, 10/16 (63%) were treated with corticosteroids, mainly receiving oral corticosteroids, whereas six (37%)

improved spontaneously, and in three patients immunotherapy was reintroduced without recurrence of liver injury [106]. These results have mostly been reproduced by other groups [107, 108]. In contrast with hepatitis induced by infliximab which is biochemically, immunologically, and histologically very similar to genuine *de novo* autoimmune hepatitis (AIH), liver injury associated with checkpoint inhibitors seems to be quite different from AIH. Patients very rarely have autoantibodies, such as ANA and SMA, rarely have elevated IgG, and characteristic features of AIH, such as severe interface hepatitis, piecemeal necrosis, plasma cell infiltration, and rosette formation, are generally lacking [109].

Diagnosis

At the current time, diagnosis of DIAIH is dependent upon combination of factors; its similarity to AIH and its resolution on drug withdrawal. In most case reports and case series, patients have developed liver injury associated with drug intake considered responsible for the liver injury and concomitant elevation in antinuclear antibodies (ANA) and/or smooth muscle antibodies (SMA) and also elevation in the serum levels of IgG. However, this is probably not an adequate basis for making this diagnosis. Some drugs can lead to development of autoantibodies and/or IgG levels, in the absence of liver disease [5, 111–113]. Thus, taking into consideration serological findings alone is not reliable, and it would be possible to diagnose these patients according to the new simplified criteria for AIH [68]. In the largest series, the new simplified score of AIH was used to establish the diagnosis of DIAIH [68]. In this series, the demographics were very similar, and similar proportion of patients had positive ANA (83% vs. 70%) and SMA (50% and 45%), in DIAH and classical AIH, respectively. The only laboratory test that differed significantly between the two groups was ALP; which was higher in DIAIH than in AIH [68]. Similarly, histological features were very similar in these two groups, and no single histological finding could distinguish between them [68]. A subgroup analysis demonstrated that severity of inflammation and fibrosis and the frequency of what have been considered AIH specific findings were comparable between DIAH and AIH [51]. Marked fibrosis (Ishak score >4) was, however, only seen in patients with classical AIH, and not in DIAIH cases [51]. The most commonly used causality assessment instrument for DILI, the RUCAM score, has not been validated for DIAIH [114]. In a recent study, RUCAM was used in a series of patients with well-documented DIAIH, due to infliximab, nitrofurantoin, and imatinib that all have been linked previously with DIAIH [72].

Among the 15 patients included, overall, 12 had a probable causal relationship, one highly probable, and two possible according to RUCAM. In the 15 patients, 14 had elevated ANA, but none had positive SMA, and 40% had elevated IgG levels [72]. However, only 6/15 (40%) fulfilled the new simplified criteria for AIH [52]. The new simplified criteria did not seem helpful in these cases, and they have not been validated in this clinical context [52]. Only six patients had a liver biopsy undertaken. However, it was very difficult to distinguish between histological features that favored DILI and those favoring AIH potentially requiring steroids. Thus, liver biopsies did not seem to guide therapy. These results are in agreement with a study of the use of liver histology in discrimination between DILI and AIH [51]. The results of this recent study [72] do not suggest that liver histology is likely to change management. Some of the patients not fulfilling the new simplified criteria for AIH clearly required corticosteroids, as the liver tests did not normalize despite discontinuation of the implicating agent [72].

Therapy

In most case reports and case series, corticosteroids have been used in DIAIH as in other forms of AIH. However, in some DIAIH cases, immunosuppression has not been considered necessary. In one series, 2/11 (18%) of patients with minocycline-induced AIH achieved clinical and biochemical resolution without any immunosuppression [68]. It is also clear that all patients with hepatitis due to checkpoint inhibitors do not need therapy with corticosteroids and recover spontaneously [106–108]. Out of 4/9 (30%) patients with DIAIH developing after a second exposure of drugs leading to DILI in the Spanish registry [61], liver tests normalized in two patients without requiring immunosuppression, and smooth muscle antibody became negative after drug discontinuation [61]. Thus, some of these patients have a rather rapid resolution without immunosuppression, whereas if this does not happen, most agree that there is an indication for corticosteroids. However, it is unknown how long the immunosuppression is required. In the majority of patients with idiopathic AIH, relapse can be expected after withdrawal of immunosuppression. Therefore, it is conceivable that many physicians are hesitant to withdraw immunosuppression also in this type of AIH. However, successful withdrawal of steroid therapy has been reported in most cases of DIAIH in patients where this has been tried and/or reported [61, 68, 72]. In the largest series of patients with DIAIH, discontinuation was tried in 14 DIAIH cases (median follow-up 36 months), with no relapse, whereas 65% of the AIH patients relapsed [68]. This argues for the concept that at least minocycline and nitrofurantoin can induce AIH, and not only unmask otherwise sporadic cases of AIH. Thus, in the vast majority of DIAIH cases reported, withdrawal of immunosuppression has been successful when this has been tried. However, in many case reports and case series, patients were still on

immunosuppression at the time of these reports, and the authors did not mention any plans of trying to discontinue that therapy in the future. To our knowledge, only three cases of DIAIH were associated with a relapse when immunosuppression was withdrawn [60, 81, 115]. However, although a discontinuation of immunosuppression should be tried in all patients, a possibility of a relapse of liver injury cannot be excluded which requires monitoring of liver tests after discontinuation of immunosuppression. If a relapse occurs, this argues against being induced by the drug and might have been *de novo* AIH. Although it is conceivable that the AIH could have been triggered by drugs in these cases, the AIH patient should be managed and treated like other AIH cases.

Conclusion

Our understanding of relationship of drug metabolism in the development of primary immune response has improved substantially [116]. Recent studies propose that the drug metabolism within the antigen presenting cell itself may generate functional antigens [22]. Adduct formation beyond a threshold level would stimulate cell death, which provides a maturation signal for dendritic cells, as well as co-stimulatory signals to initiate and drive the pathogenic immune response. Recent discovery of HLA alleles as risk factors for DILI due to increasing number and variety of drugs has undoubtedly highlighted the role of adaptive immunity in the pathogenesis. When considered in the context of other complex traits, the association between DILI and HLA class I or class II alleles are unusually strong. Interestingly, alleles that have been associated with DILI caused by several chemically unrelated drugs, such as flucloxacillin, ximelagatran, lapatinib, and antituberculosis drugs, reside on similar haplotypes. A recent report concluded that DILI caused by at least nine different drugs can be related to two main haplotypes [117]. Understandably, potential application of these associations in preempting DILI has been considered. One study estimated *HLA-DQA1*0102* allele to have a sensitivity of 74% and negative predictive value of 99% to identify subjects at risk of developing hepatotoxicity secondary to lumiracoxib [118]. However, the HLA genotypes and haplotypes are common in the general population. Considering the low incidence of DILI is the cohort of patients undergoing therapy, genotyping would have a limited value in pretreatment screening. In the context of DILI due to flucloxacillin, despite the strong association with *HLA-B*5701,* only 1 in every 500–1000 individuals with this genotype will develop DILI when exposed to the drug [23]. However, high negative predictive value of genotyping as a diagnostic test may still be useful in patients where exclusion of DILI as a possibility would allow continuation of an effective therapy.

Further understanding of drug, environment, and host factors that contribute to the development of DILI will improve detection of hepatotoxicity during drug development and allow early diagnosis of clinically significant DILI. Effective preemption and primary prevention should remain the goal of translational research.

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Hepatobiliary Cancers and Immunology

31

Takahiro Kodama and Tetsuo Takehara

Abbreviations

APCs	antigen-presenting cells
BCLC	Barcelona Clinic Liver Cancer
Breg	regulatory B cells
CAFs	cancer-associated fibroblasts
CAR	chimeric antigen receptor
CCRK	cell cycle-related kinase
CIK	cytokine-induced killer
COX	cyclooxygenase
CTLA	cytotoxic T lymphocyte antigen
CTLs	cytotoxic T cells
DCs	dendritic cells
ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2
FDA	Food and Drug Administration
GITR	glucocorticoid-induced tumor necrosis factor receptor
GP73	Golgi protein 73
GWAS	genome-wide association study
HCC	hepatocellular carcinoma
ICGC	International Cancer Genome Consortium
IDO1	indoleamine-2,3-dioxygenase 1
IFN	interferon
IL	interleukin
ILCs	innate lymphoid cells
LAG	lymphocyte-activation gene
MAGE	melanoma-associated antigen
MDSCs	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MICA	major histocompatibility complex class I chain-related protein A
MICB	major histocompatibility complex class I chain-related protein B
M-MDSCs	monocytic MDSCs
NAFLD	nonalcoholic fatty liver disease
NK	natural killer
NO	nitric oxide
NOX1	NADPH oxidase 1
NSCLC	non-small-cell lung carcinoma
PD-1	programmed cell death 1
PGE2	prostaglandin E2
PMN-MDSCs	polymorphonuclear MDSCs
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SCF	stem cell factor
SCNA	somatic copy number alterations
TAA	tumor-associated antigens
TACE	transarterial chemoembolization
TAMs	tumor-associated macrophages
TCGA	The Cancer Genome Atlas
TCR	T-cell receptor
TGF	transforming growth factor
TIBs	tumor-infiltrating B cells
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIM	T-cell immunoglobulin and mucin-domain containing
TNF	tumor necrosis factor
Treg	regulatory T cells
ULBP	UL16-binding proteins
VEGF-A	vascular endothelial growth factor A
WHO	World Health Organization
α-GalCer	α-galactosylceramide

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Key Points

- Hepatocellular carcinoma (HCC) is the major leading cause of cancer-related death worldwide and mostly occurs in people with chronic liver disease or cirrhosis due to HBV and HCV infection, alcohol addiction, and nonalcoholic steatohepatitis.
- HCC is classified into subclass based on molecular profile as well as immune status.
- HCC cells evade or suppress the anti-tumor immune system by altering their characteristics and producing immunosuppressive molecules including cytokines/chemokines in concert with stromal cells in their tumor microenvironment (TME).
- In the HCC TME, regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) play immunosuppressive and tumor-promoting roles, whereas cytotoxic T cells (CTLs) and natural killer (NK) cells, central players of the immune control of HCC, are functionally impaired.
- Several molecular targeted agents and immune checkpoint inhibitors are currently available for the treatment of advanced HCC, and their combination therapy could be a more potent new therapeutic.
- Alternative immunotherapeutic strategies including vaccine, immune cell-based therapy, or elimination of immunosuppressive cells are still actively under investigation toward HCC.

Introduction

Hepatobiliary cancers include hepatocellular carcinoma (HCC), as well as biliary tract malignancies such as intra- and extrahepatic cholangiocarcinoma, gallbladder cancer, and cancer of the ampulla of Vater [1]. There are also various rare cancer types in this category including fibrolamellar HCC, epithelioid hemangioendothelioma, and angiosarcoma as well as benign liver tumors (focal nodular hyperplasias, hepatic adenomas, and hemangiomas) and benign biliary neoplasms (biliary cystadenoma, biliary hamartoma, and granular cell tumors) [1]. Since HCC accounts for 80–90% of hepatobiliary cancers cases, this chapter focuses on discussing the epidemiology, molecular pathogenesis, diagnosis, treatment, immunology, and immunotherapy for HCC [2].

Epidemiology

According to the World Health Organization (WHO) statistics in 2018, the yearly worldwide burden of liver cancers is 841,080 new cases and 781,631 liver cancer-related deaths [3]. It is considered the fourth leading cause of cancer-related death and ranks sixth regarding incidence rate [3]. Incidence rates are different between Asian and Western regions, and approximately 80% of cases occur in sub-Saharan Africa and eastern Asia [4]. HCC rarely occurs in people without chronic liver disease. The incidence of HCC is twice higher in males than females. The major risk factors for HCC include HBV and HCV infections, alcohol addiction, nonalcoholic fatty liver disease, and dietary exposure to aflatoxin B1 and aristolochic acid [5]. HCC also develops from patients with rare disorders such as α-1 antitrypsin deficiency and hemochromatosis. The prevalence of risk factors varies according to the geographic area. Chronic hepatitis B is still the main cause of HCC worldwide, regardless of the advancement of universal vaccination program. The WHO estimates that 257 million people were living with HBV infection in 2015. In Western countries and Japan, HCV infection is the main cause of HCC [5]. The development of effective antiviral therapies toward chronic hepatitis C including interferon and direct-acting antivirals greatly helps to eradicate HCV infection and may reduce the incidence of HCC [5]. In contrast, the incidence of HCC from nonalcoholic fatty liver disease (NAFLD) is increasing worldwide [5]. Metabolic syndrome, diabetes, and obesity may increase the risk of HCC development in patients with NAFLD [4].

Molecular Pathogenesis and Classification

Hepatocarcinogenesis involves a complex multistep process including sustained hepatic necroinflammation, fibrogenesis, and compensative hepatocyte regeneration, all of which are induced by continuous hepatotoxic stimuli such as viral infection, ethanol, and lipid. The liver has a unique ability to fully regenerate itself by proliferation of differentiated hepatocytes in case of acute damage. However, sustained chronic necroinflammation in the liver causes the activation of non-parenchymal cells, promotion of liver fibrosis, and alteration of immune response, all of which contribute to promote tumorigenesis. In addition, aberrant hepatocyte proliferation induces replication stress, DNA damage, and genetic instability, leading to the accumulation of a variety of genetic events in hepatocytes including somatic mutations, InDel, somatic copy number alterations (SCNA), and chromosomal rearrangements in addition to epigenetic modifications, which result in their malignant transformations. Recent advancement of next-generation sequence technology enabled us to explore the global landscape of cancer genome. International collaborative projects of cancer genome sequence such as The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) performed whole genome or exome sequence of several hundreds of HCC genomes [6, 7].

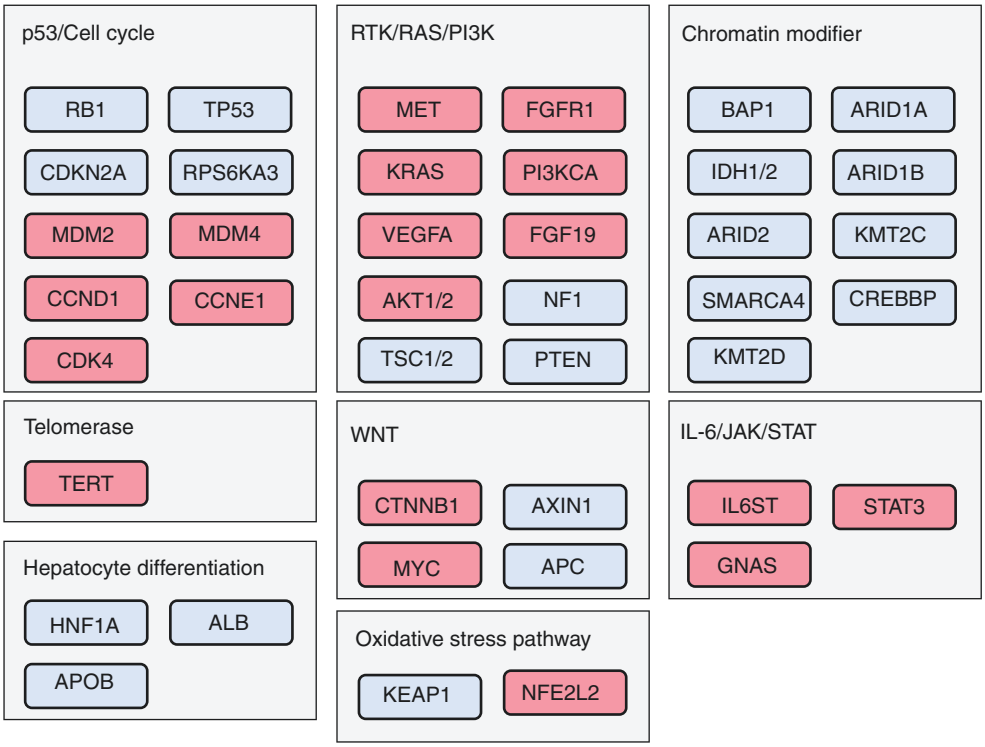

Fig. 31.1 The landscape of altered genes and pathways driving HCC. Red indicates activated genes and blue indicates inactivated genes

Most frequent genetic alterations are mutations in the TERT promoter region, which account for approximately 60% of HCC [6]. HBV genome is also reported to be integrated into this region, leading to TERT activation. Other major mutated genes are TP53 and CTNNB1, both of which account for approximately 30% of cases. Aflatoxin B1 is known to cause specific mutagenesis in TP53 gene [8]. On the other hand, approximately 20 genes are defined as genetic drivers with much less frequent mutations (less than 10% of cases) such as AXIN1, ARID1A, BAP1, KEAP1, and RB1. Most frequent SCNA are observed in chromosomes 1q and 8q as copy number gain regions and 8p and 17p as their losses [6]. The major driver oncogenes dysregulated by SCNA include CCND1 and FGF19 (11q13.3), MYC (8q24.21), MET (7q31.2), VEGFA (6p21.1), and MCL1 (1q21.3). Regarding epigenetic modifications, CDKN2A was identified as the tumor-suppressor gene silenced by hypermethylation in about a half of cases [6]. Integrated molecular analysis of somatic alterations revealed dysregulated signaling pathways driving HCC including telomerase, p53/cell cycle, WNT, receptor tyrosine kinase (RTK)/RAS/PI3K, chromatin modifiers, and oxidative stress pathways [8] (Fig. 31.1).

HCC is classically classified into two different subtypes based on molecular signatures, which correlate well with clinical features (Fig. 31.2) [9]. One is the proliferation class which clinically shows high serum levels of alpha-fetoprotein, poor differentiation, and more vascular invasion and results in worse prognosis. Its molecular features include chromosomal instability, TP53 mutations, and

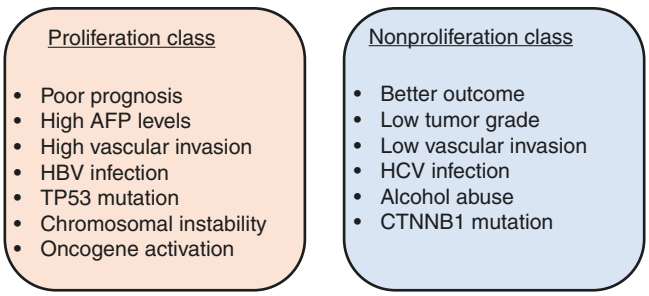

Fig. 31.2 Molecular classification of HCC with genomic and clinical features

activation of oncogenic pathways including RAS/MAPK, MET, and AKT-mTOR. The other is the nonproliferation class which clinically shows a better outcome and molecularly includes more CTNNB1 (beta-catenin) mutations. In addition, recent analysis proposed the new classification of HCC based on immune status in the TME, which may be helpful to determine patient prognosis and response toward immunotherapy [10, 11]. This new classification will be further described below.

Diagnosis and Treatment

HCC is typically diagnosed by a variety of imaging techniques [12]. Ultrasonography is often used for the surveillance. Once a mass is detected in a cirrhotic liver,

confident diagnosis of HCC can be established by contrast-enhanced CT or MRI [5]. Typical HCCs show hyperenhancement in the arterial phase and washout in venous or delayed phases, which reflects the difference of vascular supply between benign lesions, supplied by the portal vein, and malignant lesions, supplied by the hepatic artery [13]. Contrast-enhanced ultrasonography is also used in European and Asian countries. Tumor biopsy is performed for nodules in case they do not show a typical pattern on imaging. To consider treatment of HCC, it is important to incorporate liver function as well as tumor stage, since most patients have chronic liver diseases or cirrhosis [5]. The Barcelona Clinic Liver Cancer (BCLC) system is the most widely used staging system worldwide (Fig. 31.3) [4]. Early-stage HCC patients (BCLC 0 or A) can undergo curative treatment including local ablation, surgical resection, or liver transplantation. Intermediate-stage HCC patients (BCLC B) undergo transarterial chemoembolization. For patients with advanced-stage HCC (BCLC C), sorafenib, a multi-kinase inhibitor, was the only treatment option for more than a decade. However, recently several other molecular targeted drugs have shown the positive results in phase III trial and can be used for these advanced patients. These include lenvatinib as the first-line setting and regorafenib, cabozantinib, and ramucirumab as the second-line setting [4]. In addition, immune checkpoint inhibitors have been shown to be effective in some cases and can be used in some regions, which will be further discussed below.

Overview of Cancer Immunology

Immune defense mechanism plays a central role in the prevention and progression of cancer. But tumors can escape immune surveillance by creating an immunosuppressive environment. A variety of immune cells that resided in the tumor microenvironment modulate cancer development and progression in either favorable or unfavorable manner. This paragraph overviews the roles of each immune cell type in cancer and the cross-talks between immune cells and cancer cells in the TME.

Cytotoxic T Cells

Cytotoxic T cells (CTLs), which express the CD8 coreceptor, are the central immune cells to combat tumors [14, 15]. Naïve $CD8^+$ T cells developed in the thymus become effector $CD8^+$ T cells with cytotoxic capacity, named as CTLs, through T-cell priming which generally occurs in lymphoid tissues [15]. Antigen-presenting cells (APCs) such as dendritic cells (DCs) cross-present antigens on the major histocompatibility complex (MHC) class I molecules to $CD8^+$ T cells via their T-cell receptor (TCR), which turns the naïve $CD8^+$ T cells into the effector CTLs. $CD4^+$ T cells also help for priming of $CD8^+$ T cells through cytokine secretion [14, 16]. CTLs eliminate tumor cells by detecting antigens from tumor cells presented by MHC class I molecules through their TCRs [15].

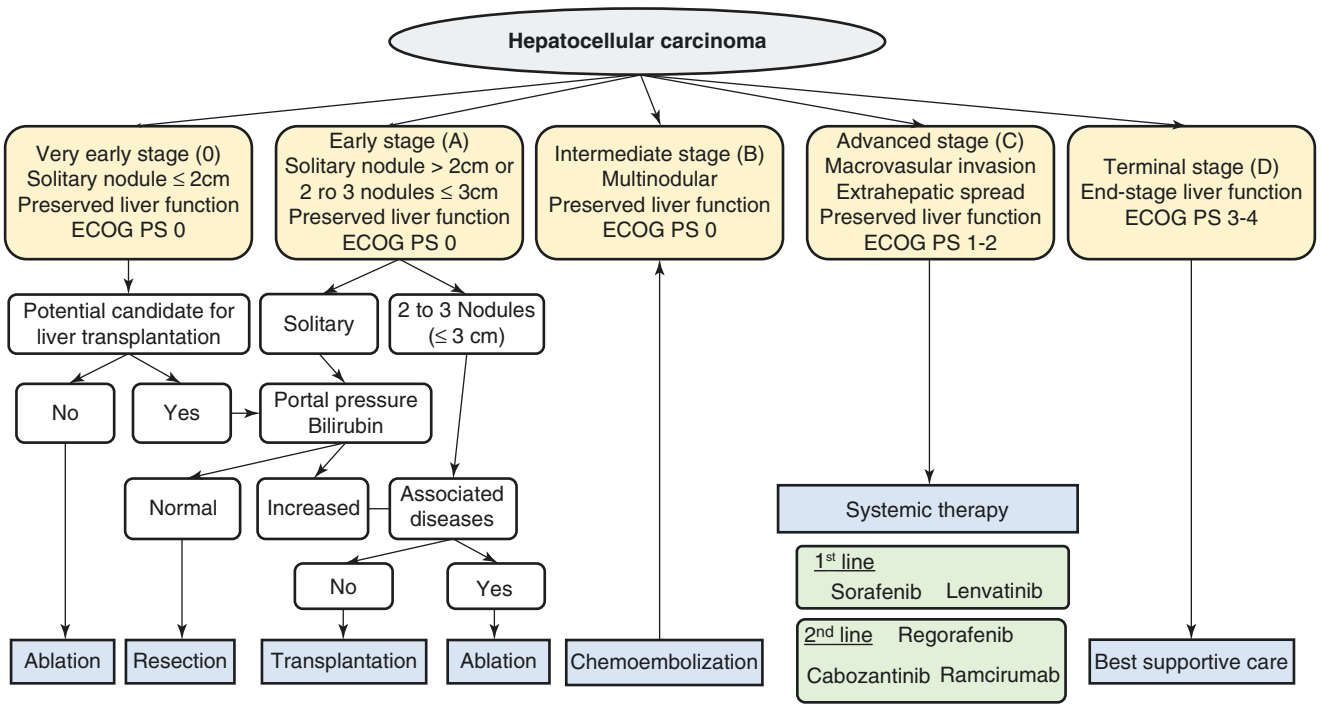

Fig. 31.3 Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy

Existence of CD8 T-cell-mediated anti-tumor immunity has been proved by the presence of tumor-specific CTL from peripheral blood or tumor tissue in patients of various cancers [17] as well as in spontaneously regressing tumors [18]. In addition, infiltration of CTLs into the TME is positively correlated with better prognosis in a variety of cancer types such as breast, cervical, colorectal, and lung cancer, glioblastoma, and melanoma [19–22]. These findings further support the importance of tumor-specific CD8 T-cell responses to exert tumor immunity.

Tumor antigen was first demonstrated in 1989 when Lurquin et al. found that tumor-specific CTLs recognized a peptide derived from a mutated intracellular protein in cancer cells in mouse [23]. Melanoma-associated antigen (MAGE)-1 was the first human tumor antigen recognized by CTLs in melanoma [24]. Since then, a variety of tumor antigens have been reported and categorized into either tumor-associated antigens (TAA) or tumor-specific antigens (TSA). TAA is also classified into at least four groups including differentiation antigens, overexpressed antigens, viral antigens, and cancer-germline antigens [25]. Differentiation antigens are expressed in tumor cells and the normal tissue of origin of the malignancy. Meanwhile, overexpressed antigens are expressed in tumor and a wide variety of normal tissues, but expression levels are much higher in tumor cells than those in normal tissues. Viral antigens are derived from infected virus, such as HBV, human papillomavirus, Epstein-Barr virus, and human T-cell leukemia virus which are involved in some cancer types including hepatocellular carcinoma, cervical carcinoma, nasopharyngeal carcinoma, and adult T-cell leukemia [26]. Cancer-germline antigens, encoded by cancer-germline genes, are expressed in tumor cells and reproductive organs including placental trophoblasts and testicular germ cells [27]. There is a database of those antigenic peptides on the https://caped.icp.ucl.ac.be/about website. TSA, also known as neoantigens, are generated in tumor cells by somatic mutations in genes that are ubiquitously expressed and thus uniquely expressed in tumor cells [25]. Most of these neoantigens are not shared between patients and therefore may be considered patient specific.

Upon recognition of these antigens, CTLs kill cancer cells either directly or indirectly. Activated CTLs release perforin and granzymes through exocytosis of cytotoxic granules, which make pores in the plasma membrane of tumor cells and cleave their intracellular substrates. CTLs can activate intracellular caspases of tumor cells through the Fas ligand/receptor interaction and induce their apoptosis [15]. CTLs secrete cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF)-α [27].

Although CTLs have a powerful ability to kill tumor cells in many ways, they fail to effectively eliminate cancer cells because their effector functions are impaired by a broad spectrum of immunosuppressive mechanisms that are evoked by cancer cells and their microenvironment that consists of a variety of immune cells and cancer-associated stromal cells [28]. These dysfunctional T cells, also known as exhausted T cells, were first described in chronic murine lymphocytic choriomeningitis virus infection but later found in cancer [29, 30]. One of the most famous hallmarks of exhausted T cells is upregulation of inhibitory receptors, called as immune checkpoints, including programmed cell death 1(PD-1), cytotoxic T lymphocyte antigen (CTLA)-4, T-cell immunoglobulin and mucin-domain containing (TIM)-3, lymphocyte-activation gene (LAG)-3, T-cell immunoreceptor with Ig and ITIM domains (TIGIT), CD160, and CD244 [29–32]. Cancer cells release a number of immunosuppressive factors into their TME. Cancer cell-derived adenosine activates the adenosine receptor A2aR in T cells, leading to the T-cell dysfunction, in addition to activate regulatory T (Treg) cell and myeloid-derived suppressor cells (MDSCs) [33]. Indoleamine-2,3-dioxygenase 1 (IDO1) in the TME catalyzes the degradation of the essential amino acid tryptophan and produces its metabolite kynurenine, inducing Treg cell activation and $CD8^+$ T-cell exhaustion [34]. Vascular endothelial growth factor A (VEGF-A) causes T-cell exhaustion through a variety of mechanisms such as inhibiting functional maturation of DCs, inducing Treg cell proliferation, MDSC accumulation, and upregulation of inhibitory checkpoints including PD-1, Tim-3, and CTLA-4 on $CD8^+$ T cells [35–37]. Cancer cells also produce transforming growth factor (TGF)-β and cyclooxygenase (COX)-2 as well as express PD-L1, contributing to T-cell dysfunction [15, 27]. A variety of regulatory immune cells in the TME, such as Treg cells, MDSCs, and tumor-associated macrophages (TAMs), exert immunosuppressive effects and inhibit T-cell activity.

$CD4^+$ T cells help for priming of $CD8^+$ T cells to express cytotoxic effector molecules, downregulate inhibitory receptors, and increase migration capacities [14, 27]. It is reported that $CD4^+$ CTLs kill tumor cells in several cancer types, including non-small-cell lung carcinoma (NSCLC), cutaneous T-cell lymphoma, and melanoma through death receptor signal such as Fas and TRAIL [27, 38].

Treg Cells

Treg cells are a subset of $CD4^+$ T cells and are characterized by positive expression of Foxp3 [39]. Treg cells specialize in maintaining self-tolerance and preventing autoimmunity through suppression of immune response. Treg cells induce T-cell exhaustion through the production of immunosuppressive molecules including interleukin (IL)-10, IL-35, TGF-β, IDO, VEGF, and adenosine and also cellular mechanisms such as CTLA-4-mediated suppression of APCs and expressing CD73 on their surface [32, 39–41]. IL-35 facilitates intratumoral T-cell exhaustion through the expression

of multiple inhibitory receptors such as PD-1, TIM-3, and LAG-3 or BLIMP1-inhibitory receptor axis in combination with IL-10 [42]. Treg cells are also reported to kill tumor-infiltrated effector T cells directly through the FasL-Fas signaling pathway and perforin/granzyme B secretion [43, 44] or indirectly through the deprivation of IL-2 which is indispensable for the survival of effector T cells [45]. Treg cells prevent functional maturation of DCs by CTLA-4 and lymphocyte function-associated antigen 1-dependent depletion of co-stimulatory signals on DCs. Tregs transform M1-like macrophages (M1) into M2-like immunosuppressive macrophages (M2) and suppress natural killer (NK) cell function [46, 47].

NK Cells

NK cells are one of the most characterized innate lymphoid cells (ILCs) and named in the basis of their strong cytotoxic ability to kill tumor cells [48]. NK cells are a central player of the innate immune system and kill cancer cells without any priming or prior activation. Their important defensive ability against cancer was first proved by the experimental evidence that xenograft tumor growth was accelerated in the mice by antibody-dependent NK cell depletion [49]. There have been also several significant positive correlations reported between impaired function of NK cells and poor clinical outcome regarding metastasis, postoperative recurrence, and survival, further indicating the protective role of NK cells against cancer [50].

NK cells present a variety of activating and inhibitory receptors on their surface [51]. Activation status of NK cells is determined by the balance of signals from these receptors [52]. NKG2A is the most common inhibitory receptor, interacting with HLA-E, and NKG2D is the activating receptor, interacting with major histocompatibility complex class I chain-related protein A (MICA) and MHC class I chain-related protein B (MICB) and UL16-binding proteins (ULBPs). Most normal healthy cells express MHC class I, which is recognized by NK cells and prevents it from killing by them. Cancer cells often lose their MHC I and thus become vulnerable to NK cell killing through secretion of cytotoxic granules containing perforin and granzymes. NK cells also produce pro-inflammatory cytokines including IFN-γ and TNFα, activating other immune cells. NK cells are activated by the several cytokines including IL-12, IL-2, IL-18, and IFN from macrophages and DCs [53].

Cancer cells escape the immune surveillance from NK cells in various manners. For instance, tumor cells express inhibitory molecules such as PD-L1 or shed ligands activating NK cells such as MICA, MICB, and ULBPs [52]. Tumor cells secrete immunosuppressive molecules such as IL-10 and prostaglandin E2 (PGE2), which activate immunosuppressors including MDSCs and Treg cells, leading to the inactivation or exhaustion of NK cells [50].

NKT Cells

NKT cells are a subset of CD1d-resricted T cells that have a characteristic of both conventional T cells and NK cells [54]. They are also divided into two subgroups, types I and II, based on the TCR repertoire. Type I NKT cells are well-defined and known to express the Vα14Jα18 invariant TCR α-chain in mice and Vα24Jα18 in humans. NKT cells recognize glycolipids and stress-related proteins via their TCR in the context of CD1d molecules and play the modulatory roles toward various immune cells by cytokine secretion and direct cell-cell contact. The most well-known glycolipid antigen to type I NKT cells is the marine sponge-derived α-galactosylceramide (α-GalCer) [55]. Activation of NKT cells depends on a balance between activating and inhibitory signals similar to NK cells.

The anti-tumor activity of NKT cells was first demonstrated by the mouse model with intravenous inoculation of melanoma cells or intraperitoneal inoculation of lymphoma cells [56]. These mice showed a significant prolongation of their survival when treated with the glycolipid α-GalCer [55, 56]. Furthermore, depletion of type I NKT cells accelerated tumor development in p53-deficient mice [57], indicating the important anticancer role of type I NKT cells. Type I NKT cells directly kill tumor cells via CD1d interaction by cytolysis using perforin, granzyme B, and FasL, upon their activation by tumor-derived glycolipids cross-presented by APC. They are also thought to exert anti-tumor activity indirectly through secretion of IFN-γ and activation of other immune cells.

Type II NKT cells have diverse TCRs and their roles are still not well-documented. They are not reactive to α-GalCer and are thought to make up an oligoclonal population that recognizes a diverse repertoire of lipid antigens [58]. Several papers describe the suppressive roles of type II NKT cells in tumor immunity through the production of IL-13, which activate MDSCs [59].

Macrophages

Macrophages are not a single-cell population with the uniquely defined characteristic but rather heterogeneous groups of innate immune cells with a diverse functional role [60]. They originated from either embryonic progenitor or hematopoietic stem progenitor cells and derived from monocytes in response to inflammation. Macrophages were historically classified into M1 and M2, representing pro-inflammatory and anti-inflammatory macrophages, respec-

tively [60, 61]. However, now macrophages are revealed to be more heterogeneous and dynamic, and their characteristics are continuously altered by their tissue microenvironment.

Macrophages that reside in the TME are called as TAMs [62]. Several experimental evidences reveal the pro-tumorigenic role of TAMs [62], and recent transcriptome analysis of 25 human cancer types showed the positive correlation between the presence of TAMs and poor prognosis [63]. TAMs support tumor angiogenesis by secreting pro-angiogenic factors including VEGFA, CXCL8, and CXCL12 and also help tumor cells metastasize [62, 64]. TAMs suppress immune cells especially CTLs in the TME via direct contact by immune checkpoint ligands such as PD-L1, PD-L2, CD80, and CD86, or secretion of cytokines such as TGF-β and IL-10, or depletion of metabolites such as L-arginine and production of ROS [60].

MDSCs

MDSCs are a poorly differentiated heterogeneous population of immature myeloid cells (IMCs) that have a strong immunosuppressive activity [65]. IMCs normally differentiate into macrophages, DCs, or granulocytes under healthy conditions and also rapidly expand into activated neutrophils and monocytes upon strong pathogenic stimuli, which is called myelopoiesis [66]. However, their appropriate differentiation is impaired in the presence of long-lasting low-strength signals from chronic infection, inflammation, and cancer, leading to the accumulation of MDSCs [67]. A variety of tumor-derived factors contribute to covert IMCs into MDSCs such as IL-6, IL-10, IL-1β, TGF-β, VEGF, and stem cell factor (SCF) [26, 65]. In addition, several chemokines in TME recruit MDSCs into TME including CCL2, CXCL5, CCL15, and CXCL12 [65, 68–70].

MDSCs are classified into two cell types, polymorphonuclear MDSCs (PMN-MDSCs), which inherit the feature of granulocytes, and monocytic MDSCs (M-MDSCs), which are similar to monocytes. PMN-MDSCs are dominant in most case of cancer among MDSCs [66]. M-MDSCs and PMN-MDSCs induce immunosuppression using different mechanisms. PMN-MDSCs mainly induce antigen-specific T-cell suppression or tolerance through the production of reactive nitrogen and oxygen species [nitric oxide (NO), reactive oxygen species (ROS), and peroxynitrite], which eliminate key nutritional factors for T cells [66]. Meanwhile, M-MDSCs suppress T-cell responses in antigen-specific as well as nonspecific manners such as expression of Arg1, NO, TGF-β, and IL-10. MDSCs induce the generation of immunosuppressive Treg cells and M2 macrophages through the secretion of IL-10 and IFN-γ [66]. MDSCs also express several immune-regulatory molecules such as PD-L1 and FasL.

B cells

The role of B cells in cancers is less well investigated and seems to be controversial. There are a substantial number of B cells in the TME of several cancer types [71, 72]. Early murine study showed the depletion of B cells increased the resistance of the mice to the inoculation of syngeneic fibrosarcoma [73]. Tumor-infiltrating B cells are reported to secrete pro-angiogenic factor lymphotoxin and contribute to promote prostate cancer progression. A subset of B cells, named as B regulatory cells (Breg), have been reported and secreted immunosuppressive cytokines, TGF-β and IL-10 [74]. These data supported the pro-tumorigenic role of B cells. On the other hand, clinical studies of several cancer types showed the positive correlation between the presence of B cell in the TME and better patient outcome [73]. Antibodies against intracellular tumor antigens are frequently found in cancer patients. Indeed, experimental evidence shows that B-cell-derived antibodies have been shown to bind tumors in an antigen-specific manner and induce complement-dependent lysis [73].

HCC Immunology

Immunology and immunological microenvironment of HCC have been recently extensively studied [75–79]. Immune dysregulation, including changes in the number and/or function of immune cells, expression of their receptors or ligands, altered cytokine/chemokine levels contribute to HCC development and progression (Fig. 31.4). This paragraph summarizes the roles of each immune cell type in HCC and the cross-talks between immune cells and cancer cells in the HCC TME.

Cytotoxic T Cells

Meta-analysis including 21 studies with approximately 3500 HCC patients shows that the existence of high magnitude of $CD8^+$ TILs is associated with a better prognosis [80], suggesting the importance of CTLs for the immune control of HCC and also the promise of T-cell-based immunotherapy. On the other hand, recent single-cell RNA-sequence analysis of 5000 T cells in combination with T-cell repertoire analysis in HCC patients revealed that clonally exhausted $CD8^+$ T cells and CTLA-4^{high} Treg cells were enriched in the HCC TME, preventing from eliciting sufficient T-cell-mediated killing of tumor cells [81]. Furthermore, tumor-infiltrating $CD8^+FOXP3^+$ regulatory T cells shared the same TCRs with other exhausted $CD8^+FOXP3^-$ T cells, suggesting that HCC tumor microenvironment altered tumor-infiltrating $CD8^+$ T cells into exhausted status and occasionally into suppressive cells. PD-1^{high} $CD8^+$ exhausted TILs tend to express multiple

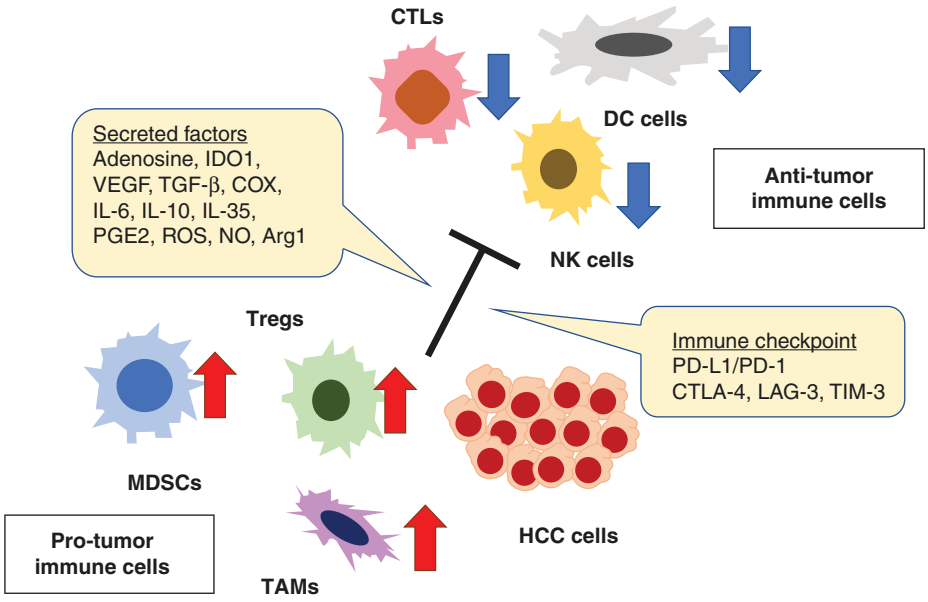

Fig. 31.4 Immunosuppressive microenvironment of HCC

immune checkpoint receptors including TIM-3 and LAG-3, and HCC patients with PD-1^{high} CD8$^+$ exhausted TILs show the aggressive tumor features and higher PD-L1 expression on tumor [82]. The large transcriptional heterogeneity of CD8$^+$ TILs exists among HCC patients, affected by HBV levels and antiviral treatment. IL-12-mediated pathway is associated with the functional status of CD8$^+$ TILs in HCC, and its activation indicates a better prognosis [83]. Several mechanisms of CD8$^+$ T-cell exhaustion in HCC have been considered including acidic and hypoxic TME, metabolic competition with tumor cells, and interaction with immunosuppressive molecules (e.g., IL-10, VEGF, IDO) and cells (e.g., Tregs, MDSCs) [84]. Abundant expression of TOX in CD8$^+$ T cells was observed in the HCC TME and promotes PD1 translocation to their cell surface [85]. Upregulation of long noncoding RNA Lnc-Tim-3 was detected in the HCC TME and promotes T-cell exhaustion in HCC via binding to Tim-3 [86]. T-bet transcriptional factor negatively regulates PD-1 expression in CD8$^+$ T cells in the HCC TME [87].

Among 547 HCC patients, circulating and liver-infiltrating CD4$^+$ CTLs increased in early-stage HCC patients but decreased in advanced-stage HCC patients [88]. The loss of CD4$^+$ CTLs was significantly associated with poor survival and high recurrence rates of HCC patients, suggesting the tumor-suppressive role of CD4$^+$ CTLs.

Tregs

Meta-analysis including 3854 HCC patients from 27 cohort studies shows that higher levels of Treg cells in the tumor and peripheral blood but not in the peritumoral area are significantly associated with shorter OS and DFS of HCC patients [89]. In addition, the patients with higher levels of Treg cells have multiple tumors, higher AFP levels, poorly differentiated tumors, advanced TNM stage, and vascular invasion, suggesting the potent immunosuppressive functions of Treg cells in HCC. Accumulation of Treg cells is also associated with reduction of CD8$^+$ T cells in HCC [90–92]. Treg cells from HCC patients inhibit proliferation, activation, degranulation, and production of granzyme A, granzyme B, and perforin of CD8$^+$ T cells [91]. Treg cells also promote angiogenesis and downregulate the expression of co-stimulatory molecules CD80/CD86 on DC cells and suppress their production of TNF-α and IL-12 in HCC [93–95]. The immunosuppressive function of Treg cells is partly via the upregulation of glucocorticoid-induced tumor necrosis factor receptor (GITR) [96]. CXCL10/CXCR3 signaling and CCL28 contribute to recruit the Treg cells into the HCC TME [95, 97].

NK Cells

The percentage of NK cells among total lymphocytes is much higher in the liver compared to that in the peripheral blood or spleen [51]. The number of infiltrating CD56$^+$ NK cells is positively correlated with better patient survival of HCC [98–100], suggesting that NK cells play critical roles in the surveillance and prevention of HCC. Meanwhile, the number of intrahepatic NK cells is reduced in HCC patients, and the function of NK cells in the TME is impaired [51]. NK cells in the TME show a lower capacity of secreting IFN-γ and TNF-α than non-tumor NK cells [100]. A variety of mechanisms of NK cell dysfunction in HCC patients are reported. We have previously shown that HCC cells shed membrane-bound MICA and escape from immune surveillance of NK cells [101]. Furthermore, we and others showed that soluble MICA, shed by ADAM10 and ADAM17, works

as a decoy of NKG2D receptor and prevents the activation of NK cells [101–103]. The importance of impaired anti-tumor surveillance by NK cells via blockade of NKG2D/MICA signal was later highlighted by the genome-wide association study (GWAS) showing the strong association between the locus in the 5′ flanking region of MICA and HCC occurrence in chronic hepatitis C patients [104]. We have shown that a subset of HCC cells with positive expression of CD133, one of the cancer stem cell markers, has high ADMA9 protease activity and thereby becomes insensitive to NK cell cytolytic activity via MICA shedding [105]. NK cell dysfunction is induced in HCC by a variety of molecules including TGF-β1, AFP, PGE2, IDO, and hepatocyte growth factor (HGF) and the interaction with other cell types in the TME such as MDSCs, monocytes/macrophages, and cancer-associated fibroblasts (CAFs) [106–110]. MDSCs suppress cytokine production and cytotoxicity of NK cells by direct contact via the NKp30 receptor [111]. Monocytes/macrophages induce NK cell dysfunction through CD48/2B4 interaction [100]. There are several markers for dysfunctional/exhausted NK cells in the HCC TME. CD11b-CD27 NK cells exhibit an inactive and immature phenotype, and its frequency is positively correlated with tumor progression [112]. $CD96^+$ NK cells induced by TGF-β1 in the TME are functionally exhausted, and its frequency is positively correlated with poor prognosis of HCC patients [113].

NKT Cells

Although NKT cells are enriched in the liver, relatively fewer studies have addressed to elucidate their role in HCC and are still somewhat controversial. One report shows that high levels of intratumor NKT cells are correlated with better OS and PFS after surgical resection, suggesting that NKT cells suppress tumor recurrence in HCC [114]. To support this finding, NKT cells activated by α-GalCer- or DC-mediated ex vivo stimulation suppress HCC growth in mice [115, 116]. On the other hand, one study reports that $CD4^+$ iNKT cells increased in HCC show higher Th2 cytokine production and lower cytolytic activity, inhibiting the expansion of tumor antigen-specific $CD8^+$ T cells [117]. Furthermore, iNKT cells are experimentally shown to contribute to HCC development in murine NASH liver [118]. Taken together, NKT cells might have context-dependent dual roles in HCC, and further studies are necessary to clarify its role in HCC.

B cells

Tumor-infiltrating B cells (TIBs) are observed in close proximity to tumor-infiltrating T cells, and their presence is correlated with better survival of HCC patients [119]. The density of TIBs is also associated with the enhanced expression of IFN-γ and granzyme B, suggesting that the presence of B cells enhances the local T-cell activation [119, 120]. Animal experiments using syngeneic mouse model showed that B-cell depletion abrogated $CD4^+$ T-cell activation and induced $CD8^+$ T-cell exhaustion, impairing the tumor control [119]. These data strongly suggest the anti-tumor activity of TIBs in HCC. Meanwhile, a subset of B cells defined as Breg have been identified as contributors to the pathogenesis of neoplastic diseases. Peripheral blood $CD19^+CD24^+CD38^+$ Breg cell frequency is significantly higher in HCC patients and correlated with advanced disease status. $CD19^+CD24^+CD38^+$ Breg cells produce IL-10 and induce HCC cell proliferation [121]. Similarly, $CCR6^+CD19^+CD5^+$ Breg cells respond to tumor cell-derived CCL20 and enhance angiogenesis, leading to HCC progression [122]. Activated CCL20/CCR6 axis is correlated with poor prognosis of HCC patients. Recent report shows that plasma cells undergo IgM-to-IgA class-switch recombination in the NASH liver in response to TGF-β [123]. Liver-infiltrating IgA-expressing plasmocytes express PD-L1 and secrete IL-10, inducing the exhaustion of $CD8^+$ T cells [123]. Taken together TIBs may be a heterogeneous population, and their further subclassification may be necessary to understand their roles in HCC.

DC Cells

DC cells are mostly immature state and reside adjacent to the portal spaces in the liver. In the murine liver, four distinct DC subsets are found including conventional myeloid DCs, plasmacytoid DCs, $CD8\alpha^+$ DCs, and NK DCs [124]. While pDCs are predominant in the mouse liver, mDCs seem to be predominant in the human liver [124]. The number of peripheral mDCs decreases in HCC patients with impaired function of IL-12 secretion [125, 126]. Increase in systemic IL-10 levels may cause the functional impairment of circulating DCs in HCC [127]. Meanwhile, there is a new immunosuppressive subset of human $CD14^+CTLA\text{-}4^+$ regulatory dendritic cells in the peripheral blood of HCC patients [128]. These cells suppress T-cell response via expression of IL-10 and IDO. However, another group did not see the association between this subtype and HCC [129]. Therefore, the significance of this DC subset needs to be further validated using larger cohorts.

Macrophages (TAMs, Kupffer Cells)

There are a handful of experimental evidences to support the pro-tumorigenic roles of TAMs in HCC via section of TNF-α, IL-6, and IL-1β in DEN-driven HCC model and orthotopic HCC tumors [130–132]. $PD\text{-}L1^+$ TAMs co-localized with exhausted $PD\text{-}1^+CD8^+$ T cells are increased in HCC tumor tissues and are positively correlated with poor survival [133]. The high density of TAMs is also associated

with the increase in Treg cells in the TME and correlated with poor prognosis in HCC patients [134]. Collectively, TAMs play the immunosuppressive and tumor promoting roles in human HCC.

