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Liver Immunology

Principles and Practice

Second Edition

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The editors and authors of this book dedicate the text and its contents to Linie Moore, symbolic of her courage, dedication, imagination, and enthusiasm. Linie is one of a kind and is a true star in the struggle to find a cure for autoimmune disease.

"Gutta cavat lapidem"

Foreword

Meeting the Challenge of Patients with Immune-Mediated Liver Disease

Medicine, health, and health care are in a period of rapid evolution. As expenses continue to soar, societal pressure will push us to provide better health for less money to more people. These lofty aims are achievable, although not likely with our present delivery system.

New systems will require even more data-driven decisions and a more tailored, personalized approach to individuals. This personalized approach will in part depend on understanding data available in the individual genetic code which can help direct risk assessment as well as help us make better diagnostic and treatment decisions.

Our new, data-driven approach will be based on a better understanding of underlying mechanisms, with an increased focus on more directed or targeted therapy. This new edition of this textbook will help set the stage for those exciting changes.

Advances in liver disease include the emergence of imaging modalities to assess liver fibrosis (magnetic resonance elastography and ultrasound elastography) improved imaging of liver masses and noninvasive methods to assess the biliary tree and vascular structures. Immunohistochemical staining allows differentiation of tumors and infections as well as characteristics of some immune-mediated diseases.

The understanding of the genetic code has led to genome-wide association studies which hold the promise of new insights into potential pathogenetic pathways that can be then explored with more directed, functional studies. These associations are already being used to target therapies designed to ameliorate the inflammatory response. In cancer therapy, whole genome sequencing of tumors has now allowed specifically targeted therapy which may yield greater efficacy with fewer adverse side effects. Genetic polymorphisms have also been used to predict treatment responses and predict development of steatosis, among other uses.

Therapy for liver disease has emerged quickly in the past decade. Hepatitis B is suppressed long term with excellent clinical results while the vast majority of patients with Hepatitis C may soon be curable. Primary biliary cirrhosis is now treatable with ursodeoxycholic acid while primary sclerosing cholangitis is still lacking the effective therapy.

Our understanding of the basic and clinical aspects of immune-mediated liver disease is rapidly progressing and an excellent update, such as provided in the second edition of this classic textbook, is timely. There has been increased attention on immune diseases of the liver because of a rising increase in prevalence in some cases, in others because of new discoveries that may give us clues to pathogenesis, while in other instances improved management has been established. Several diseases among this collection of illnesses remain without effective therapy, spurring further research to find sorely needed treatment options. Furthermore, several of these diseases are accompanied by increased risk of malignancy, adding more to the urgency to better understand and treat these conditions.

This second edition begins with important information about the epidemiology and mortality of liver disease worldwide. These are followed by chapters related to basic immunology, application of liver immunology for diagnosis, and several excellent chapters that provide a

solid foundation for understanding immune-mediated liver disease including those associated with the biliary tree. A chapter on non-hepatic manifestations of immune-mediated liver disease helps provide context for how these diseases affect the patient overall.

There are chapters that discuss various discrete immunologically mediated infectious liver disorders including those related to bacteria, parasites, and all of the classic viruses. Chapters on the traditional autoimmune liver diseases; primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis as well as overlap syndrome are included. The breadth of this second edition is highlighted by chapters on alcoholic liver disease, nonalcoholic fatty liver disease, and drug-induced liver disease among others, which have potentially immunologic features, yet are not usually included among the immune-mediated liver diseases. More classic immune-mediated liver disease occurring in the setting of transplantation, whether graft vs. host disease or liver transplantation, are also included.

The edition ends with a forward-looking view of future directions and how we might meet the challenge of refractory patients, written by the editors jointly.

The editors of this second edition have consolidated an outstanding group of authors who are responsible for the various chapters. The book will serve as a comprehensive textbook for many liver diseases, especially those that have an immune-related pathogenesis. The text does not cover malignant, vascular, congenital, or cystic diseases. It leverages the focus on immune-mediated diseases to provide an in-depth and comprehensive overview of this important aspect of liver disease.

This book will serve as an excellent overview for this rapidly evolving field and should add to our understanding of the pathogenesis of these diseases, as well as provide insights that can be harnessed into helping improve the care of patients afflicted with these various immune-mediated diseases. This book will be valued by those learning about this field in training as well as by established experts in the field. The editors are to be congratulated for this important contribution.

Rochester, MN, USA Keith D. Lindor, MD

Preface

Recognition of the importance of the liver to health by Babylonians in the nineteenth century BCE stands in stark contrast to the relative obscurity of the liver in the minds of most educated adults today. Medical appreciation of the vital nature of the liver's diverse functions continues to evolve along with our efforts to better understand a multitude of hepatobiliary diseases caused by alcohol, xenobiotics, viruses, autoimmunity, and genetic diseases. The unanticipated success of liver transplantation in the absence of histocompatibility matching between donor and recipient showed that the hepatic environment is immunosuppressive. Further studies proved that liver transplantation also protected other transplanted organs from being rejected, indicating that the liver is truly an immunologic organ. Recent data provide new insights into the physiological roles of hepatocytes, sinusoidal lining cells, activated macrophages (Kupffer cells), cholangiocytes and stellate cells, and their modulation of T cells, natural killer (NK) cells, and NKT cells. Concurrently, studies of the pathogenetic mechanisms involved in hepatobiliary diseases have provided unequivocal evidence that the pathogenesis of virtually all hepatobiliary diseases involves inflammation involving the innate and/or adaptive immune responses. Progress in our understanding of the liver as an immune organ and immunopathogenesis of diverse hepatobiliary diseases provides hope that this knowledge will rapidly be translated into more effective therapies in the near future. These factors were the impetus for the third edition of Liver Immunology: Principles and Practice, which is directed to clinicians, investigators, and students. The editors are indebted to all of the authors who have donated their talents, intellects, and expertise to provide "state-of-the-art" contributions. All of us hope that this book will provide new perspectives of hepatobiliary physiology and pathophysiology and stimulate creative approaches to accelerate the pace of research progress in the field. Time has validated our belief that continued studies of immunology of the liver will ultimately improve the care and the prognosis of patients afflicted with a diverse array of hepatobiliary diseases. The editors have many people to thank, not the least of which are the contributors, all of whom worked very hard to have their manuscripts delivered on time and in the style we requested. However, we especially want to thank Nikki Phipps and Kathy Wisdom, our assistants at UC Davis, who worked so hard to make this book a reality.

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Introduction

1

Ian R. Mackay

The Arousal of Immunology

The invitation to prepare a foreword to "Liver Immunology Second Edition" is indeed an honor, given the successes of the first two editions of the book. The young and exuberant science of immunology, having just passed its first centennial and undergone detachment from microbiology in the 1950s, has enjoyed accelerating progress, at the laboratory and medical levels. Selection of the most influential advances in immunology in the "modern" era (post-1940s) is of course subjective, but would span topics as diverse as self-recognition to the immunological role of the intestinal microbiota. My list of the more significant advances is shown in Table 1.1.

The title of this text, *Liver Immunology*, subsumes notions of a "lymphoid liver" [1] and the liver as a "unique immunological organ" (Chap. 4) and thus a legitimate constituent among tissues intrinsic to the immune system. The liver sturdily fulfils essential immune defensive needs as a "gatekeeper" located strategically between the intestinal/portal and systemic blood circulations. Therefore, it can intercept influxes via the portal vein of microbial escapees or incompletely digested dietary constituents. This role depends on the liver being well equipped within its fenestrated vascular sinusoids, with all cellular elements of innate immunity, macrophages, myeloid dendritic cells, Kuppfer cells and mucosal-associated invariant T (MAIT) cells [2], with all these complemented by barrier functions of liver sinusoidal epithelial cells. Notwithstanding the tolerogenic capabilities of these sinusoidal cellular elements, our "lymphoid liver" can, and does, succumb to diseases due to dysfunction of its protective immune armory, whether as a result of ineffectual responses to hepatotropic viruses, or loss of self-tolerance with troublesome autoimmunity affecting hepatocytes or terminal cholangioles, or adverse reactivities to drugs disposed of by the intra-hepatic cytochrome P450 (CYP450) family of oxidative enzymes. Each of these dysfunctions can induce ongoing destructive inflammation—and it doesn't end there! Thus, various "degenerative" liver diseases exist, some common and others rare, in which endogenous products of hepatocellular injury resulting from ethanol abuse, metabolic steatosis, genetic deficiencies of the serpin (serum protease inhibitor) *alpha*-1 anti-trypsin provoke a cytokine-dependent auto-inflammatory response by defensive cells of the innate immune system. These issues are so expertly covered in the Chapters herein to follow that this Foreword could well conclude simply by commending the Editors on their judicious selection of contributing authors. However, I will use the Foreword as a rationale for the notion of "liver immunology" and explore some of the refractory questions that continue to challenge us.

Virus-Induced Chronic Inflammatory Hepatitis

In times long past, the only recognized type of persisting hepatitis was that known as "chronic active hepatitis" for which an autoimmune basis was eventually proposed. The very different situation today is that chronic viral hepatitis has become the overwhelmingly prevalent type, attributable to changed social customs and lifestyles, and readily available sensitive laboratory tests for the now identified causative viruses. Although many viruses have hepatotropic potential, it is only hepatitis viruses B and C (HBV, HCV) that do establish a chronic infection due to a non-eliminative host immune response to persisting intracellular virus provoking inflammation, recurring liver cell necrosis, regeneration, and fibrosis, culminating in cirrhosis and, sometimes, hepatocellular carcinoma. HBV and HCV infection of the liver in some respects are similar, but differ substantially including their capacity to establish a persistent infection.

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Table 1.1 Some of the major discoveries in immunology 1940–2000

Timeline	Discovery
1945	Demonstration of specific cell (lymphocyte)-based immune reactivity in contrast to serum-based reactivity
1948	Immune tolerance as basis of self-non-self discrimination
1957	Clonal selection theory of acquired immunity
1960s	Failure of tolerance and ensuing autoimmunity as a cause of many diseases
1961–1963	Thymus as the site for progenitors of lymphocytes and tolerogenesis
1960s	Increasing interest in genetic determinants of immunological expressions—in mice (beginning 1940s) and in humans (beginning 1950s)
1969	The T and B lymphocyte paradigm
1970s	"Molecularization" of immunology—massively influential—e.g., multiple gene recombinations explained diversity of B and T cell antigen receptors
1970s	New T-cell subset (suppressor/regulatory T cells) down-regulates immune reactivities and explains peripheral immune tolerance—homeostasis
1980s	Identification in tissue extracts of "factors" antecedent to characterized cytokines, receptors, and intracellular signaling in immune responses
1990s	Essential inter-dependence of innate and adaptive immune systems broader visions of "innate" immunity
1990s	Recognition of importance of apoptosis and mechanisms as a component of immunological function
2000s	Role of intestinal commensal microbiota in shaping physiological and pathological immune responses

Chronic hepatitis B Carrier rates of HBV globally differ depending on socio-cultural lifestyles, routes of viral transmission, and racial-genetic background. In high prevalence regions transmission can be by vertical infection mother to fetus, or by close perinatal contact, whereas in low prevalence regions transmission is mostly parenteral, often in the setting of intravenous drug use. The vigor of adaptive immunity in healthy individuals ensures clearance of infection in some 95 % of instances, while failure to clear infection depends on immunodeficiencies associated with developmental immaturity, nutritional deficit, or coexisting chronic illness, e.g., renal failure. However, we need far more knowledge on the nature of the permissive immune deficiency states that underlie susceptibility such as general debility, malnutrition associated with poverty, alcohol or drug abuse, or chronic illness. A decreased T cell responsiveness to HBV appears important, perhaps by limiting the capacity for engagement of multiple antigenic epitopes presented by the virus. However, even so, among chronically infected individuals, there is a 2 % per annum viral clearance rate associated with demonstrable HBV-specific T cells and anti-HBs in blood [3].

Interestingly, with failure of viral clearance, a default option for the host is tolerogenesis resulting in a "healthy carrier" state. Immunogenetic factors have some influence on the occurrence or outcome of infection with HBV, and also the response to the normally highly effective HBV vaccine, noting that different HLA alleles appear to provide protection or susceptibility among different populations [4], and there are small effects of polymorphisms of the promoter for cytokine genes, IL-10 and TNF-alpha [5].

Since direct correlation has been drawn between viral load and propensity to progress to cirrhosis [6], therapeutic reduction of viral load is beneficial. However, the relative participation of CD4 and CD8 T cells in hepatocyte injury requires more study, while B cells enter the picture due to ongoing antigenic stimulation by viral antigens, leading to immune complex disease and/or essential mixed cryoglobulinemia [7].

Chronic hepatitis C HCV is less complex genetically and structurally than HBV, but equally illustrates the complexity of immune interactions between a "survival-adapted" virus and its human host [8]. In healthy individuals acute infection with HCV versus HBV is less readily cleared (~30 % versus ~95 %); and although debility-related immune deficiency predisposes, it is not necessary for chronic HCV infection. Nor is there a tolerance option as with HBV infection, since most if not all HCV carriers have some degree of hepatic inflammation. HCV hepatitis seems to be facilitated by various comorbidities, and particularly by effects of alcoholic or nonalcoholic steatosis, noting the propensity of HCV itself to induce fat deposition in liver cells [9]. Innate immunity provides a first line of defense against HCV infection, since Toll-like receptors (TLR) on phagocytic cells recognize pathogen (virus)-associated molecular patterns (PAMP). The RNA of HCV engages TLR3, thereby activating signaling pathways for expression of pro-inflammatory and anti-viral cytokines, particularly interferons (IFNs), and primes the host for an adaptive immune response [8]. While ensuing IFN expression results in some reduction in levels of HCV in liver cells, full clearance requires additionally a rapid and effective adaptive immune response involving engagement by T cells and likely B cells of multiple antigenic epitopes of the virus polyprotein [9], particularly by the NS5A protein of the virus [10]. There has been good progress in defining epitopes on structural and nonstructural proteins of the HCV particle, their relative capability of being presented by different HLA molecules, and activation of protective CD4 and CD8 T cell immunity. Comparable to HBV infection, the outcome of an acute infection depends on the quality and number of HCV epitopes initially engaged and efficient development of effector/memory T cells [11].

The many explanations for the escape of HCV from the host immune attack include ongoing development of

immunologically variant quasispecies that outrun the available T cell specificities of the host, suppression of T cell activities by HCV proteins, tardiness of primed T cells to move rapidly to the newly infected liver, defective engagement of critical HCV epitopes such as NS5A that favors viral persistence by exerting anti-apoptosis effects on infected hepatocytes [10], depletion of CD8 T-cell responsiveness during evolution of infection [11], and debility-related immune impairment of T cell and NK cell performance, with limited IFN-gamma responses. Finally the first encounter between naïve T cells and HCV occurs in the tolerogenic milieu of the liver rather than in the immunogenic milieu of a regional lymph node [12]. Among genetic influences, HLA class I and class II alleles influence clearance, well illustrated for the highly protective class I allele HLA B27 that engages an epitope within the NS5B protein, although structural polymorphisms of HCV evolve to circumvent this [13].

Initially in the chronic liver-damaging phase of HCV infection CD4 helper and CD8 cytolytic T cells (CTLs) are operative with good control of viremia albeit associated with greater evidence of histologic liver damage [14], whereas later T cell activity wanes but, even then, CTL activity is still demonstrable among T cells in liver, although not in blood. B cells have received relatively less attention in the host interaction with HCV, although antibody to HCV is clearly demonstrable and is directed to multiple components of the HCV polyprotein. Anti-HCV antibody does have neutralizing capacity, at least in infected chimpanzees, and likely serves to limit cell-to-cell transfer of virus in the liver.

The B-cell response becomes relevant in the later pathology of HCV infection in dictating many of the extrahepatic features [15, 16], particularly type 2 mixed cryoglobulinemia prevalent in endemic regions of infection [17]. These cryoglobulins contain HCV, anti-HCV, and oligoclonal IgM rheumatoid factor and are pro-inflammatory, causing arthralgia, vasculitis, cutaneous purpura, and membranoproliferative glomerulonephritis. Production is antigen (HCV)-driven since therapeutic reduction of viral load is ameliorative [17]. Another B cell feature of chronic HCV infection is production of various nonorgan-specific autoantibodies (NOSA) at relatively low levels [18], as described below. Later in the course, B cells may undergo lymphoproliferative expansion towards B-cell lymphoma resulting from chronic antigen drive with lymphomagenic chromosomal translocations such as the apoptosis inhibitory gene BCl-2 from chromosome16 to the IgH locus on chromosome 14 [t(14;18)(q32;q21.3)]; however, the one study on human HCV-infected liver tissue did not confirm this [19].

Conventional treatment of chronic hepatitis C was initially with anti-viral type 1 IFN but in recent years "big pharma" has been developing anti-viral drugs of ever-increasing efficacy that, used in combinations, clearly contain or even eliminate HCV and thereby "cure" the disease. Finally those seeking clues to the causes in general of autoimmune disease find it intriguing that conventional therapy of HCV infection with type 1 IFN provokes autoimmune reactions *de novo* affecting the thyroid gland [20] andother tissues.

Autoimmune Chronic Inflammatory Liver Disease

Autoimmune hepatitis (*AIH*) Knowledge on AIH has accumulated to such an extent over the past 60 years that readers could be readily forgiven for believing that all that needs to be known is already known. Yet even after Liver Immunology 1e was published in 2003, more knowledge on AIH has accrued. Fortunately, hepatologists are beneficiaries of the wisdom of thought-leaders, the International Autoimmune Hepatitis Group (IAIHG) that evolved from a conference on AIH in 1993 and has convened regularly thereafter. The IAIHG works to rationalize and standardize nomenclature, develop criteria to assist clinical diagnosis and epidemiologic studies, adjudicate on therapies, and promote research into AIH in general [21, 22]. A recent simplification in 2008 limited the cumbersome initial diagnostic criteria to just these items: negative indices of hepatitis virus infection, hypergammaglobulinemia, compatible histologic features (interface lymphocytic hepatitis with prominent plasmacytosis), and autoantibodies at requisite levels to prescribed autoantigens [23]. These simplified criteria do perform well but validation is needed.

However, notwithstanding all the advances, several problematic aspects to AIH remain unsolved, as follows.

Hyper-immunoglobulinemia (*hyper-IgG*). Recognized from the1950s, the earliest days AIH [24], extreme polyclonal hyper-IgG associates with the activity of the disease. It even provides a useful marker of response to treatment and aligns well with the plasmacytosis in the liver (and bone marrow). The usual but not entirely convincing explanation is that this hyper-IgG is simply a polyclonal immune response to degraded liver cells. Forthcoming genetic studies may provide some answers.

AIH-associated autoantibodies. The traditional diagnostic autoantibodies were discovered in the 1960s using indirect immunofluorescence (IIF) on frozen tissues: (a) nuclear chromatin (antinuclear antibody, ANA), (b) smooth muscle in rodent gastric mucosa (SMA) with a later recognition that filamentous (F) actin was the likely reactive moiety specific for (AIH), and (c) microsomes (cytoplasm-derived elements) of liver and kidney tubular cells (LKM, later called LKM1). Subsequently a mutual exclusivity in reactivity of sera for ANA/SMA or anti-LKM led to the specification of two types, 1 and 2, of AIH (see below). These antibodies underpin

laboratory diagnosis, but, at the same time, exemplify a complex unsolved puzzle—why the association of a given autoimmune disease such as AIH with an autoantibody directed to a molecule that has no discernible correlation with the cellular pathology? Various other examples would include the diagnostic autoantibodies detected in Sjogrens disease, polymyositis, and systemic sclerosis.

Also recognized in AIH are other autoantigens of practical and/or theoretical interest [25]. These include a cytoplasmic constituent named "liver-pancreas-soluble liver antigen" (LP/SLA), identified as UGA-serine transfer (t)-RNA protein complex, to which antibody can be diagnostic in otherwise pan-seronegative cases and/or point to severe progressive disease. Another antigen of interest is the reactant for what was first called "granulocyte-specific ANA" [26], and later atypical anti-neutrophil cytoplasmic antibody (ANCA). This antibody is detectable at high prevalence in AIH but only in type 1 and not type 2. Thought has reverted to the reactant being an unidentified neutrophil nuclear rather than a cytoplasmic constituent. A further reactant of interest seen in type 2 AIH elicits an autoantibody that is a fellow-traveler with anti-LKM; this liver cytosol antigen (LC-1) has been molecularly identified as formiminotransferase cyclodeaminase. Since, occasionally, anti-LKM$^+$ ve sera react with CYP450 isoforms other than the prototypic 2D6 (often in drug-induced forms of hepatitis) and are directed against the P450 isoform, e.g., 2C9 or 3A1, that hydroxylates the drug, is there an as yet undetected molecule that, in the course of its disposal by CYP450 2D6, initiates the apparently spontaneous anti-LKM$^+$ ve AIH?

Specificity of anti-F actin for AIH. The designation "SMA" for one of the major serological reactants in AIH is so embedded that any change is unlikely, but my perception (and perhaps not all would agree) is that the true AIH-relevant reactant is filamentous (F) actin, whether detected by IIF testing by reactivity with actin microfilaments in renal glomeruli and tubules [25], or by ELISA with purified F actin, and that type 1 AIH is the single disease in which anti-F actin is regularly demonstrable. Moreover, positivity for anti-F actin helps to separate AIH from viral and other miscellaneous causes of low-level SMA reactions with other cellular filaments, and also from SLE with which AIH is occasionally aligned. F actin is relatively neglected as an autoantigenic molecule, since it has attracted little interest in its immunoreactivity or relationships between its epitope sites and the functional binding sites for some 70 cytoplasmic proteins among which is its cell motility partner, myosin.

Two serological types of AIH. The concept of two "serotypes" of AIH, 1 and 2, evolved from observations in 1987 on the mutual exclusivity in AIH of sero-positivity of ANA/SMA (type 1) and anti-LKM (type 2) [27]. The distinction is matched by differing HLA susceptibility alleles. Interestingly type 1 aligns more with multisystem nonorgan-specific diseases, whereas Type 2 more with organ-specific diseases. No convincing liver-specific autoantigen can be distinguished in type 1 AIH (despite much effort to identify a liver membrane—specific antigen), whereas the LKM1 reactant has been molecularly identified as the 2D6 isoform enzyme of CYP450 family allowing for development of useful mouse models which are lacking for the more prevalent type 1 AIH. Two different modes of immunopathogenesis for the one clinical disease in the one single organ are indeed very curious [28].

NOSA in chronic HCV infection. The possibility that the disease-defining AIH-associated autoantibodies could result entirely from a B-cell response to destruction and spillage of liver cell constituents seems untenable given the existence of the two disease serotypes, each with a distinct antibody profile. Yet issue injury in itself, e.g., ischemic infarction, is known to evoke a low-level immune response, expressed histologically by lymphocytic infiltration. This, then, likely explains the low levels of NOSA in chronic hepatitis C (CHC) as described in European studies such as that of Stroffolini et al. [19]; the prevalence in CHC of any NOSA reached approximately 36.9 %, and for ANA ~16 % and SMA ~27 %, although anti-LKM1 reached only ~2 %, similar to that (~5 %) among healthy hepatitis C virus carriers in similar locations. Disease if any in these HCV carriers is not typical of AIH. Also, and, in contrast to spontaneous type 2 AIH, the autoantibodies may react with CYP450 isoforms other than 2D6, or with epitopes on CYP450 2D6 other than those engaged by type 2 AIH sera. There being no association between any of these NOSA and hepatitis disease expressions, these pathogenetically irrelevant autoantibodies should sound a warning note to clinicians on over-interpretation of results of serological laboratory assays.

T cells in liver cell injury of AIH. In type 1 AIH T cells are prominent in the lymphocytic infiltrates in the liver and are presumed by some authors to determine liver cell damage. However, in type 1 AIH, relevant autoantigen preparations are not available, and hence assay systems for cytotoxic or cytokine-releasing T cells are inapplicable, as pertains for various of the multisystem autoimmune diseases. On the other hand, in sero-type 2 AIH, T cells in blood do respond to immunoreactive peptides derived from the characterized autoimmune reactant CYP450 2D6.

T reg cells and immunological homeostasis in AIH. In his classic monograph on clonal selection theory in 1959, FM Burnet developed the idea of forbidden (self-reactve) clones of lymphocytes and envisaged that healthy individuals must possess *homeostatic mechanisms* to render these ineffective. Now, over 50 years later, immunological homeostasis has

regained currency through the agency of regulatory T cells (Tregs) that in some way serve to nullify anti-self-reactivities in the periphery. The corollary is that defects in numbers or function of Tregs are complicit in autoimmunity. There are already hints of such processes in the pathogenesis of autoimmune liver diseases—presumably much more will be heard of Tregs in a range of autoimmune diseases.

Further details. The various issues concerning AIH engaged herein are examined in greater depth in Chaps. 7 and 19.

Primary biliary cirrhosis (*PBC*). I am rather familiar with the story of PBC. I still recall reading, even though over 50 years ago, the exemplary clinical research publication from the Rockefeller Institute that put PBC "on the map" [29], and wondering why so little was known of its cause. As fortune would have it, only several years later on we ascertained a positive result (albeit in just the one PBC case tested) with a complement fixation test for autoimmunity to a cellular cytoplasmic constituent—identified as mitochondria in London using IIF. This was telling us something! Detection of anti-mitochondrial antibody (AMA) became one of the most useful, and widely used, of all the immunoserologic diagnostic assays. But identification of the actual mitochondrial reactant progressed only slowly until the 1980s when we and others showed by immunoblot that this was a ~72 kDa polypeptide. Next a cDNA was isolated by molecular cloning from a gene expression library by one of the Editors of this text (MEG) working as a sabbatical visitor at the Hall Institute in Melbourne [30]. The elusive reactant for AMA, finally identified as the E2 subunit of enzymes of the 2-oxo-acid dehydrogenase complex (2-OADC) (chiefly pyruvate dehydrogenase), allowed for a stream of immunological studies including localization of immunodominant autoepitopes for antibody and T cells to the inner lipoyl domain of the E2 subunit (PDC-E2). This heralded novel insights into this enigmatic disease and posed the questions on why and how uncontrolled autoimmune responses to the autoepitope of PDE-E2 might occur, and how these might damage specifically the terminal cholangiolar cells, as seen in PBC.

Essentially the "core" autoepitope is a highly conserved linear sequence (residues 169–176, IETDKATIG) that includes lysine (^{173}K) to which is attached the lipoyl cofactor, although the "complete" antibody paratope might span residues within the conformational structure from ^{131}MH to F…V^{180}. Sooner or later, we may see a solved crystal structure of a monoclonal anti-PDC-E2 in a complex with purified PDC-E2. Studies on T-cells in PDC-E2 revealed reactivity to a similarly located epitope in the inner lipoyl region of PDC-E2 and, as expected, there was a very high enrichment, 150-fold, of PDC-E2 epitope-reactive CD4$^+$ T cells in liver infiltrates, and in portal lymph nodes compared with blood. Finally there is immunohistochemical evidence of invasion and destruction of biliary ductular cells by epitope-specific effector cytolytic CD8$^+$ T cells.

But there is an "elephant in the room!" That is, some 30–40 % of cases of PBC express another set of autoantibodies; these being to nuclear antigens. Why is this? These ANA, in contrast to those routinely studied in rheumatic diseases, are PBC-specific and show unique staining patterns by IIF. Mostly they are molecularly characterized. The specificities include (a) "speckled dot" representing the Sp100 molecule and related promonocytic leukemia (PML) protein, (b) "nuclear membrane" representing proteins gp210 and gp63 of the nuclear pore complex, and (c) centromeric protein (CENP) otherwise characteristic of limited cutaneous systemic sclerosis. These atypical ANAs provide no clues to provocative causes or pathogenesis of PBC and simply serve to place the disease in that "twilight zone" between Th1,Th17- dominant organ-specific and Th2-dominant multisystemic autoimmune diseases marked by deficient peripheral tolerance emanating from dysfunction of Treg cells.

The discovery of the molecular basis for AMA reactivity prompted a sustained research effort at Davis CA into all aspects of PBC, on the premise that unpicking the "genes and environment" nexus should prove fruitful. The results, compacted in Chap. 18, strengthen a belief that explanations for PBC, as for autoimmunity in general, will ultimately be resolved into effects of multiple possible genetic anomalies interactive in various ways with multiple possible environmental provocations—under conditions in which chance will have an influence of uncertain magnitude [31].

The genetic components in PBC might be seen as less prominent than those for other autoimmune diseases, yet there is a uniquely high concordance for PBC in monozygotic twins (~60 %); a strong intra-familial susceptibility, a notably high female predisposition, and data from a mouse model are supportive [32].

Coming to environmental components, attention has been directed to sources of epitope mimics of the PDC-E2 lipoyl domain autoantigen. These range from infections with microbes that carry versions of the 2-OADC enzymes to exposure to novel xenobiotics that structurally influence the PDC-E2 region so as to create an immunogenic mimic sufficiently resembling PDC-E2 to break tolerance to the natural epitope. But perhaps there is no need to invoke extrinsic agents as initiators of autoimmune disease given that products of defective (incomplete) apoptosis may serve this function, prompting use of the term "apoptope." Experimentally it was found that there are unique features to apoptosis of cholangiocytes in that these cells specifically lack the capacity for glutathionylation allowing PDC-E2 to remain intact in apoptotic blebs as a potential immunogenic apoptope [32]. Then, with tolerance broken, by whatever means, and forbidden clones established, PBC would become slowly established by ongoing reexposure of the lymphoid system to the natural autoantigen.

A still further outcome of the availability in PBC of molecularly characterized autoantigens has been the encouragement given to develop mouse models of the disease. Already, over the past decade, these have (a) indicated the likelihood that genetic influences are indeed important in pathogenesis [33]; (b) shown that environmental agents and particularly xenobiotics are candidate initiators of PBC [34]; and (c) revealed strong permissive influences exerted by defects in peripheral tolerance dependent on signaling pathways of the receptor for the polyfunctional cytokine transforming growth factor (TGF)-beta [35], with effector functions attributable to clonally restricted populations of autoantigen-specific $CD8^+$ ve T cells [36]. These mouse models illustrate that disrupted TGF signaling indeed influences immunological homeostasis—their disadvantage is the relatively short life span of the mouse, precluding close recapitulation of the slowly evolving human PBC.

Primary sclerosing cholangitis. There are unquestioned immunological accompaniments to this mysterious disease, with some suggestive of autoimmunity including a tendency to overlap with type 1 AIH, more particularly in childhood (Chap. 24), and a disease association with ulcerative colitis for which, however, an autoimmune basis is being questioned, and an association with the "autoimmune" HLA haplotype B8 DRB1*030I. However, there are too many incongruities for PSC in adults to be ascribed to autoimmunity: It is male-dominant, the cholangitic lesions are sparse in lymphocytes but rich in fibrocytes, no disease-specific marker autoantibody is demonstrable, although there is a high frequency (88 %) of an atypical pANCA (not of proteinase 3 specificity). As mentioned, children with type 1 AIH coexpress an associated cholangiolar disease called "autoimmune sclerosing cholangitis," but its relationship to adult PSC remains undefined: perhaps a preferable descriptor would be "pediatric autoimmune cholangitis."

So, in conclusion, we see adult PSC as an aberrant proinflammatory response to products of otherwise innocuous intestinal microorganisms with ensuing cytokine activation, and a periductular myofibroblast response—and thus essentially auto-inflammatory (see below).

Drug-Induced Chronic Inflammatory Liver Disease

Immune-mediated drug-induced liver injury (imDILI) (sometimes called "idiosyncratic") could depend on several mechanisms. One is conjugation of a reactive metabolite of the drug to a host protein which, in the case of liver, is likely to be the enzyme protein, e.g., a CYP450 isoform responsible for its disposal (Chap. 27). This adduct generates an antigenic moiety which, on a permissive genetic background, can promote the inductive phase of an immune response expressed as allergic sensitization to the drug, and with ensuing inflammatory reactivity by the host. The actual site of induction is uncertain, whether within the liver or regional (hilar) lymph nodes. The "executive" phase of the response is variable mechanistically, being either antibody or T cell dominant but, at present, neither in vitro nor in vivo test systems seem sufficiently well developed to define the process in each individual instance of imDILI.

Of note, imDILI is sometimes accompanied by production of autoantibodies that simulate those detected in spontaneous AIH, either of ANA/SMA positivity or of anti-LKM positivity. The former, which challenge explanation, were seen prototypically in hepatitis occurring after prolonged use of the now obsolete hypotensive drug, a-methyl dopa, and nowadays are seen (rarely) with use of minocycline, nitrofurantoin, flucloxacillin, and others. The latter anti-LKM type would intuitively be more frequent, since many drugs are enzymatically disposed of by hydroxylation by isoforms of the CYP450 family, with the antibody corresponding to the isoform that hydroxylates the drug. Examples include the uricosuric tienelic acid (no longer marketed) that is degraded by CYP450 2C9 and provoked imDILI accompanied by anti-LKM 2C9, and the anti-hypertensive hydrallazine that is degraded by CYP 1A2 and (infrequently) provokes imDILI accompanied by anti-LKM CYP 1A2. Perhaps the best diagnostic procedure is observing recovery from imDILI after identifying and ceasing therapy with the culprit drug and, if needed, a deliberate (and carefully supervised) rechallenge.

Alloimmune Chronic Inflammatory Liver Disease

Alloimmune liver disease occurs in the context of host-versus-graft (HVG) or graft-versus-host (GVH) reactions occurring after transplantation of a donor allogeneic liver, or after transplantation of donor allogeneic bone marrow (BM) cells reactive against host liver (and other tissues). The outcome is a potent immunologic response whether to the "foreign" major MHC (HLA) class 1 molecules (and likely class 2 as well) or, in the case of BM transplants from HLA-matched donors, with "minor" transplantation antigens. Reactivity is expressed as mixed combinations of inflammatory damage to hepatocytes (interface hepatitis), biliary cells with ductopenia, or blood vessels with vascular occlusive lesions. This physiological propensity for allo-antigenic reactivity is attenuated by the tolerogenic milieu of our lymphoid liver so that allogeneic liver or BM transplants tend to succeed well despite MHC barriers, compared with skin and kidney, so leading to liver and BM transplantation becoming thriving elements of applied immunology.

Liver allografts, *HVG hepatitis*. The relatively less aggressive responses by the host to liver allografts is exemplified in some species (pig and some rodent strain combinations) by success with no requirement for immunosuppression, and in humans a lesser than expected need for immunosuppressive drugs. Yet the liver cannot be regarded as "immunologically privileged" since it is freely accessed by the portal venous and arterial circulations. Finally, the inherent intra-hepatic tolerogenicity is claimed to be augmented by lymphocytic chimaerism due to leakage out from a grafted liver of donor leucocytes.

However, rejection reactions either acute or chronic against the liver allograft do occur in some 80 % of human liver allografts. Acute rejection is expressed as portal leucocytic (granulocyte, monocyte, lymphocyte) infiltration seen as interface hepatitis, biliary ductulitis with ductopenia, and vascular endothelitis. Chronic rejection is expressed particularly by biliary ductopenia and obliterative arteritis. It is intriguing that an AIH can develop in an allografted liver, expectedly if an autoimmune disease was the reason for the transplant, but surprisingly when it occurs "de novo" (as it usually does) in allografts done for diseases other than autoimmune liver disease [37]. The basis for this diagnosis is histology together with fulfillment of the other conventional AIH criteria. Indeed the occurrence of "de novo AIH" in a liver allograft pose intriguing questions for the genesis of autoimmune disease in general.

Hemopoietic cell allografts, *GVH hepatitis.* There are many applications of allogenic hemopoietic (bone marrow) cell transplantation (HCT) in contemporary practice including immunodeficiencies, hematologic malignancies, aplastic anemia and, increasingly, in treatment of intractably progressive autoimmune diseases.

GVH disease is a complication in some 30–50 % of cases of allogeneic HCT from HLA-matched siblings. It is attributable to mature T lymphocytes of the donor inoculum, having been "protected" by immunosuppression of the recipient, reacting with foreign (non-HLA) "minor" histocompatibility antigens of host origin that become exposd on the cell-surface. The tissues predominantly affected by GVH disease are skin, intestinal tract mucosal surfaces, and liver, particularly cholangiocytes. Expectedly, given the likely similarity of the mode of pathogenesis, expressions resemble those of multisystem autoimmune diseases. In the liver, as with HVG disease, the lesions are hepatitic resembling those AIH, cholangitic resembling those of PBC, or vascular and resemble (to a degree) those of primary systemic sclerosis (scleroderma). In particular, in both HVG and GVH disease, intrahepatic biliary ductular cholangiocytes appear highly vulnerable to immune attack [38], as pertains in PBC, and in both conditions there is destructive invasion of ductules by activated T lymphocytes. There was even a claimed detection of AMA in a mouse model of GVH disease with affected bile ducts, but AMA was scarce among the autoantibodies tested for among 95 cases of human GVH disease [39].

Congenital alloimmune hepatitis. This alloimmune gestational disease of the liver, previously described (erroneously) as neonatal hemochromatosis, can present as fetal death in utero or severe liver dysfunction in the neonate. Once established, the disease characteristically recurs in subsequent pregnancies. Whtington [40] proposed the likelihood of a maternal immune attack on a surface-exposed liver cell-specific alloantigen occurring mid-term in gestation, with production of IgG alloantibody that crosses the placenta and causes a complement-dependent lysis of fetal hepatocytes.

The question of course arises on the nature and identity of the provocative liver-specific alloantigen. One liver alloantigen well-known to us was first described in the 1960s in mice, as the liver-specific F antigen which is a highly conserved and abundant liver cytoplasmic protein among mammals, including humans, that carry one or other of the two allotypes, F-1 or F-2 [41]. Immunization with allotypic F breaks tolerance and raises precipitating antibody that reacts with both allelic forms, the non-self-immunogen and the self-protein, thus eliciting both allo- and autoantibody. Cloning of the genes encoding the murine F alloantigens revealed that the deduced protein products had 95 % homology with a notable sequence difference near the carboxy terminus [41], but provided no functional insights. The known properties of the F alloantigen give this protein a candidate status as the alloantigen that causes the congenital alloimmune hepatitis of human infants.

Auto-inflammatory Chronic Liver Diseases

"Autoinflammatory" is a term that is gaining currency to describe an inflammatory response *sui generis*. This response can be invoked particularly by cellular degradations and products thereof, likely involves processes of innate immunity including induction by cytokines and chemokines, and is independent of adaptive immune responses [42]. In the liver, these cellular degradations may be associated with products associated with lipid accumulations, protein misfolding diseases, heavy metals (iron, copper), or other cytoplasmic inclusions not adequately disposed of by chaperone pathways.

An exemplary "autoinflammatory" liver disease is alcoholic hepatitis in which lipid inclusions known as Mallory bodies excite a pericellular neutrophilic inflammatory reaction, T cell chemotaxis, pro-inflammatory Type 1 cytokine release, and progressive fibrosis (Chap. 22). Particular attention is now directed towards a newer entity which hepatologists became aware of in the early 1980s, styled nonalcoholic

steatohepatitis (NASH), or a wider category of nonalcoholic fatty liver disease (NAFLD) (Chap. 23). The "non-alcoholic" (NA) component of the acronym is a hangover from earlier days when fat in the liver was such a reliable marker of alcoholic liver damage abuse that any other cause could not be entertained. Although some writers have invoked an adaptive immune response to these lipid inclusions to explain progression of alcoholic hepatitis to cirrhosis, the data are tenuous and the more likely process is activation of the innate immune system with production by macrophages of pro-inflammatory chemokines and cytokines, in combination with other potentiating factors, as reviewed [42], and with a genetic component since in some cases steatosis can be quite innocuous.

The reason in the first place for fat accumulation in the liver in NASH is often (~85 % of cases) the multifactorial "metabolic syndrome," characterized by central obesity, hypertriglyceridemia, hypertension, Type 2 diabetes, and insulin resistance. There is evidence that secretion of leptin by adipocytes can contribute to attraction into adipose tissue of macrophages [43] which thereupon promote cytokine-driven pro-inflammatory responses mediated by TNF-alpha, and profibrogenic TGF beta. Thus, NAFLD (Chap. 23) that lies at an intersect of hepatology, metabolism, immunology, inflammation, and genetics has strong claims for inclusion in this contemporary text on liver immunology.

Tornada

Tornada is an Occitan literary term for a short piece at the end of a body of writing, often a poem. Here, my tornada is presented to conclude this condensed overview of the many emerging issues pertaining to immunohepatology and to commend "Liver Immunology Edition 3" enthusiastically as an authoritative and comprehensive conspectus of this burgeoning area of enquiry into liver function and pathology.

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Core Concepts in Immunology

2

Cliona O'Farrelly and Derek Doherty

Key Points

- Cells of the innate immune system recognize microbial products and altered self using highly conserved receptors.
- Activated innate cells release cytokines and chemokines which induce and mediate inflammation locally and enter the circulation.
- Circulating inflammatory cytokines induce production of acute phase proteins and complement components by the liver thus inducing systemic inflammation.
- Natural killer cells detect altered expression of cell-surface molecules induced by viral infection or malignancy and kill their targets.
- Activated dendritic cells traffic from sites of infection and inflammation to lymph nodes, bearing cargoes of phagocytosed antigen which they present to naïve T cells thus initiating activation of the adaptive immune system.
- T lymphocytes express clonotypic antigen receptors that recognize peptide fragments of protein antigens presented by major histocompatibility complex molecules on antigen-presenting cells, e.g., DCs.
- Activation of a naïve T cell requires an appropriate cytokine milieu, a signal through its antigen receptor (signal 1) as well as a danger signal through a costimulatory receptor (signal 2).
- Adaptive immune responses to danger can be either inflammatory involving cytotoxic T cells, Th1 cells, and natural killer cells or antibody-dominated, involving Th2 cells and B cells, mast cells, and eosinophils.
- Antibodies secreted by plasma cells can neutralize toxins and viruses, activate complement, direct histamine release, and target pathogens for phagocytosis and cytotoxicity.
- Regulatory T cells and myeloid cells control innate and adaptive mechanisms.
- The innate and adaptive immune systems interact with and regulate each other.

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Prologue

The immune system is constantly engaged in maintaining homeostasis while poised to respond to signs of damage or danger. Immunological homeostasis is disturbed by tissue damage and growth abnormalities as well as by infection. Throughout the body, epithelial cells and immune-surveillant cells are equipped with specific receptors to detect these signs and respond with a complex, interacting set of defense mechanisms. Highly conserved primordial "identify and destroy" strategies characterize *innate immunity*, while more sophisticated detection and targeted killing processes, that display exquisite specificity, multiple layers of regulation, and memory, characterize *adaptive immunity*. In this chapter, the fundamental concepts of innate and adaptive immunity and their interaction are briefly reviewed. Further details on individual topics can be obtained in the reviews cited.

Recognition of Danger by the Innate Immune System

The innate immune system is activated following recognition 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 is mediated by highly conserved receptors toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RIGs; Table 2.1; [2, 3]).

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Table 2.1 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

From Akira S, Uematsu S, Takeuchi O. Pathogen Recognition and Innate Immunity. *Cell* 2006; 124:783–801
Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol*. 2010; 11(5), 373–84

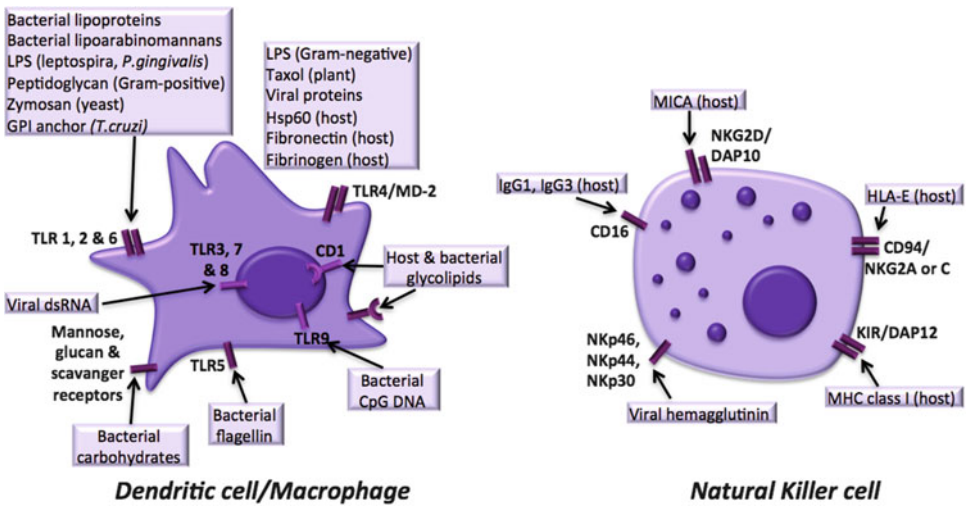

Fig. 2.1 Key phenotypic features of dendritic cell (**a**) and natural killer cell (**b**), important innate immune cells of myeloid (DC) and lymphoid (NK) lineages

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 functions, such as antimicrobial peptides (AMPs; Fig. 2.1) as well as messenger and regulatory functions (cytokines and chemokines). 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. Large populations of phagocytic cells of the myeloid lineage, expressing a wide range of detection molecules, are dispersed at strategic sites throughout the body, providing effective surveillance for potential pathogens. These tissue-specific macrophages and dendritic cells are found in the gut, lungs, skin, liver, and uterus, poised to be activated if their receptors are engaged by PAMPs (Fig. 2.1a). Epithelial cells at all these sites are also important detection and response elements of innate immunity and are potent producers of AMPs. Innate effector mechanisms activated by the above recognition systems during inflammation cause the target to be dispatched and

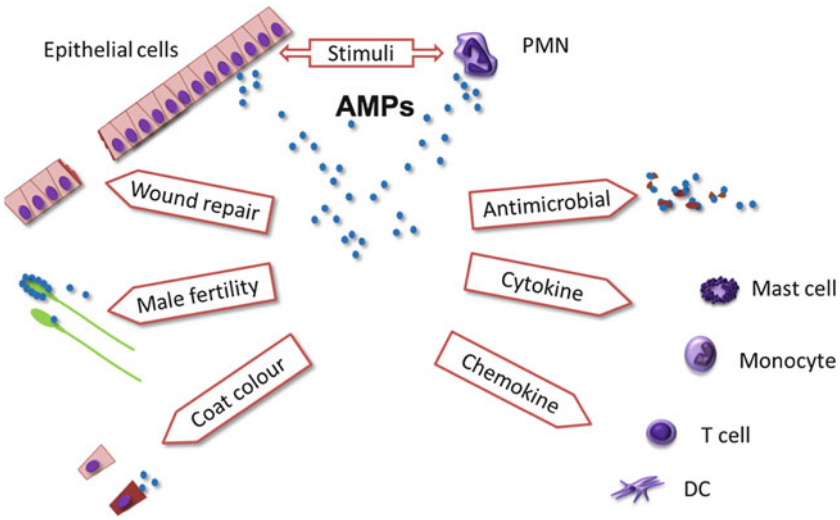

Fig. 2.2 AMP (antimicrobial peptide) functions. These small peptides have highly conserved features and are found in species as diverse as flies, molluscs, and humans. They were initially named for their ability to kill microorganisms. Several families of AMPS, including defensins and cathelicidins, have been shown to have a range of additional activities

include natural killer cell cytotoxicity, complement activation, opsonization, phagocytosis, respiratory burst, and AMP activity (Fig. 2.2).

The innate immune system is equipped with a second type of detection system, used by innate lymphoid cells, especially natural killer cells (Fig. 2.1b), 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 hemagglutin. 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] and CD94 in humans; Ly49 in mice) 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. As well as killing abnormal self-cells, subpopulations of NK cells are potent secretors of cytokines, in particular IL-22 and other growth factors with major tissue repair and remodeling potential [6]. These NK populations are thought to be particularly important in organs with high cell turnover and natural requirement for tissue repair, e.g., the liver, gut, and uterus.