TAMs are induced and/or infiltrated into the TME through a variety of mechanisms including IL-10 production from HCC cells and hypoxic tumor microenvironment [133, 135]. HCC cells secrete Golgi protein 73 (GP73) upon ER stress, which in turn activate TAMs through GRP78 [136]. DAMPs from dying hepatocytes also educate TAMs via upregulating NADPH oxidase 1 (NOX1) [137]. Tumor cell-intrinsic osteopontin activates TAM via CSF1-CSF-1R pathway [138]. CCR6/CCL20 pathways also contribute to recruit TAMs [87].

TAMs suppress cytotoxic T-cell functions via PD-L1/PD-1 interaction, galectin-9/TIM-3 interaction, and IDO production in the HCC TME [133, 139, 140]. $CD14^+$ TAMs also promote the expansion of HCC $CD44^+$ cancer stem cells via IL-6/Stat3 signaling [141]. In the hypoxic TME, Hif1-α upregulates TREM-1 expression in TAMs, and $TREM\text{-}1^+$ TAMs express high levels of PD-L1 and CCL20, inducing CTL exhaustion in combination with recruitment of $CCR6^+Foxp3^+$ Tregs [142].

MDSCs

Frequency of M-MDSCs, defined by $CD14^+HLA\text{-}DR^{-/low}$, and PMN-MDSCs, defined by $LOX\text{-}1^+CD15^+$ are both increased in the PBMCs of HCC patients and positively correlated with worse patient outcomes [143–146], suggesting that MDSCs exert a tumor-supporting function in human HCC. MDSCs suppress a variety of anti-tumor immune cells including NK cells, DCs, and CTLs in HCC [111, 147, 148]. MDSCs also decrease the anticancer function of Kupffer cells in HCC [149].

MDSCs are recruited into the HCC TME and activated by a variety of mechanisms [150]. Hypoxia activates ectonucleoside triphosphate diphosphohydrolase 2 (ENTPD2) in cancer cells, which in turn promote the maintenance of MDSCs through the conversion of extracellular ATP to 5′-AMP [151]. Decrease of receptor-interacting protein kinase 3 (RIP3), the core regulator of necrosis, in HCC cells recruits MDSCs via the CXCL1/CXCR2 axis [152]. Tumor-associated fibroblasts attract monocytes by the stromal cell-derived factor (SDF)-1a/CXCR4 pathway and turn them into MDSCs via IL-6-mediated stat3 activation [150]. Hepatic stellate cells (HSCs) also recruit MDSCs via cyclooxygenase-2 or p38 MAPK signaling [153, 154]. Obesity-based IL-6 and androgen signaling-induced cell cycle-related kinase (CCRK) also recruit MDSCs via G-CSF in HCC [155].

HCC Genetic Drivers and Immune Regulation

The association between immune dysregulation and molecular feature of HCC has been recently investigated (Fig. 31.5) [10]. Analysis of 956 HCC patient samples identifies the immune class in 25% of cases, characterized by extensive intratumor infiltration of various immune cells. This class is further divided into two subtypes, exhausted and active immune. The former shows TGF-β signaling activation accompanied with high levels of T-cell exhaustion makers. The HCC patients with active immune subtype show longer survival than others. In contrast 25% of HCC patients show fewer immune cell filtration, and they are associated with CTNNB1 mutation, suggesting the involvement of WNT/CTNNB1 pathway signaling in suppression of antitumor immunity. Very recently, using a novel mouse model that rapidly induces autochthonous and mosaic liver tumors with customizable genetic drivers and certain immunogenicity, it is shown that activation of WNT/CTNNB1 signal-

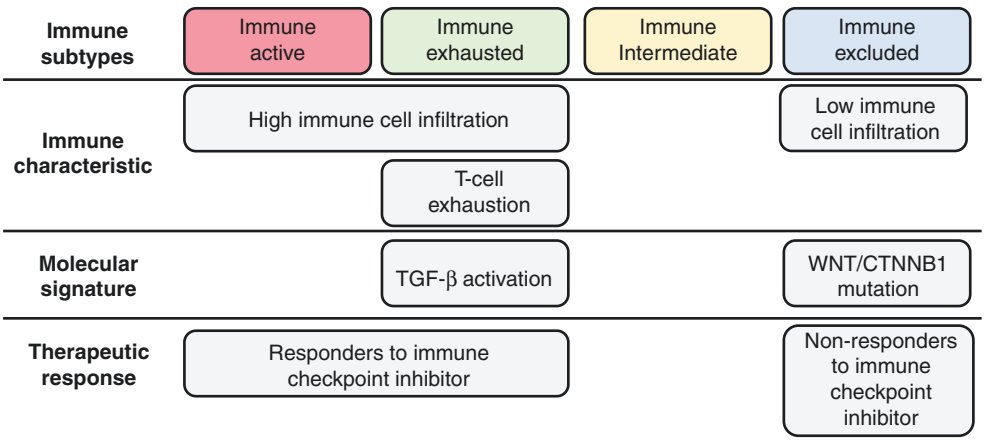

Fig. 31.5 Immune classification of HCC with genomic and clinical features

ing promotes immune escape of HCCs from CD8 T cells via suppression of CCL5-mediated DC recruitment [156]. Anti-PD-1 therapy is not effective for CTNNB1-driven mouse liver tumors because of the absence of CD8 T-cell intratumor infiltration. Prospective tumor sequences of 31 HCC patients who undergo checkpoint inhibitors show that WNT/CTNNB1 signaling activation is indeed associated with lower disease control rate and shorter median PFS and OS [157]. Taken together, these findings suggest that the CTNNB1 mutation/activation status could be the indicator of immune "hot" or "cold" tumors and thus might be the efficacy biomarker of immune checkpoint inhibitors in HCC.

Immunotherapy of HCC

In general, cancer cells acquire hundreds of genetic mutations, which produce high antigenic neoantigens, leading to activation of anticancer immune system. However, as mentioned above, HCC cells cleverly evade or suppress the immune system by altering their characteristics and producing immunosuppressive molecules including cytokines/chemokines in concert with stromal cells in their TME. Restoring/reactivating anti-tumor immune systems has been considered to be a powerful therapeutic, and a variety of strategies have been tested experimentally or clinically. Among them, checkpoint inhibitors have achieved the biggest success and are now clinically used in several cancer types. In this paragraph, we will review the current state of cancer immunotherapy toward HCC.

Checkpoint Inhibitors

Under physiological conditions, inhibitory checkpoint molecules such as PD-1, PD-L1, CTLA-4, and Tim-3 prevent unwanted T-cell hyperactivation and maintain homeostatic immune-tissue network. On the other hand, cancer cells induce the upregulation of these inhibitory immune checkpoints on their own or on immune cells and suppress anti-tumor immune system. Checkpoint inhibitors take the brakes off the immune system and unleash anti-tumor immune responses. This approach has been a great success and the main breakthrough in cancer treatment during the last years.

Among these inhibitory checkpoints, PD-1 is mainly expressed on T cells and bound with its ligand PD-L1 and PD-L2. This binding suppresses T-cell migration, proliferation, and cytokine secretion. PD-L1 is expressed on APCs and tumor cells. In HCC, PD-L1 is mainly expressed in Kupffer cells and less expressed in other APCs or HCC tumor cells [133, 158]. Higher PD-L1 expression levels in tumors, peritumoral hepatocytes, and circulation are reported to be associated with disease aggressiveness and/or poor prognosis of HCC patients [159–161], suggesting a checkpoint-dependent T-cell exhaustion mechanism in HCC. Nivolumab, a fully human IgG4 monoclonal anti-PD-1 antibody, was tested for advanced HCC as the phase I/II Checkmate-040 trial and showed promising results (20% response rate, 64% disease control rate, mild AEs). This trial scaled up and showed the median survival time of 28.6 months and 15 months for advanced HCC patients as a first-line and second-line therapy, respectively. Based on these results, the FDA approved nivolumab as a second-line agent after sorafenib in September 2017. Subsequently, the phase III CheckMate-459 study of nivolumab for unresectable HCC as a first-line therapy was conducted, but the recently announced results revealed that nivolumab did not achieve a statistical significance for improved OS compared with sorafenib, although it showed a trend toward improvement. Pembrolizumab, another recombinant human IgG4 monoclonal anti-PD-1 antibody, was tested for advanced HCC as the phase II KEYNOTE-224 study and achieved accelerated approval by the FDA in November 2018 with similar response to nivolumab (17% response rate and the median survival time of 12.9 months). However, pembrolizumab also missed the primary endpoint of the subsequent phase III KEYNOTE-240 trial for unresectable HCC as a second-line therapy compared to the best supportive care, although results showed that the pembrolizumab did improve OS versus placebo (HR, 0.78; 95% CI, 0.611–0.998; $p = .0238$).

CTLA-4 is another inhibitory checkpoint expressed on effector T cells and bound with B7 ligand. This binding delivers an inhibitory signal to T cells. CTLA-4 is constitutively expressed in Treg cells, which exert immunosuppression [93]. Tremelimumab is a human monoclonal anti-CTLA-4 antibody and was tested for advanced HCC as a phase II trial. RR was 17.6% and median OS was 8.2 months [162]. TIM-3 is another inhibitory receptor, and increased infiltration of TIM-3-positive cells in HCCs is associated with poor prognosis [163, 164].

Although checkpoint inhibitors achieved complete or durable response for some of HCC patients, three-quarters of patients cannot obtain the benefit. To further enhance the treatment efficacy of checkpoint inhibitors, a variety of combinational therapies have been tested. Potential synergistic combinations include checkpoint inhibitors with conventional locoregional therapies or anti-angiogenic drugs or other checkpoint inhibitors. Especially, the combination therapy between checkpoint inhibitor and anti-angiogenic drug has shown very promising results. The response rate of phase Ib trial results of combination therapy between atezolizumab, anti-PD-L1 antibody, and bevacizumab, anti-VEGF

antibody for advanced HCC reached 65%, which let FDA designate this combination trail as a breakthrough therapy in July 2018. In October 2019, media release has been just recently posted, announcing that the following phase III IMbrave150 study met its primary endpoints demonstrating statistically significant and clinically meaningful improvement in OS and PFS compared with sorafenib, although its detail is not publically presented yet. Similarly, phase Ib KEYNOTE-524/Study 116 trial of combination treatment with pembrolizumab and lenvatinib, a multi-tyrosine kinase inhibitor, was conducted for advanced HCC. The response rate was 42.3% with no PD patients [165]. Based on the results, this combination therapy was also designated as a breakthrough therapy by the FDA in July 2019. Since anti-angiogenic drugs such as bevacizumab and lenvatinib may exacerbate tumor hypoxia, these drugs may potentially enhance the expression of immune checkpoint molecules and suppress anti-tumor immune response. Simultaneous checkpoint inhibitor may reactivate immune response and exert a potent synergistic effect [166]. The immune response of combination therapy between PD-1 blockade and lenvatinib has been investigated in the syngeneic mouse HCC model, showing that the combination treatment increases CD8 T-cell population but decreases monocyte and macrophage population, which might contribute to its synergistic anti-tumor effect [167]. Considering favorable recent clinical results, these combination therapies may replace the current first-line therapy toward advanced HCC anytime soon.

Cancer Vaccines and DC-Based Immunotherapy

Cancer vaccines aim to activate tumor-specific immune responses of cytotoxic effector cells, mainly CTLs. A variety of cancer vaccines are being tested including proteins, peptides, tumor lysates, and viral vectors. The most well-studied target antigen for HCC is GPC3, because it is over-expressed in the majority of HCC but not in normal tissue [163]. GPC3 peptide induces a GPC3-specific CTL response in advanced HCC but failed to show statistically significant reduction of the recurrence rate in patients at adjuvant setting [168]. Similarly, hTERT and AFP vaccine clinically failed in HCC [84].

Among APCs, DCs have been used together with tumor vaccine, because of the most potent ability to induce an antigen-specific T-cell response. Indeed, a DC vaccine called sipuleucel-T was already approved by the Food and Drug Administration (FDA) for metastatic prostate cancer in the USA [169]. Several groups tried AFP-derived peptides to pulse DCs in HCC patients, but clinical results were not satisfactory because of the weak immune activation due to the self-nature of AFP [170]. Infusion of mature autologous DCs pulsed with lysates of liver cancer cell line ex vivo shows a partial radiological response in advanced HCC [171, 172]. DC infusion was also tested in adjuvant setting [173, 174]. Recent meta-analysis of DC-based immunotherapy including 19 clinical trials of 1276 cases reveals that DC-based therapy significantly prolonged PFS and OS of HCC patients with no severe adverse events [175].

NK Cells-Based Immunotherapy

NK cells often become dysfunctional in many cancer types, so several approaches are performed to explore NK cell-based cancer immunotherapy including cytokines and antibodies to activate NK cells or adoptive NK cell transfer. We have previously shown that epirubicin, used in transarterial chemoembolization (TACE), and sorafenib augmented NK cell activity via suppression of MICA shedding in HCC cells [176, 177]. Several other groups also showed that NKG2D ligand including MICA is upregulated by sodium butyrate and several histone deacetylase inhibitors in HCC [178–180]. We have shown that intrahepatic delivery of alpha-GalCer-pulsed or IL-12-treated DCs suppress murine liver tumor via hepatic NK cell activation [181, 182]. The anti-tumor effect of IL-12 via NK cell activation was shown by other groups [183, 184]. Regarding cell-based therapy, allogenic natural killer cell immunotherapy combined with cryoablation significantly improved PFS without severe adverse side effects [185]. In addition, glypican-3-specific chimeric antigen receptor (CAR)-modified NK cell-based immunotherapy was reported to show a strong anti-tumor activity toward HCC xenografts [186]. There are currently several clinical trials ongoing to test the efficacy of NK cell-based immunotherapy [187].

Cytokine-Induced Killer (CIK) Cell-Based Immunotherapy

CIK cells consist of $CD3^+CD56^+$ cells, $CD3^-CD56^+$ NK cells, and $CD3^+CD56^-$ cytotoxic T cells and are created by incubation of peripheral blood mononuclear cells with IL-2 and anti-CD3 antibody [188]. CIK cells are mainly T effector memory $CD8^+$ T cells with NK-like cytotoxicity [188]. A significant prolongation of recurrent-free survival was achieved by CIK cell therapy in a multicenter, randomized, open-label, phase 3 trial for HCC as adjuvant setting [189]. A follow-up study of enrolled patients in this phase III trial confirmed that the significant improvement in RFS and OS in the adjuvant CIK cell-based immunotherapy group lasted over 5 years without boosting [190]. Meta-analysis of adjuvant CIK therapy for HCC including eight randomized clinical trials concludes that CIK therapy shows a higher survival

rate and a significant reduction of relapse rate compared to non-CIK therapy [191]. On the other hand, CIK therapy lacks efficacy in advanced HCC, because CIK therapy increases MDSCs, leading to the impairment of cytotoxic activity of CIKs [192]. PDE5 inhibitor is reported to enhance CIK cell therapy via suppression of MDSC activity [192].

Target MDSCs

Since MDSCs promote immunosuppression and angiogenesis in the TME, several attempts have been made to target MDSCs. Strategies to target MDSCs include their depletion, blockage of their trafficking and migration into TME, and inhibition of their immunosuppressive functions. Among the clinically available drugs for HCC, cabozantinib was reported to reduce intratumoral PMN-MDSCs in a prostate cancer model. But it has never been assessed in HCC patients yet [193]. At the preclinical level, TRAIL receptor 2 (TRAIL-R2/DR5) agonistic antibody DS-8273a eliminated MDSCs in the phase I clinical trial for patients with advanced cancers including HCC [194]. To block the recruitment of MDCSs in the TME, CCR5 and CXCR2 inhibitors were tested for some solid tumor types and showed the inhibition of MDSC trafficking, but they have never been tested in HCC [195]. Tadalafil, the FDA-approved PDE5 inhibitor, reversed the suppressor function of MDSCs in the HCC mouse models [192].

Target TAMs

Because TAMs facilitate HCC progression through directly affecting tumor cells as well as modulating the immune system in the TME, targeting TAMs could be a promising therapeutic to HCC. Current approaches are still preclinical but aim to eliminate TAMs, block their recruitment, and reprogram TAM to have an anti-tumoral phenotype. For depletion of TAMs, clodronate-encapsulated liposomes can be used. Liposomes upon phagocytosis by macrophage release clodronate and induce apoptosis of macrophages. Their administration was shown to inhibit tumor growth in the orthotopic HCC model [196]. For the inhibition of the monocyte recruitment, CCL2-CCR2 signaling axis is an important target. Several groups demonstrated that CCR2 antagonist or CCL2-neutralizing antibody inhibited HCC growth by blocking TAM-mediated immunosuppression [197–199]. On the other hand, this treatment strategy could be potentially harmful, because senescent hepatocyte-derived CCL2 attracts monocyte-derived macrophages, which result in the clearance of senescent hepatocytes, thereby preventing HCC development [200, 201]. For the reprogramming polarization of TAMs, baicalin, a natural flavonoid, and CSF-1R inhibitor were experimentally shown to polarize TAMs into an M1-like phenotype and thereby suppressed HCC growth in xenograft model [202, 203].

Target Treg Cells

There are still only a few experimental data available to target Treg cells in HCC. Treatment with soluble GIRT ligand suppresses the anti-tumor function of Treg cells, leading to the restoration of the proliferative capacity and cytokine production of $CD4^+CD25$- T cells in vitro [204]. TGF-β blockage suppressed the Treg cells in the HCC TME, leading to the suppression of HCC metastasis in mice [205]. In the clinical setting, sorafenib significantly decreased the frequency and absolute number of $Foxp3^+$ Tregs in blood of HCC patients [206]. Furthermore, OS was improved in patients with a greater reduction of the number of $Foxp3^+$ Tregs upon sorafenib treatment [206].

TCR-Engineered T-cell-Based Immunotherapy

Immunotherapy using T cells with engineered TCR has been rigorously investigated. There are several clinical trials including TCRs specific for tumor-associated antigens of AFP, p53, CEA, NY-ESO, etc. [207–210]. These TCR genes were isolated from the T-cell clones by repeated in vitro antigen stimulation or in vivo immunization in the HLA-A2 transgenic mice [211]. Recent report shows that HCC cells in chronic hepatitis B patients contain short-length HBV mRNAs and produce HBV-derived epitopes that can be recognized by TCR and thus activate T cells [212]. Therefore, autologous T cells engineered to express TCRs specific for these epitopes could be used for personalized immunotherapy.

CAR-Engineered T-cell Immunotherapy

CAR-T cells are genetically engineered T cells expressing CAR on their surface [169]. CAR-T cells recognize tumor cell surface antigens via the extracellular antigen recognition domain of CAR that have single-chain variable regions composed of the heavy and light chains of a tumor surface antigen-specific monoclonal antibody. Intracellular signaling domains of CAR consist of binding of co-stimulatory molecules to the intracellular portion of TCR, which are required for signal transduction and their own activation [169]. CAR-T cells have achieved a big success in treating CD19-positive hematological malignancies [163]. For HCC, several groups examined the efficacy of GPC3-CAR-T cells and reported the anti-tumor effect on HCC xenograft or mouse models

[213–215], but its efficacy for HCC patients is still very limited [215]. A robust anti-tumor activity of the AFP-CAR-T cells is also shown in HCC xenograft [216]. However, it efficacy has never been studied for HCC patients yet.

Conclusion

Innate and adaptive immune systems are important for cancer prevention and progression. However, their functions are dysregulated due to the sustained necroinflammation in patients with chronic liver disease or cirrhosis, creating a tumor-promoting environment [75]. In HCC TME, Tregs, TAMs, and MDSCs predominantly suppress anti-tumor immunity via the downregulation of the effector and cytotoxic activities of CTL and NK cells. Immune checkpoint inhibitors in combination with anti-angiogenic drugs exert a potent anti-tumor effect toward advanced HCC, probably through breaking immunosuppressive TME. Further studies are warranted to understand the cross-talk and interplay among these immune cells and cancer cells, which facilitate to develop better immunotherapeutics against HCC.

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Acute-on-Chronic Liver Failure

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Abbreviations

AARC	APASL ACLF research consortium
ACLF	Acute-on-chronic liver failure
AD	Acute decompensation
AIH	Autoimmune hepatitis
APACHE	Acute Physiology and Chronic Health Evaluation
APASL	Asian Pacific Association for the Study of the Liver
CANONIC-CLIF	Acute-on-chronic liver failure in cirrhosis
DAMPs	Damage-associated molecular patterns
EASL-CLIF	European Association for the Study of the Liver Chronic Liver Failure consortium
HE	Hepatic encephalopathy
MELD	Model for end-stage liver disease
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failures assessment

Key Points

- Acute-on-chronic liver failure (ACLF) is a distinct syndrome characterized by high 28-day mortality.
- ACLF is characterized by acute hepatic insult in a patient with diagnosed or undiagnosed chronic liver disease/cirrhosis.
- Acute insult can be inflicted by alcohol, virus (hepatitis B, hepatitis A or E, or a nonhepatotropic virus), drug, herbal supplement, autoimmune, or Wilson's flare.
- Postacute insult, pathogenesis of ACLF is based upon systemic inflammatory response, persistent inflammation, gut dysbiosis, and increased gut permeability, leading to cytokine storm in the portal and systemic circulation and organ failure.
- "Golden window" of 7 days usually precedes development of sepsis, organ failure providing opportunity for interventions, supportive care, organ support, and guiding management.
- Abstinence, steroids, and antivirals may be used as specific etiology-based therapies in ACLF, and GCSF as a nonspecific regenerative therapy.
- Plasma exchange or artificial liver support system such as MARS or Prometheus may help as adjunctive therapies.
- Liver transplant is the definitive therapy, and nearly 80% 1-year survival can be achieved with optimal selection and timing.

Acute-on-chronic liver failure (ACLF), as a term, first came into existence in 1995 when the Japanese review described alcoholic hepatitis, case of acute liver injury superimposed on cirrhosis, a condition different from acute liver failure [1]. Acute liver failure (without coexistent liver failure), acute-on-chronic liver failure (on background of underlying chronic liver failure), and acute worsening of decompensated cirrhosis denote the spectrum of liver failure and are usually associated with extrahepatic organ failure and high short-term mortality [2]. There are at least 13 definitions being propagated to define ACLF [3], owing to an overlap between the terminologies; however, the most commonly cited remain the Asian Pacific Association for the Study of the Liver (APASL) [4] and the European Association for the Study of the Liver (EASL) Chronic Liver Failure (EASL-CLIF) consortium (Fig. 32.1) [5, 6].

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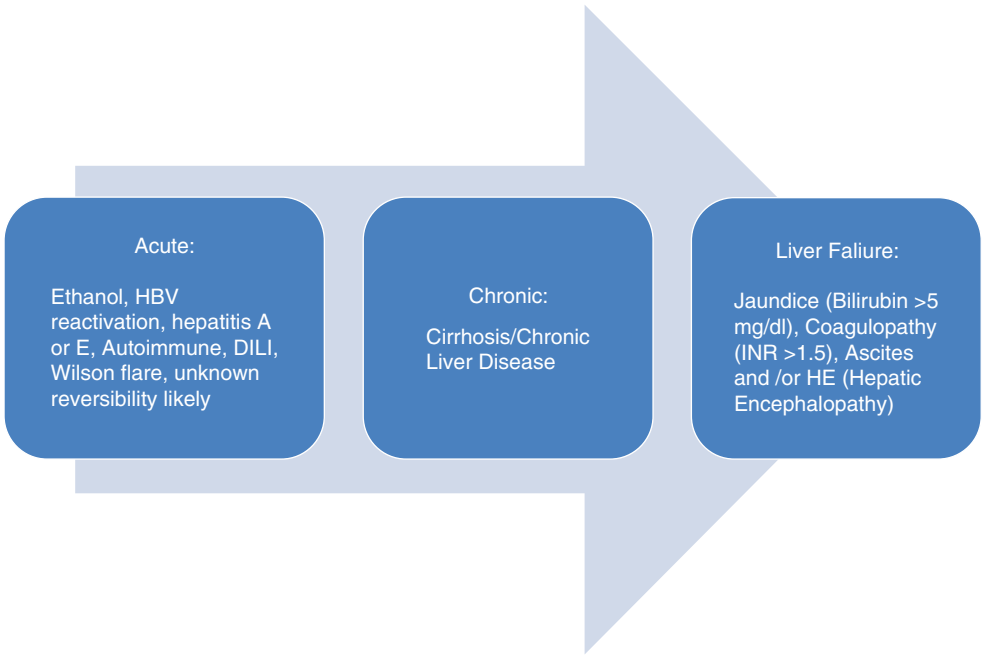

Fig. 32.1 Outline and concept of ACLF. Hepatic insult is the acute insult that leads to ACLF in patient with underlying chronic liver disease. Severity and extent of the acute insult and the stage of underlying chronic damage to liver helps in determining the outcome

Table 32.1 Comparison of the commonly accepted ACLF definition

	APASL	EASL/CLIF	NASCELD
Definition	Acute hepatic insult manifesting as jaundice and coagulopathy complicated within 4 weeks by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis associated with high mortality	An acute deterioration of preexisting chronic liver disease usually related to a precipitating event and associated with increased mortality at 3 months due to multisystem organ failure	A syndrome characterized by acute deterioration in a patient of cirrhosis due to infection presenting with two or more extrahepatic organ failure
Diagnosis	Early reversibility is likely and nontransplant interventions may affect outcome	Too late, reversibility is unlikely and nontransplant interventions may not affect outcome	Too late, reversibility is unlikely and nontransplant options may not affect outcome
Time frame	4 weeks	4–12 weeks (variable)	Not defined
Acute insult	Hepatic	Hepatic or extrahepatic (systemic)	Infection, i.e., systemic (extrahepatic)
Sepsis	Consequence/complication of liver failure	Cause/precipitant of liver failure	Cause/precipitant of liver failure
Golden window	Well defined for therapy, i.e., by 7 days SIRS or sepsis as well as for decision regarding liver transplant	No such concept	No such concept
Reversibility	Yes	Not described	Not described
Decompensated cirrhosis	Excluded	Included	Included

Defining Acute-on-Chronic Liver Failure

ACLF is defined as a clinical syndrome characterized by severe and acute hepatic dysfunction from varying insults and carries high short-term mortality [7]. The first consensus definition of ACLF was proposed by APASL in 2009 [8], and main distinguishing feature from rest of the definition remains the use of hepatic insults in defining liver failure. The APASL ACLF Research Consortium proposed a new definition in 2014 consensus statement, that is, "ACLF is an acute hepatic insult manifesting as jaundice (serum bilirubin ≥5 mg/dL (85 micromol/L) and coagulopathy (INR ≥ 1.5 or prothrombin activity <40%) complicated within 4 weeks by clinical ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease/cirrhosis, and is associated with a high 28-day mortality" (see Fig. 32.1). Moreau et al. defined the ACLF on the basis of the CANONIC study as "an acute deterioration of pre-existing chronic liver disease, usually related to a precipitating event and associated with increased mortality at 3 months due to multi-system organ failure." Subsequently the duration of mortality has been reduced to 4 weeks in Western definition [9]. Main difference in various commonly used definitions has been highlighted in Table 32.1.

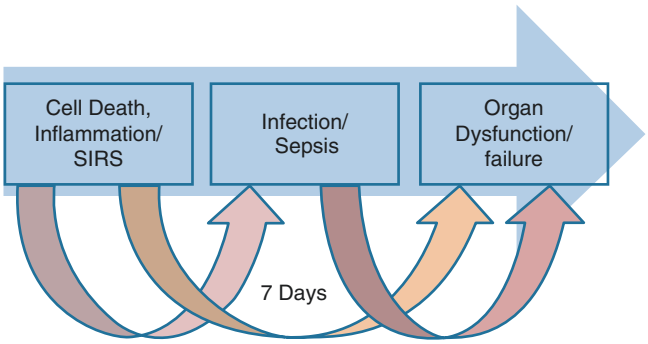

Fig. 32.2 Patients with ACLF within a period of 7 days develop SIRS, which can progress and lead to sepsis, organ failure, and mortality. This window of 7 days is known as the therapeutic window for antibiotics, organ supportive measures, nutrition, and prioritization for the liver transplantation should be done. (Modified from [2])

Concept of Functional Reserve or Critical Mass

Underlying functional reserve and severity of acute insult dictate the course of patient, that is, sudden acute insult on the healthy liver precipitates acute liver failure however, in the presence of underlying chronic liver disease; it may precipitate progressive liver failure (ACLF).

The "Golden Window" concept refers to the time in which acute insult, if removed, may lead to the reversal of the underlying liver failure, preventing extrahepatic organ failure and promoting hepatic regeneration (Fig. 32.2). This provides the window for introduction of therapies like steroids for alcoholic hepatitis and autoimmune hepatitis, antiviral therapies for HBV-related ACLF, and role of plasma exchange or other modalities that may help to tide over the acute insult and result in better transplant-free or short-term survival.

Differentiating ACLF From Acute Decompensation

Controversy remains between the East and the West in defining ACLF. As per the APASL Research Consortium (AARC) definition, when the ACLF is diagnosed, there is still significant hepatic reserve, so removing the acute insult may help in reserving the hepatic injury and improving survival. However, in prior decompensated cirrhotics, underlying functional reserve is poor, so even after removal of the acute injury, the transplant-free survival usually remains poor. Time frame for defining the acute decompensation is 3 months while ACLF is defined by a time period of 4 weeks. Acute decompensation has clumped together ACLF, hepatic, extrahepatic, sepsis-related ACLF creating confusion between the East and the West. Differentiating between the two groups will help in determining the homogenous group, guiding therapy, and prognosis of the disease (Table 32.2).

Table 32.2 Differentiating between acute-on-chronic liver failure and acute decompensation

Parameter(s)	Acute-on-chronic liver failure (ACLF)	Acute decompensation (AD)
Presentation	Hepatic insult Index	Hepatic or nonhepatic Can be index or subsequent
Identifiable precipitant	In up to 95% cases	In up to 70% cases
Time from insult to presentation	Within 4 weeks	Up to 12 weeks
Underlying cirrhosis	May or may not be present	Always present
Prior decompensation	No	With or without prior decompensation
Mortality at 1 and 3 months	33–51%	23–29%
Reversal or recovery	In half of cases	Uncommon
Clinical manifestations	Jaundice with ascites/HE/ coagulopathy	Ascites/HE/GI bleed/ sepsis/AKI, coagulopathy

What Constitutes an Acute Insult?

Origin of the acute insult forms the important difference between the two definitions. While in APASL definition it has to be hepatic insult that constitutes the acute insult, in EASL CLIF it can be hepatic or extrahepatic. Sepsis is the initial precipitating event or part of the liver failure still remains a controversial point between the two definitions. As the primary affected organ is liver, by default the insult should be directed to the primary organ, that is, the liver, such as acute exacerbation of COPD would not be called acute-on-chronic liver failure if it leads to worsening of liver functions. Similarly, patients with upper GI bleed developing renal failure, followed by jaundice or encephalopathy, would be difficult to be called ACLF.

Organ failures are an important component of ACLF; greater is the number of dysfunctional organs, poor is the outcome, and an overall increase in mortality is noted as shown by the CLIF sequential organ failures assessment (SOFA) score. Similarly, the chronology of the organ failures is also important, which may help in distinguishing between the two definitions. CLIF-SOFA score is being used in the West; however, it has been shown that simple organ failures may be helpful as simple bedside prognostication [10]. If we take the same patient, CANONIC definition will wait for the extrahepatic organ failure to set in before the diagnosis of ACLF. Since the rate or incidence of organ failure can be variable, diagnosis of ACLF could be delayed; hence, ACLF could be diagnosed with APASL definition earlier and prognostication and treatment options could be EASL CLIF definition.

Differentiating between the ACLF precipitated by the direct hepatic insult as by the extrahepatic source/sepsis is important as the cytoprotective therapy may be more relevant

in the direct insult, while anti-inflammatory therapy may be relevant in those accompanied by the extrahepatic organ failure [11]. In subgroup analysis in the CANONIC study, difference in survival was noted in the patients having hepatic insult as the precipitant compared to extrahepatic source, indicating difference in response to varying therapies [12]. In a study by Mahmud et al., of 80,383 patients with cirrhosis with a follow-up of 3.35 years, both EASL and APASL ACLF were seen in 783 patients while EASL ACLF in 4296 developed EASL ACLF alone, and APASL ACLF in 574 cases. Combined mortality was more in patients with both EASL and APASL ACLF, indicating severe disease. Median bilirubin was 2 mg/dL in EASL ACLF. It was stated that patients with APASL ACLF have higher short-term mortality, and have higher liver-related mortality, while nonhepatotrophic organ involvement was more common in EASL ACLF. This may lead to late diagnosis and can be clinically cumbersome to apply. Therefore, it was proposed that bilirubin should be reduced from >12 to ≥5 mg/dL, which may help in early diagnosis and liver-directed therapies can be assessed to reduce the mortality [13].

Etiology and Pathogenesis of the Acute Insult

Nature and severity of the acute insult determine the development and progression of the ACLF. Ascites and hepatic encephalopathy complicating liver failure are usually associated with a higher mortality (51% [Asian studies] [7] vs. 33.9% in the European counterparts [5]).

Alcohol-Related ACLF

Underlying chronicity is determined by the dose and duration of alcohol intake, which recent intake or binge usually accounts for the acute insult. Ethanol causes gut dysbiosis, causes hepatotoxicity, and promotes apoptosis secondary to an increase in reactive oxygen species, activation of the innate and adaptive immunity [14, 15]. There is an increase in the proinflammatory mediators (TNF, IL-1, IL-6, IL-17), which is noted with alcohol consumption, while a decrease in anti-inflammatory mediators (adiponectin and adenosine) is seen [16]. Impaired regeneration of liver is noted by limiting DNA synthesis. Chronic alcohol consumption leads to deranged proliferation of the liver progenitor cells as seen with low levels of tumor necrosis factor and IL-6 [17].

Hepatitis B Infection

Reactivation of hepatitis B on the background of underlying compensated cirrhosis or acute infection with hepatitis B in underlying CLD can precipitate ACLF. Eight percent of the patients with acute flare may develop decompensation [18]. Genetic heterogeneity plays an important role in response to acute insult; risk of HBV-related ACLF was increased with rs3129859 at human leukocyte antigen [19]. Similarly, presence of HBV basal core promoter/pre-core mutations, such as T1753V, A1762T, G1764A, A1846T, G1896A, and G899A, was related with an increased risk [20]. Changes in the immunological control and reconstitution of host response account for the reactivation of hepatitis B. An increase in the number of HBeAg and HBcAg specific T cells mediates the liver injury [21]. It can be seen spontaneously or secondary to intensive chemotherapy or immunosuppressive therapy [22] or following rituximab therapy [23].

Acute Viral Hepatitis

Hepatitis E virus (HEV) has been associated with ACLF and high mortality in India while cases from the West are usually sporadic [24]. Role of hepatitis E in precipitating ACLF in the West is not known. HEV induces cell-mediated immunity damage and increase in type I and II helper cells [25]. Increase in cytokines such as IFNγ, IL-2, and TNF is noted mediating the liver damage. Superinfection with hepatitis A and E has been associated with ACLF and hepatitis E has been associated with more severe form of ACLF and with higher mortality [26].

Drug-Induced Liver Injury

Hepatotoxic drugs and complementary and alternative medications have been implicated as a causative factor for drug-induced liver injury. Antitubercular remains an important cause for drug-induced liver injury. Up to 1.8–5.7% of the ACLF cases have been attributed to drug-induced liver injury. Owing to aberrant metabolism, reduced hepatic clearance, and altered excretion, patients with cirrhosis are prone to DILI [27]. High mortality has been attributed to DILI [28].

Sepsis and ACLF

Patients secondary to cirrhosis have deficient innate and adaptive immunity, which denotes inability to clear the infection [29]. Sepsis is a consequence or part of ACLF remains a controversial issue. Sepsis is defined as an extrahepatic insult in EASL CLIF definition. The term "infection-related ACLF" (I-ACLF) has been proposed; however, liver failure remains a late event and extrahepatic organ failures remain the major cause of mortality [30, 31]. Reduced HLD-DR expression, reduction in myeloid and plasmacytoid dendritic cells, and increased interferon production increase the risk of sepsis [32]. APASL defines sepsis as part or consequence of liver

failure and preventing sepsis by modulating the immune system should help in preventing organ failure.

Acute Variceal Bleed

Acute variceal bleed has been taken both as the precipitating event and as a defining event for acute decompensation. In CANONIC study, acute variceal bleed was the acute event in 13.8% of the patients [5]. However, if the acute variceal bleed results in jaundice and coagulopathy that fulfills the criteria of liver failure, the term ACLF can be used.

Autoimmune Hepatitis

Severe autoimmune hepatitis (AIH) can present as jaundice, encephalopathy, and coagulopathy, manifesting as ALF or ACLF. It is seen in up to 20% of the patients [33]. The spectrum of AIH as acute insult has not been clearly defined in Western studies. AIH is usually seronegative, with normal to high serum immunoglobulin G levels, and is characterized by parenchymal collapse, and advanced fibrosis (F3/F4), ductular reactions, and lymphoplasmacytic inflammation are predominant findings [34, 35].

Other Insults

Other nonhepatotrophic insults such as TIPS, TACE, or any surgery that can also lead to direct hepatic injury can account for ACLF.

Defining the Chronic Etiology

Diagnosis of underlying chronicity can be difficult in setting of the ACLF. Clinical History, physical examination to look for signs of portal hypertension, imaging (ultrasonography or CT), endoscopy can help in identifying underlying cirrhosis. If there is no conclusive evidence of cirrhosis, transjugular liver biopsy may be done to ascertain the cause [36]. There have been changing trends in etiologies of the chronic liver disease, that is, initially hepatitis B was the commonest etiology for the chronic liver disease; however, recent data suggest that etiology of the chronic etiology remains same in the West and the East [37, 38].

Pathophysiology of ACLF

Inflammation developing due to cell death remains the hallmark of ACLF, with an increase in white cell count, C-reactive protein, and cytokines, such as interleukins (IL)-6, IL-1β, and IL-8 [39]. Acute stress is an inducer that leads to tissue injury and releases DAMPs, and leads to damage via inflammation and immune-mediated mechanism. Increase in both pro- and anti-inflammatory cytokines is noted in ACLF, that is, TNF-a, sTNF-aR1, sTNF-aR2, IL-2, IL-2R, IL-6, IL-8, IL-10, and IFN-ϒ.

Inflammation

Inducers of the inflammation engage with the effectors, leading to the generation of the inflammatory response. Failed immune-tolerant mechanism, direct virulence of the microorganism, and excessive immune-mediated damage lead to tissue damage. Endogenous or exogenous inducers can initiate the immune response.

ACLF is usually complicated by the infections that are associated with significant mortality and morbidity.

Secondary to portal hypertension, altered bowel flora, and direct damage to the intestinal barrier, increased translocation of bacteria is noted. With the increase in severity of the liver dysfunction, increased migration of the bacteria is noted [40]. Increased cytokines like IL-6 and TNF-α and modulation of the cytokines with changing the gut bacteria, that is, Ruminococcaceae and Lachnospiraceae support the role of the cytokine in mediating the damage and altering the gut bacteria as means of therapeutic strategy [41] (Fig. 32.3).

Immunological Basis of ACLF

Dysfunctional immune system, over exaggerated immune response, altered in the processing of the antigen and altered effector response leads to increased systemic inflammatory response and sepsis like state in ACLF characterized by increased IL-6 and reduced HLA-DR expression [42]. Increased reactive oxygen species and oxidative burst are noted secondary to an increase in neutrophils, which are predominantly dysfunctional. A decrease in synthesis of TNF-α is noted secondary to HLA-DR expression, which is noted in ACLF patients [43]. MER receptor tyrosine is increased in the ACLF, and it is the negative regulator of immune cells and is expressed on the monocytes/macrophages, DCs, and epithelial cells. Increase in the former is associated with poor outcomes [44]. It has been correlated with levels of inflammatory cytokines and increased predisposition with infections. Increase in T-regulatory cells (T-reg) that cause inhibition of the monocyte and macrophages via an increase in interferon-ϒ production and higher ratio of T-reg to Th17 cells is correlated with survival. Ammonia levels and DAMPs have been shown to modulate the immune system, and high ammonia reduces the neutrophil activation, monocyte HLA DR expression, and migration capacities of the neutrophils [45]. Increased expression of the CXCR1/CXCR2 receptors

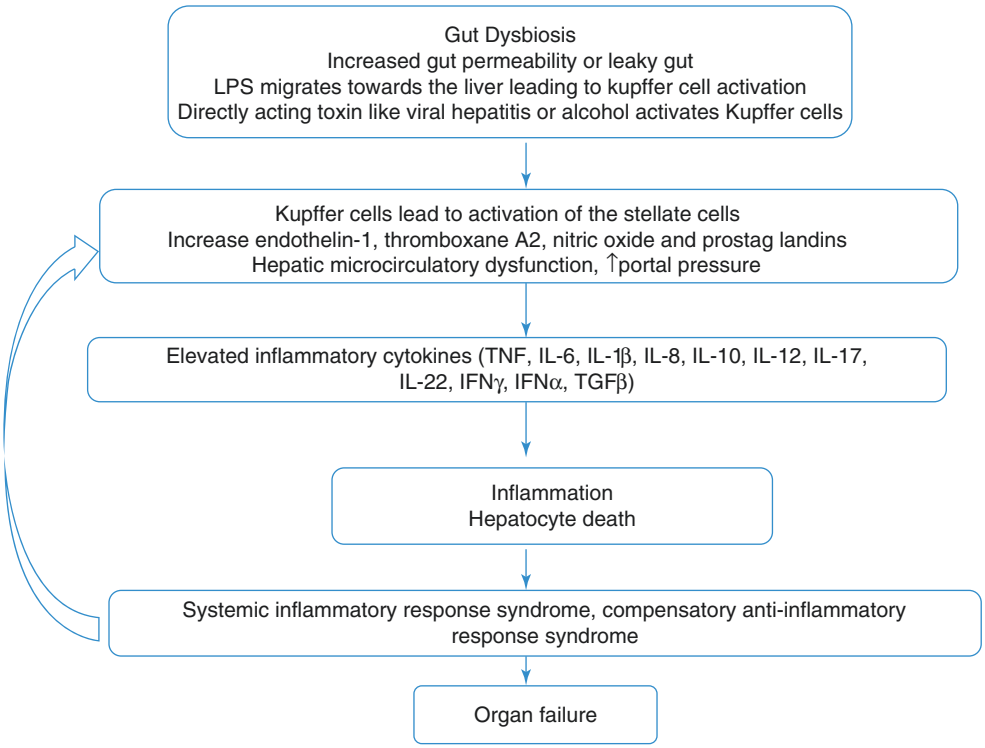

Fig. 32.3 Mechanism of injury in ACLF. Acute insult activates the Kupffer cells in hepatic sinusoids, leading to the release of the inflammatory mediators. Increase in gut permeability allows immune cells and endotoxin to migrate toward liver. An increase in endothelin-1, thromboxane *A2*, nitric oxide, and prostaglandins leads to hepatic microcirculatory dysfunction and increases portal pressure. An increase in hepatic necrosis and apoptosis is noted secondary to neutrophil infiltration and ROS species. These changes lead to injury, state of immunoparalysis, SIRS, and CARS and predispose to organ failure. (Modified from [7])

Table 32.3 Pathophysiology of sepsis in *ACLF* and immune changes (pro- and anti-inflammatory response

Sepsis in ACLF				
Mechanism of sepsis – bacterial translocation, bacterial infection	Proinflammatory response	Anti-inflammatory response	Upregulation of genes that regulate the innate immune response – Neutrophils: phagocytic defect, Monocytes: DR loss, NK cells	Downregulation of genes that regulate the adaptive immune response – T-cell exhaustion, Inability to proliferate, Increased apoptosis
Phases of sepsis	Early phase–hyper inflammation response (SIRS/sepsis)		Late phase: Immunosuppressive phase (CARS)	
Results of sepsis	Organ failure		Organ dysfunctions	

and reduction of the phagocytic capacity of the neutrophils in alcoholic hepatitis contribute to organ failure and high mortality [46].

There is a defect in the innate immunity also. There is activation of Kupffer cells via toll-like receptors and damage-associated molecular patterns (DAMPs) in response to the lipopolysaccharides (LPS). M2 variants of Kupffer cells are activated and cause anti-inflammatory effect via an increase in transforming growth factor-β (TGF-β) [47]. Stimulation of Kupffer cells induces activation of the hepatic stellate cells leading to release of endothelin-1 and thromoboxane-A2 causing disturbances of hepatic microcirculation and rapid aggravation of portal hypertension [48].

There is an immunological imbalance between pro- and anti-inflammatory responses and this leads to a sepsis-like state in ACLF. Activated immune cells in ACLF are dysfunctional and are in a state of immune paralysis leading to an increase in SIRS and increased predisposition to infections (Table 32.3).

Role of Histology in Predicting Outcome in ACLF

Biopsy in ACLF is done through the transjugular route owing to the presence of ascites and underlying coagulopathy. Poor prognostic markers on biopsy are marked ductular proliferation, coarse inspissated ductular bile plugs, eosinophilic degeneration of hepatocytes, foci of confluent/bridging necrosis, higher apoptosis, pericellular fibrosis, Mallory's hyaline, and advanced fibrosis [49]. In a cohort of 107 patients, a score derived from ballooning degeneration and Mallory-Denk bodies in the presteroid biopsies samples, helped in predicting the response to steroids. Area under the

curve for combined Mallory-Denk body and ballooning degeneration with a score >5 for predicting nonresponse was 0.731 [50]. Risk of infection is increased with a high degree of bilirubinostasis.

Disease Prognostication and Scoring Models

Severity of ACLF, underlying multiorgan failure, and progression of organ failure and ACLF should be taken into account while considering for early LT. MELD score ≥28 and APACHE ≥ 12 are associated with high mortality. Nonresponse to steroid at day 7 is associated with high mortality and early transplant is associated with high survival rate at 6 months (77 ± 8 vs. 8%, $p < 0.001$) [51]. In autoimmune hepatitis, MELD score >27 (83.3% sensitivity, 78.9% specificity, area under the receiver operating characteristic curve 0.86) and presence of hepatic encephalopathy, ≥F3 fibrosis (advanced fibrosis) were associated with poor response to steroids and should be referred to early transplantation [52].

Sequential Organ Failure Assessment (SOFA), Acute Physiology and Chronic Health Evaluation (APACHE II), model for end-stage liver disease (MELD), and CLIF-SOFA score have been used to assess disease severity and disease prognostication at the baseline [5]; however, they take into account mortality after the inclusion of the extrahepatic organ failure and are bit cumbersome. Simple organ failure is easy to recall and can be used as bedside assessment tool for predicting mortality [53].

APASL has established a more accurate *ACLF specific score*, *AARC score*, for prognostication of ACLF that has shown to provide better performance than other scores. It is a dynamic model consisting of bilirubin, creatinine, PT-INR, lactate, and hepatic encephalopathy and has been proposed on the basis of AARC database of 1402 patients. It is a dynamic score with an increase in score at day 4 and day 7 from 5 to 6 to 11 indicates high mortality, while persistent grade I or II organ failure indicates improved survival (Table 32.4). AARC score has good predictability with area under the curve of 0.81 and sensitivity and specificity of 75 and 81% negative predictive value for 28 and 90 days [54].

Similarly seen in the Western study, grade of ACLF at the time of diagnosis may help in guiding resolution of the disease, resolution of ACLF was noted in 55% of grade 1 ACLF while 15% of the grade 3 ACLF, and final grade is usually reached by the end of day 7; hence, calculation of the score at day 7 could help in predicting the 28- and 90-day mortality [55].

Baseline MELD > 28, AARC score > 10, advanced HE in the absence of overt sepsis, or multiorgan failure indicates poor prognosis.

Management of ACLF and Organ Failures

Bridge therapies, specific therapies, and definitive therapies along with general measures and nutrition form the basis of management of ACLF. Differentiating ACLF from decompensated cirrhosis is necessary as the two carry different prognosis. Acute insult should be evaluated, preventing inflammatory injury, and protecting organ failure should be hallmark of underlying management (Fig. 32.4).