Local and Systemic Inflammation: Central Role for the Liver (Fig. 2.3)

Inflammation is a general term given to the mobilization and effector activities of the innate immune system that are activated by responses to signals of "danger" and there is growing appreciation for its additional roles in physiological and metabolic homeostasis [7]. Chemical messengers from activated cells of the innate immune system and from pathogen-infected and tumor cells are responsible for mediating inflammation. These chemical messengers include chemokines (e.g., macrophage inflammatory protein-α [MIP-1α], MIP-β, CXCL8 [IL-8], Regulated on Activation, Normal, T-cell Expressed and Secreted [RANTES]) and cytokines such as tumor necrosis factor-α (TNF-α), the interleukins IL-1, IL-6, IL-12, and IL-18, and the interferons IFN-α and IFN-β, as well as growth factors G-CSF (granulocytic colony-stimulating factor) and GM-CSF (granulocyte–monocyte colony-stimulating factor). Secretion of some cytokines, e.g., IL-1 and IL-18, requires activation of inflammasomes, large complexes of proteins whose function is to activate the caspases required for cleavage of pro-forms of cytokines to their mature forms IL-1 [8]. Inflammatory cytokines act locally and also diffuse rapidly through the tissues and into the circulation. A key function of this activity is the recruitment of additional inflammatory cells from other sites of the body. Chemokines direct monocytes, neutrophils, and lymphocytes bearing the appropriate chemokine receptors to sites of inflammation, infection, or metastasis [9, 10]. 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 [11, 12]. 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 ([13]; Fig. 2.3).

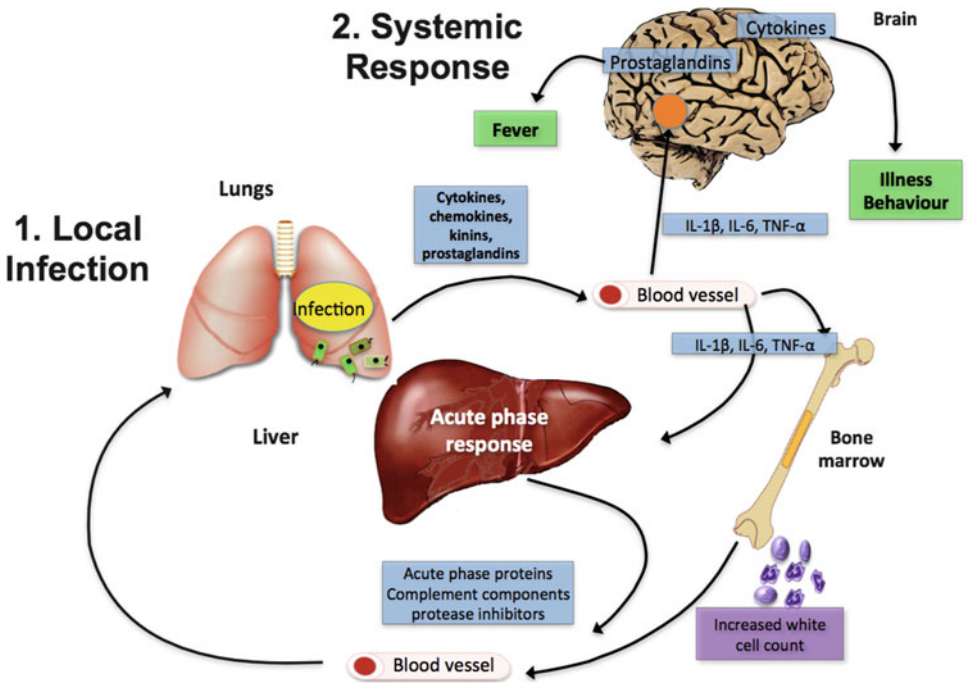

Fig. 2.3 Systemic inflammation. The liver has a key role in detecting circulating inflammatory cytokines, producing acute phase proteins and alerting the body to inflammation. Induction of the acute phase response has significant metabolic implications

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 [14, 15]. MicroRNAs are major regulators of the inflammatory response [16] 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 [17]. Resolution of inflammation is accompanied by activation of extensive tissue repair and remodeling mechanisms; e.g., the IL-10 cytokine family is now known to have major effects on epithelial cell biology [14, 18]. In some situations, activatory and effector functions fail to be regulated, leading to chronic inflammation which results in permanent scarring, tissue damage, or fibrosis, such as joint destruction in rheumatoid arthritis or fibrosis and cirrhosis in chronic hepatitis. Effective regulation and resolution of inflammation is therefore intensely complex and will only be understood when genetic influences [19] are studied in the context of systems biology.

Adaptive Immunity

If a microorganism or tumor evades or overcomes innate defense mechanisms and continues to grow unchecked, inflammation is not resolved and an adaptive immune response is initiated. The first and crucial step is the activation of T lymphocytes. Naïve, 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 (Fig. 2.4). 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.

T-Cell Receptors and Antigen Recognition (Fig. 2.5)

Naïve T cells can only be activated by "professional" antigen-presenting cells (APCs), which are myeloid cells, capable of capturing, processing, and displaying antigen on their

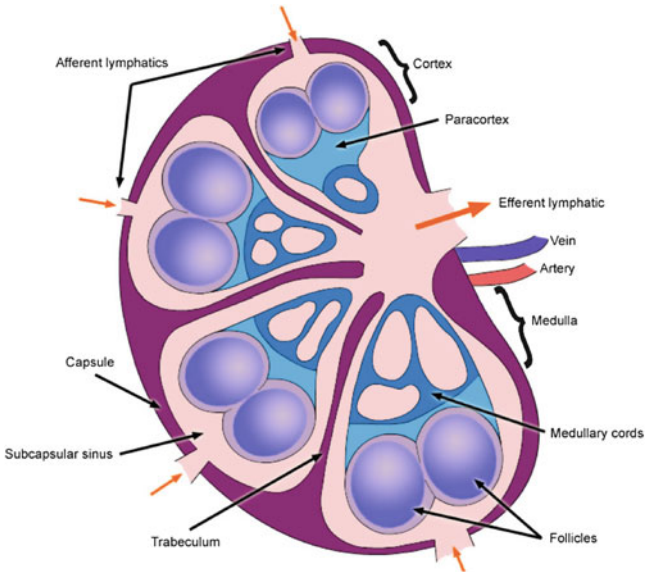

Fig. 2.4 Lymph node. Lymph nodes are small lymphoid organs tightly packed with resting T and B lymphocytes dispersed throughout the body and connected via the lymphocytic and blood circulation systems. Large numbers of these organs are positioned close to sites of potential infections: throat, lungs, gastrointestinal tract, and genito-urinary tract. Activated dendritic cells traffic to lymph nodes from sites of inflammation and induce the activation and proliferation of specific T and B cells leading to significant increase in size

cell surface [20, 21]. 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. 2.5). 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 [22, 23]. 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

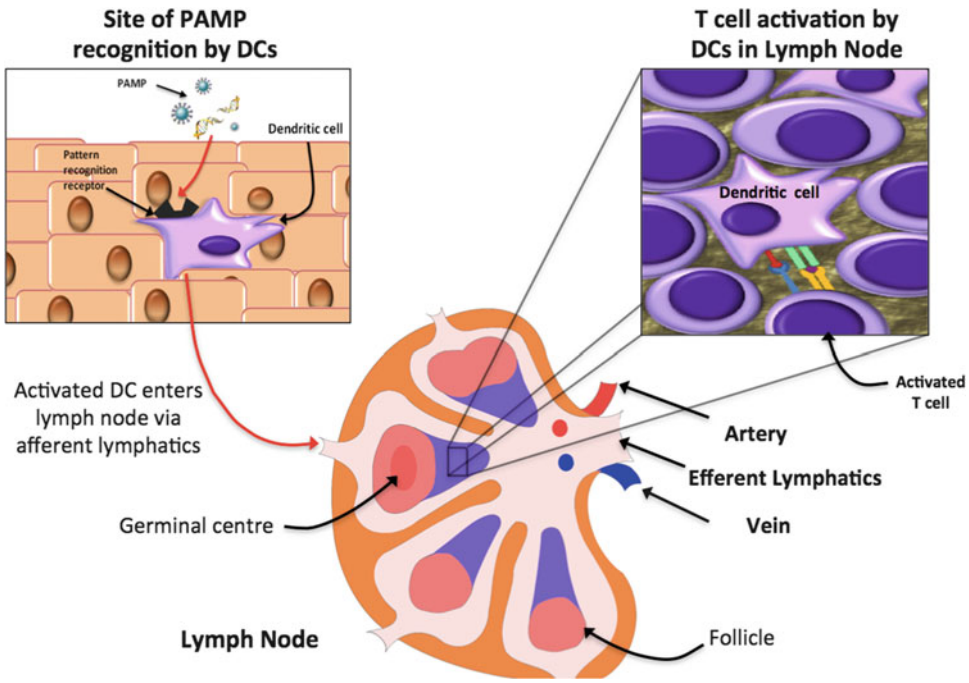

Fig. 2.5 T-cell activation 1. Dendritic cells are activated on recognition of pathogen-associated molecular patterns (PAMPs) by specialized receptors such as TLRs (toll-like receptors). They phagocytose and undergo phenotypic changes before trafficking to lymph nodes and present antigen to naïve T cells

recognize self-MHC molecules are allowed to survive (positive selection). These processes occur during T-cell maturation in the thymus.

T-Cell Activation

Distinct classes of T cells recognize intracellular and extracellular antigens presented by class I and class II major 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 IFN-γ, which disrupts viral replication [24, 25]. 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 [17].

Engagement of the TCR by peptide/MHC complexes, in the absence of additional signals, is insufficient for the activation of naïve 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 naïve T cell requires the simultaneous engagement of a series of accessory molecules on the T cell with corresponding costimulatory molecules on the APC that are induced by danger signals from the innate immune system [26]. The B7 family of molecules, CD80, CD86, and B7-homolog expressed by an APC, transduce costimulatory signals to T cells through CD28 and inducible costimulatory 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. 2.6).

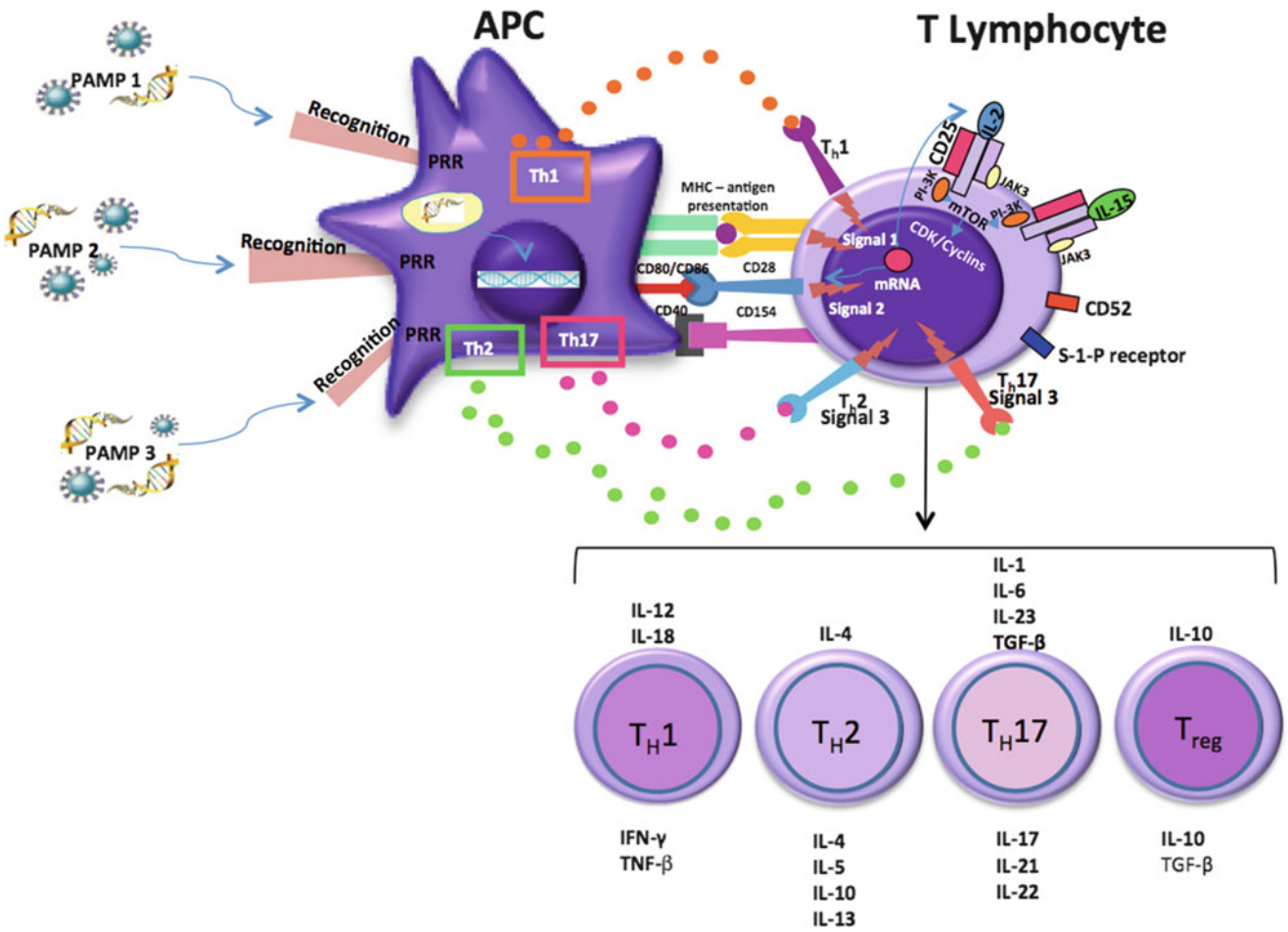

Fig. 2.6 T-cell activation 2. An activated dendritic cell presents antigen to T cells in the context of major histocompatibility complex class II molecules. A second signal is provided through engagement of CD80 and CD86. Effective T-cell activation and proliferation will only occur in the appropriate cytokine environment

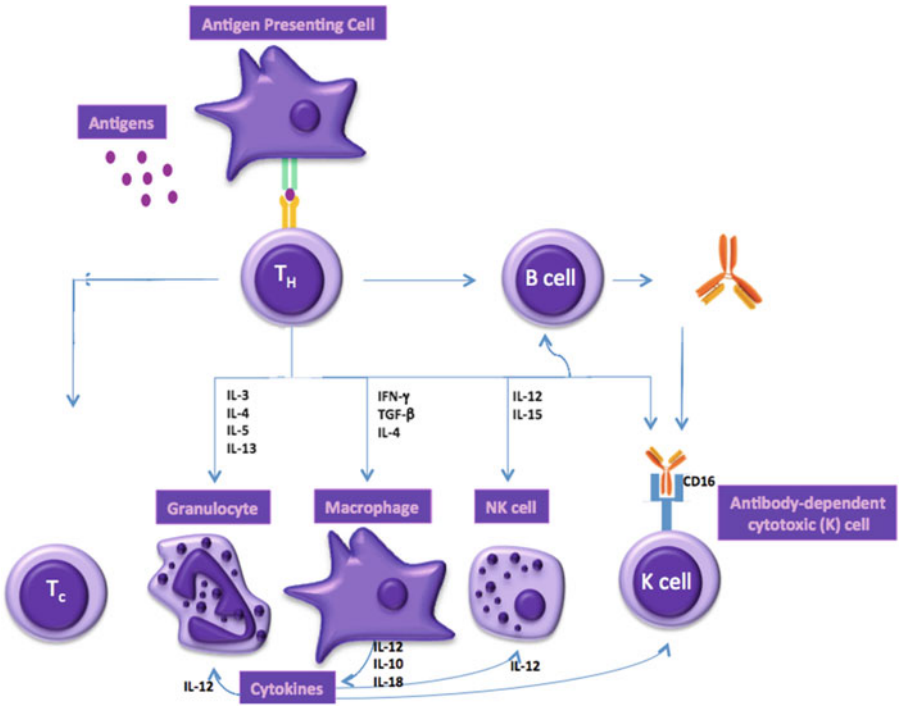

Fig. 2.7 Cytokines and T-helper cell function. The cytokine environment created by the activated dendritic cell determines the phenotype, transcription factor profile, and cytokine profile of the responding T-cell subpopulations

If the interaction between the TCR and the peptide/MHC is maintained over a threshold amount of time, the naïve T cell is activated, and it undergoes clonal proliferation and differentiation into effector T cells. Full activation of naïve 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 PRR engagement, and ultimately induce different subpopulations of cytokine-secreting T cells including TH1, TH2, T regulatory cells, and TH17 cell populations (Fig. 2.7). 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 T cells can then respond in a variety of ways to the same peptide/MHC complexes, alone, without the need for co-stimulation.

Effector Functions of the Adaptive Immune System and Their Regulation (Fig. 2.6)

The differentiation of naïve T cells into functional effector cells is controlled by signals from the innate immune system [21, 24, 26]. 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 of the IgG2a isotype in mice. 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 [21, 24, 26]. Other populations of $CD4^+$ T cells with regulatory function (Fig. 2.8), 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 [27–29]. Myeloid cells have also shown to have regulatory activity in particular myeloid-derived suppressor cells (MDSCs) which secrete IL-10, TGF-β, as well as arginase and IDO which modify T-cell behavior by catabolizing arginine and tryptophan, respectively [30].

B-Cell Antigen Receptors (Antibodies)

Antibodies, like TCRs, are coded for by sets of rearranging gene segments (Fig. 2.9) and thus possess as much diversity and specificity for antigen as the TCR [31]. Antibodies released in soluble form can neutralize toxins and viruses and also opsonize pathogens for phagocytosis by macrophages,

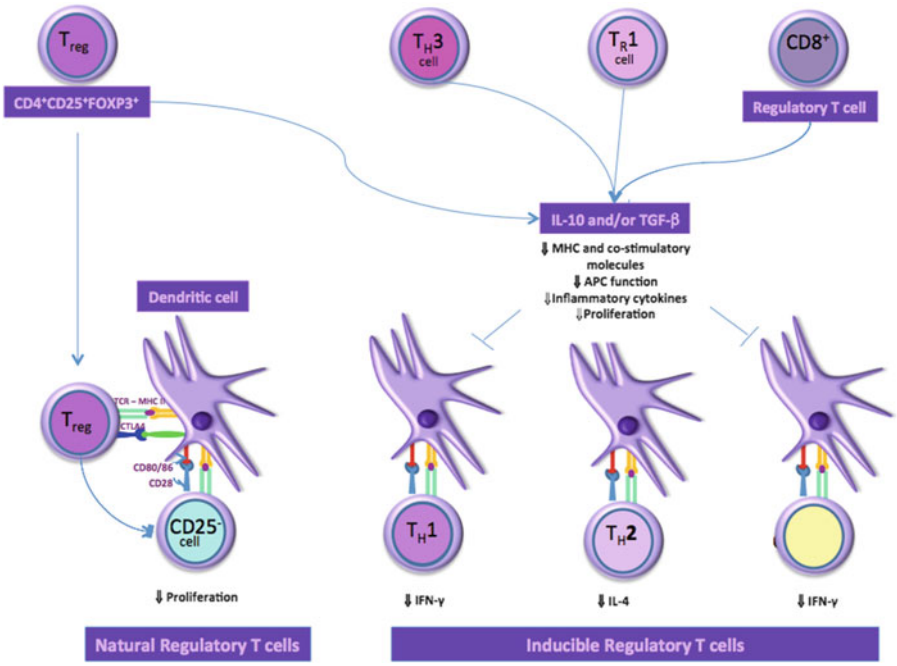

Fig. 2.8 Regulatory T-cell populations. T regs (T regulatory cells) can be constitutive or inducible. They are all characterized by the transcription factor FOXp3. Most are $CD4^+$, express the IL2r chain CD25, and secrete IL-10 and/or TGFb. Some express the catabolic enzymes arginase and (IDO)

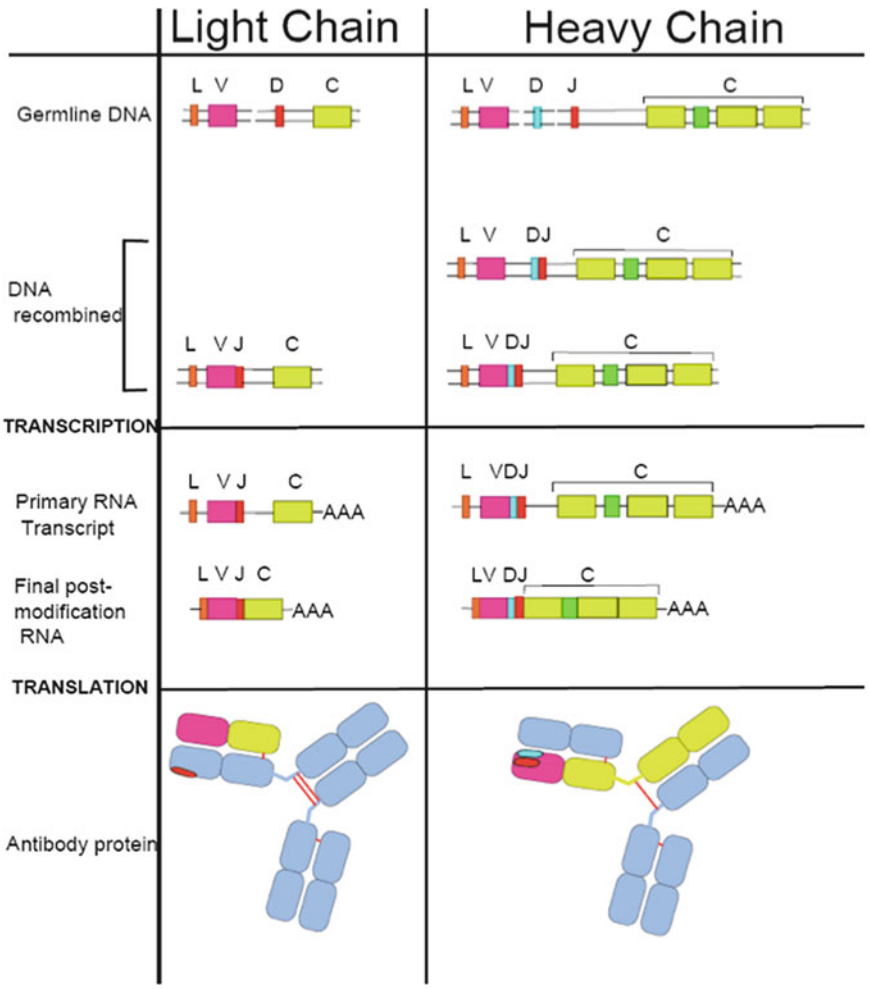

Fig. 2.9 Gene rearrangement required for the generation of antibodies [and T-cell receptors]. During B-cell development, families of immunoglobulin gene segments undergo rearrangement to generate a unique DNA sequence for each B-cell antigen receptor. On differentiation to a plasma cell, additional posttranslational modification results in the generation of secreted forms of the molecule (antibodies)

cytotoxicity by NK cells, and directed histamine release by mast cells and basophils [32]. Antibodies can also activate complement leading to the lysis of bacteria [11]. 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 [33].

Interaction and Interdependence of Innate and Adaptive Immune Systems

Until recently, innate and adaptive immunity were thought of (and certainly taught as) two independent, almost mutually exclusive systems. However, innate and adaptive immune systems are in continuous dialogue, with each regulating the other. Myeloid cells, in particular, macrophages and DCs of the innate immune system, act as APCs for T cells in the initiation of adaptive immune responses. The selective differentiation of naïve T cells into Th1, Th2, TH17, or T regulatory cells is controlled by signals from cells of the innate immune system, such as DCs and macrophages (Fig. 2.7). Immature DCs internalize antigens in the tissues and migrate to the lymph nodes, where they act as PCs for the activation of T cells. DCs are capable of directing T-cell maturation into distinct T-cell subtypes [33–37]. The nature of the antigen influences the pattern of cytokines produced by the DCs, which in turn determines the type of T cell expanded from naïve precursors. Release of IL-12 and IL-18 by DCs stimulates Th1 induction, whereas IL-10 production by DCs stimulates the generation of T regulatory populations. PRR ligation of immature DCs induces them to mature into one of two mutually inhibitory DC subsets, DC1 or DC2 cells, which promote Th1 or Th2 responses, respectively. NK cells also regulate Th1 or Th2 cell differentiation by the selective production of IFN-γ, IL-5, or IL-13.

In addition to the cross talk between the cells of the innate and adaptive immune systems, some populations of adaptive immune lymphocytes are equipped with antigen recognition and effector mechanisms characteristic of innate immune cells. Natural killer T (NKT) cells express NK markers and also TCRs that recognize glycolipid antigens presented by the nonclassical antigen-presenting molecule CD1d [38–40]. γδ (gamma-delta) T-cell populations can directly recognize small metabolite molecules (prenyl pyrophosphates, thymidine metabolites, alkyl-amines, and glycoproteins) and stress-inducible proteins (nonclassical MHC class I molecules and heat shock proteins) without the need for MHC restriction [41]. γδ T cells can also recognize glycolipid antigens presented by molecules of the CD1 family. Upon activation, NKT cells and γδ T cells rapidly kill tumor cells, regulate Th1/Th2/Tr1 cell differentiation by the selective production of IFN-γ, IL-4, or IL-10, and induce maturation of DCs into APCs. B1 B cells also have a limited repertoire of antigen receptors and are considered the B cell equivalent of γδ T cells.

Immune Cell Production and Differentiation

All immune cells differentiate from hematopoietic stem cells (HSCs) along either myeloid or lymphoid pathways (Fig. 2.10). In the first weeks of fetal development, this process occurs in the yolk sac but quickly moves to the fetal liver. The bone marrow becomes the predominant site of immune cell production before birth where it continues throughout life. Myeloid, NK cell, and B-cell populations develop to maturity in the bone marrow. Growth factors and cytokines, including GM-CSF, G-CSF, IL-3, IL-6, IL-7, and IL-15, produced by stromal cells and neighboring immune cells regulate the rate and specificity of immune cell production. T-cell progenitors traffic to the thymus, under the "direction" of chemokines secreted by thymic epithelial cells, where they undergo a rigorous selection process before emerging as mature naïve T lymphocytes that populate the lymph nodes and other sites. HSCs, as well as myeloid and lymphoid progenitors, have been found in adult liver, gut, and uterus suggesting that region-specific populations of immune cells may develop locally, thus contributing to the specialized immune repertoires seen in each of these organs [42].

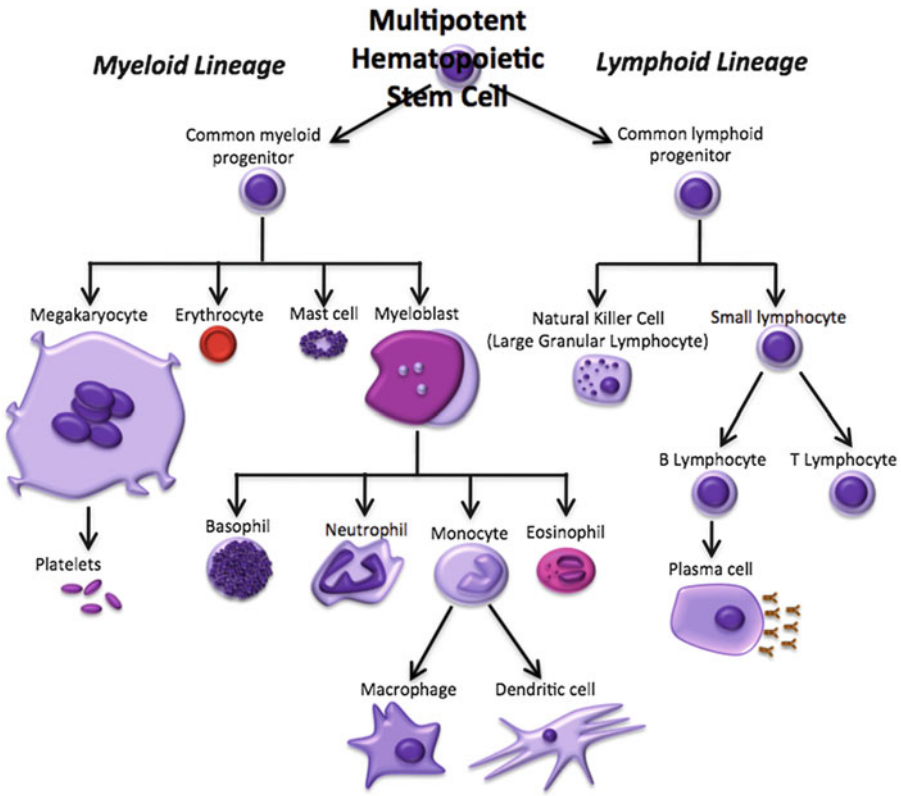

Fig. 2.10 Generation of immune cells from matopoietic stem cells (HSCs). All white cells (leucocytes) are derived from HSCs, predominantly in the bone marrow. Under the influence of growth factors and cytokines generated by stromal cells in the bone marrow, differentiation occurs along myeloid and lymphoid pathways. This process is ongoing during life, influenced and regulated by cytokines, growth factors, and acute phase proteins generated during infection and inflammation

Glossary

Accessory cell A cell that aids an adaptive immune response but does not mediate specific antigen recognition

Acute phase proteins Series of blood proteins produced by hepatocytes in response to inflammatory cytokines that participate in the early phases of host defense against infection

Adaptive immune response The response of antigen-specific lymphocytes to antigen and the development of immunological memory

Adhesion molecules Mediate the binding of one cell to another

Adjuvant A substance that enhances the immune response to an antigen with which it is mixed

Alleles Variants of a single gene

Allergy An immune response to an innocuous antigen

Alloreactivity The stimulation of T cells by nonself-MHC molecules; can only occur in vitro or during transplantation

Anergy A state of T-cell non-responsiveness to antigen

Antibodies Antigen-specific receptors found on the surfaces of B cells or secreted by plasma cells; heterodimers (two heavy and two light chains); 5 classes IgM, IgG, IgA, IgD, and IgE

Antigen Molecule (usually peptide) recognized by T-cell or B-cell antigen receptor

Antigen presentation The display of peptide fragments of protein antigens bound to MHC molecules for T-cell recognition

Antigen-presenting cells (APCs) Specialized cells that can internalize, process, and present antigens to T cells, e.g., DCs, some macrophage populations; B cells

Antigen processing The intracellular degradation of proteins into peptides for inclusion into MHC class I and MHC class II molecules for presentation to T cells

APC See antigen-presenting cell

Apoptosis Programmed cell death

Autoimmune disease Pathology caused by immune responses to self-antigens

Basophils Granulocytes; white blood cells with functions similar to those of mast cells

and antibody plasma proteins (immunoglobulins) that bind specifically to antigens and mediate neutralization, opsonization, and complement activation

B cells Lymphocytes with antigen-specific immunoglobulin receptors; B7, see CD80 and CD86 (Appendix 2.1)

Bone marrow The site of hematopoiesis

CD Cluster of differentiation (see Appendix 2.1)

Cell-mediated immunity Immune responses involving immune cells, e.g., T cells and NK cells

Chemokines Small peptides with conserved cystines that bind to specific receptors and influence white cell trafficking; chemokines direct monocytes, DCs, granulocytes, and lymphocytes bearing the appropriate chemokine receptors to sites of inflammation, infection, or metastasis

Clonal expansion Proliferation of antigen-specific lymphocytes, allowing rare cells to increase in number

Complement Set of plasma proteins that function in a protein cascade leading to the formation of a lytic complex and production of chemotactic factors and anaphylotoxins

Complement receptors Cell-surface receptors that bind pathogen-bound complement, resulting in their phagocytosis

Complementarity-determining regions Regions of the T-cell receptor or immunoglobulin molecules that make contact with antigens

Co-receptor Cell-surface proteins found on T lymphocytes that have ligands on antigen-presenting cells required for additional signaling necessary for effective T-cell activation

Co-stimulation A signal from an APC required in addition to antigen for full activation of lymphocytes

C-reactive protein An acute phase protein that binds to phosphatidylcholine on bacteria and opsonises them for phagocytosis

C gene segment Constant gene segment, coded for by Ig and TCR genes

CTLA-4, see CD152 (Appendix 2.1) Cytokine proteins secreted by cells that affect the behavior of other cells (see Appendix 2.2)

Cytokine receptors Cellular receptors for cytokines, cytotoxic T cells, T cells that can kill other cells

D gene segment Diversity gene segment, coded for by Ig and TCR genes

Damage-associated molecular patterns (DAMPs) Molecules released by stressed or damaged cells undergoing necrosis that act as danger signals to promote or exacerbate the inflammatory response

DC, see dendritic cell Dendritic cell; cells of the innate immune system that capture antigens and present them to T cells and direct T-cell subtype differentiation

Diapedesis Movement of cells from blood across blood vessel walls into tissues

Digest (*Context*) targeted destruction of engulfed particles or cells by phagocytes

Effector cells Lymphocytes that mediate the removal of pathogens from the body without the need for further differentiation

ELISA See enzyme-linked immunosorbent assay

ELISpot assay An adaptation of ELISA in which individual cells are placed over a bound antibody or antigen that trap the cells' secreted products and are detected with an enzyme-coupled antibody

Endotoxin A bacterial toxin that is released when the cell is damaged

Enzyme-linked immunosorbent assay (ELISA) Serological assay in which bound antigen or antibody is detected by a linked enzyme that converts a colorless substrate to a colored product

Eosinophil Granulocyte; white blood cell of myeloid lineage involved in immunity against parasites

Epitope The region on an antigen that is recognized by a lymphocyte antigen receptor

Fas See CD95 (Appendix 2.1)

Fc receptors Cellular receptors for the constant portions of immunoglobulins; mediate their biological function

Flow cytometry Automated characterization of cells in single cell suspensions with regard to cell size, cell granularity, and fluorescence owing to bound fluorescent antibodies

Gamma-delta (γδ) T cells Potent innate effector lymphocytes involved in antitumor immune surveillance

Gene segments Segments of TCR and immunoglobulin genes that undergo somatic recombination resulting in the generation of diversity of antigen recognition molecules

Germinal centers Sites in secondary lymphoid tissues of B-cell proliferation, selection, and maturation

Granulocytes See polymorphonuclear leukocytes

Haplotype Set of genes associated with one haploid genome

Granzyme A type of serine protease released from granules by $CD8^+$ T cells and NK cells that induces death

Helper T cells $CD4^+$ T lymphocytes

Hematopoiesis Generation of all blood cells from their precursors

Histamine A vasoactive amine stored in mast cell granules that is released upon antigen binding to IgE molecules on mast cells

Histocompatibility The ability of tissues to coexist without eliciting immune responses; HLA (human leukocyte antigens) encoded by the MHC (major histocompatibility complex)

Humoral immunity Soluble immune-related molecules, mostly found in the serum, e.g., acute phase proteins, antibodies

Hypersensitivity Immune responses to innocuous antigens that occur repetitively; mediated by IgE

ICOS (inducible costimulatory receptors) Molecules found on the surface of T cells required for T-cell activation after engagement of the TCR

Ig See immunoglobulin

Immunization The deliberate provocation of an immune response by introducing antigen

Immunoblotting A technique in which proteins are separated by electrophoresis and detected by antibodies

Immunofluorescence A technique for detecting molecules in tissue sections using antibodies labeled with fluorescent dyes

Immunoglobulin (Ig) Antigen-specific receptors found on the surfaces of B cells or secreted by plasma cells; heterodimers (two heavy and two light chains); 5 classes IgM, IgG, IgA, IgD, and IgE (see antibodies)

Immunoglobulin superfamily Proteins with domains that have the sequence and structural features that characterize immunoglobulins/antibodies; usually cell-surface receptors

Immunohistochemistry A technique employing enzyme-labeled or fluorescent antibodies to detect specific molecules in tissue sections

Immunological memory The ability of antigen-specific effector T cells and B cells to persist for years

Immunoprecipitation Detection of soluble proteins using specific antibodies

Immunoreceptor tyrosine-based activation motifs (ITAMs) Tyrosine residues on the cytoplasmic domains of signaling proteins that upon phosphorylation trigger cell activation; important for NK function

Immunoreceptor tyrosine-based inhibitory motifs (ITIMs) Similar to ITAMs except they signal inhibition of cellular functions; important for NK cell function

Inflammasome Large complex of proteins that activate caspase-1 which is required for cleavage of pro-IL-1 to allow it to be secreted from the cell

Inflammation Early phase of an immune response involving the local accumulation of plasma proteins and leukocytes at a site of infection; systemic inflammation is characterized by acute phase protein production

Innate immunity A variety of defense mechanisms that non-specifically target pathogens in the early stages of an immune response

Integrins A family of adhesion molecules found on the surfaces of immune and endothelial cells

Interferons A family of cytokines with antiviral activity

Interleukins Cytokines produced by leukocytes (see Appendix 2.2)

J chain Protein used to hold the pentamer of IgM and the dimer of IgA together, coded for by a non-immunoglobulin gene

J segment Joining gene segment, found amongst Ig and TCR gene segments

Knockout mice Mice with heritable targeted disruptions of specific genes

Kupffer cell Specialized macrophages (phagocytic cells) in the liver

Langerhans cells Macrophages found in the skin

Leukocyte General term for white blood cells

Lymphatic system A series of vessels that drain fluid from the tissues to the blood, carrying lymphocytes and other immune cells and molecules

Lymph nodes Secondary lymphoid organs where adaptive immune responses are initiated

Lymphocytes Mononuclear leukocytes that mediate adaptive immune responses; include T and B lymphocytes and natural killer cells

Lymphokines Cytokines produced by lymphocytes

Macrophage Myeloid cell of the innate immune system with APC function found in the tissues (e.g., Langerhans cells in the skin; Kupffer cells in the liver)

Major histocompatibility complex (MHC) Highly polymorphic gene complex found on chromosome 6 in the human; codes for class I and class II antigen-presenting molecules as well as other molecules of immunological importance

Mannose binding lectin Acute phase protein synthesized in the liver early in inflammation

Mast cells Histamine-releasing cells of myeloid origin with IgE receptors found fixed in tissues

Membrane attack complex Complement components that can disrupt membranes of pathogens

MHC See major histocompatibility complex

MHC restriction Recognition of peptide antigens presented by MHC molecules by T cells

MICA, MICB MHC class I-related stress proteins expressed by epithelial cells recognized by NK cells and some T cells

Minor histocompatibility antigens Antigens that can lead to graft rejection when recognized by T cells

Minor lymphocyte stimulatory (Mls) loci Mammary tumor virus genes integrated into the mouse genome that code for superantigens

MIP-1α and -β Macrophage inflammatory proteins α and β chemokines

Monoclonal antibodies (MAbs) Antibodies produced by a single clone of B cells

Monocyte Myeloid phagocytic cell found in the circulation

Myeloid cells Macrophages and granulocytes

Myeloid-derived suppressor cells Cells of the myeloid lineage capable of suppressing T-cell activity by secreting IL-10 and TGF-β

N nucleotides Extra nucleotides that are inserted into the junctions between gene segments of TCR and Ig DNA, by terminal deoxynucelotidyl transferase (Tdt) to create further diversity

Naïve lymphocytes Lymphocytes that have never encountered antigen

Natural cytotoxicity Spontaneous killing of cells by NK cells

Natural killer (NK) cells Lymphoid cells of the innate immune system that kill virus-infected and tumor cells

Natural killer T (NKT) cells Cells that combine the phenotypic and functional characteristics of NK cells and T cells

Necrosis Death of cells owing to physical or chemical injury, as opposed to apoptosis

Negative selection Intrathymic deletion of developing T cells that recognize self-antigens

Neutralization Inhibition of infectivity of a virus or toxicity of a toxin by antibodies

Neutrophil Polymorphonuclear, phagocytic leuckocyte; most numerous in the circulation

NK cell See natural killer cell

NK1.1+ T cell T cells that express the NK cell stimulatory receptor NK1.1

NKG2D Activating receptor found on NK cells and some T cells

Kp46 Natural cytotoxicity receptor found on NK cells that recognizes viral hemagglutinin

NKT cells See natural killer T cells

NOD-like receptors (NLRs, NODs) Family of intracellular PRRs that recognize cytoplasmic PAMPs

Nude mice A mutant strain of mice with no hair and defective thymic formation so they have no mature T cells

Opsonization Alteration of the surface of a pathogen, e.g., due to binding of acute phase proteins, so that it can be recognized and ingested by phagocytes

Pathogen-associated molecular patterns (PAMPs) Conserved antigenic structures present on microorganisms that are recognized by the innate immune system

Pattern recognition receptors (PRRs) Receptors on cells of the innate immune system that recognize common structures (PAMPs) found on infectious agents

Perforin A protein produced by T cells and NK cells that can polymerize to form a pore in a target cell as part of cell killing

Peyer's patches Aggregates of lymphocytes in the small intestine

Phagocytic cell Cells of the myeloid lineage that have the ability to engulf particles and cells

Phagocytosis Engulfment of particles, microbes, and dying cells by cells of the myeloid lineage

Plasma cell A terminally differentiated B cell capable of producing antibodies

Polygenic Several gene loci code for multiple proteins of similar function

Polymerase chain reaction (PCR) A technique for amplifying specific sequences of DNA

Polymorphic A gene locus with multiple alleles

Polymorphonuclear leukocytes White cells of myeloid lineage, characterized by their granules and the shape of their nuclei with potent phagocytic and microbial killing potential; see neutrophils, basophils, eosinophils

Positive selection Selective maturation of T cells that can recognize self-MHC molecules in the thymus

Priming Initial interaction between a lymphocyte and an antigen

Professional APC Cells that are capable of presenting antigen to naïve T cells

Programmed death receptor-1 (PD-1) A receptor on activated lymphocytes that mediates inhibition of lymphocyte effector functions

Proteosome A multifunctional protease that degrades antigenic proteins into peptides for antigen presentation

Radioimmunoassay (RIA) A technique in which an antigen or antibody is bound to a solid support and specific radiolabeled antibody or antigen in a preparation is quantified by binding to these molecules

RAG1 and RAG2 Recombinase activating gene products required for TCR and Ig gene rearrangement

RANTES (Regulated on Activation, Normal, T-Cell Expressed and Secreted) A chemokine responsible for influencing the migration of T lymphocytes

Receptor-mediated endocytosis Internalization of molecules by cells using specific receptors for the molecules

Receptor repertoire The totality of lymphocyte receptors present in an individual

Regulatory T cells (Tr cells) T cells that suppress the activity of effector T cells through secretion of IL-10 and/or TGF-β

Respiratory burst (oxidative burst) Following phagocytosis, the sharp increase in the uptake of oxygen, which facilitates the production of superoxide and hydrogen peroxide, potent killers of microbes

RIG-I-like receptors Family of RNA helicase enzymes that specifically recognize viral derived RNA in the cytoplasm

Secondary immune response The more rapid, potent, and specific lymphocyte response elicited by second exposure to antigen, characterized by higher affinity antibodies of the IgG class

Second signal A costimulatory signal required for lymphocyte activation

Selectins A family of adhesion molecules

Sero-conversion The phase of an infection during which antibodies are produced

Serology The use of antibodies to identify antigens

Somatic recombination Rearrangement of TCR or Ig gene segments

Superantigens Molecules that stimulate whole families of T cells by binding to MHC class II molecules and Vβ domains of the TCR

Suppressor T cells See T regulatory cells

Syngeneic Between two genetically identical individuals; T-cell lymphocytes that mature in the thymus and recognize antigen by a TCR associated with the CD3 protein complex

T-cell clone Cultured T cells expanded from a single cell

T-cell line Cultures of T cells grown by repeated stimulation

T-cell receptor (TCR) Antigen-specific receptors on T cells

Terminal deoxynucleotidyl transferase (Tdt) Enzyme which inserts extra nucleotides into the junctions between gene segments of TCR and Ig DNA, to create further diversity; also used in TUNEL, assay for apoptosis

T lymphocyte See T cell

TCR See T-cell receptor

TGF-β See Appendix 2.2

Th1 cells $CD4^+$ T cells that secrete IFN-γ, TNF-β, and IL-2, activate macrophages, and promote inflammation

Th2 cells $CD4^+$ T cells that secrete IL-4, -5, -9, -10, and -13 and promote B-cell differentiation

Th3 cells T regulatory cells; $CD4^+$ T cells that secrete TGF-β and or IL-10 and suppress Th1 cell responses

Th17 cells $CD4^+$ T cells that secrete IL-17, -17F, -21, and -22; important during inflammation

Thymus Organ where T cells differentiate from bone marrow-derived lymphoid progenitor cells and undergo positive and negative

TNF (tumor necrosis factor) An inflammatory cytokine (see Appendix 2.2)

Tolerance The failure of the immune system to respond to antigen

Toll-like receptors Receptors on macrophages and dendritic cells that recognize common components of microorganisms and mediate signaling pathways (analogous to the Toll receptor in Drosophila)

Transgene Introduction of foreign genes to the genome of an organism

T regulatory cells $CD4^+$ T cells that secrete TGF-β and or IL-10 and suppress T-cell responses; can be inducible or constitutive

V gene segments Variable gene segment, coded for by Ig and TCR genes

Vaccination The deliberate induction of immunity against a pathogen by immunization with a dead, attenuated, or defective form of the pathogen

Western blotting A technique for detecting proteins separated by gel electrophoresis using labeled antibodies

Xenogeneic Immune response elicited between organisms of different species

Appendix 2.1: Relevant Cluster of Differentiation (CD) Antigens

CD1	MHC class I-like lipid-presenting molecule expressed by APCs and other cells
CD2	Adhesion/costimulatory molecule expressed by T cells and NK cells
CD3	TCR-associated molecular complex necessary for TCR-mediated signal transduction
CD4	Co-receptor for MHC class II molecules found on T cells, monocytes, and macrophages
CD8	Co-receptor for MHC class I molecules found on T cells and some NK cells
CD11	Family of adhesion molecules found on lymphocytes, granulocytes, monocytes, and macrophages
CD14	Receptor for lipopolysaccharide and other molecules found on DC and macrophages
CD16	Immunoglobulin Fc receptor found on neutrophils, macrophages, and NK cells
CD18	Adhesion molecule found on leukocytes that associates with CD11
CD19	Costimulatory receptor found on B cells
CD20	Costimulatory receptor found on B cells
CD25	High-affinity IL-2 receptor (α-chain) found on activated T cells, B cells, and monocytes
CD28	Naïve T-cell receptor for costimulatory molecules CD80 and CD86
CD34	Adhesion molecule found on hematopoietic precursors
CD35	Complement receptor found on most leukocytes
CD40	B-cell receptor for costimulatory molecule CD154
CD44	Leukocyte adhesion molecule
CD45	Signaling molecule that augments signals through T-cell and B-cell antigen receptors
CD49	Family of adhesion molecules found on leukocytes
CD50	Family of adhesion molecules found on leukocytes
CD54	Family of adhesion molecules found on hematopoietic cells
CD56	Adhesion molecule found on NK cells
CD58	Adhesion molecules found on hematopoietic cells CD64 immunoglobulin Fc receptor found on monocytes and macrophages
CD69	Lectin of unknown function found on activated T cells, B cells, NK cells, and macrophages
CD74	MHC class II chaperone molecule found in APCs
CD79	B-cell antigen receptor-associated molecular complex required for Ig-mediated signal transduction
CD80	Costimulatory molecule found on APCs
CD81	B-cell co-receptor
CD86	Costimulatory molecule found on APCs
CD94	Stimulatory/inhibitory receptor for HLA-E found on NK cells and some T cells
CD95	Apoptosis-inducing molecule found on a wide variety of cells (Fas)

(continued)

(continued)

CD102	Adhesion molecule found on resting lymphocytes, monocytes, and endothelial cells
CD106	Adhesion molecule found on endothelial cells
CD116	Receptor for granulocyte–macrophage colony-stimulating factor found on myeloid cells
CD117	Stem cell factor receptor found on hematopoietic cell precursors
CD119	IFN-γ receptor found on macrophages, monocytes, and B cells
CD120	TNF-α and -β receptor found on many cell types
CD121	IL-1 receptor found on T cells, B cells, macrophages, and monocytes
CD122	IL-2 receptor β-chain found on NK cells and some T cells and B cells
CD124	IL-4 receptor found on mature T cells and B cells
CD125	IL-5 receptor found on eosinophils, basophils, and activated B cells
CD132	Common γ-chain receptor for IL-2, IL-4, IL-7, IL-9, and IL-15; CD134 costimulatory molecule found on activated T cells
CD152 (OX40)	Negative regulator of T-cell activation that interacts with CD80 and CD86 (CTLA4); CD154 costimulator of B-cell activation found on activated T cells
CD158	Stimulatory/inhibitory receptor (KIR) found on NK cells
CD161	Costimulatory receptor found on NK cells and some T cells

Appendix 2.2: Cytokines

Inflammatory cytokines	
IL-1α, β	Pluripotent inflammatory cytokine; induce T-cell and macrophage activation and increase body temperature
TNF-α	Tumor necrosis factor-α: induces local inflammation, activation of macrophages, and nitric oxide production; influences metabolism
IFN-α, -β	Interferons-α and -β: type 1 interferons important in antiviral immunity; stimulate NK, stimulate MHC class I expression, and inhibit viral replication
IFN-γ	Interferon-γ: stimulates Th1 cell, NK cell, and macrophage activation and MHC expression by APCs; inhibits Th2 cell differentiation
IL-6	Inflammatory cytokine; stimulates acute phase protein production by the liver and leukocyte production in the bone marrow
IL-8	Chemotactic factor for neutrophils
IL-12	Activates NK and NKT cells and promotes Th1 cell differentiation
IL-17	Important cytokine for mediating the inflammatory process; acts through receptors expressed by many cell types
IL-18	Promotes Th1 cell differentiation
IL-22	Promotes tissue regeneration and repair
Th1 cytokines	
IL-2	Stimulates T-cell growth and proliferation and cytotoxicity by NK cells
TNF-β	Tumor necrosis factor-β: important regulatory cytokine; mediates cell killing; also has other metabolic effects
IFN-γ	Interferon-γ: stimulates Th1 cell, NK cell, and macrophage activation and MHC expression by APCs; inhibits Th2 cell differentiation
Th2 cytokines	
IL-4	Stimulates production and class switching of IgG1 and IgE and growth of mast cells
IL-5	Stimulates IgA production and growth of eosinophils
IL-6	Stimulates lymphocyte growth and acute phase protein production by the liver
IL-9	Enhances mast cell activity
IL-10	Suppresses Th1 cell and macrophage activity and costimulates mast cell growth
IL-13	Stimulates B-cell growth and differentiation and inhibits macrophage activity
Tr1 cytokines	
IL-10	Suppresses Th1 cell and macrophage activity and costimulates mast cell growth
TGF-β	Transforming growth factor-β: inhibits Th1 cells
Hematopoietic growth factors	
IL-3	Growth factor for hematopoietic progenitor cells
G-CSF	Granulocyte-colony-stimulating factor: stimulates proliferation and differentiation of cells in the bone marrow to granulocytes
GM-CSF	Granulocyte–macrophage colony-stimulating factor: stimulates growth and differentiation of myeloid cells
IL-7	Induces lymphocyte differentiation, induces RAG1 and RAG2 expression, which is required for TCR and Ig gene rearrangement
IL-15	Induces differentiation of NK and NKT cells

(continued)

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The Geoepidemiology of Autoimmune Liver Disease

3

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Key Points

- Autoimmune liver diseases often coexist with other rheumatological conditions and virtually all rheumatological diseases can impact the liver: in particular, a clinically relevant injury is rare while liver enzyme abnormalities are observed in up to 43 % of patients.
- Understanding the epidemiology of primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), and autoimmune hepatitis (AIH) is crucial to the clinician in the rheumatology practice, but data are burdened by the lack of symptoms until later stages of cirrhosis while definitive diagnostic criteria are lacking for PSC.
- The prevalence and incidence rates of autoimmune liver diseases are derived from descriptive case-finding studies which are poorly reliable or comparable along with the absence of established diagnostic criteria in the case of PSC.
- The most frequent cause of biochemical liver abnormalities among rheumatic patients is provided by medication-induced hepatotoxicity, particularly caused by nonsteroidal anti-inflammatory drugs, opioids, antidepressants, disease-modifying antirheumatic drugs, and even anti-TNFα and anti-IL6 biologics.
- Similar to other rheumatological conditions, PBC and AIH recognize a frank predominance of female patients while PSC affects men more frequently.
- The incidence and prevalence of PBC appear to be increasing worldwide, possibly secondary to longer survival in the former case and to physician awareness and more sensitive antibody testing in the latter.
- According to international guidelines, a screening for viral hepatitis should be performed before starting treatment with an immunosuppressive agent, in order to avoid virus reactivation.