Need for ICU Care

Patients with ACLF should be looked for presence of sepsis, organ failures, and underlying shock or hypotension. Presence of SIRS should be taken as a sign of occult sepsis. Antibiotics (prophylactic or therapeutic) should be guided by local hospital or community data, severity of infection, and nosocomial or community-acquired infections. Patients with ACLF and sepsis carry grave prognosis with mortality reaching up to 80% [56]. Terlipressin in combination is used in septic shock, which may help in reserving the shock and improving tissue microcirculation [57]. Patients with ACLF are predisposed to paracentesis-induced circulatory dysfunction (PICD) even with less than 5 L of paracentesis (modest volume paracentesis), and albumin has shown to reduce the

Table 32.4 AARC scoring and grading system

AARC score and ACLF grade						
Points	*Total bilirubin (mg/dL)*	*INR*	*Creatinine (mg/dL)*	*Lactate (mmol/L)*	*HE grade*	*Score maximum 15, minimum 5*
1	<15	<1.8	<0.7	<1.5	0	
2	15–25	1.8–2.5	0.7–1.5	1.5–2.5	I–II	
3	>25	>2.5	>1.5	>2.5	II–IV	
Grade	*Score*	*28-day mortality*	*Action required*			
I	5–7	12.7%	A potentially recoverable group			
II	8–10	44.5%	Needs special monitoring			
III	11–15	85.9%	Demands immediate interventions for improved outcome			

For a baseline AARC score of ≥10, with each one-unit increase, the day 7 mortality increased sharply compared to the patients who presented with a score of <10 at baseline (20% vs. 4%). The AARC score also predicts the day 28 and day 90 survival

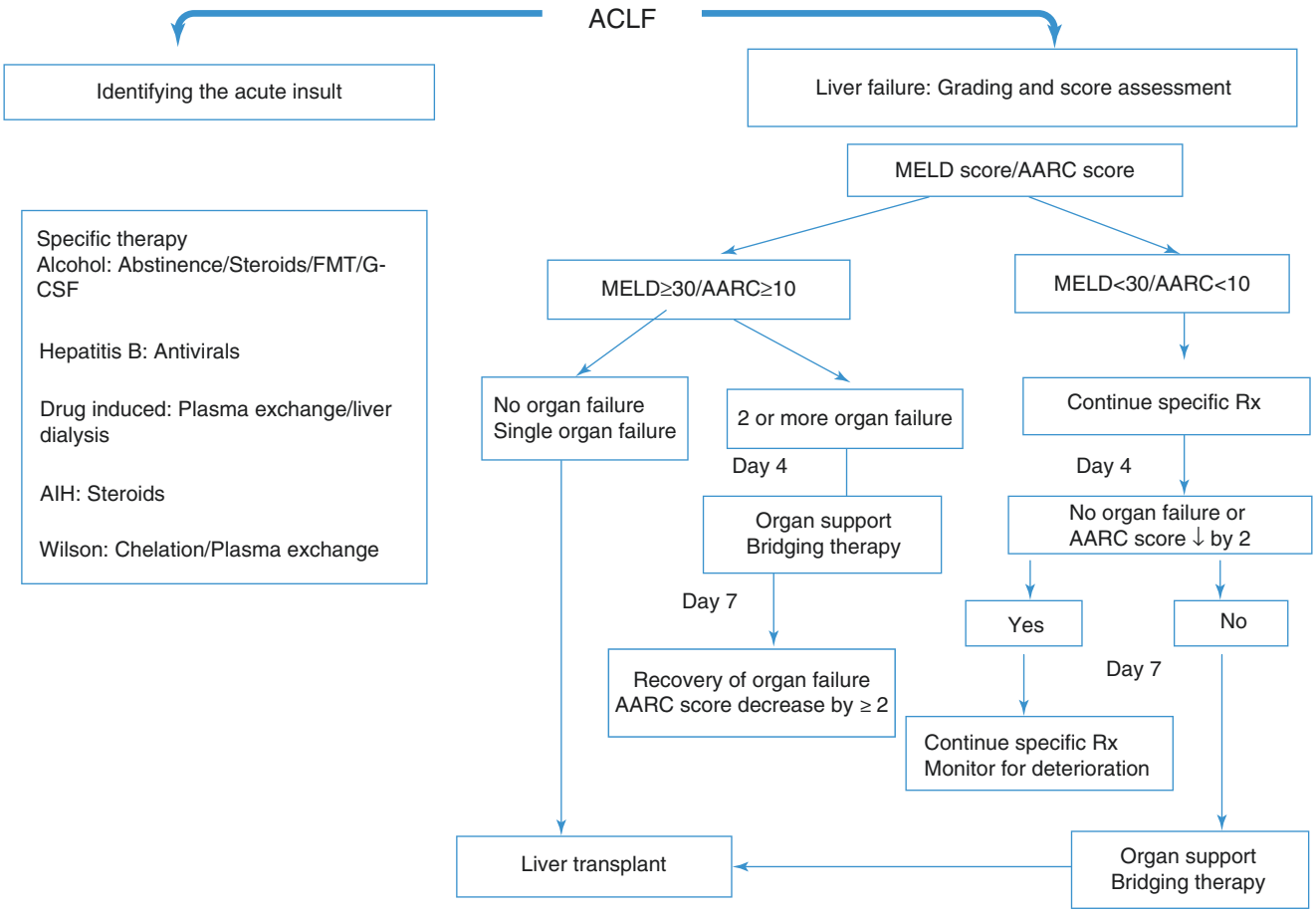

Fig. 32.4 Algorithmic management of patients with acute-on-chronic liver failure

incidence of PICD (70% vs. 30%, $p = 0.0010$) [58]. Besides being used as a plasma expander, albumin binds to prostaglandin E2 (PGE2), reduces the risk of infections, and has ROS scavenging activities, protecting endothelial integrity. Albumin has immune-modulatory effects, binding molecular patterns (i.e., lipopolysaccharide [LPS], DNA fragments), inflammatory mediators, DAMPs (hyaluronic acid, mitochondrial DNA), and reactive nitrogen species. Albumin has been shown to have effect on innate immune system. Guiding serum concentration of albumin could be a therapeutic target [59, 60].

Hepatic encephalopathy is noted in 40% of the patients and requires ICU care [4]. Increasing grade of encephalopathy indicates poor prognosis and higher mortality. Inflammation and impairment of brain energy kinetics play a part in pathophysiology of encephalopathy in ACLF. Baseline ammonia levels correlate with severity of encephalopathy and targeted reduction in ammonia may be given empirically.

Renal dysfunction is noted in 30% of the ACLF and causes include hepatorenal syndrome (HRS), acute tubular necrosis, sepsis, or hypokalemia and carries high mortality at day 7. Only one-third of the patients show response to terlipressin and albumin [61]. Terlipressin, given as continuous infusion, has been shown to be superior to noradrenaline in the management of ACLF AKI [62]. AARC score, severity of AKI, and MELD have been shown to be predictors of response [63].

Concept of Organ Failure and Dysfunction

Differentiating between dysfunction and organ failure is useful in determining the extent of organ damage, determining the progression or reversal of the organ damage, which may help in listing for the transplant or need for the palliative care. Organ dysfunction may be initial and reversible stage of the sepsis that may be reversible and progression to failure is predictive of increased mortality. They are not part of definition but may be used in prognostication of the patients. Liver, kidney, and brain are the organs of utility and involvement of circulatory and respiratory organs may be sign of futility, contraindicating liver transplant (Table 32.5).

Table 32.5 Defining the kidney and cerebral failure/dysfunction in ACLF

Organ	Organ dysfunction	Organ failure
Renal	Serum creatinine >1.5 mg/dL Early use of vasoconstrictors Targeting inflammation Combination of vasoconstrictors	Serum creatinine >1.1 mg/dL May benefit from anti-inflammatory strategies (albumin, *N*-acetylcysteine) and maintaining MAP Role of biomarkers (urine NGAL and IL-18) needs to be evaluated
Cerebral	Grade III/IV hepatic encephalopathy High-volume plasma exchange or albumin dialysis Decreasing systemic inflammation	Grade I/II hepatic encephalopathy Neuroinflammation plays a role Early detection of cerebral edema by DTI/DWI Ammonia-targeted therapy require more trials and validation

Specific Treatment

Alcoholic Hepatitis

Aggressive nutrition (1.5–2.0 g protein/kg per day and 35–40 kcal/kg), suppression of inflammation (corticosteroids, pentoxifylline, IL-1 receptor antagonist [Anakinra] is in phase II RCT, apoptosis signal regulating kinase-1 (ASK-1) inhibitor, modulating gut-liver axis, drug targeting regenerative pathways, that is, granulocyte colony-stimulating factor (G-CSF); antioxidants, that is, *N*-acetylcysteine are being used for management of alcoholic hepatitis.

HBV Treatment

With early reduction of hepatitis B DNA (reduction of 2-log of DNA achieved with 2 weeks), improved survival is noted [64]. Nucleos(t)ide should be started immediately at presentation in HbsAg-positive patients presenting with reactivation without waiting for HBV DNA report.

Autoimmune Hepatitis

Twenty percent of the patients with severe AIH can manifest as acute liver failure or ACLF [33]. As per the AARC cohort, AIH as etiology of ACLF is seen in 2.8% of the total ACLF cohort [65]. Steroids can be used in autoimmune hepatitis and have been shown to improve 90-day survival [47]. As mentioned before, advanced age, MELD > 27, fibrosis ($F \geq 3$), and hepatic encephalopathy are predictors of poor response to steroids [47].

Liver Support Devices

Removing the toxins and reducing the liver injury and promoting regeneration of the liver form the basis of artificial liver support devices. Liver injury is primarily driven by the cytokine burst [66]. The toxins, cytokines, and vasoactive substances accumulate secondary to the failing liver in addition to the toxins produced by the gut microbiota. These toxins promote inflammation, dysfunction of the innate, and adaptive immunity.

Data on the use of artificial liver support devices in ACLF are limited. There is no clarity regarding the use of liver support as per the APASL and EASL guidelines for ACLF. ALSS (the Molecular Adsorbent Recirculating System, MARS®; Gambro, Sweden) and the fractionated plasma separation and adsorption (FPSA; the Prometheus System®; Fresenius Medical Care, Germany) are the commonly used liver dialysis devices. These devices are based on albumin dialysis and are aimed at protecting the clinical and neurological status of individual. However, these devices, despite showing reduction in ammonia and bilirubin, have failed to show any survival benefit [67, 68] (Table 32.6).

Plasmapheresis has been used to aid the recovery of the failing liver, and as a bridge to transplant, and acts by removal of a wide range of toxins [69]. In a retrospective analysis by Wan Yue-Meng et al., a sicker cohort of patients in plasma exchange group has shown a better survival compared to those managed with the standard therapy [70]. In a study by Maiwall et al., plasma exchange was compared with Prometheus, which has shown to improve the hepatic encephalopathy and MELD score; however, no survival benefit or change in transplant free survival was noted [71] (see Table 32.6).

However, these treatment modalities require strict protocol and can be used in a selected group of patients. Further RCTs are required to prove the beneficial effect of the liver support systems.

Liver Transplantation in ACLF

Definite treatment for ACLF remains liver transplantation. In the absence of any obvious contraindications, patients should be counselled regarding the need of liver transplantation. ACLF is characterized by high short- and medium-term mortality, ranging from 34 to 50% [2, 5]. Patients develop infection, sepsis usually within first week, so before the patients are "too sick to transplant," serial assessment should be done for prioritization for liver transplantation [72]. Underlying sepsis, vasopressor requirement, psychological support, respiratory failure, or renal failure leads to high waitlist mortality. Recently one study showed mortality in the range of 67% in ACLF patients on waitlist for transplantation [73].

Table 32.6 Artificial liver support system in acute-on-chronic liver failure

Study	Population(n)	Device	Results
Ash et al. (1994) [79]	Mixed (some with ACLF and others with ALF) (56)	Liver dialysis vs. SMT	Improved HE and hemodynamic profile Increased bleeding in patients with DIC
Sen et al. (2004) [80]	ACLF—severe alcoholic hepatitis (18)	MARS + SMT vs. SMT (9 MARS; 9 controls)	Improvement of HE No hemodynamic changes No changes in plasma cytokines and ammonia levels
Laleman et al. (2006) [81]	ACLF—severe alcoholic hepatitis (18)	MARS + SMT vs. Prometheus + SMT or SMT alone (3d)	Better hemodynamic improvement in MARS with less bilirubin reduction than Prometheus or SMT alone
Banares et al. (2013) [65]	ACLF: bilirubin >20 mg/dL and/or HE greater than grade II and/or HRS (189)	MARS + SMT vs. SMT Up to 10 sessions (6–8 h)	No changes in survival Improvement in HE Improvement in HRS No differences in overall adverse event
Kribben et al. (2012) [66]	ACLF (145)	Prometheus + SMT vs. SMT Up to 8–11 sessions	No changes in overall survival Survival benefit in post hoc analysis in type I HRS and MELD score >30
Maiwall et al. (2017) [69]	ACLF (636)	Prometheus vs. plasma exchange vs. SMT	Improves HE and MELD No change in transplant free survival
Deshpande et al. (2018) [82]	ACLF (16)	Plasma exchange	No change in 28-day survival

HE hepatic encephalopathy, *HRS* hepatorenal syndrome, *SMT* standard medical therapy, *MARS* molecular adsorbent recirculating system, *MELD* model for end-stage liver disease

Patients with MELD more than 28, AARC score >10, and > grade 2 encephalopathy in the absence of any contraindication should be listed for early transplantation. Analysis of ACLF-AARC cohort of 1021 patients showed that MELD > 27 requires listing and presence of MELD > 30 and advanced stage of encephalopathy is associated with high mortality [74]. Dynamic scores such as AARC score can help in better prediction model for listing for liver transplant. Many studies have shown excellent outcomes with transplant in ACLF with 5-year survival more than 80% [75].

Newer Therapeutics in ACLF

The definitive therapy for ACLF, that is, liver transplant is often limited and newer options like regenerative therapy, stem cell mobilization, or immunomodulatory therapies have been proposed.

Garg et al. used G-CSF for ACLF patients. Forty-seven patients were randomized to G-CSF (n = 23) and standard (n = 24) and found that the 2-month survival was 66% compared to 26% (p = 0.001) [76]. Similarly, in the study by Duan et al., 3-month survival was 48% in G-CSF group vs. 21% in the standard treatment group [77]. Similarly, mesenchymal stem cell therapy was used by Shi et al. in hepatitis B-related ACLF, and 3-month mortality was 79.2% on the UC-MSC survived vs. 52.5% in the control group [78].

Prevention of ACLF

Identification of acute insult, universal immunization against hepatitis B, screening for hepatitis before starting immunosuppressants, mitigating the gut flora in NASH, alcoholic hepatitis, and obesity can help in preventing the ACLF. Educating the patients, attendants, the primary care physician about ALT level can help in preventing the DILI. Early referral can help the patient reach the tertiary care center in the "golden window," without sepsis or any organ failure, and can help in decreasing mortality and early referral for transplant.

Conclusion

ACLF is a serious and often a progressive form of liver failure with high short-term mortality. There are large studies from the East and the West, which may help in defining the homogeneity and having a universal acceptable definition. The aim of the management of ACLF patients should be to ameliorate the acute insult, achieve immune homeostasis by countering the systemic inflammatory response, and early diagnosis of organ dysfunction to prevent organ failure. Liver transplant remains the definitive option, and the role of bridge therapies and artificial liver support system remains to be evaluated in a greater detail.

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The Pathogenesis of Liver Diseases in Pregnancy

33

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Key Points

- Liver diseases in pregnancy can include acute fatty liver of pregnancy (AFLP); hyperemesis gravidarum; hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome; severe eclampsia; and hepatic cholestasis of pregnancy.
- Acute fatty liver of pregnancy is a serious disease, with current mortality rates still as high as 5%.
- While hyperemesis gravidarum usually occurs early in pregnancy, the others occur in the later trimesters.
- The pathogenesis of AFLP may be defect in fatty acid metabolism, such as a deficiency of long-chain 3-hydroxyl acyl CoA dehydrogenase (LCHAD).
- The pathogenesis of HELLP may be similar to that of severe preeclampsia and may involve a microangiopathy and activation of the coagulation system, but other factors such as complement, fatty acid metabolism, and the renin-angiotensin system may play a role.
- The pathogenesis of hyperemesis gravidarum is unknown but is probably multifactorial, with genetic, environmental, and epigenetic factors involved.
- The incidence of intrahepatic cholestasis of pregnancy varies widely, suggesting a genetic component to the pathogenesis, although estrogen levels may play a role as well.

Introduction

Pregnancy is a normal physiologic process. However, pregnancy is associated with changes in multiple organ systems in order to adapt to a growing fetus. Physiologic changes resulting from pregnancy are shown in Table 33.1. In addition to changes in vascular circulation and flow, the state of pregnancy also affects the gastrointestinal, cardiovascular, and respiratory systems. But changes also occur in other systems, including the skin and musculoskeletal, neurological, and psychological systems. Changes in lipid metabolism [1, 2] and liver enzymes [3] also occur although structurally the liver is unchanged [4].

New-onset liver disease can present during pregnancy, while patients with preexisting liver disease may experience an increase or exacerbation of their disease [5, 6]. The commonly recognized liver diseases that are associated with pregnancy include acute fatty liver of pregnancy (AFLP); hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome; hyperemesis gravidarum; intrahepatic cholestasis of pregnancy; preeclampsia/eclampsia; and pregnancy-related hemolytic-uremic syndrome (HUS) [5, 7–10]. There is some

Table 33.1 Physiologic and immunologic status related to the liver during pregnancy

Liver
Normal liver structure
Gall bladder
Normal biliary tract
Increased fasting and residual gallbladder volume
Serum chemistries and liver function
Reduced serum albumin beginning in first trimester (resulting from hemodilution)
Increased serum cholesterol
Increased serum triglycerides
Increased alkaline phosphatase levels
Reduced gamma-glutamyl transpeptidase
Slightly increased ALT levels in serum
Unchanged AST levels in serum
Reduced total and free bilirubin levels

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evidence that HELLP may be merely a more severe form of preeclampsia, in which case the two conditions may share a common pathogenesis. However, our knowledge of the pathogenesis of most of these conditions is far from complete.

Physiologic Changes in the Liver During Pregnancy

Any liver disease that occurs in pregnancy must be assessed in the context of normal physiologic changes that occur in the liver and other organs or systemically during pregnancy. For example, spider angiomas and palmar erythema are two skin changes that can occur in liver diseases but are also found in higher frequency during normal pregnancy. On the other hand, there has been no evidence that there are histological or structural changes within the liver during pregnancy [4]. On physical examination, the liver is pushed upward by the uterus; therefore, if the liver is palpable on examination, then this is considered to be abnormal. Biochemical changes can also occur during pregnancy; hemodilution can cause a reduced albumin level [3], but cholesterol and triglycerides are significantly increased [1, 2].

Liver enzymes may also change during normal pregnancy [3]. Alkaline phosphatase levels are usually at least twice that of normal. Serum alanine aminotransferase (ALT) is slightly elevated but not serum aspartate aminotransferase (AST). Serum gamma glutamyl transferase levels decrease during pregnancy, accompanied by increased 5'nucleotidase levels. Overall, indirect and direct bilirubin levels tend to trend lower during pregnancy.

The clinical presentation of hepatobiliary disease includes nausea, vomiting, abdominal pain, jaundice, malaise, and pruritus. When there is jaundice, such as in intrahepatic cholestasis of pregnancy, the itch can be very severe and debilitating. Unfortunately, itching can be very difficult to treat. The correct diagnosis of liver diseases that occur during pregnancy depends on timing [11], as some, such as hyperemesis gravidarum, are more frequently seen early, as in the first trimester, whereas others, such as intrahepatic cholestasis of pregnancy, occur more often in late pregnancy. Acute fatty liver of pregnancy and HELLP usually occur late in the second or in the third trimester, but earlier onset has been reported [11]. A comparison of the different liver diseases seen in pregnancy is shown in Table 33.2.

Table 33.2 Comparison of the different liver diseases seen in pregnancy

Parameter	AFLP	Hyperemesis gravidarum	HELLP	Intrahepatic cholestasis	Preeclampsia with severe features (liver involvement)
Incidence	1 in 7000–20,000 pregnancies	Approximately 1 in 300 pregnancies	0.1–1% of pregnant women, 1–2% of preeclampsia	0.32–5.6% in the United States, 0.5–1.5% in Europe, 27.6% in Araucanos Indians in Chile	1% of all pregnancies
Trimester	Third	First	Late second to third	Late second to third	Late second to third
Presentation	Nausea, vomiting, abdominal pain, malaise, headache, anorexia, hypertension, jaundice, ascites, encephalopathy, DIC	Vomiting persistent enough to lead to a 5% weight loss	Abdominal pain, nausea, vomiting, malaise	Pruritus, right upper quadrant pain, nausea, poor sleep, reduced appetite	Hypertension, proteinuria, end-organ dysfunction, headache, may have pulmonary edema, renal insufficiency
Laboratory findings	Elevated ALT; AST usually below 500 μ/l; elevated WBC; decreased platelets; elevated uric acid, creatinine, and ammonia; decreased glucose, fibrinogen, and antithrombin levels; increased APTT, PT; burr cells on peripheral smear; proteinuria	Ketonuria, elevated ALT, less elevated AST, usually both below 1000 μ/l	Elevated AST, platelets less than 100,000/mm^3, elevated LDH, Total bilirubin greater than 1.2 mg/dl, elevated uric acid	Elevated ALT, AST, elevated total bilirubin (usually less than 6 mg/dl), direct bilirubin, elevated serum bile acids	ALT, AST greater than 2 times normal range, platelets less than 100,000/mm^3, elevated uric acid, increased serum creatinine, protein/creatinine ration in urine is increased
Defined criteria	Swansea criteria	No	Yes	No	Yes (ACOG)

Table 33.2 (continued)

Parameter	AFLP	Hyperemesis gravidarum	HELLP	Intrahepatic cholestasis	Preeclampsia with severe features (liver involvement)
Mortality	Decreased from 75% to less than 5% over past decades	Never	Variable	Rare (though prematurity and stillbirth have been reported)	1 per 100,000 live births, case-fatality rate of 6.4 deaths per 100,000, liver involvement present in 10–15% of maternal deaths associated with preeclampsia
Pathogenesis	20% associated with LCHAD deficiency, presumed defective fetoplacental fatty acid metabolism		Unclear, thrombotic microangiopathy, complement dysregulation, LCHAD deficiency	Unknown, possibly related to estrogen and progesterone metabolism (reduced 5-alpha and 3-alpha progesterone and saturation of liver transport system)	Poor placental perfusion, shallow placentation, failure to remodel spiral arteries of decidua and myometrium, antiangiogenic factors such as sFLT-1, endoglin
Predominant gender of fetus	Male	Female	N/A		N/A
Risk factors	Fetal LCHAD deficiency, multiple gestation, previous AFLP, low BMI (less than 20 kg/m^2), preeclampsia or HELLP, male fetus	Young maternal age, primigravida, female fetus	Previous history of preeclampsia or HELLP	History of liver disease, in vitro fertilization, multiple pregnancy, personal or family history of IHC	Risk factors or preeclampsia: past history of preeclampsia, multiple gestation, primigravida, family history of preeclampsia, advanced maternal age, prior pregnancy associated with placental insufficiency, existing medical conditions including pregestational diabetes, chronic hypertension, SLE, APS, prepregnancy BMI greater than 25 kg/m^2, chronic kidney disease
Treatment	Delivery of the fetus		Delivery of the fetus, antihypertensive therapy, magnesium sulfate, steroids	UDCA, S-adenosyl-methionine, cholestyramine, rifampin, hydroxyzine or steroids	Delivery of the fetus, antihypertensive therapy

AFLP acute fatty liver in pregnancy; *APTT* activated partial thromboplastin time; *ALT* alanine aminotransferase; *AST* aspartate aminotransferase; *BMI* body mass index; *DIC* disseminated intravascular coagulation; *HELLP* hemolysis, elevated liver enzymes, and low platelet count; *IHC* intrahepatic cholestasis of pregnancy; *LCHAD* long-chain 3-hydroxyl CoA dehydrogenase; *PT* prothrombin time; *UDCA* ursodeoxycholic acid

Liver Diseases Unique to Pregnancy

Acute Fatty Liver of Pregnancy

Acute fatty liver of pregnancy (AFLP) is estimated to occur in about 1 in every 7000–20,000 pregnancies [12–15]. It is a severe illness, and while mortality rates have decreased significantly with improved recognition and care, it is still a potentially fatal condition. Mortality rates for the mother and child used to be up to 75% and 85%, respectively. This has dropped to a still significant 18% and 23%, respectively, and some reports suggest that with improved care, the mortality rates have now dropped to 5%. AFLP usually presents in the third trimester. The symptoms include nausea and vomiting, abdominal pain, tiredness, loss of appetite, and headache. Patients may suffer from hypertension, but this is more common in HELLP. Risk factors include a history of AFLP in previous pregnancies, a history of HELLP, low body mass index, and multiple gestation [16, 17]. In contrast to hyperemesis gravidarum, it is more common in those with male fetuses.

AFLP can be confused with HELLP. One differentiating factor is the fibrinogen level, wherein a fibrinogen level greater than 300 mg/dl is more common in patients with AFLP. Other biomarkers that can differentiate AFLP from HELLP include prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT), low blood sugar, and abnormally high creatinine levels. The Swansea criteria were introduced and validated in the United Kingdom for the diagnosis of AFLP. In a small study of women who underwent liver biopsies, positive and negative predictive values were established at 85% and 100%, respectively [18].

Table 33.3 Swansea criteria for the diagnosis of AFLP

Clinical criteria
Abdominal pain
Encephalopathy
Polydipsia or polyuria
Vomiting
Laboratory criteria
Acute kidney injury with creatinine over 1.7 mg/dl
Elevated ammonia greater than 47 μmol/l
Elevated bilirubin over 0.8 mg/dl
Elevated transaminases over 42 IU/l
Elevated uric acid greater than 5.7 mg/dl
Hypoglycemia less than 72 mg/dl
Leukocytosis over 11,000 cells/μl
Prothrombin time greater than 14 s or other evidence of coagulopathy
Imaging
Ascites or bright liver diagnosed by ultrasound
Biopsy
Microvesicular steatosis determined by liver biopsy

Any six of the above criteria are required to make the diagnosis of AFLP

The criteria are shown in Table 33.3. Six of the fourteen criteria in the absence of an alternative cause of liver disease must be present to make the diagnosis of AFLP [19].

AFLP can be more dangerous than HELLP, in that AFLP can progress to liver failure, severe hypoglycemia, and encephalopathy. One study reported that in 46 patients with liver failure or imminent liver failure, 70% had AFLP and 15% had HELLP [20].

Pathogenesis

The pathogenesis of AFLP is unknown. It has been postulated that it may be related to an abnormality in fetal fatty acid metabolism. In the latter stages of pregnancy, there is an increase in free fatty acids. This is necessary to support fetoplacental growth. Women with an inherent defect in fatty acid metabolism, such as a deficiency of long-chain 3-hydroxyl acyl CoA dehydrogenase (LCHAD) [21], are not able to keep up with the metabolism of the increased fatty acid burden and fatty acids accumulate in hepatocytes, leading to cellular damage [22].

LCHAD catalyzes the conversion of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA. If a fetus is homozygous for a pathogenic variation in LCHAD, then the fetoplacental unit is incapable of the beta-oxidation of mitochondrial fatty acids, and intermediate products are released into the maternal circulation. Since the mother is heterozygous for a pathogenic variant, she is unable to metabolize long-chain fatty acids and this leads to maternal liver dysfunction, which then causes a coagulopathy, along with electrolyte imbalances and eventually multi-organ failure.

It is estimated that LCHAD deficiency is responsible for up to 20% of AFLP [13, 14]. The most common pathogenic mutation is G1528C, p.Glu474Gln. Though not as frequently seen as LCHAD, deficiencies in short-chain acyl-CoA dehydrogenase [23], medium-chain acyl-CoA dehydrogenase [24], and carnitine palmitoyltransferase [25] can also lead to AFLP.

The role of the placenta in the pathogenesis of AFLP cannot be discounted. An animal model was developed to study AFLP. Microvesicular steatosis was induced using sodium valproate, and this led to structural alterations in the mitochondria and evidence of oxidative stress in organelles of the liver. These changes were also seen in the placenta of patients with AFLP [26].

HELLP Syndrome

HELLP is an acronym for hemolysis, elevated liver enzymes, and low platelet, a syndrome that occurs during pregnancy. The term was first coined in 1982 by Louis Weinstein following a review of 29 patients with common complications of pregnancy [27]. The pathophysiology of HELLP syndrome is unknown. HELLP is sometimes thought of as a more severe form of preeclampsia, although it has not been definitively determined that these two conditions are related. There are observations seen in preeclampsia, such as a higher incidence with nulliparity, which are not seen in HELLP [28]. HELLP patients have more inflammation of the liver and abnormalities in coagulation than patients with preeclampsia [29–31]. The incidence of HELLP is between 0.1% and 1.0% of pregnancies [32]. Risk factors include a history of preeclampsia or HELLP during a previous pregnancy. Complications of HELLP include liver hemorrhage in the mother and prematurity in the fetus.

Criteria for the Diagnosis of HELLP Syndrome

Criteria for diagnosing HELLP are shown in Table 33.4. There are two sets of criteria, the Mississippi and the Tennessee criteria. In addition to a peripheral smear demonstrating evidence of hemolysis, such as schistocytes and burr cells, there should also be anemia, increased serum bilirubin, and low haptoglobin. To satisfy the features of thrombocytopenia and liver disease, the platelet count should be less than 100,000 cells per microliter and the AST or ALT two times more than the normal. Other biomarkers include an elevated lactate dehydrogenase (LDH).

The differential diagnosis of HELLP includes AFLP, thrombotic thrombocytopenic purpura (TTP), and pregnancy-related atypical hemolytic-uremic syndrome (HUS). How to distinguish HELLP from AFLP was already discussed in the

Table 33.4 Criteria for the diagnosis of HELLP

Criteria for the diagnosis of HELLP (Tennessee) [32]		
Hemolysis	Elevated liver enzymes	Low platelets
At least two of the following	*And*	*And*
Severe anemia	AST or ALT must be at least 200% of upper limits of normal	Less than 100,000 platelets per microliter
Serum bilirubin greater than or equal to 1.2 mg/dl		
Peripheral smear showing burr cells and schistocytes		
Low serum haptoglobin (less than 25 mg/dl) or elevated lactate dehydrogenase (greater than twice upper limits of normal		

Criteria for the diagnosis of HELLP (Mississippi) [130]			
Class	Liver enzymes	LDH	Platelets[a]
1	AST or ALT greater than or equal to 70 IU/ml	Greater than or equal to 600 IU/ml	Less than 50,000 platelets per microliter
2	AST or ALT greater than or equal to 70 IU/ml	Greater than 600 IU/ml	Platelet count less than or equal to 100,000 and greater than 50,000 per microliter
3	AST or ALT greater than or equal to 40 IU/ml	Greater than 600 IU/ml	Platelet count less than or equal to 150,000 or greater than 100,000 per microliter

[a]Platelet count is the nadir during the course of the disease

section above. TTP can also present with elevated LDH, but transaminases are generally normal. Clotting parameters are more likely to be prolonged in HELLP compared with TTP, where only platelet counts are decreased. TTP also tends to occur earlier in pregnancy than HELLP, predominantly in the second trimester compared with the third trimester for HELLP. Pregnancy-related atypical HUS usually presents with a higher incidence of renal disease. The renal disease is often severe enough to lead to dialysis, whereas liver involvement in Pregnancy-related HUS is generally absent to mild. The distinction between preeclampsia, pregnancy-related HUS, and HELLP is not always clear.

A case of transfusion-related acute lung injury (TRALI) has been reported after a Caesarean section in a patient with HELLP [33].

Histology in HELLP Syndrome

Pathologic changes of the placenta in HELLP syndrome have been studied, comparing the normal placenta with the placenta in patients with preeclampsia with and without HELLP. In one study, the preeclampsia patients with HELLP syndrome tended to have higher placental weights than those without HELLP [34], but this was not confirmed in another study [35]. Small-sized villi with increased syncytial knotting may occur, which may be indicative of poor placental perfusion [36]. Additional changes in the placenta are discussed in the next section.

Pathogenesis of HELLP

HELLP may represent more than one disease that may have varying pathogenetic mechanisms. For example, there is a small number of HELLP patients with a fetal long-chain 3-hydroxyacyl CoA dehydrogenase deficiency, similar to AFLP [21]. Two series of 6 and 19 cases found that 100% and 79% of pregnancies in which there was a deficiency of LCHAD developed HELLP, respectively [22, 37].

The pathogenesis of most cases of HELLP is thought to be related to a microangiopathy and activation of the intravascular coagulation pathway. The pathological changes include vascular spasms, platelet aggregation, vascular endothelial damage, platelet consumption, deposition of fibrin, and ultimately, end-organ ischemia and failure.

Complement may play a role in pathogenesis of HELLP. The activation of complement may be triggered by an immunological rejection of the fetus by the mother. This loss of tolerance leads to activation of complement and the release of C3a, C5a, and the later components of the complement cascade. Complement has many functions in the human immune system, but those relevant to HELLP may include stimulation of macrophages and leukocytes leading to the release of vasoactive substances, ultimately causing those pathological changes noted in the mentioned previously. This leads to the clinical manifestations including thrombocytopenia, hemolysis, and liver enzyme elevation [38–40]. Complement activation appears to be involved in the pathogenesis of preeclampsia and spontaneous abortion in women with systemic lupus erythematosus or positive antiphospholipid antibodies. This study utilized the PROMISSE database of 250 pregnant patients with lupus or positive antiphospholipid antibodies to demonstrate mutations in three complement regulator proteins (complement factors H and I and membrane cofactor protein (MCP)) [41]. In a case report, a woman with severe HELLP that developed relatively early in pregnancy was treated with eculizumab, a monoclonal antibody directed against C5, and displayed significant clinical improvement and resolution of laboratory abnormalities. The period of remission lasted 16 days, but then the symptoms of HELLP recurred [42].

Other factors that may play a role in the pathogenesis of HELLP include the renin-angiotensin system. Elevated angiotensin II levels are known to play a role in hypertension and renal disease [43]. Agonistic autoantibodies to the type 1 angiotensin II receptor leads to their activation, which can potentially lead to regulation of the activity of intracellular Protein Kinase C, leading to angiotensin II-induced vascular

abnormalities. It has also been shown that the renin-angiotensin system plays a role in hepatic fibrosis and chronic liver disease. Inhibitors of this pathway have been shown to reduce fibrosis scores compared to controls [40]. Also reduced were the serum fibrosis markers including TGF-β1, collagen I and IV, TIMP-1, and MMP2. In other studies, investigators noticed an improvement in mean arterial pressures in patients treated with renin-angiotensin system inhibitors compared with controls.

Some authors have suggested that HELLP syndrome originates as a result of placental ischemia which may either cause or result from aberrant placental development and abnormal function. It has been proposed that liver ischemia causes release of mediators that leads to endothelial damage including vasoconstrictive agents. Platelet activation ensues [44]. Which involves remodeling of the placental arteries and defective placentation, placental infarction and abruption [34].

Hyperemesis Gravidarum

Hyperemesis gravidarum is a pregnancy-related condition that is characterized by excessive nausea and vomiting, weight loss, and dehydration. It usually occurs in the earlier phases of pregnancy and usually resolves by about the 20th week of pregnancy but in some cases may persist throughout pregnancy. It is fairly common, occurring in about 1 in every 300 pregnancies. Risk factors include young age of the mother, primigravida, and multiple pregnancy. It is also associated with elevated serum aminotransferase and bilirubin levels. Given that nausea and vomiting are so common in the early stages of pregnancy (up to 90% incidence) and are part of normal expected changes in physiology, the name hyperemesis gravidarum must be applied only to those patients on the extreme end of the symptom spectrum. In many cases, hyperemesis gravidarum affects quality of life, leading to direct and indirect work performance issues.

The risk factors associated with hyperemesis gravidarum are unclear [45–47]. There may be a genetic risk, based on the increased occurrence in family members and on twin studies [48–55]. One of the most intriguing observations is that hyperemesis occurs significantly more frequently with female fetuses, with an odds ratio of 1.27, 95% CI 1.21–1.34 [56]. The things that you should never do during pregnancy, including drinking alcohol and cigarette smoking, are ironically associated with a lower risk of hyperemesis gravidarum [47].

In addition to symptoms of nausea and vomiting, patients with hyperemesis gravidarum also may present with elevated liver enzymes. In one study, this occurred in about half of the patients admitted for hyperemesis [57]. The ALT elevation is generally higher than that of AST. The levels typically do not go over 1000 units/l. The total bilirubin level may also be elevated but generally it is mild [58].

Many of the patients with hyperemesis also have elevated thyroid levels, which has been blamed on the higher activity of human chorionic gonadotropin and its ability to stimulate the thyroid gland [59]. Other laboratory findings in hyperemesis gravidarum include elevated serum amylase and lipase [60], decreased magnesium and calcium levels, electrolyte derangements, and elevated hematocrit; the latter are due to persistent vomiting and hypovolemia, respectively [61]. In cases where liver biopsies were done to exclude other liver diseases, findings were either normal or showed nonspecific findings including a lack of inflammation but with areas of necrosis and central vacuolization [58, 62].

Pathogenesis of Hyperemesis Gravidarum

The mechanisms by which nausea and vomiting occur in pregnancy is unknown. Hormones such as estrogen and progesterone have been implicated, but there is no formula for their relative amounts that have been found to definitively cause these symptoms. Estrogen levels have been blamed, but these are highest in the third trimester which is contrary to this argument. Human chorionic gonadotropin (hCG) levels are highest in the first semester, so this suggested a possible role in early pregnancy nausea and vomiting, but studies have not shown a correlation between high hCG levels and these symptoms.

One proposal mentions *Helicobacter pylori* as a cause of nausea and vomiting. However, the results are inconsistent. A systematic review and meta-analysis published in 2014 found that, collectively, the studies showed a significant higher incidence of *H. pylori* in patients with hyperemesis gravidarum than in normal pregnant controls, but it was also noted that there was a large variability between studies. The studies also did not distinguish between past or active infection.

Abnormal gastrointestinal motility has also been cited, as well as reduced esophageal sphincter integrity, suggesting that in pregnancy, there is increased gastrointestinal reflux [63]. However, this does not explain why nausea and vomiting would improve as the pregnancy progresses.

Finally, genetic studies have identified certain alleles that convey a higher risk of nausea and vomiting, including the placental proteins GDF15 and IGFBP7 and the hormone receptors GFRAL and PGR [64, 65].

Intrahepatic Cholestasis of Pregnancy

Intrahepatic cholestasis presents during the late second or early third trimester and resolves quickly upon delivery of

the fetus. The incidence is variable and ranges from less than 1% in European studies [66] to 27.6% in Araucanos Indians in Chile [67]. While variability depends on geography and environmental factors, there are no specific triggers that have been identified. Some studies have found a seasonal predominance in the winter months [66].

Even within the United States, variability is high and can range from 0.32% to 5.6%, the higher incidence being found in a primarily Hispanic population [68, 69]. Patient-related risk factors have been identified to include advanced maternal age, a family history of intrahepatic cholestasis, chronic hepatitis C infection, and a prior pregnancy with the disease [70]. Intrahepatic cholestasis is the most common liver disease that is unique to pregnancy.

Pathogenesis of Intrahepatic Cholestasis of Pregnancy

It is believed that the etiology of intrahepatic cholestasis of pregnancy is multifactorial, with genetic, environmental, and hormonal factors all playing a role. The genetic component is supported by a familial predisposition to develop the condition. There are also increased risks in certain ethnic groups and in first-degree relatives.

Although no specific environmental factors have been identified, a contribution of the environment to the pathogenesis is supported by a seasonal pattern seen in some studies as well as the wide-ranging geographic variation mentioned above. Other environmental factors that have been implicated are low vitamin D levels resulting from a lack of sunlight exposure and low selenium levels resulting from poor diet [71].

Hormonal factors include elevated estrogen levels. This seems to be consistent with the timing of intrahepatic cholestasis. It most often occurs in the second trimester when estrogen levels are at their highest, and it often occurs in twin pregnancies which are also associated with higher estrogen levels compared to uniparous pregnancies [72, 73].

A 2019 randomized study of 600 women with intrahepatic cholestasis of pregnancy showed that ursodeoxycholic acid was better than placebo in controlling pruritus, but did not perform better than placebo in composite measures including neonatal unit admissions, perinatal deaths, and preterm delivery and also did not change the incidence of stillbirth [74].

Preeclamptic Liver Dysfunction (Preeclampsia with Severe Features)

Particularly severe cases of preeclampsia are labeled as preeclampsia with severe features. This terminology was changed from severe preeclampsia in 2013. As mentioned above, there is considerable overlap between preeclampsia and HELLP. Criteria for the diagnosis of severe preeclampsia are shown in Table 33.5. With preeclampsia with severe features, the overlap is even more significant. The severe features include seizures, pulmonary edema, hypertensive encephalopathy, stroke, retinal detachment, cortical blindness, disseminated intravascular coagulation, placental abruption, renal failure, and hepatic failure or rupture. Death can ensue from many of these complications. Liver disease can be present in both HELLP and in preeclampsia with severe features [75]. The incidence of preeclampsia with severe features in the United States is about 1% of all pregnancies [76]. The incidence is higher in women who have never given birth [77, 78].

Table 33.5 Criteria for the diagnosis of preeclampsia

Systolic blood pressure greater than or equal to 160 mmHg, diastolic blood pressure greater than equal to 110 mmHg (after 20 weeks' gestation, confirmed[a])
or
Systolic blood pressure greater than or equal to 140 mmHg, diastolic blood pressure greater than or equal to 90 mmHg (after 20 weeks' gestation, confirmed[a]), plus new onset of at least one of the following:
Cerebral or visual symptoms (including headache, blurry vision, scotomas, photophobia)
Liver transaminases higher than twice the upper limit of normal for local laboratory
Platelet count less than 100,000/mm^3
Proteinuria greater than 0.3 g in a 24-h urine collection or greater than 0.3 mg/mg in a random urine sample
Pulmonary edema
Serum creatinine greater than 1.1 mg/dl or twice the normal creatinine

[a]Confirmed means detected on two occasions 4 hours apart

Pathogenesis of Preeclampsia

While the pathophysiology of preeclampsia is most likely dependent on both maternal and fetal factors, there is evidence that the placenta plays a significant role as well. The development of preeclampsia is dependent on the placental tissue, not the fetus. The disease resolves after delivery of the placenta, but full recovery may take weeks. Abnormal structural development of the placenta has been demonstrated in patients with preeclampsia. Normal placental development involves remodeling of the spiral arteries during the late first trimester [79, 80]. Maternal spiral arteries are the terminal branches of the uterine artery which supplies nutrients to the fetus and placenta. It has been shown that in preeclampsia these spiral arteries do not penetrate the myometrium and do not develop into the normal large vascular channels, instead remaining narrow and causing reduced perfusion to the placenta [81, 82]. In addition, ineffective trophoblast develop-

ment has been shown to be related to defective spiral artery invasion. The pseudo-vasculogenesis from adhesion molecule expression to endothelial cell expression [83] that is seen in normal placental development is impaired in women with preeclampsia [79, 80]. The impaired trophoblast differentiation may be under the control of semaphorin 3B, which inhibits the vascular endothelial growth factor signaling pathway [84]. All this leads to decreased perfusion to the placenta. The ischemic placenta can generate factors such as soluble frns-like tyrosine kinase 1 (sFLT-1) which may play a role in the maternal features of preeclampsia [85, 86].

Immunological factors that have been shown to play a role in preeclampsia are analogous to those seen in transplant rejection. After all, the fetus is to some extent foreign to the mother, and the mother must be able to develop tolerance to the fetus to carry it to term. The development of tolerance is dependent on the expression of HLA class I antigens HLA--A–C, HLA-E, and HLA-G by extravillous trophoblast cells and the ability for natural killer cells to express the appropriate receptors such as killer immunoglobulin receptors (KIR) to recognize these class I molecules. One study showed polymorphisms in KIR and HLA-C were associated with a higher risk of preeclampsia [87].

The role of antibodies to the angiotensin II type 1 (AT-1) receptor has also been suggested in the pathogenesis of preeclampsia. The angiotensin AT-1 receptor may stimulate the sFLT-1 receptor mentioned above and may also play a role in the mobilization of intracellular free calcium, increased plasminogen activator-1 production, and the defective trophoblast invasion that occurs in preeclampsia [88–91]. In addition, signs of exaggerated levels of inflammation have been detected in mothers with preeclampsia, including complement activation or dysregulation [92, 93], nitric oxide production [94], increases in cell-free DNA [95, 96], and circulating syncytiotrophoblast debris [97, 98].

Genetic factors in preeclampsia have been identified. Variants in the DNA sequence near the FLT1 locus on chromosome 13 in the human fetal genome has been associated with preeclampsia [99]. Genetic evidence has also suggested that the genetic factors that play a role in HELLP may be different than those in preeclampsia with severe features [100]. Other polymorphisms reported to play a role in preeclampsia include those in the SERPINE1 (PAI-1) 4G/4G insertion/deletion promoter [101, 102].

The critical function of the endothelium in pregnancy is demonstrated by the potential role of endothelial dysfunction in the development of preeclampsia. Deficiency in the function of VEGF (as mentioned earlier) and placental growth factor (PIGF) has been implicated in the pathogenesis of preeclampsia [103]. sFLT-1 has a direct role in inhibiting the biological activity of these growth factors. Another endothelial factor that may play a role in preeclampsia is soluble endoglin. Endoglin is a co-receptor of transforming growth factor (TGF)-β that is highly expressed in the vascular endothelium and syncytiotrophoblasts. Soluble endoglin function as an anti-angiogenic protein which may affect the trophoblast development within the placenta [104–106].

Liver Diseases Not Unique to Pregnancy

Neonatal Lupus

While heart block is the most well-known manifestation of neonatal lupus, the disease can also affect the skin, liver, spleen, as well as hematological and neurological systems. The liver diseases that occur in the neonate as a result of neonatal lupus are most commonly intrahepatic cholestasis and/or hepatitis [107, 108], usually manifested by jaundice, icterus, and transient elevation of liver enzymes. The incidence of liver involvement in neonatal lupus is between 10% and 24%. This is usually mild and resolves spontaneously within the first 6 months of life, as do other noncardiac manifestations of neonatal lupus [109–111].

Pathogenesis of Neonatal Lupus

The pathogenesis of neonatal lupus can be traced to the presence of autoantibodies to Ro/SSA and La/SSB. U1-RNP autoantibodies can also be seen in neonatal lupus [112–114]. However, it is unclear if these autoantibodies are pathogenic. Mothers who have infants with neonatal lupus do not necessarily have lupus themselves at the time of delivery, but they frequently show positive autoantibodies. The risk of having a child with neonatal lupus is related to the presence of these autoantibodies and is about 2% [115]. If the mother has had a child with neonatal lupus, then the incidence increases with subsequent pregnancies. Other pathogenic mechanisms that have been proposed in neonatal lupus include maternal-fetal microchimerism. In one study, maternal cells were found in the myocardium of male fetus or neonates of Ro and La antibody-positive mothers who developed heart block [116]. Fetal HLA-alleles have been associated with neonatal lupus, with high risk alleles being HLA-DRB1*04 and HLA-Cw*05 and low risk alleles being DRB1*13 and Cw*06 in one Swedish study [117]. It is likely that the pathogenesis of neonatal lupus, as in many other autoimmune diseases, is a combination of genetic and environmental factors [112, 118]. The pathogenesis for developing liver disease in neonatal lupus is not known.

Patients with Preexisting Liver Disease

Autoimmune Hepatitis and Pregnancy

Autoimmune hepatitis is a risk factor for pregnancy and has been associated with a higher frequency of stillbirth, prema-

turity, and spontaneous abortions. Moreover, patients with AIH can experience flare-ups during and after pregnancy. In addition, if the AIH is advanced and there is evidence of portal hypertension, the risk of bleeding is increased. In spite of this, the prognosis for the newborn is generally favorable. A report of nine pregnancies in seven patients who had autoimmune hepatitis revealed 22.2% exacerbations [119]. Six of the pregnancies resulted in live births (two premature), and three were first trimester miscarriages. Flares were treated with azathioprine and prednisolone in 2/3 of the patients.

Pathogenesis of Autoimmune Hepatitis

Like most autoimmune diseases, the pathogenesis of autoimmune hepatitis is multifactorial, with genetic and environmental factors playing a role. Autoimmune hepatitis presents with elevated liver enzymes resulting from inflammation of the liver. There are two forms of autoimmune hepatitis, type 1 and type 2, which are classified according to the autoantibodies detected. Anti-nuclear antibodies and anti-smooth muscle antibodies are characteristic of type 1 autoimmune hepatitis, whereas liver kidney microsomal antibodies (LKM-1) are found in type 2 autoimmune hepatitis.

The role of autoantibodies in the pathogenesis of autoimmune hepatitis is unclear. Other factors can precipitate autoimmunity or a breakdown in tolerance that leads to invasion of the hepatic parenchyma by a dense mononuclear infiltrate that is seen on histological examination of the liver in autoimmune hepatitis. CD4+ helper cells have been shown to be able to recognize an autoantigen that is presented by MHC class 1 molecules, leading to their activation and differentiation into Th1 helper cells. These Th1 cells can activate macrophages and further enhance HLA class 1 expression, priming hepatocytes to be vulnerable to attack by cytotoxic CD8+ T lymphocytes. At the same time, HLA class 2 expression leads to the production of various cytokines that favor the development of autoantibodies. All this occurs in the face of reduced Treg cell numbers and functions which further allows autoreactive T cells to differentiate and proliferate unchecked [120–123].

Multiple studies have shown that patients with autoimmune hepatitis can safely deliver a fetus [124, 125]. However, poor pregnancy outcomes occur more often when disease control of a patient's autoimmune hepatitis is suboptimal [126]. Risk factors for adverse outcomes include the presence of autoantibodies against soluble liver antigen (SLA), Ro (SSA), and liver/pancreas antigen (LP) [127]. Twenty percent of pregnant females have a flare during their pregnancy. The mechanisms that lead to flares during pregnancy is not clear. The postpartum period carries a greater risk for flare-ups in up to 52% of mothers who have delivered their baby [128, 129].

Conclusion

Several different liver diseases can occur during pregnancy. Some of these diseases are unique to pregnancy, whereas others can be exacerbated during pregnancy. The diseases that are unique to pregnancy include acute fatty liver of pregnancy, HELLP, intrahepatic cholestasis, and hyperemesis gravidarum. These disorders can be distinguished by their features and timing. Hyperemesis gravidarum usually presents early in pregnancy, but AFLP, HELLP, and severe preeclampsia are more commonly seen in late pregnancy. Intrahepatic cholestasis can also occur during late pregnancy and is associated with pruritus. The prognosis varies for the different liver diseases, and patients with AFLP can develop severe liver dysfunction and liver failure. The treatment of the more severe forms of liver diseases in pregnancy is to deliver the fetus. The pathogenesis of liver diseases in pregnancy is multifactorial, with genetics and the environment potentially playing relative roles.

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Graft-Versus-Host Disease

34

Zunirah Ahmed and John M. Vierling

Key Points

- Following allogeneic hematopoietic cell transplantation (HCT), engraftment of donor leukocytes and stem cells to reconstitute the hematopoietic and immunologic systems occurs within an allogeneic ("foreign") host, despite efforts to match donors and recipients for HLA alleles.
- The success of HCT depends on sufficient engraftment of donor cells to reconstitute hematopoietic and immunologic systems within the host and the ability of donor cells to mediate potent graft-versus-tumor (GVT) alloreactions to eliminate residual tumor cells and prevent relapse mortality.
- Graft-versus-host disease (GVHD) represents the deleterious extension of the GVT alloreaction to include inflammatory destruction of nonmalignant host tissues and organs.
- GVHD can be subdivided into acute GVHD and chronic GVHD, which constitute distinct clinicopathological syndromes that differ in time of onset, clinical features, therapeutic responses, prognosis, and immunopathogenic mechanisms.
- Acute and chronic GVHD are the leading causes of morbidity and non-relapse mortality after HCT.
- GVHD can also occur after solid organ transplantation when donor leukocytes within the allograft engraft in the allogeneic recipient host.
- No prophylactic strategies reliably prevent GVHD, and standard therapies for severe GVHD have limited efficacy.
- Further advances in our understanding of the immunopathogenesis of both acute and chronic GVHD are required to develop safe and effective strategies for the prevention and treatment of aGVHD and cGVHD.

Introduction

Allogeneic hematopoietic cell transplantation (HCT) remains the curative option of choice for patients afflicted with several malignant and nonmalignant diseases of the lymphatic and hematopoietic system [1]. To be clinically successful, engraftment of donor stem cells and mature B and T lymphocytes present in the graft must reconstitute a competent lymphoid immune system. Failure of donor cells to engraft in the host results in severe immunodeficiency and risks of severe infections from opportunistic organisms (i.e., viruses, bacteria, fungi, and protozoa). Conversely, post-HCT engraftment of donor innate immune cells (e.g., neutrophils, monocytes, macrophages, dendritic cells, NK cells, and NKT cells) along with polyclonal, poly-antigen-specific donor T and B cells reconstitutes host immunity, reduces the risk of infection and mediates essential graft-versus-tumor (GVT) responses required to eliminate residual malignant cells [2]. However, generation of robust donor GVT responses that prevent relapse of malignancy substantially increases the risk of donor alloreactions against nonmalignant host tissues and organs that result in acute or chronic graft-versus-host disease (aGVHD or cGVHD) (Fig. 34.1) [3–6]. The heterogeneity of clinical features of GVHD suggests that donor, recipient, transplant and pharmacological factors modulate the phenotype.

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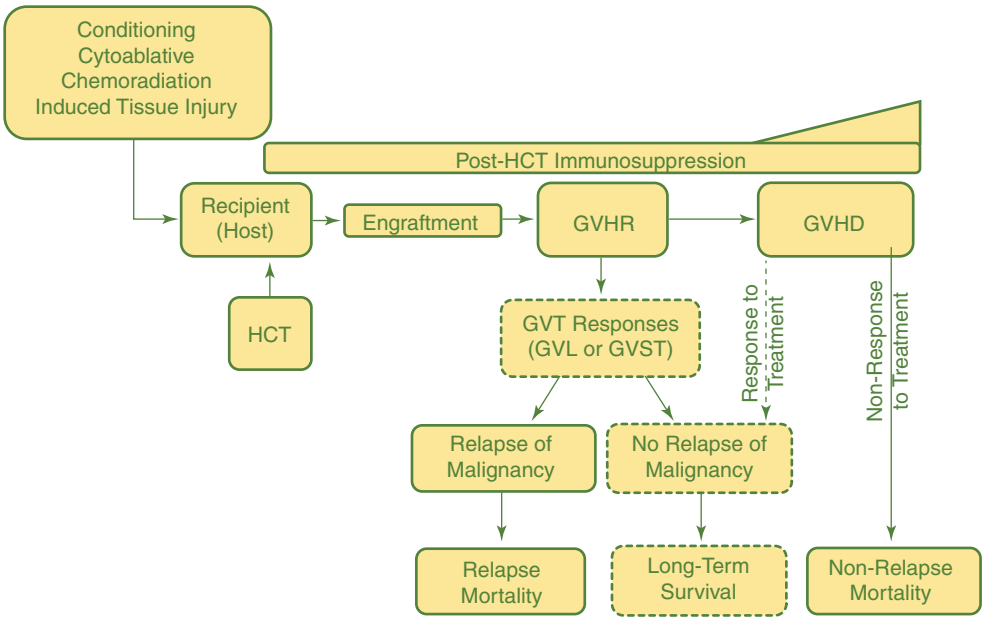

Fig. 34.1 Generation of the graft-versus-host reaction and its consequences of beneficial graft-versus-tumor effects and harmful GVHD graft-versus-host disease

aGVHD) and cGVHD are potentially devastating complications of HCT and are the leading causes of non-relapse mortality (NRM, i.e., mortality not due relapse of malignancy) after HCT [3–6]. Multiple strategies to prevent GVHD have been devised, but none has been successful in preventing GVHD, while preserving vital GVT responses [7, 8]. To prevent rejection of donor cells and minimize the number of surviving malignant cells, all recipients of HCT undergo preconditioning with cytoablative chemotherapy and/or radiation [3–6]. Recipients also require prophylactic immunosuppression after HCT that allows sufficient alloreactivity for GVT responses, while suppressing development of aGVHD [3–6]. The systemic, effects of preconditioning chemoradiation and post-HCT immunosuppression predispose recipients to infections and delay reconstitution of donor-derived host immunity.

The goals of this chapter are fourfold. The first is to describe the physiology and pathophysiology of engraftment after HCT or, more rarely, after blood transfusions or solid organ transplantation. The second is to discuss the clinical manifestations, diagnostic criteria, grading systems, and outcomes of aGVHD and cGVHD. The third is to describe current concepts of the immunopathogenesis of beneficial GVT responses and deleterious aGVHD and cGVHD responses. The fourth is to review current and future strategies to prevent and treat aGVHD and cGVHD.

Engraftment After Hematopoietic Cell Transplantation

Overview

Successful engraftment after HCT generates common lymphoid progenitors of donor innate and adaptive immune cells in the host [3–6]. Unfortunately, recovery of innate and adaptive immunity in the host occurs gradually after HCT because of the inhibitory effects of preconditioning chemoradiation and post-HCT immunosuppression. Innate immunity typically recovers during the first several months. In contrast, reconstitution of adaptive immunity often requires 1–2 years. The kinetics of immune recovery are affected by differences in the cytoablative chemotherapy and radiation regimens, sources of donor cells (e.g., peripheral blood, bone marrow, umbilical cord blood), and type and intensity of prophylactic immunosuppression used to preserve GVT responses while preventing GVHD. Delayed recovery of host immunity results in prolonged immunodeficiency and substantial morbidity and mortality.

Composition of Donor Grafts

Donor grafts contain mature innate immune cells, mast cells, both naïve and antigen-primed T and B cells and progenitor stem cells for innate and adaptive lineages. The sources of

donor grafts include peripheral blood, bone marrow, umbilical cord blood, and stem cells [3–6]. The most common grafts used in HCT come from (1) HLA-identical sibling donors, also designated as matched related donors; (2) unrelated donors with ≥7 of 8 HLA-A, HLA-B, HLA-C, and HLA-DRB1 alleles matching the recipient, referred to as matched unrelated donors; and (3) donors of a T cell-replete haploidentical grafts treated with post-HCT cyclophosphamide, referred to as haploidentical donors. Both antigen-specific mature and naïve T cells can proliferate in host peripheral lymphoid tissues in the presence of surviving host APCs [9, 10]. Thus, engraftment involves both mature T cells derived from proliferation of infused donor T cells and T cells derived from donor lymphoid progenitor cells that have undergone conditioning in the host thymus. When engraftment of mature T cell populations exceeds the engraftment of T cells produced by donor lymphoid progenitors, the total TCR repertoire is more limited and more alloreactive [9, 10]. This results in robust GVT reactions with a lower risk of relapsing malignancy but increases the corresponding risk of GVHD [9, 10].

Recovery of Immune Functions in the Host

Recovery of Innate Immunity

The first cells to engraft are monocytes, followed by granulocytes, NK cells, and platelets [11]. Full recovery of functional monocytes may take up to 1 year. Host macrophages and dendritic cells (DCs) that survived cytoablative conditioning are gradually replaced over several months by macrophages and DCs produced by donor stem cells. Neutrophil counts often normalize within 2–4 weeks; however, neutrophils remain dysfunctional for up to 4 months. NK cell levels normalize within several weeks [9, 10, 12]. Normal NK cells express inhibitory receptors (killer Ig-like receptors, KIRs), lectin-like CD94:NKG2 heterodimers, and activating receptors. Following HCT, most NK cells express CD94:NKG2, but only a minority express KIRs. NK cells regain normal differential expression of KIRs > CD94:NKG2 within 1–3 years.

Recovery of Adaptive Immunity

Following HCT, donor B and T cells must reconstitute the cellular and humoral immune systems of the host. This process typically requires several years, especially in immunosuppressed patients with cGVHD [9–11].

Cellular Immunity

Following HCT, T cells recover in two independent ways: (1) expansion of donor memory T cells, resulting in a limited TCR repertoire, and (2) migration of donor T-cell progenitors to the host thymus for subsequent generation of naïve T cells with a robust allospecific TCR repertoire [9–11]. Donor-derived T cells produced in the host thymus express differentiation markers of mature CD3/CD4 or CD3/CD8 T cells and are present in peripheral blood approximately 3 months after HCT. These donor-derived naive T cells subsequently populate peripheral lymphoid tissues, where they respond to peptide antigens presented by donor-derived antigen-presenting cells (APCs). Thus, functional T-cell recovery in the host depends upon the quantity and TCR repertoire of memory T cells in the donor graft and the capacity of the host thymus to generate donor-derived naïve T cells. The host thymus is particularly crucial for recovery of functional CD4 T cells, which is problematic in adults >40 years of age because of natural age-related atrophy of the thymus. In both young and older recipients, CD8 T cells rapidly recover due to clonal expansion outside the thymus [9–11].

Humoral Immunity

Production of antibodies to mediate humoral immunity after HCT requires both B cells and T cells. B-cell counts normalize by 6 months after autologous HCT but often require 9 months after allogeneic HCT [9–11]. B-cell progenitors differentiate in the eradicated host bone marrow and migrate to peripheral host lymphoid tissues where helper cytokines from antigen-specific donor CD Th1, Th2, and T follicular helper (Tfh) cells cause differentiation into antibody secreting B cells and plasma cells [13]. The post-HCT microenvironment of alloantigens, T helper cytokines, including high levels of soluble B-cell activation factor (sBAFF), results in inappropriate survival and aberrant activation of B cells that escape negative selection mechanisms meant to prohibit survival of alloreactive B cells [13]. Deficiencies in the antigen-specific TCR repertoire of donor CD4 T cells resulting from atrophy of the host thymus causes a deficient repertoire of donor B cell antibodies remains deficient after HCT [13].

Factors Affecting Host Immune Reconstitution

Multiple factors affect the kinetics and sufficiency of host immune reconstitution (Table 34.1) [11]. The clinical outcomes depend on the combined effects of these factors. Thus, the relative impact of any one factor cannot be determined with certainty.

Biomarkers of Host Immune Reconstitution

Multiple biomarkers of host immune reconstitution have been proposed (Table 34.2) [11]. Routine assessments include immunoglobulin titers and CD4 T-cell counts 1 year post-HCT. Even when both values return to normal, they do not constitute proof of fully functional T-cell or

Table 34.1 Factors influencing immune reconstitution after allogeneic hematopoietic cell transplantation The text in the Effects Row start at different levels. Should they not all start at the same level?

	Donor-recipient factors	Source of hematopoietic stem cells	Cell dose and graft conditioning	Preconditioning and immunosuppression	GVHD
Variables	1. HLA Matching 2. Donor age 3. Recipient age 4. CMV status	1. Peripheral blood progenitor cells 2. Bone marrow 3. Umbilical cord blood	1. Dose and quantity of stems cells 2. T-cell depletion	1. Cytoablative potency 2. Immunosuppression potency and spectrum	1. Presence 2. Absence
Effects	1. Best results with HLA-matched donors 2. Reduced stem cell function and engraftment with donors > 35 years old 3. Reduced T-cell development due to thymic dysfunction beginning at puberty 4. Risk of CMV reactivation in CMV (+) recipients, which delays diverse T cell reconstitution	1. Faster reconstitution but 10X increase in T cell quantities increase risk of GVHD 2. Innate immunity recovers in months, but T and B cell recovery takes 1–2 years 3. Alternative source of enriched stem cells with longer duration of neutropenia, delayed immune reconstitution, and increased infection-related morbidity (but not mortality)	1. Dose-dependent rate of reconstitution influenced by source and proportion of stem cells 2. Depletion of mature T cells from graft may decrease incidence of GVHD but can increase risk of infections due to delayed reconstitution and reduce GVT responses	1. Myeloablative regimens require immune reconstitution to restore hematopoiesis. Reduced intensity of preconditioning facilitates survival of host stem cells 2. Prevention of host rejection of donor grafts 3. Both preconditioning cytoablative regimens and post-HCT immunosuppression can damage the thymus and reduce recovery of donor-derived adaptive T-cell responses	1. Both aGVHD and cGVHD, as well as therapies used to treat them, delay immune reconstitution 2. Absence of GVHD optimizes potential for immune reconstitution

Table 34.2 Biomarkers of hematopoietic reconstitution after allogeneic hematopoietic cell transplantation

Lymphocytes	T cells	B cells	Vaccine response	Advanced molecular testing
Recovery of normal absolute lymphocyte counts	1. Absolute CD4 count 2. CD4/CD8 ratio	1. Quantification 2. Serum immunoglobulin levels of IgG and IgM	Increasing titer of antigen-specific immunoglobulin after immunization	1. Quantification of functional T cell subset phenotypes using flow cytometry 2. Identification of TCR rearrangement excision circles (TRECs) as biomarkers of newly generated antigen-specific T cells 3. Testing of TCR diversity using spectratyping 4. Deep sequencing of TCR to assess diversity of repertoire

B-cell adaptive immune responses. Conversely, failure of CD4 T cells to exceed 200/μL within 3 months of HCT is associated with increased rates of infection, NRM, and poor overall survival. The most reliable biomarker of immune function after HCT is production of antigen-specific antibodies to vaccines.

Graft-Versus-Host Reaction and Graft-Versus-Host Disease

In 1966, Billingham defined three criteria (Table 34.3) for development of the graft-versus-host reaction (GVHR) against allogeneic antigens [14]. Since the initial events occur in lymphoid compartments, Sackstein astutely added chemokine-mediated trafficking of activated donor T cells to the Billingham criteria to recognize that transendothelial migration was obligatory for formation of inflammatory infiltrates mediating tissue-specific pathology [15]. The GVHR is the obligatory precursor for generation of the allogeneic effector mechanisms mediating both beneficial GVT and destructive GVHD responses.

Graft-Versus-Tumor and Graft-Versus-Leukemia Responses

The term GVT encompasses both the graft-versus-leukemia (GVL) responses against hematologic malignancies and the graft-versus-solid tumor (GVST) responses against multiple

Table 34.3 Billingham criteria for development of graft-versus-host reaction

Criteria	Comment
Donor grafts must contain immunologically competent cells	GVHR is mediated by functional donor immune cells
Recipients must express tissue antigens that are not expressed by the donor	Donor immune cells recognize alloantigens expressed by the recipient host
Recipients must be incapable of mounting an effective response to destroy transplanted donor cells	Effective alloreactive responses of the recipient host against donor cells would prevent donor cell engraftment

non-hematologic malignancies [2, 16, 17]. GVT responses represent alloimmune reactions of donor NK cells, T cells, and antibodies against hematologic or non-hematologic malignant cells that persist in the host after HCT. Since the donor adaptive immune GVT response is against host allogeneic HLA and/or minor histocompatibility antigens (miHA) expressed by residual tumor cells, it is tightly linked with the risk of GVHD (see Fig. 34.1). Cytotoxic donor innate immune NK cells also mediate GVT responses by recognizing tumor cells injured by conditioning chemoradiation therapy [18]. GVST responses are directed against a wide variety of malignant cell types and are mediated by cytotoxic donor NK and alloactivated T cells. Recent studies indicate that host-derived immune cells may contribute to GVST responses, presumably due to a break in the tolerance of host effector cells to autoantigens expressed by the autologous tumor. Overall, GVST response rates of up to 53% have been reported [16]. However, GVST responses produce significant risks for aGVHD (16–65%) and cGVHD (14–54%) [16].

The balance between GVT responses and GVHD is determined by multiple factors, including the degree of immunogenetic differences between donor and host; the inflammatory environment created by conditioning regimens; the quantity of APCs of donor and host origin; the antigen specificity of the malignant cells; the kinetics, diversity, and magnitude of the response; and the effectiveness of inhibitory factors to reduce or silence the response [2]. Manipulation of specific factors to eliminate malignancy without provoking severe GVHD is one of the most important goals in HCT research [17].

Standardized Definitions for Acute and Chronic GVHD

Originally, the distinction between aGVHD and cGVHD was based on the time of clinical onset being before or after 100 days post HCT [3–6]. Recognition that signs of aGVHD or cGVHD can occur before or after 100 days post-HCT led to revised definitions and guidelines incorporating both clinical manifestations and the elapsed time to onset [19, 20]. The revised definitions include classic aGVHD; persistent, recurrent, and de novo late-onset aGVHD; classic cGVHD; and overlap cGVHD (Fig. 34.2).

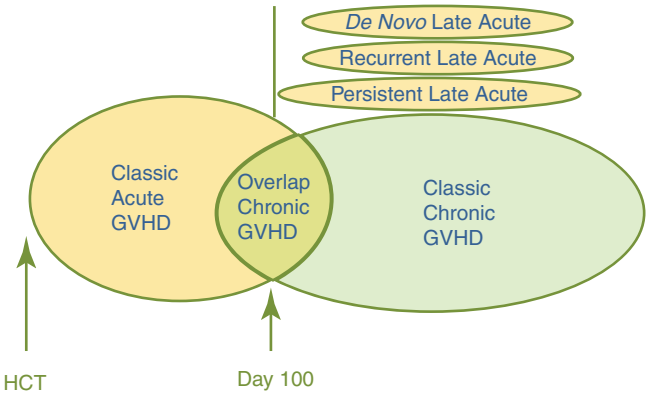

Fig. 34.2 Occurrence and timing of different types of graft-versus-host disease based on standardized definitions. Classic acute GVHD: Onset ≤100 days after HCT with clinical features of aGVHD and absence of "diagnostic" or "distinctive" features of cGVHD. Persistent, recurrent, and de novo late-onset acute GVHD: Onset >100 days after HCT with clinical features of aGVHD in the absence of "diagnostic" or "distinctive" features of cGVHD. Classic chronic GVHD: Presence of "diagnostic" or "distinctive" features of cGVHD in the absence of features of aGVHD. Can present at any time after HCT. Overlap chronic GVHD: Presence of cGVHD with aGVHD manifestations in skin, intestine or liver. This has also been referred to this as "acute on chronic" GVHD [6, 19, 20]

Epidemiology of Acute and Chronic GVHD

Acute GVHD

The exact incidence of aGVHD after allogenic HCT remains unknown because of confounding issues of identification and verification in the literature [3]. The reported incidence ranges from 9% to 50% in recipients of allogeneic HCT from HLA-identical siblings. The incidence even may be higher in recipients of HCT from matched unrelated donors, haploidentical related donors or haploidentical related donors [3].

Chronic GVHD

Patients may develop classic cGVHD as a de novo disease without prior aGVHD, following aGVHD or as overlap syndrome (see Fig. 34.2) [20]. The exact incidence remains unclear, and older estimates ranged from a 6% to 80% with a long-term prevalence of 40%. However, a recent systematic review of studies reporting the cumulative incidences of cGVHD in populations with >100 patients diagnosed with modern criteria provides more accurate data [21]. Importantly, the data showed that the cumulative incidence of cGVHD increases with time and varies with severity. The 1-, 2-, and

5-year cumulative incidences ranged from 14% to 58.3%, 13% to 83.8% and 44% to 70%, respectively. The 1-year cumulative incidence of cGVHD (grouped by disease severity) was 21.6% for mild and 23.8% for moderate cGVHD. The cumulative incidence of moderate to severe cGVHD ranged from 8.8% to 42.6%. A single center reported the incidence by severity as 29% in mild, 42% in moderate and 28% in severe [20]. The 1-year cumulative incidence of severe cGVHD ranged from 2.2% to 13%. In other studies, the incidence of severe cGVHD increased after 1 year, ranging from 8.3% to 27.6% at 2 years. These data clearly show that the incidence of cGVHD increases from the time of HCT through 5 years and varies among patients with mild, moderate or severe cGVHD.

Risk Factors for Acute and Chronic GVHD

Table 34.4 compares the risk factors for aGVHD and cGVHD. Risk factors for aGVHD vary according to the underlying disease and require distinct risk models for each. The presence of comorbid diseases in recipients prior to HCT also increases the risk of aGVHD, and an HCT-Specific Comorbidity Index (HCT-CI) has been developed to predict the incidence of severe aGVHD and mortality [22]. The presence of ≥1 risk factors for cGVHD portended development of cGVHD. For example, the cumulative prevalence of cGVHD 5 years after HCT in a large retrospective study was proportional to four specific risk factors: high recipient age, prior aGVHD, HCT from female donor to male recipient, and chronic myelogenous leukemia. The cumulative incidence of cGVHD was 9% in the absence of any of the risk factors, 29% with one risk factor, 53% with two risk factors, 68% with three risk factors, and 75% with all four risk factors. The cumulative incidence of cGVHD was 57% in recipients of HLA-identical allogeneic peripheral blood precursor cell transplants. Prior aGVHD significantly increased the risk of cGVHD (hazard ratio [HR], 1.67; 95% confidence interval [CI], 1.0–2.8), while prophylactic treatment with methotrexate and tacrolimus significantly reduced the risk (HR, 0.35; 95% CI, 0.2–0.6). Currently, immunotherapy for cancer using checkpoint inhibitors is commonly used to increase the activity of cytotoxic T cells against malignant cells. Unfortunately, checkpoint inhibitors, either before or after HCT, have increased significantly the risk of GVHD [23]. Mechanistically, checkpoint inhibitors likely increased the pathogenic cytotoxicity of donor alloreactive T cells mediating GVHD (see Pathogenesis below).

Table 34.4 Comparison of risk factors for acute GVHD and chronic GVHD

Risk factors	Acute GVHD	Chronic GVHD
Degree of HLA mismatch	Yes	Yes
Sex disparity: female donor into male recipient	Yes	Yes
Older age of donor or recipient	No	Yes
Intensity of Pre-HCT conditioning regimen	Yes	No
Type of prophylactic regimen to prevent aGVHD	Yes	No
Source of stem cells	Yes PBPC or BM > UCB	Yes PBPC rather than BM or UCB
Prior alloimmunization of donor (i.e., history of pregnancy or blood transfusions)	No	Yes
Prior aGVHD	NA	Yes
Donor lymphocyte infusions	No	Yes
Splenectomy	No	Yes
Cytomegalovirus seropositive donor and/or recipient	No	Yes
Epstein-Barr virus seropositive donor and/or recipient	No	Yes

Abbreviations: *aGVHD* acute GVHD, *PBPC* peripheral blood precursor cells, *BM* bone marrow, *UCB* umbilical cord blood

Clinical Features, Diagnosis, Grading, Therapy, and Prognosis of Acute and Chronic GVHD

Prophylaxis to Prevent Acute GVHD

A variety of immunosuppressive therapies have been used as prophylaxis after HCT to prevent or moderate development of aGVHD [24]. Current approaches are summarized in Table 34.5. As our understanding of pathogenic mechanism involved in aGVHD increases, additional prophylactic strategies will likely emerge.

Acute GVHD

Diagnostic Criteria for Acute GVHD

Patients with aGVHD can be staged and graded on the basis of clinical and biochemical features [1, 3, 4]. Table 34.6 summarizes the diagnostic features of patients with aGVHD and compares them with diagnostic features of patients with cGVHD. The onset of aGVHD is most often between 14 and 35 days after HCT [25]; however, aGVHD may also occur within 10 days of HCT (see Fig. 34.2). Rarely, hyperacute GVHD occurs 1 week after HCT with fever, diffuse erythroderma, desquamation, and edema that is most often fatal.

Target Organs in Acute GVHD

The principal target organs in aGVHD are the skin, gastrointestinal tract, and liver (Fig. 34.3). GVHD initially attacks specific cell types within these three organs: keratinocytes in

Table 34.5 Current strategies for the prevention of graft-versus-host disease

Strategies	Agents and mechanisms
Reduction of donor effector T Cells	1. ATG: Depletion of all naïve and mature donor T cells 2. Alemtuzumab: Depletion of T and B cells expressing CD52 3. Post-HCT cyclophosphamide: Depletion of rapidly proliferating, alloreactive T cells 4. Depletion of CD45 naïve T cells ex vivo
Inhibition of TCR and costimulatory signaling	1. CSA or TAC: Inhibition of mitogenic IL-2 production required for proliferation of activated CD4 and CD8 T cells 2. MMF: Inhibition of de novo purine synthesis required for cell proliferation with enhanced antiproliferative effects in T and B cells lacking compensatory purine salvage pathways 3. SIR: Inhibition of mTOR signaling by IL-2 and growth factors required for T cell clonal expansion 1. MTX: Folate antagonist causing non-specific antiproliferative effects 2. Abatacept or Belatacept: CTLA-4 Ig inhibitors of costimulatory signaling through CD28 on T cells
Disruption of cytokine production or signaling	1. Filgotinib or itacitinib: JAK1 inhibitors that block T cell activation, cytokine secretion and proliferation 2. Ruxolitinib: JAK1/2 inhibitor that blocks T-cell activation, cytokine secretion and proliferation. FDA-approved for myelofibrosis 3. Pacritinib: JAK2 inhibitor that blocks T-cell activation, cytokine secretion and proliferation 4. Tofacitinib: JAK1/3 inhibitor that blocks T-cell activation, cytokine secretion and proliferation. FDA-approved for rheumatoid arthritis 5. Tocilizumab (anti-IL-6 mAb) + CSA: Inhibitors of proinflammatory IL-6 signaling and IL-2 production 6. Alpha-1-antitrypsin: Inhibition of proinflammatory cytokine production and neutrophil elastase 7. Vorinostat: Inhibition of histone deacetylase resulting in reduction of proinflammatory cytokines. Also causes expansion of Tregs
Inhibition of chemokine-mediated transendothelial trafficking of leukocytes	1. Maraviroc: Inhibition of CCR5-mediated donor T-cell infiltration into tissues 2. Natalizumab: Inhibition of donor T cell binding to α4β7 integrin 3. Vedolizumab: Inhibition donor T cell binding to the α4 integrin subunit
Immunosuppression and immunomodulation of donor T and B cells to host allogeneic antigens	1. CD4 nTregs: Modulation of naturally occurring alloantigen-specific donor T cell reactions against host tissues 2. CD4 iTregs: Prevention of alloantigen-specific donor T-cell reactions against host tissues using protocols to induce the antigen-specificity required 3. CD4 Tr1: Prevention of alloantigen-specific donor T-cell reactions to promote peripheral tolerance 4. Alpha-1-antitrypsin: Induction of Treg expansion 5. Vorinostat: Inhibition of histone deacetylase resulting in expansion of Tregs, upregulation of IDO expression in DC, and immunomodulation of APC functions 6. Extracorporeal photopheresis: Expansion of CD4 Tregs 7. Low dose IL-2: Preferential induction and expansion of CD4 Tregs 8. MSCs: Non-immunogenic promotion of immunosuppression, hematopoiesis, and tissue regeneration 9. MAPCs: Non-immunogenic promotion of immunosuppression, immunomodulation and tissue regeneration

Abbreviations: *ATG* anti-thymocyte globulin, *CD* cluster of differentiation, *IL* interleukin, *CSA* cyclosporine, *TAC* tacrolimus, *MMF* mycophenolate mofetil, *SIR* sirolimus, *mTOR* mechanistic target of rapamycin, *MTX* methotrexate, *CTLA-4* cytotoxic T lymphocyte antigen-4, *JAK* Janus kinase, *mAb* monoclonal antibody, *Treg* T regulatory, *nTreg* natural T regulatory, *iTreg* inducible T regulatory, *Tr1* type 1 regulatory T cell, *DC* dendritic cell, *IDO* indoleamine 2,3-dioxygenase, a potent immune-suppressive enzyme in DCs, *MSCs* mesenchymal stem cells, *MAPCs* multipotent adult progenitor cells
Created with data from [7, 8, 24, 64, 139, 140]

the skin, enterocytes lining the gastrointestinal tract, and cholangiocytes lining small- to medium-caliber bile ducts. Sustained necroinflammation and fibrosis lead to progressive organ damage. Table 34.6 compares the organ involvement in patients with aGVHD and cGVHD.

Organ-Specific Manifestations of Acute GVHD

Skin

The skin is the most commonly affected organ in aGVHD [26]. It presents as a maculopapular rash, often involving the palms, soles of the feet, and ears, with symptoms of pruritus, burning, or tightness of the skin. The rash spares the scalp. Severe cases may progress to erythroderma with bullae, desquamation, and ulceration. Biopsy is mandatory to detect diagnostic features of apoptosis in the dermal crypts, dyskeratosis, epidermal keratinocytes, and lymphocytic infiltration of the dermal vasculature and to exclude other causes such as drug-induced hypersensitivity or viral infections.

Intestine

aGVHD can involve the upper and/or lower gastrointestinal tracts [27–29]. Symptoms include as anorexia, nausea, vomiting, diarrhea (with or without blood), abdominal pain, or ileus. Vomiting without nausea is a feature of intestinal aGVHD, but aGVHD does not cause gastroparesis. Secretory and/or exfoliative diarrhea with bleeding ulcerations may occur, and ulcerations increase the risk of sepsis. The differential diagnosis of diarrhea includes CMV or herpes virus

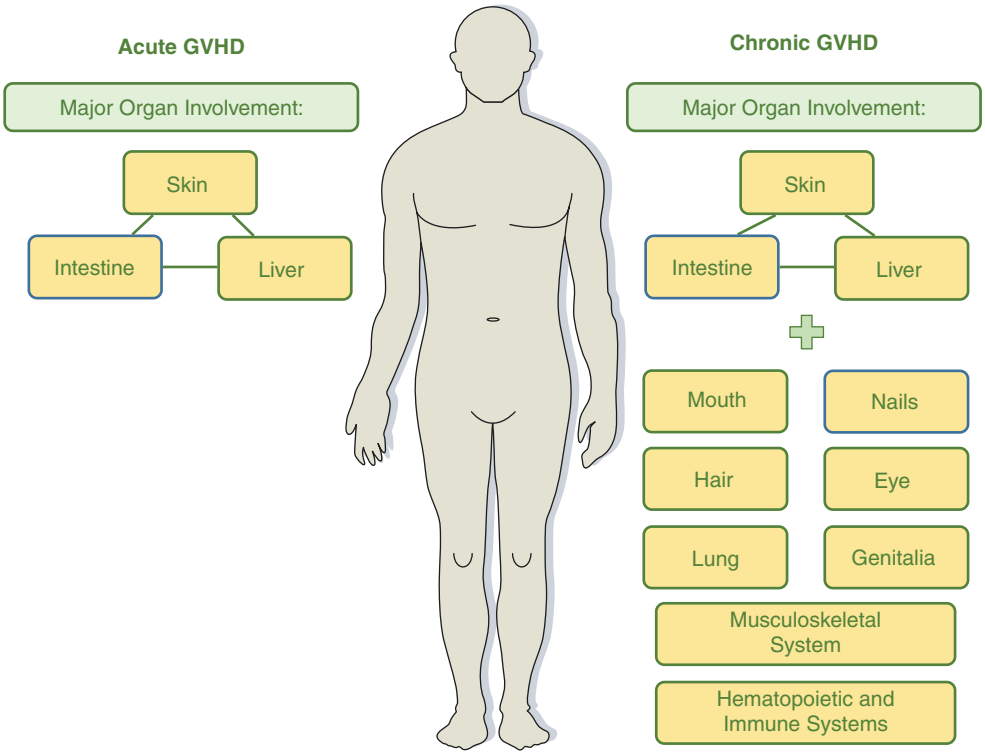

Fig. 34.3 Comparison of major organ involvement associated with acute graft-versus-host disease and chronic graft-versus-host disease. HCT hematopoietic cell transplantation, GVHD graft-versus-host disease

Table 34.6 Comparison of diagnostic features of acute GVHD and chronic GVHD

Organ or site	Diagnostic features of aGVHD	Diagnostic, distinctive, or unclassified features of cGVHD	Features observed in aGVHD or cGVHD
Skin	Maculopapular ash	*Diagnostic:* Poikiloderma Lichen planus-like features Sclerotic features Morphea-like features Lichen-sclerosis-like features *Distinctive:* Depigmentation Papulosquamous lesions *Unclassified:* Ichthyosis Keratosis pilaris Hyperpigmentation Hypopigmentation Sweat impairment	Erythema Pruritus (Maculopapular rash)
GI Tract	Diarrhea Nausea Vomiting Anorexia Weight loss	*Diagnostic:* Esophageal web Upper esophageal stricture or stenosis *Distinctive*: None *Unclassified*: None	Diarrhea Nausea Vomiting Anorexia Weight loss
Liver	Elevated ALP >2X ULN Elevated total bilirubin Elevated ALT >2X ULN	*Diagnostic*: None *Distinctive*: None *Unclassified*: None	Elevated ALP >2X ULN Elevated total bilirubin Elevated ALT >2X ULN
Mouth	None	*Diagnostic*: Lichen planus-like lesions *Distinctive*: Xerostomia Mucosal atrophy Mucoceles Ulcers Pseudomembranes *Unclassified*: None	Mucositis Gingivitis Erythema Pain

Table 34.6 (continued)

Organ or site	Diagnostic features of aGVHD	Diagnostic, distinctive, or unclassified features of cGVHD	Features observed in aGVHD or cGVHD
Nails	None	*Diagnostic*: None *Distinctive*: Dystrophy Ridging, splitting, brittleness Onycholysis Pterygium unguis Symmetric loss *Unclassified*: None	None
Hair	None	*Diagnostic*: None *Distinctive*: Scarring or non-scarring scalp alopecia (after recovery from chemotherapy) Scaling of scalp Loss of body hair *Unclassified*: Patchy thinning of scalp hair Premature graying	None
Eyes	None	*Diagnostic*: None *Distinctive*: New-onset dry, gritty painful eyes Keratoconjunctivitis sicca Cicatricial conjunctivitis Confluent punctate keratopathy *Unclassified*: Photophobia Periorbital hyperpigmentation Blepharitis	None
Lung	None	*Diagnostic*: Bronchiolitis obliterans on biopsy Bronchiolitis obliterans syndrome *Distinctive*: Air trapping and bronchiectasis on chest CT *Unclassified*: Cryptogenic organizing pneumonia Restrictive lung disease	None
Genitalia	None	*Diagnostic*: Lichen planus-like features Lichen sclerosis-like features *Females*: Vaginal scarring or clitoral/labial agglutination *Males*: Phimosis or urethral/meatus scarring or stenosis *Distinctive*: Erosions Fissures Ulcers *Unclassified*: None	None
Musculoskeletal	None	*Diagnostic*: Fasciitis Joint stiffness or contractures due to fasciitis *Distinctive*: Myositis or polymyositis *Unclassified*: Edema Muscle cramps Arthralgia or arthritis	None

(continued)

Table 34.6 (continued)

Organ or site	Diagnostic features of aGVHD	Diagnostic, distinctive, or unclassified features of cGVHD	Features observed in aGVHD or cGVHD
Hematopoietic and Immune	None	*Diagnostic*: None *Distinctive*: None *Unclassified*: Thrombocytopenia Lymphopenia Eosinophilia Hypogammaglobulinemia Hypergammaglobulinemia Autoantibodies Raynaud's phenomenon	None
Miscellaneous	None	*Diagnostic*: None *Distinctive*: None *Unclassified*: Pleural or pericardial effusions Ascites Peripheral neuropathy Nephrotic syndrome Myasthenia gravis Cardiomyopathy Cardiac conduction abnormality	None

Abbreviations: *aGVHD* acute GVHD, *cGVHD* chronic GVHD, *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *ULN* upper limit of normal.
Created with data from [5, 6]

infections, parasitic infections, and pseudomembranous colitis caused by *Clostridium difficile* toxin. Mucosal biopsies are diagnostic [28] when characteristic histologic features of apoptotic bodies in the base of intestinal crypts, crypt abscesses, loss of crypts, and the flattening of the villi are present.

Liver

aGVHD of the liver typically causes hyperbilirubinemia and increased serum alkaline phosphatase (ALP) and alanine or aspartate aminotransferase (ALT or AST) levels [1, 3, 4, 30]. Severe cases can progress to liver failure with coagulopathy and hepatic encephalopathy. Hepatic aGVHD must be distinguished from other hepatic complications observed after HCT [30]. These include drug-induced liver injury (DILI) caused by cytoablative chemotherapies, immunosuppression regimens, or more recently check point inhibition of CTLA-4 or PD-1/PD-1L; viral infections, especially non-hepatotrophic viruses such as CMV, EBV, or herpes viruses and more rarely hepatotropic viruses such as HAV, HBV ± HDV, HCV, and HEV; cholestasis associated with sepsis, the systemic inflammatory response syndrome, or total parenteral nutrition; primary hepatic lymphoma; and post-transplant lymphoproliferative disease caused by EBV-transformed B cells [30]. Certain conditioning regimens or chemotherapies for leukemia cause sinusoidal obstructive syndrome (SOS) with acute hepatic congestions presenting as rapid onset of ascites and right upper quadrant pain [31].

Liver biopsy should be performed to establish or refute the diagnosis of hepatic aGVHD [30]. Histologic features of hepatic GVHD include lymphocytic cholangitis with segmental apoptosis of cholangiocytes lining the proximal small to medium-caliber interlobular bile ducts, lymphocytic infiltrates in the portal tracts and endothelialitis of the portal and/or terminal hepatic veins. Progression of cholangiocyte destruction culminates in ductopenia (loss of the interlobular duct) (Fig. 34.4) [30, 32, 33]. The exact incidence of hepatic aGVHD remains unclear, since many patients do not undergo liver biopsy and the differential diagnosis contains so many alternative etiologies.

Clinicians should keep in mind useful facts about hepatic GVHD [30]. First, aGVHD rarely (4%) manifests solely as liver dysfunction; thus, isolated hepatic dysfunction is more likely due to another etiology. Second, histopathology is not pathognomonic in aGVHD because immunosuppression prophylaxis or treatments for aGVHD may alter biopsy findings. Third, the probability of hepatic aGVHD is highest when accompanied by features of skin or intestinal involvement.

Grading of Severity in Acute GVHD

Experts from the European Society for Blood and Marrow Transplantation, the National Institutes of Health, and the Center for International Blood and Marrow Transplant Research recently reviewed definitions and guidelines with the goal of reducing confusion [19]. They concluded that the

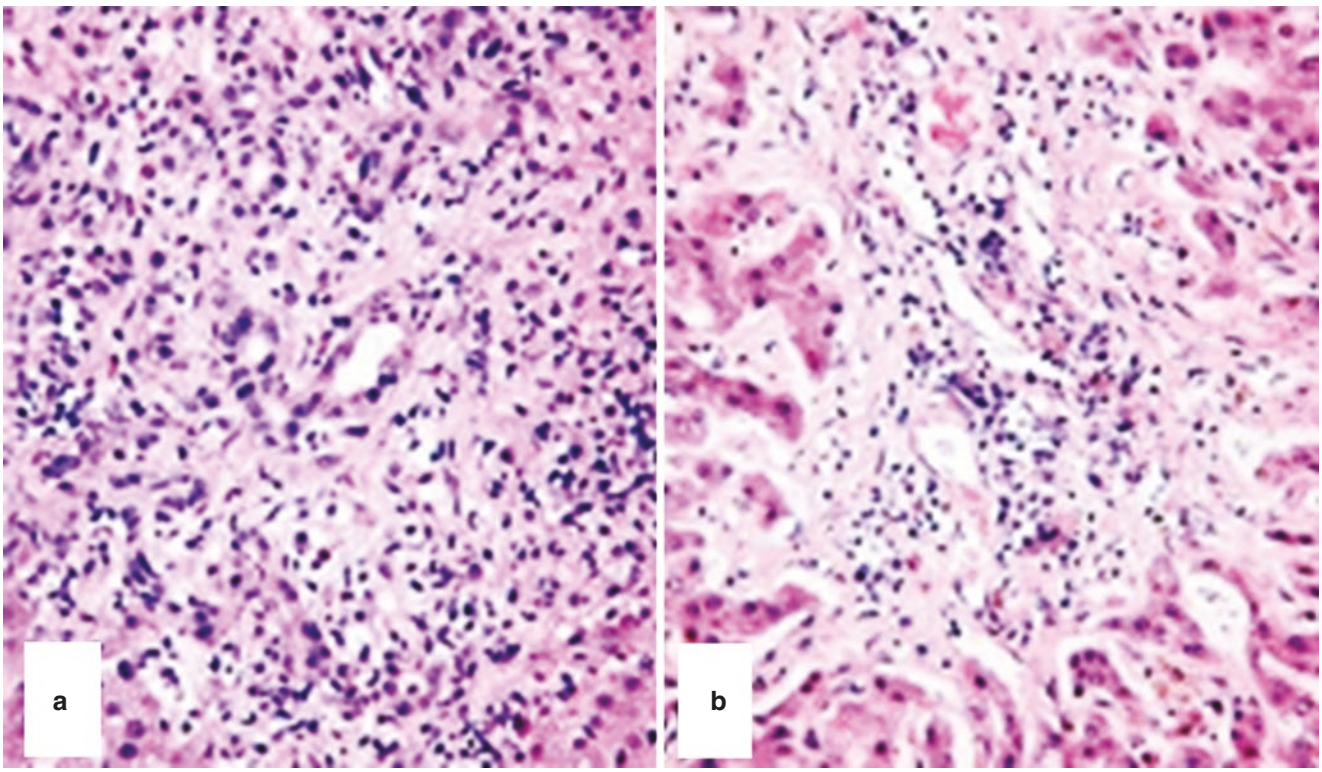

Fig. 34.4 Histopathology of hepatic graft-versus-host disease. (**a**) Late-onset acute GVHD, day 123. The portal tract contains an infiltrate composed of lymphocytes and scattered eosinophils. The interlobular bile duct exhibits lymphocytic infiltration of the biliary epithelium, segmental loss of nuclei, cytoplasmic vacuolization, and nuclear dyspolarity. Ductular proliferation is evident at the margin of the portal tract (original magnification ×250). (**b**) Refractory untreated GVHD day 350. The portal tract contains an infiltrate composed of lymphocytes and plasma cells. The interlobular bile duct is absent (ductopenia) and there is fibrosis (original magnification ×250). (Photomicrographs adapted from [32] and used with permission)

most comprehensive and detailed criteria available are the Mount Sinai Acute GVHD International Consortium (MAGIC) criteria for aGVHD and the NIH 2014 criteria for cGVHD (Table 34.7). The revised Ann Arbor Score, based on an algorithm incorporating plasma levels of TNFR1, ST2, and REG3α (Table 34.8), also accurately predicted the probability of NRM and identified high-risk aGVHD at the time of onset independently of the initial clinical presentation [34].

Biomarkers for Acute GVHD

The currently recommended grading systems used in aGVHD are based on validated biomarkers (see Table 34.7) [19, 34]. Table 34.8 lists biomarkers with diagnostic and prognostic value, based on their likely roles in the pathogenesis of GVHD [35, 36]. As noted above, the Ann Arbor score predicting NRM was derived from the quantities of TNFR1, ST2, and REG3α detected in plasma [34]. Discovery and validation of novel biomarkers for diagnosis, grading, prognosis, and prediction of response to therapy and NRM represent urgent unmet needs in aGVHD. Among promising candidates are microRNAs (miRNAs) that regulate gene expression at the post-transcriptional level; however, their status remains exploratory [36].

Treatment of Acute GVHD

The goals of therapy for aGVHD include control and resolution of manifestations of aGVHD, preservation of the GVT effect, and minimization of the risk of infection. The choice of initial therapies for aGVHD depends on the severity of symptoms, the number of organs involved, and the composition of the prophylactic regimens used at the time of onset of aGVHD [3, 4, 37]. Onset of aGVHD occurs in the setting of prophylactic immunosuppression, which unfortunately varies among HCT centers. The most commonly used prophylactic regimens are combinations of a calcineurin inhibitor (cyclosporine or tacrolimus) and methotrexate. Other approaches have included anti-thymocyte globulin to deplete T cells and temporary cyclophosphamide dosing to deplete highly proliferative alloreactive T cells while preserving Tregs [3, 4, 37]. Results of randomized controlled trials support the use of steroid monotherapy for initial therapy of aGVHD [3, 4, 37]. In contrast, evidence for efficacy and safety of non-steroidal treatments is poor. A 2016 systematic review and meta-analysis of clinical trials of steroids versus steroids plus an additional agent could identify any additional agents (e.g., anti-thymocyte globulin, infliximab [anti-TNFα], dacli-

Table 34.7 Grading of severity in acute graft-versus-host disease

Severity staging by organ	Original Glucksberg criteria	"Modified Glucksberg" or "Keystone" criteria and IBMTR criteria	MAGIC criteria
Liver			
0	Bilirubin (<2 mg/dL) or AST/ALT (150–750 IU)	Bilirubin (<2.0 mg/dL)	Bilirubin (<2 mg/dL)
1	Bilirubin (2–3 mg/dL)	Bilirubin (2.0–3.0 mg/dL)	Bilirubin (2–3 mg/dL)
2	Bilirubin (3.1–6 mg/dL)	Bilirubin (3.1–6.0 mg/dL)	Bilirubin (3.1–6 mg/dL)
3	Bilirubin (6.1–15 mg/dL)	Bilirubin (6.1–15.0 mg/dL)	Bilirubin (6.1–15 mg/dL)
4	Bilirubin (>15 mg/dL)	Bilirubin (>15.0 mg/dL)	Bilirubin (>15 mg/dL)
Skin			
0	No rash	No rash	No rash
1	Rash < 25% of BSA	Rash < 25% of BSA	Rash < 25% of BSA
2	Rash 25% to 50% of BSA	Rash 25% to 50% of BSA	Rash 25% to 50% of BSA
3	Rash > 50% of BSA	Rash > 50% of BSA	Rash > 50% of BSA
4	Generalized erythroderma with bullous formation	Generalized erythroderma with bullous formation	Generalized erythroderma (>50% BSA) plus bullous formation and desquamation >5% of BSA
Lower GI			
0	Diarrhea < 500 mL/day	Diarrhea < 500 mL/day	Diarrhea < 500 mL/day or <3 episodes/day
1	Diarrhea > 500 mL/day	Diarrhea > 500 mL/day	Diarrhea 500 to 999 mL/day or 3–4 episodes/day
2	Diarrhea > 1000 mL/day	Diarrhea > 1000 mL/day	Diarrhea 1000–1500 mL/day or 5–7 episodes/day
3	Diarrhea > 1500 mL/day	Diarrhea > 1500 mL/day	Diarrhea >1500 mL/day or >7 episodes/day
4	Diarrhea > 2000 mL/day	Severe abdominal pain with or without ileus	Severe abdominal pain with or without ileus or grossly bloody stools (regardless of stool volume)
Upper GI			
0	NA	No persistent nausea and no histologic evidence of GVHD in the stomach or duodenum	No or only intermittent anorexia[a] or nausea or vomiting
1	NA	Persistent nausea with histologic evidence of GVHD in the stomach or duodenum	Persistent anorexia or nausea or vomiting

Abbreviations: *AST* aspartate transaminase, *ALT* alanine aminotransferase, *BSA* body surface area), *GI* gastrointestinal tract, *GVHD* graft versus host disease, *IBMTR* International Bone Marrow Transplantation Registry, *IU* international units, *MAGIC* Mount Sinai Acute GVHD International Consortium, *NA* not applicable

[a]To suggest GVHD, anorexia should be accompanied by weight loss and nausea should last at least 3 days or be accompanied by at least two vomiting episodes/day for ≥2 days [19]

Table 34.8 Biomarkers of acute GVHD categorized according to potential roles in pathogenesis

	Pre-HCT conditioning	Developmental phase of aGVHD	Onset of aGVHD
Intestinal injury and dysbiosis of gut microbiome	Picornavirus	Blautia (protective) Low urinary 3-IS, viruses	REG3α, fecal calprotectin, fecal α-1-antitrypsin, miRNAs
Vascular endothelial damage	VEGF-A, PlGF, Follistatin, Ang2	ST2, REG3α	VEGF/ Ang2 ratio, Follistatin, REG3α, low EGF, amphiregulin
Innate immune activation	IL-1β, IL-6, TNFα, NK cell sCD226	IL-6, TNFα, CRMF44	IL-6
Adaptive immune activation of cytotoxic T cells	IL-33	TNFR1, CD25, IL-7, IL-8, sTIM3, CD4/CD8 ratio, elevated serum CXCL9	TNFR1, ST2, sTIM3, REG3α

Abbreviations: *IL* interleukin, *VEGF* vascular endothelial growth factor, *PlGF* placental growth factor, *s* soluble, *CD* cluster of differentiation, *sCD226* soluble NK cell adhesion molecule, *IS* indoxyl sulfate (biomarker of gut microbiome diversity), *IL-33* inducer of CD4 Th2 cytokine production, *ST2* member of IL-1 receptor family acting as a receptor for IL-33, *REG3α* C-type lectin belonging to family of antimicrobial peptides, *CRMF44* surface marker of activated dendritic cells, *sTIM3* T-cell immunoglobulin and mucin-domain containing-3 expressed on IFNγ producing dendritic cells, CD4 Th1, Th17,Treg cells and CD8 T cytotoxic cells, CD25, IL-2 receptor, *miRNA* microRNA, *Ang2* angiopoietin-2, a vascular growth factor, *EGF* epidermal growth factor, *TNFR1* tumor necrosis factor receptor-1 (CD120a)

zumab [anti-CD25], CD-5-specific immunotoxin or mycophenolate mofetil) that significantly increased the efficacy above steroid alone [38]. Thus, therapies for initial management, tissue-specific management, and steroid-resistant aGVHD are largely empiric (see Table 34.9). The combined effects of organ damage due to aGVHD, immunosuppression, cytopenia, and prophylactic antimicrobials substantially increase the risks for infections with bacteria or fungi resistant to prior courses of prophylactic or therapeutic antimicrobials.