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The Continuum Between Epidemiology and Comorbidities

The liver plays a pivotal role not only in the induction of the immune response against pathogens, but also in the maintenance of tolerance against self-molecules, being one of the largest lymphoid organs [1]. It is therefore not surprising that the liver may be targeted by a tissue-specific inflammatory process as observed in primary liver autoimmune diseases, namely autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). Primary immune diseases of the liver are characterized by peculiar histopathology and progressive courses, while virtually all rheumatologic diseases can affect the liver. A clinically significant liver involvement is rare; conversely, liver enzyme abnormalities may be observed in up to half of rheumatology patients. In most cases, a liver biopsy will only demonstrate minor changes and the biochemical findings can be ascribed to the primary affection, while in a small number of patients with rheumatic diseases an overlap syndrome with a coexisting

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primary liver disease can be diagnosed. In this setting the liver damage is usually progressive, frequently complicated by cirrhosis and portal hypertension. It should, however, be reminded that the most frequent cause of biochemical liver abnormalities among rheumatic patients is provided by medication-induced hepatotoxicity. This chapter is designed to review the wide spectrum of liver involvement that can be seen in the clinical management of patients with extrahepatic autoimmune diseases, but we are convinced that a preliminary discussion of the incidence and prevalence of PBC, PSC, and AIH is a necessary step to understand and manage rheumatological comorbidities in clinical practice [2, 3]. While autoantibodies have different roles in the three conditions, the histological pattern is quite specific, and liver biopsy is a key point for the diagnosis of difficult cases, albeit not recommended in all patients.

The Epidemiology of Autoimmune Liver Disease

PBC

PBC is a chronic cholestatic liver disease characterized by lymphocytic infiltrate of the small bile ducts, along with the frequent finding of non-caseous granulomas. The vast majority of PBC cases are asymptomatic at diagnosis, and serum autoantibody detection and cholestatic enzymes (i.e., alkaline phosphatase, gamma glutamyl transferase) usually prompt the diagnosis [4, 5]. Serum anti-mitochondrial (AMA) antibodies are the most specific marker detected in PBC and may also cluster among first-degree relatives (1–13 %), thus supporting a genetic component involved in PBC. The pathogenesis of PBC is unknown, but the association with AMA and antinuclear antibody (ANA) in most cases prior to the clinical onset and the detection of autoreactive T cells supports the hypothesis that PBC is an autoimmune disease mediated by a humoral response against mitochondrial enzymes. PBC is significantly more common in women with a female/male ratio estimated as 9/1 and it is commonly diagnosed in middle-aged postmenopausal women [6].

PBC in the General Population

A recent systematic review on epidemiologic studies reported PBC incidence rates ranging between 0.33 and 5.8 per 100,000 inhabitants/year, and prevalence rates between 1.91 and 40.2 per 100,000 inhabitants and that both figures have been increasing over the past years [7]. Both yearly incidence (0.33–5.8/100,000) and point prevalence (1.91–40.2/100,000) rates manifest a wide variability with median values of 1.55 and 13.7, respectively. These rates are the result of the analysis of studies with variable criteria for case selection, populations, countries, ethnic backgrounds, and other variables, thus making the results poorly comparable. This may also explain the wide range of incidence and prevalence rates with the highest reported in Olmsted County (USA) and Newcastle upon Tyne (UK) and attributed to geographical factors or genetic risks as well as to the presence in these areas of dedicated medical systems [7] (Table 3.1). Of note, studies performed with different approaches in later time periods in the Australian state of Victoria [8, 9] and in the Canadian States of Ontario and Alberta [10, 11] show

Table 3.1 Synopsis of population-based epidemiological studies of PBC

Year	Location	No. of cases	Annual incidence (per million)	Prevalence (per million)
1980	Sheffield, UK	34	5.8	54
1980	Dundee, UK	21	10.6	40.2
1983	Newcastle, UK	117	10	37–144
1984	Malmoe, Sweden	33	4–24	28–92
1984	Western Europe	569	4	23 (5–75)
1985	Orebro, Sweden	18	14	128
1987	Glasgow, UK	373	11–15	70–93
1990	Umea, Sweden	111	13.3	151
1990	Ontario, Canada	225	3.26	22.4
1990	Northern England	347	19	129–154
1995	Victoria, Australia	84	–	19.1
1995	Estonia	69	2.27	26.9
1997	Newcastle, UK	160	14–32	240
2000	Olmsted county, MN (USA)	46	27	402
2005	Sabadell, Spain	87	17	195
2009	Alberta, Canada	137	30	227
2012	Southern Israel	138	20	238
2012	Iceland	168	22.5	383

For further details please refer to Boonstra et al. [7]

3–10-fold prevalence increases. The epidemiology of PBC is largely dependent on the discrimination of serum AMA as, depending on the type of assay used, these are the hallmark of PBC and are identified in nearly 95 % of patients (versus <0.5 % of healthy subjects, commonly at low titers) [12–14]. The autoantigens most commonly recognized by AMA are respiratory chain enzymes, particularly the lipoylated domains of the PDC-E2 and 2-oxo glutaric acid dehydrogenase complex [15]. Detectable AMA are one of the criteria for diagnosis, and it is thus important that the routine tests by indirect immunofluorescence are negative AMA in a fraction of established cases, and this may also affect the epidemiological studies available [16]. Of note, AMA may precur the occurrence of PBC by decades, but appear to have a significant predictive value in asymptomatic subjects without evidence of cholestasis. When data are cumulatively considered, a surprisingly high prevalence rate ranging from 0.25 to 1 % should be considered likely for healthy general population. Furthermore, serum ANA are positive in about 50 % of PBC cases, and recent works have identified specific target antigens [17], but data on their case-finding capacity are lacking as these markers have not been used for case-finding purposes. PBC affects women significantly more commonly than men, but one should also note that the sex imbalance among AMA-positive subjects (and not fully recognized PBC cases) in large serum collections is significantly lower (2–3:1), thus suggesting that a discrimination bias may apply also in this case, similar to the disease prevalence [18] (Table 3.1). The proposed mechanisms for PBC female predominance will be discussed in a later paragraph. Finally, the natural history of PBC largely affects its epidemiology and survival rates are related to serum bilirubin levels while the impact of medical treatments remains controversial. Liver transplantation is the ultimate curative treatment with adequate survival rates despite recurrence may occur in 18 % and 30 % of cases at 5 and 10 years, respectively [19].

PBC in Family Members and Monozygotic Twins

The study of twins is a powerful tool to estimate the role of genetic predisposition and environmental influence in the onset of complex diseases [20]. Concordance rates for PBC have been reported as 63 % in monozygotic (MZ) sets and 0 % in dizygotic (DZ) twins and the same series also included one pair of MZ twins with significantly different PBC phenotypes despite the concordant diagnosis [21]. Of note, the MZ twin concordance rate is among the highest reported for autoimmune diseases [22]. To further stress the importance of genetics, we note that the occurrence of PBC among first-degree relatives of patients (coined "familial PBC") is common and these have a 50–100-fold higher risk to develop the disease [23]. First-degree relatives in general and mothers, sisters, and daughters in particular have a significantly higher prevalence of presenting serum AMA [24]. Indeed, the sibling relative risk, which is the odd ratio for PBC of a subject with a sibling affected by the disease, is 10.5, among the lowest for autoimmune diseases. The cumulative prevalence of familial PBC (i.e., the presence of multiple cases in one family set) varies within the 1–6 % range according to geographical areas, possibly due to the same case-finding differences that have been previously discussed, while a higher prevalence is proposed for serum AMA [24].

PBC Sex Predominance

As mentioned before, PBC is characterized by female preponderance as in the case of most autoimmune diseases. Only 7–11 % of PBC cases are reported to develop in men with a resulting female/male ratio of approximately 9–10/1. It is interesting to note that PBC sex ratio is shared with frequently coexisting conditions such as Sjogren syndrome and autoimmune thyroid disease. PBC symptoms are similar in men and women, but data on natural history and liver transplantation suggest that men may have a worse disease progression [25]. Genetic studies are limited for men with PBC while twin studies did not include male pairs, while immunological studies in male and female PBC cases show that sex hormones play a different role in inflammation and autoantibody production without differences in serum autoantibody profiles [25]. Discussing in detail the possible mechanisms influencing female preponderance are outside the aims of this discussion [26, 27], but we will address the current sex chromosome hypothesis. Cholestasis is frequently found in patients with defects of the X-chromosome causing Turner's syndrome, premature ovarian failure, and IPEX syndrome [25]. Skewed X chromosome inactivation is associated with late-onset autoimmune diseases, but not in PBC itself [28, 29]. Further, peripheral lymphocytes of patients with PBC are characterized by an increased rate of X chromosome monosomy [23] and a different methylation status was identified in two genes, CLIC2 and PIN4 in the twin affected by PBC, and this epigenetic status may account for the disease expression. Taken altogether, the available evidence supports the view that X chromosome gene defects may cause a haploinsufficiency (secondary to gene deletion or silencing) which may predispose to PBC onset [18, 30, 31].

Risk Factors for PBC Onset

As mentioned before, PBC etiopathogenetis remains to be determined and risk factors may indicate new pathways or support previously reported ones (Table 3.2). The largest epidemiological studies agree on the observation that having a first-degree relative with PBC, a history of recurrent urinary tract infections, past smoking, or the use of hormone replacement therapies are significantly associated with an increased risk of PBC [32, 33]. Most recently, a NIEHS-sponsored workshop reviewed the epidemiology of autoimmune diseases [34] and in particular focused on chemicals contained

Table 3.2 Main risk factors and comorbidities in PBC, PSC, and AIH

	PBC	PSC	AIH
Risk factors	• First-degree relatives with PBC	• Concomitant presence of IBD, mainly ulcerative colitis	• HLA genes
	• Genetic factors	• Continuous exposure to endogenous and exogenous toxins	• Autoimmune polyendocrine syndrome type 1 with AIRE mutations
	• History of recurrent urinary tract infections	• Ischemic injury	• Environmental factors
	• Past smoking	• Bile toxicity	
	• Hormone replacement therapies	• HLA alleles A1, B8, and DR3	
	• Frequent use of hair dye and nail polish		
Comorbidities	• Sjögren's syndrome	• Ulcerative colitis (up to 75 % of cases)	• IBD (mainly ulcerative colitis)
	• Scleroderma	• Colorectal and hepatobiliary malignancies (i.e., cholangiocarcinoma)	• PSC
	• Rheumatoid arthritis		• Systemic autoimmune diseases (i.e., lupus, scleroderma, and myositis)
	• Mixed connective tissue disease		• Celiac disease
	• Autoimmune thyroiditis		• Viral infections (i.e., HIV, Epstein-Barr)
	• Celiac disease		
	• Higher risk of overall cancer		

in hair dye and nail polish as two previous studies showed higher risk to develop PBC in women who were using these chemicals [32, 33]. However, the epidemiologic studies reporting the highest incidence of PBC in Northern European countries (namely UK and Scandinavia) and Northern American countries (i.e., Minnesota) may be biased by different and sometimes better methodologies for diagnosis and may thus undermine these associations. Of most importance is the observation that scholarity and family income are significantly associated with the risk of developing PBC and should be further investigated [33].

PBC Comorbidities

PBC is frequently associated in up to 30 % of patients to one or more additional autoimmune diseases, and this association seems to influence the disease prognosis. Such common coexistence supports the numerous theories on a common etiological background between autoimmune diseases as well as represented by the shared female predominance. Further, autoimmune diseases commonly associated with PBC also share the limited response to immunosuppressants. A paradigmatic example comes from systemic sclerosis (SSc), the autoimmune disease most frequently associated with PBC being found in approximately 3–50 % of cases [35], mostly as the ACA-positive limited cutaneous form. One hypothesis of the frequent SSc-PBC association is the common pathogenesis against circulating microchimeric fetal cells that may trigger the abnormal autoimmune response, but data are unconclusive. Of note, patients with PBC and SSc appear to have a more benign course of the liver disease [36, 37]. Further, Sjögren's syndrome is diagnosed in 15–20 % of patients with PBC [6] and the two conditions can be defined as "autoimmune hepitelitis" with obvious tissue specificity differences [26]. Other comorbid conditions that may be identified in PBC patients are rheumatoid arthritis, mixed connective tissue disease, autoimmune thyroiditis, and celiac disease [38], but their incidence in PBC patients is much lower than the one observed for SSc and Sjögren's syndrome. Osteopenia is frequently encountered in postmenopausal women and may be more severe if PBC coexists; this should be addressed in the clinical management with calcium and vitamin D supplementation along with other dedicated treatments [39, 40]. Finally, despite low prevalence rates in PBC series, self-reported systemic lupus erythematosus (SLE) was found as a significant risk factor for PBC in one study [33]. Similar to other chronic liver diseases, PBC is associated with a higher risk of hepatocellular carcinoma which occurs with similar incidence compared to other etiologies of liver cirrhosis [41].

PSC

PSC is a chronic cholestatic liver disease characterized by the chronic inflammation of the intrahepatic and/or extrahepatic biliary ducts and fibrosis, leading to large duct stenosis and eventually liver cirrhosis from long-standing cholestasis [42]. The etiopathogenesis of PSC is largely unknown, but genetic (HLA-B8 and HLA-DR3) and immune factors are involved in the disease onset, as supported by the significant association with inflammatory bowel disease (IBD), particularly ulcerative colitis. As many as 2.4–7.5 % of patients

Table 3.3 Prevalence and incidence rates reported for PSC since 2000

Year	Country	Prevalence (yearly)	Incidence	References
2010	Sweden	16.2	1.22	[130]
2008	UK	3.04–4.8	0.34–0.48	[131]
2007	Canada	n/a	0.92	[132]
2004	UK	12.7	0.91	[133]
2003	USA (Olmsted County)	13.6	0.9	[134]
2002	USA (Alaska)	0	0	[135]
2000	Singapore	1.3/100,000	n/a	[136]

with IBD, primarily ulcerative colitis, have PSC [43]. Apart from the clinical suspicion raised in patients with symptomatic IBD, the symptoms of uncomplicated PSC are similar to those seen in PBC and are nonspecific, including pruritus, fatigue, and upper abdominal discomfort, and the diagnosis can be incidental [42]. On the other hand, complications include infectious cholangitis, jaundice, or cholangiocellular carcinoma and are thus more suggestive of the diagnosis. Different from PBC, PSC is a male-predominant disease by a 3/1 ratio, and the peak age for PSC diagnosis is 20–30 years.

PSC in the General Population

In 2011, a systematic review and literature meta-analysis on the incidence of PSC was published, showing a cumulative incidence of 1.0 (0.82–1.17) per 100,000 inhabitants in six population-based studies of North America and Europe [44]. On the other hand, the prevalence of PSC is estimated to range within 0.22–8.5 per 100,000 inhabitants, but these rates may be influenced by the higher risk of ulcerative colitis and different HLA haplotype representation among ethnic groups [38]. Similarly to PBC, PSC seems to have higher incidence in Northern Europe, particularly Scandinavia, and the Northern US, including the Olmstead county also characterized by high PSC rates (Table 3.3), with the lowest numbers in South America, Africa, and Asia [45]. A recent work on patients listed for liver transplantation demonstrates that different PSC phenotypes characterize ethnic and racial groups [46], similar to what observed for IBD, with African Americans developing an end-stage liver disease at an earlier age. This observation confirms that genetic background plays a significant role in the etiology and global distribution of the disease, but it is not sufficient to determine the phenotypic manifestation of PSC [46]. Most patients with PSC have serum autoantibodies, but these are not specific, as in the case of anti-neutrophil cytoplasm antibody (ANCA) (80 %) [47], ANA, and anti-smooth muscle antibody (SMA) (20–50 % of PSC patients) [48]. Newer autoantibodies directed at p53 have been identified recently in autoimmune liver diseases, but there is no report in PSC [49]. Only limited data are available on the survival rate of PSC patients. A report published in 2007 estimated the mean survival time since the diagnosis to be approximately 25 years, with a significantly shorter median time to either death or liver transplantation of approximately 10 years [50]. A different study reported median survival rates of 12–18 years until liver transplantation or death [51]. The occurrence of cholangiocellular carcinoma is likely the major determinant of survival and its occurrence is not influenced by fibrosis and manifests a 10–15 % lifetime risk of development. Current treatments are largely unsatisfactory, particularly in the mass-forming intrahepatic variant that does not cause jaundice and is thus diagnosed at advanced stages.

PSC in Family Members and Monozygotic Twins

Different from PBC, data on family cases of PSC are limited to few reports. MZ twins concordant for PSC and ulcerative colitis have been described with significantly different severity for both conditions [52]. In one report, one twin had severe PSC but mild ulcerative colitis and died of infectious cholangitis, while the other twin had severe ulcerative colitis and mild PSC through determination of cholestasis index. The second report included three families with members affected by PSC and ulcerative colitis, and in each family two siblings were affected, including a set of twin brothers in one case. All six cases had both PSC and ulcerative colitis, with the exception of one individual who had PSC only. A third report was published in 2005 and included two brothers concordant for PSC and underlined their concordant HLA haplotypes DR3-DQ2 and DR6-DQ6 [53]. In a more recent study from Sweden, first-degree relatives of patients manifested a PSC prevalence of 0.7 %, or a 100-fold increased risk of disease compared with the general population [54], supporting the hypothesis that genetic factors are of importance for development of PSC [55], in agreement with the data from genome-wide association studies. Lastly, a second study from Sweden also confirmed this hypothesis and demonstrated that the risk of PSC was statistically significantly increased in the offspring, siblings, and parents of the PSC patient cohort, 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 [56].

PSC Sex Predominance

PSC recognizes a 2–3/1 male predominance that is unique among autoimmune liver diseases. The sex ratio mirrors what observed in IBD in which we have significantly more data compared to PSC only. Similar to PBC, however, sex chromosome changes have been advocated based on a small number of X-linked hyper-IgM syndrome cases, a rare condition caused by mutations in the X-linked CD40 ligand gene, with normal or elevated serum IgM but reduced levels of IgG and IgA, and defective T-cell function, leading to high risk of severe infections and neoplastic transformation. Indeed, a subgroup of patients with X-linked hyper-IgM syndrome develops hepatitis (9 %) and sclerosing cholangitis induced by *Cryptosporidium* [57]. Therapy in these patients is based on intravenous immunoglobulin and ursodeoxycholic acid, but there is an established risk to develop hepatocellular and cholangiocellular carcinoma.

Risk Factors for PSC Onset

The etiology of PSC is unknown, and several triggers have been proposed for the immune-mediated response against the bile ducts (Table 3.2). Studies on autoantibody production in PSC have also identified the increased prevalence of HLA alleles A1, B8, and DR3 in PSC patients [58], which may represent genetic factors predisposing to PSC at least in some ethnic groups, while more recent genome-wide association studies have proven less conclusive than what observed in PBC with only limited associations [59–61]. As mentioned, the strongest risk factor is indeed a concomitant IBD, mainly ulcerative colitis, that usually is characterized by a mild and quiescent course. However, both PSC and ulcerative colitis require follow-up also for the high risk of colorectal malignancy that requires routine colonoscopic surveillance. Other factors frequently associated with higher PSC risk are the continuous exposure to endogenous toxins like the LPS component of bacteria in the portal system, or even to exogenous toxins as demonstrated by the high incidence of PSC in a region with toxic waste areas [62].

PSC Comorbidities

As mentioned before, PSC is strongly associated with IBD, most often ulcerative colitis, that is diagnosed in up to 75 % of cases when endoscopy with histology is performed also in the absence of symptoms [63], while only a minority of patients with ulcerative colitis will develop PSC. On the contrary, the comorbidity with Crohn's disease is uncommon and only a few cases are reported in literature [64]. Screening of serum liver tests should be performed in IBD as PSC may be asymptomatic and early treatment could impact the clinical evolution of PSC. More importantly, the presence of both conditions leads to a poor prognosis and higher risk to develop both colorectal and cholangiocellular carcinoma [65, 66]. As in other cases of chronic cholestasis, the possibility of increased bone loss and osteoporosis should not be overlooked and warrants an adequate preventive treatment [67].

AIH

AIH is a chronic hepatitis caused by the autoimmune injury of hepatocytes, rapidly progressing to liver cirrhosis and failure. Two major types are recognized based on the autoantibody profiles, with type 1 showing ANA and SMA positivity and affecting adult patients and type 2 characterized by liver kidney microsomal antibody (LKM) antibodies in pediatric patients. The etiology of AIH is unknown, and it is not clear what breaks tolerance to self and elicit an immune response specific to the hepatocellular parenchyma [68]. The histologic features are mainly represented by "interface hepatitis," and the liver biopsy allows to rule out overlap syndromes with PBC or PSC and determine the stage of liver injury [69]. More important and different from PBC and PSC, AIH responds significantly to immunosuppressants, particularly glucocorticoids and azathioprine.

AIH in the General Population

Epidemiology studies on AIH are limited, but the disease is believed to have an annual incidence of 2/100,000 people and a point prevalence of 15/100,000 in the Caucasian population of Northern Europe [70]. As for PBC, AIH has a striking female predominance and is more frequent in women of younger age (<40 years), including children. Autoantibodies are crucial for AIH diagnosis, being a part of the currently accepted diagnostic criteria [71]. AIH is divided in two main types according to the autoantibody specificity: type 1-AIH manifests ANA and SMA, and type 2-AIH anti-LKM-1 and anti-LC1 antibodies [72]. Some autoantibodies also seem to have a prognostic value, as for anti-soluble liver antigens (SLA) that are usually predictive of more severe disease and worse prognosis, while identification of ANCA is common in AIH but poorly specific [72]. The 10-year survival rate for AIH patients is estimated to be 96 % also for those patients who are not responsive to medications and must undergo liver transplantation [73]. The majority of patients usually respond to steroids and azathioprine within 6–12 months as represented by the improvement in biochemical parameters of disease activity and significant improvement in histological disease activity. However, 20–40 % of patients will not achieve disease remission and will require alternative therapies with other immunosuppressants.

AIH in Family Members and Monozygotic Twins

Familial reports of AIH are limited to one family in which out of five members with similar human leukocyte antigen haplotypes, two developed AIH, one was ANA-positive, and the remaining two had no features of autoimmunity [74].

Data on the concordance for AIH in twins are limited to one report by Nolte et al. [75] on the case of acute hepatitis of unknown origin, associated with high titer anti-LKM1 characteristic of type 2 AIH in a pair of identical twin brothers. Data on specific genes associated with AIH are also limited and one recent study investigated children with type 1 and type 2 AIH for variants of the AIRE gene involved in the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a rare autosomal recessive disorder typically presenting with chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal failure variably accompanied by other symptoms. The heterozygous transversion c.961C>G (p.Ser278Arg) located in exon 7 was identified in four patients with AIH type 1, and mostly in those presenting with a positive family history for autoimmune diseases [76].

Risk Factors for AIH Onset

Among AIH predisposing factors, HLA haplotypes genes have been widely investigated, but results vary due to the analysis of different ethnic groups. AIH is also a complex disease recognizing a genetic background and the role of an environmental trigger [68]. As previously mentioned, AIH has been studied in the setting of the "autoimmune polyendocrine syndrome type 1" that is characterized by AIRE mutations and consequent high susceptibility to mucocutaneous candidiasis and autoimmune manifestations [77]. Vogel et al. demonstrated that AIRE geneti mutations could influence mechanisms of immunologic tolerance, and thus may be candidate etiologic factors for the onset of autoimmune liver diseases [77]. About 20 % of patients with the "autoimmune polyendocrine syndrome type 1" may develop AIH, but the majority of AIH cases in children and adults are sporadic and not associated with the most studied AIRE mutations, so they may reflect different phenotypic expressions of the disease [78]. Finally, there are reports of AIH induced by anti-TNFα treatment with monoclonal antibodies [79, 80], while the same treatments may prove beneficial in selected cases [81].

AIH Comorbidities

Similar to PSC, also AIH is more prevalent in patients with IBD, mainly represented by ulcerative colitis detected in up to 16 % of patients with AIH. A small subgroup of patients manifests signs of AIH-PSC overlap syndrome and the management of these patients depends on liver histology, the serum autoantibody profile, the degree of biochemical cholestasis, and cholangiography, because some of these patients will respond to immunosuppression. Other diseases described in association with AIH are systemic autoimmune diseases (i.e., lupus, scleroderma, and myositis), celiac disease [82], and viral infections (i.e., HIV, EBV) [83, 84]. The risk of osteopenia in patients with AIH is secondary to the prolonged use of steroids, along with the possibility to cause metasteroidal diabetes. The risk of hematological adverse events from azathioprine use should not be overlooked and when the use of azathioprine is foreseen, the screening for thiopurine methyltransferase deletions is recommended [85, 86].

Liver Involvement in Systemic Rheumatic Disease or Secondary Immune Liver Diseases

Liver involvement in systemic rheumatic diseases is common, even though the liver is generally not the major target organ (Table 3.4). These conditions will be separately discussed in further details.

Sarcoidosis

Sarcoidosis presents the highest frequency of liver involvement with hepatic granulomas observed in virtually all patients. Granulomas are usually small and mainly located in the portal spaces and hepatic sarcoidosis is generally clinically silent. Rare clinical manifestations of sarcoid liver disease include cholestasis, Budd-Chiari syndrome (BCS), and extrahepatic biliary obstruction from enlarged granulomatous lymph nodes. In a minority of patients, the disease can ultimately lead to portal hypertension and cirrhosis and it has been suggested that such serious complications may be due to the increased intrahepatic resistances secondary to arterial-venous shunts and to elevated resistances in the intrahepatic sinusoids. Another hypothesis suggests that

Table 3.4 Prevalence of liver injury in the most common systemic rheumatic diseases

	LFT alteration prevalence (%)	Predominant biochemical profile	Histological alterations prevalence (%)
Sarcoidosis	50–90	Hepatocellular	99
Sjogren's syndrome	50	cholestatic	18
Systemic lupus erythematosus	30	hepatocellular	20
Systemic sclerosis	1	cholestatic	9
Rheumatoid arthritis	77	cholestatic	65
Polymyalgia rheumatica	62	cholestatic	–

ischemia secondary to primary granulomatous phlebitis of the portal and hepatic veins may be responsible of cirrhosis and focal fibrosis [87]. Rare cases of ductopenia-related liver sarcoidosis have been reported; a total of 32 cases of hepatic sarcoidosis with chronic cholestasis resembling PBC or PSC have been described in the literature and this possibility should not be overlooked [88].

Connective Tissue Diseases

Liver involvement is considered to be the most common non-exocrine feature in *primary* Sjögren's syndrome (pSS) [89, 90]. Hepatomegaly occurs in 11–21 % of patients presenting with pSS, while elevated liver function tests are described in 27–49 % of the patients. These alterations are usually mild and of little clinical significance, may be either persistent or intermittent. A cholestatic biochemical profile is detected in 30 % of cases, but predominantly hepatocellular or mixed patterns are also observed [91]. To note, 47–73 % of patients with PBC report sicca symptoms and 26–93 % of these subjects manifest histological changes compatible with pSS at salivary gland biopsy. Interestingly, the salivary gland ducts of all PBC patients, independently from the presence of sicca symptoms, manifest a PBC-like immunohistochemical monoclonal AMA staining specific for the self-antigen PDC-E2. As a matter of fact, the two conditions share many similarities, both affecting as preferential target the epithelium. In PBC the major target is bile duct, salivary gland epithelia, and the uroepithelium, while salivary gland, bile duct, bronchial, alveolar, and tubular epithelium provide the main target in pSS. Therefore, the two conditions are often referred to as "generalized autoimmune epithelitis." Histology shows a predominance of lymphocytic infiltrate, mainly CD4+, which is located around the bile duct in PBC and around the salivary duct in pSS. Even though the serum antibodies detected in the two diseases are directed against ubiquitous proteins expressed in all nucleated cells, disease manifestations are organ-specific in PBC and—to a minor extent—in pSS, suggesting that the epithelia are active participants in the pathogenesis of both conditions. A cell-specific lack of glutathionylation has been described in the biliary epithelial cells in PBC and in the salivary duct epithelium in pSS. As a consequence, antigens remain intact and retain their immunogenicity during cell apoptosis. In both PBC and pSS, it has been shown that IgA against self-antigen derived from local plasma cells are internalized into the epithelial cells as complexes with poly-Ig-receptor, then transported to the apical surface of the cell through a process called transcytosis to be secreted at the mucosal surface after poly-Ig-receptor cleavage. More importantly, in PBC IgA AMA have been detected not only in the bile, but also in saliva and urine from patients. Noteworthy, IgA AMA have been demonstrated to be produced locally, supporting the hypothesis that epithelial tissues other than cholangiocytes are involved in PBC. PBC and pSS do share some other similarities: infectious agents have been proposed as triggers for tolerance disruption in both diseases. *E. coli* and *N. aromaticivorans* have been identified as the best candidates in PBC, while in pSS the evidence favors viruses as EBV, CMV, and retroviruses. Conversely, the recently completed genome-wide association studies performed in PBC reported an association with polymorphisms of HLA, interleukin (IL)-12A, IL-12RB2, and a less significant one with STAT4; a similarity with pSS could be detected only in the minor association with STAT4. Non-concordant findings have also been provided by epigenetics, an emerging link between genomics and environment in generating disease susceptibility and phenotype variability in adult life. A different methylation in hemidesmosome gene has been identified in PBC, while a different methylation at X-linked promoters has been reported in pSS [26].

In SLE, abnormal liver function tests are frequently observed, found in up to 50 % of patients at some point in the disease course [89]. In 20 % of cases, liver test abnormalities occur during disease flares, while in 23 % of SLE cases with abnormalities in liver functions, no cause for pathological liver tests could be identified [92]. In these cases, the increase in serum ALT was generally mild. When a liver biopsy was performed, histology showed portal inflammation. Increase in liver tests has been shown to correlate with disease activity and to improve upon steroid treatment. A chronic active hepatitis—termed "lupoid hepatitis" by some authors—is described in up to 5 % of patients with SLE. Antibodies to ribosomal P protein have been shown to strongly correlate with lupus hepatitis, being detected in a significant proportion of patients (69 %). In this setting, histology demonstrates predominantly mild lobular inflammation without piecemeal necrosis. A number of different histopathological patterns can be found in liver biopsies of SLE patients: small artery vasculitis has been reported in up to 21 % of cases [91], nonalcoholic fatty liver diseases in 20–73 %, nodular regenerative hyperplasia (NRH) in 5.7 %, chronic persistent or active hepatitis in 2.4 %, cirrhosis in 1.1 %, and fibrosis in 0.8 % [93, 94]. Rare cases of giant cell hepatitis, granulomatous hepatitis, massive hepatic necrosis, cholangitis, isolated portal hypertension, and liver infarction have also been described.

A wide range of hepatic diseases have been reported in association with the presence of anti-phospholipid antibodies (aPL), the serum markers of anti-phospholipid syndrome (APS). APS-related hepatic manifestations are mainly of vascular origin, ranging from thrombosis of major arterial or venous beds to microthrombotic conditions. However, non-thrombotic liver diseases have also been reported.

The most striking association is that of aPL positivity with BCS, the first report dating back to 1984. Since then

few more cases have been described in literature. BCS is a clinical and pathological entity characterized by structural and functional abnormalities of the liver resulting from obstruction of the outflow of hepatic venous blood [95]. BCS is clinically characterized by abdominal pain, hepatomegaly, and ascites, and the clinical presentation may range from almost asymptomatic to fulminant liver failure. The pathogenic role of aPL in BCS is controversial: some authors have suggested that autoantibody production is just an epiphenomenon secondary to the liver damage. However, in some cases aPL were detected before the onset of BCS strongly suggesting that the aPL are not a mere consequence of liver abnormalities. It should be considered that BCS may be the first clinical manifestation of APS: this syndrome should be taken into account in the differential diagnosis of hepatic vein thrombosis.

After the first report of a possible association of aPL with hepatic-veno-occlusive disease (HVOD), an unusual hepatic disorder characterized by hepatomegaly and ascites, only sporadic cases have been documented. On the other hand, several cases of histologically proven occlusion of small hepatic veins, which differs from HVOD by the absence of endophlebitis, were reported.

Hepatic infarction is a rare entity thanks to the dual blood supply to the liver; nevertheless, several cases of hepatic infarction have been reported in association with aPL.

aPL positivity has also been linked to hepatic artery thrombosis (HAT), a main cause of graft loss and patient mortality after liver transplantation. In literature, there is no consensus about the necessity of screening for aPL in the pre-transplant workup. Even though some authors claim that aPL positivity does not identify patients at high risk for post-transplant vascular thrombosis, aPL testing in liver pre-transplant patients may be recommended and a close follow-up for signs of HAT in aPL-positive patients may be warranted. Idiopathic portal hypertension has also been rarely reported in association with aPL: microthrombi may represent a possible cause for the occurrence of portal hypertension.

Among the non-thrombotic liver diseases, several reports have documented a relationship between aPL and NRH, an uncommon disorder characterized by the transformation of the liver parenchyma into nodules of hyperplastic hepatocytes without fibrosis. In one study, sera from 13 patients with histologically defined NRH were tested for aPL: 77 % of the NRH patients had aPL compared with 14 % of the patients with autoimmune liver diseases. Although a causal relationship between aPL and NRH is not clearly established, determination of these antibodies may still be advisable in NRH population. aPL positivity in patients with liver cirrhosis has been reported in sporadic cases; few authors have depicted an association between the severity of alcoholic liver cirrhosis and the presence of aPL. However, definite conclusions about this relationship cannot be drawn as in this setting aPL may reflect liver lesions and immunological dysfunction. Finally, aPL are frequently detected in PBC and PSC—being associated with a more severe hepatic disease. In particular, in a recent study, IgG and/or IgM anti-cardiolipin antibodies (aCL) have been detected in 40 % of PBC and PSC patients, compared to a mere 2.25 % among healthy individuals. In PBC, IgG aCL were associated with cirrhosis, increased Mayo risk score, and thrombocytopenia, while among PSC patients a relationship with longer disease duration and biochemical activity emerged [96].

In SSc, a mild liver involvement has been reported in 1.1 % of patients, while at autopsies liver fibrosis was found in 8.8 % of patients, slightly more prevalent than among non-SSc controls [97].

Arthritis

A clinical evidence of liver disease is generally lacking in patients with rheumatoid arthritis (RA). Hepatomegaly is reported in 25 % of patients by ultrasound and correlates with elevations in rheumatoid factor. Even though liver involvement is uncommon, abnormalities in liver tests have been described in up to 77 % of RA cases. As ALT and bilirubin are generally documented as normal, elevations in ALP and γ-glutamyl-transpeptidase (γGT) levels are the predominant biochemical abnormalities to mirror a cholestatic pattern of injury. ALP was shown to be increased in 18–46 % of patients with RA, while an elevation in γGT was observed in 23–77 % of RA subjects. In a clinical study, 65 % of unselected RA patients had abnormal liver biopsies. Mild portal chronic inflammatory infiltrate of the portal tract with small foci of necrosis was the most common histologic pattern, described in 50 % of patients. Fatty liver was diagnosed in 20 % of RA cases, while other histologic abnormalities included periportal fibrosis, sinusoidal dilatation, and rarely cirrhosis. Rheumatoid hepatitis is a rare complication of RA and tends to be mild with transiently elevated liver function tests. These abnormalities usually correlate with the activity of the underlying disease [98].

Hepatomegaly has been documented in 42–67 % of patients with Felty's syndrome, a rare clinical condition characterized by the triad of RA, leukopenia, and splenomegaly. Abnormal liver function tests were found in 10 of 18 patients in a prospective study. A rise in serum ALP was found in 25 % of patients; other abnormalities regarded aminitransferases, bilirubin, prothrombin time, and γGT. Histology showed non-specific changes as diffuse lymphocyte infiltrate, periportal fibrosis with lymphocytic infiltration, and portal hypertension.

Hepatosplenomegaly and liver test abnormalities, predominantly elevated aminotransferases and ALP, occur frequently in patients with adult onset Still's disease. Therefore,

these manifestations have been included in the diagnostic criteria for this clinical condition. Increases in liver enzymes are generally mild (2–5 times the upper limit of normal [ULN]), transient, and usually associated with disease activity. Fulminant hepatitis requiring liver transplantation has been reported, while chronic liver disease has never been described [99].

Psoriatic Arthritis (PsA) may manifest a high prevalence of liver steatosis, which is significantly associated with disease activity. Increased levels of serum ALP are a common finding among patients with ankylosing spondilitis (AS), occurring in 14–48 % of cases. ALP elevation is usually isolated, with normal aminotransferases and bilirubin levels. The clinical significance of the increased ALP levels in AS setting is rather debated: some authors have suggested that ALP may be a biochemical marker of disease activity, being a nonspecific reaction to inflammation. Consistently, ALP levels were found to correlate directly with ESR in untreated patients, while a reduction was observed after treatment with nonsteroidal anti-inflammatory drugs (NSAIDs).

Vasculitis

A cholestatic pattern with elevated ALP and γGT levels characterizes as many as 62 % of patients with polymyalgia rheumatica [100]. Interestingly, polymyalgic patients with elevated liver enzymes are at increased risk to have Horton's arteritis [101].

Liver involvement occurs in 16–56 % of patients with polyarteritis nodosa. Liver biopsy usually shows necrotizing hepatitis, while hepatic arteriograms may show caliber changes with corkscrew vessels and distal microaneurisms. When involving the portal veins and hepatic arteries, vasculitis can lead to atrophy of a liver lobe, liver infarction, acute liver failure, or nodular regenerative hyperplasia. Vasculitis of small and medium-sized arteries suppling the small bile ducts lead to intrahepatic sclerosing cholangitis [102].

There are only occasional case reports of granulomatous necrotizing hepatic involvement and mild non-specific lobular hepatitis in Wegener's granulomatosis [103].

In Behçet's disease, the most common hepatic complication is BCS: Behçet's disease accounts for 42.4 % of the cases of BCS in Turkey. Other hepatic conditions reported to occur in cases of Behçet's disease comprise hepatomegaly due to fatty liver or congestion, cirrhosis, acute hepatitis, and hepatic abscess [103].

IgG4-related cholangitis is one of the manifestations of IgG4-related systemic disease, a recently recognized clinical entity for which a significance remains debated. Commonly, patients present with obstructive jaundice and display an elevation in serum immunoglobulin G4. Histologically, an abundant infiltration of IgG4-positive plasma cells is detected in the biliary duct wall, with evidence of dense lymphoplasmacytic infiltration of the bile duct wall, transmural fibrosis, lymphoplasmacytic infiltration, and fibrosis in the periportal area [104].

Finally, liver involvement at autopsy is reported in 55–95 % of patients with amyloidosis, a condition which may complicate the course of some autoimmune conditions. Presenting symptoms include abdominal pain, hepatomegaly, fatigue, anorexia, and ascites. The most striking pathologic feature of hepatic amyloidosis is the massive deposition of amyloid in the hepatic parenchima, along the sinusoid within the space of Disse or in vessel walls, with subsequent cellular atrophy and diminished cell number [105].

Overlap Syndromes

Overlap syndromes are autoimmune connective tissue diseases characterized by the combination of features typical of at least two clinical entities. Overlap syndromes may frequently include AIH, PBC, or PSC (Table 3.5): two primary immune liver diseases may also coexist, or a primary immune liver disease and a systemic rheumatic disease may be present in the same patients.

AIH, PBC, and PSC may be found with higher prevalence rates in patients with systemic rheumatic diseaes. Accurate estimates of the prevalence of such overlap diseases are not available in literature: most studies are case reports or case series, with liver histology derived from autoptical investigations or liver biopsies performed on selected patients with liver enzyme abnormalities [106]. Several studies have confirmed a higher prevalence of

Table 3.5 Reported frequency rates for liver disease overlap syndromes

	Autoimmune hepatitis (%)	Primary biliary cirrhosis (%)	Primary sclerosing cholangitis (%)
Autoimmune hepatitis	–	4.2–9	1.4–49.1
Systemic lupus erythematosus	2.7–20	2.7–15	1 case
Primary Sjogren syndrome	6–47	35–57	11 cases
Systemic sclerosis	11 cases	51.2	1 case

primary autoimmune liver diseases among pSS patients. In a study on 45 patients, a diagnosis of PBC was established in 8.8 % of cases, 4.4 % of subjects were found to have AIH [33, 107, 108]. More recently, PBC was diagnosed in 6.6 % of 410 subjects with pSS. Ninety-two percent of pSS patients with a positivity for AMA demonstrated histologic feature consistent with PBC, suggesting the importance of AMA screening. Secondary liver disease in pSS is associated with elevated inflammatory markers, similarly to what is observed in the systemic manifestations of the diseases. There are only occasional reports in the literature describing patients with pSS and PSC, suggesting that this association may be sporadic rather than causative. It should also be remembered that the prevalence of hepatitis C virus (HCV) infection is much higher among patients with pSS than in the general population, ranging from 12 to 19 % [109].

In patients with SLE, overlap syndromes with AIH or PBC have been reported with a similar prevalence (2.7 %) and a family history for SLE was found to be independently correlated with PBC in a large case control study. It may be difficult to differentiate between AIH and lupus-associated hepatitis, given the common clinical and serologic presentation; histologic findings may be helpful [109]. The strong association between PBC and SSc is well characterized, being first described in 1934. Seven to 12 % of PBC patients present SSc, while PBC has been reported in 2.5 % among SSc patients. PBC prevalence in SSc population rises up to 51.2 % when considering solely patients with liver dysfunction [110]. Most subjects with PBC-SSc overlap syndrome have limited cutaneous SSc. Generally, these patients display positive AMA [111]. Liver disease in PBC-SSc patients may progress to cirrhosis and results in liver-related morbidity; however, mortality is more commonly ascribed to other scleroderma-related complications. SSc overlap syndromes with AIH and PSC have been reported only occasionally, in 11 cases and 1 case, respectively. Recently, it has been suggested that antibodies against centromeric protein I (CENPI, a protein localized in the inner kinetochore structure) may be a marker of concurrent autoimmune liver disease in SSc patients [112]. In addition, single case reports of AIH are found in polymyositis, dermatomyositis, RA, Still's disease, polymyalgia rheumatica, and polyarteritis nodosa [113]. Similarly, PBC has been anecdotally reported in polymyositis, dermatomyositis, RA, Still's disease, polymyalgia rheumatica, Churg-Strauss's syndrome, microscopic polyangiitis, Behçet's disease, and Schonlein-Henoch purpura. Finally, PSC has been reported in association with rheumatic diseases only exceptionally.