Patients with aGVHD require daily evaluation for symptoms and infectious and nutritional or metabolic complications [37]. Formal grading of severity should be performed on days 5 and 7 after initiation of therapy for aGVHD (see Table 34.7). Patients are deemed steroid-refractory if they experience progression by day 5 or show non-response by day 7. No superior regimen has been identified for treatment of steroid-refractory aGVHD (Table 34.9). Therefore, all patients with steroid-refractory aGVHD should be encouraged to enroll in a clinical therapeutic trial.

Table 34.9 Systemic treatment options for acute GVHD

Regimens	Systemic effect(s)	Tissue-specific effects	Comments
Optimized prophylaxis Typically, CSA + MTX or TAC + MTX	Broad immunosuppression inhibiting proinflammatory genes and production of mitogenic IL-2 required for clonal expansion of CD4 and CD8 T cells and MTX antiproliferative effects on dividing innate and adaptive immune cells Systemic immunosuppression, HTN, DM, nephrotoxicity Increased risk of infections	Varies according to composition of regimen and dosages Antiproliferative effects injure IECs	Interpretation of safety and efficacy hampered by absence of a consensus regimen for treatments of aGVHD
Steroid monotherapy			
Methylprednisolone for Grade II to IV aGVHD	Reduces transcription of proinflammatory genes causing broad immunosuppression Increased risk of infections	Systemic effects, except for topical steroids for skin lesions	Efficacy in aGVHD shown in RCTs
Oral budesonide	First-pass extraction by liver eliminates systemic steroid effects	Increased intraluminal effects for GI aGVHD due to poor absorption	Suitable for predominantly GI symptoms of aGVHD First pass extraction by liver causes hepatospecific immunosuppression
Oral beclomethasone	Lack of absorption prevents systemic steroid effects	Increased intraluminal effects for GI aGVHD due to poor absorption	GI effects similar to budesonide
Steroid + additional agent for steroid-refractory high-grade aGVHD			
Mycophenolate mofetil	Antiproliferative agent with enhanced effects on lymphocytes Antimicrobial effects	Antimicrobial effects AE of diarrhea may confuse assessment of GI aGVHD	Prodrug converted in the liver to mycophenolic acid. Failed in RCT for treatment of Grade II-IV aGVHD
Ruxolitinib	Risks of severe cytopenias, infections Elevation of ALT SIRS after abrupt withdrawal in patients with myelofibrosis	No specific GI toxicities	Selective JAK 1/2 inhibitor, FDA approved for treatment of myelofibrosis, polycythemia vera and aGVHD
Sirolimus	T cell immunosuppression Increased risk of infections Compromises wound healing Absence of risks of HTN, DM or nephrotoxicity unless combined with a CNI	mTOR inhibition of IL-2 and T cell growth factor signaling decreases proliferation of activated T cells	Safety and efficacy established in solid organ transplantation
Etanercept	Inhibits immune-mediated pathology caused by excessive TNFα High risk of serious infections Risks of reactivation of tuberculosis or HBV infection	No tissue or organ specificity Binds soluble TNFα, reducing its immunopathological effector functions No effect on TNFα production	FDA-approved for rheumatological diseases and psoriasis
Anti-thymocyte globulin	Intense immunosuppression with depletion of T cells Increased risk of infections Risk of anaphylaxis	No specific GI toxicities	Rabbit anti-thymocyte globulin FDA-approved to prevent rejection after kidney transplantation

(continued)

Table 34.9 (continued)

Regimens	Systemic effect(s)	Tissue-specific effects	Comments
Non-pharmacologic therapy			
Extracorporeal photopheresis	Immunosuppression of donor T cell allogeneic responses Risks of phlebitis at infusion site, thrombocytopenia	No tissue or organ specificity	Infusion of autologous peripheral blood lymphocytes after incubation with 8-methoxypsoralen and exposure to ultraviolet-A light Infused irradiated monocytes differentiate into immature, tolerogenic DCs that inhibit propagation of donor T cell responses
Psoralen and ultraviolet A irradiation (PUVA)	Immunomodulatory effects on mechanisms of cutaneous inflammatory injury	Skin specificity	Used for steroid-resistant aGVHD and cGVHD

Abbreviations: *CSA* cyclosporine, *TAC* tacrolimus, *MTX* methotrexate, *IL* interleukin, *HTN* hypertension, *DM* diabetes mellitus, *IECs* intestinal epithelial cells, *RTC* randomized controlled trial, *GI* gastrointestinal, *ALT* alanine aminotransferase, *SIRS* systemic inflammatory response syndrome, *JAK* Janus kinase, *mTOR* mechanistic target of rapamycin, *TNFα* tumor necrosis factor-alpha, *DCs* dendritic cells, *PUVA* psoralen and ultraviolet A irradiation

Prognosis of Patient with Acute GVHD

The rates of NRM vary widely among patients with aGVHD, indicating that multiple patient-specific factors determine NRM. Maximal clinical severity of aGVHD and the number of organs involved when admitted for critical care increase NRM [34, 39]. Most deaths occur after failure of therapy and result from complications of aGVHD involvement of the gastrointestinal tract [29]. A prospective study of biomarkers in 492 patients newly diagnosed with aGVHD identified three subgroups of patients whose probabilities of NRM varied according to aGVHD severity [34]. Three of the prognostic biomarkers had been previously validated (see Table 34.8) and had biological relevance to the gastrointestinal tract (TNFR1, ST2, and REG3α). The study also employed the new Ann Arbor GVHD scoring system for severity of aGVHD and probability of NRM. Overall, NRM increased significantly with each Ann Arbor severity score: 8% (95% CI 3%–16%) for score 1; 27% (95% CI 20%–34%) for score 2, and 46% (95% CI 33%–58%) for score 3. Correspondingly, rates of therapeutic response significantly decreased as severity scores increased: 86% for score 1; 67% for score 2; 46% for score 3 ($p < 0.0001$). The Ann Arbor scores also predicted a positive treatment response, risk of developing intestinal aGVHD, and stratified risk of NRM independently of clinical symptoms. MAGIC, the collaboration of 25 HCT centers, recently validated the MAGIC algorithm probability (MAP) predictor of response to treatment and maximum GVHD severity on the basis of the two gastrointestinal biomarkers ST2 and REG3α [19] (see Table 34.8). At the onset of aGVHD, the MAP accurately assigned patients to Ann Arbor groups 1, 2, or 3 and predicted NRM. In addition, after the first week of treatment for aGVHD, the MAP accurately predicted the risk of NRM for steroid-resistant patients. The accuracy of MAP reflects the fact that gastrointestinal complications of aGVHD trigger and amplify GVHD (see Pathogenesis) and are the major cause of NRM.

Chronic GVHD

cGVHD is a major cause of late mortality after HCT that is not attributable to relapse of malignant disease [21, 40]. Table 34.6 summarizes differences between aGVHD and cGVHD based on the NIH Consensus Statement [5]. The risk of cGVHD is substantially increased in patients with any prior manifestation of GVHD [41]. The reported incidence of cGVHD ranged from 30% to 60% with bone marrow derived HCT and may be higher using peripheral blood stem cells. The NIH Consensus criteria for diagnosis and grading of severity have been validated but have had limited value in predicting clinical outcomes [42].

Diagnostic Criteria for Chronic GVHD

The clinical manifestations of cGVHD are more protean than those caused by aGVHD (see Fig. 34.1 and Table 34.6) [5, 6]. Clinicopathological features of cGVHD are categorized as diagnostic, distinctive, and unclassified (see Table 34.6) [6]. Significant dysfunctions of the immune system in cGVHD increases susceptibility to viral, bacterial, fungal, and protozoal opportunistic infections. Histopathological changes in the immune system include involution of the thymic epithelium, loss of Hassall corpuscles, lymphopenia, and absence of secondary germinal centers in lymph nodes. The diversity of clinical and laboratory abnormalities often leads to delays in diagnosis and therapy [6, 20]. Thus, clinicians must diligently assess HCT recipients on a serial basis and consult specialists whenever suspicious of cGVHD.

Target Organs in Chronic GVHD

As in aGVHD, the principal target organs in cGVHD are the skin, gastrointestinal tract, (including esophagus) and liver [5, 6]. However, cGVHD also involves many additional organs, including the eye, lacrimal glands, oropharyngeal mucosa, salivary glands, lungs, female and male genitourinary tracts, musculoskeletal system, hematological and immune systems (see Fig. 34.3 and Table 34.6) [6, 42]. Involvement of multiple organs differentiates the clinical manifestations and morbidity and mortality risks of cGVHD from those of aGVHD. Table 34.6 compares differences in organ-specific and clinical manifestations between aGVHD and cGVHD. Most patients with cGVHD have ≤3 tissues or organs involved [43].

Organ-Specific Manifestations of Chronic GVHD

Skin

Skin manifestations of cGVHD differ from those observed in patients with aGVHD Table 34.6 [6]. Lesions resembling diffuse lichen planus, papulosquamous dermatitis, fibrous plaques, desquamation, altered pigmentation, and vitiligo occur in up to 80%. Alopecia and onychodysplasia may occur as a result of the destruction of dermal appendages. Changes may mimic scleroderma with induration and tightening of the skin, joint contractures, cutaneous atrophy, and chronic ulcerations.

Intestine

Gastrointestinal symptoms in patients with cGVHD can mimic a variety of intestinal diseases [41, 44]. In addition to manifestations observed in aGVHD, patients with cGVHD experience symptoms and signs of dysmotility, pancreatic insufficiency, lactose intolerance, or infectious gastroenteritis or colitis. Esophagitis may result in dysphagia or odynophagia. Gastrointestinal disease often occurs in conjunction with oral manifestations [45], and malnutrition occurs in 29% [43].

Liver

Hepatic disease associated with cGVHD classically manifests with cholestatic biochemical abnormalities with elevated serum ALP and gamma-glutamyl transferase (ggt) and variable elevations of total and direct bilirubin and AST or ALT [41]. Initial histopathological findings of portal inflammation and lymphocytic cholangitis of interlobular bile ducts are similar in both aGVHD and cGVHD (Fig. 34.4) [33]. However, chronic lymphocytic cholangitis can result in senescent changes in the biliary epithelia [46], which contribute to progression of ductopenia of interlobular bile ducts, and worsening of chronic cholestasis [6, 21, 30]. Progressive ductopenia obstructs bile flow and cholestasis stimulates periportal fibrosis. However, progression to biliary cirrhosis is rare, in part due to the fact that death often occurs before development of cirrhosis.

Eye

Destruction of the lacrimal gland results in keratoconjunctivitis sicca with symptoms of ocular dryness, photophobia, and burning pain [47]. The conjunctivae are rarely involved in severe cGVHD.

Mouth and Oropharynx

Destruction of the epithelia of the salivary glands leads to xerostomia [48]. Oral mucosal erythema is common, and the occurence of white plaques can lead to a misdiagnosis of candida or herpes infections. Lichenoid plaques occur with advanced disease. Food sensitivity caused by oral mucosal lesions often limits oral nutrition.

Lung

cGVHD is associated with bronchiolitis obliterans, which has a poor prognosis [21, 49]. It presents as cough and/or dyspnea, and pulmonary function tests show obstructive airway disease and reduced diffusion capacity for carbon monoxide (DLCO). Computed tomography of the chest typically shows hyperinflation with a ground glass appearance, but it may also appear normal. Severe sclerotic cutaneous disease may constrict the chest wall, producing dyspnea without pulmonary disease.

Female and Male Genital Tract

cGVHD affects the vulva and vagina in 25–49% of long-term survivors of HCT [50].Vulvar involvement presents a median of 7–10 months after HCT, but vaginal involvement can present concurrently or independently up to 8 years later. Sclerotic stenosis of the vagina can lead to hematocolpos. Genital GVHD may be the initial manifestation of cGVHD in up to 27% of women. Phimosis and ureteral or meatal scarring with stenosis are the most common genital manifestation of cGVHD in males.

Musculoskeletal System

Musculoskeletal manifestations often occur in conjunction with skin changes in cGVHD [51]. Fasciitis may restrict the range of motion of joints. Muscle cramping is common, but myositis or elevated creatine kinase are rare. Chronic treatment with steroids after HCT may cause avascular necrosis, osteopenia, or osteoporosis.

Hematopoietic System

cGVHD is commonly associated with chronic cytopenias [1, 6]. Stromal bone marrow damage can decrease production, and autoimmune-like neutropenia, anemia, and/or thrombocytopenia may occur. Thrombocytopenia caused by cGVHD is a poor prognostic sign [52]. In children, eosinophilia is a biomarker for development of cGVHD [53].

Immunologic System

cGVHD is intrinsically immunosuppressive and occurs in the setting of chronic immunosuppressive therapies [41, 44]. Specific abnormalities of cellular immunity occur in cGVHD, including decreased production of antibodies against specific antigens, defective numbers and functions of CD4 T cells, and defective Tregs. Functional asplenia and hypogammaglobinemia also occur.

Grading of Severity in Chronic GVHD

The National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in cGVHD proposed a new scoring system for individual organs and an algorithm for calculating global severity (mild, moderate, severe) in 2005. In 2014, a second NIH consensus conference updated the global scoring criteria and explicitly eliminated scoring of any feature caused by an alternative etiology [6]. The 2005 NIH consensus scoring system was assessed prospectively by the Chronic GVHD Consortium in a group of 298 patients, evaluated every 3–6 months [42]. At the time of enrollment, the global cGVHD severity was mild in 10% ($n = 32$), moderate in 59% ($n = 175$), and severe in 31% ($n = 91$). In the majority, scores for involvement of skin, lung, or eye disproportionately determined the global severity score, while only 16% of global severity scores were attributed to other organs (Table 34.10). NIH global severity scores did not correlate with risk factors predicting development of cGVHD or NRM. However, NIH global severity scores for cGVHD at the time of patient enroll-

Table 34.10 Severity scoring for chronic graft-versus-host disease

Organ[a]	Score 0	Score 1	Score 2	Score 3
Skin				
	0% BSA	1–18% BSA	19–50% BSA	>50% BSA
	No sclerotic lesions	NA	Superficial sclerotic changes	Deep sclerotic changes
Mouth—with or without lichen planus-like lesions	No symptoms	Mild symptoms not limiting oral intake	Moderate symptoms partially limiting oral intake	Severe symptoms significantly limiting oral intake
Eyes	No symptoms	Mild dry eye not affecting ADL but requiring lubricant drops ≤3 X/d	Moderate dry eye partially affecting ADL and requiring lubricant drops >3 X/d. No impaired vision	Severe dry eye significantly affecting ADL Impaired vision
GI tract	No symptoms	Symptoms without significant weight loss (i.e., <5%)	Symptomatic with mild to moderate weight loss (i.e., 5–15%) *or* moderate diarrhea without significant impact on ADL	Symptomatic with severe weight loss (i.e., >15%), requirement for nutritional supplements for calorie needs *or* esophageal dilation for stricture *or* severe diarrhea with significant disruption of ADL
Liver[b]	Normal total bilirubin and ALT or ALP <3 x ULN	Normal total bilirubin with ALT ≥3–5 X ULN or ALP ≥ 3 x ULN	Elevated total bilirubin ≤3 X ULN or ALT >-5 X ULN	Elevated total bilirubin >3 X ULN
Lungs[c]	No symptoms FEV1 ≥ 80% predicted	Mild symptoms (e.g., SOB after climbing one flight of stairs) FEV1 60–79% predicted	Moderate symptoms (e.g., SOB walking on level ground) FEV1 40–59% predicted	Severe symptoms (e.g., SOB at rest requiring O_2) FEV1 ≤ 39% predicted
Joints and fascia	No symptoms	Mild tightness of arms or legs; normal or mildly decreased ROM **AND** not affecting ADL	Tightness of arms or legs **OR** joint contractures, erythema due to fasciitis; moderate decrease in ROM **AND** mild to moderate limitations of ADL	Contractures **WITH** significant decrease of ROM **AND** significant limitations of ADL (e.g., unable to tie shoelaces, buttons shirts, dress self, etc.)
Genital Tract	No signs	Mild signs[a] and for females with or without discomfort on examination	Moderate signs[a] and may have symptoms with discomfort on examination	Severe signs[a] with or without symptoms

Abbreviations: *BSA* body surface area, *ADL* activities of daily living, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *ULN* upper limit of normal, *FEV1* forced expiratory volume in the first second, *SOB* shortness of breath, *ROM* range of motion

[a]See Table 34.6 for details of diagnostic, distinctive and unclassified features in cGVHD

[b]In the absence of any alternative etiology of liver test abnormalities

[c]Lung scores should include both symptoms and FEV1 score. If discordant, the FEV1 score be used for final scoring

ment were significantly associated with survival and NRM ($p < 0.0001$for both).

Biomarkers for Chronic GVHD

Discovery and validation of biomarkers for the diagnosis, grading, prognosis, prediction of response to therapy, and NRM are urgently needed in cGVHD [43, 54, 55]. Table 34.11 lists promising exploratory biomarkers for cGVHD.

Treatment of Chronic GVHD

Tragically, ≥50% of HCT recipients, whose GVT responses have eliminated their malignant tumors, develop cGVHD [56]. The results of treatment outcomes studies remain disturbingly poor [57]. Currently, <20% of treated patients achieve durable partial or complete responses 1 year after initiating therapy and most require additional systemic therapy to survive [58]. In 2017, the FDA approved ibrutinib for the treatment of steroid-refractory cGVHD. Ibrutinib binds to Bruton's tyrosine kinase (BTK) expressed on B cells and is used to treat B-cell malignancies, including mantle cell lymphoma, chronic lymphocytic leukemia, and Waldenström's macroglobulinemia. While novel therapeutic agents are being developed, clinicians must use existing drugs as initial therapies or as salvage therapies for refractory cGVHD. The initial choice of therapy is based on assessment of the specific organs involved and the severity. Some manifestations can be managed with organ-specific treatments. However, systemic therapies are required if risk factors for cGVHD-related morbidity or mortality exist. In 2015, a consensus recommendation called attention to the fact that many studies showed bias in the reported outcomes of treatment for steroid-refractory cGVHD [59]. Table 34.12 summarizes options for systemic treatment of patients with cGVHD [8, 60–65].

Table 34.11 Candidate biomarkers for chronic GVHD

Biomarker	Physiological role
IL-1ra	Inhibitor of IL-1 receptor signaling by proinflammatory IL-1
sIL-2R (CD25)	Soluble biomarker of activated T cells
IL-6	Proinflammatory CD4 Th2 cytokine; mediator of hepatic acute phase reaction
IL-10	Anti-inflammatory CD4 Th2 cytokine
IL-15	Cytokine augmenting CD8 T cell anti-tumor effects
TNFα	Potent proinflammatory CD4 Th1 cytokine
TGFβ1	Cytokine with anti-inflammatory and profibrotic activities. Obligatory for Treg activation
sBAFF and BAFF/B cell ratio	Soluble B cell growth factor promoting B cell activation and proliferation
sCD13	Soluble aminopeptidase N with a role in peptide antigen presentation
CRP	Non-specific acute phase reactant biomarker of systemic inflammation
Cystatin B	Inhibitor of cathepsin proteases
CXCL9	Chemokine secreted by activated T cells
sMICA	Soluble ligand for NKG2D cytotoxic receptors on NK, γδ T, and $CD8^+$ αβ T cells
Lactoferrin	Glycoprotein that binds iron
Lactoperoxidase	Antimicrobial enzyme
ST2	Receptor for IL-33

Abbreviations: *IL* interleukin, *IL-1ra* interleukin-1 receptor antagonist, *sIL-2R* soluble IL-2 receptor (AKA CD25), *TNFα* tumor necrosis factor alpha, *TGFβ1* transforming growth factor beta-1, *sBAFF* soluble B-cell-activating factor, *sCD13* soluble aminopeptidase N located in the small-intestinal and renal microvillar membranes and responsible for digestion of peptides, *CRP* C-reactive protein, a biomarker of systemic inflammation, *Cystatin B* inhibitor of cathepsin proteases, *CXCL* chemokine C-X-C motif ligand, *sMICA* soluble major histocompatibility class I-related chain A membrane bound ligand that activates NKG2D receptors expressed natural killer (NK), γδ T and $CD8^+$ αβ T cells, *ST2* member of IL-1 receptor family acting as a receptor for IL-33, *Treg* regulatory T cell

Prognosis of Patient with Chronic GVHD

Based on the scoring system of the NIH Health Consensus Development Project on Criteria for Clinical Trials in cGVHD, patients with mild chronic GVHD have a good prognosis, while patients with severe chronic GVHD have a poor prognosis [42]. Based on the prospective study using the 2005 NIH global severity score at enrollment, 2-year overall survival was 97% for patients classified as mild, 86% for those classified as moderate, and 62% for those classified as severe [42], Notably, liver involvement in cGVHD represented an independent risk factor for NRM [66]. A systematic review of outcomes studies containing ≥100 patients with cGVHD identified overall survival rates of 66–94% after year 1, 59% to 83% after year 2, and 53–71% after year 4 [21]. While cGVHD was associated with higher NRM, it reduced the risk of relapse of malignancy, resulting in a net increase in overall survival.

A new composite endpoint of GVHD-free, relapse-free survival (CGRFS) has been proposed to define the probability of a patient being alive, in remission and free of significant cGVHD (i.e., moderate to severe severity scores) at any time post-HCT [67]. When used to assess prospectively 422 consecutive patients followed for a median of 36 months (2010–2015) at a single center, the actuarially estimated 3-year overall and disease-free survivals were 60% and 54%, respectively [67]. The CGRFS after 1, 2, 3, and 4 years was 45%, 46%, 47%, and 49%, respectively. Patients surviving with moderate to severe cGVHD decreased over 1, 2, 3, and 4 years of follow up from 23% to 14%, to 7% and to 4%, respectively. Based on the CGRFS score, nearly half of patients had no morbidities attributable to active cGVHD.

Table 34.12 Systemic treatment options for chronic GVHD

Regimens[a]	Systemic effect(s)	Tissue-specific effects	Comments
Steroid monotherapy			
Prednisone	Reduces transcription of proinflammatory genes causing broad immunosuppression Increased risk of infections	Systemic effects without tissue specificity	Primary therapy of choice for broad anti-inflammatory effects in multiple organs
Steroid + additional agent for steroid-refractory cGVHD			
Prednisone + CNI Either CSA or TAC	Broad immunosuppression inhibiting proinflammatory genes and production of mitogenic IL-2 required for clonal expansion of CD4 and CD8 T cells Systemic immunosuppression, HTN, DM Increased risk of infections	Varies according to composition of regimen and dosages Antiproliferative effects injure IECs	Limited evidence of efficacy in cGVHD
Prednisone + CNI + MMF	Broad immunosuppression inhibiting proinflammatory genes and production of mitogenic IL-2 required for clonal expansion of CD4 and CD8 T cells and MMF antiproliferative effects on dividing T and B cells Systemic immunosuppression, HTN, DM, nephrotoxicity Increased risk of infections	Varies according to composition of regimen and dosages MMF antiproliferative effects include IEC injury and bone marrow suppression with cytopenias MMF-induced diarrhea may cause confusion with GI involvement in cGVHD	Limited evidence of efficacy in cGVHD. MMF effect enhnaced in lymphocytes because of absence of purine salvage pathways in T and B cells
Prednisone + SIR or Prednisone + TAC + SIR	Broad immunosuppression inhibiting proinflammatory genes and signaling and/or production of mitogenic IL-2 required for clonal expansion of CD4 and CD8 T cells Systemic immunosuppression. Prednisone + SIR not associated with HTN, DM, nephrotoxicity Prednisone + SIR + TAC associated with reduced risk of HTN, DM, and nephrotoxicity Increased risk of infections	mTOR inhibition of IL-2 and T cell growth factor signaling decreases proliferation of activated T cells	Safety and efficacy established in solid organ transplantation
Ibrutinib	B-cell immunosuppression mediated by binding to Bruton's tyrosine kinase (BTK) on B cells Inhibits B-cell chemotaxis by CXCL12 and CXCL13, cellular adhesion and B-cell receptor (BCR) signaling Promotes B-cell apoptosis Increased risks of infections, mucositis, cytopenias	B cells and Ig-mediated pathology	FDA-approved for steroid-refractory cGVHD, mantle cell lymphoma, chronic lymphocytic leukemia, and Waldenström's macroglobulinemia
Ruxolitinib	Inhibits cytokine and growth factor signaling involved in multiple types of inflammation mediated by JAK 1 and 2 receptors	Potentially broad effects without tissue specificity	FDA-approved for myelofibrosis, and polycythemia vera
Rituximab	Chimeric monoclonal antibody against CD20 expressed on immature and mature B cells, but not plasma cells. Induces profound B-cell apoptosis Risks of infusion reactions, infections, reactivation of HBV infection, fatal progressive multifocal leukoencephalopathy	B-cell depletion reduces Ig-mediated pathology	FDA-approved for the treatment of autoimmune diseases caused by B-cell Ig production and B-cell malignancies

Table 34.12 (continued)

Regimens[a]	Systemic effect(s)	Tissue-specific effects	Comments
Imatinib	BCR-ABL tyrosine kinase inhibitor. Risks of neutropenia, thrombocytopenia, anemia, diarrhea and reactivation of HBV infection	Partial specificity for pulmonary and cutaneous sclerotic cGVHD. Diarrhea may cause confusion with GI involvement in cGVHD	Second line therapy for steroid refractory pulmonary or cutanteous sclerotic cGVHD. Third line therapy for other cGVHD organ involvement
Ursodeoxycholic acid	Reduces toxicity of hydrophobic bile acids by enriching bile acid pool with hepatoprotective UDCA Inhibits apoptosis Induces choleresis Mildly anti-inflammatory	Improves biochemical abnormalities in cholestatic liver diseases	FDA-approved for treatment of primary biliary cholangitis Frequently used empirically in other cholestatic liver diseases without proof of beneficial outcomes
Etanercept	Inhibits immune-mediated pathology caused by excessive TNFα High risk of serious infections Risks of reactivation of tuberculosis or HBV infection	No tissue or organ specificity Binds soluble TNFα, reducing its immunopathological effector functions No effect on TNFα Production	FDA-approved for rheumatological diseases and psoriasis
Thalidomide	Immunomodulation of inflammation Inhibits production of TNFα Stimulates T cells Risks of teratogenicity, blood clots, cytopenias, tremors	Inhibition of TNFα secretion reduces pathological effects in multiple tissues and organs	FDA-approved first-line therapy for multiple myeloma and pediatric GVHD Treatment option for adult cGVHD
Pentostatin	Immunosuppression of rapidly dividing immune effector cells or cancer cells Risks of hypersensitivity, rash, peripheral neuropathy, muscle twitching, AKI, cytopenias	Cell specificity based on susceptibility to antiproliferative effects as a purine analog that inhibits adenosine deaminase	FDA-approved for treatment of hairy cell leukemia
Non-pharmacologic therapies			
Extracorporeal Photopheresis	Immunosuppression of donor T-cell allogeneic responses Risks of phlebitis at infusion site, thrombocytopenia	No tissue or organ specificity	Infusion of autologous peripheral blood lymphocytes after incubation with 8-methoxypsoralen and exposure to ultraviolet-A light Infused irradiated monocytes differentiate into immature, tolerogenic DCs that inhibit propagation of donor T-cell responses
Psoralen and ultraviolet A irradiation (PUVA)	Immunomodulator effects on mechanisms of cutaneous inflammatory injury	Skin specificity	Used for steroid resistant cutaneous aGVHD and cGVHD

Abbreviations: *CNI* calcineurin inhibitor, *CSA* cyclosporine, *TAC* tacrolimus, *IL* interleukin, *CD*, *mTOR* mechanistic target of rapamycin, cluster of differentiation, *MMF* mycophenolate mofetil, *SIR* sirolimus, *HTN* hypertension, *DM* diabetes mellitus, *CXCL* chemokine CXC ligand, *JAK* Janus kinase, *FDA* US Food and Drug Administration, *UDCA* ursodeoxycholic acid, *TNFα* tumor necrosis factor-alpha, *PUVA* psoralen and ultraviolet A irradiation

Created with data from [8, 60–65]

[a]All patients with cGVHD requiring systemic therapy should be encouraged to enroll in RCTs

GVHD After Solid Organ Transplantation

Overview

Solid organ transplants contain variable numbers of donor passenger leukocytes, including dendritic cells, T and B cells, NKT cells, and NK cells that can react against the allogenic recipient. Generally, the host alloimmune response destroys donor T, B, and NK cells, despite being partially immunosuppressed to prevent allograft rejection. However, persistence of donor cells within the allograft, such as dendritic cells, may result in clinically silent chimerism within lymphoid organs [68]. Rarely, alloreactive donor cells from the transplanted organ engraft and expand to cause GVHD in the recipients [69–73]. In contrast to other solid organ transplantation, OLT requires only ABO blood group compatibility, but not matching for HLA Class I or II antigens. When OLT is performed between a donor and recipient with serendipitous matching of HLA alleles, there is a risk of GVHD due to failure of the recipient to eliminate donor cells present in the allograft [74]. As in GVHD after HCT, donor effector cells, including macrophages, T cells, and NK and NKT cells, can attack the recipient's skin, intestine, bone marrow, and lymphoid tissue.

Incidence

The incidence of GVHD after solid organ transplant remains unclear because there is no central registry and most cases are not reported. One review stated that GVHD occurred in 5.6% of recipients of small intestine transplants and 1–2% of recipients of OLT [75]. In adult recipients of OLT, the incidence of GVHD was inversely proportional to the number of HLA mismatches: ≤1% with 3–4 HLA Class I A and B mismatches; 7.4% with 0–1 HLA Class I A and B mismatches; and 12.5% with 0–1 HLA Class II DR mismatches [75]. In a recent review of pediatric recipients of solid organ transplantation, the mean incidence of GVHD after small intestine transplantation was 11% (range of 8.3–13.4%) and 1.5% after OLT [73].

Risk factors include older recipient age, African American race, mismatched sex, and CMV infection. A single-center, case-controlled multivariate analysis of 8 cases in 2775 recipients of OLT identified two significant risk factors: a difference of >20 years in the age of donor and recipient and matching for HLA class I alleles [76].

Clinical Features

In a review of 156 adults with aGVHD after OLT, the median time to onset was 28 days [77]. Clinical features included skin rash (92%), pancytopenia (78%), and diarrhea (65%). Since engrafted T cells and the liver allograft are autologous, donor leukocytes recognize hepatic tissue as self and do not cause hepatic GVHD. The mortality was 73% within 6 months, and sepsis caused by bacterial and fungal infections was the most frequent cause of death (60%). Only recipient age >50 years was identified as a risk factor. In pediatric GVHD [73], commonly affected sites were the skin (87%), gastrointestinal tract (43%), lungs (7%), eye (4%), and kidney (1%). Among pediatric recipients of non-hepatic solid organ transplants, 18% developed hepatic GVHD.

Treatment and Prognosis

In adults, empiric treatments of aGVHD have included intensified corticosteroids and calcineurin inhibitors, neutralization of TNFα, and reduction of immunosuppression to facilitate host destruction of engrafted donor cells [77]. In the review of 156 adults with aGVHD after OLT, mortality rates, based on the therapy for GVHD, were 84% for high-dose steroid monotherapy, 75–100% for increased doses of calcineurin inhibitors, and 55% for antagonists of IL-2 signaling [77]. The mortality rate was 25% in a small case series using alefacept or TNFα antagonists [77].

Overall, the prognosis for adults with GVHD post-OLT is poor [77]. The retrospective review of 156 adults with post-OLT GVHD documented a high mortality rate within 6 months of onset of a GVHD with long-term adult mortality ranging from 68% to 85%. In contrast, the average reported mortality for pediatric patients was 33% (range 0–100%) [73]. Among the 85% of pediatric patients treated with high-dose steroids, 75% achieved remissions, ranging from partial to complete.

Pathogenesis of Acute and Chronic GVHD

Overview

The three requirements for successful HCT are (1) robust GVT responses to prevent relapse, (2) development of functional donor tolerance to host alloantigens, and (3) ability of donor immunity to respond to foreign microbial and tumor antigens. The initial alloactivation of donor lymphocytes against host antigens that generates the GVHR (see Fig. 34.1) occurs in an obligatory proinflammatory setting of systemic tissue injury caused by preconditioning chemoradiation. This results in massive release of cell, tissue, and organ-specific host antigens, damage-associated molecular patterns (DAMPs), and translocation of pathogen-associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs) through the injured gut mucosa to activate donor innate and adaptive immunity following HCT [10, 78, 79]. Differentiation and maturation of alloactivated donor cells generates cytotoxic effector functions required for the beneficial GVT responses that can also recognize normal host tissues as foreign and instigate GVHD (Figs. 34.1, 34.3 and Table 34.3). Studies in animal models and humans have identified differences in the immunopathogenic mechanisms of aGVHD and cGVHD [10, 78, 79]. While aGVHD is characterized by cytotoxic effects of innate immune cells (neutrophils and NK cells), infiltrating donor T cells and antibodies, cGVHD is characterized by fibroproliferative tissue responses in multiple organs and a paucity of inflammatory infiltrates.

Genetic Factors in HCT

Role of HLA Matching

The human MHC, designated as HLA, is located on the short arm of chromosome 6 and expresses class I, II, and III gene products [1, 80, 81]. Class I HLA molecules are gene products of the A, B, and C loci, expressed by virtually all cell types. Class II HLA molecules are encoded by the DR, DQ, and DP loci, which are primarily expressed on hematopoietic

cells. Importantly, Class II HLA expression can also be induced on other cell types by inflammatory cytokines. Since the incidence of aGVHD is directly proportional to the degree of HLA histoincompatibility, serological and molecular allelic testing are performed to quantify the extent of HLA matching between the donor and recipient. As expected, the most common grafts used in HCT are from HLA-identical sibling donors (matched related donor); an unrelated donor with ≥7 of 8 HLA-A, HLA-B, HLA-C, and HLA-DRB1 matched alleles with the recipient (matched unrelated donor) or donors of T cell-replete haploidentical grafts treated with post-HCT cyclophosphamide (haploidentical donor).

Minor Histocompatibility Antigens

miHA are produced by the protease degradation of normal cellular proteins into antigenic peptides [1, 82]. Intracellular proteins processed in proteasomes of nucleated cells are presented in antigen-binding grooves of HLA class I molecules on the cell surface. In contrast, peptides derived from the extracellular environment are proteolytically processed in lysosomes and presented by HLA Class II molecules. Epithelial cells activated by proinflammatory cytokines, DAMPs, and PAMPs aberrantly express HLA class II molecules, whose antigen-binding grooves contain self rather than exogenous peptides. Even when the donor graft and the host are HLA-identical, the miHAs of the donor and host likely differ [83]. miHA differences may be the result of differential expression of genes in the recipient or expression of genes with single-nucleotide polymorphisms (SNPs). In mice genetically engineered to be MHC-identical, there are large numbers of miHA that differ among strains. A classic example of a human miHA is the H-Y antigen exclusively produced by the male Y chromosome, which is recognized as foreign by female donor cells [1, 84]. HA-1 is an example of a miHA derived from a recipient SNP, and its predominant expression by hematopoietic cells is believed to induce greater graft-versus-leukemia reactions after HCT [83, 85]. miHAs may be widely expressed among different cell types or be expressed uniquely by specific cells within tissues or organs. Tissue-specific expression of miHAs has been postulated to explain the restriction of target organ involvement in GVHD. Despite using HLA-identical grafts and optimal post-HCT prophylactic immunosuppression, presentation of host miHAs by host professional APCs to donor T cells results in aGVHD in ~40% of such recipients [83, 86].

Non-HLA Genes

Genetic polymorphisms in non-HLA genes may also influence the incidence and/or severity of aGVHD [1, 87]. Examples may include toll-like receptors (TLR), polymorphisms in killer inhibitor receptor (KIRs) on NK cells, cytokines IL-12 and TNFα [88], and nucleotide-binding oligomerization domain containing 2 (NOD2) genes [89, 90]. Polymorphisms in KIRs dictate whether a receptor has an inhibitory or an activating potential [91]. Experimental deletion or inhibition of TLR or NOD-like receptor pathways (critical for NOD-like receptor P1, P3, and C inflammasomes) significantly decreased aGVHD, while other NOD2 SNPs have been associated with severe grades of aGVHD [90]. Interestingly, polymorphisms of proteins involved in innate immunity also influence clinical outcomes of HCT. For example, SNPs in the NOD2/caspase-activating recruitment domain 15 (CARD15) genes of donors and recipients have been associated with intestinal GVHD and all-risk mortality after HCT from either related or unrelated donors [89, 92]. SNPs in the *NOD2/CARD15* gene, which encodes the intracellular sensor of the PAMP muramyl dipeptide expressed by IECs and monocyte/macrophages, profoundly impacted post-HCT mortality among 168 consecutive patients receiving HCT from related or unrelated donors [89]. *NOD2/CARD15* SNPs were present in 14% of donors and 21% of recipients. Cumulative 1-year HCT-related mortality was 20% in donor/recipient pairs without SNPs but rose to 49% in pairs with recipient SNPs only, to 59% in pairs with donor SNPs only, and to 83% of the 12 pairs with both donor and recipient SNPs. A recent meta-analysis of studies of the association between NOD2 SNPs and grade III-IV aGVHD showed that pairs of NOD2 SNPs (but not solitary SNPs) in HCT recipients were associated with these higher grades of aGVHD, especially in Caucasians [90]. Subgroup analyses showed a higher risk in recipients of HCT from HLA-identical siblings (OR = 4.00; 95% CI 1.50–10.69, p = 0.012) but not recipients of HCT from matched unrelated donors (OR = 1.16; 95% CI 0.45–2.98, p = NS). The effects of non-HLA gene SNPs in the pathogenesis of GVHD are likely influenced by variables such as the type and intensity of pre-HCT conditioning regimens, the degree of HLA matching, and the source of the donor graft (e.g., core blood versus bone marrow versus peripheral blood stem cells).

Cytokine Genes

Multiple cytokines are involved in the pathogenesis of aGVHD and cGVHD [9, 10, 79, 88, 93–95]. Hence, SNPs in cytokine genes would be expected to impact pathogenesis. Indeed, donor and recipient SNPs in the *TNF* gene encoding TNFα have been implicated in GVHD [1, 94]. These include TNFd3/d3 in the recipient, TNF863 and TNF857 in donor and/or recipients, and TNFd4, TNFα-1031C, and TNFRII-196R in donors [96]. However, a recent study showed no association between TNFα (rs1800629–308 G/A) genotypes and alleles risk for development of GVHD [88]. Three subtypes of IL-10 gene promoters in recipients dictate high, intermediate, and low production of the anti-inflammatory IL-10 [97] . These promoters have been associated with differences in the occurrence of aGVHD in HCT from HLA-

identical sibling donors. SNPs of the 2/2 genotype of IFNγ have been associated with high IFNγ production, while the 3/3 genotype has been associated with low IFNγ production. These SNPs have been associated with increased and decreased rates of aGVHD, respectively [98].

The IL-12 cytokine family and its T-cell receptor family play important roles in the pathogenesis of aGVHD and cGVHD [88, 93]. The IL-12 family of cytokines includes IL-12, IL-23, IL-27, IL-35, and IL-39. These cytokines are heterodimers that interact with cognate receptors on T cells composed of heterodimers of Janus kinase (JAK) 1 and 2 proteins or a JAK protein and a tyrosine kinase 2 (Tyk2) protein. Receptor signaling is mediated by phosphorylation of specific pairs of signal transducer and activator of transcription (STAT) molecules. Specific JAK-STAT signaling induced by individual cytokines of the IL-12 family induce differentiation of CD4 T-cell subsets. IL-12 and IL-23 are proinflammatory inducers of CD4 Th1 and CD4 Th17 differentiation, respectively. IL-27 exhibits contradictory biologic functions by inducing type 1 regulatory (Tr1) cells to secrete immunosuppressive IL-10 while also promoting inflammation by inhibiting CD4 T regulatory (Treg) cell development and increasing differentiation of proinflammatory CD4 Th1 cells. Both CD4 Tregs and regulatory B (Breg) cells produce IL-35, which mediates potent anti-inflammatory effects. The pathogenic role of IL-39 remains incompletely understood, but it does induce proinflammatory B-cell responses. A recent study found significant difference in the distribution of IL-12 (rs3212227 +1188 A/C) genotypes and alleles between stem cell transplant recipients with or without GVHD [88].

Microbiome and Disruption of Intestinal Functions

The composition and functions of the gut microbiome play key roles in maintenance of health, and variations in composition have been implicated in the pathogeneis of multiple diseases. Recent evidence indicates that nutrients processed and released by the gut microbiota also directly modulate host immune functions [99]. Data from animal and human studies indicate that the gut microbiota may influence development of GVHD, but the mechanisms remain incompletely defined [9, 78]. Ongoing studies in humans link the gut microbiota to development of GVHD, and possibly the severity of aGVHD [78]. However, the detailed findings are heterogeneous, likely reflecting differences in exposures to broad spectrum antibactrial and antifungal drugs, the types of underlying malignancies, prior chemotherapies, comorbid diseases, HCT protocols, diet, and regimens to prevent or treat GVHD. Humans treated with non-absorbable antibiotics or metronidazole targeting anaerobes for gut decontamination appeared to be protected from GVHD, while in other studies protection form GVHD was associated with high alpha diversity and high abundance of anaerobes in the *Clostridia* class and *Lachnospiraceae* family [100]. A plausible unifying explanation for these findings is that in the absence of sufficient gut decontamination to remove microbial PAMPs and MAMPs as triggers of inflammation, the diversity and abundance of the remaining gut microbiota determine the risk of GVHD with some species increasing the risk (e.g., *Enterococci*) and others decreasing the risk (e.g., *Blautia* species) [78].

Pathogenesis of Acute GVHD

Experimental models and human studies indicate that pathogenesis of aGVHD can be divided into five interrelated steps (Fig. 34.5): (1) systemic priming effects of conditioning chemotherapy and radiation; (2) activation of host antigen presenting cells (APCs) and processing and presentation of host peptide antigens; (3) activation of donor T cells with subsequent proliferation and differentiation of effector cell functions; (4) chemokine-mediated transendothelial migration into target tissues resulting in inflammatory cytotoxic destruction of specific target cells; and (5) perpetuation of donor-medicated destruction of target cells due to reactivation of donor T and B cells by host antigens released from injured, inflamed tissues.

Step 1: Systemic Priming Effects of Conditioning Chemotherapy and Radiation

Conditioning chemoradiation prior to HCT directly damages cells of the gut mucosa, including (1) intestinal epithelial cells (IECs); (2) innate lymphoid cell 3 (ILC3) cells that produce IL-22 required for epithelial cell repair after injury; (3) intestinal stem cells (ISC) required for epithelial regeneration; (4) Paneth cells responsible for secretion of antimicrobial peptides (AMPs) that regulate the gut microbiota; and (5) goblet cells responsible for maintenance of the mucus barrier separating gut microbes from the epithelium and immune cells [9, 78]. Conditioning also damages cells in other host tissues, resulting in release of DAMPs and activation of NOD-like receptor inflammasomes (e.g., NLRP3) in both immune cells and host epithelial cells [95].

Tissue injury releases DAMPs from intestinal mucosal cells and gut-associated lymphoid tissues and destroys the integrity of the gut barrier, allowing translocation of intestinal microbes, DAMPs, PAMPs, and MAMPs to enter the portal venous blood and circulate to the liver. The loss of bacterial inhibition mediated by AMPs and impairment of mucosal regeneration create a prolonged vicious cycle [9, 78]. Treatment of neutropenic HCT recipients with prophylactic broad-spectrum antimicrobials to prevent bacterial and fungal infections reduces microbial diversity; alters the proportions of the major bacterial phyla, as well as the fungi, protists, archaea, and viruses; and promotes survival of antimicrobial-resistant species. Overgrowth of bacteria that degrade mucin retards regeneration of the mucus barrier, perpetuating microbial contact with IECs and activation of immune cells. DCs

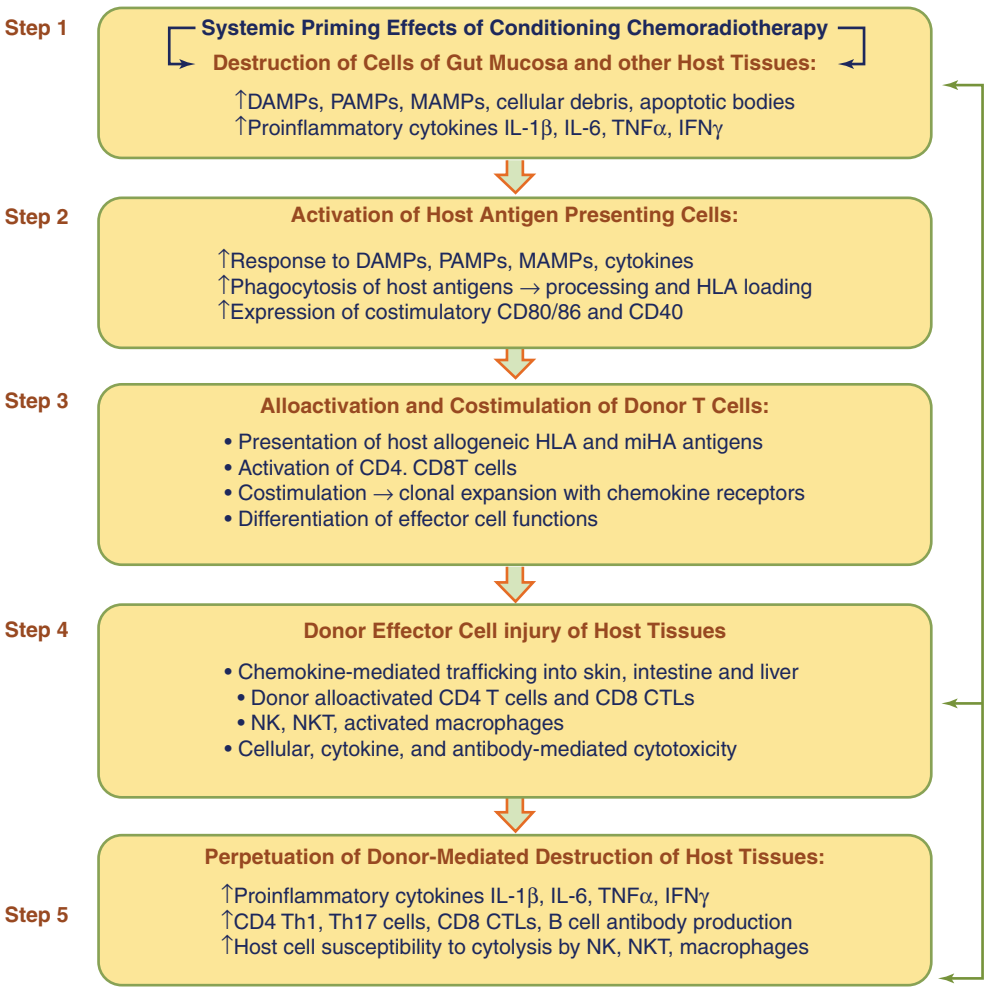

Fig. 34.5 Working model of the pathogenesis of acute graft-versus-host disease. DAMPS Damage-associated molecular patterns, PAMPs pathogen-associated molecular patterns, MAMPs microbe-associated molecular patterns, IL interleukin, TNFα tumor necrosis factor alpha, CD cluster of differentiation, NK natural killer, IFNγ interferon gamma

activated by PAMPs and MAMPs induce CD4 Th1 and CD4 Th17 responses that contribute to gut injury. These effects, often combined with poor oral nutrition, decrease production of riboflavin metabolites and short-chain fatty acids (SCFAs) by the gut microbiota. Reduced riboflavin metabolites impair activation of anti-inflammatory mucosal-associated invariant T (MAIT) cells, while reduced levels of SCFAs are insufficient to stimulate immunoregulatory CD4 Tregs [9, 78, 99].

Reduced diversity of gut bacterial species also results in impaired bacterial metabolism of bile acids and decreased activation of nuclear farnesoid X receptor (FXR) expressed by normal IECs and G-protein-coupled bile acid receptor 1 (aka, TG5) expressed by normal IECs and macrophages [9, 78]. Reduced FXR signaling by bile acids impairs mucosal barrier functions, facilitating translocation of microbial species, PAMPs, and MAMPs. Reduced TG5 signaling in macrophages results in a loss of nuclear factor κB (NFκB) inhibition of the secretion of proinflammatory cytokines TNFα and IL-1β. In addition, reduction of polyamines generated by the gut microbiota impairs their ability to augment the barrier function of IECs. Metabolites produced by gut microbiota also include ligands for the aryl hydrocarbon receptor (AhR), which is critical for maintenance of the gut epithelial barrier and regulation of gut innate immunity.

Step 2: Activation of Host Antigen-Presenting Cells

Factors contributing to intense activation of host APCs before HCT include comorbid diseases; infections; the type of underlying malignancy; cytotoxic destruction of tissues and organs (especially IECs) caused by preconditioning therapies that release DAMPs, PAMPs, and MAMPs that activate inflammasomes in both immune cells and epithelial cells; and release of cellular antigens for processing and presentation [1, 9, 10, 78, 79]. Destruction of host tissues, including malignant cells, enhances secretion of cytokines (IL-1β, IL-6 and TNFα, IFNγ) and chemokines. Activated host APCs (e.g., DCs, macrophages, and B cells) express increased densities of adhesion and HLA molecules, as well as costimulatory molecules CD80/86 (aka B71/B72) and CD40. Signaling mediated by DAMPs, PAMPs, MAMPs through pattern recognition receptors (PPRs), and chemokines through chemokine receptors induce changes in the gene expression of immune cells and of epithelial cells activated by cytokines and chemokines.

Recognition of viral DNA by TLRs on activated host APCs may explain why cytomegalovirus (CMV) infection is a risk factor for GVHD [101].

Step 3: Alloactivation and Costimulation of Donor T Cells

GVHD results from activation, costimulation, clonal proliferation, and differentiation of HLA-matched naïve donor T cells to host allogeneic antigens presented by host APCs [9, 10, 79]. In contrast, B-cell production of antibodies play no specific roles in the pathogenesis of aGVHD [13]. Early after HCT, mature donor T cells with TCRs capable of reacting to host alloantigens mediate immediate or "direct" cytotoxicity against host cells expressing these alloantigens. Later, naïve donor T cells that have undergone host thymic selection are activated by host HLA and miHAs presented by APCs of host, and later by APCs of donor origin. Replacement of host APCs by donor-derived APCs and activated macrophages (e.g., Kupffer cells in hepatic sinusoids) facilitates processing and presentation of both host non-polymorphic and miHA peptide antigens [102]. Such newly activated T cells mediate so called "indirect" cytotoxicity, the dominate mechanism for longer term alloantigen-specific tissue cytolysis. Clonal proliferation and differentiation of donor CD4 and CD8 T effector cell functions requires potent costimulation produced by binding of CD28 on the T cell with CD80/CD86 (aka B7.1/B7.2) on the APC and/or binding of CD154 (aka CD40-ligand) on the T cell with CD40 on the APC. Activation of host APCs increases their expression of CD80/86 and CD40 costimulatory molecules. Secondary costimulation is also critical for sustaining proliferation of activated donor T cells and increasing production of cytokines required for the pathogenesis of aGVHD [103]. Secondary costimulation results from the binding of CD134 (OX40) on the activated T cell with CD252 (OX40L) expressed on APCs [104]. It is called secondary costimulation because CD134 (OX40) expression requires induction by TNFα and IFNγ and is delayed approximately 3 days after initial T-cell activation and primary costimulation. OX40-OX40L binding generates a survival signal for activated T cells and promotes development of memory T cells. T-cell activation also induces production of coinhibitory molecules, including cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death receptor-1 (PD-1), and PD-ligands 1 and 2 (PD-L1/2). However, in the final balance between costimulatory and coinhibitory signaling, the upregulated expression of costimulatory molecules in aGVHD overwhelms the effects of these coinhibitory molecules [103].

Production of large amounts of Th1 cytokines IL-2, TNFα, and IFNγ is required for development of aGVHD [9, 10, 79] [105–108]. IL-2, the primary mitogen for CD4 and CD8 T cells, drives clonal proliferation of alloreactive donor effector T cells. In contrast, low concentrations of IL-2 preferentially induce clonal expansion of antigen-specific Tregs [9, 10]. IFNγ amplifies GVHD [106], increasing the sensitivity of APCs to PAMPs, augmenting intracellular signaling induced by DAMPs, increasing expression of HLA molecules, adhesion molecules, and chemokine receptors required for transendothelial migration and target cell recognition by donor T cells. IFNγ and TNFα also cause damage to the intestine and skin [9, 10, 79]. Paradoxically, IFNγ can also suppress aGVHD by inducing apoptosis of activated donor T cells [109, 110]. aGVHD is also associated with production of copious amounts of proinflammatory cytokines IL-1β, IL-6, and TNFα, as well as IL-11, IL-12, IL-15, IL-17, IL-18, IL-21, and IL-33 [9, 10, 79]. Collectively, these proinflammatory cytokines promote NK, CD4 Th1, and CD8 T-cell cytotoxicity, chronic inflammation and secretion of other cytokines and chemokines [9, 10, 79]. However, it is important to recognize that these cytokines act as part of a larger network of concurrent stimuli in aGVHD that mediate diverse pathogenic consequences [95].

In humans and animal studies of GVHD, both CD4 and CD8 regulatory T cells (CD4 Tregs and CD8 Tregs) can modulate GVHD by suppressing antigen-specific proliferation of activated donor T-cell subsets [111–114]. CD4 Tregs secrete anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGFβ) to inhibit effector CD4 and CD8 T-cell subsets, and TGFβ transforms CD T effector cells to CD 8 Tregs [115]. The NOTCH signaling pathway induces T cells to proliferate in aGVHD and is an important target of Treg inhibition [116]. In contrast to the immunosuppressive effects of TGFβ in aGVHD, TGFβ appears to play a pivotal role in the pathogenesis of fibroinflammatory tissue injury in cGVHD as a profibrotic cytokine. CD8 Tregs immunoregulate immune responses either by secretion of the immunosuppressive cytokines IL-10 and TGFβ or by direct cell contact, causing cytotoxic death of destructive effector cells or providing inhibitory signaling through CTLA-4 or PD-1 [117]. The role of B regulatory cells (Bregs) in human aGVHD is poorly understood [13]. DCs have dichotomous roles in normal adaptive immunity: activation of CD4 and CD8 T cells versus a tolerogenic effect mediated by apoptotic deletion of activated T cells and promotion of Treg proliferation [118]. In aGVHD, the tolerogenic effects of donor-derived DCs are significantly impaired, favoring immunopathology.

Donor innate lymphoid cells (ILCs), which include NK and ILC1, ILC2, and ILC3 cells, may play important immunomodulatory roles in aGVHD and cGVHD [12]. For example, NK cells can modify or prevent GVHD by direct cytolysis of activated effector T cells, by depletion of host APCs resulting in inhibition of T-cell activation or by secretion of immunosuppressive IL-10 [12]. Migration of ILC1 cells to the skin can potentially alleviate cutaneous aGVHD, while ILC2 cells can induce myeloid-derived suppressor cells (MDSCs) and can directly inhibit CD4 Th1 and Th17 effector cells in murine aGVHD. As noted in the discussion

of the gut, ILC3 cells secrete IL-22 required for repair of injured host epithelial cells. Type 1 invariant NKT (iNKT) cells can also suppress GVHD in an IL-4-dependent manner [119]. In animal models, donor type 2 NKT cells promoted cytotoxic GVL responses while reducing GVHD [120].

Step 4: Donor Effector Cell Injury of Host Tissues

Transendothelial migration of effector T, NK, and NKT cells and activated macrophages from the blood into the target tissues of the skin, intestine, and liver is the first essential step in tissue cytotoxicity and organ damage in aGVHD [10, 15, 121]. In aGVHD, the skin, intestine, and liver differentially express chemokine ligands and chemokine receptors (Table 34.13) that not only mediate chemoattraction of effector cells but also induce their functional terminal differentiation. Transendothelial migration of activated donor effector cells from the blood to the tissues requires cytokine and chemokine activation of vascular endothelial cells to express adhesion molecules and cell surface pili that concentrate chemokines from the adjacent tissue. Activated donor CD4 T cells, CD8 CTLs, NK and NKT cells, and activated macrophages express chemokine receptors and counter-receptors for these endothelial adhesion molecules, allowing them to bind to the activated endothelium and migrate between the endothelial cells into the tissues. Once in the tissue, effector cells migrate along the gradients of chemokines secreted by specific target cells [9, 10]. For example, expression of the α4, β7 integrin and its ligand, the mucosal addressin cell adhesion molecule 1 (MadCAM-1), direct the transendothelial migration of donor T cells to Peyer's patches during intestinal GVHD [122].

Activation of donor CD4 T cells in HCT preferentially generates a Th1 phenotype, providing the helper functions for the proliferation and differentiation of CD8 T cells, which function as antigen-specific cytotoxic T lymphocytes (CTLs) [9, 10]. Donor NK cells also mediate cytotoxicity by recognition of stressed or injured target cells expressing reduced amounts of KIR on their membranes [12]. Cytotoxic inflammatory mediators include IL-1β, IFNγ, TNFα, and nitric oxide. Circulating and cellular effector cells act synergistically to augment local tissue injury and promote target cell destruction. The role of type 2 cytotoxic NKT cells in aGVHD pathology is unclear.

Table 34.13 Expression of chemokines and chemokine receptors in skin, intestine and liver

	Skin	Intestine	Liver
Chemokines	CXCL1, 2, 9, 10, 11 CCL2, 5, 6, 7, 8, 9, 11, 12, 17, 19, 20, 27 XCL1	CXCL9, 10, 11, 16 CCL2, 3, 5, 20 XCL1 CX_3CL1	CXCL1, 9, 10, 11, 16 CCL2, 3, 5, 20 XCL1
Chemokine receptors	CXCR3 CCR1, 2, 4, 5 CCR10 XCR1	CXCR3, 6 CCR1, 2, 5, 6 CX CX_3R1	CXCR2, 3, 6 CCR1, 2, 5 XCR1

Donor cells of the innate and adaptive immune responses, specifically NK cells and CD8 CTLs, are the primary effector cells of aGVHD. CD8 CTLs mediate cytolysis in hepatic GVHD primarily through the binding of CD95 (aka Fas) to CD95L (CD95-ligand, aka FasL). In contrast, CD8 CTLs mediate cytolysis of enterocytes and keratinocytes through the perforin-granzyme pathway [123, 124]. Resistance of hepatocytes to perforin-granzyme mediated cytolysis [125] may partially explain the relative paucity of hepatocyte cytolysis in hepatic aGVHD [33].

Step 5: Perpetuation of Donor-Mediated Destruction of Host Tissues

Migration of activated cytotoxic effector cells into the skin, intestine, and liver in aGVHD, along with continued production of proinflammatory cytokines IL-1β, IL-6, TNFα, and IFNγ results in injury and destruction of host cells within target organs of the skin, intestine, and liver in aGVHD [9, 10]. Tissue destruction maintains high concentrations of DAMPs required to activate inflammasomes in immune cells and cytokine-activated epithelial cells. Clearance of apoptotic bodies and cellular debris perpetuates high concentrations of cytokines and chemokines and inhibits cellular repair. The net effects are similar to those caused by conditioning chemoradiation therapy (see Step 1), except that the immunologic effector mechanisms produce more tissue-specific pathology. These processes produce a positive feedback loop capable of not only sustaining the activation of donor T cells but also of generating new populations of effector cells that react against a broader array of host miHAs.

Pathogenesis of Chronic GVHD

cGVHD is characterized by fibroinflammatory pathology involving not only the skin, intestine, and liver but also many additional tissues and organs (see Fig. 34.3 and Table 34.6). Biopsies characteristically show dense fibrosis and a paucity of infiltrating inflammatory cells. Since these pathological features differ from those in aGVHD, it follows that the pathogenic mechanisms of cGVHD must differ significantly from those causing aGVHD. Yet, the pathogenic mechanisms responsible for cGVHD remain poorly defined. In contrast to multiple animal models of aGVHD, experimental models of cGVHD are less numerous and less representative of human cGVHD [43]. To date, no animal models fully mimic the contributions of conditioning chemoradiation, prophylactic and therapeutic immunosuppression, increased intestinal permeability, altered gut microbiota, multiple courses of antibiotics and antifungals, nutritional deficits, comorbid diseases, and polypharmacy.

Three-Phase Model of Chronic GVHD Pathogenesis

Experimental studies and observations in human cGVHD support a three phase working model of pathogenesis (Fig. 34.6) that differentiates the pathogenic mechanisms of cGVHD from those of aGVHD [126]. Although cGVHD can be preceded by aGVHD, the onset of classic cGVHD most often occurs 100 days to 2 years after HCT (see Fig. 34.2). Key strengths of the working model are its explanation for the delay between HCT and onset of cGVHD and the differential contributions of cellular and humoral immunity to the unique pathology of cGVHD.

Phase 1: Control of Initial Inflammation and Tissue Injury After HCT

All patients who develop classic cGVHD initially experience systemic injury due to conditioning chemoradiation therapy; disruption of the gut mucosa; release of DAMPs, PAMPs, and MAMPs; and processing and presentation of host antigens by host APCs resulting in activation of alloreactive donor T cells. Thus, the key question is why do patients who develop classic cGVHD fail to develop intense aGVHD?

To explain this, the working model postulates quantitative and qualitative differences in the spectrum and intensity of donor-derived immune responses to host alloantigens in a necroinflammatory microenvironment (see Fig. 34.6). In contrast to patients who develop aGVHD, those who develop cGVHD produce only transient necroinflammation of host tissues mediated by mature donor T cells capable of direct cytotoxicity against allogeneic HLA or miHAs and by the donor innate immune responses of neutrophils, NK, NKT, DCs, and activated macrophages. The combined ability of CD 4 or CD8 Tregs and prophylactic immunosuppressive drugs to control further alloimmune reactions results in suppression of production of effector cells and in decreased levels of proinflammatory cytokines IL-1β, IL-6, TNFα, and IFNγ. The net effect is failure to generate intense donor T-cell allogeneic responses against host HLA and miHAs required to produce aGVHD, despite activation of endothelial cell expression of adhesion molecules and chemokines capable of mediating transendothelial migration of alloantigen-specific donor T cells into target tissues of the skin, intestine, or liver.

Recent evidence that functional Tregs metabolically rely on oxidative phosphorylation, rather than the glycolysis

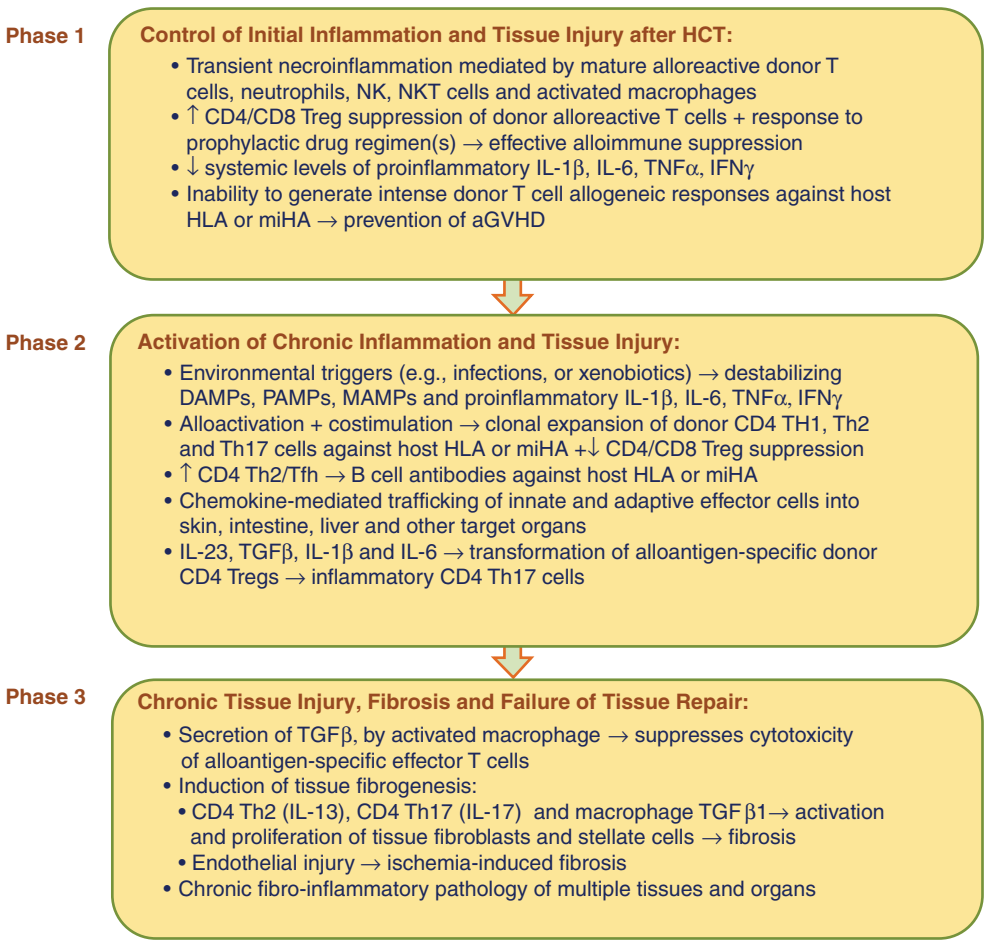

Fig. 34.6 Working model of the pathogenesis of chronic graft-versus-host disease. NK natural killer, reg regulatory, HLA human leukocyte antigen, miHA minor histocompatibility antigens, IL interleukin, Th T helper, Tfh T follicular helper, TNFα tumor necrosis factor alpha, IFNγ interferon gamma, DAMPs damage-associated molecular patterns, PAMPs pathogen-associated molecular patterns, CD cluster of differentiation, TGFβ transforming growth factor beta

required by cytotoxic allogeneic T cells [127], suggests that nutritional and metabolic differences may also dictate the occurrence of aGVHD versus cGVHD. Observations that depletion of T cells in vivo or short courses of cyclophosphamide after HCT reduce the incidence and severity of cGVHD circumstantially support the working model [43].

In contrast with aGVHD, alloreactive donor B cells are pathogenic in cGVHD [13, 43] as a result of their functions as APCs and/or as producers of antibodies specific for miHAs [128, 129]. High levels of BAFF promote survival of immature and mature donor B cells, leading to inappropriate rescue of B cells with allo- or autoreactive potential [13]. Furthermore, increased BAFF to B-cell ratios in patients with cGVHD are associated with deranged peripheral B-cell homeostasis. The increased levels of BAFF may be a physiologic response to B-cell cytopenia, since most patients with cGVHD have lower quantities of total B cells [43]. In patients with cGVHD, the quantities of Bregs are low and their secretion of immunosuppressive IL-10 is deficient. The net result is production of high levels of auto- and alloimmune antibodies and hypergammaglobulinemia in cGVHD [13, 43]. The pathogenic effects of allo- or auto-antibodies to DNA or nuclear antigens or the male HY antigen in male recipients of female donor HCT are poorly characterized, and there is no evidence that immune complexes are produced [13, 43]. Another antibody commonly found in cGVHD is anti-platelet-derived growth factor receptor (anti-PDGF-R), which leads to accumulation of reactive oxygen species and induces type 1 collagen secretion by fibroblasts [43]. The efficacy of rituximab depletion of B cells as treatment for cGVHD is the best evidence of the important role of B cells in the pathogenesis of cGVHD [43]. However, the inability of rituximab depletion of B cells to prevent cGVHD suggests that a B cells in are not the primary cause of its pathogenesis [130].

Phase 3: Chronic Fibrosis, Tissue Injury, and Failure of Tissue Repair

Several pathogenic mechanisms contribute to the fibroinflammatory tissue and organ damage characteristic of cGVHD. Activated macrophages within inflammatory infiltrates secrete TGFβ, which mediates two processes: downregulation of effector CD8 T-cell cytotoxicity and potent induction of collagen secretion by activated fibrocytes and stellate cells [43]. Alloactivated CD4 Th2 and Th17 cells within the inflammatory infiltrates also promote tissue fibrosis by secreting IL-13 and IL-17, respectively [126]. In addition, chronic inflammatory injury of the vascular endothelium and reduced perfusion of target tissues and organs may result in ischemic fibrogenesis [131]. These combined effects promote the fibroinflammatory injury of the skin, intestine, liver, and multiple other tissues and organs characteristic of cGVHD.

GVHD Predilection for the Skin, Intestine, and Liver

The primary target cells in both aGVHD and cGVHD are keratinocytes in the skin, IECs in the intestine, and cholangiocytes of small-caliber interlobular bile ducts in the liver [9, 10]. Donor effector T cells also preferentially target these cells in models mismatched for MHC Class I or II, where multiple other tissues should be comparable targets based on their ubiquitous expression of allogeneic MHC molecules [82].

Two non-mutually exclusive mechanisms have been proposed to explain donor effector cell predilection for the skin, intestine, and liver. First, tissue specificity could reflect the expression of immunodominant allogeneic miHAs by keratinocytes, IECs, and cholangiocytes. In murine GVHD, patterns of tissue expression of miHAs were qualitatively and quantitatively altered in the three target tissues [132], which support this postulate. In humans, UGTIIB17 (resulting from a gene deletion in the UDP-glycosyltransferase 2 family) represents a candidate miHA that is expressed in the liver, the IECs of the small and large intestine, and the pancreas [82]. Additional evidence in humans and mice is provided by the finding of oligoclonality of TCR Vα and/or Vβ chains on donor T cells infiltrating target tissues [133, 134], which indicates that the TCRs recognize only a limited number of alloantigens. The second postulated mechanism for specific tissue targeting is qualitative or quantitative differences in the secretion of cytokines and chemokines that chemoattract and terminally differentiate donor effector T cells and promote cytotoxic cytokine-induced injury. The skin, intestine, and liver express multiple, identical chemokine ligands (see Table 34.13) capable of chemoattracting similar populations of donor effector T cells bearing receptors for these ligands [82, 135, 136]. Indeed, IFNγ, IL-1β, IL-6, and TNFα can induce keratinocytes, IECs, and cholangiocytes to secrete chemokines and polarizing Th1 cytokines, such as IL-12. Immunohistochemical studies of biopsies and studies of immortalized cholangiocytes in a murine model of cGVHD provide support the second mechanism [135, 137].

Future Directions

Prevention of the Need for HCT

The highest priority in oncology is development of novel, effective primary therapies that can ultimately eliminate the need for HCT as a rescue therapy. Until that becomes a reality, work will continue on strategies to improve the success of HCT and prevent the significant morbidity and mortality caused by aGVHD and cGVHD.

Improvement in the Success of HCT

Three outcomes define the clinical success of HCT. The first is effective donor cell engraftment and hematopoietic reconstitution to achieve protective cellular and humoral immunity in the host. The second is effective GVT responses that prevent relapse mortality by eradicating all residual malignant cells in the host. The third is prevention of both aGVHD and cGVHD as causes of morbidity and NRM. Each of these outcomes is the subject of active research. Table 34.5 summarizes current strategies for the prevention of aGVHD or cGVHD. Oncolytic virotherapy being developed for hematological malignancies was recently reported to abrogate murine GVHD, indicating the exciting prospect of eliminating residual hematological malignancies after HCT without the risk of aGVHD [138].

Development of Therapies for Acute and Chronic GVHD with Greater Safety and Efficacy

Currently, clinicians must rely on therapies that are only partially successful and often poorly tolerated (see Tables 34.9 and 34.12). Multiple clinical trials are ongoing worldwide to improve prophylactic regimens to prevent aGVHD and to assess the safety and efficacy of new first-line therapies, as well as therapies for steroid refractory aGVHD and cGVHD [63]. These clinical trials are based on our mechanistic understanding of pathogenesis [8] and include studies of inhibitors of alloactivation, proliferation, and effector functions of donor innate and adaptive immune cells, JAK-STAT cytokine signaling, proteasomes, cytokine effector functions, and chemoattraction to generate inflammation (Tables 34.9 and 34.12). Other approaches include fecal microbiota transplantation to restore a favorable microbiota and infusions of regulatory cells or low-dose IL-2 to promote effective immunoregulation. In the absence of proven therapies, it is imperative that all patients consider participation in clinical trials of new therapeutics to advance the field. All available trials can be reviewed at www.clinicaltrials.gov.

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Immunopathogenesis of Liver Cirrhosis

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Abbreviations

ACLF	Acute-on-chronic liver failure
ASK1	Apoptosis signal – regulating kinase 1
BAMBI	Bone morphogenetic protein (BMP) and activin membrane-bound inhibitor homolog
CCR2	C–C motif chemokine receptor 2
DAMP	Danger-associated molecular pattern
ER	Endoplasmic reticulum
EV	Extracellular vesicle
HMGB – 1	High – mobility group box 1
HSC	Hepatic stellate cell
IFN	Interferon
IL	Interleukin
MAIT	Mucosal-associated invariant T cells
Mdr2	Multidrug resistance gene 2
mtDNA	Mitochondrial DNA
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PDGFR	Platelet-derived growth factor receptor
STAT3	Signal transducer and transcription factor 3
TGF	Transforming growth factor
TIMP – 1	Tissue inhibitor of metalloproteinases-1
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
UPR	Unfolded protein response

Key Points

- Liver cirrhosis represents the end stage of chronic liver disease, characterized by excessive scar tissue (fibrosis), intense inflammatory cell infiltration, and liver loss-of-function, leading to multiple organ failure.
- Fibrogenic cells in the liver may be of different origins, but activated stellate cells represent the main source of extracellular matrix components.
- Fibrogenic cells sense changes in their microenvironment especially under inflammatory conditions and respond to a plethora of inflammatory stimuli including cytokines, danger-associated molecular patterns, and pathogen-associated molecular patterns.
- Both innate and adaptive immune cells are actively participating in the initiation, progression, and resolution of liver fibrosis. Current efforts are made to decipher the complex interplay between the immune system and liver disease.
- Immunomodulation represents a promising therapeutic approach in the control of liver fibrosis.

The classical development of chronic liver diseases, whatever the etiology, follows a well-established pattern. Chronic or severe acute liver injury leads to the initiation of various inflammatory processes that comprise the activation of local immune cells, as well as the recruitment and activation of circulating cells. The liver also possesses potent regenerative capacities, characterized by the ability for parenchymal or liver-resident progenitor cells to proliferate in response to hepatic function alteration. Additionally, fibrogenic cells of different origins may be activated and deposit scar tissue. When tissue scarring is excessive, this is termed "fibrosis" and serves as the soil for advanced liver disease or cirrhosis, ultimately leading to liver failure.

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A considerable amount of data supports the limitless relevance of studying the interactions between liver fibrosis and inflammation, in terms of virtually all immune cell types and phenotypes as well as immunological processes performed by professional or non-professional immune cells. Accordingly, new discoveries are made on a regular basis in the field of liver immunology and fibrosis and will further increase our knowledge of all the finely tuned cellular processes implicated and consequently lead to numerous advances for patients in the near future.

What Is Liver Fibrosis, and How Does It Evolve to Liver Cirrhosis?

Liver cirrhosis is defined as a final stage of chronic liver disease in which excessive parenchymal cell necrosis and scar tissue accumulation impedes blood flow, leading to liver loss-of-function and, consequently, the accumulation of toxics in the blood mainstream. Every year about 5–7% of previously asymptomatic cirrhotic patients exhibit multiple organ failure when decompensation has occurred [1, 2]. Complications include ascites, peritonitis, hepatic encephalopathy, hepatorenal syndrome, hepatopulmonary syndrome, and hypersplenism. Another important risk for cirrhotic patients is acute-on-chronic liver failure (ACLF), described as acute decompensation and a high short-term mortality occurring after an acute insult (e.g., drug-induced liver injury, viral hepatitis, or alcohol consumption) on a compensated cirrhotic liver [2, 3]. Importantly, systemic inflammation is constantly observed in patients with decompensated cirrhosis and ACLF patients, emphasizing the close interaction between immune cells and pathogenesis [2, 4]. Histologically, cirrhosis is characterized by regenerative nodules representing an attempt of the remaining liver cells to regenerate the organ and restore liver functions. These regenerative nodules are surrounded by fibrous septa made of extracellular matrix bridging the portal tracts. The fibrotic tissue is densely composed of fibrogenic cells, innate and adaptive immune cells, and pseudo-ductular structures, in a process termed as ductular reaction (see below) [5]. Hence, liver fibrosis and accompanying inflammation and ductular cell proliferation may be regarded as unbalanced regenerative processes [6]. At later stages, liver transplant may represent the only therapeutic option for cirrhotic patients, which represents a challenge due to organ donor shortage.

Virtually any chronic liver disease can lead to cirrhosis, whether caused by alcohol abuse, viral hepatitis, chemical toxicity, autoimmune liver diseases, non-alcoholic fatty liver disease, or genetic predispositions, among others. Whatever the etiology, the common soil for liver cirrhosis is fibrosis [3]. Thus, a lot of efforts are being put toward limiting or even reversing liver fibrosis. Among the different strategies, fibrogenic cell inhibition and immune system modulation represent the most promising approaches.

Liver fibrosis is defined as an excessive accumulation of extracellular matrix, mainly consisting of type I and III collagens, laminin, and hyaluronic acid. This scar tissue progressively occupies larger areas and replaces functional liver parenchyma. Myofibroblasts derived from activated hepatic stellate cells (HSCs) are considered to be the main source of extracellular matrix in the liver [7, 8]. HSCs are located in the space of Disse in the healthy liver, between the hepatic sinusoids lined by liver sinusoidal endothelial cells, and the basolateral surface of the hepatocytes. One of their main functions in the healthy liver is the storage of retinoids (vitamin A) in perinuclear droplets. Upon activation by various stimuli (detailed below), HSCs progressively lose their vitamin A droplets and adopt a myofibroblast phenotype notably defined by intense extracellular matrix deposition, alpha-smooth muscle actin expression, as well as migratory properties [8]. Myofibroblasts are characterized by their contractility, participating in the increase of portal resistance observed upon liver fibrosis. This contractility is induced by enthothelin-1 and angiotensin-II [9, 10]. The signals that activate or inhibit HSCs have been extensively reviewed previously [11].

Collagen deposition typically occurs around the remains of hepatic parenchyma and fibrotic septa expand from the periportal area in advanced stages of liver fibrosis, a feature referred to as bridging fibrosis. It is thus remarkable that HSCs, considered to be the main producers of extracellular matrix, seem to migrate to the perilobular areas before laying down collagen fibers. These facts raised doubts on the origin of the putative fibrogenic cells in the liver. Thus alternative cellular sources of fibrogenic cells have been identified, namely, portal fibroblasts and bone-marrow-derived circulating fibrogenic cells, or fibrocytes, among other potential candidates [12–16]; even epithelial cells (i.e., hepatocytes and biliary epithelial cells) undergoing epithelial-to-mesenchymal transition have been studied [15, 17, 18]. Although there is still some debate regarding the relative contribution of each cell type, HSC-derived myofibroblasts are still considered as the main collagen-producing cells in chronic liver diseases.

A process called ductular reaction is a hallmark in most chronic liver diseases [5]. It is defined as immune cell accumulation, fibrosis, and ductular cell proliferation in the periportal area. Numerous studies have reported close interactions between these three events. In brief, liver injury leads to the recruitment of immune cells including monocyte-derived macrophages and T lymphocytes, which in turn favor fibrogenic cell activation, as well as ductular cell (liver progenitor cells, or biliary cells) proliferation [19–28]. Fibrogenesis has been suggested to favor ductular cell proliferation, and reciprocally, ductular cells are known to release fibrogenic factors

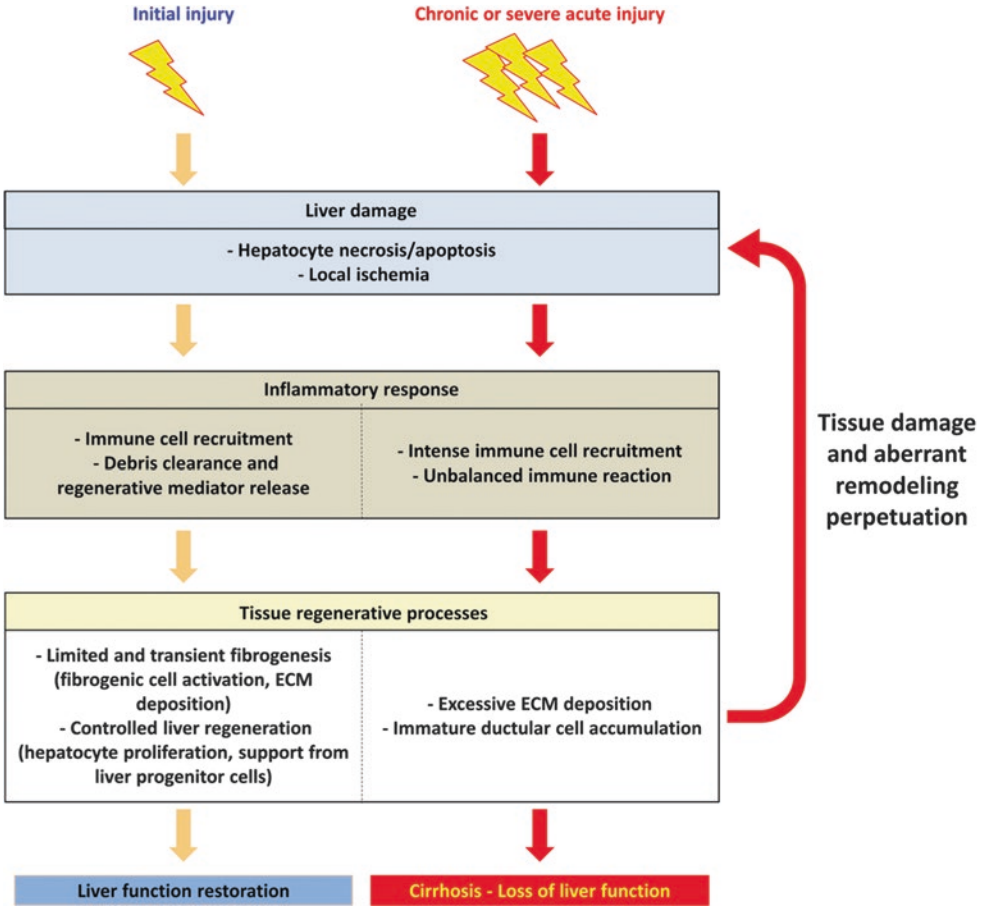

Fig. 35.1 Classical liver disease history. Inflammation plays a critical role not only in inducing liver fibrogenesis during chronic liver injury but also in promoting liver fibrosis resolution and liver regeneration. *ECM* extracellular matrix

[5, 29–31]. Moreover, fibrogenic cells and ductular cells release pro-inflammatory mediators, thus participating in the maintenance of an inflammatory microenvironment and tissue injury [32–35]. This vicious circle may hold the key to the control of chronic liver disease progression, and numerous efforts are put toward identifying key therapeutic targets that may impede these processes (Fig. 35.1).

HSCs Sense Changes in Their Microenvironment Under Inflammatory Conditions

HSCs are well located and equipped to sense changes in their microenvironment. Indeed, their cytoplasmic protrusions expand toward the liver sinusoidal cells and hepatocytes [8, 36, 37]. HSCs, and by extension myofibroblasts, possess a complete arsenal of sensing receptors that detect changes in their microenvironment especially inflammatory conditions (please see reference [38]). Toll-like receptors (TLRs) are among these key receptors, and TLR types 1–9 have been proposed to be expressed by HSCs [33, 39, 40]. Most notably, direct TLR 2, 3, 4, and 9 activation on HSCs have been described as some of the mechanisms leading to inflammation and fibrosis progression (as detailed below) [41]. The TLRs and injury-related changes in the liver microenvironment have been described as crucial mediators of numerous inflammatory and fibrogenic processes through immune or parenchymal cell stimulation, but that will not be discussed in this section (reviewed elsewhere [42, 43]).

Danger-Associated Molecular Patterns (DAMPs) (Such as High-Mobility Group Protein 1 [HMG-1], Mitochondrial DNA [mtDNA])

DAMPs are molecules that are released upon cell death or exposed atypically at the cell membrane under stressing conditions. DAMPs are often described as the mediators of sterile inflammation, a process that initiates immune responses and tissue regeneration/fibrosis, independently of pathogens [44]. A variety of DAMPs have been implicated in liver disease and fibrosis, from nucleus or mitochondrial DNA to acute-phase proteins and protein chaperones [42]. For instance, TLR9 stimulation on HSCs, by apoptotic hepatocyte-derived nucleus DNA, led to the immobilization of migrating HSCs at the site of injury and to their activation into a collagen-producing phenotype [45]. Another example

has been the proposed mechanism that TLR3 activation would induce the release of exosomes from HSCs, which would then stimulate interleukin (IL)-17A production by γδ T cells – a potent pro-inflammatory and pro-fibrogenic cytokine [46]. High mobility group box 1 (HMGB1) is similarly regarded as a crucial enhancer of liver fibrosis. Indeed, it has been shown that HMGB1 released by damaged hepatocytes activates HSCs toward a pro-fibrogenic phenotype, through TLR4 activation and endoplasmic stress induction [47]. However, liver (i.e., hepatocyte and biliary cell) HMGB1-deficient mice had similar liver inflammation and fibrosis in a hepatocarcinogenesis model [48].

Pathogen-Associated Molecular Patterns (PAMPs) (Such as Bacterial Products)

It has been shown that LPS-mediated TLR4 stimulation on HSCs favors their activation toward collagen-producing cells through reduced TGFβ pseudo-receptor bone morphogenetic protein (BMP), bone morphogenetic protein (BMP), and activin membrane-bound inhibitor homolog (BAMBI) expression, thus rendering them more responsive to transforming growth factor (TGF)β1 stimulation [33]. This study also demonstrated the importance of systemic inflammation and more specifically of intestinal bacterial product leakage in initiating and perpetuating liver fibrosis. Indeed, antibiotic treatment reduced bile duct-ligation-induced liver damage and tissue fibrosis [33]. These effects have been specifically attributed to TLR4 expression on HSCs and not Kupffer cells. Moreover, TLR9 stimulation by bacterial- or mitochondrial-derived DNA is known to promote liver fibrosis [49]. Lastly, it has been demonstrated that lipopolysaccharides (LPS) treatment (i.e., TLR4 activation) on HSCs downregulates miR-29 expression, favoring HSC activation and increased collagen expression [50].

Other Inflammatory Mediators (Such as Apoptotic Bodies, Extracellular Vesicles)

HSCs can be activated when phagocytosing damaged hepatocyte-derived apoptotic bodies [51]. Of note, macrophages that phagocyte apoptotic bodies also adopt an anti-inflammatory phenotype, notably characterized by enhanced TGFβ1 release [52]. This macrophage polarization has been questioned by another study reporting that cell debris phagocyting monocyte-derived macrophages adopt a phenotype favoring fibrosis resolution, through increased matrix-metalloproteinase expression [53]. Extracellular vesicles (EVs) allow for intracellular component sharing among cells, and EVs are increasingly studied in the field of liver diseases and fibrosis [54–56]. EVs are implicated in cell-to-cell communication and can also be used to deliver therapeutic agents to targeted cell populations. Indeed, studies using mesenchymal stromal/stem cell-derived EVs have reported promising results in ameliorating liver fibrosis and inflammation in rodent models [57, 58]. More recently, it has been shown that liver stem cell-derived EVs reduced ductular reaction and liver fibrosis in the multidrug resistance 2 knockout (*Mdr2*−/−) mice, via Lethal-7 microRNA and notably by reducing NF-κB and IL-13 signaling pathways in liver tissue [59]. EV cargos may also prove to be detrimental, since another group demonstrated that HSC-derived platelet-derived growth factor receptor (PDGFR) α-enriched EVs directly promote liver fibrosis in vivo [60]. As stated above, it has been demonstrated that apoptotic body engulfment by HSCs leads to fibrosis progression, thus shedding the light on the need for state-of-the-art EV isolation protocols to appropriately discriminate between EVs and apoptotic bodies [61, 62].

Cytokines Regulate Liver Fibrosis Initiation, Progression, or Resolution

Liver fibrosis is the consequence of a multitude of events, to include chronic tissue injury, inflammation, and fibrogenic cell activation. Here, we mainly discuss several cytokines that play an important role in regulating HSC activation in the liver. Some factors are produced by multiple cell types and have been shown to have redundant functions. Table 35.1 summarizes the main cytokine implications in liver fibrosis.

Major Cytokines That Promote Liver Fibrosis

Transforming growth factor beta 1 (TGFβ1) and platelet-derived growth factor (PDGF) are the two major cytokines that promote HSC activation and proliferation, respectively. TGFβ1 is considered the most prominent fibrogenic factor that favors HSC activation and fibrogenesis through the activation of Smads 2 and 3. Many types of cells can release TGFβ1, including HSCs, hepatocytes, T cells, and macrophages [63, 64]. In addition, TGFβ1 possesses potent anti-inflammatory properties that may direct immune cells toward a pro-fibrogenic response.

PDGF is long recognized as a potent mitogen for HSCs by targeting PDFGRα on these cells [65, 66]. Different sources of PDGF have been identified, such as Kupffer cells and proliferating cholangiocytes [67]. PDFGRα expression is highly upregulated after HSC activation and is induced by TGFβ1 [68]. Moreover, it has been shown that targeting PDFGRα in hepatocytes may result in lowering PDGFRα expression on HSCs, thus reducing their activation and liver fibrosis [69]. Therapeutic approaches such as the use of dom-

Table 35.1 Main cytokines regulating HSC activation and liver fibrosis

Cytokine	Main source(s)	Direct effects on HSCs/MFBs	Other effects on the liver
IL-1β	Macrophages	Induces fibrogenic gene expression	Pro-inflammatory
IL-4	Granulocytes, NK cells, Th2 lymphocytes	Stimulates collagen production	Protection against infection
IL-6	Macrophages	HSC survival and proliferation	Hepatoprotective and pro-inflammatory
IL-10	Macrophages, dendritic cells, T cells	HSC senescence	Anti-inflammatory
IL-13	Granulocytes, Th2 lymphocytes	Stimulates collagen production	Protection against infection
IL-17A	Th17 lymphocytes, γδT cells, neutrophils, MAIT cells	Induces collagen production and release of pro-inflammatory mediators, sensitizes HSC to TGFβ1	Pro-inflammatory
IL-22	Th17, Th22 lymphocytes	Induces HSC senescence	Hepatoprotective
IL-33	LSECs, activated HSCs	Induces HSC activation and collagen production	Biliary cell proliferation
TNFα	Macrophages	Increases HSC survival and increases TGFβ1 signaling	Pro-inflammatory
TGFβ1	Macrophages, myofibroblasts	Induces HSC activation and collagen production	Anti-inflammatory
PDGF	Kupffer cells, proliferating cholangiocytes	Mitogenic on HSCs	Angiogenic
IFNγ	CD8+ T cells, NK cells, Th1 lymphocytes	Decreases fibrogenic gene expression	Increases liver damage and inflammation, thus fibrosis

Abbreviations: *HSC* hepatic stellate cell, *IL* interleukin, *IFN* interferon, *LSECs* liver sinusoidal endothelial cells, *NK* natural killer, *PDGFR* platelet-derived growth factor receptor, *TGF* transforming growth factor, *TNFα* tumor necrosis factor alpha

inant negative soluble PDGFβ receptor or PDGF receptor signaling inhibitor (imatinib) have generated promising results in counteracting liver fibrosis [70, 71].

IL-17A (IL-17)-producing cells are frequently observed in the liver of patients suffering from alcoholic steatohepatitis and a variety of other chronic liver diseases associated with liver fibrosis [19, 72]. IL-17 levels are strongly increased upon liver fibrosis, and IL-17 has also been shown to correlate with disease progression and a poor prognosis in a variety of liver diseases [19, 73, 74]. More specifically, IL-17A-deficient animals exhibited reduced fibrosis and inflammation in the bile duct ligation model [73, 74]. Recombinant IL-17 strongly increased production of pro-inflammatory mediators such as IL-6 and TNFα in macrophages [74]. IL-17-receptor is ubiquitously expressed in the organism and has been shown to directly induce fibrogenic cell activation by favoring HSC to myofibroblast activation and enhancing collagen expression [73]. Furthermore, IL-17 directly induced collagen type I production in myofibroblasts through signal transducer and transcription factor 3 (STAT3) activation. Another study reported that IL-17A sensitizes HSCs to TGFβ1-mediated activation [75]. Interestingly, Th17 cells are also a potent source of IL-22, which exerts anti-fibrotic effects (see below) [73, 76].

Defined as Th2-profile cytokines, IL-4 and IL-13 are often associated and display similar functions [77]. Although both cytokines are crucial in host defense against infection, they also exert potent fibrogenic functions that have been long described. Indeed, IL-4 has been shown to increase TGFβ1 production in fibroblasts, and IL-13 is a potent pro-fibrogenic cytokine that directly acts on myofibroblasts [78–80]. Both IL-4 and IL-13 have also been shown to directly stimulate collagen expression and production in cultured fibroblasts [81, 82]. Lastly, IL-4 levels have been correlated with advanced fibrosis development in HCV patients [83].

Major Cytokines That Attenuate Liver Fibrosis

Interferon-gamma (IFN-γ) is considered a major negative regulator of liver fibrosis. IFN-γ directly reduces myofibroblast activation and collagen production in culture [84–87]. Moreover, IFN-γ is known to induce a cytotoxic NK cell phenotype in the liver, directed against activated HSCs [88]. Additionally, IFN-γ is known to increase liver injury in acute models such as Concanavalin A or lipopolysaccharides [89, 90]. Accordingly, in a model of methionine- and choline-deficient high-fat, or in a model of primary sclerosing cholangitis (Mdr2−/− mice), it has been reported that IFN-γ-deficient mice had reduced liver inflammation and fibrosis as compared to IFN-γ-expressing mice, possibly due to reduced tissue injury [91, 92]. These results highlight the complex roles of IFN-γ in favoring both tissue injury and repair mechanisms and orientating the immune system toward an anti-fibrotic response and inhibiting fibrogenic gene expression on HSCs.

IL-22, mainly produced by Th17 and Th22 cells, opposes the anti-fibrogenic effects of IL-17 by inducing HSC senescence and protecting against hepatocellular damage [76]. Mechanistically, IL-22-induced HSC senescence was pre-

vented when STAT3 signaling was blunted. Accordingly, IL-22 deletion exacerbated liver fibrosis and IL-22 administration prevented bile-duct ligation liver fibrosis [73]. In addition, IL-22 has potent hepatoprotective roles, by favoring hepatocyte survival through STAT3 activation, thereby inhibiting liver fibrosis [93, 94].

Other Cytokines That May Have Dual Roles in the Control of Liver Fibrosis

TNFα, one of the most potent inflammatory cytokines, is upregulated during tissue injury responses and is participating in tissue injury by favoring hepatocyte apoptosis. TNFα administration enhanced liver fibrosis through increasing HSC survival through induction of tissue inhibitor of metalloproteinase 1 (TIMP-1) expression, an effect that has been reported to be mediated by Kupffer cell activation [95]. TNFα and LPS stimulated HSCs harbored reduced BAMBI expression, resulting in increased TGFβ1 signaling [96]. Additionally, HSCs isolated from TNR-receptor 1- and/or TNR-receptor 2-deficient mice showed reduced proliferation in response to PDGF and a reduced expression of collagen [97]. Contrastingly, direct treatment of isolated HSCs by TNFα led to reduced collagen expression but increased cell proliferation in other studies [98–100]. Therefore, TNFα seems to have contradictory effects on HSCs, which may be linked to their integration into a more complex microenvironment that exposes fibrogenic cells to a multitude of activating and inhibitory signals. TNFα stimulation induced activation of apoptosis signal-regulating kinase 1 (ASK1) and subsequently activated the p38/JNK signaling pathway, promoting liver fibrosis [101, 102]. Although an early phase II trial reported that selonsertib, an ASK1 inhibitor, showed promising results in reducing liver fibrosis in nonalcoholic steatohepatitis patients [103], selonsertib failed to reduce liver fibrosis in a phase III clinical trial (STELLAR-4, NCT03053063).

IL-6 not only plays an important role in protecting against hepatocellular damage and promoting liver regeneration but also acts as a pro-inflammatory cytokine. However, IL-6 can also promote HSC activation and survival via the activation of STAT3, thereby enhancing liver fibrosis [104–106]. Therefore, the effect of IL-6 on liver disease progression depends on the balance between its beneficial and detrimental functions.

Several studies reported that IL-1-receptor or IL-1β deficient mice were resistant to liver fibrosis [107, 108]. It has been questioned, however, whether these effects were due to direct effects of IL-1β or IL-1α on HSCs or indirect by favoring a pro-inflammatory environment. In addition, macrophage-derived IL-1β was demonstrated to induce fibrogenic gene expression in myofibroblasts from the liver [109].

IL-10 is a potent anti-fibrotic cytokine, and accordingly, IL-10-deficient mice develop a stronger immune response and a more severe fibrosis than wild-type mice following repeated CCl4 injections [110, 111]. Direct effects of IL-10 on HSCs were related to senescence induction and a decrease in HSC viability [112].

IL-33 is generally associated with tissue regeneration. For instance in the liver, it has been shown to promote biliary cell proliferation [113]. Moreover, IL-33 expression is higher in human and mouse fibrotic livers as compared to normal liver samples [114]. Accordingly, IL-33 has been characterized as a pro-fibrogenic factor being produced by activated HSCs and increasing their collagen production, as well as a pro-fibrogenic immune environment [114–118].

Immune Cells Regulate Liver Fibrogenesis

Liver fibrosis is seemingly always associated with liver inflammation (with the apparent exception of hemochromatosis) [119, 120]. Virtually all myeloid and lymphoid immune cells are implicated in liver fibrosis initiation, progression, and/or resolution (Fig. 35.2) [55, 121]. While immune cells respond to tissue injury by clearing DAMPs and PAMPs, an unbalanced response or chronic inflammation can enhance tissue damage and lead to liver fibrosis. Indeed, inflammatory processes are tightly regulated, and a slight imbalance results in either aggravated pathology or recovery. While specific factors produced by immune or parenchymal cells have been discussed (see above), we herein briefly describe the putative and sometimes contradictory roles of immune cell populations in the liver over the course of liver fibrosis.

Liver-Resident Macrophages

Kupffer cells are the liver-resident macrophages and are renowned to exert sentinel functions in healthy conditions. Kupffer cells are thus considered to be among the first immune cells to sense changes associated with liver injury [122]. During liver disease initiation, they are notably a potent source of chemokines for other immune cell types. Due to the difficulties in distinguishing Kupffer cells and monocyte-derived macrophages upon chronic liver injury, some reports may inadvertently confound these two cell types, and macrophage-depleting approaches may impact both compartments [123]. Accordingly, macrophage depletion by using clodronate-loaded liposomes at early stages of liver fibrosis prevents excessive scarring, while macrophage depletion at later stages dampens fibrosis resolution in the CCl4 model [124].