Viral Hepatitis in the Rheumatology Setting

A concomitant liver disease displays several implications on the therapeutic management of a rheumatologic condition. Indeed, hepatitis virus reactivation and drug-related liver injury are among the major concerns in rheumatology [114, 115]. Currently, there is no consensus on which patients should be screened before the institution of an immunosuppressive therapy. Two main strategies are available: screening patients considered at high risk for hepatitis B virus (HBV) infection or universal screening. Given the absence of risk factors in many patients with HBV infection and the possibility of adequate prevention, it is advisable to screen all patients requiring any immunosuppressive agent. The risk of HBV reactivation is well documented in HBsAg-positive patients who receive methotrexate and leflunomide. In addition, treatment of RA patients with low-dose methotrexate has been associated with fatal HBV reactivation in HBsAg-negative/anti-HBc-positive patients, even the immunological reconstruction after MTX withdrawal has been linked to fatal HBV reactivation. Severe cases of hepatitis flares have been described during treatment with anti-tumor necrosis factor α (anti-TNFα) agents in RA patients with chronic HBV infection. Other biological therapies, particularly rituximab (anti-CD20), have been involved in cases of HBV reactivation in HBsAg-positive individuals and HBsAg-negative/anti-HBc-positive patients with hemato-oncologic diseases. The risk of HBV reactivation with rituximab is higher when it is used in combination with chemotherapy, but it can occur with rituximab alone. Conversely, there are no reported cases of HBV reactivation with abatacept, anakinra, and tocilizumab. The use of steroids at medium or high dose (>7.5 mg/day for long periods), cyclophosphamide, calcineurin antagonists, mycophenolate mofetil, and azathioprine is associated with high risk of reactivation (14–70 %), while patients receiving glucocorticoids <7.5 mg/day, sulphasalazine, hydroxychloroquine, and gold compounds are considered at low risk for HBV reactivation.

Detailed recommendations on the use of immunomodulatory molecules in RA patients with chronic liver disease were reported by the American College of Rheumatology (ACR) in 2008 [116], then revised in 2012 [117]. The American Association for the Study of Liver Diseases also presented practice guidelines in 2009 for the management of patients with chronic HBV or HCV infection requiring immunosuppressive therapy [118, 119], and clinical guidelines are also available for viral hepatitis and IBD treatment [120]. These guidelines recommend that ALT levels, anti-HBsAg, anti-HBsAb, anti-HBcAb IgG and possibly HBV DNA, anti-HCV antibodies, and subsequently HCV RNA should be tested before an immunosuppressant treatment is started.

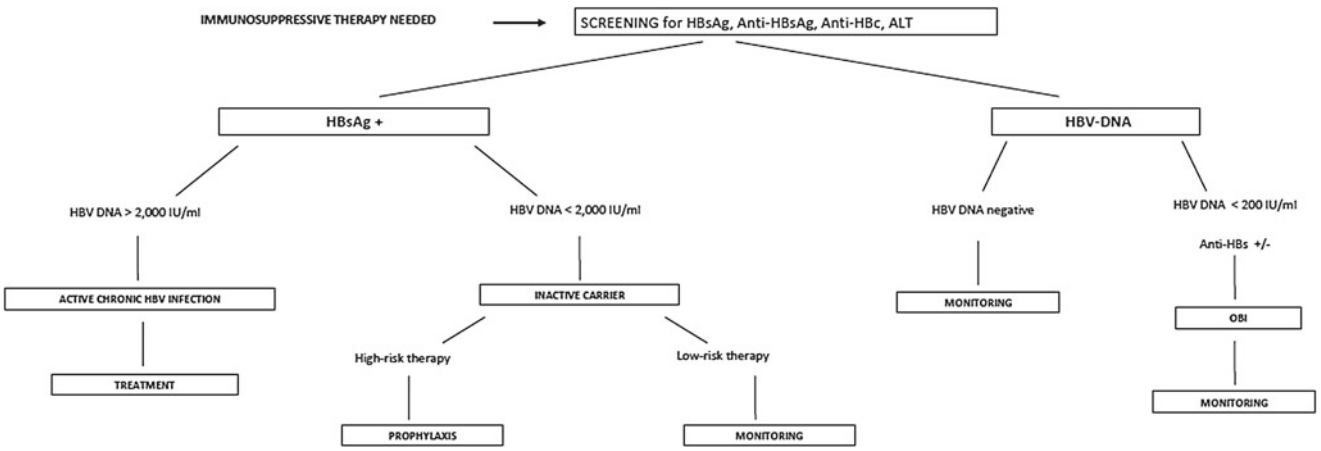

Fig. 3.1 Flow-chart of recommended HBV testing and management in patients candidate to immunosuppressive agents

Recommendations for HBV testing among patients that should be started on an immunosuppressive medication and for clinical management are presented in Fig. 3.1. Currently, a preventive antiviral treatment is recommended in patients with an active chronic HBV infection (HBsAg-positive, elevated ALT, and serum HBV DNA levels >2,000 IU/mL) and in patients with chronic HCV infections without extrahepatic contraindications. Patients with inactive HBV (HbsAg-positive, HBeAg-negative, normal ALT, and HBV DNA <2,000 IU/mL) present a risk of HBV reactivation which is related with the type of immunosuppression used. Prophylactic treatment is recommended in patients needing nonbiologic or biologic disease-modifying anti-rheumatic drugs (DMARDs) at higher risk of HBV reactivation, regardless of viral load. In patients candidate to treatment with immunosuppressive agents at lower risk of HBV reactivation, HBV DNA, ALT, AST, and HBsAg should be monitored every 6 months, starting prophylaxis/therapy in the case of HBV reactivation (HBV DNA >2,000 IU/mL and/or seroreversion of HBsAg). Prophylaxis should be started 2–4 weeks before the beginning of immunosuppressive therapy and maintained for at least 6–12 months after its suspension. It is currently recommended to use nucleoside/nucleotide drugs (NA). These antiviral agents are orally administered, well-tolerated, and safe. There are several NA approved for the therapy of chronic HBV: lamivudine and adefovir, which are first-generation drugs having high levels of resistence, and entecavir and tenofovir, with high potency and low resistance profile. Lamivudine is the most studied prophylactic agent; however, given the high rates of resistance, it is no longer recommended as first-line treatment of HBV. Drugs with high potency and high genetic barrier such as entecavir and tenofovir should be now considered first [121].

While the use of antiviral agents as prophylaxis against HBV in HBsAg-positive patients who are undergoing cytotoxic chemotherapy is a standard strategy, there is no consensus about how to manage patients with occult HBV infection (OBI). OBI is defined by the presence of HBV DNA in the liver tissue of individuals who test negative for HBsAg, by currently available assays, regardless of the detection of HBV DNA in the serum. When detectable, the level of HBV DNA in the serum is usually very low (<200 IU/mL). Depending on the HBV antibodies detected, OBI may be seropositive (anti-HBc and/or anti-HBs positive) or seronegative (anti-HBc and anti-HBs negative). If serum HBV DNA levels are similar to those detected in serologically evident HBV infection, it should be considered as false OBI, usually due to infection by HBV variants with mutations in the S gene. These variants produce a modified HbsAg that is not recognized by commercially available detection assays. It is recommended to use a highly sensitive and specific test, like HBV nucleic acid amplification testing (NAT), a PCR technique with a detection limit of less than 10 copies of HBV DNA per reaction. To date, available data are insufficient to recommend routine prophylaxis in OBI. OBI patients, as well as subjects who are HBV DNA-negative and anti-HBc-positive, should be closely monitored for eventual HBV reactivation. HBV DNA NAT (low limit of detection <10 IU/mL) should be monitored every 4 weeks and antiviral therapy should be instituted when the result is above 30 IU/mL; otherwise it is advisable to monitor HBsAg at 4-week intervals with a highly sensitive assay (low limit of detection <0.1 ng/mL) and begin antiviral therapy when the test becomes positive. However, further studies are needed to clarify the clinical usefulness, safety, and cost-effectiveness of these strategies in OBI [122].

DMARDs such as methotrexate and leflunomide are contraindicated in cirrhosis secondary to chronic HBV and HCV infections, whether treated or untreated, for all Child-Pugh stages, while biologics are contraindicated in both chronic HBV and HCV, whether treated or untreated, for those with significant liver injury (defined as chronic Child-Pugh

classes B or C). In recent ACR recommendations, etanercept could be potentially used in HCV [117]. Immunosuppressant regimens including high-dose glucocorticoids appear to have the highest risk of HBV reactivation and HCV replication, so steroid-sparing treatment should be adopted when possible, although low doses appear to be safe. Finally, the use of NSAIDs should be carefully evaluated in patients with liver cirrhosis regardless of the etiology based on the risk of renal injury secondary to tubular ischemia, while the risk of idiosynchrasic liver failure is not increased.

Rheumatic Drugs and Liver Toxicity

Many of the medications currently used in the management of rheumatologic conditions may induce hepatotoxicity. Drug-induced liver injury may vary from mild biochemical abnormalities to progressive fibrosis and cirrhosis, chronic liver failure, or fulminant liver failure. Painkillers such as NSAIDs, opiods, and paracetamol, anti-depressants, neuromodulators, and almost all DMARDs can affect the liver [123]. Nearly all NSAIDs have been reported to cause liver test abnormalities; however, serious hepatotoxicity is uncommon. NSAID related liver injury is usually mild, reversing at drug cessation. The appearance of NSAID-induced liver injury appears to be dose-independent, while the risk of liver injury following acetaminophen intake is now well-defined and recognized a dose-dependent increase [124]. Methotrexate may induce an increase in liver enzymes and has been associated with an elevated risk of fibrosis and cirrhosis in patients receiving long-term treatment. However, recent data on the impact of methotrexate on liver function tests demonstrated a reasonably safe profile for this medication if properly used [125]. A transient aminotransferase elevation is documented in 5.4–14.8 % of patients on leflunomide, but typically liver abnormalities resolve in the course of treatment and serious hepatotoxicity is rare. Sulfasalazine and penicillamine have been linked to a hypersensitivity hepatitis usually occurring within the first weeks of treatment. ACR recommendations suggest that, in case of transaminases levels greater than two-fold the upper normal limit, the initiation of DMARDs such as methotrexate, leflunomide, and sulfasalazine is contraindicated, while recommendations on when to discontinue the drug are unclear [116]. Finally, liver function test abnormalities are frequently reported with the use of all TNFα inhibitors, including the more recent golimumab (about 6 % of RA patients independently from DMARDs treatment). Such alterations are slightly more prevalent among patients receiving infliximab and adalimumab than among etanercept users [126]. Elevations are usually mild to moderate (<3 ULN), but severe elevations (>5 ULN) have been reported in less than 0.9 % of patients. Transaminase elevations are usually asymptomatic, resolving spontaneously or after medication adjustment or discontinuation. The mechanisms underlying anti-TNFα-mediated liver toxicity are not yet elucidated and the causes of the differences between pharmacological compounds are not known. Moreover, cases of anti-TNFα-induced AIH have been described [127]. Tocilizumab, a humanized antibody against the human IL-6 receptor, often resulted in mild to moderate (>1 to 3 ULN) ALT/AST increases in patients with normal baseline levels, which often occurred during concomitant DMARDs therapy. Rates of elevations of ALT and AST >3 ULN were 9.5 % and 3.1 %, respectively, in the all-exposed population and led to dose reduction or temporary interruption of treatment in 9.3 % of patients; rates remained stable and were not associated with clinical sequelae. There have also been reports of patients with normal enzyme levels, but elevated indirect bilirubin levels. On the other hand, IL-6 inhibition may confer benefit in certain forms of liver disease, such as fatty liver. Indeed, IL-6 is associated with insulin resistance and fatty liver. Finally, recent works have provided evidence for a link between the development of certain hepatobiliary malignancies, particularly cholangiocarcinoma, and upregulation of interleukin-6.

Abnormal liver enzymes or bilirubin levels have been also reported with anakinra. To note normalization of liver biochemistry has been achieved with dose tapering or drug discontinuation [128]. To date, no hepatic side effects have been reported with rituximab, abatacept, an inhibitor of T cell costimulation, and belimumab, which specifically targets B lymphocyte stymulator protein (BlyS) [129].

Conclusions

Patients with systemic rheumatic diseases often manifest transient mild to moderate liver test abnormalities. Usually, liver involvement does not display a specific biochemistry profile nor peculiar histopathological features. A hepatocellular injury pattern, a cholestatic profile, or a mixed pattern have all been described. Further evaluation is often unrevealing and a cause of the biochemical abnormality cannot be identified, therefore representing a diagnostic dilemma. However, most commonly these nonspecific abnormalities have no clinical significance, neither requiring a specific treatment. Pertinent to everyday's clinical practice, progressive liver involvement is frequently related to viral hepatitis reactivation or to a concomitant autoimmune liver disease. Therefore, it is of pivotal importance to screen patients for HBV and HCV infection, in order to provide the ideal therapeutic regimen and avoid life-threatening reactivations. Moreover, considering that autoimmune liver diseases present an aggressive course when left untreated, patients with unexplained persistent alteration of liver biochemical profile

should undergo further investigations. Therefore, testing for ANA, AMA, and SMA should be performed in patients with signs of chronic liver injury. Pivotal to a better understanding of liver involvement in rheumatoloigical conditions is the epidemiology of autoimmune liver diseases. Recent reviews and meta-analyses have shown an increased incidence and prevalence of immune liver diseases worldwide, but mainly in Northern Europe and USA. The continuous search of predisposing or risk factors that may trigger PBC, PSC, and AIH is a very important area of study and new candidates are reported continuously, as in the case of AIRE genetic variants in AIH [76]. However, a critical appraisal is required in the interpretation of epidemiological data, as population selection and study criteria are frequently biased. Along this hypothesis, the time increase of prevalence of autoimmune liver diseases could be explained with physician (and patient) awareness, more frequent and more extensive laboratory testing (i.e., serum AMA), drug availability for treatment and better survival, wide use of databases in healthcare with digitalized laboratory and pathology databases, hospital notes. Ultimately, we may expect true prevalence rates in the general population to be significantly higher compared to what currently reported in areas with a high level of healthcare, applying stringent search strategies, while the significance and predictive value of isolated autoantibody positivity in the general population should be clarified by larger cohorts and adequate follow-up periods. In general terms, drug-induced liver injury should be considered as it is significantly more common than primary and secondary disease-related liver involvement. Nevertheless, concurrent opportunistic infections have to be ruled out in rheumatic patients on immunosuppressive medications. In conclusion, it is extremely important in patients with rheumatic conditions to closely monitor liver function tests; a careful clinical evaluation and eventual further investigations should be performed whenever an hepatic abnormality is detected.

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Diagnostic Liver Immunology

4

Christopher L. Bowlus

Key Points

- Most forms of both acute and chronic liver disease 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 clinical practice of hepatology.
- Anti-mitochondrial antibodies remain one of the key diagnostic hallmarks of primary biliary cirrhosis with extremely high sensitivity and specificity.
- Autoantibodies are common in primary sclerosing cholangitis and autoimmune hepatitis, 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. Primary biliary cirrhosis (PBC) and nonalcoholic steatohepatitis (NASH) are representative of the former while viral hepatitis B and C are typical of the latter. Historically, the diagnosis of a liver disease was based primarily on histology and in large part the types and locations of inflammatory cells within the liver parenchyma. This remains the case for alcoholic liver disease and the now epidemic fatty liver disease. However, increasingly specific liver disease diagnoses are made based upon specific immune responses signified by the presence of specific antibodies and other serologic findings. Currently, genetic tests in liver immunology are limited to HLA class II alleles in autoimmune hepatitis and hereditary hemochromatosis, the latter a result of variants in the HLA class I-like *HFE* gene resulting in dysregulation of the antimicrobial peptide hepcidin and subsequent iron overload. However, as our understanding of the genetic basis of liver diseases progresses, the use of individualized genetics will likely become increasing important in diagnosing many liver diseases and personalizing their treatments. In this chapter, we will review the common liver diseases with an immunologic basis with an emphasis on the diagnostic tools in current use (Table 4.1).

Liver Biochemistries

Central to the diagnosis of any liver disease are the liver biochemistries, particularly the aminotransferases, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase. Except in severe cases of acute hepatitis or advanced fibrosis, the majority of chronic liver diseases cause few if any signs or symptoms. 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, 2]. Evaluation of the pattern of liver biochemistry abnormality is the first step in the diagnosis of any liver disease. Elevation primarily of the ALT suggests a primary injury to the hepatocyte due to a viral hepatitis, autoimmune hepatitis, or other infectious causes. A cholestatic pattern of liver biochemistries is typical of diseases affecting the bile ducts such as PBC and primary sclerosing cholangitis (PSC), but may also be present in granulomatous disease such as sarcoidosis.

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Table 4.1 Etiologies of several immune-mediated liver diseases

Infectious liver diseases	Autoimmune diseases	Granulomatous liver diseases
Hepatitis A	Primary biliary cirrhosis	Primary biliary cirrhosis
Hepatitis B	Primary sclerosing cholangitis	Sarcoidosis
Hepatitis C	Autoimmune hepatitis	Common variable immunodeficiency
Hepatitis D	IgG4-sclerosing cholangitis	*Mycobacterium*
Hepatitis E		*Leshmania*
Brucellosis		*Schistosoma*
Entamoeba histolytica		*Listeria*
Echinococcus		*Yersinia*
		Tularemia
		Psittacosis
		Bartonella
		Cytomegalovirus
		Epstein Barr virus
		Hepatitis A, B, C
		Histoplasma
		Coccidioides
		Cryptococcus
		Nocardia
		Candida
		Coxiella burnetii
		Allopurinol
		Diltiazem
		Interferon alpha

Infectious Liver Diseases

Humoral responses, whether directed to foreign agents or self-antigens, are central to 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 lead to diagnoses 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.

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 and in rare case may develop a relapsing cholestatic illness. The diagnosis is dependent upon the presence of anti-HAV IgM, which appears within 2–4 weeks of infection [3]. Although IgM is lost in the majority of cases 6 months after infection, persistence of anti-HAV IgM beyond 9 months has been reported [4, 5]. 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 [6]. Anti-HAV IgG is not measured separately, but rather it is measured along with IgA and reported as total antibody. 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.

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 4.2). 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. However, it is important to recognize that during reactivation of chronic HBV infection, anti-HBc IgM may become positive [7, 8]. In addition, the mere presence of anti-HBc neither confers immunity to HBV infection nor indicates active HBV infection, but only past exposure to the virus. Further, detectable levels of HBV DNA can be found in up to 15 % of individuals with anti-HBc but undetectable HBsAg [9, 10] and immunosuppression, particularly with anti-CD20 antibody, can lead to severe reactivation of occult HBV infection [11–13]. In chronic HBV infection, 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 and is 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 [14, 15]. Anti-HBs developed either through natural infection or immunization is protective against infection. The titer of anti-HBs after vaccination wanes over time, and booster immunization is ineffective in eliciting an anamnestic response in more than 20 % of those previously immunized [16].

Table 4.2 Interpretation of hepatitis B tests

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

HBs hepatitis B surface antigen, *Anti-HBs* hepatitis B surface antibody, *Anti-HBc* hepatitis B core antibody, *HBV DNA* hepatitis B virus DNA, *HBe* hepatitis B e antigen, *Anti-HBe* hepatitis B e antibody, *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 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 serologic testing for HCV has been recommended by the US Centers for Disease Control and Prevention for all persons born between 1945 and 1965 [17]. 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 posttransfusion hepatitis but lacked sensitivity and specificity [18]. Second-generation assays incorporated epitopes from the core and NS3 regions followed by the addition of epitopes from NS5 [19]. 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.

Hepatitis D

Hepatitis D virus (HDV) is a defective RNA virus which requires infection with HBV for HDV to replicate [20]. HDV RNA can be detected in serum by 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). However, in clinical practice in the USA, these tests are not available leaving diagnosis to the presence of anti-HDV IgM and IgG antibodies.

Hepatitis E

Similar to HAV infections, short-lived anti-HEV IgM is detectable in serum within 2–6 weeks of infection and are followed by long-lived IgG antibodies. Currently available assays for both IgM and IgG classes of anti-HEV vary considerably in their performance and none are currently licensed in the USA [21]. Although initially characterized as a disease of developing countries with both endemic infections and sporadic outbreaks, there has been an increasing recognition of autochthonous infections in developed countries. Perhaps due to the lack of easy access to testing, HEV infection is rarely reported in the USA despite a reported seroprevalence of 21 % and annual incidence of 0.7 % [22, 23].

Bacterial and Parasitic Infections

Nonviral infections of the liver span bacterial, mycobacterial, and parasitic organisms and are often difficult, if not impossible, to diagnosis by culture. *Brucellosis* is a small Gram-negative coccobacillus which is the most common zoonotic infection worldwide and often causes a granulomatous hepatitis. Culture of *Brucellosis* 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 of *Entamoeba histolytica* is 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.

Autoimmune Liver Diseases

The major autoimmune liver diseases have historically included PBC, PSC, and autoimmune hepatitis (AIH). More recently, a variant of autoimmune pancreatitis with biliary involvement has been recognized and is associated with elevated levels of serum IgG4, thus the term IgG4-sclerosing cholangitis. PBC, PSC, and IgG4-scleroing cholangitis must be distinguished not only from each other but also from other causes of cholestasis. In addition to the clinical setting, the diagnosis is based upon autoantibodies, imaging studies, and liver histology (Table 4.3). Similarly, AIH may present with all the features typical of an acute or chronic viral hepatitis. Imaging is less useful in this setting with the diagnosis of AIH established by serologic and histologic findings. In rare cases, patients may present with features of two of these 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 are lacking.

Primary Biliary Cirrhosis

Diagnosis of PBC is based primarily on the highly sensitive and specific anti-mitochondrial antibodies (AMA) reacting to the precisely defined epitope of lipoic acid of the E2 subunit of pyruvate dehydrogenase. AMA are present in upwards of 95 % of cases and their presence is one of the three key criteria to diagnosis, the other two being an elevated alkaline phosphatase 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. In addition to AMA, PBC is also associated with specific antinuclear antibodies, namely gp210 and Sp100 giving a nuclear rim or multiple nuclear dot pattern, respectively. In cases of AMA-negative PBC, these antinuclear antibodies (ANA) can assist in making the diagnosis [24].

Elevated levels of serum IgM are typical of PBC 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 [25, 26]. Serum IgM levels in PBC have also been inversely correlated with methylation of the CD40L promoter in CD4+ T cells suggesting a mechanisms 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 [27]. While an elevated IgM is frequent in PBC and may be useful in AMA negative cases, its clinical significance remains unclear.

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 criterion [28]. Peri-nuclear anti-neutrophil 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. Although a specific atypical pANCA, also termed peripheral anti-neutrophil nuclear antibody (pANNA), has been associated with these conditions and a putative self-antigen has been reported, confirmation of the antigen and identification of the epitope remain unresolved [29, 30]. Other autoantibodies including ANA and anti-smooth muscle antibodies 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. 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 [31, 32].

Autoimmune Hepatitis

Specific diagnostic criteria for AIH have been established by an international panel of experts and revised twice [33–35]. All three versions of the International Autoimmune Hepatitis Group guideline have included the presence of specific autoantibodies reflecting their importance in the diagnosis of AIH, yet it remains clear that autoantibodies are neither necessary nor sufficient to establish the diagnosis of AIH. In addition to ANA, antibodies to smooth muscle (SMA), liver–kidney microsome type 1(LKM-1), and soluble liver antigen (SLA) have been the primary autoantibodies used in the diagnosis and classification of AIH. 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 labs rather than indirect immunofluorescence even though 20 % of type 1 AIH patients with SMA are negative for F-actin [36]. In contrast, the molecular target of LKM-1 antibodies has been identified as the cytochrome P450 2D6 subunit and commercially available immunoassays utilizing the antigen are available. The presence of anti-LKM-1 indicates type 2 AIH, which typically presents in children and young adults. 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 these antibodies has been identified as UGA tRNA suppressor-associated

Table 4.3 Diagnostic features of autoimmune liver diseases

	Age	Sex	ALP	AST/ALT	AMA	ANA	pANCA	SMA	Ig
Primary biliary cirrhosis	>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 63 % (type I)	Elevated IgG in >90 %
IgG4-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

ALP alkaline phosphatase, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ANA* antinuclear antibody, *SMA* smooth muscle antibody, *Ig* immunoglobulin

antigenic protein and the presence of these antibodies is associated with severe disease and poor outcomes [37–39]. Hypergammaglobulinemia is present in greater than 90 % of AIH cases and is a major diagnostic criterion [35]. The mechanism underlying this phenomenon is unclear, but the level does correlate with disease activity making the serial testing of IgG levels useful for monitoring disease activity. Hypergammaglobulinemia may also be useful in distinguishing AIH from nonalcoholic fatty liver disease in which ANA and SMA are frequently present [40].

IgG4-Sclerosing Cholangitis

More recently, IgG4-sclerosing cholangitis has been recognized as one of many systemic sclerosing diseases associated with elevated levels of serum IgG4 and lymphoplasmacytic infiltration of IgG4-positive cells. IgG4-sclerosing cholangitis is often associated with autoimmune pancreatitis and can resemble PSC with sclerosing lesions of the bile ducts [41–44]. Differentiating IgG4-sclerosing cholangitis from PSC can be problematic and is made more difficult by the lack of sensitivity of serum IgG4 for IgG4-sclerosing cholangitis. 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 [45, 46]. It remains unclear if these PSC patients actually represent IgG4-sclerosing cholangitis or a true subgroup of PSC [47–49]. 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 [43, 50–54].

Granulomatous Liver Diseases

Granulomas are aggregates of modified macrophages which develop through antigen stimulation and interactions of T lymphocytes and macrophages. Relatively rare, granulomas are found in only 2–15 % of liver biopsies either as an isolated granulomatous disease or a systemic disease [55–59]. Biochemically, hepatic granulomas typically present with elevated serum levels of alkaline phosphatase and γ-glutamyltransferase (γGT). Rarely do these diseases result in portal hypertension or cirrhosis. Although the list of potential causes of liver granulomas is too numerous to list 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 [55–57]. In contrast, infectious causes including *Mycobacterium tuberculosis*, visceral leishmaniasis, and schistosomiasis are common in the Middle East and South Asia [58, 60].

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 more frequently involved with granulomas being found in 67–70 % of cases, with a greater percentage found in African–Americans compared to Caucasians [61]. Up to one-quarter of patients can have liver without lung involvement [62]. The clinical presentation can include hepatomegaly, pruritus, and rarely jaundice. Sarcoidosis may even present with biliary obstruction mimicking PSC [63].

Common Variable Immunodeficiency

CVID, a heterogeneous disease characterized by impaired B cell differentiation resulting in defective immunoglobulin production and chronic infections expected of an immunodeficient state, frequently manifests with autoimmune disorders. 24–90 % of CVID patients develop epithelioid granulomas in the liver which may be isolated or involve multiple organs [64–66]. Typically these patients present with elevated serum alkaline phosphatase. Given that granulomas are also typical of PBC, it is not surprising that cases of PBC in CVID have been reported [67]. In addition, nodular regenerative hyperplasia (NRH) has been reported in 84 % of CVID patients [66].

Summary and Future Directions

Establishing the diagnosis of immune-mediated liver diseases requires a comprehensive knowledge of liver immunology and pathology. Through a combination of 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. Already, tissue typing, either serologically or molecularly, for HLA-DR is helpful in some cases of AIH. Recent genome-wide

association studies have identified significant non-HLA genetic associations in PBC and PSC and will likely be incorporated into new treatment trials in order to identify subgroups of patients more likely to respond to specific therapies. In addition, as the cost of sequencing drops with new technologies, these markers will likely make the transition from research to the clinical realm. The greatest barrier at present to accomplishing this is not the lack of knowledge but the lack of infrastructures and systems to deal with the growing volume and complexity of data.

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The Liver as a Lymphoid Organ

5

Percy A. Knolle

Key Points

- The liver has unique immune functions and behaves as immune organ.
- The liver is both target and modifier of antigen-specific immune responses.
- The gut–liver axis and the gastrointestinal microbiome shape intrahepatic immunity and local organ-damage.
- Organ-resident liver cells function as antigen-presenting cells skewing local and systemic immune responses revealing lymphoid organ function.
- The hepatic microenvironment modifies the functional properties of bone marrow-derived immune cells resulting in unique regulation of immune responses in the liver.
- Unique regulation of adaptive immunity in the liver and its relevance for hepatic infections and therapeutic vaccination.

Lymphoid Tissues

The first paragraphs of this chapter review the salient functions of lymphoid tissue, which is important in order to evaluate the unique function of the liver as lymphoid organ. This is followed by a thorough review of the current knowledge of hepatic immune functions that render the liver a lymphoid organ and will provide a conceptual framework for a better understanding of the more detailed chapters on particular hepatic immune functions in this textbook.

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Primary Lymphoid Tissue

Lymphoid tissues are considered highly specialized tissues for particular functions of the immune system that cannot be achieved in parenchymal tissues or in the blood. One key feature of primary lymphoid tissue is first the generation of T and B cells and also the selection of lymphocytes (T cells) that are not autoreactive but can recognize peptide antigen in the context of MHC I as well as MHC II molecules. This function is localized within primary lymphatic tissues such as bone marrow (generation of lymphocytes) and the thymus (differentiation of T cells). In the thymus, thymic epithelial cells together with dendritic cells present tissue-specific antigens on MHC I or MHC II molecules. The generation of tissue-specific antigens is facilitated by action of the transcription factor AIRE in thymic epithelial cells that allows for random transcription across the entire genome and thereby generates a spectrum of peptide antigens that is representative for the entire organism [1]. Interestingly, also stromal cells in secondary lymphatic tissues such as lymph nodes express AIRE and thereby contribute to eliminate autoreactive T cells outside of the thymus [2]. The process of T cell selection can be categorized in distinct steps called negative and positive selection, where negative selection through induction of cell death by apoptosis occurs as a consequence of lack of recognition of any signal via the T cell receptor (meaning T cells cannot interact via the T cell receptor with MHC molecules on other cells) or by very strong signals indicating that T cells recognize autoantigens [1]. The process of negative selection in the thymus generates a T cell repertoire that does not cause autoimmunity and has been termed central tolerance; as the AIRE-mediated expression of tissue antigens in stromal cells in lymph nodes also contributes to the absence of autoimmunity, both peripheral and central tolerance mechanisms contribute to prevention of autoimmunity under physiological situations. Positive selection is the result of a balanced signaling process through the T cell receptor and additional co-receptors that result in survival of the T cells while migrating from the thymic

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cortex to the medulla. While the function of thymus and lymph nodes in shaping the organism's T cell repertoire trying to avoid generation of autoreactive T cells is well recognized, recent publications indicate that autoreactivity may be a prerequisite for the function of T cells to respond to stimulation by antigen-presenting cells [3]. It appears that such autoreactivity allows for tonic T cell receptor signaling to occur, which is a prerequisite for T cells to respond to environmental signals [4].

Secondary Lymphoid Tissue

Secondary lymphoid tissues such as lymph node and spleen are organized in particular compartments that execute distinct functions, which has led to the paradigm that "form follows function" [5]. The lymph nodes and spleen share certain anatomic areas such as B cell zones (germinal centers) and T cell zones but other areas are distinctly organized. These areas comprise the marginal zone in the spleen, peri-capsular areas in the lymph nodes, and the lymph node conduit system, which are all critical for delivery of antigen to secondary lymphatic tissue and which are populated by specific immune cell populations that function as antigen-presenting cells. The presence of secondary lymphatic tissue is absolutely required for the generation of B cell immunity, because B cell activation, maturation, proliferation, and differentiation strictly require germinal centers that organize the interactions with $CD4^+$ T helper cells and other immune cell populations relevant for B cell differentiation. The generation of T cell responses is less dependent on the presence of secondary lymphatic tissues, because T cell immunity is also observed in the absence of spleen and lymph nodes in mice carrying a genetic defect in NIK signaling demonstrating that other organs substitute lymphoid tissue in generation of T cell responses [5].

It is believed that the low frequency of antigen-specific lymphocytes and the resulting small clone size of lymphocytes with a particular antigen-specificity necessitate the optimized interaction with antigen-presenting cells. Such optimized interaction between antigen-presenting cell populations and lymphocytes is a key feature of secondary lymphatic tissue. Chemokine-mediated migration of antigen-presenting cells such as dendritic cells and naïve as well as central memory T cells or B cells finally leads to their colocalization in T cell zones or B cell zones, respectively [5]. The close interaction between antigen-presenting cells and different lymphocyte populations is required for appropriate lymphocyte activation and differentiation leading to T cell immunity [6]. Thus, positioning of lymphocytes in anatomically defined areas of secondary lymphoid tissues and interaction with other cell populations are important for development of adaptive immunity.

Mechanisms Determining Induction of Adaptive Immunity in Secondary Lymphatic Tissue

The induction of adaptive immunity requires activation of innate immune cells and inflammation. Innate immune activation can result from triggering of immune sensing receptors that comprise membrane-bound Toll-like receptors, cytosolic DNA receptors, helicases, or the different types of inflammasomes. Alternatively, pro-inflammatory signaling can result from the paracrine activation through inflammatory mediators released from activated innate immune cells or from dying cells. The appropriate maturation of antigen-presenting cells, i.e., dendritic cells and monocytes/macrophages, by such cell-autonomous activation or paracrine inflammatory signaling results in the improved migration of those cells into secondary lymphatic tissue, better antigen-presentation, and increased expression of co-stimulatory molecules that are important for T cell activation [6]. The balance between co-inhibitory and co-stimulatory signals in combination with signals through the T cell receptor delivered from antigen-presenting cells is believed to determine the outcome of T cell differentiation leading to either immune tolerance or protective immunity. Thus, induction of innate immunity is a prerequisite for successful induction of adaptive immunity and works in a tiered-fashion to improve immune cell clustering, better antigen-presentation, and co-signaling processes.

Lymphoid tissue has previously been considered to be a sterile environment where information about the state of inflammation or infection with microbes in the periphery are conveyed via immune cells migrating into lymphoid tissue. Similarly, antigen presented in lymphoid tissues was believed to be transported via antigen-presenting cells from the periphery into lymph nodes and then either be presented by these migrating cells to lymphocytes or be cross-presented by specialized lymph node-resident plasmacytoid dendritic cells [7]. Alternatively, antigen can enter lymph nodes through a network of conduits operating by capillary pressure resulting in complex distribution of soluble antigens to several compartments within draining lymph nodes [8].

It has been recently recognized, however, that secondary lymphatic tissue also allows for controlled infection by bacteria and viruses. A $CD169^+$ subset of monocytic cells that lacks certain interferon responsive signaling elements is preferentially infected by viruses thus permitting controlled expression of microbial antigens in the marginal zone of the spleen [9], which facilitates development of subsequent virus-specific immunity. Bacterial or viral infection of lymph nodes has been shown to occur during local and systemic infection and to contribute to the induction of protective adaptive immunity as well as supporting improved memory T cell responses [10, 11].

Tolerance Induction in Secondary Lymphatic Tissue

In the absence of innate immune stimulation and development of inflammation the outcome of antigen-presentation to T cells is tolerance than immunity. As already mentioned above, the presentation of peptide antigens induced through the action of the AIRE transcription factor in lymph node stromal cells leads to contact of T cells with antigen-presenting stromal cells or cross-presenting dendritic cells in the absence of inflammatory signals [2]. Such interaction results in the clonal deletion of T cells and thereby contributes to peripheral immune tolerance. Similarly, expression of antigen under physiological noninflammatory situations in peripheral tissues such as the pancreas leads to cross-presentation of these antigens in the draining lymph node by dendritic cells, which leads also to clonal deletion of antigen-reactive T cells, a process termed cross-tolerance [12]. Lack of innate immunity and inflammation therefore determines induction of tolerance rather than immunity by failing to induce functional maturation of antigen-presenting cells.

Tertiary Lymphoid Tissues

It is of interest to note that the molecular signals required for development of lymphoid tissues during ontogeny and also later in life are essentially pro-inflammatory in nature, e.g., signaling via the lymphotoxin receptor or the IL-17 receptor.

Lymphoid tissue can also develop in situations of chronic inflammatory processes in any non-lymphoid organ such as liver, pancreas, or central nervous system. This lymphoid tissue arising typically during situations of chronic infections with viruses or bacteria is termed tertiary lymphoid tissue. This tertiary lymphoid tissue may either arise from persistent pro-inflammatory signaling via the lymphotoxin receptor or the IL-17 receptor or arise as a consequence of chronic infection and development of a granulomatous tissue, e.g., after infection with intracellular bacteria such as *Listeria* spp. or *Mycobacteria* spp. Whereas tertiary lymphoid tissue shares many of the typical anatomic and functional features of secondary lymphoid tissue, i.e., the presence of B cell zones (germinal centers) and T cell zones as well as immigration of antigen-loaded antigen-presenting cells, granuloma have a completely different architecture that is defined by a ringwall of fibrocytes and regulatory macrophages located around the infection locus. There is intensive cross talk among T cells and macrophages in granuloma that leads to complex regulation of adaptive T cell immunity within the structures [13]. The occurrence of tertiary lymphoid tissue or granuloma is considered a hallmark for the presence of chronic infectious and inflammatory processes. The relevance of these tissues for clearance or persistence of microbial infection still awaits clarification.

The Liver as Lymphoid Organ

In the following paragraphs the key immune features of the liver with relevance to its function as lymphoid organ will be discussed and compared to the structure/function correlation in the different types of lymphoid tissues. The knowledge reviewed here supports the view that the liver complements the functions of lymphoid tissues and contributes both to maintenance of immune tolerance towards antigens presented to the immune system in the liver and to induction of immunity and immune memory towards antigens circulating in the blood.

Immune Functions of the Liver

Induction of immune tolerance in the liver has been first reported in 1967 by transplant surgeons [14, 15]. Three main points demonstrate the ability of the liver to induce antigen-specific immune tolerance. (a) Liver transplants are accepted by the recipients immune system despite MHC-discrepancies even in the absence of immune suppression [14, 15]. (b) Simultaneous transplantation of the liver and another organ from the same donor leads to increased graft acceptance of the co-transplanted organ. Organ-transplants from another donor lead to graft rejection, demonstrating antigen-specific induction of immune tolerance by the transplanted liver [16]. (c) Drainage of a transplant directly into the portal vein or direct application of donor cells into the portal vein leads to increased acceptance of the graft [17].

Linking Hepatic Anatomy to Immune Function

The liver holds a unique position with regard to the blood circulation. It receives venous blood draining from almost the entire gastrointestinal tract via the portal vein and from the systemic circulation via the hepatic artery. More than 2,000 L of blood stream daily through the human liver; peripheral blood leukocytes pass on average more than 300 times per day the liver. These simple facts clearly demonstrate that the liver is a “meeting-point” for antigens and leukocytes circulating in the blood.

Among the many physiological functions of the liver, clearance of the blood from macromolecules and it’s metabolization are important for the understanding of the liver as a lymphoid organ. Nutrients are extracted from portal venous blood and further used for hepatocellular metabolism, but at the same time the liver eliminates toxic waste products and pro-inflammatory agents, such as endotoxin or other gut-derived bacterial degradation products, from portal venous and arterial blood without eliciting an immune response to these antigens.

Table 5.1 Sinusoidal cell populations

Hepatic cell population	Percent of liver volume[a]	Percent of total liver cells
Kupffer cells	2.1	15
Liver sinusoidal endothelial cells	2.8	19
Stellate cells	1.4	5–8
Liver-associated lymphocytes	n.d.	n.d.
Hepatocytes	78	60
Dendritic cells	n.d.	n.d.

[a]Sinusoidal lumen 10.6 %, space of Dissé 4.9 %, adapted from [18]

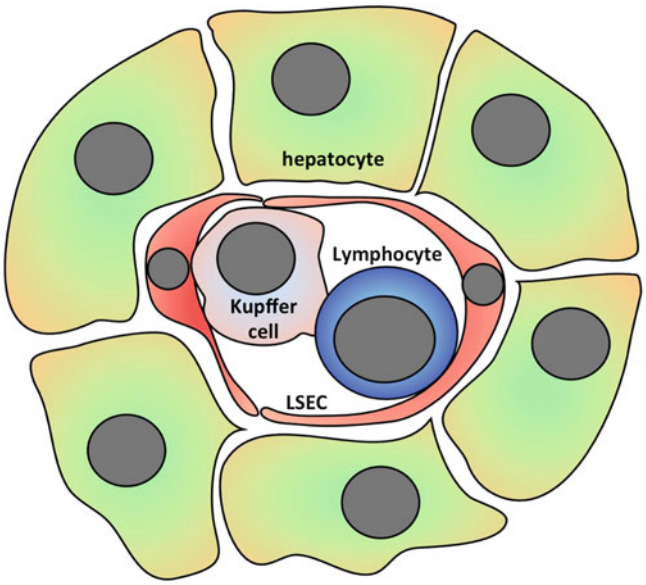

Fig. 5.1 Schematic drawing of the hepatic sinusoidal cell populations and their close interaction with each other and hepatocytes. Within the sinusoidal lumen, Kupffer cells, liver-associated lymphocytes (LAL), as well as dendritic cells and circulating lymphocytes are found. Liver sinusoidal endothelial cells (LSECs) separate the sinusoidal lumen from hepatocytes

The liver is optimally structured to function as metabolic organ, i.e., to clear macromolecules from blood and release metabolic products from hepatocytes into the blood. Nutrient-rich blood from the gastrointestinal tract enters the liver via the portal vein, which drains after extensive ramifications into the so-called portal field comprising one portal venous vessel, one arterial vessel, and a bile duct surrounded by connective tissue. Portal venous and arterial blood drain into the hepatic sinusoids, which form a three-dimensional meshwork of vessels generating a mixed arterial-venous perfusion of the liver. Blood flows from the portal tract to the central veins, which convene to hepatic veins draining into the inferior vena cava. The hepatic sinusoids are composed of several cell populations (Table 5.1).

Although hepatic sinusoidal cell populations contribute only to 6.3 % of total liver volume they represent approximately 40 % of the total number of hepatic cells, 26 % of total membrane surface (mainly liver sinusoidal endothelial cells (LSEC)), 58 % of total endocytotic vesicles (mainly LSEC), and 43 % of total lysosomal volume (mainly Kupffer cells and LSEC) [18]. The sinusoidal cell populations are most prominent in mediating the unique immune functions to the liver as they have immune competence and can easily establish interaction with passenger lymphocytes in sinusoidal blood.

LSEC physically separate lymphocytes passing through sinusoids from hepatocytes [19]. In contrast to endothelial cells in other organs, there is no basement membrane in the liver. The space between hepatocytes and LSEC is called the space of Dissé, which contains abundant extracellular matrix produced by LSEC and is populated by stellate cells (see Fig. 5.1), that span around the LSEC and control sinusoidal blood flow by contraction leading to reduction of the sinusoidal diameter [20]. Kupffer cells are located predominantly in the periportal region and are in close contact with LSEC. The blood flow in the hepatic sinusoids is slow [21], which is ideal for clearance of macromolecules from the blood and initiation of contact between liver sinusoidal cells and passenger lymphocytes (see Fig. 5.1).

Liver-associated lymphocytes form a heterogeneous population of hepatic lymphocytes showing an unusual repertoire of surface molecules and a restricted TCR-repertoire [22]. These cells are found in close association with LSEC and Kupffer cells, engaging in concert with these cells in local defense mechanisms against invading pathogenic microorganisms or tumor cells [23]. The liver harbors a large population of CD1- and MHC I/II-restricted T cells bearing NK cell markers, the so-called NKT cells, which have an activated phenotype and rapidly release substantial amounts of soluble mediators upon TCR-induced activation [24]. NKT cells patrol hepatic sinusoids and arrest upon recognition of their cognate antigen on sinusoidal cells suggesting the presence of a local intravascular immune surveillance system [25].

Within the periportal region, a rather specialized population of dendritic cells is found which together with Kupffer cells is ideally situated to scavenge pathogenic agents from portal venous blood [26]. The liver is connected to the lymphatic system, as particles injected via the portal vein are found within a few hours in retroperitoneal lymph nodes inside dendritic cells suggesting that dendritic cells had ingested the particles and had migrated to lymphatic tissue [27]. Certainly, liver dendritic cells play a key role in regulating immune responses to antigens delivered via the blood stream to the liver [28, 29].

Taken together, the localization of bone marrow-derived immune cells in the hepatic sinusoids does not resemble the typical anatomic compartments found in secondary or tertiary lymphatic tissues. However, the question remains

whether the unique hepatic microvascular structure results in slow blood flow and facilitates interaction of lymphocytes with sinusoidal cells and thereby helps those sinusoidal cell populations in exerting potent immune functions.

Local Antigen-Presentation in the Liver

Similar to secondary or tertiary lymphoid tissue, the liver harbors cell populations that bear the immune competence to stimulate naïve or already activated T cells and function as antigen-presenting cells [30]. Two situations must be discriminated: first, the situation where antigen is first seen by naïve T cells on local antigen-presenting cells in the liver, i.e., the priming of an immune response occurs in the liver. This is the situation where the liver functions as lymphoid organ in actively regulating the quality of adaptive immune responses. Second, the condition where already activated T cells recognize their antigen again on local antigen-presenting cells in the liver. Such antigen re-encounter is unlikely to result in functional skewing of immunity but rather triggers the execution of T cell effector functions. This is the situation where the liver serves as target of immunity.

Hepatocytes as Antigen-Presenting Cells

Hepatocytes as most abundant hepatic cell population bear immune competence and can stimulate naïve CD8 T cells in an antigen-specific fashion. The outcome of such stimulation by antigen-presenting hepatocytes is bim-mediated apoptotic death of such stimulated lymphocytes [31]. Also, hepatocytes engulf naïve T cells in a process called suicidal emperipolesis that eventually leads to death of the T cell [32]. Therefore, antigen-presentation by hepatocytes and its consequences for the fate of such activated T cells is considered to contribute to the tolerizing phenotype of the liver [33]. It is important to note that hepatocytes do not present circulating antigens thereby limiting the spectrum of peptide presented to T cells to those being present endogenous to hepatocytes. It is unclear whether viral infections of hepatocytes lead also to functional skewing of T cell responses and would modify the course of antiviral immunity against infected hepatocytes.

There is an interesting parallel to the controlled replication of viruses in CD169$^+$ macrophages in the spleen. Herpes viruses that infect hepatocytes do not further disseminate from infected hepatocytes by release of progeny virus to other organs, because there is no release of infectious virus from infected hepatocytes indicating that this viral infection is readily contained within the liver [34]. In contrast, the infection of hepatocytes by hepatotropic viruses such as hepatitis B virus (HBV) or hepatitis C virus (HCV) clearly leads to release of infectious virus from infected hepatocytes, which may be the result of a viral immune escape mechanism that has so far not been studied in detail.

Antigen-Presentation by Sinusoidal Cell Populations

A key feature of all sinusoidal cell populations that are competent for antigen-presentation is that they can (cross)present soluble circulating antigens to CD4$^+$ and CD8$^+$ T cells. Consequently, sinusoidal cells can cross-present hepatocyte-derived antigens as well as systemically circulating antigens and therefore influence adaptive immune responses not only against local antigens but also against antigens that are systemically distributed.

Kupffer cells are a heterogenous population of liver-resident macrophages derived from the bone marrow or from so far ill-defined hepatic progenitor cells [35]. These cells can present antigen to naïve CD4$^+$ and CD8$^+$ T cells but the outcome of such antigen-presentation is rather induction of tolerance than immunity indicating that Kupffer cells actively contribute to the tolerogenic function of the liver as a lymphoid organ [36, 37]. However, little data exist whether innate activation through Toll-like receptors or cytosolic immune sensing receptors can lead to functional maturation of Kupffer cells and consequently induction of immunity rather than tolerance. Irradiation, which causes profound changes in the composition of bone marrow-derived cells in the liver and leads to generation of inflammation, results in loss of T cell tolerance towards liver transplants [38]. More research is required in order to assign specific immune functions to the different populations of Kupffer cells and to investigate whether they bear functional plasticity to switch from tolerogenic to immunogenic antigen-presentation upon appropriate activation by innate or inflammatory stimuli.