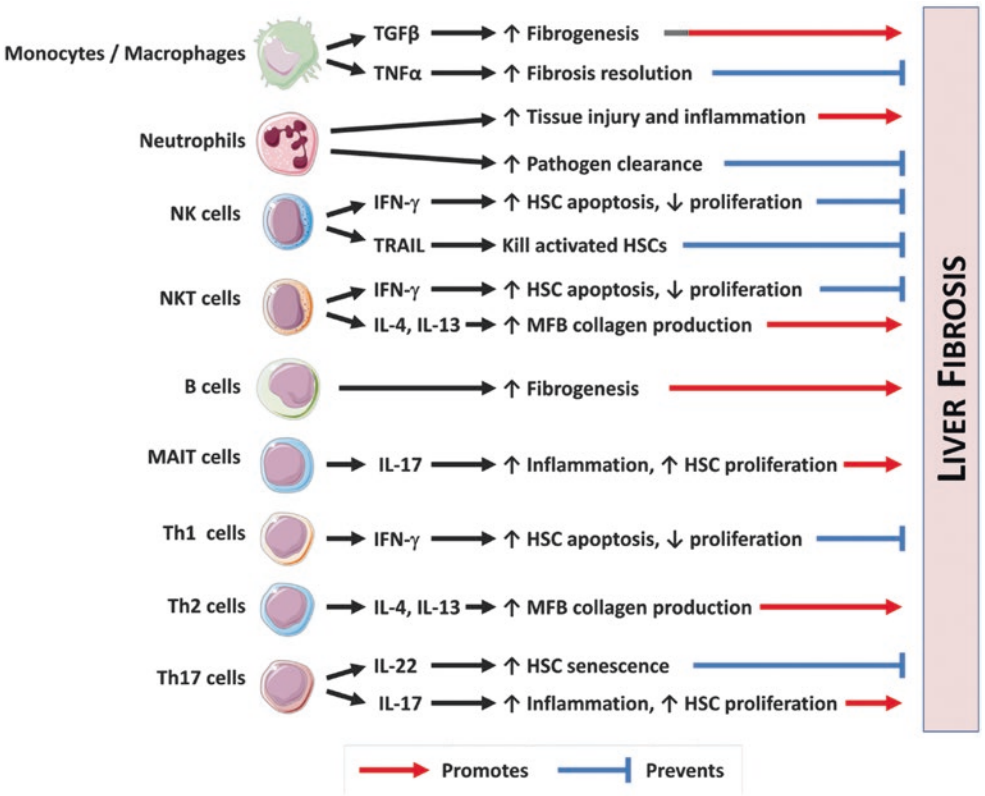

Fig. 35.2 Immune cell implication in liver fibrosis prevention or promotion. This figure represents the main immune cell types and subpopulations implicated in liver fibrosis progression or resolution. *HSC* hepatic stellate cell, *IL* interleukin, *IFN* interferon, *MAIT* mucosal-associated invariant T cells, *MFB* myofibroblast, *NK* natural killer, *TGF* transforming growth factor, *Th* Helper T cell, *TNFα* tumor necrosis factor alpha

Monocyte-Derived Macrophages

Mononuclear cell infiltration (more specifically monocyte accumulation) is a classical feature of liver fibrosis. Monocytes can activate toward a plethora of phenotypes, including pro- or anti-inflammatory and pro- or anti-fibrogenic phenotypes [123, 125]. They are thus regarded as crucial orchestrators of liver disease due to their potent cytokine secretion. C–C motif chemokine receptor 2 (CCR2) is crucial for monocyte recruitment, since CCR2-deficient mice had reduced numbers of liver macrophages after bile duct ligation [126]. Accordingly, the use of a CCR2/CCR5 antagonist (cenicriviroc) has shown promising effects for the treatment of nonalcoholic steatohepatitis with fibrosis [127]. Nonetheless, monocytes are also crucial in liver regeneration, and monocyte/macrophage-depleting methods have been proven to sometimes delay or prevent tissue repair mechanisms [128–132].

Neutrophils

Neutrophils are among the first responders to liver injury and are mainly characterized by their potent roles in aggravating tissue injury through reactive oxygen species release [133, 134]. Consequently, neutrophil recruitment and activation are generally regarded to promote chronic pro-inflammatory and pro-fibrogenic environment. On the other hand, neutrophils are crucial in pathogen clearance that is necessary for inflammation resolution [135].

Natural Killer (NK) and NKT Cells

A clear role of NK cells in liver fibrosis is to control fibrosis progression through killing activated HSCs and producing IFN-γ that induces HSC apoptosis and cell cycle arrest [88, 136, 137]. In contrast, CD1d-dependent invariant NKT cells play dual roles in regulating liver fibrogenesis; for example, NKT cells not only can promote fibrosis progression, through IL-4 and IL-13 production [138], but may also attenuate liver fibrosis by killing HSCs and producing IFN-γ [139, 140].

T Lymphocytes

$CD4^+$ T lymphocytes, also termed T-helper cells, are regarded as immune response orchestrators due to their very distinct and intense immune system-mediating cytokine release. Th1, Th2, and Th17 are the most studied and well-characterized activation phenotypes in liver disease. While Th1 cells are classically regarded as anti-fibrogenic through the promotion of anti-fibrogenic responses and IFN-γ-mediated fibrogenic cell death, Th2 cells are considered to be pro-fibrogenic

through IL-4 and IL-13 production [141–143]. Th17 cells, on the other hand, have been described as having contradictory roles in liver disease. Th17 cells are mainly characterized by IL-17A and IL-22 production, which exert opposing functions in liver disease, IL-17A being pro-inflammatory and pro-fibrogenic and IL-22 favoring tissue regeneration and inducing HSC senescence (discussed above) [74, 76]. Cytotoxic CD8+ T lymphocytes play detrimental roles in alcoholic liver disease, notably by directly killing parenchymal cells [144]. Furthermore, autoreactive CD8+ T cells are considered to be the main drivers of biliary cell damage in primary biliary cholangitis, by targeting biliary epithelial cells [145].

B Lymphocytes

B lymphocytes represent a major lymphocyte population in the liver [146]. Despite identical initial injury, B-cell-deficient (*JH−/−*) mice showed reduced fibrosis 16 weeks after CCl4-induced liver fibrosis [146]. Furthermore, B cells accumulate in the liver from Mdr2−/− mice, and B-cell ablation by intravenous injections of anti-mouse CD20 monoclonal antibody promoted HSC senescence-mediated fibrosis resolution and was also associated with reduced TNFα and NF-κB activation [147].

Mucosal-Associated Invariant T (MAIT) Cells

MAIT cells have recently gained a lot of interest due to their antibacterial activity and are especially enriched in the human liver [148]. They are innate-like T cells mostly characterized as CD161+CD8+ T-cells and by the invariant TCR-chain, Vα7.2-Jα33 [149, 150]. MAIT cells have notably been shown to accumulate at the portal tracts around biliary ducts in human cholangiopathies and have thus been suggested to play a role in bile duct diseases [151]. Moreover, IL-7 is produced by hepatocytes under inflammatory conditions, and IL-7-stimulated MAIT cells dramatically upregulated their IL-17A production [148]. Similarly, repetitive IL-12 stimulation upregulated IL-17A production by MAIT cells [152]. In this same study, the authors demonstrated that although MAIT cells are less frequent in fibrotic than in the healthy liver, the remaining MAIT cells have adopted a pro-fibrogenic phenotype that further accentuates liver fibrosis, notably through IL-17A [152].

Roles of Immune Cells in Fibrosis Resolution

Despite the crucial roles of inflammation in initiating liver fibrosis, there is considerable amount of data enlightening the role of immune cells in fibrosis resolution. Accordingly, it has been demonstrated that macrophage depletion at early stages prevents CCl4-induced liver fibrosis, while macrophage depletion during liver fibrosis resolution stage, on the other hand, leads to fibrosis perpetuation [124]. The role of macrophage-mediated fibrosis resolution could be explained by the fact that bone-marrow-derived macrophages are required for natural killer (NK) cell recruitment. NK cells will then release TNF-related apoptosis-inducing ligand (TRAIL) and IFN-γ and subsequently induce fibrogenic cell apoptosis [88, 153]. Another major function of NK cells is the production of IFN-γ, which is known to oppose TGFβ1 fibrogenic signaling and inhibit HSC fibrogenicity through STAT1 activation [154].

Potential Anti-fibrotic Therapeutic Approaches by Targeting Immune Components

Withdrawal of the causative agents of liver injury has been shown to effectively prevent disease worsening and even allow for fibrosis or cirrhosis regression in hepatitis B and C infected patients, autoimmune diseases, non-alcoholic steatohepatitis, and more disputably in alcoholic patients [155–158]. As discussed above, many immune components have been implicated in the pathogenesis of liver fibrogenesis. Some of these components could be used as therapeutic targets for the treatment of liver fibrosis. For example, one potential approach to modulate HSC activation is to alter the TGFβ1 and unfolded protein response (UPR) [159]. More specifically, upon extracellular matrix protein assembly, fibrogenic cells experience increased ER stress, leading to the UPR and allowing for a proper protein folding and trafficking out of the cell while favoring cell survival. TGFβ1 is known to increase ECM protein production and to induce ER stress as well as UPR [159]. Procollagen I export blockade through transport and Golgi organization protein 1 (TANGO1) impairment led to HSC death in basal conditions due to enhanced ER stress, which was further increased upon concomitant TGFβ1 stimulation [159]. In addition, targeting the UPR through pharmacological inhibition of C/EBPβ-p300 may result in limiting fibrogenic cell activation and liver fibrosis [160].

IFN-γ is one of the most potent anti-fibrotic cytokines and was examined in clinical trials for the treatment of liver fibrosis with some beneficial effects, but long-term benefits were not observed [161, 162]. These disappointing results were likely due to low efficacy and adverse effects from IFN-γ therapy because IFN-γ strongly inhibits liver regeneration by targeting hepatocytes and induces inflammation by targeting immune cells. Researchers have been trying to develop fibroblast-targeted IFN-γ via the fusion of PDGF-β

receptor recognizing peptide and IFN-γ, which had increased anti-fibrotic potency and improved safety profile in experimental models of liver fibrosis in vivo [163].

IL-22 has many beneficial functions in the liver, including hepatoprotective, proliferative, anti-oxidative, and anti-fibrotic functions [76]. More importantly, IL-22 therapy may generate limited side effects because IL-22 mainly targets epithelial cells as well as HSCs without affecting immune cells. Thus, IL-22 is a promising drug for the treatment of liver failure and may also have therapeutic potential for the treatment of liver fibrosis [164]. Indeed, a clinical trial shows promising results regarding IL-22Fc treatment for severe alcoholic hepatitis [165].

In summary, many immunological factors play important roles in controlling liver fibrogenesis. Further understanding of their functions may help identify novel therapeutic targets and effective strategies for the treatment of liver fibrosis in the future.

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Immune-Mediated Liver Disease in the Transplanted Liver

36

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Key Points

- Liver transplantation remains the therapy of choice for patients with end-stage liver disease. However, the liver allograft is susceptible to a range of complications, many of which involve immune-mediated components, including rejection, graft hepatitis, as well as recurrent and de novo autoimmune disease.
- The increasing burden of chronic liver disease has not been paralleled with an increase in donor pool, and as a result many procedures are performed using "marginal donor organs" that have an increased risk for poor function.
- The use of marginal donors is a risk for ischemia–reperfusion injury (IRI), the most common reason for retransplantation in the early postoperative period.
- Understanding the molecular mechanisms responsible for rejection and IRI will allow development of new therapeutic treatments.
- Antibody-mediated rejection (both acute and chronic) is becoming better defined and understood, but better diagnostic and therapeutic algorithms are required.
- Acute T-cell-mediated rejection remains the most common form of rejection although it does not necessarily translate into poor long-term outcome.
- T-cell- and antibody-mediated chronic (ductopenic) rejection remains a risk factor for immune-mediated graft loss.
- De novo autoimmune hepatitis, now termed "plasma cell hepatitis," may occur in a small proportion of recipients and may progress despite increased immunosuppression.
- The liver allograft remains relatively resilient to immune-mediated injury compared to other solid-organ allografts, in part due to its inherent tolerogenic properties and large hemopoietic organ mass.
- The reported incidence of recurrent autoimmune liver disease varies largely due to a lack of codified diagnostic criteria; liver biochemistry can remain within the normal range on a background of recurrent autoimmune liver injury.

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Introduction

Liver transplantation (LT) has evolved as the treatment of choice for many patients with end-stage liver disease (Fig. 36.1). Indications are broadly an anticipated survival in the absence of transplantation of 1 year or less or a quality of life that would be corrected by LT (such as intractable encephalopathy or pruritus) and if the recipient has a greater than 50% probability of being alive and well 5 years after transplantation. With the introduction of highly effective direct-acting antivirals against hepatitis C virus (HCV), both the need for transplantation for end-stage HCV and graft loss from recurrent disease are decreasing and changing the epi-

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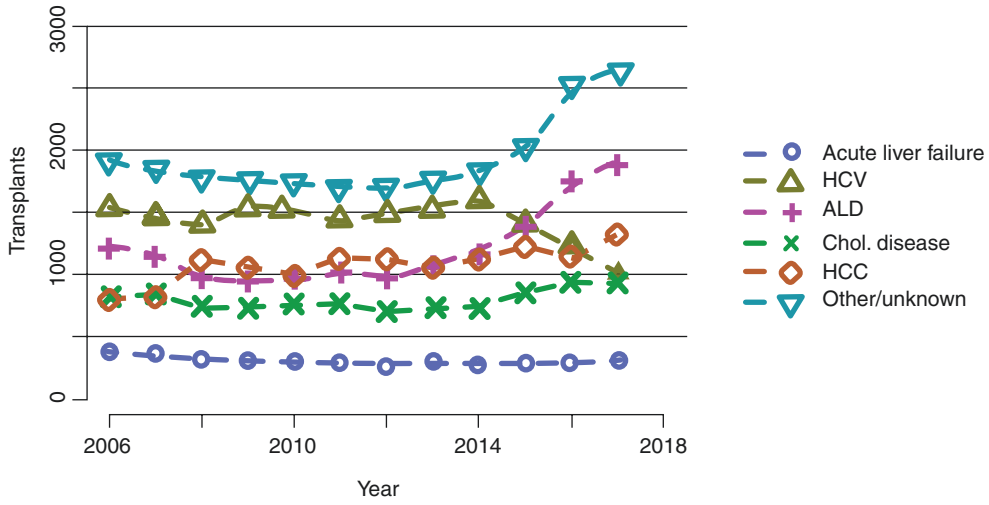

Fig. 36.1 Indications for liver transplantation. (Source: UNOS OPTN/SRTR (2018) Annual Data Report [1]). HCV hepatitis C virus, ALD alcohol-related liver disease, Chol cholestatic (such as primary biliary cholangitis), HCC hepatocellular carcinoma

demiology of transplantation. End-stage liver diseases from alcohol and from nonalcoholic fatty liver disease are the leading indications in many jurisdictions.

Approaches to organ allocation vary but are based on either need (estimated probability of death) or benefit (estimated gain in survival because of transplantation). The Model for End-Stage Liver Disease (MELD) score, or a modified version, is the most widely used scoring system for estimating survival without a transplant (Table 36.1). Transplant benefit outweighs risk when the MELD score reaches 15.

While both the number of patients awaiting LT and deceased organ donors have shown a steady rise over the last decade, there remains a gap between the number of listed candidates and the number of grafts. Up to 15% of adults electively listed for a deceased donor transplant will die or become too ill for a transplant [1]. Consequently, the use of marginal grafts—defined as an organ with increased risk for poor function or failure that may subject the recipient to greater risks of morbidity or mortality—has become more common. The advent of newer techniques such as in situ perfusion and either pulsatile or static, normothermic or hypothermic perfusion may allow better selection of organs, improved quality of organs and allow for ex situ interventions to modulate the immune responses.

Short-term and, to a lesser degree, long-term graft outcomes improve year on year. Data from the United States show that in 2017, graft failure occurred in 7% at 6 months and in 10% at 1 year (for deceased donor liver transplants done in 2016), in 16% at 3 years (for transplants done in 2014), in 24% at 5 years (for transplants done in 2012), and in 43% at 10 years (for transplants performed in 2007) [1]. The main causes of death (with or without a functioning graft) are recurrent disease, cardiovascular disease, de novo or recurrent malignancy, renal failure, and graft failure.

Table 36.1 Medical urgency scoring systems for prioritizing receipt of a liver transplant

Score	Components/formula
CTP[a]	Ascites, encephalopathy, bilirubin, albumin, prothrombin time
MELD[b]	3.78[Ln serum bilirubin (mg/dL)] + 11.2[Ln INR] + 9.57[Ln serum creatinine (mg/dL)] + 6.43
MELD − Na[c]	MELD − Na [0.025 × MELD × (140 − Na)] + 140
UKELD[c]	[5.395 × ln(INR)] + [1.485 × ln(creatinine)] + [3.13 × ln(bilirubin)] × [81.565 × ln(Na)] + 435

[a]The Child-Turcotte-Pugh (CTP) score integrates five empirically selected variables with a range of 5–15 points. The CTP score has several pitfalls in this regard, not least the nature of ascites and encephalopathy as subjective variables

[b]The Model for End-Stage Liver Disease (MELD) score has a discriminative ability for 3-month survival of greater than 80%, regardless of the severity of liver disease, without any significant improvement by adding etiology or complications of cirrhosis

[c]MELD – Na and the United Kingdom for end-stage liver disease (UKELD) are modifications of the MELD score. In the liver transplant setting serum sodium is an independent factor of mortality, particularly for lower sodium values (120–135 mmol/L). Within this range, a decrease of 1 mmol/L corresponds to a 12% increase in 3-month mortality independently of MELD score. Compared to standard MELD, the MELD – Na and UKELD scores provide better statistical performance for the risk of death among potential transplant candidates. Newer scores integrating sodium as a variable perform superior to MELD alone and have thus superseded the latter in clinical practice

While the number of immunosuppressive agents in routine clinical practice has not increased over the past decade, practice has continued to evolve. Data from the United States show that the number of recipients having no induction therapy is falling (currently about 65%), with IL2R antagonists such as basiliximab and T-cell-depleting

agents being most commonly used. There is currently little evidence for a beneficial effect of such therapy [2]. While maintenance immunosuppressive regimens vary between centers, the most common regimen at 1 year is based on tacrolimus, mycophenolate, and corticosteroids, with tacrolimus dual therapy (with either mycophenolate or steroids) being used less. In most units, the tacrolimus trough levels targeted during the initial posttransplant period have been drastically reduced. Mammalian target of rapamycin inhibitors (mTORi) is used increasingly. Of note, complete withdrawal of immunosuppression is possible in a small proportion of highly selected LT recipients [3], as discussed later.

Acute allograft rejection is now seen less commonly, in approximately 10–30% of the recipients. Age and indication for LT are significant risk factors, with acute rejection seen in around 23% in recipients aged 18–34 years, compared with 9% in those aged over 63 years. Early (<6 months) rejection is associated with a better prognosis than later rejection [4].

The liver allograft is susceptible to a range of complications, including ischemia–reperfusion injury, technical issues, acute and chronic rejection, and recurrent disease. However, compared to other solid-organ allografts, the liver is less susceptible to immune-mediated damage, in part because the liver has an inherent ability to attenuate immune-mediated rejection targeted toward alloantigens.

Ischemia–Reperfusion Injury (IRI)

IRI is the main cause of both primary nonfunction and delayed graft function accounting for 80% of retransplantations during the first week. This results from a multifaceted process that combines elements of *warm* and *cold* injury. *Warm IRI* occurs when perfusion is reduced after clamping or when there is reduced liver perfusion from shock, heart failure, respiratory failure, hemorrhage, trauma, or sepsis. *Cold IRI* occurs when the organ is preserved in hypothermic fluid and is followed by reperfusion after implantation. IRI is often unpredictable but is seen more in marginal and steatotic liver allografts [5]. Although immune mechanisms are involved in IRI, the association of IRI with clinical graft rejection is conflicting.

Molecular Mechanisms of Ischemia–Reperfusion Injury

The ischemic injury is a localized process of cellular metabolic disturbances resulting from a lack of oxygen and adenosine triphosphate (ATP), whereas reperfusion injury involves both direct and indirect cytotoxic mechanisms.

Altered Redox Status and Reduced Microcirculatory Blood Flow

Injury begins with reduced organ perfusion leading to a lack of ATP production with consequent impairment of Na^+/K^+-ATPase membrane pump function resulting in an increase of intracellular Na^+, followed by swelling of hepatocytes, Kupffer cells, and hepatic sinusoidal endothelial cells (HSEC), and leads to narrowing of the liver sinusoids. Elevated levels of reactive oxygen species (ROS), such as superoxide (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH−), can be detected early. The source of ROS in hepatic IRI is controversial. It used to be assumed that xanthine oxidase was a significant intracellular source of ROS formation. However, there is little direct evidence for an intracellular oxidant stress by xanthine oxidase during the early reperfusion phase. Other putative cellular sources for these ROS are mitochondrial metabolism and HSEC-associated NADPH oxidase. However, most of the oxidant stress appears to occur in the vasculature, with Kupffer cells as the main source [6]. This is accompanied by reduced nitric oxide (NO) production and aggravated sinusoidal narrowing.

The release of ROS causes damage to cellular membrane lipids resulting in cellular swelling and death, an increase of vasoconstrictors endothelin and thromboxane A2, and adhesion and aggregation of platelets and leucocytes. These changes exacerbate the narrowing of sinusoids and reduction in microcirculatory blood flow, perpetuating hypoperfusion-induced injury.

Ionic and Mitochondrial Disturbances

The ROS generated lead to an increase in cytosolic and mitochondrial calcium (Ca^{2+}). This reduces the mitochondrial transmembrane potential, and as a result, the activity of the mitochondrial enzyme ATP synthase becomes reversed in an effort to hydrolyze ATP to provide energy for the different ionic pumps in the mitochondrial membrane. However, this further increases the Ca^{2+} influx resulting in ATP consumption instead of production in the mitochondria, a process enhanced by the oxidative damage to enzymes of the mitochondrial respiratory chain caused by ROS.

Cytosolic and mitochondrial Ca^{2+} and other ionic disturbances lead to damage of plasma and mitochondrial membranes including the formation and opening of mitochondrial permeability transition pores (MPTPs). Mitochondria affected by MPTPs are permanently damaged due to depolarization and are removed from hepatocytes, thereby increasing ROS production and ATP consumption. With the number of damaged mitochondria increasing, cytochrome C is released from the mitochondria into the cytosol triggering cellular apoptosis. When the majority of mitochondria are

damaged by MPTPs, ATP levels decline resulting in hepatocyte necrosis. The intrinsic lack of oxygen leads to anaerobic respiration of hepatocytes and intracellular acidosis. pH changes activate the Na^+/H^+ exchanger in an effort to reduce cytosolic H^+ concentration and further increase Na^+. However, the Na^+/K^+ exchanger is ATP-dependent; therefore, the ATP-depleting mechanisms block this exchange exacerbating the increase in intracellular Na^+ resulting in cell death. These effects counteract the potentially protective nature of an acidic pH during reperfusion.

Cellular Cascade

The primary cells initiating IRI in the liver allograft are Kupffer cells [7]. Besides inducing direct damage by ROS release, Kupffer cells are also activated by ROS, thereby entering a perpetuating "vicious cycle" of self-activation and destruction. Kupffer cells are also activated by complement proteins, which themselves lead to further hepatocyte damage by formation of a membrane attack complex in the plasma membrane. Activated Kupffer cells secrete interleukin (IL)-1β and tumor necrosis factor-α (TNFα), which activate and induce migration of neutrophils and $CD4^+$ T-cells. These proinflammatory cytokines also stimulate HSEC and hepatocytes to produce more ROS and induce the expression of functional adhesion molecules such as ICAM-1 and VCAM-1, which leads to adhesion and aggregation of leucocytes and platelets, further influencing the microcirculatory blood flow in the liver. The activation of Kupffer cells is enhanced by interferon γ (IFNγ) and IL-17 released by activated lymphocytes. These cytokines also activate natural killer (NK) T-cells that directly damage liver tissue and themselves produce IFNγ with further activation of KC and hepatocytes. The net result of this circular, reciprocal cellular activation is the destruction of hepatocytes and HSEC. These cytokines can also lead to an alteration of downstream transcription factors including activator protein-1 (AP1), heat shock factor, signal transducer and activators of transcription (STATs), cycloxygenase-2 (COX2), antiapoptotic proteins (Bcl-2, $Bcl\text{-}_{XL}$), and the nuclear factor kappa B (NFκB) pathway [8]. These modifications are followed by the release of danger-associated molecular patterns (DAMPs) that bind to toll-like receptors (TLRs), specifically TLR4, and the receptor for advanced glycation end (RAGE) products (Fig. 36.2).

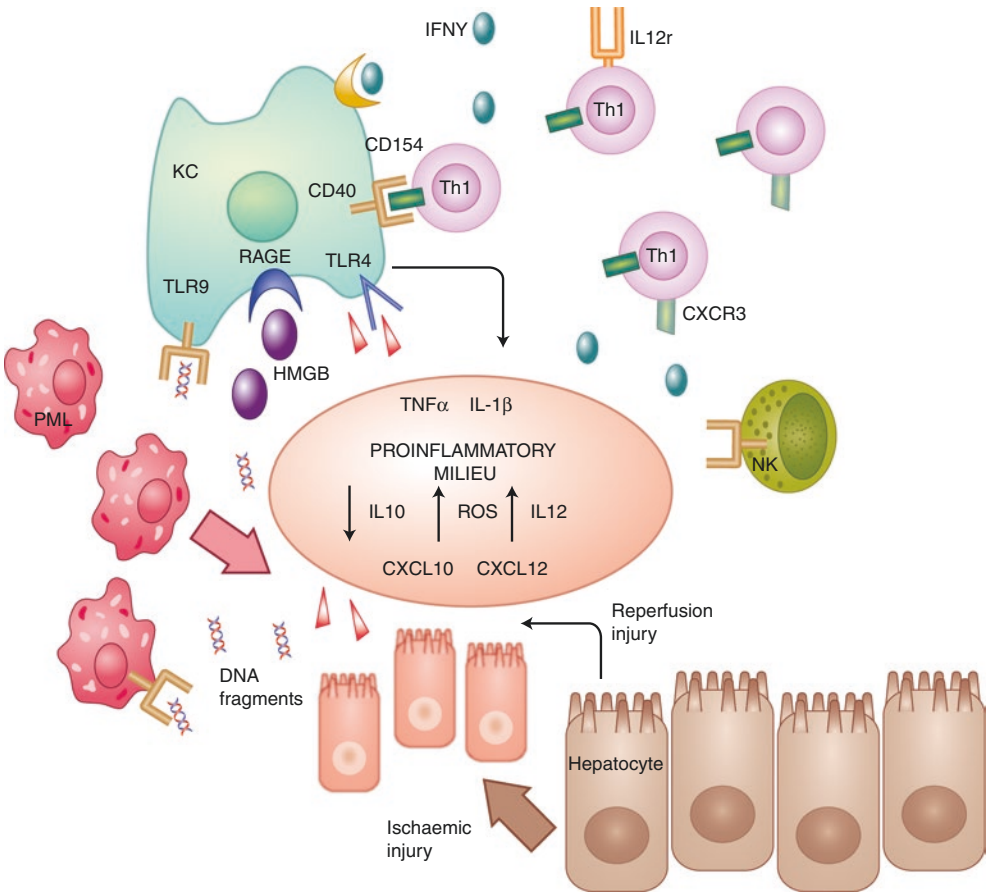

Fig. 36.2 Immune activation in hepatic ischemia–reperfusion injury (IRI). The ischemic insult induces necrotic cell death, which in turn provides diverse "danger"-associated molecules (e.g., HMGB1 and DNA fragments) to activate innate TLR4, RAGE, and TLR9 signaling on Kupffer cells (KC) as well as dendritic cells (DC) and neutrophils. T-cells, particularly Th1-effector cells, may also facilitate local innate immune activation via CD154–CD40 interactions. Interferon-gamma produced by T-cells and NK-cells enhances innate immune activation, and the proinflammatory milieu composed of TNFα, IL-1β, CXCL10, CXCL2, and ROS recruits and activates local and circulating immune cells that promote cytotoxicity against the liver parenchyma

The endogenous TLR ligands are classified as follows:

- Those released from necrotic cells: heat-shock proteins (HSPs), high-mobility group box-1 (HMGB1), and DNA or RNA complexes. HMGB1 protein is the most well-characterized DAMP with its target being TLR4 in liver.
- Derived from degraded extracellular matrix: heparan sulphate, hyaluronan, fibrinogen, fibronectin A domain, and tenascin C.

RAGE plays a major role in the pathogenesis of IRI by regulating CXCL2 production via early growth response protein-1 (Egr1), as well as influencing cell death and TNFα production via Egr1-independent mechanisms. TLR9 detects bacterial and endogenous DNA, serving as a sensor of tissue necrotic cell death that exacerbates liver innate immune activation. TLR9 signals exclusively via the myeloid differentiation primary response gene-88 (MyD88) pathway, in contrast to TLR4-mediated hepatocellular damage. MyD88-independent activation of Kupffer cells by DAMPs occurs in the early phase of liver injury (1–6 hours) and may depend on the direct cytotoxic effect of a soluble TNFα-enriched inflammatory milieu. In later stages (>12 hours), newly recruited and activated polymorphs require MyD88 signaling through TLR9. Thus, different TLRs operate at distinct stages and in different cell types. Liver recipients infected with hepatitis C virus (HCV) with specific TLR4 mutations have significantly worse long-term graft outcomes than recipients lacking this mutation [9], whereas another TLR3 polymorphism may confer protection from acute rejection. Non-TLR innate receptors (e.g., NOD-like receptor [NLR], RIG-I-like receptor [RLR]) recognize PAMPs existing within the cytosol that can also trigger local inflammatory responses and immune activation.

Potential Therapeutic Targets for IRI

Prosurvival genes and antioxidants involved in direct scavenging of ROS have been shown to be highly protective when induced before or shortly after the start of ischemic injury. Many such genes are controlled by the transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)–Kelch-like ECH-associated protein 1 (Keap1) system [8–10]. Many Nrf2 target genes are thus potential therapeutic targets.

Glutathione is a highly effective antioxidant present in high concentrations in hepatocytes, levels being regulated by the Nrf2-dependent gene glutamate–cysteine ligase. Administration of *N*-acetylcysteine (NAC) increases intracellular glutathione levels, which also allows for the detoxification of hydrogen peroxide as well as other ROS such as hypochlorous acid and peroxynitrite. As glutathione is continuously released from hepatocytes into the vascular space, it can detoxify ROS generated by Kupffer cells. Intravenous infusion of glutathione effectively protects against the vascular oxidant stress during reperfusion after warm or cold ischemia [10]. Moreover, high doses of NAC may also support mitochondrial energy metabolism, and gene transfer studies of glutathione synthesis components, suggest that glutamine cysteine ligase catalytic subunit (gclc), glutamine cysteine regulatory subunit (gclm), and glutathione synthase are protective against IRI by increasing intracellular glutathione levels [11].

Clinical trials assessing NAC have failed to provide convincing evidence that the clinical outcome is improved, despite a reduction in the biochemical markers of liver injury [12]. This may, in part, be attributable to the relatively short plasma half-life of glutathione in vivo. Induction of certain HSPs such as haem-oxygenase-1 (HSP32), a Nrf2-inducible gene, has also been shown to increase survival and protect against IRI in the liver, and haem-oxygenase-1 induction is another promising therapeutic avenue in experimental models of hepatic warm and cold ischemia [13].

One of the most commonly investigated methods of reducing IRI has centered around ischemic preconditioning whereby the liver is exposed to a brief period of ischemia and then reperfusion before a longer period of hepatic ischemia [14]. This may lead to a reduced inflammatory response as well as reduced oxidant stress. There are a number of common mechanisms involved in preconditioning therapies, including activation of the p38/MAPK cascade by cAMP-activated protein kinase and induction of antioxidant survival genes such as HSP32. Ischemic postconditioning has also been shown to be protective against the ischemic insult and exerts its beneficial effects through mechanisms similar to those observed in preconditioning, such as activation of the prosurvival PI3K/Akt pathway and induction of antioxidant superoxide dismutases and NO [15]. While NO can combine with superoxide to form peroxynitrite, a potent oxidant and nitrating species, it exerts a dualistic role and serves as a vasodilator during ischemic injury (see earlier), and the presence of glutathione limits serves to limit the harmful effects of peroxynitrite.

Numerous other interventional strategies that indirectly reduce the inflammatory oxidant stress, including NADPH inhibition, blocking adhesion molecules, depletion of Kupffer cells or neutrophils, and mitochondrial permeability transition inhibition, have been highly effective against experimentally induced IRI, but despite their efficacy in the experimental setting, clinical results have so far been disappointing [10].

Allograft Rejection

Despite the availability of a range of immunosuppressive agents with different mechanisms of action, immune-mediated damage remains a significant cause of graft and patient loss. The preferred terminology for the patterns of liver allograft rejection are as follows:

- Antibody-mediated rejection (AMR), early/acute and late/chronic
- T-cell-mediated rejection (TCMR), early/acute and late/chronic
- Plasma cell rich–rejection

This classification replaces the previous terminology of acute and chronic rejection, ductopenic rejection, and de novo autoimmune hepatitis (AIH).

Immunobiology of Rejection

Allograft rejection involves a host-versus-graft reaction whereby antibodies, complement, lymphocytes, and other immune cells mediate immune responses to allogeneic cells leading to damage of the graft (Table 36.2).

Several immune pathways can trigger allograft rejection. A hyperacute vasculitic form of rejection can be observed when transplant recipients have preformed antibodies to ABO antigens or less commonly in the presence of other donor-reactive antibodies such as those against major histocompatibility complex (MHC) class I-encoded antigens. Antibodies to donor MHC class I and MHC class II can also be associated with acute and chronic graft damage that can take the form of a vasculopathy whereby antibodies injure the graft by activating complement and mononuclear cells and recipient leucocytes expressing F_c receptors are activated by antibody-coated donor cells. It remains unclear whether antibodies are a cause or consequence of rejection; however, recent research indicates that, in addition to the pathogenic mechanisms outlined earlier, anti-HLA antibodies can contribute to alterations in endothelial cell function through complement-independent mechanisms by transducing both proinflammatory and pro-proliferative intracellular signals. This supports a mechanistic role in antibody-mediated rejection (AMR) [16].

With the exception of preformed antibodies to ABO antigens, liver allografts are relatively resilient to the development of AMR compared to other solid-organ transplants. Indeed, it has taken some time for clinicians to accept that AMR may be a significant cause for graft damage [17, 18]. More commonly, acute allograft rejection is driven by recipient T-cells that recognize donor organ alloantigens. The accumulated injury caused by donor disease (such as hepatic steatosis), the procurement process, cold ischemia, surgical trauma, and reperfusion injury initially leads to the release of proinflammatory cytokines such as IL-6, IL-1β, TNFα, and endothelial cell activation. In the peri-transplant period, the production of such factors fosters the differentiation of recipient $CD4^+$ T-cells into destructive T_H1 and T_H17 phenotypes and concurrently blocks development and suppressive function of immunosuppressive regulatory T-cells (T_{reg}). This process has been shown to involve three key immune pathways [19]:

- *The direct pathway*: Recipient T-cells recognizing intact allogeneic MHC molecules on the surface of donor APCs.
- *The indirect pathway*: Recipient APCs trafficking through the allograft phagocytose allogeneic antigens (predominately derived from MHC) shed by donor cells and present donor peptides to recipient T-cells in the context of recipient MHC molecules.
- *The semi-direct pathway*: Recipient APCs acquire intact MHC molecules following direct contact with donor

Table 36.2 Immune responses in allograft rejection

Immune response	Key pathological findings
AMR-*hyperacute*	Widespread hemorrhage
	Microvascular thrombosis
	Hepatocyte necrosis
	Variable infiltration of neutrophil polymorphs
AMR-*acute*	Portal tract edema
	Ductular reaction
	Neutrophil-rich portal infiltrate
	Portal venular endothelial inflammation (neutrophil-rich)
	Severe cases: portal and periportal hemorrhage secondary to severe endothelial injury [93]
AMR-*chronic*	Low-grade portal, periportal and perivenular lympho-plasmocytic inflammation
	Low-grade interface and perivenular necro-inflammatory activity
	Non-inflammatory portal/periportal, interface and/or perivenular fibrosis
	Portal tract inflammation (mixed inflammatory infiltrate)
Acute TCMR-*early*	Centrilobular necro-inflammatory lesions involving hepatic venules and liver parenchyma (central perivenulitis) may be observed
	Bile duct inflammation varies from mild to severe
Acute TCMR-*late*	Portal tract inflammation—mainly mononuclear cells
	Interface hepatitis and central perivenulitis are more prominent than in early ACR, whereas portal and bile duct inflammation is less severe
TCMR-ductopenic	Early features include inflammation and atrophic/dysplastic appearances of the bile ducts
	Later features may include a ductular reaction and periportal fibrosis
	Centrilobular fibrosis progressing to cirrhosis (typically veno-centric) with persistent injury

AMR antibody-mediated rejection, *ACR* acute cellular rejection, *CR* chronic rejection

APCs and/or through fusion with donor APC-derived exosomes. These "chimeric" recipient APCs or "cross-dressed" APCs stimulate recipient T-cells that recognize intact allogeneic MHC–peptide complexes.

Both $CD4^+$ and $CD8^+$ T-cells participate in cellular rejection, although the traditional view is that allograft rejection is driven predominantly by T_H1 and T_H17 immune responses. Cells of the innate immune system are also frequently found in the allograft during rejection and there is a growing body of interest in the role of NK cells in rejection and graft tolerance [20]. Eosinophils also play a role in both acute and chronic rejections. Their effect is likely mediated through IL-5 and regulated on activation, normal T-cells expressed and secreted (RANTES).

The liver demonstrates important differences when compared to other transplanted organs in its response to immune-mediated injury. Its unique structure and APC populations allow the liver to act as a site for lymphocyte activation [21]. The portal blood supply from the intestinal circulation leads to "endotoxin tolerance" [22] and under many circumstances, T-cell activation in the liver results in tolerance rather than effector responses. The mechanisms that switch the local micro-environment to promote an effector immune response are incompletely unveiled although in part this depends on the site of lymphocyte activation. It has been hypothesized that activation by dendritic cells (DCs) in draining lymph nodes leads to a vigorous immune response, whereas local activation by HSEC or hepatocytes favors tolerance [23].

Once hepatic inflammation is triggered, this leads to activation of resident immune cells as well as recruitment of leucocytes from the periphery. The initiating step during leucocyte recruitment is interaction with the vasculature and migration of leucocytes into tissue. In the liver, the key interaction is with sinusoidal endothelial cells where leucocytes are captured from flowing blood and undergo firm adhesion and transendothelial migration. This contrasts with leucocyte extravasation in many other organs that takes place in the postcapillary venules. Because of the relatively low levels of shear stress present in the sinusoids, classical selectin-mediated rolling is not necessary; rather, there is a brief tethering step. Besides being mediated by integrin interactions with the immunoglobulin family members ICAM-1 and VCAM-1, firm adhesion in the liver is mediated by nonclassical adhesion molecules that are widely expressed within the hepatic sinusoids including CD44 [24], common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) [25], and vascular adhesion protein-1 (VAP-1), which have also been demonstrated to mediate transendothelial migration [26]. VAP-1 has been shown preferentially to mediate the recruitment of T_h2 cells, whereas CD44 contributes to neutrophil recruitment. Conversely, CLEVER-1 preferentially recruits T_{reg}.

Activation of graft endothelium leads to an upregulation of classical and nonclassical adhesion molecules, and chemokine secretion follows the sequence seen in other proinflammatory settings and coincides with the infiltration of lymphocytes into the liver allograft. In inflammatory liver diseases involving lymphocyte recruitment into the parenchyma via the hepatic sinusoids, liver infiltrating effector lymphocytes express high levels of the chemokine receptor CXCR3 that is associated with increased expression of the chemokines CXCL9-11 on hepatic endothelium. In human allografts, CXCR3 ligands are also present in the hepatic sinusoids and graft infiltrating lymphocytes express high levels of CXCR3 [27]. CCL2-5 (ligands for CCR5) can also be detected on portal endothelium in liver rejection, whereas CXCL12 (ligand for CXCR4) is restricted to biliary epithelium. Variations in adhesion molecule and chemokine expression are likely to contribute to the characteristic differences detectable in various posttransplant immune-mediated liver injuries.

Clinicopathological Features

Acute Antibody-Mediated Rejection (AMR)

Acute AMR was first described in ABO-incompatible LT, when, as a result of the presence of preexisting natural antibodies directed against ABO blood-type antigens expressed by the graft endothelium, recipients developed microvasculitis, focal fibrinoid necrosis, and platelet-fibrin thrombi 2–6 hours post-LT, leading to early allograft failure. Even more infrequently, acute AMR can also be mediated by antibodies specific to donor-type HLA antigens. Less severe changes may be observed in the presence of other donor-reactive antibodies (such as anti-Kell, anti-Duffy, lymphocytotoxic antibodies) and present in a manner similar to acute rejection. Mechanistically, during acute AMR, antibodies recognize and bind to antigens located on the vascular endothelium of the graft, activating the complement cascade of the host, which results in endothelial cell damage by the membrane attack complex. Besides directly mediating damage, complement deposition also leads to neutrophil recruitment, the rapid onset of inflammation, initiation of the procoagulant cascade, platelet activation with thrombosis, vascular occlusion, and hemorrhagic necrosis of the graft.

The clinic–pathological feature of ABO-compatible and ABO-incompatible liver allografts shows some differences [28].

ABO-incompatible LT is usually done in the context of living-donor LT and recipients are treated with a variety of regimens that may include the use of rituximab, plasmapheresis, and splenectomy. Early allograft liver histology with significant levels of antibody may show sinusoidal and portal

vein platelet-fibrin thrombi as well as erythrocytes and neutrophil sinusoidal sludging, with hemorrhage into the space of Disse and portal connective tissue and hepatocyte apoptosis. In later biopsies, there is also focal fibrin deposition, portal edema, periportal hepatocellular coagulative necrosis, and erythrocyte hemorrhage. C4d staining may show C4d deposition on the surface of endothelial cells of the portal vein and capillaries.

ABO-Compatible Allografts

A similar clinical pattern may be seen in those who, despite having an ABO-compatible allograft, have a high titer of antidonor specific antibodies (DSA). The allograft histology may show platelet aggregates in portal and central veins, associated with diffuse portal C4d staining. Other features include capillary dilatation and leucocyte sludging/margination involving portal vein branches, portal and peribiliary plexus capillaries, and inlet venules, rarely extending into sinusoids and central veins. Hyperacute AMR can develop in a recipient with preformed antidonor antibodies usually against ABO blood group antigens. Severe cases, which are very rare, present as acute fulminant hepatic failure within the first few hours to days after transplantation but are rarely seen beyond 2 weeks.

Histological changes are usually identified in the explanted liver and include intrasinusoidal neutrophil and platelet aggregates and platelets lining vessels, with portal edema, ductular reaction, and a neutrophil-rich inflammatory infiltrate resembling changes seen in biliary obstruction. Portal hemorrhage occurs in more severe cases and is associated with a worse graft survival. Periportal coagulative necrosis occurs rarely and also represents an adverse prognostic feature. This can progress to widespread infarction associated with large-vessel thrombosis, variably affecting portal and hepatic veins, hepatic arteries, and the inferior vena cava. In failed allografts, it is not uncommon to find large bile duct necrosis, sclerosing cholangitis, and hepatic artery thrombosis.

Treatment Considerations

The outcomes of ABO-incompatible liver transplants are inferior to those of ABO-compatible donor–recipient pairing. Treatment has focused on the prevention of antibody- and complement-mediated damage to the vascular endothelium and include attempts to reduce the DSA titer (<1:8–16) with various combinations of preoperative high-dose intravenous steroids (methylprednisolone), depletion of donor-reactive antibodies through plasmapheresis or intravenous polyclonal immunoglobulin infusion, administration of the protease inhibitor gabexate mesilate and anti-CD20 antibodies, portal infusion of prostaglandin E1 and splenectomy [29, 30]. Emergency retransplantation remains sometimes the only viable option.

Massive hemorrhagic necrosis (MHN) is a distinct form of hyperacute liver injury and characterized by an uneventful postoperative period, only to be followed by a sudden deterioration in graft function and graft failure, hemorrhage, and hepatocyte necrosis but with only mild graft inflammation and without occlusive lesions in large arteries or veins. These distinctive features differ from other recognized patterns of graft damage and comprise a unique form of graft dysfunction [31]. Histologically these livers have associated small-vessel veno-occlusive lesion disease, ductopenia, and foam cell arteriopathy.

Chronic Antibody-Mediated Rejection (AMR)

Chronic liver allograft AMR lacks a typical clinical or biochemical presentation and specific diagnostic criteria. Many histopathological features potentially associated with chronic AMR have in fact been described in protocol liver biopsies of clinically stable liver transplant recipients with normal liver tests. Identification of specific lesions of chronic AMR is slowly emerging from studies including liver biopsies of long-term follow-up of pediatric recipients, and recipients suboptimally immunosuppressed or off immunosuppression [32]. The common histological findings of chronic AMR after LT resemble those seen in chronic AMR after kidney transplantation, that is, vascular inflammation (portal and perivenular in the liver), tissue damage (interface activity in the liver), and fibrosis with C4d deposits that are less prominent after LT compared to kidney transplantation.

According to the 2016 Comprehensive Banff Update [28], the diagnostic criteria of chronic AMR are based on histological findings with less emphasis on C4d deposition, circulating DSA, and exclusion of other causes of liver injury. Low-grade portal, periportal, and perivenular lymphoplasmacytic inflammation, with low-grade interface and perivenular necro-inflammatory activity and non-inflammatory fibrosis, are the main features of chronic AMR. However, the diagnosis may be difficult because the deposition of complement is weak and the histological criteria may overlap with those of late TCMR (see below). Indeed, in a recent study employing both histopathology and gene expression, which included surveillance liver biopsies from stable pediatric liver transplant recipients exhibiting portal inflammation, interface activity and fibrosis, the transcriptional profiles observed were indistinguishable from those typically detected at the time of conventional TCMR [33]. This is in contrast to what has been observed in the setting of chronic AMR in kidney transplantation, where the presence of circulating DSA is clearly associated with a distinct transcriptional pattern [34]. Finally, it should be emphasized that while the 2016 Comprehensive Banff Update criteria for acute AMR define

three levels of diagnosis certainty (definite, suspicious, and indeterminate), the criteria for chronic active liver allograft AMR include only two levels of diagnosis certainty (probable or possible). In this context, especially in chronic AMR, there remains an unmet need for more robust and unequivocal diagnostic criteria for AMR in LT.

Acute T-Cell-Mediated Rejection (TCMR)

The majority of cases (~65%) develop within the first year, with a median time of 8 days posttransplantation. Acute TCMR may be early (within the first 90 days following transplantation) or late (appearing >90 days).

The incidence of acute rejection has fallen from 60–75% to 10–30%. In part, this is attributable to regimens using tacrolimus rather than cyclosporine [35]. Risk factors associated with acute TCMR include the following:

- Indication for transplantation: Chronic HCV (69%) infection, primary biliary cholangitis (63%), and autoimmune hepatitis (61%) are associated with a greater frequency of severe acute rejection. In contrast, transplantation for fulminant hepatic failure secondary to acetaminophen (37%) and alcoholic liver disease (42%) has a lower incidence of acute TCMR.
- Use of anti-cytomegalovirus (CMV) prophylaxis: This is associated with a reduced risk of developing rejection (HR = 0.78).
- An increased risk associated with preoperative renal impairment (serum creatinine >2.0 mg/dL).
- Low levels of immunosuppression: Rapid corticosteroid withdrawal (<2 weeks) and subtherapeutic levels of calcineurin inhibitors are associated with late acute TCMR.
- High degree of intrapatient variability of tacrolimus levels [36].
- Specific IL10 [37] and CTLA4 [38] genetic polymorphisms are associated with a lower risk of ACR.
- Ethnicity: Acute TCMR is more likely in patients of Black race than Caucasians (1.91 vs. 0.74 episodes per year).
- Autoantibodies: Patients with anti-biliary epithelial cell (BEC) antibodies are more likely to develop an episode of acute TCMR than those who do not (65.9% vs. 42.5%).
- Preformed and de novo anti-HLA antibodies.
- Longer cold ischemia time (>15 hours).
- Use of drugs such as checkpoint inhibitors [39].
- Poor recipient performance status.

The patient with acute TCMR may report nonspecific symptoms of malaise and ill health, fever, asthenia, and abdominal pain but can often be asymptomatic in the early phase. Other signs, also of limited clinical utility, include tender (graft) hepatomegaly and pale bile.

Molecular Mechanisms of Acute TCMR

In the early sensitization stage of cellular rejection, $CD4^+$ and $CD8^+$ T-cells, via their T-cell receptors (TCR), recognize the alloantigens expressed on the cells of the foreign graft. Two signals are needed for recognition of an antigen: the first being provided by the interaction of the TCR with the antigen presented by MHC molecules and the second by costimulatory receptor/ligand interactions on the T-cell/APC surface. Of the numerous costimulatory pathways, the interaction of CD28 on the T-cell surface with its APC surface ligands, B7-1 or B7-2 (also known as CD80 or CD86), remains the most widely studied [40]. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) also binds to these ligands and provides an inhibitory signal. Other costimulatory molecules include the CD40 and its ligand CD40L (CD154).

During T-cell activation, membrane-bound inositol phospholipid (IP) is hydrolyzed into diacylglycerol (DAG) and IP3 with a resultant increase in cytoplasmic calcium. The elevation in calcium promotes the formation of calcium–calmodulin complexes that activate a number of kinases as well as protein phosphatase IIB or calcineurin. Calcineurin dephosphorylates a cytoplasmic nuclear factor of activated T-cells (NFAT), permitting its translocation to the nucleus, where it binds to the IL-2 promoter sequence and then stimulates transcription of IL-2 mRNA. Numerous other intracellular events, including protein kinase C (PKC) activation by DAG and activation of nuclear factor kappa B (NFκB), also occur.

The cellular inflammatory response in acute TCMR is initiated by alloreactive T-cells following activation by donor HLA molecules and consists of infiltration of the allograft by T-cells, eosinophils, monocytes, and NK cells in addition to professional APCs such as DCs. The response is characterized by a predominant intrahepatic T_H1/T_H17 cell immune response and a reduced frequency of intrahepatic T_{reg}. Alloreactive T_H1 effector $CD4^+$ T-cells can affect allograft damage by providing help to antibody-secreting B-cells and through a delayed hypersensitivity-like response involving the activation and recruitment of macrophages that subsequently release inflammatory mediators such as IL-1, TNF, complement components, and free radicals. T_H1 cells also aid the activation and recruitment to the graft of cytotoxic $CD8^+$ T-cells that recognize alloantigens on donor tissue and kill graft cells through the release of perforin and granzyme and through Fas/FasL interactions. Antibodies can also injure the graft in acute TCMR, although to a lesser extent than that mediated by T-cells.

Antigen presentation to T-cells is increased as the expression of adhesion molecules, class II MHC, chemokines, and cytokines is upregulated and promotes the shedding of intact, soluble MHC molecules that may activate the indirect allorecognition pathway. Various T-cells and T-cell-derived

cytokines such as IL-2 and IFNγ are upregulated early after transplantation. Subsequently chemokines such as RANTES, CXCL10, and CCL2 are expressed promoting intense macrophage infiltration of the allograft. IL-6, TNFα, inducible nitric oxide synthase (iNOS), and growth factors, including TGFβ and endothelin, cause smooth muscle proliferation and intimal thickening. Endothelial cells activated by T-cell-derived cytokines and macrophages express class II MHC, adhesion molecules and costimulatory molecules. These can present antigen and thereby recruit more T-cells, amplifying the rejection process. $CD8^+$ T-cells mediate cell-mediated cytotoxicity reactions either by delivering a "lethal hit" or, alternatively, by inducing apoptosis.

NK cells also provide help to CD28-positive host T-cells and are increasingly recognized as active participants in the acute and chronic rejections of solid tissue grafts [41, 42]. NK cells can mount a potent effector immune response without prior sensitization and are activated by the absence of MHC molecules on the surface of target cells. This recognition process is mediated by various inhibitory receptors and stimulatory receptors that are triggered by antigens on non-self cells. These effector responses include both cytokine release and direct toxicity mediated through perforin, granzymes, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL).

Pathological Findings

Early acute TCMR is characterized by a predominantly cholestatic biochemical profile (elevated ALP/γGT and bilirubin), whereas late acute TCMR is often more of a hepatitic picture. However, changes in liver biochemistry are nonspecific and cannot reliably be used to determine either the presence or severity of TCMR. Peripheral blood eosinophilia has been reported to be associated with acute cellular rejection but is affected by the concomitant use of steroids and its sensitivity and specificity remain uncertain. A fall in peripheral blood eosinophils may be an independent predictor of histological resolution of acute TCMR [43]. To date, few biomarkers have been widely assessed but IL-2 receptor expression demonstrated the highest diagnostic accuracy. No specific radiological finding has been associated with acute rejection although a reduction of liver microperfusion during early acute rejection (thermodiffusion method) may precede the onset of abnormal liver biochemistry [44]. Reduced portal blood flow velocity and an increase in splenic pulsatility index are also recognized features of early acute TCMR (accuracy 88%) [45].

The histological features of acute TCMR have been extensively defined although graft rejection may coexist with other causes of graft damage such as recurrent HCV infection, IRI, or drug toxicity. Thus, interpretation of liver histology is often complex. The histological diagnosis of early TCMR centers on the triad of portal inflammation, endothelialitis, and nonsuppurative destructive cholangitis (Snover's triad).

Demetris proposed and developed a score for acute TCMR, called "Rejection Activity Index" (RAI). The score is an aggregate of the severity of three aspects of histopathology. Each component is scored from 1 to 3. The severity is described as mild (3–6), moderate (7–9), or severe (10–12). It should be noted that the aggregation of the three aspects of rejection is not validated and the total score does not correlate well with either response to increased immunosuppression or graft outcome [46].

The liver histology in those with acute TCMR shows: portal inflammatory response consisting of a mixed cellular infiltrate consisting of eosinophils, monocytes, neutrophils, and $CD4^+$ and $CD8^+$ lymphocytes. Interface hepatitis is rarely more than mild.

- Vascular endothelialitis: Inflammation primarily affects the venules of the portal tract although it can occasionally affect the hepatic veins. Lobular inflammation (in the form of a variable central perivenulitis) is occasionally associated with hepatic vein endothelialitis, although in more severe cases, hepatic arteritis is observed.
- Biliary infiltration: This is predominantly a $CD8^+$ lymphocytic infiltrate. A ductular reaction may be present, the extent of which correlates with the severity of bile duct injury and cholestasis. Ballooning and bilirubinostasis are common features in the first few weeks posttransplant and related to preservation–reperfusion injury.

From a transcriptional standpoint, liver TCMR shows a significant overlap with the gene expression changes associated with TCMR in other transplant settings such as kidney and heart transplantation, and includes canonical transcriptional pathways such as nuclear factor-kB, STAT1/interferon-γ, tumor necrosis factor-α, chemokine receptor networks, and immune effector networks.

Late acute TCMR has some histological differences, notably the presence of central perivenulitis [47]. Furthermore, fibrosis can be present in late TCMR but is not really a feature of early acute TCMR.

- Portal inflammatory response: Predominantly a mononuclear cell infiltrate consisting of lymphocytes, monocytes, and plasma cells, with a variable degree of interface hepatitis.
- Vascular endothelialitis: In contrast to early acute TCMR, portal vein inflammation and hepatic vein inflammation are rarely more than mild in late ACR, and arterial lesions are not readily seen in the latter.
- Central perivenulitis is more frequent than in early acute TCMR and typically occurs without hepatic vein endothelialitis.
- Biliary infiltration: Bile duct inflammation is rarely more than mild in late acute TCMR. Bilirubinostasis is uncom-

mon, although mild degrees of fibrosis (periportal or centrilobular) may be present and can progress with time.

Treatment Considerations and Outcome

There are many potential causes of allograft damage, some of which may be exacerbated by treatment for rejection. Thus, the presence and severity of rejection should normally be confirmed histologically before treatment is instigated. Moreover, treating the patient with acute TCMR should be individually tailored and requires an expert multidisciplinary approach. Concerns about the effect of high-dose corticosteroids on the replication of HCV have become less significant with the ready availability of effective antiviral therapies.

In contrast to the cardiac and renal transplantation settings, the development of early acute TCMR is not necessarily harmful to the liver allograft. Indeed, only 5% of patients develop graft failure due to acute TCMR [48], and early immunological engagement may help enhance allograft tolerance [49]. Early immune events may actually be beneficial for long-term liver allograft survival and early rejection responding to treatment increases the chance of survival. It is likely that immune activation is necessary for subsequent graft infiltration of cells that eventually promote tolerance. Moreover, liver allografts with histologically more severe rejection tend to have a longer survival than those with milder forms, possibly because liver allograft acceptance may be associated with an early active immune response.

Current immunosuppressive protocols, although largely successful in preventing rejection, have the theoretical potential to inhibit tolerance. Calcineurin inhibitors and corticosteroids block anti-CD40 ligand-induced graft acceptance suggesting that such agents block early activation-associated tolerance processes, thus preventing the induction of long-term tolerance. The window for immunological engagement (WOFIE) occurs in the first 24–48 hours posttransplantation and relates to events completed by the end of the first 2 weeks [49]. Thus, by attempting to block rejection early, there is a greater potential that induction of long-term tolerance will also be abrogated. Whether delay in the introduction of such therapies, perhaps under the cover of agents that still allow early activation (e.g., mycophenolate or sirolimus/everolimus), will increase the likelihood of long-term graft acceptance without continued immunosuppression is unclear.

Various approaches have been used for the grading of hepatic allograft rejection; the Banff RAI is the most widely used.

1. *Portal inflammation:*

   Mostly lymphocytic inflammation involving, but not noticeably expanding, a minority of the triads (score 1).

   Expansion of most or all of the portal triads, by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils, and eosinophils (score 2).

   Marked expansion of most or all of the triads by a mixed infiltrate containing numerous blasts and eosinophils with inflammatory spillover into the periportal parenchyma (score 3).
2. *Bile duct inflammation:*

   A minority of the ducts are cuffed and infiltrated by inflammatory cells with mild reactive changes such as increased nuclear: cytoplasmic ratio of the epithelial cells (score 1).

   Most or all of the ducts infiltrated by inflammatory cells. More ducts show degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmic vacuolization of the epithelium (score 2).

   As above but with most or all of the ducts showing degenerative changes or focal luminal disruption (score 3).
3. *Venous endothelial inflammation:*

   Subendothelial lymphocytic infiltration involving some, but not a majority of the portal and/or hepatic venules (score 1).

   Subendothelial infiltration involving most or all of the portal and/or hepatic venules with or without confluent hepatocyte necrosis/dropout involving a minority of perivenular regions (score 2).

   As above but with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis involving a majority of perivenular regions (score 3).

Although useful as a marker of the histological severity of rejection, neither the total score nor the individual components reliably predict the response to treatment in acute TCMR [46]. In the individual transplanted for nonviral hepatitis with histologically mild rejection and minimal biochemical abnormalities, it is reasonable to increase the tacrolimus dose maintaining a trough whole blood level of 8–12 μg/L. Where liver tests are within the near-normal range, no change in treatment may be indicated. For those in whom the tacrolimus level is already within the target range, mycophenolate can be substituted for azathioprine and corticosteroids can be added/increased.

The first episode of moderate or severe rejection should be given short-term, high-dose corticosteroids, which are then tapered. The majority (75–80%) of cases respond to this approach, and recurrent and nonresponsive episodes of acute TCMR can be treated with further cycles of corticosteroid therapy. However, repeated or nonresponsive acute TCMR is associated with an increased risk of developing chronic graft dysfunction. Although current tacrolimus-based regimens have reduced the incidence of steroid-resistant rejection (SRR) by 50%, up to 35% of acute TCMR episodes may fail to respond to high-dose corticosteroids, and several possible approaches using anti-T-cell-targeted therapies have been used, leading to resolution in 60–70% of cases. Up to 60 and 77% of steroid-resistant rejection patients respond to rabbit

anti-thymocyte globulin (ATG) or OKT3, respectively. However, these treatments are associated with an increased risk of infection. Anti-IL2 receptor antibodies are well tolerated and effective (response rate 48–71%) in those with SRR and no evidence of chronic rejection. However, the median time to respond may be in excess of 3 weeks.

Single and responsive episodes of early acute TCMR do not significantly affect long-term graft survival and are not associated with an increased probability of chronic rejection. However, episodes of late acute rejection respond less well to enhanced immunosuppression (51% compared with 80% for early ACR), progress to liver fibrosis more frequently and, unlike early acute TCMR, are associated with a worse outcome and a significant risk of progression to chronic ductopenic rejection. Early acute TCMR is not related to the development of late acute TCMR [50].

Chronic T-Cell-Mediated Rejection

Chronic TCMR is usually diagnosed in the second half of the first year but may occur at any time. The incidence of chronic TCMR has fallen over the last few decades to approximately 4%, most likely as a result of more effective immunosuppression regimens and early detection and treatment. More cases now occur later (>12 months posttransplant) with a more insidious presentation and an indolent course. However, the clinical phenotype is variable, and several distinct presentations have been described:

- Following recurrent, late, or nonresponsive TCMR: Although not the end stage of TCMR, both acute-late and chronic rejection may share a temporal relationship, and late acute and chronic TCMR have several overlapping histological features. While over 25% of patients treated for late acute TCMR develop chronic TCMR, only 5–10% of patients treated for early TCMR develop chronic TCMR.
- Late chronic rejection and progressive cholestasis: The patient is asymptomatic but with biochemical evidence of cholestasis. As the serum bilirubin becomes elevated, the patient may develop symptoms of cholestasis such as pruritus and fatigue.
- Resolving chronic rejection: Although many cases progress to graft failure, some patients with histological features of chronic rejection can recover with increased immunosuppression. This is more common in tacrolimus-based regimens but is rarely seen in those patients with more than 50% portal tracts that are devoid of bile ducts.
- Decompensated liver disease: Patients present with ascites and other features of decompensation in cases with hepatic veno-occlusive lesions.

Molecular Mechanisms

The pathways leading to chronic TCMR are less well understood than for acute TCMR although it is hypothesized that chronic TCMR is mediated by a low-grade, persistent, delayed hypersensitivity response involving both humoral and cell-mediated alloimmune mechanisms. Persistent viral infection can also induce cellular immune responses that synergize with donor-specific alloreactive T-cells within the allograft.

BECs express high levels of class II HLA antigen and are a prime target of the immunological attack in chronic TCMR. A recognized feature of chronic TCMR is loss of small bile ducts as a result of a lymphocyte-mediated attack on biliary epithelium. The characteristic vascular lesions are intimal aggregates represented by homing of activated "foamy" macrophages that secrete mesenchymal growth factors (e.g., PDGF, TGFβ) that lead to smooth muscle proliferation in the intima of arterial walls. Chronic TCMR therefore reflects vascular occlusion and chronic ischemia secondary to the injury of blood vessels by antibody- or cell-mediated mechanisms.

Pathological Findings

The biochemical features are of progressive cholestasis, bilirubin rising in later stages with eventual decline in liver synthetic function. Anti-tissue antibodies (ANA and ASMA), although detected in >70% of patients, are neither specific nor sensitive for the diagnosis. The main histological features of chronic TCMR are a loss of bile ducts and an obliterative arteriopathy [51]. Specifically:

- Portal inflammation: This is of a variable severity during the early stages and may encompass features of acute TCMR, but the degree of inflammatory activity will subside as disease progresses.
- Vascular endothelialitis: The arteriopathy mainly involves a loss of small hepatic arteries (an early feature) that precedes the development of bile duct disease, whereas medium-/large-vessel arteriopathy may not always be seen on percutaneous biopsy specimens. Arterial lesions are mainly inflammatory and include lymphocytes (mainly T-cells) and lipid-laden macrophages. A variable degree of portal vein and hepatic vein inflammation is also present in the early stages, whereas hepatic venous and portal veno-occlusive disease develops later.
- Central perivenulitis is common early.
- Biliary inflammation: Bile duct inflammation is variable although bile duct atypia and senescence are recognized phenomena during the early stages and result in progressive duct loss. In contrast to acute rejection, ductular reactions are typically absent in chronic TCMR presenting within the first-year posttransplantation but may be pres-

ent in cases that develop later, particularly in those with coexisting biliary fibrosis.

- Centrilobular hepatocyte damage: Ballooning and bilirubinostasis are common findings in chronic TCMR. Centrilobular hepatocyte loss persists as inflammation subsides during later stages and progresses to centrilobular fibrosis. Subsequently, there are increasing numbers of myofibroblasts associated with varying degrees of intimal fibrosis.
- Fibrosis: Fibrosis is variable and most likely progressive. Distinct patterns of fibrosis are recognized and include the following:
  - Veno-centric: Related to obliteration of hepatic and/or portal vein branches
  - Periportal/biliary: Associated with duct loss and ductular reaction
  - Centrilobular: As a consequence of central perivenulitis
  - Bridging: Leading to cirrhosis (rare but recognized)

The Banff classification distinguishes early and late chronic TCMR based on the potential reversibility of rejection-related events [52]. Early chronic TCMR is characterized by inflammatory and degenerative changes in bile ducts. However, in contrast to acute rejection, chronic TCMR is not typically associated with a biliary ductular reaction, significant inflammation or periportal fibrous expansion. Moreover, duct loss can be heterogeneous in distribution, and the assessment of bile duct numbers should be interpreted with caution, particularly in small-sized biopsy samples. While liver biopsy evaluation is essential for diagnosing chronic TCMR, the histopathologic features comprising the Banff classification overlap with obstructive cholangiopathy as well as other non-rejection-related causes of ductopenia. In addition, the evolution and progression are variable, possibly reflecting different pathophysiological mechanisms. Moreover, even after a histological diagnosis of chronic TCMR has been made, features used to define late disease are not uniformly present in all cases. For instance, arteriopathy can occur without bile duct loss and vice versa. Similarly, bridging perivenular fibrosis may be present without significant bile duct loss or obliterative arteriopathy. Therefore an individual patient may have late features of biliary disease and early features of perivenular fibrosis or significant perivenular fibrosis and relatively well-preserved biliary architecture.

Thus, although histological findings and severity as graded by Banff provide useful information about the likelihood of reversal (those with >50% of portal tracts having well-preserved biliary architecture being more likely to have reversible disease), these findings should be combined with the clinical and biochemical phenotype before any decision to alter medical therapy or retransplantation is made.

Treatment Considerations

Therapeutic strategies may be effective in the ductopenic stage although the evidence supporting their use in chronic TCMR is small. Nevertheless, episodes may resolve if >50% of portal tracts have intact bile ducts, and in patients with early chronic TCMR and mild/moderate cholestasis (with serum bilirubin <1 mg/dL), regimens using tacrolimus are more effective than those using cyclosporine. Sirolimus/everolimus is effective in up to 50% of patients in the ductopenic stage [53] and may also prevent intimal narrowing of the arteries through its action on smooth muscle. Mycophenolate has also proven efficacious in stabilizing liver function in small numbers of patients [54]. End-stage chronic TCMR usually warrants retransplantation, although there is a high risk of recurrent chronic TCMR in the subsequent graft.

Graft Hepatitis

Unexplained inflammatory changes in late posttransplant biopsies are common with the incidence ranging from 10 to 50% in patients undergoing liver biopsy more than 1 year posttransplant [55]. The term idiopathic "graft hepatitis" has been adopted where biopsies demonstrate a chronic hepatitis without an otherwise obvious cause, characterized by a portal infiltrate of predominantly mononuclear cells often with lobular changes located mainly in the perivenular regions. Graft hepatitis is likely a variant of chronic hepatitic rejection and some of the lobular changes that are seen in this condition could also be classified as centrilobular acute rejection. This is supported by the finding that increasing immunosuppression in graft hepatitis can lead to prevention of fibrosis and that graft hepatitis is more likely to occur in recipients with late acute TCMR [56]. Another possibility is the presence of an as-yet unidentified viral trigger driving the immune response.

There is increasing interest in hepatitis E virus (HEV) as an underrecognized cause of chronic hepatitis in solid-organ transplant recipients. Studies from Europe demonstrate that despite its low prevalence, the presence of HEV infection in the immunosuppressed individual can be associated with graft hepatitis and rapid progression to advanced fibrosis or cirrhosis requiring retransplantation [57]. Recent studies show that the prevalence of HEV infection in the immunosuppressed liver allograft recipients is about 0.5% but this is likely to fall as most jurisdictions now exclude blood donors with HEV RNA detectable in blood [58]. Now, infection is most commonly acquired through contaminated food, especially pork. Treatment is initially with lowering of the burden of immunosuppression, where possible, and addition of ribavirin (unlicensed use).

Unexplained hepatitis can also be an early manifestation of recurrent autoimmune disease (see later); this may precede a definitive diagnosis by many years, and organ nonspecific autoantibodies can be found in 24–73% of patients. There is no association with blood-type compatibility, gender mismatch, or HLA donor–recipient mismatch.

The clinical significance of graft hepatitis is unclear as patients are generally well at the time of diagnosis with minimal liver biochemistry abnormalities and good graft function. However, some studies suggest that it can lead to significant tissue injury over time. Data from the pediatric literature have demonstrated that unexplained chronic hepatitis can progress to bridging fibrosis/cirrhosis in 50–70% of cases in children over a 10-year period [59]. Significant fibrosis or cirrhosis has also been demonstrated in up to 27% of adult liver recipients with graft hepatitis [60].

Treatment with corticosteroids improves the biochemical abnormalities with disappearance of interface inflammatory activity and a reduction in fibrosis despite the persistence of autoantibodies in just under half of all cases.

The Liver as a Tolerogenic Allograft

The immune system has developed a natural ability to discriminate between "self" and "nonself' antigens by deleting immature autoreactive bone-marrow-derived T-cells in the thymus by negative selection, before they enter the peripheral circulation (*central tolerance*). Although durable and efficient, central tolerance does not allow exposure to the full range of tissue-specific self-antigens encountered outside the lymphatic and circulatory systems. Thus, several peripheral mechanisms (*peripheral tolerance*) have evolved to control potentially autoreactive T-cell clones through the processes of immunological ignorance, apoptosis, anergy, and the action of T_{reg} or other regulatory cell subpopulations. The liver is constantly exposed to food antigens and bacterial degradation products via the portal vein and has consequently evolved its own, inherent tolerogenic mechanisms to prevent it being constantly inflamed by immune activation [61]. A vigorous intrahepatic immune response, in part, depends on activation of effector T-cells by fully activated DCs within secondary lymphoid tissues. Conversely, direct activation within the liver by hepatic-resident APCs results in tolerance and the generation of intrahepatic T_{reg} via the action of IL-10 and TGFβ. The reasons that local antigen presentation in the liver results in tolerance are multifactorial and discussed more fully elsewhere (see Chap. 6). Consequently, the liver allograft is relatively less susceptible to immune-mediated damage and rejection compared to many other transplanted solid organs, a property that is also in part due to its larger size and inherent ability to regenerate. This implies that the liver has a unique ability to attenuate immune-mediated rejection targeted to alloantigens. This is evidenced by the lower frequency of clinically significant rejection episodes and the need for lower global immunosuppression in liver transplant recipients, as compared to other solid-organ transplant recipients.

Following LT, induction of tolerance may occur similarly to that seen in tolerance to self-antigen whereby alloantigen-reactive effector T-cells are eradicated or disabled before the establishment of immunoregulatory networks that maintain tolerance. The inherent, natural tolerogenicity of the liver allograft can be attributed to the persistence of several donor APC populations with tolerogenic properties such as an absence of costimulation, which initially depletes alloreactive effector cytopathic T-cells through apoptosis. Once effector T-cells are depleted, tolerance is, at least in part, maintained via the action of immunosuppressive T_{reg} that restrain nondeleted alloreactive cells and alloreactive thymic emigrants, and the ultimate outcome of graft rejection or tolerance depends on the relative balance between rejection-causing effector T-cells and rejection-blocking T_{reg}. During organ transplantation, both donor and recipient T_{reg} are involved in allograft tolerance as donor T_{reg} are carried across within the liver allograft, and recipient T_{reg} develop following recognition of alloantigen presented by liver APC, as detailed earlier. The liver allograft is also an abundant source of soluble MHC class I antigens that are able to bind to alloreactive $CD8^+$ T-cells as well as DSA, and induce activation and apoptosis in the absence of appropriate costimulation [62].

Microchimerism

The ability to function as a hemopoietic organ has a profound influence on the inherent tolerogenicity of the transplanted liver. During transplantation, donor passenger stem cells (including precursor/immature DCs) migrate out of the liver allograft and seed/integrate into host lymphoid and nonlymphoid tissues. This phenomenon is known as "microchimerism" and occurs, to a certain degree, with all vascularized organ allografts. However, the potential for microchimerism is markedly increased with liver transplants compared to other solid-organ transplants due to the liver possessing a greater organ mass, the intrinsic hematopoietic capacity of the liver itself, and a larger inherent leucocyte load. Peripheral tolerance will be maintained as long as donor alloantigen is available, and the liver's ability to function as a renewable source of donor stem cells enhances its tolerogenic properties [63].

Microchimerism-induced tolerance involves both a direct and an indirect pathway: in the *direct* pathway, donor DCs migrate into host spleen or lymph nodes and engage alloreactive T-cells. Here they promote activation-induced cell death, thereby deleting alloreactive T-cell clones. In the *indi-*

rect pathway, persistence of donor antigen provides a constant source of alloantigen that can be presented by nonprofessional recipient APC in the periphery resulting in the elimination/inactivation of donor-reactive recipient T-cells. The hemopoietic potential of the liver is further supported by the ability of hepatic stromal cells to provide a wealth of growth factors and cytokines necessary for precursor stem cell development (e.g., GM-CSF, TGFβ, IL-10). Their presence assists in the production of immature precursor leucocytes, all of which contribute to the tolerogenic properties of the liver, secondary to nonprofessional presentation of alloantigen.

Potential Barriers to Developing Tolerance

Donor–Recipient HLA Matching

Given its greater tolerogenicity, tissue-typing is not routinely done in routine LT. However, while some patients may gain an advantage from high degrees of HLA matching, concern has been voiced about a possible increased likelihood of recurrence of primary disease with good HLA compatibility, as well as an increased risk of CMV infection when HLA-DR is well-matched. Although a lower number of HLA mismatches (0–2 vs. 3–6) may reduce the incidence of acute rejection, the degree of HLA mismatching has no significant effect on 1-year and 5-year graft survival or patient outcome. This is likely due to the development of tolerance to donor antigens with time through the factors highlighted earlier.

Memory T-Cell Responses

Following T-cell activation and proliferation, homeostasis of the adaptive immune system is usually restored following clearance of antigen by cell death of most effector T-cells. However, a small number of effector T-cells escape deletion and survive to become long-lasting memory T-cells that expand and acquire effector function more rapidly than naïve T-cells upon reexposure to antigen, thus ensuring protective immunity against pathogens upon reinfection. It is now clear that the normal pool of memory T-cells contains alloreactive T-cells even in patients who have received no prior exposure to alloantigen. It is likely that such alloreactive T-cells have been generated following cross-reaction with pathogen-associated antigens encountered through infection ("heterologous immunity"). Because of their capacity to rapidly generate effector immune responses following reactivation, memory T-cells are particularly efficient at mediating allograft rejection.

Compared with naive T-cells, memory T-cells are less sensitive to therapeutic T-cell-depleting antibodies, conventional immunosuppression as well as costimulatory molecule blockade and therefore represent a real issue for antirejection therapies. Furthermore, aggressive T-cell depletion therapy can amplify this phenomenon by inducing homeostatic T-cell proliferation in response to lymphopenia.

Infection

Following an infectious insult, tolerance can be reversed and result in T-cell immunity against micro-organisms such as hepatotropic viruses. NK cells are present in greater numbers in the human liver than in other organs and contribute to pathogen-induced immune responses. Given the difficulties in generating efficient adaptive immune responses within the liver, the role of the innate immune system in the induction of defensive and antimicrobial reactions is greater compared to that observed in most other organs. NK cells possess potent cytolytic activity and thus the capacity to induce tissue inflammation by producing powerful proinflammatory cytokines. In this way they can behave as effector cells mediating the process of transplant rejection. However, recent research has demonstrated a dual role for these cells and suggests that they can also play a part in the induction of transplant tolerance [64]. In this manner, NK cells can mediate the balance between survival of graft-derived donor cells and killing of donor DC subsets, thereby inhibiting the direct priming of alloreactive T-cells. Moreover, NK cells can also directly suppress the activation of effector T-cells and regulate the induction of T_{reg}. These dualistic effects of NK cells may be mediated by differences in their activation status, an avenue that possesses potential for future therapeutic intervention in the induction of transplant tolerance.

Operational Tolerance Following Transplantation [65]

The immunosuppressive drugs most frequently given to patients in the early posttransplantation period include a calcineurin inhibitor, azathioprine or mycophenolate, and corticosteroids (see Chap. 32). In recent years, the goal of immunosuppressive therapy has shifted from the prevention of acute rejection toward the preservation of long-term graft function and minimization of the side effects/complications from the use of long-term immunosuppression. Therefore, several interventional studies are currently underway, with specific aim of enabling graft acceptance through minimal dosage of conventional immunosuppressants, a concept known as *"prope" tolerance* [66]. Prope tolerance protocols have been tested in several clinical trials of kidney and liver recipients although it remains unclear whether they produce better long-term outcomes than conventional immunosup-

pressive regimens. Strategies that have shown tolerogenic effects in experimental models include the combination of costimulatory blockade reagents and T-cell depletion, as well as adoptive T_{reg} therapy. Concerns have been raised about testing such approaches in the clinic for an episode of acute steroid-resistant rejection could severely affect graft survival.

Spontaneous long-term acceptance of transplanted livers following complete discontinuation of conventional immunosuppression has been observed in a small but significant proportion of liver transplant recipients. Although not routinely attempted in day-to-day practice, rare circumstances may necessitate withdrawal of immunosuppression. This should only be attempted in expert hands in highly selected recipients and with the patient fully informed. The intrinsic tolerogenic nature of the liver allograft may allow liver allograft recipients to successfully withdraw immunosuppressive drugs completely, and such patients are termed "*operationally tolerant*." From an immunological perspective, spontaneous operational tolerance (SOT) has arisen to define a state of immune nonreactivity toward a specific set of antigens that is indefinitely maintained in the complete absence of ongoing immunosuppressive treatment/following treatment withdrawal.

Clinical Experiences of Immunosuppressive Withdrawal Following Liver Transplantation

Elective withdrawal of immunosuppression is possible in up to 20–40% of highly selected recipients. Favorable clinical markers for successful withdrawal include the following:

- An increasing time since transplant (at least 3 years of stable function for patients older than 50 years or at least 6 years for patients younger than 50 years)
- Low incidence of previous acute TCMR episodes
- Nonautoimmune primary liver disease
- Normal preweaning histology

More recent studies suggest that in stable recipients of liver transplants, operational tolerance might occur more frequently later on after LT. In one study in particular, 79% of the patients who were more than 10.6 years post-LT were weaned off their immunosuppression with success [67]. The incidence of acute TCMR during weaning of immunosuppression ranges from 12 to 76%. These episodes are usually mild and often resolve with reinstitution of baseline immunosuppression, with or without a short course of corticosteroid treatment. At the time of writing, only two cases of graft loss due to chronic rejection have been reported following medication withdrawal in patients with operational tolerance and those two cases occurred in uncontrolled studies. Longer-term data are needed to see whether the complete absence of immunosuppressive therapy increases development of subclinical rejection-related histologic lesions.

Applied Immunology and Future Prospects

At present, few clinicians consider routine withdrawal of immunosuppression a feasible option. Without better predictive tools or clinical guidance, the risks of withdrawing immunosuppression usually outweigh the benefits. The key for the future lies in determining which specific clinical, serological, immunological, and/or molecular characteristics identify those most likely to succeed without immunosuppression, so that immunosuppression withdrawal would only be considered in a suitable preselected group.

The comprehensive interrogation of the human genome has led to the development of a myriad number of strategies for monitoring transplant patients through measurements of immunological gene markers, and there is now an emerging interest in defining specific immune and genetic signatures in patients who successfully undergo complete immunosuppression withdrawal. These molecular biomarkers, which have been reviewed elsewhere [68], may serve as a predictive tool for the immunosuppressive management of the posttransplant population in the near future.

- Operationally tolerant renal allograft recipients have recently been identified as having increased total B-cell numbers and naive B-cells in the peripheral blood, suggesting that these cells may be important regulators of the antidonor immune response [69].
- Reports from the renal transplant literature have also identified increased expression of multiple B-cell differentiation genes, and a set of three genes (IGKV4-1, IGLL1, and IGKV1D-13) distinguishes tolerant from nontolerant recipients [69]. These genes encode kappa or lambda light chains that are upregulated during the transition from premature to mature (antibody-secreting) B-cells and during class switch and receptor editing that occurs after stimulation of mature B-cells with antigen. This B-cell signature is associated with upregulation of CD20 (a B-lymphocyte surface marker) mRNA in urine sediment cells and elevated numbers of peripheral blood naive and transitional B-cells in tolerant participants compared with those receiving immunosuppression. These results point to a critical role for B-cells in regulating alloimmunity and provide a candidate set of genes for screening in kidney transplant recipients.
- Similar studies in liver transplant recipients have identified, by means of the analysis of peripheral blood mononuclear cells, NK cells as well as gene signatures of the TCR belonging to a subset of gamma delta (γδ) T-cells as exerting a strong influence on tolerance. Functional analysis of these data revealed that tolerance-related expression

profiles were significantly enriched in transcripts associated with NK and γδ T-cells (CD94, NKG2D, NKG7, KLRC2, CD160, KLRB1, and KLRC1).

- Higher levels of T_{reg} as well as upregulation of the transcription factor FoxP3 also exist in peripheral blood and liver tissue from tolerant liver recipients. Furthermore, circulating T_{reg} numbers are significantly lower during rejection and negatively correlate with the rejection activity index. Pediatric liver transplant recipients on minimal or no immunosuppression (prope tolerant) have also been demonstrated to have low levels of TNFα and high IL-10 gene polymorphism profiles compared to control patients on maintenance immunosuppression [70].
- There also exists a marked difference in a set of genes involved in iron homeostasis, with the master regulator of iron metabolism, hepcidin, being overexpressed in operationally tolerant liver patients [71]. Levels of soluble HLA-G are also significantly higher in tolerant pediatric recipients compared to those with rejection or on stable immunosuppression therapy [72].
- Hepatocytes express a distinct set of miRNAs of which miR-122 is the most abundant. Levels of miR-122 as well as miR-148α are increased 9- to 20-fold during an episode of rejection, and levels of the former fall rapidly after institution of methylprednisolone treatment. Moreover, these potential biomarkers may help discriminate episodes of rejection versus other causes of graft dysfunction [73].
- Recent studies have suggested that patients with low pretransplant levels of soluble CD86 are more likely to suffer acute rejection, whereas levels of soluble Fas become increased during an episode of acute rejection. A better understanding of these molecular mechanisms could favor their potential as new therapeutic targets, as well as in the design of new drugs directed at controlling their levels in serum [74].

Novel and Ongoing Therapeutic Approaches to Promote Liver Transplant Tolerance

Several innovative cell therapy approaches, including infusion of stem cells, DCreg or Treg, to promote liver allograft tolerance have been developed. In a phase I–II study [75], ten liver transplant recipients who received 1.5–3 × 10^6/kg third-party unrelated mesenchymal stem (stromal) cells (MSC) on postoperative day 3 ± 2 were compared with ten liver transplants without MSC. There were no side effects; however, the infusion did not promote tolerance. A phase I study [76] showed that two infusions of 1.5 × 10^8 third-party multipotent adult progenitor cells into living-related liver transplant recipients on days 0 and 2 posttransplant was feasible and safe; however, no follow-up data (>1 year) have been reported. A combination of donor-derived stem cell infusion on day 7 posttransplant, with ATG induction on days 1–5 post-transplant, led to early IS drug withdrawal in living donor KT [77]. An open-label, prospective pilot trial of two intravenous doses of 10^6 donor-derived MSC/kg in pediatric living-donor transplant recipients given standard IS is currently ongoing.

A first-in-human clinical trial of donor-derived DCreg infusion 1 week before transplant in combination with standard-of-care IS to achieve early complete IS withdrawal and potentially tolerance induction in living donor liver transplant patients is ongoing at the University of Pittsburgh (NCT 03164265); no side effects of cell infusion have been observed. Several registered clinical trials of Treg cell therapy have recently been reviewed. The uncontrolled trial reported by Todo and colleagues [78] is the only one that has demonstrated to date successful long-term induction of liver transplant tolerance. In brief, ten consecutive splenectomized living-donor liver transplant recipients were treated with conventional IS, cyclophosphamide, and a single dose of a non-GMP lymphocyte product with donor-specific IS properties and enriched in Tregs. IS weaning was initiated 6 months posttransplant and successfully completed in seven out of ten recipients by month 18 posttransplant.

Several groups in the United States, Europe, and Japan are planning to conduct similar trials to confirm these encouraging results.

Recurrent Autoimmune Disease Following Transplantation (Table 36.3)

Recurrent Primary Biliary Cholangitis (PBC)

Approximately one-third of patients with PBC may not respond to therapy with ursodeoxycholic acid (UDCA), obeticholic acid, and other agents (such as bezafibrate) and develop progressive cholestasis, fibrosis, and cirrhosis for

Table 36.3 Immune responses in recurrent autoimmune disease

Disease phenotype	Key pathological findings
Recurrent PBC	Lymphocytic or granulomatous bile duct destruction
	Portal inflammatory infiltrates and ductopenia (plasmacytic portal infiltrate can be an early feature)
Recurrent PSC	*Early stages:*
	Mild acute–chronic pericholangitis
	Portal tract infiltrate (neutrophils and eosinophils)
	Later stages:
	Cholestasis
	Intralobular foam cell clusters
	Copper deposits with Mallory's hyaline in periportal hepatocytes
	Biliary fibrosis/cirrhosis with ductopenia
Recurrent AIH	Portal lymphoplasmacytic infiltration
	Lobular and interface hepatitis ± necroinflammation

PBC primary biliary cholangitis, *PSC* primary sclerosing cholangitis, *AIH* autoimmune hepatitis

which LT remains the only viable option. Outcome following transplantation for PBC is excellent with reported 5-year survival between 77 and 86% [79]. Of interest, while the pruritus rapidly resolves, the lethargy persists [80, 81]. However, recurrent PBC in the liver allograft has been reported to occur in up to 6–33% of recipients [82, 83]—the large variance in the reported literature is likely to be a representation of heterogeneous diagnostic criteria and inconsistent use of protocol biopsies between units. Diagnosis of recurrent PBC is challenging, for antimitochondrial antibodies (AMA) remain detectable following LT, even with no evidence of recurrent disease [84]. Moreover, histological features such as mononuclear inflammatory infiltrates, formation of lymphoid aggregates, epithelioid granulomata, and bile duct damage, although supportive of a diagnosis of recurrent PBC, can occur in the context of a normal posttransplant liver biochemistry and may not be of clinical significance.

Etiopathogenesis and Molecular Mechanisms

Tacrolimus usage is a risk factor for early and aggressive recurrence of PBC compared with cyclosporine [85], and some have suggested that the protective effects of the latter are secondary to its putative antiviral properties [86]. Thus, the Birmingham series showed tacrolimus as initial immunosuppression was associated with recurrence (HR = 2.3) over a median time of 5.1 years, compared to 10.2 years for patients taking cyclosporine [85]. Other reported risk factors of recurrent PBC include family history, age and gender of donor, lack of corticosteroids in the immunosuppressive regimen, and prolonged ischemic time, although reproducible data for many of these are lacking.

There is increasing evidence for a genetic predisposition for PBC, but the effect of HLA matching on disease recurrence in the allograft remains controversial. Morioka proposed that a lower number of HLA donor–recipient mismatches are an independent risk factor for disease recurrence following living-donor LT, whereas others have suggested a relationship between DR-locus mismatch and diseased donor transplantation [87–89]. A study of serial liver biopsies from a patient shortly after transplantation also showed BEC expression of robust markers of epithelial–mesenchymal transition (EMT) at the point of diagnosis of recurrent PBC [90]. However, whether BEC or their progenitors undergo EMT to become matrix-producing myofibroblasts during biliary fibrosis has been subject to significant ongoing controversy [91].

Treatment Considerations and Outcome

Recurrent PBC does not appear to influence long-term patient survival or graft loss [92, 93], at least in the medium term. In patients who develop recurrence, survival remains excellent with only few reported cases of graft loss or retransplantation. In the Birmingham cohort, the proportion of grafts lost to recurrent disease was 4% of all grafts lost in those transplanted for PBC, with a median time from recurrent disease of 7.8 years [94]. The limited available data from other centers suggest that UDCA does not seem to influence patient or graft survival although numbers are small and follow-up period is relatively short [92].

Recurrent Primary Sclerosing Cholangitis (PSC)

There is no available medical treatment for PSC which has consistently been proven to improve outcome [95]. The reported incidence of recurrent PSC following LT differs widely between studies, with quoted rates between 10 and 27% [96]. As with PSC in the native liver, the diagnosis of recurrent PSC is based on a combination of biochemical, radiological, and histological findings and the exclusion of other causes. Proposed diagnostic criteria for the diagnosis of recurrent PSC have been put forward by the Mayo Clinic [97], which consists of a confirmed diagnosis of PSC prior to liver transplant and either a cholangiogram demonstrating nonanastomotic biliary strictures of the intrahepatic and/or extrahepatic biliary tree with beading and irregularity occurring >90 days posttransplantation or a liver biopsy initially demonstrating pericholangitis ± an infiltration of neutrophils and eosinophils in the portal tract as well as small bile duct loss. Cholestasis, intralobular foam cell clusters, and deposits of copper with Mallory's hyaline in periportal hepatocytes may be detected in the later stages, largely in the context of biliary fibrosis or cirrhosis, and the associated bile duct loss may be associated with fibrous cholangitis and/or fibro-obliterative lesions [97].

Despite well-defined criteria, the diagnosis of recurrent disease remains challenging. As with diagnosis of PSC in the native liver, histology is used more commonly as a supportive tool in the diagnosis of recurrent PSC in the liver graft. This is largely due to the patchy involvement of the liver in recurrent disease and the disproportionate representation of liver pathology by biopsy. More importantly, histological features that are suggestive of recurrent PSC can also be present in other complications of LT. These include ischemia, recurrent biliary sepsis, and reperfusion injuries. In particular, chronic cellular rejection may share very similar findings, making it difficult to distinguish between the two, but it is unusual for chronic rejection to cause multiple nonanastomotic strictures as seen in recurrent PSC [98]. However, other diseases can result in cholangiopathic appearances similar to recurrent PSC; these include hepatic

artery thrombosis/stenosis (HAT/HAS), established ductopenic chronic rejection, reperfusion injury, biliary sepsis, anastomotic strictures, and ABO incompatibility between donor and recipient [99].

Etiopathogenesis and Molecular Mechanisms

Risk factors for the development of recurrent PSC have been proposed, which include donor–recipient gender mismatch, the presence of specific HLA haplotype (e.g., HLA-DRB1*08), episodes of steroid-resistant rejection, the use of OKT3 for the treatment of cellular rejection, recurrent acute TCMR, and cytomegalovirus infection. There are also data to indicate that PSC patients transplanted with living-donor allografts, especially those from genetically related donors, have a higher risk for PSC recurrence [100]. The absence of an intact colon seems to favor the absence of recurrence [101]. The meta-analysis of 14 retrospective cohort included 2159 recipients of whom 17.7% developed recurrent PSC. Colectomy before LT (HR = 0.65), cholangiocarcinoma before LT (HR = 2.42), inflammatory bowel disease (HR = 1.73), donor age (HR = 1.24 per 10 years), MELD score (HR = 1.05 per point), and acute TCMR (HR of 1.94) were associated with the risk of recurrent PSC.

The observation that transplantation in a patient with a history of colitis and an intact colon increases the risk of recurrence, while colectomy before or during transplantation significantly reduces the risk of recurrence suggests that the recipient gut continues to play an important role after LT [102]. This has been reinforced by the suggestion that recurrent PSC may occur with the onset of de novo colitis [103]. The aberrant lymphocyte homing hypothesis of PSC involves a sequence of events initiated by activation of innate immune receptors in the liver and bile ducts by gut-derived PAMPs in the portal venous blood. This leads to the expression of gut-specific chemokines and adhesion molecules such as CCL25 and mucosa-associated cellular adhesion molecule (MAdCAM)-1 on hepatic sinusoids, and is accompanied by transendothelial migration of gut-primed memory T-cells into the liver that express the chemokine receptor CCR9 and the integrin α4β7 [104, 105]. These events stimulate production of pro-inflammatory and profibrotic cytokines by the chemoattracted T-cells and activated macrophages and result in concentric peribiliary fibrosis and progressive displacement of the arterial peribiliary capillary plexus away from the bile ducts. Such a sequential pathogenesis would be favored by the presence of an intact colon in the transplant recipient. However, this would also require transplantation of an allograft from a donor whose cholangiocytes are susceptible to gut-derived stimuli. Antibodies against beta-tubulin isotype 5 detected in patients with PSC cross-react with its evolutionary bacterial precursor protein FtsZ, a component found in virtually every commensal organism comprising enteric flora, further supporting a link to the mucosal immune system and PSC [106].

Treatment Considerations and Outcome

There is no established medical therapy for recurrent PSC posttransplant and disease progression or development of complications may require retransplantation. Unlike PBC, there exist many reports that indicate an increased risk of graft loss in recurrent PSC, with a median survival before retransplantation of approximately 39 months (CI: 28–51) [107]. Furthermore, PSC patients have a higher rate of retransplantation for graft loss and a lower survival rate compared with patients transplanted for PBC [108].

Recurrent Autoimmune Hepatitis (AIH)

The majority of patients with AIH respond well to immunosuppression, usually a combination of corticosteroids and azathioprine. The reported 10-year patient survival posttransplant is approximately 75% [109, 110]. However, features of AIH can recur in spite of posttransplant immunosuppression [111]. Recurrence rates vary between 12 and 50% over 8–10 years posttransplant (median time ~2 years), depending on diagnostic criteria [96, 112]. Diagnosing recurrent AIH is challenging, and abnormal serum transaminases can be the consequence of many different insults directed toward the liver graft. Histological evidence of disease recurrence includes mononuclear inflammatory infiltrates, interface hepatitis (piecemeal necrosis), and abundant plasma cells. However, such findings can also be detected in the absence of abnormal liver biochemistry or in cases of recurrent viral hepatitis and cellular rejection [113]. Conversely, high-titer autoantibodies and hypergammaglobulinemia may remain detectable following LT, irrespective of recurrence of disease.

Etiopathogenesis and Molecular Mechanisms

Native AIH is putatively mediated by HLA-restricted, autoantigen-reactive $CD4^+$ and $CD8^+$ T-cells [114], and data from studies looking at HLA matching in living-related donor transplantations have shown an increased risk of recurrent AIH when the recipient shares common HLA typing with family donors. Recipient HLA DR3-positive genotype and donor HLA DR3-negative genotype have also been identified to correlate with an increased risk of recurrent AIH although it is somewhat paradoxical that AIH recurs across donor–recipient HLA mismatches. This suggests that recurrent disease is mediated by recipient memory T-cells against conserved autoantigenic peptides expressed by mismatched donor HLA molecules in the allograft [112]. There is also evidence that recurrent AIH is dependent upon the severity of disease prior to transplantation and that chronic ductope-

nic and acute cellular rejections are both more commonly seen in patients with recurrent disease [115]. Recurrent AIH is significantly increased in recipients with high pretransplant levels of immunoglobulin G and severe hepatic inflammation in the native liver, indicating a role for intense proinflammatory mechanisms. However, it is unusual that AIH recurs in the presence of immunosuppression sufficient to prevent rejection, although one plausible hypothesis would be that immunosuppression inhibits autoantigen-specific T_{reg} as well as effector cell populations [116].

Treatment Considerations and Outcome

Although the frequency of recurrent AIH increases with time (12% at 1 year; 36% after 5 years), disease progression and cirrhosis are uncommon with adequate immunosuppression [109]. Significant risk factors for recurrence have not been fully elucidated although patients transplanted for AIH frequently require continued steroids compared to those for other etiologies (64% vs. 17%). As recurrence of AIH is an important risk factor for graft loss [94], the practice in many centers is to keep all patients transplanted for AIH on low-dose corticosteroids, in addition to a calcineurin inhibitor and either azathioprine or mycophenolate.

Plasma Cell Hepatitis: De Novo Autoimmune Hepatitis

Features of AIH developing in the donor liver have been reported in patients transplanted for non-immune indications [117, 118], but it remains uncertain whether this represents an autoimmune response or whether the syndrome represents a variant of rejection. The former hypothesis stems from the association of de novo AIH with suboptimal immunosuppression, prior history of acute TCMR, the use of immune modulating drugs (such as pegylated interferon) and good response to increasing doses of immunosuppression [119]. Other risk factors reported include HLA-DR3 positive recipient, HLA-DR3-negative donor, HLA-DR3/HLA-DR4-positive recipient, episodes of acute rejection, retransplantation for recurrent AIH, concomitant autoimmune disease, pretransplant high levels of transaminases or IgG, and findings in the explant of moderate to severe inflammatory activity and a plasma cell infiltrate [120].

The condition is relatively uncommon and some have suggested significant differences between adults and children [121]. One recent multicenter study [122] of 1833 children found de novo hepatitis in 31 (1.7%). Fifty-two percent had no rejection preceding the diagnosis. Liver tests improved following treatment with corticosteroids and antimetabolites was not universally sustained. Portal hypertension was seen in four patients and associated with severe fibrosis and cirrhosis. Regraft was done in two patients for chronic rejection and uncontrolled portal hypertension with gastrointestinal bleeding, respectively. There were no deaths.

To support further the notion of rejection over AIH, the atypical antibody glutathione S-transferase T1 (GSTT1) can be detected in a subgroup of patients diagnosed with de novo AIH. This has been shown to be the result of a GSTT1 genotype mismatch between donor and recipient. Hence, one hypothesis is that the immune-related damages seen in GSTT1-positive patients are directed against the graft and not the host, suggesting an alloimmune over autoimmune response [123–125]. Others have reported anti-CYP antibodies in association with disease [126]. This form of graft hepatitis may also be seen in association with treatment of HCV [127, 128].

There is usually a good response to additional immunosuppression with corticosteroids, but in some cases there is progression to cirrhosis and subsequent graft failure.

Conclusion

Immune responses within the transplanted liver can take the form of hyperacute, acute, or chronic hepatobiliary injury. The most common form of injury is in the form of acute TCMR although in contrast to other solid-organ allografts this does not influence graft or patient survival in the absence of steroid-resistant rejection and may actually be beneficial to the recipient. In contrast, episodes of chronic rejection, although encountered infrequently, do adversely affect long-term graft function and remain a cause for concern. Management in this setting should focus on the recognition of features of reversibility, the so-called early chronic TCMR, as progressive chronic rejection is a significant risk factor for graft loss. Nevertheless, the transplanted liver remains relatively resilient to allograft dysfunction, in part due to its inherent tolerogenic properties. This characteristic has focused on the field of research into identifying immunological and genetic signatures associated with operational tolerance in an effort to minimize the long-term complications associated with prolonged immunosuppressive therapy.

In contrast to acute rejection, an understanding of the molecular methods giving rise to recurrent autoimmune disease in the transplanted liver remains embryonic. Recurrent autoimmune disease does not have a uniformly benign prognosis, and in particular recurrent PSC remains a significant cause of graft dysfunction and graft loss. Implications for understanding the mechanisms of autoimmune liver disease may be enhanced by identification of mechanisms associated with a loss of self-tolerance, although whether this can be applied to recurrent disease after liver transplantation (in the face of adequate immunosuppression) remains unclear as loss of tolerance is also an important aspect of alloimmune responses in the liver allograft.

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