Dendritic cells in the liver also comprise a heterogenous population of myeloid and plasmacytoid dendritic cells [30]. These cells resist functional maturation upon stimulation through Toll-like receptors or cytosolic NOD-like receptors and thereby also contribute to tolerance induction within the liver. The continuous exposure to Toll-like receptor signaling as a consequence of permanent exposure to gut-derived bacterial degradation products may contribute to the unique functional behavior of dendritic cells that reside within the liver [30]. The hepatic microenvironment appears to modulate the function of bone marrow-derived immune cells and compromises their capacity to function as professional antigen-presenting cells. Furthermore, LSEC inhibit the function of dendritic cells to act as antigen-presenting cells to stimulate naïve T cells locally in the liver [39]. Stellate cells veto naïve T cell activation [40] further substantiating the notion that the liver as lymphoid organ actively prevents

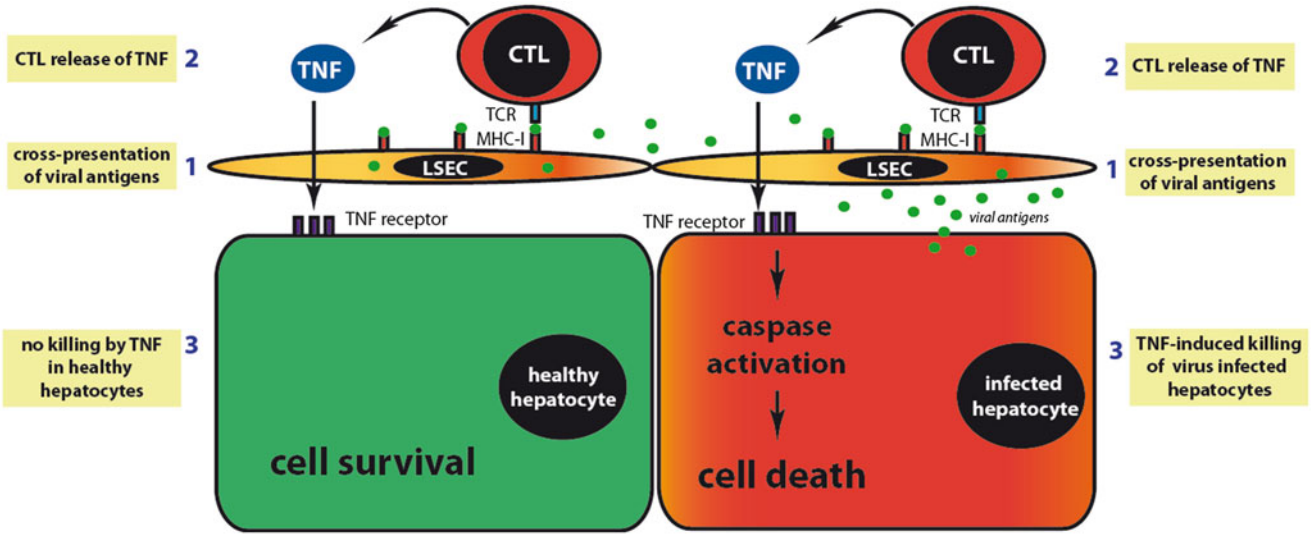

Fig. 5.2 Description of the novel noncanonical cytotoxic T cell (CTL) effector function exerted by TNF. CTLs activated by LSEC cross-presenting hepatocyte-derived viral antigens release TNF that acts in a selective fashion to induce apoptotic death in virus-infected hepatocytes

the maturation and functions of antigen-presenting cells and directly prevents immune cells from locally activating naïve T cells, a feature that is not observed in secondary or tertiary lymphoid tissue.

Finally, LSEC function as truly organ-resident antigen-presenting cells in the liver. This cell population has extraordinary scavenger function to endocytose soluble circulating antigens and process such antigens for (cross)presentation on MHC I and MHC II to naïve $CD8^+$ and $CD4^+$ T cells [30]. Again, the outcome of such antigen-presentation is induction of T cells that are non-responsive to subsequent stimulation through the T cell receptor. The molecular mechanisms determining such T cell non-responsiveness are delivery of co-inhibitory signaling via the B7H1-PD1 ligand–receptor interaction. Interestingly, LSEC resist functional maturation in response to activation through innate sensing receptors but are rendered immunogenic upon viral infection [41]. Although T cells are rendered non-responsive by LSEC towards T cell receptor signaling they are not clonally eliminated, which represents a fundamental difference towards stimulation of naïve T cells by immature dendritic cells or by lymph node stromal cells in secondary lymphoid organs.

Taken together, these results demonstrate that local antigen-presenting cell populations attribute lymphoid organ function to the liver to stimulate naïve T cells. The outcome of such stimulation in most situations is non-responsiveness or immune tolerance, which indicates that the liver as lymphoid organ has less plasticity to switch from tolerogenic to immunogenic programming of adaptive immunity compared to secondary or tertiary lymphatic tissues.

Immune Surveillance in the Liver Triggered by Antigen-Presenting Cells

The control of viral and bacterial infections must be accomplished in both lymphoid and parenchymal organs and involves the local interplay between innate and adaptive immunity. The execution of T cell effector functions in the liver has been shown to oscillate indicating that regulatory cues from the hepatic microenvironment or from regulatory liver cell populations interfere with adaptive immunity [42]. Antigen-presentation by sinusoidal cell populations and by hepatocytes contributes to adaptive immunity during experimental viral infection [43]. Recently, a novel noncanonical CD8 T cell effector function was discovered in the liver. Cross-presentation of hepatocyte-derived antigens by LSEC initiates local activation of T cells specific for those antigens and leads to release of tumor necrosis factor (TNF) from such stimulated T cells. TNF in turn acts specifically on virus-infected hepatocytes to induce apoptotic cell death, but the molecular mechanisms that determine this selective sensitivity of virus-infected but not uninfected hepatocytes to the death-inducing effect of TNF remain to be discovered [44] (see Fig. 5.2). Also, it remains unclear why hepatic infections sometimes are not cleared although innate and adaptive immune mechanisms eliminate infectious microorganisms early after entering the liver [45]. It is possible that the large number of hepatocytes, that represent the key cell population in the metabolic function of the liver, renders the liver vulnerable to persistence of infection, because detection of infected hepatocytes within the maze of hepatic sinusoids is a very difficult task for T cells. Moreover, immune-mediated

attack during microbial infection of hepatocytes may compromise the liver to function as lymphoid organ because sinusoidal cell populations are substantially altered during chronic inflammation and fibrosis. Notwithstanding, it is of interest to note that persistent viral infection leading to chronic inflammation generates tertiary lymphoid tissue in the liver; the relevance of the generation of tertiary lymphoid tissue within a lymphoid organ for clearance or persistence of infections has not been properly addressed.

The Gut–Liver Axis: Communication Between Two Lymphoid Organs

The gut is recognized as lymphoid organ that harbors large numbers of immune cells including the majority of the organism's memory T cell populations. The interaction between the gut and the liver has been suspected to play a role in the unique immune functions of the liver. Recently, it was found that loss of the cytosolic innate immune sensor NLRP6 from intestinal epithelial cells leads to a change in the composition of the gut microbiota [46]. In such mice, immune-mediated liver injury occurs during metabolic challenge situations that assigns functional relevance to gut-derived microbiota contained within portal venous blood. The change in gastrointestinal microbiota influencing immune regulation in the liver also demonstrates that the liver functions as safeguard or filter to prevent systemic dissemination of gut-derived microbiota. Another example of the intricate interaction between the two lymphoid organs is the entero-hepatic recirculation of $CCR9^+$ T cells that migrate in response to CCL25 being expressed by intestinal epithelial cells and LSEC and biliary epithelial cells [47]. As $CCR9^+$ T cells have been implicated in the pathogenesis of inflammatory bowel disease their recirculation to the liver may provide an explanation for the concomitant occurrence of autoimmune liver disease such as primary sclerosing cholangitis together with inflammatory bowel disease [47].

The Liver as Lymphoid Organ Inducing Immunity

Although many publications have reported on the tolerogenic function of the liver as lymphoid organ [30], the clearance of most bacterial and viral infections in the liver strongly suggests that the liver can also contribute to immunity [45]. Indeed, two observations support this assumption: first, antigen-presentation in the liver contributes to generation of a so far unknown protective memory T cell population and second, the liver serves as expansion hub for previously primed T cells.

For a long time it has been a conundrum in immunology how adaptive T cell immunity is generated against pathogens that circumvent or inhibit the generation of innate immunity and inflammation during infection. This becomes most obvious during viral infections, where subviral particles are rapidly disseminated systemically after the infection in the absence of inflammation. Antigen-presentation in the absence of innate immune activation and inflammation by immature antigen-presenting cells typically leads to clonal elimination of T cells. This mechanism is believed to prevent inadvertent induction of autoimmunity against tissue antigens or innocuous antigens such as food antigens. However, it will also lead to elimination of pathogen-specific T cells during the early phases of infection, when pathogens escape innate immunity such as HBV.

It was found that cross-presentation of circulating antigens by LSEC during noninflammatory conditions, e.g., during systemic dissemination of viral antigens, does not lead to clonal elimination of T cells and also rescues these T cells from cross-tolerance by antigen-presenting immature dendritic cells [48]. In contrast, such stimulated T cells relocate after priming in the liver to secondary lymphoid tissues in a CCR7-dependent fashion, similar to central memory T cells. These liver-primed T cells have memory-like functions, because they have the plasticity to generate new effector T cells after reactivation with combinatorial signaling through the T cell receptor, the co-stimulatory CD28 molecule, and the IL-12 receptor. Thus, LSEC-primed T cells are not terminally committed to their non-responsive state but require co-stimulatory signals typically generated during inflammation for their reactivation and delivered by functionally matured antigen-presenting dendritic cells. Upon appropriate activation LSEC-primed T cells have memory function and generate effector T cells in secondary lymphatic tissues that are protective to control and eliminate bacterial and viral infection [48]. This demonstrates for the first time the generation of memory T cells in the absence of inflammation outside secondary lymphatic tissues and assigns novel immunogenic properties to the liver as lymphoid organ (see Fig. 5.3).

The clearance of infections from the liver requires large numbers of antigen-specific effector CD8 T cells, but several regulatory feedback loops prevent expansion of this population of effector T cells in secondary lymphoid tissues and in the liver microenvironment also restricts T cell proliferation by constitutive expression of the enzymes arginase, IDO, and the co-inhibitory molecule B7H1 [45]. However, upon signaling through particular Toll-like receptors inflammatory monocytes adhere within hepatic sinusoids and form a cocoon-like structure. These myeloid cell aggregates arise rapidly after the initial Toll-like receptor signaling throughout the liver parenchyma and dissolve within several days. CD8 T cell proliferation in the liver occurs exclusively in these structures, which are termed iMATEs for intrahepatic

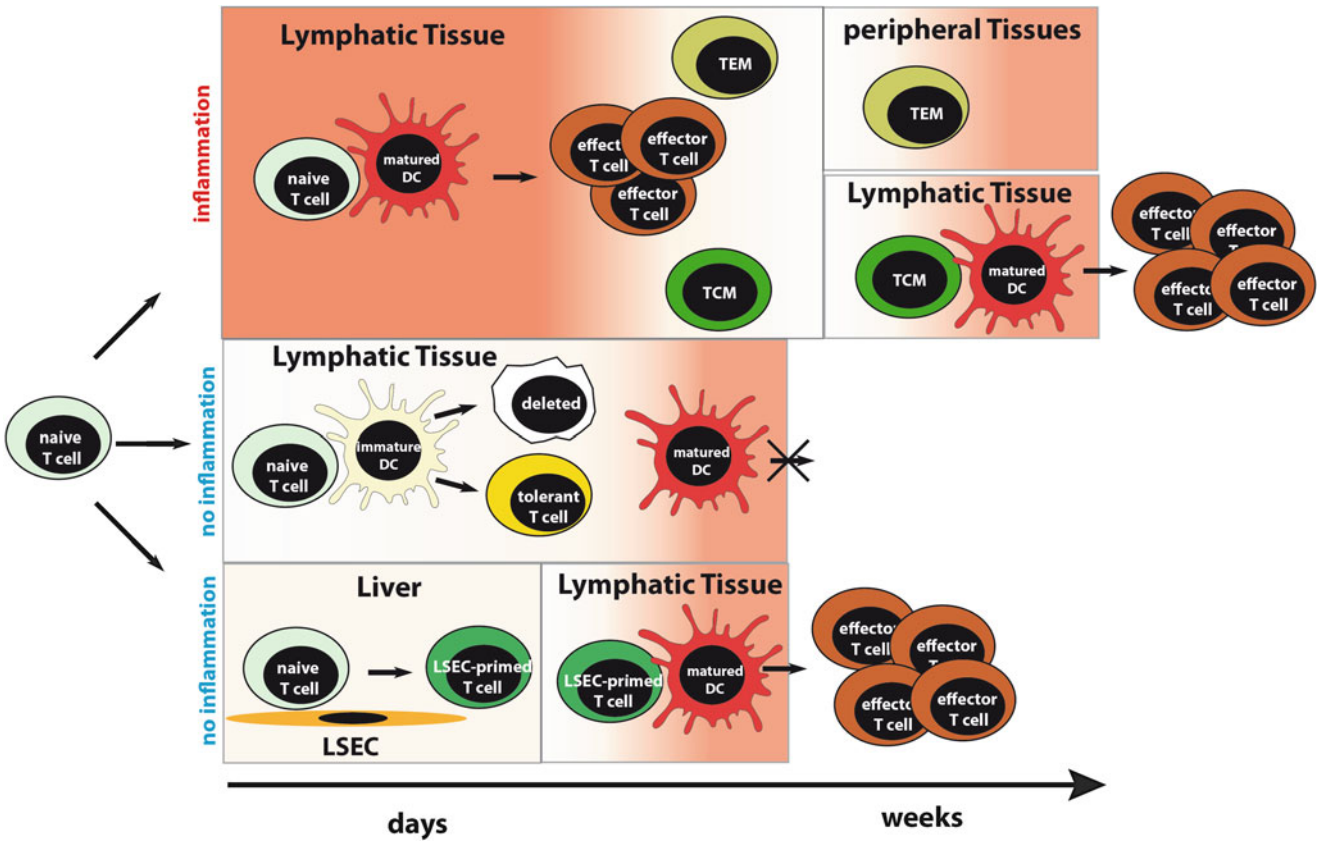

Fig. 5.3 Liver-primed T cells have memory-like functions. Naïve CD8⁺ T cells that are primed by cross-presenting LSEC in the liver under noninflammatory conditions are not deleted and also resist cross-tolerance inflicted by immature dendritic cells. Liver-primed T cells relocate to secondary lymphoid tissue and home to the same anatomic compartment, i.e., T cell zones, as naïve or central memory CD8⁺ T cells. Upon combinatorial stimulation via the T cell receptor, CD28, and IL-12 or upon stimulation by matured immunogenic antigen-presenting dendritic cells liver-primed T cells are reactivated and show central memory T cell-like features to produce effector CTLs. Such CTLs derived from liver-primed T cells have protective function

myeloid cell aggregates for T cell expansion. The iMATEs presumably provide a place where T cells are sheltered from the regulatory cues of the hepatic microenvironment. Such T cell proliferation within iMATEs results in a 50- to 100-fold expansion of the effector CD8 T cell population and requires prior stimulation of the T cells by matured professional antigen-presenting cells [49]. The iMATEs facilitate co-stimulation-dependent expansion of effector CD8 T cell populations that have previously been activated in secondary lymphatic tissue. Such jump expansion of effector T cells clears chronic viral infection from the liver [49]. The formation of iMATEs is therefore essential for the novel function of the liver to amplify T cell responses and support successful therapeutic vaccination. The anatomic structure of iMATEs does not share any similarity to secondary or tertiary lymphatic tissues. Taken together, unique anatomic structures within the liver allow for T cell proliferation and expansion and strengthen the notion that the liver functions as lymphoid organ.

Summary and Conclusion

The definition of the liver as a lymphoid organ is based on functional properties not on similarities to the microanatomy of secondary or tertiary lymphoid organs. The liver has the capacity to prime naïve T cells, to generate memory CD8 T cells, and to expand effector CD8 T cells, all recognized key features of lymphoid tissues. Such lymphoid tissue functionality of the liver, however, is facilitated by organ-resident antigen-presenting cells together with the unique hepatic microenvironment, which skews the immune function of bone marrow-derived immune cell populations. The liver as lymphoid organ is more vulnerable to immune-mediated injury because the large population of hepatocytes serves as target for innate and adaptive immunity. The induction of innate and adaptive immune tolerance in the liver may serve to protect the organ from immune-mediated damage that threatens to result from the continuous exposure to gut-derived microbiota and

their degradation products contained physiologically in portal venous blood. Similar to secondary lymphoid tissue, the liver allows for influx of bone marrow-derived immune cells, in particular inflammatory monocytes, that upon appropriate stimulation facilitate induction of strong T cell immunity in the liver. This functional dichotomy may provide the liver with the ability to fulfill its dual function as metabolic organ and lymphoid organ at the same time. Moreover, the liver complements memory T cell differentiation in secondary lymphoid tissues by generating an unique memory T cell population under noninflammatory conditions by organ-resident nonprofessional antigen-presenting cells. More research effort is warranted to characterize in more detail the unique immune features that render the liver a lymphoid organ, because such knowledge will help to overcome chronic viral infection of the liver and help to combat liver cancer.

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Innate Immunity and Disorders of the Liver

6

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Key Points

- Innate immune responses in the liver are induced by pathogens, toxins, and aberrantly accumulating metabolites.
- The liver as the central organ in metabolism and detoxification is continuously exposed to innate immune stimuli.
- Numerous innate immune cells physiologically reside in the liver.
- Innate immune receptors are expressed on various liver-resident cell types as well as on circulating cells passing the liver.
- The liver is fully competent for immune sensing but during the physiological situation there is no constitutive activation of innate immunity in the liver leading to organ inflammation.
- Innate immune responses in the liver support tissue regeneration and hepatocyte survival.
- Innate immune responses in the liver combat infection, but may also lead to acute and chronic inflammation.
- Chronic liver inflammation leads to tissue damage and contributes significantly to hepatocellular cancer development.

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Innate Immunity

The liver as an organ, which is continuously exposed to intestinal microbiota-derived innate immune stimuli contained within portal venous blood. This chapter will review our knowledge on innate immune responses in the liver and will discuss the impact of hepatic innate immunity on liver disorders.

Innate Immune Stimuli Recognized by Immune Sensory Receptors

The field of innate immunity belongs to the most rapidly evolving fields in biomedical sciences, which has not only advanced the molecular knowledge on receptors and ligands but has also broadened the understanding how innate immunity impacts on both physiological organ function and disease states. Coming from the simple view that "pathogen-associated molecular patterns" (PAMPs) are recognized by germ line-encoded pattern recognition receptors (PRRs) innate immune sensing is now understood as a complex process that integrates recognition of typical constituents of pathogens with recognition of abnormal metabolites and cell stress or cell death (danger-associated molecular patterns; DAMPs). This recognition is achieved by a large number of immune sensory receptors whose functionality is adapted to the particular tissue context.

Innate immune stimuli recognized by immune sensory receptors range from molecules that are selectively expressed by infectious microorganisms, such as hypo-methylated oligonucleotides or pathogen-typic carbohydrates and lipids like lipopolysaccharide (LPS), to molecules that are detected within cellular compartments where they should not be localized, such as double-stranded or triphosphated RNA within the cytosol or free RNA or DNA in the endosome. In addition, immune sensory receptors recognize tissue damage in the form of extracellular ATP released by dying cells

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Hepatic cell populations in a healthy liver

Hepatic cell population	% of liver volume *	% of hepatic cells	Pattern recognition receptors
Kupffer cells	2.1	15	TLR1/2, TLR3,TLR4, TLR6, TLR7/8,CD14, CD1, Fc-R, RIG-I, MDA5, AIM2
LSECs	2.8	18	TLR1/2, TLR3, TLR4, TLR6, TLR7/8, L-SIGN, Fc-R, RIG-I, MDA5, AIM2
Stellate cells	1.4	5-8	TLR4 (other receptors ??)
Hepatocytes	78	58-60	TLR3 RIG-I, MDA5, AIM2
Other cells	<1	1-2	receptors cell-type dependant

*Space of Disse: 4.9%;
*Sinusoidal lumen 10,6%

Fig. 6.1 Innate immune cells in the liver. Scavenger sinusoidal cell populations, such as Kupffer cells, phagocytose circulating bacteria, and cross talk with natural killer T (NKT) cells to generate strong intravascular pathogen-specific immune responses. Inhibiting the access of pathogens to hepatocytes may have an important role in preventing the development of persistent hepatic infections. The death of infected hepatocytes during viral replication may cause the activation of Kupffer cells or dendritic cells (DCs), which in turn promote the killing of other hepatocytes through CD95 (also known as FAS) and the release of pro-inflammatory mediators. Material from dying virus-infected cells increases cross-priming by DCs and thereby augments pathogen-specific adaptive immunity. Combinatorial stimulation by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), such as ATP, may allow immune-mediated control of established hepatic infections. *CXCL9* CXC-chemokine ligand 9, *CXCR3* CXC-chemokine receptor 3, *IFN* interferon, *IL* interleukin, *LSEC* liver sinusoidal endothelial cell, *TCR* T cell receptor, *TNF* tumor necrosis factor. Adapted from Protzer U et al., Living in the liver: hepatic infections. Nat Rev Immunol. 2012 Feb 24;12(3):201–13

(Fig. 6.1) demonstrating that immune sensing also detects tissue damage inflicted by infectious microorganisms as well as sterile tissue damage brought about by trauma or by metabolic disturbances.

Several classes of immune sensory receptors are now recognized (see Fig. 6.1), such as Toll-like receptors (TLRs) that are expressed as membrane-anchored molecules at the cell surface plasma membrane or in endosomal compartments (TLR 3,7,8,9). C-type lectin receptors (CLECs) complement the function of TLRs being also expressed on plasma membranes. Importantly, macrophages and DCs do not have to be infected by the pathogen in order to be activated via PRRs. Instead, they constantly sample material from the outside, including remnants of apoptotic cells and intact microorganisms. Degradation processes in the endosomes then expose, e.g., the viral nucleic acids to recognition by TLRs.

Finally, a substantial number of receptors, such as the nucleotide-binding oligomerization domain-like receptors NOD1/2, the retinoic acid-inducible gene-I (RIG-I)-like helicases RIG-I and MDA5, DNA recognition receptors DAI or AIM2 and STING are located in the cytosol or ER membrane, respectively. The large number of these immune sensory receptors and their complex localization pattern allows individual cells to sense the presence of infectious microorganisms and to discriminate between different stages of "danger," i.e., the presence of a pathogen compared to infection, meaning cytosolic localization and gene expression of the pathogen [1].

The signaling cascades of virtually all immune sensory receptors converge in the activation of either one or both of two central transcription-regulatory systems: (1) the nuclear factor κB (NFκB) system, promoting inflammatory responses primarily upon bacterial infections, and (2) the interferon regulatory factor (IRF) 3/7 system, initiating the antivirally active interferon (IFN) response. While in humans IRF7 is only expressed in specialized immune cells types, IRF3 is expressed constitutively. Alternatively, different cytoplasmic innate immune receptors can nucleate individual protein complexes known as inflammasomes to regulate the production of active IL-1β or IL-18.

Innate Immune Cells in the Liver

The absence of any obvious inflammation in the liver during the physiological situation may suggest that there is no innate immune sensing in the liver. Yet, this is not the case, because the liver is populated with many cell types that are capable of immune sensing (Fig. 6.1). These cell types are either liver-resident, like Kupffer cells, liver sinusoidal endothelial cells (LSECs), liver dendritic cells or hepatic stellate cells (HSCs),

or are rapidly recruited from the blood upon induction of inflammation-like natural killer (NK) cells, natural killer T (NKT) cells, neutrophil or eosinophil granulocytes, and monocytes.

These cells do not only help in fighting pathogens but also support liver regeneration, enable detoxification and support systemic immune-related functions. In the following, we focus on the liver-resident cells and circulating cells with specific functions in the liver.

Liver-Resident Cells

Kupffer cells. Kupffer cells constitute the largest population of tissue-resident macrophages in the body. They form a heterogenous population of macrophages that can be divided in bone marrow-derived and sessile populations [2]. Further subpopulations are likely to exist but cannot be identified due to the lack of discriminatory molecular markers. Kupffer cells are predominantly located in the periportal area and firmly adhere within hepatic sinusoids preferentially at vascular bifurcations and are the main phagocytic population of the liver that eliminates particles larger than 200 nm and aged erythrocytes from the circulation [3].

Kupffer cells belong to the reticulo-endothelial cell system that is known for its pronounced scavenger activity and as such Kupffer actively contribute to clearance of gut-derived bacterial degradation products such as LPS from portal venous blood [4]. Kupffer cells are endowed with many immune sensing receptors including numerous TLRs, RIG-I-like helicases, and CLECs [5], through which they contribute to the induction of inflammation in the liver by the release of pro-inflammatory mediators and to the development of immune-mediated liver disease. They are involved in the chemokine- and adhesion molecule-mediated recruitment of inflammatory monocytes to the liver that further accelerate inflammation and worsen immune-mediated organ damage.

However, Kupffer cells not only release pro-inflammatory mediators upon triggering of TLRs but also express the potent anti-inflammatory cytokine interleukin (IL)-10 [6] or PGE_2 [7] and contribute to local regulation of innate and adaptive immune responses. By removing apoptotic hepatocytes in a manner largely dependent on scavenger receptors, they prevent the attraction of, e.g., neutrophils and limit the severity of liver immunopathology [8]. Kupffer cells can also stimulate the suppressive activity of regulatory T cells inducing IL-10 expression, which is crucial for the induction of tolerance to hepatocyte-expressed antigens [9]. This suppressive function may be overcome in the presence of TLR ligands, when Kupffer cells can override regulatory T cell activity [10].

Thus, Kupffer cells contribute to innate immunity in the liver, yet show a complex pattern of immune responses that cannot be generalized but is rather a function of the Kupffer cell subpopulations being activated.

LSECs. LSECs are also part of the reticulo-endothelial cell system of the liver and possess extraordinary scavenger function that allows them to rapidly eliminate ligands for their various scavenger receptors from the circulation via receptor-mediated endocytosis. They express immune sensing receptors and release pro-inflammatory cytokines or anti-inflammatory eicosanoids [11]. Interestingly, the scavenger function of LSEC is not compromised by inflammation suggesting that the hepatic clearance function is not altered during local innate immunity.

HSCs. Hepatic stellate cells are located in the space of Dissé between LSECs and hepatocytes. By virtue of their contractile properties they control the diameter of hepatic sinusoids. HSCs are also endowed with immune sensing receptors. Following their activation during inflammation, HSCs differentiate into myofibroblasts that produce extracellular matrix and contribute to hepatic fibrosis. In culture, they carry out endo- and phagocytosis and express MHC class I and II molecules, lipid-presenting molecules (CD1b and CD1c) and T cell co-stimulatory molecules, such as CD86, the expression of which on human stellate cells is markedly upregulated by pro-inflammatory cytokines [12]. Recognition of gut-derived LPS by TLR4 or activation of TLR7 on HSCs contributes, e.g., to alcoholic liver disease. The contribution of stellate cells to innate immunity in the liver, however, is still badly characterized and requires further research.

Immune Cells Recruited to the Liver Under Physiological Conditions

Liver dendritic cells (*DCs*). The liver contains more DCs than other parenchymal organs, which may be a consequence of the innate immune stimuli contained in portal blood. DCs in the liver are restricted largely to the perivenular region, portal space, and beneath Glisson's capsule with a few cells scattered throughout the parenchyma [3]. The cytokines fms-like tyrosine kinase 3 ligand (FLT3L) and granulocyte macrophage colony-stimulating factor (GM-CSF) that mobilize DCs from the bone marrow are markedly enriched in the liver [13]. Mouse liver-resident bulk DCs or purified myeloid DCs (mDCs) are less mature, phenotypically and functionally, than those from secondary lymphoid tissue. Under steady-state conditions mouse and human liver DC exhibit tolerogenic properties [3].

The innate immune sensing in dendritic cells typically leads to their functional maturation that is characterized by expression of co-stimulatory molecules. Myeloid or plasmacytoid dendritic cells (pDCs) in the liver, however, fail to

undergo such functional maturation but rather become regulatory cells upon contact with ligands for TLRs or cytosolic NOD receptors [3]. Liver stromal cells drive hematopoietic progenitor cells to differentiate into IL-10^{hi}IL-12^{low} regulatory/tolerogenic DCs [14, 15]. Thus, the liver microenvironment influences the induction of tolerogenic DCs [3].

Immature DCs are recruited from the circulation to the rat liver in response to CCL3 secreted by Kupffer cells in the sinusoidal area [16]. DCs bind to Kupffer cells through N-acetylgalactosamine-mediated interactions [17]. Several liver DC subsets have been characterized, mainly in mice. The dominant populations are conventional mDCs [18] which produce substantial amounts of IL-10, and CD8α$^+$ DCs as in the spleen, but pDCs, that originate in the bone marrow from myeloid and lymphoid progenitors [19] and detect viral RNA or DNA [20], are more prominent in the liver than in secondary lymphoid tissue in mice. Inflammation can convert liver DCs from a tolerogenic to an activating phenotype, but the decisive molecular mechanisms involved remain elusive [3].

NK cells. Hepatic lymphocytes are enriched in NK and NKT cells that play important roles in antiviral, antimicrobial, and antitumor defense, in liver injury, but also liver fibrosis and repair [21]. Both cell types can accelerate liver injury by producing proinflammatory cytokines and killing hepatocytes. But NK cells can also inhibit liver fibrosis via killing early-activated and senescent-activated stellate cells and producing IFNγ [22].

NK cells make up 20–30 % of human and rat, and 5–10 % of mouse hepatic lymphocytes [22]. They were originally described as "pit cells" in the liver because they contain highly characteristic cytoplasmic granules [23]. NK cells generally reside in the hepatic sinusoids, express chemokine receptors and can migrate to inflamed tissue sites. Kupffer cells have been proposed to be the main source of CCL2/MCP-1 expression and to recruit CCR2 expressing NK cells to the liver [24]. NK cells arrive very early at the site of inflammation and lyse their target cells (e.g., tumor cells, virus-infected hepatocytes) by releasing cytotoxic granules containing perforin and granzymes [25].

Liver NK cells show phenotypic and functional characteristics that are distinct from their circulating counterparts. In particular, intrahepatic NK cells are more activated and the majority have a CD56^{bright} phenotype, thought to be an earlier stage of differentiation than the CD56^{dim} phenotype predominating in the periphery [26].

NK cell biological function is tightly regulated by the balance of signals provided by their diverse array of cell surface receptors, combined with the cytokine milieu. In the liver, their activation is likely to be heavily influenced by the ligands they encounter on the cells lining the extensive sinusoidal network. The interaction, e.g., of galectin-9 expressed on Kupffer cells [27] with Tim-3, upregulated on NK cells in chronic hepatitis B virus (HBV) infection, can downmodulate NK cell function [28].

NK cells also express TLRs which can modulate their function. In addition, particular cytokines (e.g., IL12, 15, and 18) as well as Type-I IFN secreted by activated DCs or infected hepatocytes activate NK cells [29]. On the other hand, NK cells may also indirectly maintain hepatic tolerance via activation of dendritic cells, which can then induce tolerogenic regulatory T cells [30]. Thus, NK cells are not only important innate effector cells with cytolytic activity, but also contribute to maintaining tolerance in the physiological situation.

NKT cells. Innate lymphocytes, which collectively are a substantial fraction of total lymphocytes, are viewed as distinct lineages carrying out "hardwired" innate rather than adaptive strategies of immune defense allowing rapid immune responses. NKT cells, which share properties of both T cells and NK cells, are one of the most prominent populations of innate lymphocytes and are mainly found in the liver.

NKT cells are recruited to the liver through CXCR6. NKT cells include both NK1.1$^+$ and NK1.1$^-$, as well as CD4$^+$, CD4$^-$, CD8$^+$, and CD8$^-$ cells. Upon stimulation, they secrete not only large quantities of IFNγ, IL-4, and GMCSF, but also other chemo- and cytokine. Most NKT cells recognize the non-polymorphic CD1d molecule, an antigen-presenting molecule that binds self- and foreign lipids and glycolipids. Some of these NKT cells co-express a heavily biased, semi-invariant T cell receptor, while others have a diverse T cell receptor repertoire. A third group is MHC-restricted and recognizes MHC-presented peptides but still carries the NK marker NK1.1. However, the field is moving fast and novel classes of innate lymphoid cells constantly emerge. In humans, mucosal-associated invariant T cells have recently been identified as a novel sinusoidal NKT population [31].

NKT cells constitute only approximately 0.1 % of all peripheral T cells, but about 30–40 % of lymphocytes in the mouse and 10–25 % in the human livers [22]. NKT cells are involved in the several kinds of liver injuries: Con-A-induced liver injury [32, 33], autoimmune liver disease, alcohol consumption-induced liver injury [34], nonalcoholic fatty liver disease [35], LPS-induced liver injury [36] as well as carrageenan (a food additive)-induced hepatotoxicity [37]. This indicates that NKT cells play an important role in liver disease development. NKT cells are, e.g., activated by HBV via the CD1 receptor [38]. They can inhibit not only HBV replication [39], but also hepatitis C virus (HCV) replication in hepatocytes by secretion of IFNγ, and their activity correlates positively with the outcome of acute HCV infection [40] and the efficacy of IFNα treatment in chronic HCV infection [41].

Besides their role in microbial clearance and liver damage, NKT cells have various regulatory functions: they can,

e.g., enhance cross-priming by upregulating co-stimulatory molecules on DCs [42], remove circulating tumor cells from the body [43], and prevent tumor metastases in the liver. Thus, NKT cells possess various regulatory functions upon activation and provide an important bridge between innate and adaptive immunity in the liver.

Immune Cells Recruited to the Liver in Inflammatory Situations

Monocytes. Monocytes constitute around 5–10 % of peripheral blood leukocytes. Originating from a myeloid precursor in the bone marrow, circulate in the blood, bone marrow, and spleen, and then enter various tissues. Monocytes are circulating precursors for tissue macrophages and dendritic cells. Depending on the inflammatory milieu (e.g., in hepatitis), they are recruited to the liver where they then differentiate. Block of particular chemokines (e.g., CCL2) can reduce the recruitment of monocyte subsets into the liver [44].

Circulating monocyte subsets are involved in various physiological processes also including to support the elimination of pathogens or damaged hepatocytes but also to drive liver pathology if persistently activated [44]. Different subtypes of monocytes exist in mice and men [45], based on the expression of particular surface markers. The differential expression of CD14 (part of the receptor for LPS) and CD16 (also known as FcγRIII) are used to characterize the two major groups in peripheral blood in humans. On the one hand, the $CD14^{hi}CD16^{neg}$ monocyte population, representing around 95 % of monocytes in healthy individuals, and, on the other hand, the "non-classical" $CD14^{pos}CD16^{pos}$ cells comprising the remaining fraction of monocytes. In patients with liver cirrhosis, $CD14^{pos}CD16^{pos}$ monocytes are activated in blood and liver and promote pro-inflammatory along with pro-fibrogenic actions by the release of distinct cytokines and direct interactions with HSCs, indicating that findings from murine models can be translated into pathogenesis of human liver fibrosis [44].

Neutrophils. Neutrophilic granulocytes are essential in the defense against invading microorganisms and the importance of neutrophil-mediated liver injury has been demonstrated in a variety of liver diseases and chemical or drug hepatotoxicity [46]. Hepatic infiltration of neutrophils is an acute response to recent or ongoing liver injury, hepatic stress, or systemic inflammatory signals [47]. Once neutrophils have reached the liver, they produce matrix metalloproteinases and attract other antigen-nonspecific innate immune cells [48]. Neutrophils can cause mild-to-severe tissue damage and consequent liver failure [46]. For neutrophils to appear in the liver, neutrophils have to undergo systemic activation (priming) by inflammatory mediators such as cytokines, chemokines, complement factors, immune complexes, opsonized particles, and other biologically active molecules, e.g., platelet-activating factor [46]. Neutrophils accumulated in the hepatic microvasculature can transmigrate into the hepatic parenchyma if they receive a signal from distressed cells [46]. Transmigration can be mediated by a chemokine gradient established towards the hepatic parenchyma and generally involves orchestration by adhesion molecules on neutrophils (2 integrins) and on endothelial cells (intracellular adhesion molecules, ICAM-1). After transmigration, neutrophils adhere to distressed hepatocytes through their β2 integrins and ICAM-1 expressed on hepatocytes. Neutrophil contact with hepatocytes mediates oxidative killing of hepatocytes by initiation of respiratory burst and neutrophil degranulation leading to hepatocellular oncotic necrosis [49]. In addition, neutrophils mediate bacterial clearance through various mechanisms, including the release of mesh-like DNA structures or neutrophil extracellular traps (NETs) that capture viruses or bacteria. Recent data indicate an interesting interplay with platelets.

Platelets. Systemic administration of virus analogs or poxvirus infection induces neutrophil and platelet recruitment to the liver microvasculature and the release of NETs that protect the liver from virus infection [47]. Circulating platelets interact with, roll along, and adhere to the surface of adherent neutrophils, forming large, dynamic aggregates. Upon activation, platelets contribute to liver disease and viral clearance by promoting the recruitment of virus-specific cytotoxic T lymphocytes (CTL) into the liver [50] mots likely by the release of serotinin [51]. Thus, platelet aggregation and immunothrombosis [52] are increasingly recognized immune defense mechanisms contributing to innate immunity in the liver.

Immune Sensing in the Liver

Hepatic and splenic immune cells are able to sense pathogens [53]. Importantly, TLRs and cytosolic helicases, such as RIG-I and MDA5, are expressed not only by bone marrow-derived immune cells, such as Kupffer cells and hepatic DCs, but also by liver-resident cells, such as hepatocytes, LSECs, and HSCs [11, 54–57]. Kupffer cells and LSECs can detect very low concentrations of TLR ligands. However, constant exposure of liver cells to the TLR ligand LPS present in portal venous blood causes a state of hyporesponsiveness (known as LPS tolerance) towards further pro-inflammatory immune stimulation [58]. As a potential consequence LSECs and hepatic DCs may not mature into immunogenic antigen-presenting cells [11, 59] and this may contribute to impair the local induction of CTL responses [60]. It is possible that this limits pathogen-specific defense, but experimental proof of this idea is lacking. Nevertheless,

it is very unlikely that reduced expression or dysfunction of immune sensing receptors compromises the ability of the liver to sense infection.

Besides TLR and cytosolic helicases, Kupffer cells and LSECs express additional immune sensing receptors that allow them to mount potent innate immune functions. For example, Kupffer cells express FcαRI (CD89) and CRIg (a C3 receptor that enhances complement-mediated phagocytosis) which promote the efficient removal of complement-coated blood-borne bacteria [61], generating a second line of defense against liver infection by pathogens that breach mucosal immunity in the gut. Stimulation of most immune sensing receptors by PAMPs leads to the activation of hepatic scavenger cells and the expression of pro-inflammatory mediators, mainly IL-6 and IL-10, which have been shown to exert tissue protective and immunoregulatory effects [62, 63]. IL-6 triggers hepatocellular expression of acute phase proteins, such as complement and C reactive protein that bind to pathogens and enhance phagocytosis, but decreases detrimental TNF release by Kupffer cells [3]. Hepatic immune sensing also induces the expression of adhesion molecules and chemokines by endothelial cells leading to immune cell recruitment from the blood, which modulates the induction of local tolerance or immunity, depending on the cells that are recruited—regulatory or effector T cells. Immune sensing also triggers the expression of immunoregulatory molecules such as IL-10, transforming growth factor-β (TGFβ) or prostanoids [64]. Hepatic expression of arginase and indoleamine 2,3-dioxygenase-2 (IDO) not only has antimicrobial activity, but it also impedes local adaptive immunity by the metabolism of amino acids that are essential for immune cell proliferation. Taken together, the constitutive and functional expression of immune sensing receptors by hepatic cell populations not only leads to the induction of innate immunity, allowing for local as well as systemic antimicrobial activity, but also restricts the local induction of adaptive immunity [3].

Transition from Local to Systemic Innate Immunity in the Liver

Due to its clearance function, the liver is central to elimination of PAMPs and DAMPs, which is achieved by the scavenger activity of Kupffer cells, DCs, and LSECs leading to the release of IL-6 and IL-1. Such pro-inflammatory mediators act in a paracrine fashion on hepatocytes to induce the expression of effector molecules, such as C-reactive protein, complement proteins, and other acute phase proteins [65]. Such cellular cross-talk between scavenger cell populations and the metabolically active hepatocytes therefore helps to increase innate immunity everywhere in the blood stream by providing increased concentrations of effector molecules required for antibacterial defense and for pro-coagulant activity. On the other hand, IL-6 activates crucial hepatoprotective genes in liver injury [62] and promotes liver regeneration [66].

Innate Immunity During Infection of the Liver

Innate Immunity Against Bacterial Infection in the Liver

Blood-borne bacteria are normally cleared rapidly from the liver by the scavenging hepatic cells. Following ingestion of bacteria, such as *Borrelia* spp., Kupffer cells attract NKT cells to the liver in a CXCR3-dependent manner and present bacterial glycolipid antigens on CD1 molecules to NKT cells. The concerted action of these sinusoidal immune cell populations induces an intravascular immune response that prevents further bacterial infection [67]. Rapid initiation of immune defense against circulating pathogens within the hepatic sinusoid strengthens the notion that early pathogen sensing supports successful elimination [68]. Besides phagocytosing Kupffer cells, platelets, neutrophils, and NKT cells are instrumental in this aspect as they can recognize microbial antigens rapidly exert immune effector functions thus bridging innate and adaptive immunity [47, 69]. Thus, our knowledge of successful antibacterial defense in the liver indicates a functional distinction between the hepatic sinusoidal compartment, where immune cells can eliminate pathogens and prevent them from accessing hepatocytes, and the parenchymal compartment, where infection is more difficult to eradicate and may even be facilitated through the tolerogenic properties of the local microenvironment and organ-resident cell populations [68].

Once they have reached the liver parenchyma, some bacteria, such as mycobacteria spp., and Listeria spp., can establish granulomas [70, 71]. Granuloma formation by mycobacteria is driven by infected macrophages, which secrete bacterial proteins that induce the expression of matrix metalloprotease 9, and thus tissue remodeling [72]. These granulomas can wall off infecting bacteria from noninfected surrounding tissue [70] but have also been shown to contribute to the dissemination of virulent bacteria [73]. Therefore, although primarily meant to contain and eliminate bacteria, it is possible that granulomas provide a distinct anatomical compartment in the liver that supports survival of bacteria [68].

Innate Immunity Against Parasite Infection of the Liver

The liver is the target organ of a number of parasites: *malaria* spp., *Schistosoma* spp., *echinococci* and *Fasciola* being

prominent examples with malaria being the best-studied example. *Plasmodium* spp., transmitted by *Anopheles* mosquitoes cause malaria in humans. Plasmodium sporozoites are transmitted from the saliva of a biting female mosquito. Infection by *Plasmodium* spp., provides an example in which the barrier and effector mechanisms of sinusoidal cells are overcome to infect hepatocytes [68]. Most sporozoites migrate to the liver and invade hepatocytes following migration through Kupffer cells. During the hepatic stage, sporozoites mature into schizonts that contain many merozoites. Plasmodia normally do not persist in the liver as they only require initial maturation and replication in hepatocytes and then actively egress from the liver as merozoites before they enter the erythrocyte stage and taken up again by female mosquito bites. Even repetitive infection with sporozoites in areas endemic for malaria often fails to generate protective immune responses, which are characterized by parasite-specific CTL and antibody responses [74]. Plasmodium sporozoites apparently evade innate immunity by inhibiting a respiratory burst in Kupffer cells [75] and by establishing a parasitophorus vacuole in Kupffer cells that prevents sporozoite surface molecules from being directly recognized by membrane-bound PRRs [76]. Nevertheless, hepatocytes that die following sporozoite infection as well as later parasite stages trigger innate immunity [77]. Thus, parasites seem to hide in the liver to evade innate immune recognition.

Innate Immunity as a Crucial Determinant in Viral Hepatitis

Viruses that target the liver belong to different virus families. The different hepatitis viruses mainly or exclusively target hepatocytes, whereas for adeno-, echo-, herpes-, and hemorrhagic fever viruses the liver cells only are a secondary target. We will therefore focus on hepatitis viruses that already show a very different outcome for which innate immunity may be a main determinant.

HBV and HCV are human blood-borne viruses, which may persist in hepatocytes lifelong and cause chronic infection. HBV is a small, enveloped DNA virus that deposits a covalently closed circular DNA in the nucleus to persist in the host. HBV is transmitted by sexual contact, by direct blood contact and at birth from mother to child. HCV is an enveloped virus with a plus-strand RNA genome. It is only transmitted by direct blood–blood contact. HBV persists in >90 % of infected neonates, but it is cleared in >90 % of adults. HCV persists in 50–80 % of all infected individuals.

Hepatitis A virus (HAV) and hepatitis E virus (HEV) cause self-limited infection. Both are non-enveloped viruses with a plus-strand RNA genome transmitted via the fecal–oral route, but belong to different virus families. While HAV infects only humans, HEV infects animals. HAV and HEV never cause chronic hepatitis, although HAV can persist for many weeks in the livers of infected chimpanzees even after clearance of virus from the serum or feces [78]. One may therefore speculate that a difference in inducing innate immunity may trigger different adaptive responses and a different outcome of infection.

Escape of Viruses from Immune Sensing in the Liver

Viruses that target the liver seem to actively avoid or even overcome local immune sensing. This is important at two stages: first, during entry into the liver and, second, during productive hepatocyte infection. For example, HBV and HCV capsids are recognized by TLR2, expressed by macrophages and Kupffer cells [79, 80]. Activation of PRRs by HBV leads to the release of pro-inflammatory cytokines and IL-10, but not type I IFNs, from Kupffer cells and LSECs [81]. Accordingly, patients with acute HBV infections have high plasma levels of IL-6 and IL-10, but no increase in antiviral type I IFNs [82]. This suggests that HBV and HCV may not only sneak under the "immune radar" by using a limited number of virus particles to efficiently target the liver, but also by avoiding the induction of antiviral IFNs and initiating cytokine responses that confer tissue protection [68].

Once an infection is established, pathogens can escape innate immune recognition by adapting their life cycles. For example, HBV is considered a stealth virus, as it escapes immune sensing by synthesizing its genome within the viral capsid [83]. In addition, HBV gene products suppress the response of liver cells to TLR ligands [55, 81].

HBV and HCV, although being important examples of persistent hepatic infections, can also be spontaneously controlled following acute infection. Following resolution of acute infection, HCV is eliminated by almost all patients [84], whereas HBV is controlled but not completely eliminated, and may reactivate under strong immunosuppression [85]. The initiation of immune responses and resolution of infection with HAV, HBV, or HCV are protracted compared with other acute viral infections. This suggests the occurrence of viral evasion of immune sensing and that innate immunity can be successfully overcome or avoided in the first few months after infection and during chronic hepatitis. Animal models and human studies of acute resolving infections have highlighted the importance of vigorous and multispecific CTL responses, which develop in the presence of adequate T cell help. These CTL responses, however, are absent during chronic infection [86].

Cytokines such as TNF and IFNγ released by CTL, NK, or NKT cells can control replication of various viruses in hepatocytes in a non-cytopathic fashion [39, 87, 88]. In addition, IL-6 limits HBV gene expression [81]. HCV, which as

an RNA virus relying on continuous replication, can even be cleared by the activation of IFN signaling in proliferating cells, and is reduced but persists in non- or slowly dividing hepatocytes [89].

Thus, antiviral cytokines limit viral replication in hepatocytes ensuring initial reduction in viremia without significant liver damage [90], but CTL-mediated cytotoxicity is required for final infection control [91]. The induction of CTL-responses, however, will only occur if antigen-presenting cells receive appropriate second and third signals by PRR triggers [92]—which persistent HBV as well as HCV avoid.

Active Interference with Pattern Recognition by Hepatitis Viruses

HAV and HCV have similar genome structures, share many aspects of their replication strategies and actively interfere with immune sensing [93, 94]. Both viruses replicate via a double-stranded RNA intermediate, which is recognized by endosomal TLR3 [95] and the cytosolic immune sensors RIG-I and MDA5 in infected hepatocytes. Besides the replication intermediates, a triphosphate motive at the 5′ end of the RNA and homopolyuridine or homopolyriboadenine motifs present in viral RNA genomes are the chief features of RIG-I recognition [56]. The HCV protease NS3/4a counteracts RIG-I, MDA5, and TLR3 signaling by cleaving essential mitochondrial signaling molecules IFN-promoter stimulator 1 (IPS1) [96, 97] and TIR-domain-containing adaptor protein inducing IFNβ (TRIF), thereby disrupting downstream IFN-regulatory factor 3 (IRF3) signaling [96, 98]. HAV can also disrupt RIG-I, MDA5, and TLR3 signaling pathways through cleavage of IPS1 and TRIF, by two distinct precursors of the HAV protease [99, 100].

However, the two viruses induce a different innate immune response resulting in a different outcome of infection. Expression of HCV NS3/4a in mice is not sufficient to hinder the induction of type I IFNs or expression of IFN-responsive genes [101] explaining that in chimpanzees, HCV, but not HAV, induces a strong IFN response in the liver and HCV is even cleared more rapidly than HAV [78]. While early and strong innate immune responses in HCV-infected individuals are an indicator of subsequent clearance of infection from the liver [102], immune responses fail to clear HCV infection in more than 50 % of cases despite a rapid and strong IFN response. In chronic hepatitis C, in about half of the patients, hundreds of type I or III IFN-stimulated genes (ISG) become again strongly expressed. However, in chronic infection, this innate immune reaction is ineffective against HCV. Moreover, patients with constitutive ISG expression have a poor response to treatment with pegylated IFN-α (PegIFN-α) and ribavirin [103]. Recently, genetic variations near the *IL28B* (*IFN-λ3*) were found to be strongly associated with spontaneous clearance of HCV and response to treatment with PegIFN-α and ribavirin further supporting a central role of the innate immune response in host–viral interactions. The viral escape mechanisms that protect HCV from IFN-mediated innate immune reactions are not entirely understood, but might involve blockade of ISG protein translation at the ribosome, localization of viral replication to cells with refractory IFN signal transduction pathways or to cell compartments that are not accessible to antiviral IFN-stimulated effector systems [104].

Does Hepatocyte Death Determine the Outcome of Viral Hepatitis?

Appropriate innate immune stimuli are needed to induce strong adaptive immunity [92] required for virus elimination. Hepatocyte killing is usually attributed to the cytotoxic potential of CTL. However, the direct MHC I-restricted recognition of virus-infected hepatocytes contributes not even to 50 % to the total effect of CTLs in hepatic immune surveillance. A noncanonical CTL effector function accounts for more than 50 % of the entire antiviral CTL activity in the liver. This noncanonical CTL effector function is triggered by LSECs that cross-present hepatocyte-derived viral antigens [105]. Such activated CTLs release TNF locally into the hepatic sinusoid, which then acts selectively on virus-infected but not uninfected hepatocytes to promote cell death [105]. This sensitization of virus-infected hepatocytes to TNF-induced cell death is a novel form of immune sensing that allows cells of the adaptive immune system, i.e., CTLs, to execute their effector function by making use of innate immune mechanisms. Thus, innate immunity not only operates at the beginning of an adaptive immune response but rather also facilitates the execution of effector functions of antigen-specific CTLs. This achieves immune surveillance against viral infection at several levels and presumably serves to counteract viral immune escape strategies.

Cell death may also help to explain the different outcome after. Although HAV and HCV have similar strategies that allow them to circumvent the induction of type I IFN responses, HAV and HCV infections have different outcomes. Besides differences in sensitivity to ISGs [94, 104], it is possible that the difference in clinical outcome lies in the unique properties of the virus particles. HAV, in contrast to HCV, is a non-enveloped virus that requires the disruption of host cell membranes and thus hepatocyte death to release its progeny. Hepatocyte death may provide a distinct immune stimulatory signal, that is required to overcome viral immune escape and liver-intrinsic tolerogenic mechanisms [68].

Kupffer cells and DC are activated by dying hepatocytes (Fig. 6.1b) and provide a synergistic signal to PAMP-driven hepatic inflammation [106]. Hepatic inflammation induces the recruitment of neutrophils, which increase local inflammation [107], and the production of type I IFNs by innate

immune cells contributing to the control of hepatic infection [108, 109]. In addition, uptake of antigens derived from dying virus-infected cells by DCs through the endocytic receptor CLEC9a increases DC functional maturation and efficiency of CTL cell cross-priming [110, 111].

Neither this, nor the absent response to IFNs, however, can explain the differences in the outcome of HAV and HCV infection and more efforts identifying receptors for DAMPs and characterizing the differences in innate and adaptive immunity will be required to unravel the immune sensing mechanisms that determine successful adaptive immunity, i.e., induction of strong T cell responses and elimination of viral infection from hepatocytes.

Consequences of Innate Immunity in the Liver: Transition from Acute to Chronic Inflammation

Activation of innate immune cells in the liver leads to *acute inflammation*, i.e., secretion of inflammatory mediators, and recruitment of blood-borne immune cells, mainly neutrophils, granulocytes, and inflammatory monocytes from the sinusoidal circulation into the liver tissue via expression of chemokines and adhesion molecules on liver sinusoidal cell populations. Inflammatory signals generated in the liver such as the chemokine CCL2 lead egress of inflammatory monocytes from the bone marrow and expression of adhesion molecules like CD54 (ICAM-1) by sinusoidal lining cells promotes recruitment to the liver [112].

Inflammatory reactions usually are rapidly limited to avoid tissue damage. Prolonged or repeated inflammatory reactions, e.g., if the trigger of the inflammatory reaction persists, it can result in *chronic inflammation.* Chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of liver tissue from the inflammatory process. Activated stromal cells are found and adaptive and innate immune cells are continuously recruited to the liver.

The transition of acute to chronic inflammatory reactions is a very important but still poorly defined process. The induction of innate immunity is typically associated with the induction of regulatory molecules that terminate activation and thereby immediately limit inflammation. In case of persistence of the inflammatory stimulus (e.g., a persistent virus or the accumulation of abnormal metabolites), innate immune activation may persist and inflammation cannot be resolved. It has recently been demonstrated that HCV protein NS5B, the HCV RNA-dependent RNA polymerase, triggers continuous innate immune activation and lymphotoxin expression [113]. It is also possible that recruitment of immune cell populations such as neutrophils and inflammatory monocytes from the blood perpetuate inflammation in a self-amplified feed-forward loop.

During chronic inflammation, infiltrating immune cells start to organize themselves into a micro-architecture composed of follicular structures similar to secondary follicles of the spleen. Examples driving such tertiary lymphoid follicle formation in the liver are chronic HBV or HCV infections as well as autoimmune hepatitis. It is believed that these persisting inflammatory nodes drive tissue damage and cancer development in organs with a high regenerative capacity such as the liver.

Liver Fibrosis and Hepatocellular Carcinoma as a Consequence of Chronic Inflammation

If inflammation persists locally in the liver, HSCs differentiate into myofibroblasts through the combined activity of TGFβ and platelet-derived growth factor (PDGF) signaling. As a consequence of this cell differentiation increased expression of extracellular matrix ensues that is accompanied by reduced enzymatic matrix degradation activity, which results in deposition and accumulation of extracellular matrix in the space of Dissé. The activation of HSCs and myofibroblasts leading to increased matrix production is driven by inflammatory mediators. However, HSCs are also directly activated by immune sensing receptors such as TLR4 [114] by LPS from the portal blood. This indicates that HSCs contribute to the immune competence of the liver and that immune sensing by HSCs directly promotes cell activation and development of hepatic fibrosis.

Rudolph Virchow in the mid nineteenth century—for the first time—described a possible link between chronic inflammation, wound healing, scar formation and the development of tumors. And as we know from today—he was right [115, 116]. In the last 15 years, numerous studies have corroborated the tight link between chronic inflammation and carcinogenesis in epidemiological studies, which demonstrated that chronic inflammation induced by pathogens, parasites, or toxins increases the risk to develop cancer. Consequently, Hanahan and Weinberg [117] defined a list of hallmarks of cancer which include an inflammatory environment [118]. Recently, this list was updated adding reprogramming of energy metabolism and evading immune destruction as further conceptual hallmarks [116]. It should be pointed out that chronic inflammation can drive cancer most efficiently in highly regenerative organs such as the liver.

Innate Immunity: A Double-Edged Sword in Hepatocarcinogenesis

Chronic inflammation in the liver can be induced by viruses, e.g., HBV or HCV which trigger chronic hepatitis and development of hepatocellular carcinoma (HCC) [119]. Infections with schistosoma parasites favor chronic inflammation-induced bladder and liver cancer [120]. Autoimmune hepatitis

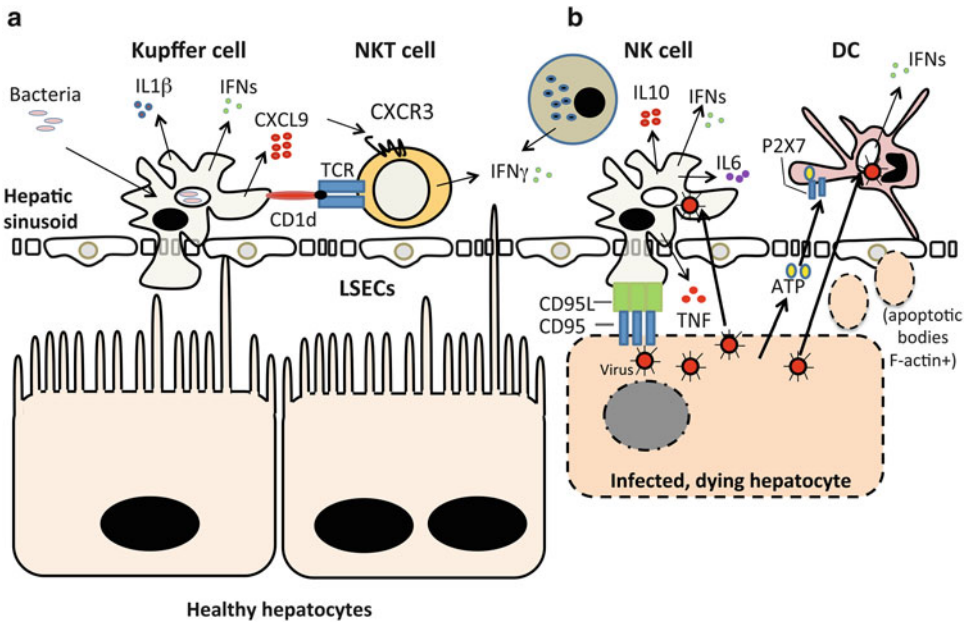

Fig. 6.2 Lymphotoxin-driven inflammation causes liver cancer. (**a**) Immunohistochemical analysis of representative 9-month-old C57BL/6 and *tg*1223 livers. B220⁺-stained B cells, CD3⁺ T cells, F4/80⁺ macrophages, Kupffer cells, and A6⁺ oval cells (scale bar, 150 μm). Ki67⁺ proliferating hepatocytes (*arrow heads*) and inflammatory cells are indicated (scale bar, 50 μm). (**b**) Macroscopy of C57BL/6 (*left panel*) and *tg*1223 livers at the age of 12 (*middle panel*) and 18 months (*right panel*). *White arrows* indicate tumor nodules. *White arrowhead* indicates a liver lobe completely affected by HCC. Scale bar size is indicated. (**c**) Histological analysis of livers derived from C57BL/6 and *tg*1223 mice. *Dashed line* depicts the HCC border. Collagen IV staining highlights the broadening of the liver cell cords and loss of collagen IV networks indicative of HCC in *tg*1223 mice (scale bar, 200 μm). High numbers of Ki67⁺ proliferating hepatocytes (*arrowheads*) are only found in *tg*1223 HCC (*right column*; scale bar, 100 μm). (**d**) Scheme of chronic inflammation-induced liver carcinogenesis in *tg*1223 mice: Transgenic hepatocytes (*brown*) express LTα and LTβ and induce chemokine production (e.g., CCL2, CCL7, CXCL1, and CXCL10) in the presence of IKKβ and intrahepatic lymphocytes. Chemoattraction and activation of myeloid cells and lymphocytes expressing particular chemokine receptors (e.g., CXCR3, CXCR2, CCR2, and CCR1) cause hepatitis: CXCL10 attracts CXCR3⁺ T and NK cells, CXCL1 CXCR2⁺ T cells, B cells, neutrophils, and CCL2 CCR2⁺ macrophages, and CCL7 attracts CCR1⁺ monocytes. Activated, infiltrating immune cells secrete cytotoxic cytokines (e.g., IL6, IL1β, TNFα, IFNγ, and LTαβ) that cause tissue destruction, hepatocyte proliferation, cell death, and tissue remodeling. In such an environment, hepatocytes are susceptible to chromosomal aberrations leading to HCC. Tissue destruction and remodeling supports the infiltration of activated inflammatory cells (e.g., myeloid cells), leading to a feed-forward loop toward chronic aggressive hepatitis. *Asterisks* indicate that genetic depletion of those components (IKKβ; T and B cells) blocks chronic hepatitis development and HCC. Blocking LTβR signaling with LTβR-Ig in 9-month-old *tg*1223 mice reduces chronic hepatitis incidence and prevents HCC. (+) indicates the fortification of a described process. (−) indicates the suppression of a described process. The transcription factor RelA is schematically depicted as a *green circle*, inducing transcription of NF-κB target genes (e.g., chemokines) (*arrow*). *B* B cells, *T* T cells, *MO* macrophages, *N* neutrophils, *NK* NK cells. Adapted from Haybaeck et al., Cancer Cell, 2009 Oct 6;16(4):295–308

[121] as well as toxins (e.g., alcohol or aflatoxin-B) [122] can induce HCC development, and primary sclerotizing cholangitis can lead to cholangiocellular carcinoma [123]. Finally, only recently—in line with epidemiological studies—it was shown in mouse models that dietary and genetic obesity promote chronic liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression [124].

Several reports have indicated that chronic HBV or HCV infection can induce upregulation of lymphotoxin, a member of the tumor necrosis superfamily, to drive chronic inflammation and liver cancer ([125]; see also Fig. 6.2). For HCV, NS5B—the viral RNA-dependent RNA polymerase—seems responsible for this phenotype since its pharmacological inhibition alleviated pro-inflammatory lymphotoxin signaling [113].

On the other hand, numerous clinical reports and experimental data exist, which support the idea that inflammation can also be anticarcinogenic [126, 127]. This was already noted more than 100 years ago when oncologists injected dead bacteria to treat cancer patients. Most likely, this inflammatory reaction drives activation of innate immune cells, which directly contribute to the destruction of tumor cells, or which generate a microenvironment that enables adaptive immune cells to efficiently attack and lyse cancer cells.

Although immense progress has been made in the last decade to understand the consequences and mechanisms of chronic inflammation on tissue integrity, chromosomal stability, apoptosis, proliferation and cancer development, the exact pathways and cellular ingredients that define inflammation as anti- or pro-carcinogenic remain unknown. Most

likely, the initial composition of the immune cells during a chronic inflammation, the cells infiltrating or surrounding the tumor tissue, the interaction of the tumor stroma with immune cells, the expression of particular cytokines and chemokines as well as the organ in which chronic inflammation occurs explain why inflammatory conditions might be a double edged-sword [116, 128]. Further, it remains elusive if the transition from chronic inflammation to cancer underlies a particular molecular pathway or rather depends on an environment that increases the stochastic likelihood to cause transformation of cells into cancer cells—presumably, both scenarios are possible depending on various genetic host factors and the etiology driving cancer.

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The Liver and Immune Tolerance

7

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Key Points

- The liver is an immunological organ with unique properties of immune tolerance, exemplified by the tolerance to digested food products and other antigens from portal vein, the acceptance of allogeneic liver transplantation across MHC barriers, the persistence of hepatotropic pathogens in liver, as well as the sustained long-term expression of specific transgene in liver with induction of the foreign protein-specific systemic immunotolerance.
- The establishment of liver immune tolerance is attributed to the unique anatomy, blood supply, cell composition, and the microenvironment in this organ. The mechanisms of liver immunotolerance include clone deletion, anergy, and unresponsiveness of various immune cell subsets. Activated $CD8^+$ T cells in liver can become functionally inhibited or undergo apoptosis as demonstrated in T cell exhaustion. The $CD3^+CD25^+Foxp3^+$ regulatory T cells (Tregs), the tolerant $CD4^+$ T cells, NK cells, NKT cells, and γδT cells also contribute to the liver tolerance.
- The hepatic antigen-presenting cells with tolerant phenotypes play critical roles in induction of liver immune tolerance by down-regulating MHC class molecules and co-stimulatory molecules, but up-regulating co-inhibitory receptor ligands (such as PD-L1) and secreting immunosuppressive cytokines (IL-10 and TGF-β). Combined blockade of co-inhibitory receptors and immunosuppressive cytokines has shown promise in reversing immune tolerance especially in persistent viral infection.
- The liver is an immunological organ with predominance of innate immunity. The enriched innate immune cells, such as NK, NKT, and γδT cells, also contribute to the formation of liver immune tolerance.
- The hepatic intrinsic immune tolerance can lead to systemic immune tolerance. Therefore, it is possible to prevent or treat systemic diseases by manipulating hepatic intrinsic immune responses. This provides a potential therapeutic strategy for systemic diseases, in which hepatic immune tolerance is either induced to control extrahepatic autoimmunity, or suppressed to reverse systemic immune tolerance.

Introduction

As the largest solid organ in the body, the liver plays a critical role in metabolism and detoxification. Recently, emerging evidence suggests that the liver is an immunological organ with unique properties of predominant innate immunity and immune tolerance. The characteristics of liver immune tolerance are associated with the unique anatomy, blood supply, the cell composition, as well as the microenvironment of this organ. The immune tolerance and predominance in innate immunity of liver are not only related to the pathogenesis of many liver diseases such as persistent hepatotropic viral infection and hepatic carcinoma, as well as the liver transplantation tolerance, but also affect the development of systemic diseases. Fully understanding the cellular and molecular mechanisms of the predominance of innate immunity, the formation of liver immune tolerance, as well as the subsequent induction of systemic tolerance, will

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provide foundation for development of novel therapeutic strategies for related diseases.

Characteristics of Liver Tolerance

The liver is located at a crossroad of the systemic circulation and receives its blood supply from two sources: approximately 20 % from the hepatic artery and 80 % from the portal vein (Fig. 7.1). Portal venous blood from intestine contains the digested products from food, aged or damaged cells, microbial products, and antigens from the intestinal bacteria, making liver the first organ that encounters these antigens. As the largest detoxification and metabolization organ in the body, liver extracts nutrients from portal venous blood for hepatocellular metabolism. Meanwhile, liver eliminates toxic waste products, including endotoxin and other bacterial degradation products from the intestine. During the processes of detoxification and metabolization, a multitude of neo-antigens might be produced. Therefore, the risk of immune activation in the liver appears to be higher than elsewhere in the body; however, the hepatic immune cells usually do not elicit any overt immune response under physiological conditions. Instead, the liver

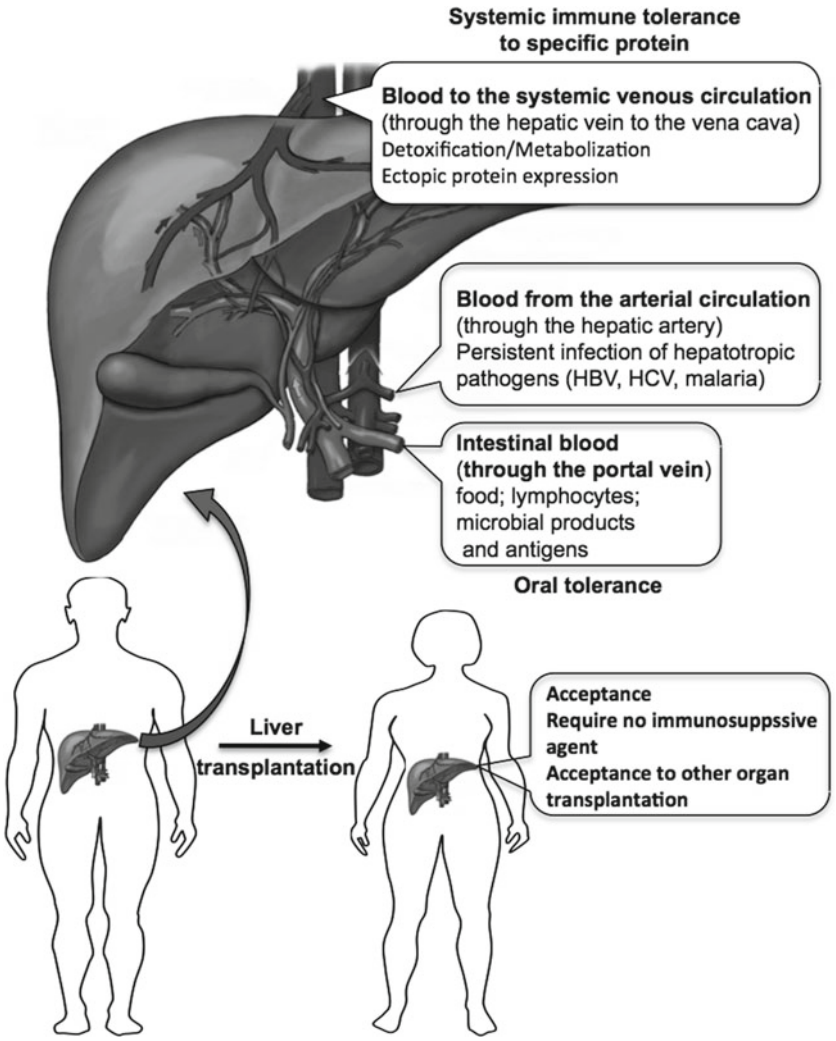

Fig. 7.1 Phenomenon of liver tolerance. The liver receives its blood supply from two sources: approximately 20 % from the hepatic artery and 80 % from the portal vein. Portal venous blood from intestine contains the digested products from food, microbial products, and antigens from the intestinal bacteria, making liver the first organ that encounters these antigens; however, the hepatic immune cells usually do not elicit any overt immune response under physiological conditions. The liver tolerance is also evident in liver transplantation, in which rejection of the allografts is in general much milder than that in transplantation of other organs. Liver allografts can induce donor-specific tolerance that further facilitates the acceptance of subsequent non-hepatic allografts, such as heart, skin, islet, and small intestine, from the same donor while rejecting third-party grafts. Liver is also an organ where many important pathogens evade immune surveillance and sustain persistent infection. Hepadnaviruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) or malaria primarily infect the liver, which usually fail to exert effective immune responses to eradicate the pathogens

is able to avoid over-activation of both innate and adaptive immune response and maintain immune homeostasis in face of the continuous antigenic challenges. Therefore, the liver has been considered as an organ of immune privilege or immune tolerance.

The immune tolerogenic properties of the liver are exemplified by its roles in oral tolerance and portal venous tolerance. Administration of antigens or donor cells by the oral route or via the portal vein induces both local and systemic tolerance to the antigen, resulting in donor antigen-specific anergy or hyporesponsiveness [1]. Oral administration of antigens is considered as an effective approach for inducing antigen-specific immune tolerance and has been used for the therapy of some immune-mediated disorders [2, 3], which has shown promise for autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis, and diabetes. Based on liver tolerance, many attempts have been made to induce donor-specific immune tolerance across MHC barriers, especially for organ transplantation. For example, inoculation with donor lymphocytes on renal or skin allograft via portal vein specifically prolonged the survival of donor grafts in rats [4]. Portal vein administration of UVB-irradiated donor spleen cells into the hepatic environment promoted peripheral allospecific hyporesponsiveness and allowed the acceptance of donor-specific heterotopic cardiac allografts. Potent and persistent donor-specific immunologic tolerance for skin grafts across MHC barriers has been successfully induced in mice by portal vein injection of allogeneic cells [5]. This induction of oral tolerance was abrogated by a portocaval shunt to bypass the liver, which confirmed the role of liver in oral tolerance induction.

The liver tolerance is also evident in liver transplantation, in which rejection of the allografts is in general much milder than that in transplantation of other organs. It was demonstrated that liver enjoyed immunological privilege compared with other transplanted organs. Allogeneic liver transplantation was accepted in the pig with no or little requirement of immunosuppressive therapy even with MHC mismatch [6]. By contrast, other organ allografts, such as skin, kidney, and heart, were rejected rapidly. Similar results were later obtained in other species such as rat and mouse. More importantly, studies showed that liver allografts can induce donor-specific tolerance that further facilitates the acceptance of subsequent non-hepatic allografts, such as heart, skin, islet, and small intestine, from the same donor while rejecting third-party grafts [7]. Combined transplantation of liver or hepatocytes with another organ from the same donor protected the non-liver graft from rejection and promoted its survival [8]. Moreover, a liver transplant could also terminate ongoing severe graft rejection of a previous organ transplant from the same donor and converted the sensitization state against donor antigens into unresponsiveness [9, 10]. Interestingly, although liver transplants are easily accepted as described above, hepatocyte transplants are usually acutely rejected, suggesting that liver nonparenchymal cells may effectively protect the parenchymal cells (hepatocytes) from immune attack [11].

Similarly, liver tolerance is also used to prevent graft-versus-host disease (GVHD) during bone marrow transplantation. Administration of oral antigen-induced immune hyporesponsiveness or tolerance prevented and ameliorated the development of chronic GVHD, without hampering the graft-versus-leukemia (GVL) effect in a murine model [12, 13]. Cotransplantation of hepatic stellate cells, which act as the important hepatic antigen-presenting cells (APCs) and contribute to induction of liver tolerance [14], has been shown to attenuate the severity of GVHD and prolong the recipient survival by suppressing alloantigen-specific T-cell proliferation [15].

Liver is also an organ where many important pathogens evade immune surveillance and sustain persistent infection. Hepadnaviruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV) or malaria primarily infect the liver, which usually fail to exert effective immune responses to eradicate the pathogens. These pathogens can not only escape the attack of hepatic intrinsic immune system, but also induce the unresponsiveness of systemic immune responses, leading to their persistent infection in the liver. These chronic infections are often associated with development of malignancies, such as hepatocellular carcinoma (HCC). Other invasive tumors such as melanoma or breast, colon, and lung cancers often preferentially metastasize to the liver.

Interestingly, increasing number of reports has shown that over-expression of certain proteins in liver by in vivo gene transfer and hepatocyte-specific transgene expression can induce transgenic product-specific systemic immunotolerance, hence leading to a long-term expression of the specific protein. $CD4^+CD25^+$ Tregs can be induced by liver-directed gene transfer and are required for tolerance induction by suppressing antibody formation and $CD8^+$ T cell responses [16]. Moreover, a gene expressed in liver can suppress humoral and cellular immune responses to the specific protein in extrahepatic sites, suggesting that the tolerance established in liver can induce systemic immune tolerance. The most studied hepatic foreign gene expression is factor IX for therapy of severe hemophilia B, in which the factor IX gene has been successfully transduced by a recombinant adeno-associated viral vector (rAAV)-2 or rAVV-8 into the liver and resulted in long-term expression of therapeutic levels of factor IX, both in dogs and in humans [17]. Antigen-specific $CD4^+CD25^+Foxp3^+$ Tregs exerts long-term regulation of antigen-specific immune responses and provides long-lasting protection and also limits recall responses induced by a second challenge in hemophilia mice [18]. The therapeutic hepatic gene transfer has been applied to treatment of other

genetic diseases, such as lysosomal storage disorders, metabolic disorders, etc. [19].

MS is an inflammatory disease that affects the central nervous system (CNS). Autoreactive T cells play a crucial role in mediating the inflammatory response by targeting myelin components. In a study using mouse EAE model of human multiple sclerosis [20], the neural autoantigen myelin basic protein (MBP) was ectopic expressed with liver-specific MBP transgenic mice or transiently expressed in liver by gene transfer to induce immune tolerance to MBP in the liver, resulting in protection against neuroinflammation. This protective role was shown to be mediated by MBP-specific $CD4^+CD25^+Foxp3^+$ Tregs. Moreover, the generation of MBP-specific Tregs depended on the expression of MBP in liver, while MBP expressed in skin did not exert protection against EAE [20]. This experiment provides important evidence that expression of auto-antigens in liver can induce intrinsic and systemic immune tolerance and may be a potential prophylactic or therapeutic strategy for autoimmune diseases.

Taken together, these findings demonstrate that the liver is a unique organ that plays a critical role in the establishment of immune tolerance. Moreover, the hepatic intrinsic immune tolerance can lead to systemic immune tolerance, hence contributing to the survival of allografts.

General Principles of Liver Immunotolerance

Central Versus Peripheral Immune Tolerance

The immune system has evolved ability to distinguish between self and non-self, by which the host eliminates invading foreign pathogens while sparing the self-normal tissues. The unresponsiveness to self-antigens maintained by normal individuals is called self-tolerance. When the tolerance is broken or lost, the self-tissues would be attacked by immune system, and autoimmune diseases may occur. The mechanisms of lymphocytes to sustain self-tolerance have been divided into two broad categories, central tolerance and peripheral tolerance, based on whether the checking mechanism operates in the central or the peripheral lymphoid organs.

Central tolerance is induced during development of immature T or B cells in the central lymphoid organs, the thymus or bone marrow. The T cell development and differentiation must undergo positive and negative selections to obtain the ability of recognizing antigenic fragments presented by the MHC molecules in a self-restricted manner and simultaneously maintaining self-tolerance. Once immature T cells have rearranged the antigen receptor genes, they become restricted to recognition of self-MHC molecules by positive selection. Those capable of recognizing self-peptide/self-MHC complex are selected for survival, while cells that fail positive selection are induced to undergo apoptosis. Further, T cells with receptors binding self-peptide/self-MHC complex too strongly are eliminated by clonal deletion, a process known as negative selection. For B cells, the antigen receptor is tested for autoreactivity; self-reactive B cells are purged from the functional repertoire during the transition from the pre-B to mature B-cell stage in the bone marrow. The mechanisms for central tolerance may include: (1) *clone deletion*, which was first proposed by Burnet et al. who demonstrated that T and B lymphocytes evolved extensive diversity during the development, generated through random rearrangement of the genes encoding antigen-specific receptors, to effectively eliminate a myriad of microbial pathogens when confronting them, while self-reactive lymphocytes are destroyed during the development of the immune system. (2) *Clone anergy*: self-reactive T or B cells become inactivated in the normal individual and cannot amplify the immune response. (3) *Receptor editing*: immature B cells in bone marrow express sIgM. If the receptor is not self-reactive, the absence of sIgM cross-linking allows gene rearrangement to cease and B cell development to continue; if the receptor is strongly cross-linked with self-antigens on cell surface, the cell reduces the surface expression of sIgM and light chain gene rearrangement continues. This secondary rearrangement can rescue immature self-reactive B cells by deleting the self-reactive light chain gene and replacing it with another light chain gene. If the new light chain is not autoreactive, the B cells continue normal development. Cells that remain autoreactive undergo apoptosis and are deleted from the repertoire (clone deletion). (4) *Immunological ignorance*: some immature T or B cells whose antigen is inaccessible to the immune cells, or their receptors bind monovalent or soluble self-antigens with low affinity, can mature normally. These cells are potentially self-reactive. Some of these ignorant cells can be activated under certain conditions such as inflammation or when the self-antigen becomes available.

Since it is unlikely that all possible self-proteins are expressed in the thymus or bone marrow, central tolerance may not be able to remove all lymphocytes reactive to self-antigens that present only in peripheral or nonlymphoid tissues or are expressed at different developmental stages. Therefore, several mechanisms operating in the periphery, named as peripheral tolerance, are required to prevent mature T or B cells from responding to self-tissue-specific antigens (TSAs). Peripheral tolerance is related to mature T or B cells that have exited from the primary lymphoid organs to circulate in blood, lymph, and secondary lymphoid organs or have entered the parenchymal tissues in response to certain stimulus. It has been suggested that central tolerance most effectively deletes those T or B cell precursors with high avidity

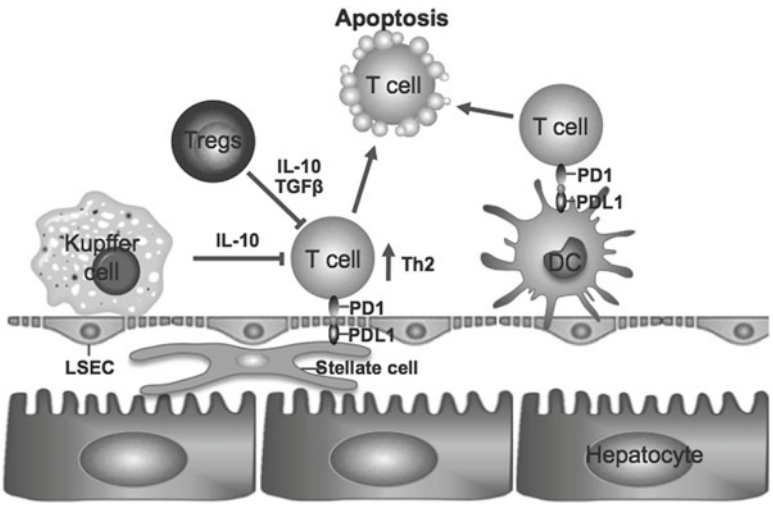

Fig. 7.2 Principles of liver immunotolerance. Peripheral tolerance is related to mature T or B cells that have exited from the primary lymphoid organs to circulate in blood, lymph, and secondary lymphoid organs or have entered the parenchymal tissues in response to certain stimulus. The mechanisms for induction and maintenance of peripheral tolerance involve immune suppression by Tregs, immune deviation from Th1 type to Th2 type, immune privilege or regulation from co-inhibitory signal (e.g. CTLA-4 and PD-1), resulting in antoreactive cells in anergy or unresponsive state. Notably, the mechanisms concerning central and peripheral tolerance are not mutually exclusive. Clone deletion, anergy, unresponsiveness, and ignorance all play important roles in both central and peripheral immune tolerance

for self-peptide-MHC complexes or self-antigens, whereas peripheral immune tolerance mechanisms play major role in controlling mature T or B cells with relatively low avidity that escape to the periphery [21]. The mechanisms for induction and maintenance of peripheral tolerance involve immune suppression by Tregs, immune deviation from Th1 type to Th2 type, immune privilege or regulation from co-inhibitory signal (e.g., CTLA-4 and PD-1), resulting in autoreactive cells in anergy or unresponsive state. Notably, the mechanisms concerning central and peripheral tolerance are not mutually exclusive. Clone deletion, anergy, unresponsiveness, and ignorance all play important roles in both central and peripheral immune tolerance.

Graveyard or Exhaustion

The liver is increasingly regarded as an immunological organ [22, 23]. The unique property of immune tolerance of liver has aroused great interest. To date, several theories or hypotheses for the mechanism of liver tolerance have been proposed.

The theory that liver is a graveyard or killing field of activated T cells was originated from the finding that high frequency of intrahepatic T cells with a phenotype characteristic of apoptosis existed in the liver at the end of an immune response in which large numbers of T cells were activated (Fig. 7.2). These cells did not undergo apoptosis diffusely throughout the immune system, but were trapped in the liver, where they underwent apoptosis. Therefore, the liver is thought to be a specific site for the trapping and destruction of activated T cells. Based on a series of experiments with transgenic mouse models, it was believed that the liver could simply sequester cells that are already starting to undergo apoptosis in the circulation (graveyard); or activated T cells accumulate in the liver where the local tolerant environment induces the apoptosis of T cells, leading to clonal deletion (killing field) [24, 25]. Hence, different from the central deletion in thymus which is due to elimination of immature T cells with higher avidity to self-antigens, the T cell apoptosis in liver is due to activation-induced cell death (AICD).

A state of T cell dysfunction named T cell exhaustion has been observed in liver with persistent chronic hepadnaviruses (such as HBV and HCV) infection. During exhaustion, T cells sequentially lose their effector functions including IL-2 and IFN-γ production and T cell proliferation. Severe T cell exhaustion results in the clone deletion of the virus-specific T cells [26, 27]. The unique negative regulatory microenvironment of liver is critical for the T cell exhaustion during chronic virus infection. First, PD-L1, the ligand of co-inhibitory receptor PD-1, is highly expressed on intrahepatic APCs, including Kupffer cells, DCs, LSECs, HSC, and hepatocytes, and signaling from PD-1/PD-L1 has been confirmed to mediate $CD8^+$ T cell exhaustion during chronic HBV and HCV persistence [28, 29]. Other co-inhibitory

signals from TIM-3, LAG-3, and CTLA-4 also contribute to this process [28]. Second, the immunosuppressive cytokines IL-10 and TGF-β induced during chronic HBV or HCV infection promotes T cell exhaustion [28]. In addition, $CD8^+$ T cell exhaustion is exacerbated by a lack of adequate $CD4^+$ T cell help, for the number of $CD4^+$ T cells is lower and the function impaired in liver, which is accentuated in chronic infection. The increased number of $CD4^+CD25^+FoxP3^+$ Tregs in liver, especially during chronic HBV and HCV infection, as well as IL-10-producing cells and myeloid-derived suppressor cells (MDSCs) further facilitate the T cell exhaustion by preventing local expansion and restricting the function of effector T cells [26]. $CD8^+$ T cell exhaustion is distinct from anergy in that the process of T cell exhaustion is progressive, with dysfunction worsening gradually and occurring after a robust initial T-cell response, while anergy is a state of nonresponsiveness when T cells are stimulated without co-stimulatory signaling.

Regulator Education

Distinct from other parenchymal organ, the unique hepatic regulatory microenvironment plays major roles in preventing the induction of immunity against innocuous antigens and maintaining immune tolerance. The liver harbors unique population of APCs, including resident liver sinusoidal endothelial cells (LSECs), Kupffer cells, HSCs, hepatocytes, and circulating DCs. The hepatic APCs possess the common immature and tolerogenic properties induced by the liver microenvironment, which are manifested with low expression of MHC class II molecules and co-stimulatory molecules but higher expression of co-inhibitory molecules such as PD-L1. On the one hand, the liver-resident APCs recruit circulating immune cells (such as naive $CD8^+$ T cells and circulating DCs) from blood into the liver where the circulating DCs are usually induced to differentiate into tolerogenic state; on the other hand, these intrahepatic APCs provide negative signals to T cells and inhibit antigen-specific T cell activation, suppress Th differentiation, and even induce tolerance in naive $CD8^+$ T cells by cross-presentation. All of these are involved in the hepatic T cell immune tolerance [14, 30]. A recent study has shed new light on the influences of liver microenvironment in priming of naïve $CD8^+$ T cells. It was shown that antigen presentation by bone marrow (BM)-derived DCs to naïve $CD8^+$ T cells in the liver led to a T cell phenotype ($CD25^{hi}CD54^{hi}Bim^{hi}$) that was distinct from the phenotypes of T cells induced by liver-resident hepatocytes ($CD25^{lo}CD54^{lo}Bim^{hi}$) or by BM-derived DCs in the lymph node ($CD25^{hi}CD54^{hi}Bim^{lo}$) [31]. Interestingly, T cells primed by either hepatocytes or BM-derived DCs in liver expressed high levels of pro-apoptotic molecules Bim and undergo Bim-dependent apoptosis [31, 32]. These results support the notion that the liver microenvironment educates the BM-derived APCs and changes their ability to prime naïve T cells.

In addition, hepatic APCs (e.g., LSECs and kupffer cells) support Treg development and differentiation, which further inhibits or prevents intrahepatic immune responses by secreting immunosuppressive cytokines IL-10 and TGF-β. Therefore, the circulating naïve T cells can be educated in the unique regulatory microenvironment of liver to become unresponsive or anergy to self-or oral antigens, or to manifest low responsiveness or dysfunction to invading pathogens, and eventually be eliminated by clone deletion.

Deviation

The differentiation of naive $CD4^+$ T cells into distinct Th cell subpopulations determines the outcome of $CD4^+$ effector T cell responses. The liver environment appears to favor prevention of extensive inflammatory T cell stimulation. The naïve $CD4^+$ T cells preferentially differentiate into Th2 effector types rather than Th1 or Th17 types after being primed by hepatic APCs, a mechanism named as immune deviation. Priming by hepatic APCs, such as LSECs, liver DCs, HSCs, and hepatocytes, all results in $CD4^+$ T cells with Th2 phenotype that secrete IL-4 and IL-10. In contrast, such priming fails to sustain Th1 responses, or selectively suppresses Th1 cytokine secretion, or even induces apoptosis of Th1 cells [33]. The tolerogenic property of hepatic APCs and the hepatic cytokine milieu contribute to the immune deviation.

Cellular Mechanisms of Liver Immunotolerance

The liver tolerance to self- and foreign antigens is attributed to the local anatomical structure and the unique cell and cytokine microenvironment. The liver-resident cells, including the parenchymal hepatocytes, LSECs, Kupffer cells, and HSCs, play major roles in inducing liver tolerance (Fig. 7.3). As an important lymphoid organ, the liver is also enriched with various types of lymphocytes, including T cells, NK cells, NKT cells, DCs, and granulocytes. Comparing with the peripheral immune organ, NK and NKT cells are very abundant in the liver, constituting up to 50 % of total intrahepatic lymphocytes. Therefore, the liver is also regarded as an organ with predominant innate immunity [22]. The liver resident $CD8^+$ T cells are generally more abundant than $CD4^+$ T cells, while activated T cells are more frequent in the liver than in blood, lymph nodes, and spleen [34]. This unique combination of liver lymphocytes is also thought to be a major cause of liver tolerogenicity.

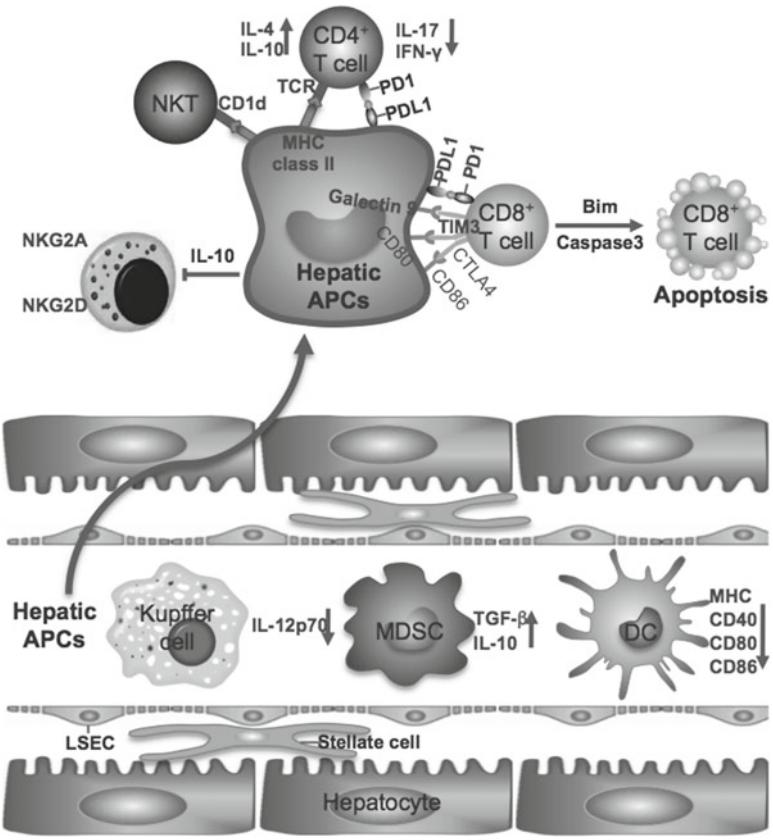

Fig. 7.3 The Cellular and molecular mechanisms of liver immunotolerance. LSECs present soluble antigens to CD4+ T cells and inhibit Th1 cell differentiation by secreting IL-10. LSECs also induce the development of Tregs and promote the proliferation of Tregs recruited to liver. LSECs also lead to CD8+ T cell exhaustion with the up-regulation of PD-L1 on LSECs and PD-1 on CD8+ T cells. Kupffer cells can make close contact with circulating lymphocytes including T cells, NK, and NKT cells in the narrow sinusoids. Kupffer cells produce IL-10 and TGF-β and activate Tregs and induce CD8+ T cell apoptosis. Hepatocytes can directly interact with circulating lymphocytes by cytoplasmic extensions penetrating the liver endothelial fenestrations. Hepatocytes are usually insufficient for inducing IFN-γ production by T cells but instead preferentially induce Th2 response, thus further impairing CD8+ T cell response. Naïve CD8+ T cells primed by hepatocytes undergo initial clone expansion, but are eventually clone-eliminated due to lack of sufficient co-stimulation followed by Bim and caspase 3-dependent apoptosis. Hepatocytes are also found to facilitate the generation of Tregs. Stellate Cells (HSCs) express PD-L1 and mediate T cell apoptosis in PD-1/PD-L1 or TRAIL-dependent manner. The liver DCs tend to induce Th2 rather than Th1 response and promote the generation of Tregs

Tolerant Residential Antigen-Presenting Cells

Liver Sinusoidal Endothelial Cells

LSECs are microvascular endothelial cells and the most abundant nonparenchymal cell population in the liver. They line the hepatic sinusoids, form a fenestrated endothelium, and are in direct contact with lymphocytes circulating through the liver. LSECs are efficient scavenger cells, not only are responsible for clearance of antigens and microbial degradation products, but also act as APCs by processing and presenting antigens to circulating CD4+ and CD8+ T cells via potent interaction. Mouse LSECs express only very low levels of MHC class II molecules and co-stimulatory molecules CD80/CD86 and do not produce IL-12 even after TLR stimulation. They present soluble antigens to CD4+ T cells and prime naïve CD4+ T cells, but are not able to promote Th1 cell differentiation. They also inhibit the expansion of mature Th1 cells [21, 35] and suppress the secretion of inflammatory cytokine IFN-γ and IL-17 by Th1 and Th17 cells. These effects depend on IL-10 secretion and the dominance of inhibitory over-activating co-stimulatory signals on LSEC [36]. LSECs also induce the development of Tregs and promote the proliferation of Tregs recruited to liver upon inflammatory stimuli [35, 37].

LSECs have the ability of cross-presentation. They can uptake exogenous antigens by mannose and scavenger receptors, arrest CD8+ T cells in liver sinusoids, and cross-present the processed exogenous antigens to the CD8+ T cells more efficiently than splenic DCs [38]. However, although the naïve CD8+ T cells primed by LSECs can proliferate, they are not fully activated for high level production of IL-2 and IFN-γ and high cytotoxicity eventually leading to CD8+ T cell tolerance rather than immunity [39, 40].

The induction of tolerance correlates with the up-regulation of PD-L1 on LSECs and PD-1 on $CD8^+$ T cells. Up-regulation of co-stimulatory signaling through CD28 can overcome the tolerogenic PD-L1/PD1 signaling. In addition, LSECs are also found to inhibit the activation and proliferation of the existing $CD8^+$ T cells in liver and to induce their apoptosis [35].

In addition to acting as APCs, LSECs are reported to contribute to induction of liver tolerance by negatively regulating other hepatic APCs. The direct contact of LSECs and hepatic DCs vetoed the ability of hepatic DCs to fully activate naïve $CD8^+$ T cells through reducing the expression of CD80, CD86, or IL-12 by DCs [41].

Interestingly, although expressing numerous pattern recognition receptors (PRRs) and constantly in contact with gut-derived microbial PAMPs from portal vein blood, LSECs did not mature after stimulation through PRR (e.g., TLRs, RIG-I, and MDA-5). Instead, LPS stimulation increased production of TGF-β and IL-10, resulting in induction of liver tolerance. However, T cell tolerance induced by LSECs can be overcome by viral infection. It was recently shown that infection with murine cytomegalovirus (MCLV) induced the functional maturation of LSECs and efficiently promoted antigen-specific differentiation into effector $CD8^+$ T cells, which was independent of DCs and CD80/CD86 [42]. These results reveal the critical role of LSECs in local induction of antiviral immunity during viral infection, which may be involved in governing the local balance between tolerance and immunity. Whether HBV or HCV infection can overcome LSECs-induced T cell tolerance is yet to be defined.

Kupffer Cells

Kupffer cells are the largest group of tissue-resident macrophages located in the liver. These cells lie within the lumen of the hepatic sinusoids where they can conveniently phagocytose and remove toxin, waste products or antigens, apoptotic cells, and microorganisms carried by portal circulation. Meanwhile, Kupffer cells can make close contact with circulating lymphocytes including T cells, NK, and NKT cells in the narrow sinusoids. They act as potential APCs by expressing MHC molecules and co-stimulatory molecules.

Under steady-state condition, Kupffer cells express no or low levels of MHC class II molecules and co-stimulatory molecules (CD80, CD86, and CD40) and act as tolerogenic APCs in inducing tolerance towards soluble antigens, oral antigens, or antigens from portal vein and liver transplants. They inhibit DC-induced antigen-specific T cell proliferation and activation by production of PGE_2 and 15-deoxy-delta12,14-PGJ_2 [43]. Although these results ruled out the possible involvement of IL-10, nitric oxide, 2,3-dioxygenase, and TGF-β in Kupffer cells-mediated T cell suppression, Kupffer cells indeed produce IL-10 and TGF-β in response to LPS, thereby probably contributes to induction of liver tolerance. Kupffer cells can also activate Tregs to induce IL-10 production, demonstrating the critical role of Kupffer cells together with hepatic Tregs in induction of tolerance to hepatocyte-expressed antigens [44]. In addition, Kupffer cells have been found to be involved in limiting the hepatic $CD8^+$ T cell response by inducing $CD8^+$ T cell apoptosis upon their entering into the liver [45]. After induction of apoptosis, Kupffer cells phagocytose apoptotic cells and further increase their production of IL-10 while reduce production of TGF-α and NO, thus provide protection of endotoxin-induced fulminant hepatitis and contribute to maintain the immune homeostasis in liver [46].

Of note, Kupffer cells can induce T cell proliferation when stimulated by TLR ligands. It is likely that Kupffer cells act as stimulatory APCs during hepatic infection. They can cross-present microbial antigens to $CD8^+$ T cells or NKT cells to mount antimicrobial immunity [47], although sometimes the immune activation induced by Kupffer cells may lead to liver injury.

Hepatocytes

Hepatocytes are the liver parenchymal cells primarily responsible for the metabolism. They also function as APCs to participate in the immunoregulation. Hepatocytes can directly interact with circulating lymphocytes by cytoplasmic extensions penetrating the liver endothelial fenestrations. Hepatocytes express low level of MHC class I molecules with no expression of MHC class II molecules. However, they up-regulate class II expression under inflammation or viral infection. Although these class II-expressing hepatocytes can present antigens to $CD4^+$ T cells leading to $CD4^+$ T cell activation, this activation is usually insufficient for inducing IFN-γ production but instead preferentially induce Th2 response, thus further impairing $CD8^+$ T cell response, promoting liver tolerance and viral persistence [48]. Naïve $CD8^+$ T cells primed by hepatocytes undergo initial clone expansion, but are eventually clone-eliminated due to lack of sufficient co-stimulation followed by Bim and caspase 3-dependent apoptosis [49]. Hepatocytes are also found to facilitate the generation of Tregs, which can suppress experimental autoimmune neuroinflammation. The antigen presentation of hepatocytes to CD1d-restricted NKT cells can induce NKT activation, which further prime IL-10-producing $CD8^+$ T cells and thus limit the local immune responses [50]. In addition, co-inhibitory molecule PD-L1 is induced on hepatocytes under viral infection or by IFN or IL-10 stimulation, which may be involved in the induction of liver tolerance [51].

However, hepatocytes also induce immunity and participate in virus clearance under certain condition. It is thought that hepatocytes contribute to induction and maintenance of liver tolerance under steady-state condition, but support the induction of T cell immunity following vaccination.

Hepatic Stellate Cells

Known as Ito cells with the function of storaging vitamin A and participating in hepatic fibrosis, HSCs have recently been proposed as professional liver-resident APCs. Activated HSCs express MHC class I and II molecules, CD1, and co-stimulatory molecules and produce a variety of cytokines. They efficiently present antigens, drive proliferation of $CD4^+$ T, $CD8^+$ T, and NKT cells, and activated antigen-specific T cells [52]. However, they have also been implicated in liver tolerance. HSCs express PD-L1 and mediate T cell apoptosis in PD-1/PD-L1 or TRAIL-dependent manner [53]. Activated HSCs secrete TGF-β and preferentially expand Tregs and induce the generation of MDSCs, which exert potent immune inhibitory activity [54]. T cell apoptosis, Treg expansion as well as induction of MDSCs are confirmed functions of HSCs in protecting islet allografts from rejection in cotransplantation experiments [54, 55]. Taken together, all these support the role of HSCs in regulating immune responses and inducing liver immune tolerance.

Tolerant Circulating Antigen-Presenting Cells

The liver contains circulating APCs, mainly circulating DCs, which participate in the liver immune responses. The number of liver DCs is usually larger than in other parenchymal organ, which may be related to the high frequency of PAMPs in portal blood. Under the steady-state condition, the liver DCs express tolerogenic phenotypes, including low expression of peptide-MHC complex and co-stimulatory molecules, expression of co-inhibitory molecule PD-L1, production of PGE_2, IL-10, and TGF-β, etc. The liver DCs tend to induce Th2 rather than Th1 response and promote the generation of $CD4^+CD25^+Foxp3^+$ Tregs [56]. The expression of tolerogenic phenotypes is associated with the local liver microenvironment. For example, liver fibroblastic stromal cells are shown to support the differentiation of $CD117^+$ hematopoietic progenitor cells or monocytes into $IL\text{-}10^{hi}$ $IL\text{-}12^{low}$ tolerogenic or regulatory DCs that inhibit T cell proliferation and induce apoptosis of activated T cells, with the participation of hepatocyte growth factor and M-CSF involved in this differentiation [57, 58].

Liver DCs include plasmacytoid DCs (pDCs), myeloid DCs (mDCs), $CD8\alpha^+$ DCs, and the less-defined natural killer DC (NKDC) subsets. pDCs are characterized by their ability to secrete large amounts of Type I IFNs in response to viral or bacterial stimuli and therefore play major roles in connecting innate and adaptive immunity and in antimicrobial immune responses. The frequency of pDCs is higher in liver than in secondary lymphoid organ. However, liver pDCs express low levels of MHC class II and co-stimulatory molecules, produce less type I IFN, and elicit insufficient T cell priming than splenic pDCs in response to viral infection or TLR stimulation. NOD2 is expressed by liver pDCs at higher levels than mDCs, while exposure to MDP weakens the stimulatory function of liver pDCs. By interaction of NOD2 and its ligand MDP, mouse liver pDCs, but not spleen pDCs, up-regulate the expression of IFN regulatory factor 4 (IRF4), a negative regulator of TLR signaling, resulting in inhibition of allogeneic T cell proliferation and IFNγ production by increased expression of PD-L1. NOD2 ligation also suppresses the production of IL-6, IL-12p70, TNF-α, and IFN-α by liver pDCs in response to stimulation with LPS or CpG [59].

Mouse liver pDCs produce more IL-10 and less IL-12p70 than splenic pDCs. They promote Th2 cell differentiation by a low ratio of expressed Delta/Jagged1 Notch ligand and induce apoptosis of allogeneic T cells. The expression of PD-L1 on liver pDCs impairs T cell stimulatory function, with the low levels of MHC and co-stimulatory molecules contributing to the induction of anergy or deletion of antigen-specific T cells. The secretion of IL-10 promotes the Tregs differentiation. In addition, liver pDCs are recently found to express high levels of IL-27p28, an IL-12 family cytokine that regulates the function of APCs and T cells, and EBV-induced protein 3 (Ebi3). IL-27 stimulation up-regulates PD-L1 expression on liver pDCs and promotes generation of $Foxp3^+$Tregs, hence promotes the immunoregulatory function of pDCs [60]. These effects are consistent with the fact that liver pDCs induce efficient systemic $CD4^+$ and $CD8^+$ T cell tolerance to orally administered antigens that reach the liver through the blood by inducing anergy or deletion of Ag-specific T cells in the liver [61].

The conventional mDCs in liver also express tolerogenic phenotypes. Upon TLR stimulation, mDCs in liver produce less IL-12, but more IL-10 and IL-27 than mDCs from spleen and other organs. In the liver microenvironment, the continuous exposure to bacterial products from the incoming portal vein blood inhibits liver mDCs maturation, leading to their unresponsive or hyporesponsive state to PAMP stimuli (known as endotoxin tolerance) and reduce the T-cell stimulatory capacity compared the DCs from other organs. LPS stimulates hepatocytes to secrete IL-6, which further activates STAT3 signaling pathway in mDCs followed by inducing increased expression of IL-1 receptor-activating kinase-M (IRAK-M), a negative regulator of TLR signaling, thereby preventing DC activation and maturation [62]. IL-10 and TGF-β secreted by liver LSECs and Kupffer cells contribute to the induction of tolerance, thus inhibiting liver mDC maturation and favoring the tolerance within the liver.

MDSCs is a phenotypically heterogeneous cell population that includes mature myeloid cells and immature myelomonocytic precursors that express both Gr-1 and CD11b in mice and with the phenotype $CD14^+HLA\text{-}DR^{-/low}$ in human [63]. MDSCs are enriched within tumors or present in blood, bone marrow, or lymph nodes in tumor-bearing host. The tumor microenvironment, such as various tumor-derived factors as well as arginase, nitric oxide, and reactive oxygen species, supports the accumulation of MDSCs, prevents their

differentiation, and induces their suppressive function, including inhibiting T cell function, blocking NK cell cytotoxicity, shifting macrophages to an immunosuppressive M2 phenotype, and inducing the development of Tregs [64, 65]. Recently, the liver has been shown to be a preferred site for the accumulation and expansion of MDSCs, which accelerates liver metastasis of the tumor [65, 66]. In addition to inhibiting effector T cell function and inducing Treg expansion, MDSCs suppress NK cytotoxicity and cytokine production through NKp30 receptor and membrane-bound TGF-β [67, 68].

Under physiological conditions, MDSCs reside in the liver and displayed suppressive effect on nonantigen-specific as well as antigen-specific T cell proliferation. Notably, the frequency of hepatic MDSCs in HBV transgenic mice was significantly higher and their capacity to suppress proliferation of HBsAg-specific T cells was significantly greater than those in normal mice [69]. The hepatic MDSCs also interact with Kupffer cells and up-regulate PD-L1 expression on Kupffer cells, which enhances the tolerogenic liver environment. These findings suggest that liver MDSCs may have a critical role in maintaining liver homeostasis under physiological conditions as well as enhancing liver immune tolerance during chronic HBV persistence and HCC. Indeed, MDSCs have been proved to prevent graft reject [55, 70] and also have been suggested as a potential target for immunotherapeutic modulation aiming at reversion of immune tolerance in tumor or chronic persistent infection of the liver [65].

Tolerant NKT Cells and γδT Cells

As described in the previous sections, both $CD4^+$ and $CD8^+$ T cells in the liver express tolerant phenotypes and are key players in the overall liver tolerance. In addition to these two T cell subsets, other T cell populations in the liver, including NKT cells and γδT cells, are also contributors to the overall tolerant environment of the liver.

NKT cells are abundant in liver, constituting 20–30 % of mouse liver lymphocytes. NKT cells act as early sentinels that convey regulatory signals to other cells by patrolling within hepatic sinusoids and interacting with other cell types such as DCs, hepatocytes, NK, and T cells, thus providing a local immune surveillance [71]. Considered as a subset of regulatory T lymphocytes, NKT cells play important roles in regulating innate and adaptive immune responses by secreting both Th1 and Th2 cytokines. Interestingly, NKT cells can either act as APCs that directly prime $CD8^+$ T cells or regulate $CD8^+$ T cell response indirectly through other APCs such as DCs or hepatocytes [50]. By closely interacting with T cells and hepatocytes, NKT cells can modulate the phenotype of $CD8^+$ T cells and facilitate the priming of IL-10-producing $CD8^+$ T cells by hepatocytes in a type I IFN-dependent manner. Thus, by modulating and limiting the specific $CD8^+$ T cell response to Ag-presenting hepatocytes, NKT cells may contribute to the tolerogenic milieu in the liver and protect the liver from immune injury [50].

Liver NKT cells have been shown to be essential for the tolerance towards transplanted antigens and allografts as well as for oral tolerance, possibly due to their regulatory functions on the Th1/Th2 imbalance [72, 73]. Adoptive transfer of regulatory NKT cells significantly ameliorated nonalcoholic steatohepatitis in ob/ob mice and the effect is related to intrahepatic CD8 trapping, which supports the role of NKT cells in sustaining liver-tolerant environment. NKT cell-derived IL-10 has also been reported to stimulate the differentiation of Ag-specific regulatory T cells that mediates systemic tolerance.

γδT cells are also enriched in liver. These cells account for 3–5 % of total liver lymphocytes or 15–25 % of total liver T cells. The role of γδT cells in the liver has not been paid much attention. A recent study showed that Vγ4 γδT cells negatively regulate the function of NKT cells in an IL-17A-dependent manner, thus mediate a protective effect against Con A-induced fulminant hepatitis [74]. An IL-10-producing γδ T cell subset was also reported to protect the liver from Listeria-elicited $CD8^+$ T cell-mediated injury [75]. γδT cells have also been demonstrated to participate in liver transplant tolerance [76].

Tolerant NK Cells

Elevated level of NK cells is present in the liver and play critical role in innate immune responses against tumors, viruses, intracellular bacteria, and parasites. The function of NK cells is regulated by both activating and inhibitory receptors on their cell surface, with inhibition being the dominant signal. The liver microenvironments are thought to be critical for the unique phenotypic and functional properties of hepatic NK cells. Our recent study showed that liver NK cells express immature phenotype [77]. The development and maturation process of NK cells is unique in liver, with IFN-γ playing an important role [78]. IL-10-producing NK cells with immunosuppressive functions in murine liver were recently reported [79]. The immature properties and the regulatory role of hepatic NK cells indicate their critical function in maintaining homeostasis and liver tolerance under normal conditions.

Accumulating evidences suggest that NK cells can develop selective defects in antiviral function during chronic infection and inflammation. Decreased number, declined activation, and attenuated cytolysis ability of hepatic NK cells were found in murine chronic HBsAg carriers [80]. In chronic HBV and HCV patients, the phenotype and function of NK cells were modified by the persistent viral infection. Although these cells retained cytotoxic potential, they fail to

produce IFN-γ, which may be mediated by IL-10-producing Kupffer cells [81]. The cytotoxic capacity of NK cells is also found to be attenuated with their decreased expression of activating receptor NKG2D and increased expression of inhibitory receptor NKG2A, while IFN-γ and TNF-α production are strongly suppressed, especially in the $CD56^{dim}$ subset. These may enhance liver tolerance that favors viral persistence. On the other hand, NK cell activation and IFN-γ production are partially restored by antiviral therapy through inhibition of viral replication [82]. In consistent with these results, our recent study showed that HBV infection increases the levels of the inhibitory receptor NKG2A on NK cells in both mice and humans. Blocking the interaction of NKG2A and its ligand increases NK Cell activity and contributes to the clearance of HBV Infection [83]. In addition, the concentration of TGF-β1 in sera from chronic HBV-persistent patients was elevated, which down-regulated the expression of NK-activating receptor NKG2D and 2B4 as well as their intracellular adaptor proteins DAP10 and SAP, leading to impaired NK cell function [84]. Together these findings demonstrate the role of dysfunctioned NK cells in HBV-induced immune tolerance as well as persistence of HBV.

Other Tolerant Cells

In addition to the previously described lymphocyte subsets, B cells have a regulatory effect on suppressing inflammatory response in a dnTGF-betaRII murine model of primary biliary cirrhosis [85]. The regulatory role of B cells on pathogenic $CD4^+$ T cells has also been reported in a murine model of inflammatory bowel disease [86]. Liver mast cells in the donor grafts are also found to play important roles in the induction and maintenance of immune tolerance and liver regeneration during orthotopic liver transplantation [87].

Molecular Mechanisms of Liver Immunotolerance

Suppressive Cytokines

The most important suppressive cytokines in liver are IL-10 and TGF-β. IL-10 has multiple effects and has been shown to reduce pro-inflammatory cytokine production, impede the functions of antigen-presenting cells, dampen T-cell responses, and also affect B cell responses. The liver is rich in IL-10, which can be secreted by Kupffer cells, LSECs, hepatic stellate cells, and Tregs. IL-10 produced in liver by hepatic APCs promotes the generation and proliferation of Tregs and Th2 cells through IL-10-dependent mechanisms. It suppresses production of inflammatory cytokines TNF-α, IL-6, and ROS. In addition, IL-10 produced in the liver down-regulates the receptor-mediated antigen uptake and suppresses the expression of MHC class II and the co-stimulatory molecules CD80 and CD86 on liver APCs, such as Kupffer cells and LSECs [88]. LSECs and liver-derived DCs can prime $CD4^+$ T cells to preferentially differentiate to tolerant type and predominantly synthesize and secrete IL-10. Several other lymphocyte subsets, such as NKT and γδT cells in the liver, also secrete IL-10. All these promote the hepatic and systemic immune tolerance. Moreover, HBV- and HCV-specific CTLs are themselves capable of producing IL-10 and can thereby attenuate antiviral immunity via an autocrine feedback loop, further aggravating immune tolerance [81].

TGF-β has been implicated in regulating the strength of the pathogen-specific T-cell responses and promoting these cells to undergo apoptosis. Liver Tregs, Kupffer cells, and LSECs can secrete TGF-β under physiological conditions, with the secretion increased upon exposure to LPS or infection. TGF-β produced in the liver is capable of suppressing the production of inflammatory cytokines and promoting the proliferation and programming of Treg cells. During acute infections, TGF-β restricts the size of both the effector and memory CD8 T-cell pools, most probably by up-regulating the pro-apoptotic gene Bim while down-regulating the anti-apoptotic gene Bcl-2. These effects are especially relevant in establishing persistent chronic infections,

TGF-β has also been shown to be involved in T cell exhaustion. Blocking TGF-β signaling by a dominant negative TGF receptor improves the function of $CD8^+$ T cells and prevents their exhaustion and deletion during chronic viral infection [26, 89].

Co-inhibitory Molecules

Inhibitory receptors play important role in regulating and controlling the strength of adaptive immune responses, including the induction of self-tolerance and prevention of autoimmunity. A main cause of T cell exhaustion is an excess of co-inhibitory signals that outweigh the co-stimulatory signals, leading to functional inhibition of T cells. The best characterized co-inhibitory receptor is PD-1, a member of the CD28 family which is mainly expressed on the surface of activated T cells and tightly regulates T cell reactivity by competing with CD28 signaling to prevent autoimmunity. Accumulating data has demonstrated that PD-1 is critical in establishing peripheral tolerance and suppressing the proliferation and function of T cells. PD-1 is markedly up-regulated on exhausted T cells during chronic viral infections including HBV, HCV, HIV, and LCMV. Blocking PD-1 signaling with anti-PD-L1 antibody promotes the proliferation of virus-specific T cells and improves their function during chronic LCMV infection, leading to reduction of viral loads [26, 27]. However, although PD-1 expression is markedly

up-regulated on hepatic HBV- or HCV-specific CD8 T cells, the function of these cells could not be restored or could only be partially rescued by single PD-1 blockade.

More recent work has revealed that other important negative co-regulation also contribute to T cell exhaustion in chronic infection. Cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), and the inhibitory molecule lymphocyte-activated gene-3 (LAG-3) have been shown to impair the functional quality of T cell response during chronic HBV and HCV infection. They also influence the exhausted state of T cells to various extents. Blockade of TIM-3 improves the proliferation of the exhausted T cells in vitro, while LAG-3 blockade alone is less effective in reversing exhaustion than a combined blockade of PD-L1 and LAG-3. Increasing number of studies has shown that virus-specific $CD8^+$ T cells in chronic infections can co-express several inhibitory receptors such as CTLA-4, TIM-3, LAG-3, and CD244 (2B4). The co-expression pattern and levels of inhibitory receptors expressed on the $CD8^+$ T cells can substantially affect the severity of functional inhibition. Therefore, persistent and/or high-level expression of multiple inhibitory receptors become the primary characteristics of the exhausted $CD8^+$ and $CD4^+$ T cells [26, 27]. It has been proposed that individual inhibitory receptors regulate distinct cellular functions, although some functions can overlap. For example, the PD-1 pathway seems to strongly affect the survival and proliferation of exhausted $CD8^+$ T cells, while LAG-3 mainly impairs the progression of cell cycle but has less influence on cell survival or apoptosis. The importance of inhibitory receptors for T cell exhaustion and liver tolerance in chronic infection has revealed new potential therapeutic targets for reversing T cell exhaustion and immune tolerance and restoring adaptive immunity. In particular, combined blockade of several inhibitory pathways, or combined blockade of inhibitory receptor and suppressive cytokines plus therapeutic vaccination could be promising novel immunotherapies for both chronic infection and cancer.

Leukocyte-associated Ig-like receptor 1 (LAIR-1) is another novel immune inhibitory receptor expressed on the majority of mononuclear leukocytes. LAIR-1 recognizes a common collagen motif and is capable of inhibiting immune cell function. The inhibitory capability of LAIR-1 has been demonstrated on several leukocyte subsets. The activation and cytolytic capacity of resting and activated NK cells was inhibited by cross-linking of LAIR-1 on human NK cells. LAIR-1 can suppress the cytotoxic activity of cytotoxic T cells and down-regulate the production of Ig and cytokine in primary B cells [90]. LAIR-1 cross-linking also inhibits the differentiation of mDCs and restrain the production of IFN-α by pDCs. Notably, LAIR-1 is differentially expressed on various T cell subsets, with higher expression on naive and effector T cells but lower expression on memory T cells. Activation of T cells down-modulate LAIR-1 [91]. However, it is yet to be clarified whether LAIR-1 is differentially expressed among different liver lymphocytes or between physiological and persistent chronic infection status, and whether it is involved in liver immune tolerance.

The autoimmune regulator (AIRE), a transcription factor that regulates the expression of TSAs, is primarily expressed by medullary thymic epithelial cells (mTECs). It promotes central tolerance in the thymus by regulating TSA expression and permitting early deletion of autoreactive T cells during negative selection stage [92]. Although most autoreactive T cells are negatively selected by interaction with $AIRE^+$ medullary epithelial cells, a few escape central tolerance and enter into the periphery, where peripheral tolerance induction is required to prevent autoimmunity. Recently, it was found that AIRE is expressed in both human and mouse peripheral lymph tissues, such as lymph nodes, tonsils, and gut-associated lymphoid tissue, and also play important role in maintaining peripheral tolerance [92, 93]. Taken together, AIRE regulates the mechanisms involved in the induction and maintenance of immune tolerance. Patients and mice defective in AIRE expression develop a multi-organ autoimmune syndrome. It will be important to determine whether AIRE or a similar molecule is expressed in liver and participate in liver tolerance.

Future Direction

Predominant of Innate Immune Cells in Liver

Although the liver has been accepted as an organ with predominant innate immunity, the underlying mechanism for the selective hepatic enrichment of innate immune cells has not been elucidated. We propose that some of the hepatic innate immune cells might be originated from intestine via the liver-gut axis. Indeed, similar in the liver, TCRγδ T cells also predominantly assemble in intestine and play critical roles in maintaining intestine homeostasis by inducing Treg cells. The percentages of NK and NKT cells are approximately 30 % and 40 %, respectively, in intestine intraepithelial lymphocytes in childhood, but decreased significantly at adult stage, probably due to their migration into liver. However, if this hypothesis is true, the liver must be able to selectively retain innate immune cells. The unique anatomy of liver sinusoids, the slow blood flow, and the lack of discrete basement membrane between LSECs enable lymphocytes in the portal vein to exit from blood and retain in the liver. Indeed, we have recently demonstrated that hepatic NK cells express specific phenotypic markers ($CD3^-NK1.1^+DX5^-CD49a^+$) and chemokine receptors ($CXCR6^+CXCR3^+$). These cells predominantly reside in hepatic sinusoids, frequently adhering to the endothelial cells or stay in the parenchyma between hepatocytes, possi-

bly due to the production of unique chemokines by organ-specific cell types such as KC-derived CCL2, CXCL4, CXCL9, CXCL11, CXCL6, and CCL3 that orchestrate specific NK cell migration into the liver. The other possibility that explains the enriched innate immune cells is they are differentiated from liver hematopoietic stem/progenitor cells. There is evidence for the existence of hematopoietic stem cells capable of multi-potent differentiation and self-renewal in adult liver [94, 95]. It is also known that fetal liver $CD34^{+}CD38^{+}$ progenitor cells can differentiate into NK cells in the presence of IL-15 [96]. Further study is warranted to clarify the exact mechanisms of the predominance of innate immunity in liver and their contribution to the liver immune tolerance, as well as their influence on various liver diseases.

The Roles of Liver Tolerance in Liver Diseases

The unique tolerogenic properties of liver render the liver an attractive target for pathogens. With their antigens presented in the liver rather than in lymphoid tissues, hepatotropic pathogens such as HBV, HCV, and malaria can escape from T cell-mediated immunity and establish persistent infections in the tolerogenic hepatic microenvironment. The immunotolerant environment further accelerates the progression of persistent HBV or HCV infection into liver fibrosis, liver cirrhosis, and HCC. Therefore, unraveling the exact cellular and molecular mechanisms of liver immune tolerance might lead to novel immunotherapies for multiple liver diseases.

It is critical to reverse or break the liver immune tolerance for successful treatment of persistent infection or cancer. Blocking inhibitory receptors, such as PD-1, ex vivo study of intrahepatic T cells from patients with chronic hepatitis B and in vivo study with a mouse model of HBV infection, has shown remarkable results in reversing the immune dysfunction of hepatic T cells and viral persistence, including increased $CD8^{+}$ cell proliferation and HBV-specific IFN-γ production in intrahepatic T lymphocytes and clearance of HBV [97, 98]. The function of exhausted HCV-specific $CD8^{+}$ T cells cannot be enhanced by PD-1 blockade in chronic HCV infection, although PD-1 expression is markedly up-regulated on these cells in the liver. The reversal efficacy for $CD8^{+}$ T cell function may also depend on the virus load, the frequency of antigen-specific T cells, and the expression level of co-inhibitory receptors on these exhausted cells. We and others have shown that a dual-function 3p-HBx-siRNAs with both HBx-RNA silencing and RIG-I activation effects can reverse HBV-induced hepatocyte-intrinsic tolerance by recovering production of type I IFNs via activation of the RIG-I pathway [99, 100]. This strategy appears to be promising in cancer therapy as well [101].

Although reversal of liver tolerance is a promising therapeutic strategy for chronic HBV and HCV infection and even for cancer, the underlying mechanisms remain to be thoroughly clarified. Up to now, most of the new interventions are still being tested in preclinical studies using animal models; there is still a long way to go before they can be eventually tested in humans. In particular, as these interventions target the regulatory pathways, they might disturb the immune homeostasis, hence need to be carefully monitored to avoid uncontrolled immunopathology or autoimmune hepatitis.

Liver Tolerance and Systemic Diseases

The hepatic intrinsic immune tolerance can induce systemic immune tolerance. The most striking example is that the liver allograft can confer protection on subsequent non-hepatic allografts from the same donor. In the liver allograft, the circulating allospecific $CD8^{+}$ T cells enter liver sinusoids with blood flow where they might encounter allo-antigens and are trapped and induced to undergo apoptosis, leading to a state of systemic tolerance. Severe hepatic intrinsic immune tolerance induced by persistent HBV infection will also lead to systemic immune tolerance, as indicated by the failure of anti-HBV antibody production and reduced circulating CTL and NK cell responses after HBV vaccination.

Based on these phenomena, strategies have been proposed to prevent or treat systemic diseases by manipulating hepatic intrinsic immune responses. In order to treat some genetic diseases, a specific protein can be long term expressed in vivo without exclusion through induction of systemic immunotolerance by over-expression of the specific protein in liver. For example, factor IX has been successfully transduced by rAAV-2 or rAVV8 in liver, which results in long-term expression of therapeutic levels of factor IX for therapy of severe hemophilia B (17). Similarly, the neuroinflammation in mouse EAE model can be protected by ectopic expression of MBP in liver. The protection is mediated by MBP-specific CD4+CD25+Tregs, which were converted from conventional CD4+T cells, thus inducing systemic immune tolerance against MBP (20). This mechanism may also occur in co-transplantation with liver. This observation that induction of immune tolerance in liver can control extrahepatic autoimmunity gives a novel promising therapeutic strategy for treatment of autoimmune diseases. On the other hand, as described above, the phenomenon that liver tolerance influences both local and systemic immune responses supports the strategy of breaking hepatic immune tolerance in order to reverse systemic immune tolerance, which shows great promise for treating persistent viral infections (such as HCV, LCMV, and HPV) and associated cancers. Deeply understanding the mechanisms governing the induction of liver immune tolerance will open new therapeutic options in the future.

Liver as an Organ for Extramedullar Hematopoiesis (EMH)

The liver is the major site of hemopoiesis during fetal life and is also the site of extra-medullary hemopoiesis in adults. The hemopoiesis function directly influences the development and differentiation of immune system. Therefore, elucidating the development and differentiation mechanisms of liver hematopoietic and immunologic tissue will help understand the pathogenesis of liver and extrahepatic (systemic) diseases. The capacity of liver hemopoiesis is associated with the unique liver immune tolerance. After liver allotransplantation, donor hematopoietic stem cells differentiate and form microchimerism in the recipients, which aid in the induction of donor-specific tolerance [17]. We propose that hepatic hematopoietic stem cells residing in adult liver may retain their differentiation characteristics similar to those in fetal liver and may be prone to differentiate into cells with inhibitory or regulatory features to sustain the immune tolerance status. Future studies on the liver hematopoiesis and lymph tissue will help uncover the mechanisms of the predominance of innate immunity in liver and the formation of liver immune tolerance as well as the subsequent induction of systemic tolerance. Fully understanding of these mechanisms will provide foundation for the development of therapeutic strategies for many related diseases.

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The Diagnosis and Classification of Immune-Mediated Hepatic Diseases

8

Fernando Alvarez

Key points

- Clinical features depend on affected cell type.
- Specificity and sensitivity of serological markers vary according to the cause.
- Histological findings orient diagnosis.
- Genetic susceptibility to immune liver diseases is highly associated to MHC locus.
- Categorization according to liver cell target defines clinical syndrome.
- Classification according to immune mechanisms of injury guides therapeutic decision.

Introduction

The liver anatomical position between the splanchnic venous system and the systemic circulation exposes the organ to food, microbiota, and self-antigens, to which immune reactivity must be avoided. Injection of antigens in the portal vein induces systemic tolerance; in contrast the presence of porto-systemic shunts leads to systemic reactivity [1, 2]. Plasmocytoid Dendritic Cells (pDCs) are important players in preventing oral T cell priming and inducing systemic tolerance [3]. In addition, the liver must also protect itself against potentially harmful pathogens and allow the development of effective immune responses. Such a delicate balance between tolerance and immunity is crucial for the integrity of the organ. Disturbances of hepatic immune homeostasis can lead to autoimmune diseases such as autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and in the case of foreign antigens to chronic viral infections such as those produced by the hepatitis B and C viruses.

The liver also plays a role in systemic CD8+ T cell immune responses. Liver expression of different chemokines and adhesion molecules attracts and retains activated T cells in the organ [4, 5]. An example of this kind of situation occurs when the stimulation of the innate immune system creates a pro-inflammatory environment resulting in bystander hepatitis [5]. Cytokines released following T cell activation mediate liver injury [6]. Concanavalin A, a non-specific stimulator of the immune system, when injected in the mouse, produces an immune "storm" leading to liver injury [6, 7]. This mouse model could be considered a form of severe bystander hepatitis; depending on the dose of the drug, with the severity of the inflammation controlled by the capacity of the organ to develop tolerance [7]. In most cases, proliferation of activated T cells in the liver is inhibited; several control mechanisms preventing chronic inflammation have been described [5]. The strong capacity of the liver to restore immune homeostasis is mediated in part through the apoptosis of infiltrating T cells and the promotion of the PD-1/PD-L1 pathway [5].

In autoimmune liver diseases the mechanisms leading to a break of immune tolerance in the liver are unknown; a viral infection or xenobiotics could contribute to the initiation of a pathological autoimmune reaction in genetically predisposed individuals. In such cases, CD4+ liver-specific autoreactive lymphocytes or those recognizing foreign antigens or haptens in the liver are activated and lead to an acquired immune response through further activation of CD8+ cytotoxic lymphocytes and B cells. Molecular mimicry between foreign antigens and self-proteins has been proposed as a possible pathogenic mechanism of autoimmunity [8, 9]. Either cytotoxic T cells or specific antibodies, autoantibodies in most cases, directly contribute to immune-mediated hepatic injury.

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Diagnosis

Clinical Findings

Fatigue is a very common finding in patients with an inflammatory-immune-related liver disease. This symptom can precede by several months the diagnosis, which becomes more evident after the development of other signs including those characteristics of a chronic hepatopathy (spider angiomas, palmar erythema). Fatigue can become invalidating for many patients, and it is difficult to manage [10].

Symptoms and signs vary as a function of the species of liver cell affected by the immune-mediated process. When hepatocytes are targeted a hepatitic syndrome is found; in contrast, a cholestatic syndrome is observed in diseases affecting the integrity of the bile canaliculi, the function of bile duct by cholangiocyte injury, or their structure. In the latter, itching and even jaundice could be the initial and more relevant feature.

Hepatomegaly is frequently present, and in severe cases cirrhosis rapidly develops, as reported in patients with AIH, and it is followed by portal hypertension and splenomegaly [10].

Female preponderance has been reported in autoimmune liver disease as AIH and PBC [10], an influence of the age on the incidence of these diseases has also been reported [10]. Clinicians must look for extra-hepatic autoimmune diseases, as well as for immune-related liver diseases in patients treated or followed for autoimmune disorders (Table 8.1). Patients and their families must be asked about autoimmune diseases in first-degree relatives. Positive antecedents are frequently found in patients with autoimmune liver diseases [10]. The presence of an active extra-hepatic autoimmune disease can also be responsible for bystander hepatitis, which resolves after treatment of the underlying disease. In other cases, a liver immune disease, such as AIH, can be part of a vast repertory of organ-specific autoimmune diseases, as observed in homozygous or composite heterozygous with a defect in the AIRE gene [11, 12], or IPEX [13].

Table 8.1 Extra-hepatic autoimmune/immune-mediated diseases in patients with autoimmune hepatitis

	Common	Rare
AIH type 1	Ulcerative colitis	Fibrosing alveolitis
	Vasculitis	
	Arthritis	
	Polymorphous erythema	
	Hypergammaglo bulineamic purpura	
	Autoimmune thrombocytopenia	
	Autoimmune hemolytic anemia[a]	
	Glomerulonephritis[a]	
AIH type 2	Vitiligo	Autoimmune lymphoproliferative syndrome
	Alopecia areata	Autoimmune enteropathy
	Nail dystrophy	IPEX
	Pyoderma grangrenosum	APECED
	Thyroiditis[a]	STAT1 deficiency
	Diabetes[a]	

[a]These autoimmune diseases could be observed in both AIH types

Antecedents of repeated episodes of infection or of systemic inflammation should orient towards the diagnosis of an inherited or acquired form of immunodeficiency, which occasionally revealed by symptoms and signs of liver inflammation. The onset of liver immune-mediated diseases associated with inherited immunodeficiency has been described in patients with IPEX, autoimmune lymphoproliferative syndromes, and several mutations in the gene coding for STAT1 [13–16].

Symptoms or signs of infection can lead to the suspicion of viral hepatitis; some of the symptoms or signs, as well as the age of the patient are helpful in the search of an etiologic agent. Chronic HBV or HCV infection can be subclinical for years, in most cases symptoms or signs become evident only after the development of cirrhosis and its complications.

It is of major importance to question the patient and the family about the possible exposure to hepatotoxic medication or toxic chemical agents. These can damage the liver through an immune-related mechanism. The clinical, biochemical, and histological picture is occasionally identical to that observed in a liver autoimmune process [17, 18]. Minocycline, a derivative of tetracycline, prescribed for the treatment of severe acne, can be responsible for liver toxicity with the presence of circulating autoantibodies. In such cases the pattern and the type of liver inflammatory infiltrate can mimic AIH [19, 20]. In addition, pruritus and jaundice can be the first symptoms of toxic liver injury and resemble the clinical picture associated with PBC and SC [19].

An increase in liver enzymes in a patient who has received a liver transplant is indicative of a rejection, due to an alloimmune reactivity against the foreign antigens. Rejection can target different species of liver cells. Depending on the preferentially targeted liver cells, different clinical, laboratory, or histological features are observed. In classical acute rejection, endothelial cells and ductal epithelial cells (cholangiocytes) are usually targeted [21], in other more chronic forms centrolobular or peri-portal hepatocytes are the primarily affected cells [22–24]. In patients receiving a bone marrow graft, the graft versus host liver disease targets mainly cholangiocytes, eventually leading to a chronic and severe form of clinically evident cholestasis. In transplanted patients, the presence of liver inflammation may be the manifestation of an opportunistic liver infection triggered by the immunosuppression

Table 8.2 Clinical and laboratory data according to the main cellular target

Cellular target	Symptoms and signs	Laboratory date	Autoantibodies		Histology predominant features
Hepatocytes[a]	Fatigue +	↑↑↑ ALT/AST	SMA		Interface hepatitis
		± GGT	ANA	Portal tracts	Lymphocytes +++
		Hyper IgG	LKM1[b]		Plasmocytes ++ (variable)
		Liver failure	LC1		Intralobular inflammation
			SLA		Bridging necrosis
Cholangiocytes/bile ducts	Fatigue +++	↑ ALT/AST	ANCA		Periductular granulomas (PBC)
	Pruritus	↑↑↑ GGT	AMA		Periductular fibrosis (SC)
	Jaundice	Hyper IgM	ANA		Portal tract fibrosis
		Hyper IgG (≅30% of SC)			

[a]In AIH: Predominance ♀/incidence variable according to age
[b]LKM with different specificity can be found in HBV HDV chronic co-infections

Table 8.3 Clinical and laboratory data in particular causes of immune-related liver injury

Causes	Symptoms and signs	Laboratory date	Autoantibodies	Histology predominant features
Innate Immune[a] system activation/cytokines storm	Related to the causing disease	↑↑ ALT/AST ↑ GGT	ANA SMA	Intralobular inflammation
Alloimmune fetal hepatitis	Hepatic failure	±↑ ALT/AST	–	Lobular disorganisation
				Rosettes +++
				Iron stockage
GCH-AIH	Fatigue	↑↑↑ ALT/AST	–	Giant cell transformation of hepatocytes
	Jaundice	Liver failure		
Drug-induced	Fatigue	↑-↑↑↑ ALT-AST	ANA	Centrolobular necrosis
	Jaundice	↑-↑↑↑ GGT	SMA	(↑) Neutrophiles
	Pruritus		LKM[b]	(↑) Eosinphiles

[a]Bytander hepatitis
[b]LKM with specific CYP reactivities

management program. Recently, a chronic viral infection caused by hepatitis E virus (HEV), leading in some cases to cirrhosis and hepatic failure, was described in immunosuppressed individuals after organ or tissue transplantation [25–27]. This is surprising since HEV in immunocompetent individuals usually lead to spontaneous resolution of the acute inflammation.

Recently, a new form of immune-mediated hepatic injury has been reported in newborns. Initially this entity was named neonatal hemochromatosis in view of iron accumulation in the liver. Currently, it is known that iron deposit in the liver is secondary to liver failure, and the injury of the organ during fetal life is the consequence of an alloimmunization of the mother against liver membrane antigen(s) [28]. Passage of IgG alloantibodies through the placenta and activation of complement on cell surfaces is the reported mechanism of hepatocyte injury [28]. Antecedents of spontaneous abortion, delay in fetal growth, prematurity, and more or less severe disease in previous children should orient to the diagnosis of fetal alloimmune hepatitis in a newborn with hepatic failure [28]. Once the mother has been immunized, the disease occurs in subsequent pregnancies. The hepatocyte alloantigen is constitutively expressed in fetal liver, in view of the fact that even with different fathers the disease can be observed, in cases where the mother has been immunized during previous pregnancies.

Laboratory Findings

The biochemical profile orients toward the target of the immune process, an increase of serum aminotransferases (ALT/AST) is characteristic of hepatocyte lysis, while an increase of serum Gamma Glutamyl Transferases (GGT) is a marker of ductular injury. Fetal alloimmune hepatitis is the first diagnosis in newborns with hepatic failure with a relatively mild increase of serum aminotransferases (Tables 8.2 and 8.3).

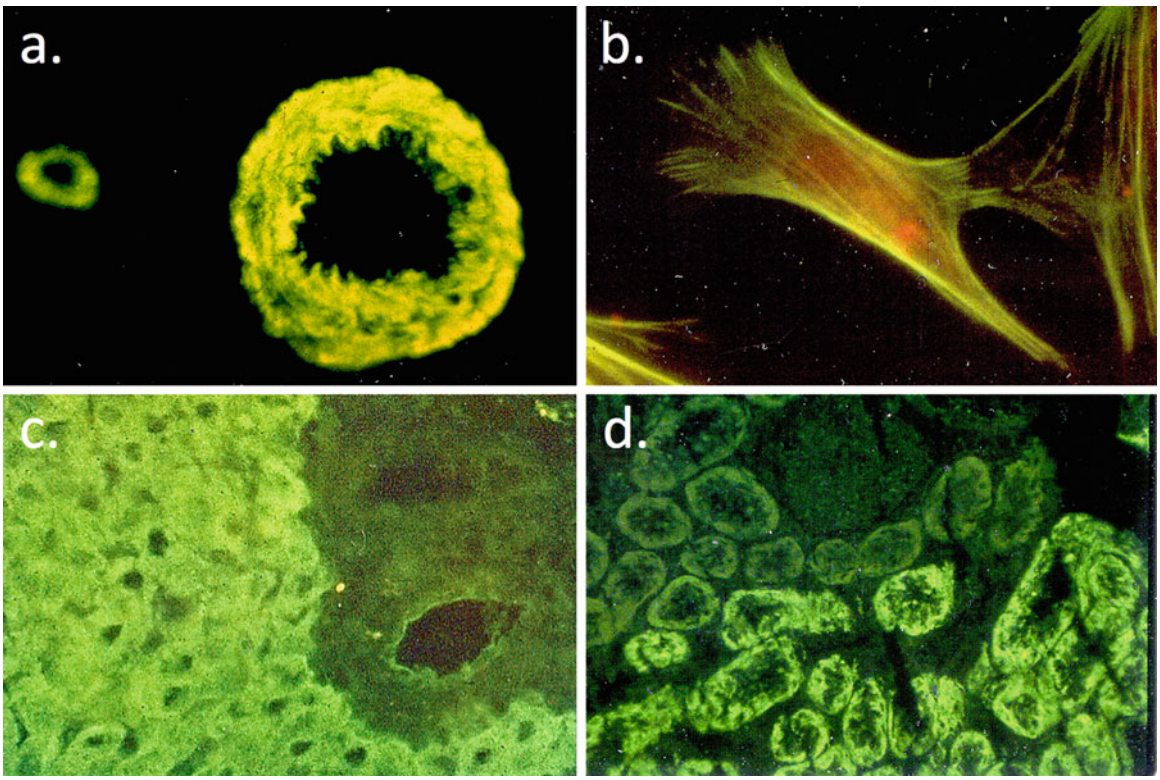


Fig. 8.1 Detection of autoantibodies by indirect immunofluorescence: (**a**) *ASMA*: positive staining of smooth muscle of arterial walls; (**b**) *ASMA*: anti-f actin in Hela cells treated by colchicine; (**c**) *LKM1*: staining of hepatocytes cytoplasm; (**d**) *LKM1*: tubular cells in the kidney

In patients with hepatitis, mainly in those in whom the disorder is of autoimmune or alloimmmune (post-liver transplant) origin, an increase in serum gamma-globulins is detected and is generally associated with the presence of circulating autoantibodies [10, 23]. In patients with AIH the increase is due to IgG subclass 1 and less of subclass 3. PBC patients usually show an increase in IgM. Hyper-IgG subclass 4 is found in a particular syndrome with pancreatitis, cholangitis, and rarely hepatitis; generally hyper-IgG4 is associated with the development of sclerosing diseases [29]. It has been speculated that the increase of IgG4 in such circumstances is secondary to the excessive production of anti-inflammatory cytokines [30].

Autoantibodies

Autoantibodies are excellent markers of several well-characterized autoimmune diseases of the liver (Tables 8.2 and 8.3). In AIH, autoantibodies allow the differentiation of the disease in two types: Type 1 showing circulating anti-smooth muscle antibodies (ASMA), which in most cases are specifically directed against actin filaments. ASMA at titers higher than 1:80 are detected in 70–90 % of patients with type 1 AIH. Anti-Nuclear antibodies (ANA) are also observed alone or associated with ASMA, in 40–70 % of patients with type 1 AIH [10, 31]. ANA is present at high titers in patients with type 1 AIH associated with inflammatory bowel diseases (IBD) [10]. Molecular targets of ANA are multiple; no specificity has been described in patients with AIH. ASMA and ANA are detected by indirect immunofluorescence, using a multi-organ substrate (Fig. 8.1a). To detect anti-Actin filaments antibodies, Hep2 cells can be used as a substrate after treatment with colchicine to disrupt microtubules and induce the formation of aggregates of vimentin [32] (Fig. 8.1b). Autoantibodies in patients with AIH type 1 are non-organ-specific and are not considered the main targets of the liver T cell autoimmune process.

ASMA have also been detected in sera from patients with acute or chronic viral hepatitis, drug-induced liver injury, and other chronic liver diseases. These groups include: (1) hepatotropic (e.g., hepatitis A, B, and C viruses) and non-hepatotropic (e.g., EBV) viruses [32]; (2) drug-induced hepatitis can display circulating autoantibodies, as ASMA and ANA (ex: drugs of the statins family or antibiotics) [17, 32]; and (3) chronic liver metabolic diseases (e.g., Wilson disease). In these cases ASMA have as their main target intermediate filaments, such as vimentin, although ASMA with actin specificity have also been found [32].

Antibodies against soluble liver antigen (SLA) have also been reported, mainly in patients with type 1 AIH, and rarely present in other liver disorders [33–36]. The presence of anti-SLA shows a significant female preponderance in children with AIH. The autoantigen has been identified as O-phosphoseryl-tRNA(Sec) selenium transferase, a 48.8 kDa protein encoded by the *SEPSEC gene*, allowing the production of sensitive and specific ELISA and immunoblot diagnostic tests [37]. Antibodies against SLA are mainly of the IgG1 subclass. They are frequently the only serological marker detected in sera from patients with type 1 AIH and are described as markers of relapses during treatment and of poor outcome [38]. The development of anti-SLA autoantibodies has been associated with HLADR3 and the susceptibility allele DRB1*0301 [39].

Liver-Kidney Microsomes antibodies of type 1 (LKM-1) directed against the Cytochrome P-450 2D6 (CYP2D6) are the most frequently found autoantibodies in sera from patients with AIH type 2 [40–44]. CYP2D6 is mainly expressed in the liver; it is also found in some tubular cells of the kidney, and in the brain (Figs. 8.1c, d). The gene coding for the CYD2D6 shows several polymorphisms. This enzyme is constitutively expressed in hepatocytes in variable individual amounts and is absent in up to 10 % of the general population. However, in patients with type 2 AIH, CYP2D6 is present and displays a normal metabolic activity [45, 46].

LKM-1 autoantibodies do not appear to play a role in hepatocyte injury. CYP2D6 is expressed in the cytosolic side of the endoplasmic reticulum membrane; the signal peptide and stop transfer sequence of this autoantigen shows no mutations in patients with type 2 AIH [47]. Therefore, if translocated to the plasma membrane, CYP2D6 would still be exposed inside the cell [47]. In patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and AIH, LKM autoantibodies, when present, are directed against CYP1A2. Other LKM types have been described in patients with viral (Herpes) and drug-induced (hydralazine) immune liver injury, in which cases LKM were directed against different CYP isozymes (e.g., CYP2C8 and CYP1A2) [32].

Some patients with type 2 AIH also display anti-Liver Cytosol type 1 (LC-1) antibodies, and in 10 % of them, it is the only serological marker of the disease. This autoantibody is directed against the Formimino-Transferase-Cyclo-Deaminase (FTCD), a cytosolic protein associated with the Golgi apparatus [48]. Titers of anti-LC1 autoantibodies correlate with the grade of liver inflammation [49].

Linear and conformational epitopes on CYP2D6 and FTCD molecules that are recognized by LKM-1 and LC-1 autoantibodies, respectively, have been reported, allowing the development of very specific and sensitive commercially available diagnostic tests using full-length proteins or specific peptides [50–54]. Human liver cytosol proteins must be used when test for anti-LC1 by immunoblot [54].

The anti-mitochondrial antibodies (AMA) characteristic of PBC are directed at members of the 2-oxoacid dehydrogenase components of multi-enzyme complexes, of which the E2 subunit of pyruvate dehydrogenase complex (PDC-E2) is the major autoantigen. AMAs are present in approximately 90–95 % of PBC sera [55].

In adults, few patients with overt AIH test positive for autoantibodies against pyruvate dehydrogenase complex E2 subunit by ELISA. Treated with standard corticosteroids they did not develop any clinical or histological evidence of PBC during follow-up [56]. However, in few patients features of PBC were detected over time [57]. In children the presence of AMA in patients with AIH is exceptional.

ANA mainly reacting with nuclear pore gp210 and nuclear body sp100 are found in approximately 20–30 % of sera from patients suffering from PBC. By indirect immunofluorescence it was shown that the sp100 autoantigen is distributed in up to 20 dot-like structures per nucleus co-localizing with the so-called nuclear bodies. By using immobilized synthetic peptides from sp100, two epitopes could be shown: SNSKVE and EPLEVFISA antigenic regions [58].

In sera from patients with sclerosing cholangitis an atypical perinuclear antineutrophil cytoplasmic antibody (p-ANCA), also called perinuclear antineutrophil nuclear antibodies (p-ANNA), can be found. p-ANCAs in autoimmune liver diseases are directed against human TBB-5 and cross-react with the bacterial protein FtsZ. The observed cross-reactivity probably reflects an abnormal immune response to intestinal microorganisms, possibly in genetically predisposed individuals [59].

Recently, new autoantigens have been detected in sera from patients with immune-related liver injury (AIH, PBC), using proteome microarrays [60, 61]. On silico peptide microarrays for high-resolution mapping of antibody epitopes will be used in the near future to replace immunofluorescence, ELISA, or Radio-immunoassays in the détection of specific and sensitive autoantibody markers of immune-related liver diseases [62].

Human Leucocyte Antigen

MHC is the most frequent susceptibility locus for immune-mediated liver diseases. Specific Human Leucocyte Antigen (HLA) haplotypes are responsible for susceptibility to the development of AIH, PBC, or Sclerosing Cholangitis (SC), while others have a protective influence. Typing of HLA can be considered as a supplementary test for the diagnosis of immune-mediated liver diseases [63].

HLA alleles associated with type 1 AIH in Europe and North America are DRB1*0301 and DRB1*0401 [63–65]; lysine at position 71 of the expressed DR molecule is the critical amino acid [63, 64]. Haplotypes associated with a higher risk of developing an AIH are: A1-B8-MICA*008-TNFA*2-DRB3*0101-DRB1*0301-DQB1*0201, and DRB4*0103-DRB1*0401-DQA1*0301-DQB1*0301, having a risk ratio of 4.6–5.51 and 3.3–3.7, respectively [63, 64, 66]. In Argentina, Mexico, and Japan susceptibility is linked to DRB1*0405 and DRB1*0404; arginine at position 71 rather than lysine is coded by these alleles [63, 65, 67]. However, they share the motif LLEQ-R with DRB1*0401 and DRB1*0301. Therefore, either K or R at position 71, in the context of LLEQ-R, are critical for susceptibility. Interestingly, the QRRAA motif at positions 70–74 is significantly increased in Korean patients ($P=0.04$, OR = 1.84) [68].

DRB1*0301 allele is in linkage disequilibrium with genes considered as additional risk factors for autoimmunity, including TNFA*2 and C4A*Q0 [64].

Hepatitis A virus (HAV) infection has been postulated as a putative trigger for AIH. A study compared HLA alleles in children who developed uncomplicated HAV infection with those in protracted HAV forms [69]. In uncomplicated hepatitis, 27 out of 69 studied patients developed anti-smooth muscle antibody (ASMA)/actin antibodies, but only 1 child had detectable antibodies 3 months after onset. In contrast, after 1 year, 27 out of 39 patients suffering from protracted forms had titers of ASMA/actin antibodies that ranged between 1:40 and 1:160, serum titers comparable to those observed in patients with type 1 AIH which suggests that the infection leads to a sustained release of liver self-antigens. DRB1*1301 haplotype, a marker for pediatric AIH, which suggests that the infection leads to a sustained release of liver self-antigens, is strongly associated with the protracted forms of HAV infection [69]. However, in the long-term follow-up none of these patients developed an AIH [69]. In conclusion, haplotypes of susceptibility are associated with a persistence of the HAV promoted inflammatory liver injury, albeit, limited in time. Therefore, in susceptible individuals a failure of liver immune hemostatic mechanism could lead to AIH [69].

HLA-DQB1 *0201 allele is found to be the primary genetic determinant of susceptibility to type 2 AIH [63, 64]. A relationship between the circulating autoantibodies and a specific HLA haplotype has been recognized in patients with type 2 AIH [70]. HLA-DRB1 *03 allele is significantly increased among patients showing both anti-LKM1 and anti-LC1 autoantibodies, as well as in those with only anti-LC1 autoantibodies. In contrast, HLA-DRB1 *07 allele was significantly associated ($P<0.0001$) with anti-LKM1(+) alone compared to groups with both anti-LKM and anti-LC1 or with LC1+ alone [70].

It should be noted that in tests using peptides representing epitopes recognized by anti-LKM1, children with the DRB1 *07 allele develop anti-LKM1 autoantibodies having a more restricted specificity (two epitopes) than those with the HLA-DRB1 *03 allele (five epitopes) [70].

In Brazil, the primary susceptibility allele for AIH-1 is DRB1*1301, but a secondary association with DRB1*0301 has also been identified [66].

IgA deficiency is common in AIH patients and is genetically linked to the MHC locus, especially with HLA susceptibility alleles HLA-DR1 and HLA-DR7 [10]. Low levels of C4a are found in more than 60 % of children with AIH. Complement factor 4a (C4a) deficiency is likely involved in AIH pathogenesis, since deletions in the C4A gene are found in patients who develop AIH at a younger age [10].

The highest risk ratio for PBC in a Northern European population is observed in carriers of the DRB1*0801-DQA1*0401-DQB1*0402 haplotype. There also exists an independent association with DPB1 [63]. In a cohort of patients with PBC from Southern Europe, specific HLA-DRB1 genes (*08, *11 and *14) account for most of the DRB1 association signal, DRB1*08 being the strongest predisposing allele, whereas DRB1*11 was protective [71]. Additionally, DRB1*14 and the DPB1 association were predisposing risk alleles [71].

Genome wide association analysis showed significant associations between PBC and 13 loci across the HLA class II region; the strongest association was found with the HLA-DQB1 locus (DQ beta chain 1) [72].

The primary association of SC with the DRB3*0101–DRB1*0301–DQA1*0501–DQB1*0201 and DRB1*1301–DQA1*0103–DQB1*0603 haplotypes has been reported. In addition, a strong protective influence of the DRB1*04–DQB1*0302 haplotype and a protective association with the DRB1*0701–DQB1*0303 haplotype have also been shown. Specific amino acids at DQbeta-87 and DQbeta-55 play a role in susceptibility and protection, respectively [73]. Authors from Southern Europe have confirmed positive and negative associations with DRB1*15 and DRB1*07, respectively, but they have not found associations with the DRB1*03, *04, or *1301 alleles typically detected in PSC from Northern Europe [74].

Haplotypes associated with the highest risk of developing a PSC are: B8-MICA*008-TNFA*2-DRB3*0101-DRB1*0301-DQB1*0201 and DRB3*0101-DRB1*1301-DQA1*0103-DQB1*0603, with a risk ratio of 2.69 and 3.8, respectively [63].

Histology

Main histological findings observed in different causes of immune-mediated liver injury are summarized in Tables 8.2 and 8.3.

Chronic Hepatitis

Inflammatory cells infiltrate portal tracts, with the limiting plates more or less destroyed by the inflammatory process depending on the severity of the inflammation. In chronic active hepatitis, inflammation affects the interface between the portal space and the plates of hepatocytes (Interface hepatitis, previously called "piece meal necrosis"). Portal tracts are often enlarged by inflammatory infiltrating cells and fibrosis [75, 76].

Infiltrating cells are mainly composed of lymphocytes, sometimes forming aggregates or follicles; such a finding is more frequently observed in biopsies from patients with a chronic hepatitis C infection [75]. Plasmocytes are frequently found, increasing in the more severe forms and constituting a particular feature of AIH (Fig. 8.2a) [75, 76].

Intralobular changes are characterized by varying degrees of inflammation and necrosis, going from isolated cell injury to confluent necrosis, leading to bridging. Hepatocytes can form rosettes, surrounded by connective tissue. In patients with AIH, some hepatocytes fuse to form multinucleated giant cells [75, 76].

Histologically, the differential diagnosis between AIH and chronic viral B or C infection is not easy (Fig. 8.3). However, the presence of follicles, some of them with prominent germinal centers, is usually found in the liver of patients with a chronic HCV infection (Fig. 8.3a) [75]; while large droplet fatty changes are more frequently found in HCV chronic infection than in other causes of chronic hepatitis [75].

"Ground glass" hepatocytes are seen in patients with chronic hepatitis B infection [75]; a high expression of the HBs antigen induces hypertrophy of the endoplamic reticulum responsible for the particular aspect of the cytoplasm (Fig. 8.3c). Immunohistochemistry using specific antibodies against viral antigens are of interest in difficult cases (Fig. 8.3d), combined with results of serological tests. In patients with HBV chronic infection and Hepatitis Delta Virus (HDV) co-infection, higher levels of inflammatory activity are recorded, as well as rapid progression to cirrhosis in almost half of these patients (Fig. 8.3e, f). Detection of

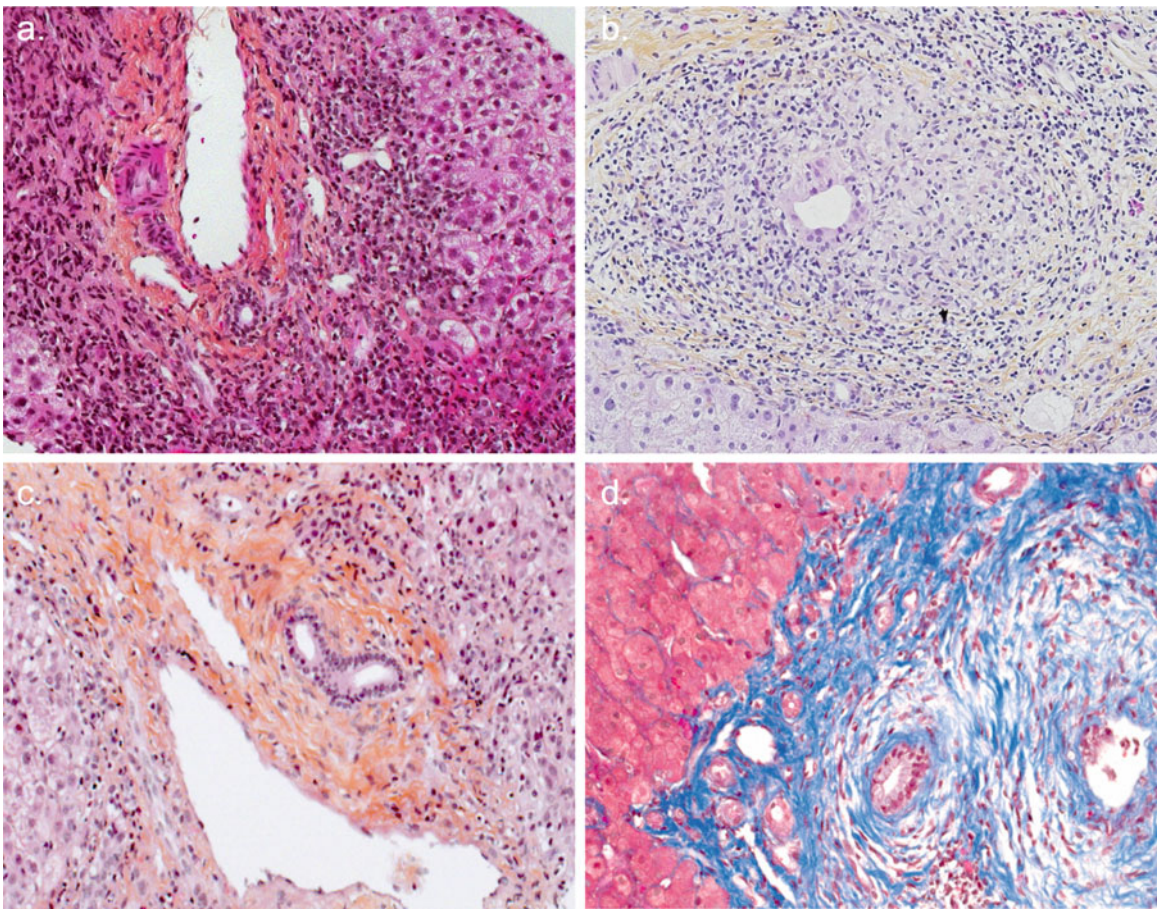


Fig. 8.2 "Classical" histological lesions of autoimmune liver diseases: (**a**) *AIH*: Portal lympho-plasmocystic infiltrate, with interface hepatitis (H&E); (**b**) *PBC*: periductular lymphocytic infiltrate (H&E); (**c**) and (**d**) *SC*: periductular fibrosis (H&E and Trichrome respectively)

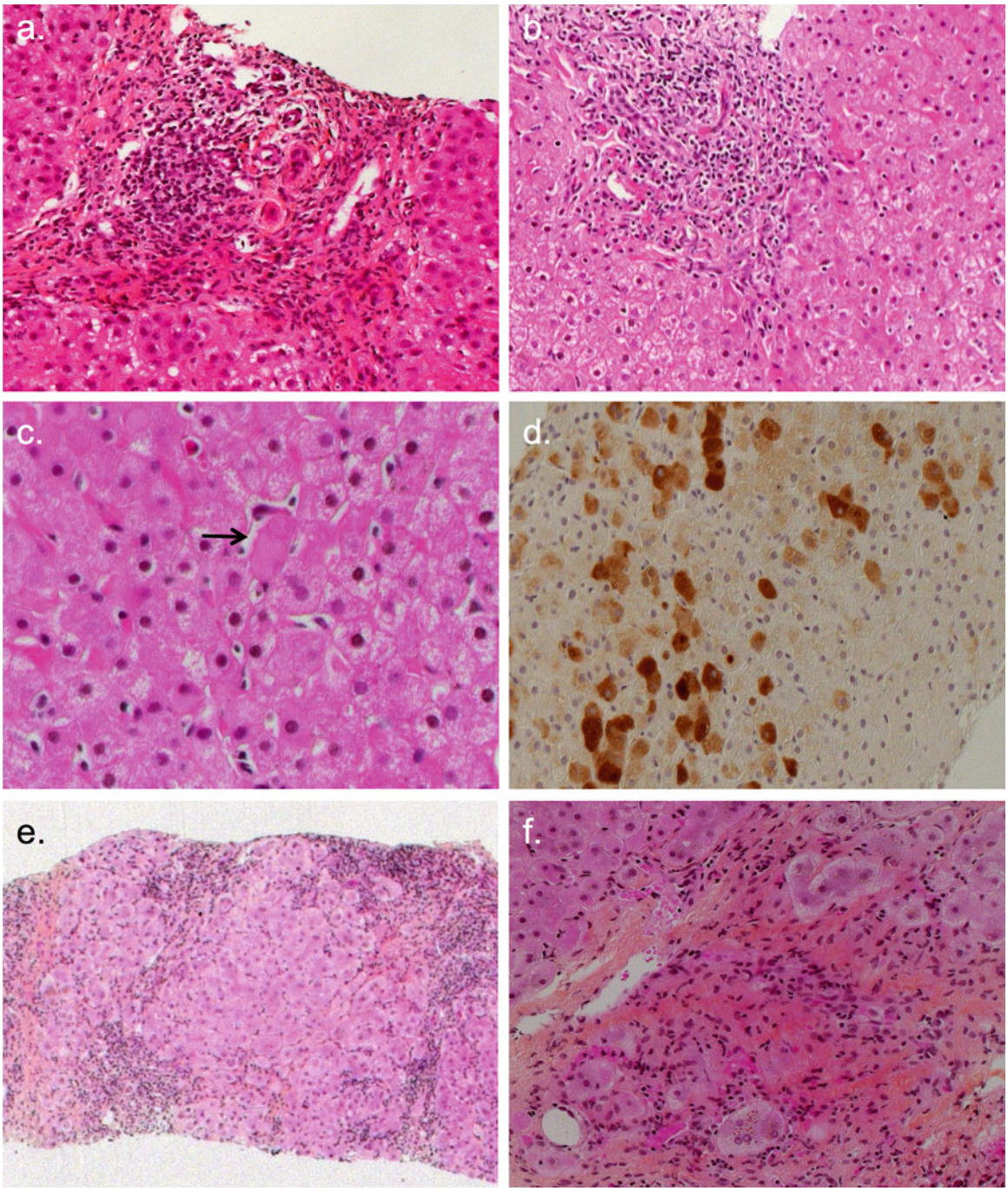


Fig. 8.3 Chronic viral hepatitis (characteristic features). (**a**) *HCV*: Small lymphocytic "nodule" in a portal tract, interface hepatitis, lipid vacuoles in hepatocytes (H&E); (**b**) *HBV*: Enlarged portal space, lymphocytic infiltrate, interface hepatitis (H&E); (**c**) *HBV*: "Ground glass" hepatocytes (arrow) (H&E); (**d**) *HBV*: indirect immunoperoxidase staining of HBs Ag, (**e**) *HBV and HDV* co-infection: enlarged portal tracts, bridging fibrosis, lymphocytic infiltrate, interface hepatitis; and (**f**) disorganization of liver lobule by portal tract expansion, Giant cell transformation

HDAg in the liver biopsy denotes active infection and confirms the diagnosis [77].

In most patients with AIH, typical clinical and laboratory features are sufficient to begin treatment without histological examination; liver biopsies should be avoided, especially in patients with hepatic failure who need frozen plasma to reduce the risk of bleeding after the procedure [78].

Giant cell hepatitis associated with Autoimmune Hemolytic Anemia (GCH-AHA) is a disease of infants and young children, in which a complete distortion of the hepatic

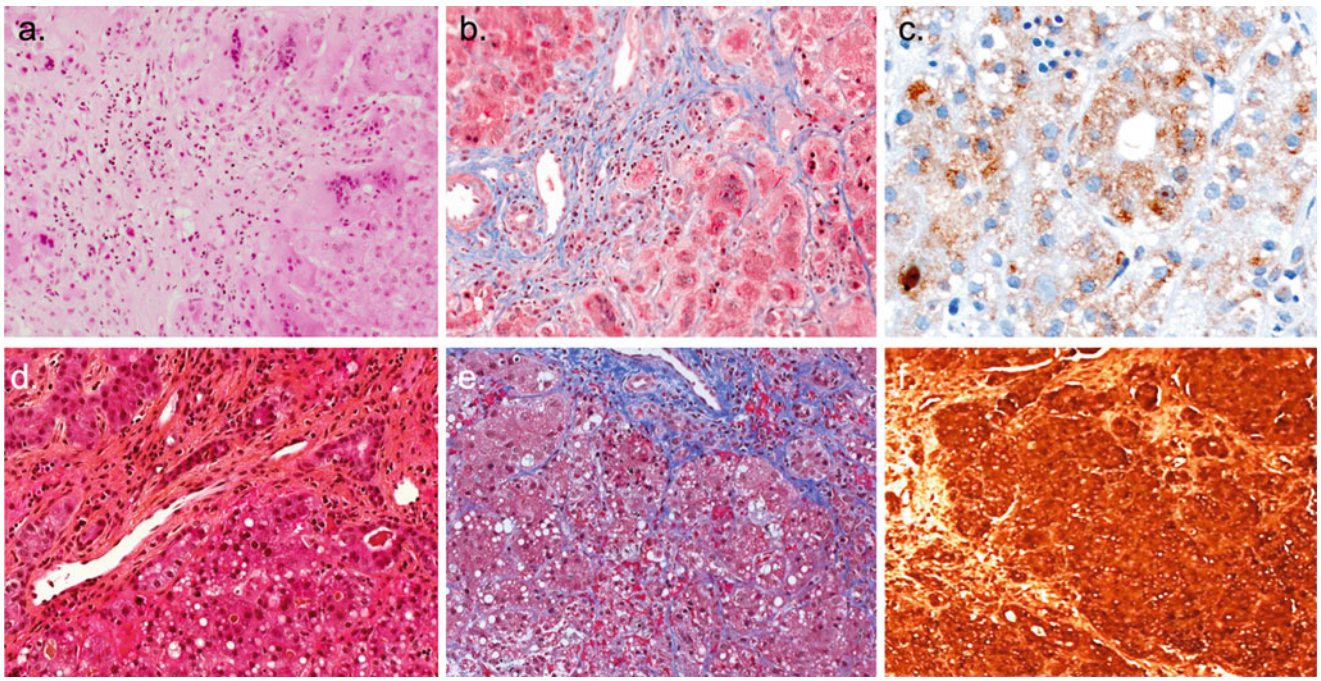


Fig. 8.4 (**a**) *Autoantibodies-mediated diseases. GCH (with AIA)*: Giant cell transformation of hepatocytes. Polymorphic cell infiltrate in portal tracts (H&E); (**b**) Giant cell transformation of hepatocytes, enlarged portal tract, bridging fibrosis, sinusoidal fibrosis (Trichrome); (**c**) Membrane Attack Complex (Complement) on cell surfaces, as detected by immunoperoxidase; (**d**) *Alloimmune fetal hepatitis*: distortion of the liver lobular structure (H&E); (**e**) with sinusoidal and portal mild fibrosis (Trichrome); (**f**) Membrane Attack Complex staining on cell surfaces (immunoperoxidase)

parenchyma is observed (Figs. 8.4a, b). Portal tract infiltrates are minimal and composed of mononuclear and polymorphonuclear cells (Fig. 8.4a). This histological picture is very unusual in other chronic liver diseases [79, 80]. These patients do not usually display abnormal serum IgG levels or circulating autoantibodies [79, 80]. Liver biopsies can be useful in the differential diagnosis between AIH and drug toxicity. In patients with drug-induced liver injury, portal neutrophils and eosinophils, with canalicular cholestasis, are predominantly found [81].

Biliary Diseases

Non-suppurative cholangitis is the hallmark of PBC, a disease that affects the interlobular bile ducts. Epitheloid cells or well-formed granuloma centered on bile ducts (granulomatous cholangitis) are found (Fig. 8.2b). Infiltration of lymphocytes within the biliary epithelium is observed (lymphocytic cholangitis), inducing cholangiocytic damage [82]. PBC can progress to bile duct loss (ductopenia). The portal inflammatory infiltrates are composed of lymphocytes, plasmocytes, and of a variable number of eosinophils. Plasmocytes around sites of non-suppurative destructive cholangitis are found in patients with high titers of AMA [82]. Fibrosis is characterized by the presence of porto-portal fibrous septa.

Periduct edema and concentric fibrosis around interlobular medium size bile ducts (« onion skin » appearance) are typical of Primary Sclerosing Cholangitis (PSC) (Fig. 8.2c, d) [10]. Ductular proliferation, portal inflammation, and disappearance of small ducts (rounded scar) are also observed. The portal inflammation in one third of the patients can reproduce the pattern described in chronic hepatitis [10].

Rejection in Transplanted Liver

In acute liver rejection, two cell types are the main targets: endothelial cells and ductular epithelium (Fig. 8.5a, b). Lymphocytes under the vascular endothelium and infiltrating the medium and small size interlobular ducts are considered as signs of acute rejection in transplanted livers (Fig. 8.5a, b) [21].

A more chronic form of rejection shows centrolobular inflammatory infiltrate and necrosis, or a portal inflammation with interface lymphocyte infiltration as described in patients with chronic active hepatitis (Fig. 8.5c, d) [22–24]. These histological pictures are also found in patients with AIH;

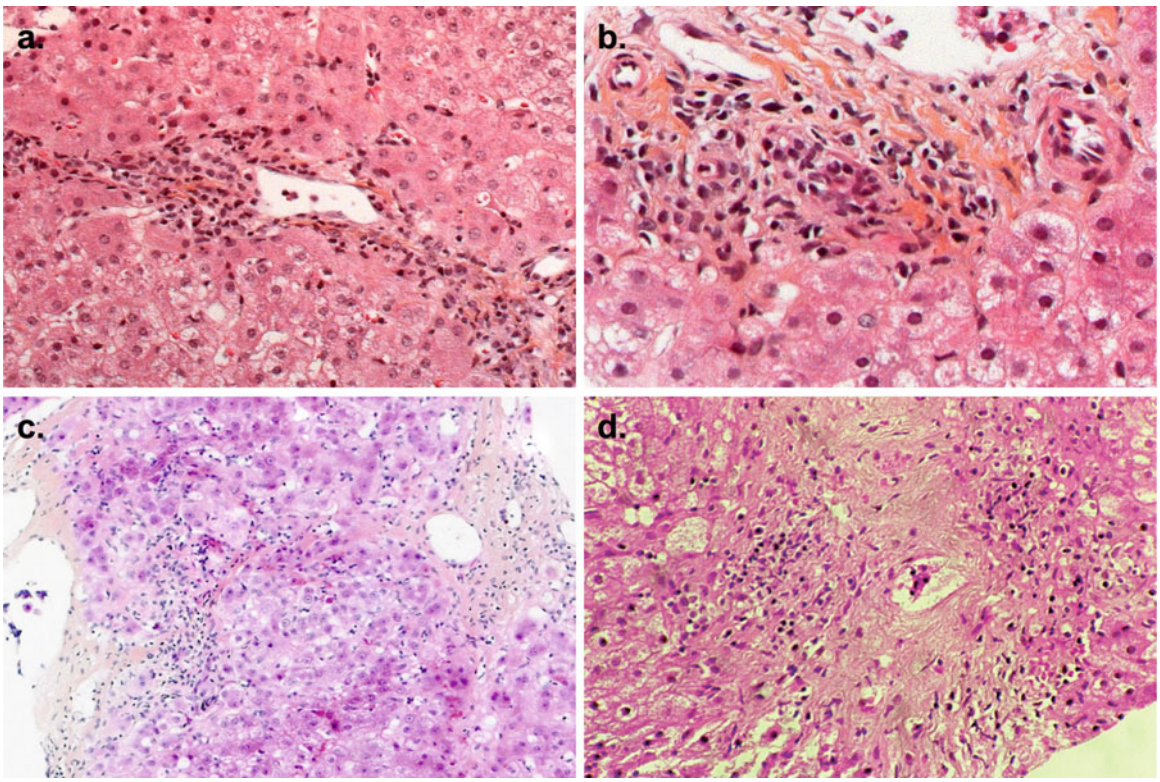


Fig. 8.5 Acute and chronic rejection. (**a**) *Acute rejection*: "endothelialitis," lymphocytes under the vascular endothelium (H&E); (**b**) lymphocytic infiltration of interlobular bile ducts; (**c**) "*Chronic" rejection/ alloimmune hepatitis*: Portal tracts infiltrated by lymphocytes, interface hepatitis, fibrosis; (**d**) centrolobular necrosis, fibrosis, and inflammation

therefore, such form of rejection could be considered an alloimmune-mediated reactivity against hepatocytes. In some of these patients circulating autoantibodies and hyper-IgG can also be present [24].

Graft Versus Host Disease

Graft versus Host disease (GVHD) can occur at variable times 3 or 4 weeks following a bone marrow transplant. Bile duct damage is a constant finding in liver biopsies (Fig. 8.6). Moderate/severe lobular hepatitis can also be present in a third of liver biopsies [83, 84]. Endothelitis is less frequent, and fibrosis is mild or absent in initial biopsies. Portal inflammation develops in the "chronic" GVHD group, associated with vanishing bile ducts and portal fibrosis [83, 84].

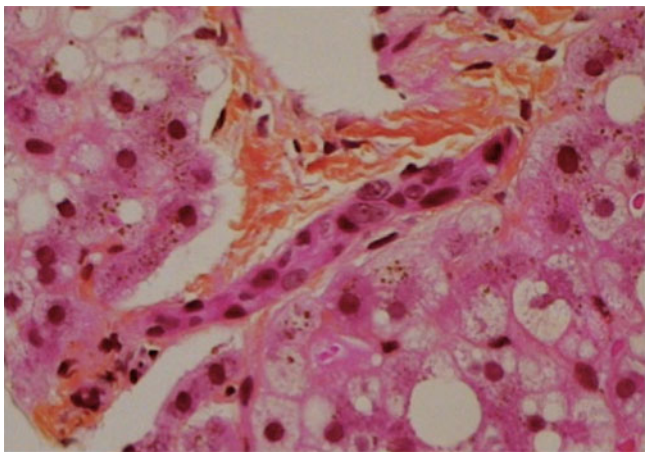


Fig. 8.6 *Graft versus Host disease* of the liver: mild inflammatory cells' infiltration of the interlobular bile duct; several cholangiocytes in "apoptosis."

Bystander Hepatitis

Liver infiltration by lymphocytes can be found in several systemic activation of the immune system, as in cases of severe extra-hepatic autoimmune diseases or infections by non-hepatotropic viruses [85, 86]. Lymphocytes accumulate in the portal tract, but mainly throughout sinusoids. Hepatocyte damage is frequent and is responsible for an increase in serum aminotransferases. Such a histological picture is the consequence of the sequestration of activated lymphocytes in the liver, which is in some cases supported by a pro-inflammatory environment triggered by infections or systemic inflammatory/autoimmune diseases [5, 85].

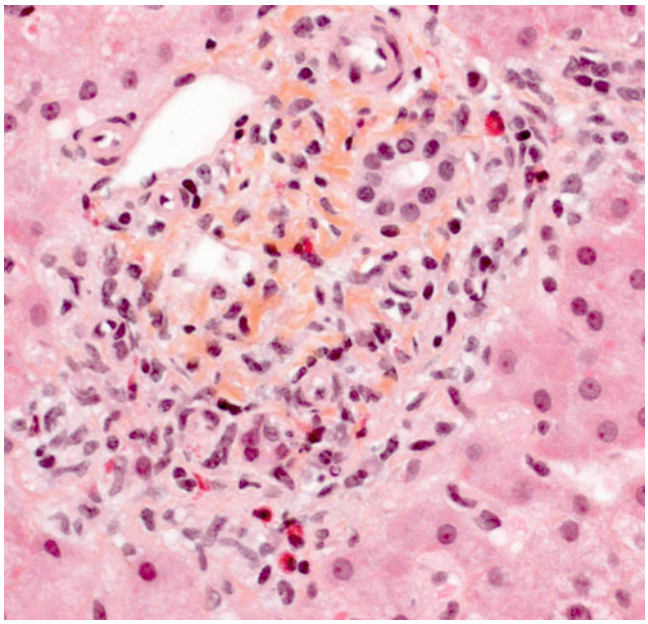


Fig. 8.7 *Drug-induced hepatitis*. Enlarged portal tract showing polymorphic infiltrate, rich in eosinophils

Drug-Induced Hepatitis

Liver biopsies can help with the differential diagnosis of other entities, mainly viral hepatitis. A high degree of centrolobular confluent necrosis and inflammation, a relatively low grade of portal inflammation, and a portal infiltrate rich in neutrophils and/or eosinophils should increase the degree of suspicion for a drug-caused disorder (Fig. 8.7) [81]. The presence of epitheloid-cell granulomas increases the possibility that the process is secondary to a drug [81].

Drugs can also be responsible for cholestasis; a differential diagnosis with other immune-related cholestatic diseases (PBC, PSC) is important. Drug-related cholestasis could be induced by an immune attack against different liver structures and present as hepatitis, bile duct injury, or cholangiopathies.

Score for Diagnosis of Autoimmune Hepatitis

The above described clinical, laboratory, and histological findings have led to the design of a scoring system for AIH diagnosis by the International AIH Group [76]. According to the aggregate score system patients can be classified as having probable or definite AIH; the response to immunosuppressive treatment can further help in the differentiation with other autoimmune diseases of the liver, such as PSC and PBC.

Simplified diagnostic criteria have been tested in adult and pediatric populations, and results were comparable in most cases at those obtained using the extended scoring system [87, 88].

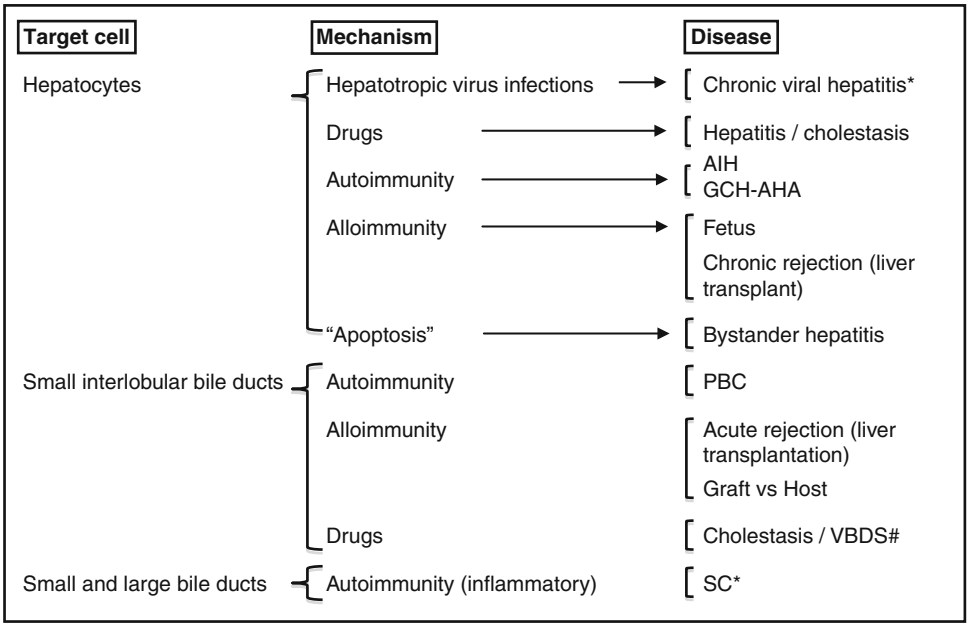
Classification

Liver Cell or Structure Targets

Immune-mediated liver diseases can be classified according to the main cellular or structural target of the immune attack (Table 8.4). This classification is useful to understand the symptoms and signs presented by the patient, including laboratory results.

Hepatocytes, the parenchymal cell of the liver, are the main target of the immune system in AIH, acute and chronic viral infection, and drug-induced immuno-allergy toxicity [10, 19]. In addition, the presence of high numbers of activated lymphocytes can be responsible for hepatocyte lysis, by cytokine toxicity, or recognition of membrane proteins by ligand or receptors expressed mainly by T cells [6, 7]. An example of bystander hepatitis is observed in cases of liver inflammation during infection by non-hepatotrpic viruses, as the Epstein-Barr virus. In other rare and particular situations, represented by inherited immunodeficiencies, in which different mechanisms can lead to the development of an immune-mediated hepatitis, the liver inflammation can be a major manifestation of the disease. At least four mechanisms are currently proposed in immunodeficient patients: (1) absence of negative selection of autoreactive T cell clones, because of mutations of the AIRE gene (autoimmune regulator), encoding for a transcription factor which plays a major role in the expression of tissue-specific proteins in thymic stromal cells, thereby leading to APECED which affects multiple endocrine glands [11, 12]. The incidence of hepatitis in APECED is variable according to the studied population, from 12 % in a series from Finland, to 27 % in a cohort from Sardinia [11, 12]. Interestingly, in the latter, AIH was a serious and surprisingly common/early/presenting feature (two deaths), with a 5:1 female bias (median age, 6 years; range, 2.5–11 years). HLA alleles appear to influence the phenotype. An interesting association is described between HLA-DRB1*0301-DQB1*0201, liver-kidney microsome autoantibodies (anti-CYP1A2), and AIH [12]; (2) mutations in the FOXP3 gene that encodes for a transcription factor required for the suppressive function of CD4+ regulatory cells [13]. Tregs development deficiency is responsible for a severe multi-organ, autoimmune phenomena including hepatitis [13]; (3) lympho-proliferation as a consequence of mutations in genes regulating apoptosis of autoreactive B and T cells [14, 15]; (4) mutations of the STAT1 (a member of the Signal Transducers and Activators of Transcription

Table 8.4 Immune-mediated hepatic diseases. Classification according to the target cell



AIH: Autoimmune Hepatitis
GCH-AHA: Giant Cell Hepatitis and Autoimmune Hemolytic Anemia
PBC: Primary Biliary Cirrhosis
SC: Sclerosing Cholangitis

AIH autoimmune hepatitis, *PBC* primary biliary cirrhosis, *SC* sclerosing cholangitis
[a]These diseases can occur in immune-competent and in immune-deficient patients (acquired or inherited immunodeficiency)
[b]Vanishing bile duct syndrome

family) gene that is involved in up-regulating genes due to a signal by either type I, II, or type III interferons [16]. Affected patients show a poor production of interferon G, interleukin-17, and interleukin-22, suggesting that a defect exists in the signaling pathways of the interleukin-12 and interleukin-23 receptors. In consequence patients have a defective Th1 and Th17 responses [16].

A chronic form of liver rejection shows the histological pattern of chronic active hepatitis and can affect centrolobular hepatocytes alone, but more frequently it is characterized by a portal lymphocyte infiltrate and interface hepatitis. Such a picture can be considered as an alloimmune hepatitis [23, 24].

A particular hepatitis in infants and young children is characterized by giant cells transformation of most hepatocytes at liver biopsy. It is associated with an autoimmune hemolytic anemia (Coombs +, of IgG type and complement) [78, 79]. This disease shows a high frequency of recurrence after liver transplantation.

The frequency of drug-induced autoimmune-like hepatitis among patients with classical features of AIH is 9 %. Minocycline and nitrofurantoin are implicated in 90 % of cases; isoniazid, halothane, and indomethacin also responsible for acute hepatitis [89]. Female predominance, acute onset, and absence of cirrhosis at presentation are important clinical characteristics [89].

Immune Mechanism of Liver Injury

Immune-mediated hepatic injury can occur through different mechanisms (Table 8.5): T cells recognizing liver antigen epitopes or hapten/proteins; B cells production of specific autoantibodies; or by sequestration and death of activated lymphocytes in the liver (bystander hepatitis). A classification of these diseases according to the specific mechanism leading to liver injury can help us in designing specific therapeutic approaches. Probably, the most persuasive example is the recent discovery that GCH-AHA in infants and young children is mediated by autoantibodies, making monoclonal anti-CD20 an optimal choice for treatment of these patients (Fig. 8.4c) [90]. Fetal alloimmune hepatitis also mediated by antibodies (Fig. 8.4f) benefits from immunoglobulin transfusions and plasmapheresis.

Conclusions

In most patients with an immune-mediated hepatic injury, no particular symptoms or signs or nonspecific ones are present. The biochemical profile can orient toward a hepatic or a cholestatic disease, depending on the main target of the immune process. Increases of serum immunoglobulins levels are frequently observed; such phenomena reflect a large activation

Table 8.5 Immune-mediated hepatic injury. Classification by main immune mechanisms responsible for the disease.[a] This mechanism remains controversial in liver-transplanted patients

Mechanism	Disease
Antibody mediated	• Fetal alloimmune hepatitis
	• Giant cell hepatitis with autoimmune hemolytic anemia (90)
	• Rejection (anti-HLA alloantibodies)*
T-cell mediated	• AIH
	• PBC
	• SC
	• Graft vs Host
	• Cellular rejection
	• Virus infections
	• Drugs toxicity
Cytokines/Apoptosis	• Bystander hepatitis

of diverse components of the immune system. Circulating autoantibodies are of great help; although in most cases are not absolutely specific of a disease, their presence do not orient toward a particular pathogenic mechanism. Studies on genes of susceptibility of large cohorts of patients with immune-related hepatic injury show that the greatest association is found with defined haplotypes in the MHC locus.

Classifications of syndromes or disease must be of help in the diagnosis, facilitate the choice of the therapeutic protocol, and promote new studies on pathogenic mechanism. Considering such orientation, taking into account currently available data, immune-mediated hepatic injury can be classified according to the main cellular target, or according to the main mechanism leading to liver inflammatory damage.

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The Diagnosis and Classification of Immune-Mediated Biliary Diseases

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Key Points

1. Immune-mediated biliary diseases encompass a range of biliary insults that occur in children and adults.
2. The consequence of biliary inflammation is often bile duct loss (ductopenia), and a resultant secondary progressive biliary cirrhosis.
3. Disease diagnosis is made by an evaluation of the patient's history, symptoms, signs, and investigations, acknowledging the epidemiology of individual diseases, and at the same time the lack of any true diagnostic test.
4. The spectrum of immune-mediated biliary disease includes, for example, biliary atresia in babies, primary biliary cirrhosis and primary sclerosing cholangitis in adults, as well as drug-induced liver injury.
5. For primary biliary cirrhosis, the tight association serologically with anti-mitochondrial antibodies is essential to appreciate.
6. Cholangiography can assist in diagnosing sclerosing cholangitis, but only careful clinical evaluation can distinguish primary from secondary disease.
7. Drug-induced liver injury is often cholestatic, and the strong HLA associations with such injury, supports involvement of immune-mediated pathways in pathogenesis.

Introduction

Immune-mediated biliary diseases are frequent clinical problems that span all ages, both genders, and all ethnicities. Whilst diseases can be classified into broad categories, mechanisms underpinning biliary damage are often shared [1–4]. Hence the study of biliary atresia (BA), a purely pediatric disease of unknown etiology but characterized by bile duct fibro-obliteration, still may mechanistically aid studies of primary biliary cirrhosis (PBC), an adult small bile duct lymphocytic cholangitis. Similarly the rare disease autoimmune pancreatitis/IgG4 cholangiopathy has potential to inform debate about the broader pathogenesis of primary sclerosing cholangitis (PSC), an idiopathic and progressive large bile duct cholangitis.

These groups of diseases continue to be described more than defined, in that unlike viral hepatitis, there remains no single definitive diagnostic test. Hence the term sclerosing cholangitis, first described in 1867, describes a group of cholangiopathies that are evident radiologically and/or histopathologically, and which are usually accompanied by classic cholestatic biochemical profiles as characterized by elevated serum alkaline phosphatase and/or gamma-glutamyl transferase levels. But the term sclerosing cholangitis describes a spectrum of chronic biliary diseases classified as *primary* if there is no known precipitating cause, or *secondary* when disease arises as the consequence of identifiable insults, such as ischemia and toxic injury, to the biliary tree. Similarly PBC, albeit better defined than PSC, is still a broad description of a highly complex immunologic process clinically more heterogeneous than the immunologic profile that characterizes its autoreactivity. The impact for patients with immune-mediated biliary disease is significant and many require liver transplantation, an effective, if albeit blunt, and scarce resource. The goal for clinicians remains the early diagnosis of disease, so that treatment can be started to prevent progression to end-stage biliary cirrhosis (with liver failure, portal hypertension, and risk of hepatocellular carcinoma), a potential end point of any chronic liver disease.

The two most significant immune-mediated biliary diseases are PBC [3, 5] and PSC [6, 7]. Patients with PBC present predominantly in middle age (>45 years) and are most commonly women (~95 %). The incidence rates vary from 0.33 to 5.8 cases per 100,000, and point prevalence from 1.91 to 40.2 cases per 100,000. Conversely, 60–71.4 % of patients with PSC are male and the annual incidence of disease is of the order of ~1.3 per 100,000 inhabitants/year in North

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America and Northern Europe. The majority of patients of European descent with PSC have coexisting inflammatory bowel disease (IBD), usually ulcerative colitis (up to 85 %). Population cohorts [8] of patients with PBC and PSC report standardized mortality ratios for all-cause mortality of 2.7 (95 % CI 1.7–4.0) and 4.1 (95 % CI 2.6–6.3), respectively, clearly highlighting unmet clinical needs. It should however also be noted that there is significant bias in the literature for both PBC and PSC, derived from an overrepresentation of reports arising from tertiary/quaternary hepatology practice, as opposed to true population cohorts including patients with likely milder disease.

Overview of Disease

There is no codified definition of what constitutes immune-mediated biliary disease but broadly speaking disease is more frequent in adults than children, and of a different nature (Fig. 9.1; Box 9.1). In pediatric practice cholestatic liver disease is the leading indication for liver transplantation, of which *biliary atresia* is most important [9, 10]. BA is immune in nature, although its triggers are unclear and may include viral infections, chronic inflammatory or autoimmune-mediated bile duct damage, and developmental abnormalities. The disease process is characterized by a progressive fibro-inflammation of the biliary tree leading to fibrosis and obliteration of both the extrahepatic and intrahepatic bile ducts. Infiltration of lymphocytes and macrophages observed in periductal spaces or along the duct epithelium in conjunction with increased expression of inflammatory cytokines supports a role for immune/autoimmune mechanisms in bile duct injury.

In adults autoimmune chronic small and large bile duct diseases dominate clinical practice as regards immune-mediated liver injury. *PBC* is the most frequent, and is estimated to occur, predominantly in women, at a prevalence of 1 in 1,000 over the age of 40. It is archetypally autoimmune

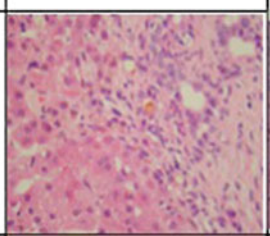
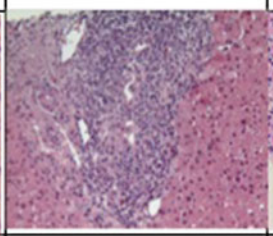
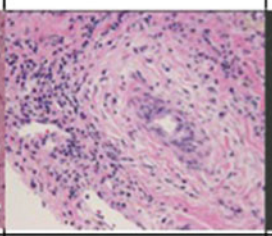
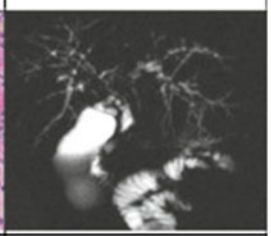
	Biliary atresia	Primary biliary cirrhosis	Primary sclerosing cholangitis	IgG4 associated autoimmune pancreatitis/ sclerosing cholangitis
Examples of Presentation	Neonatal disorder; 10-20% syndromic; persistent conjugated hyperbilirubinemia; decompensated liver disease	Asymptomatic cholestasis Fatigue/Pruritus	Asymptomatic Cholangitis Abdominal pain	Diabetes Jaundice Pancreatic mass Fleeting cholangiopathy
Specific Investigations	Cholangiography Liver biopsy Exclusion of alternate disease	Anti-mitochondrial antibodies (M2 fraction) Liver biopsy showing active granulomatous duct lesions	Cholangiography Liver biopsy showing periductal sclerosis	Elevated IgG4 levels Pancreatic imaging changes Cholangiopathy Retroperitoneal fibrosis
Typical histological or radiological appearance				
Untreated natural history	End stage liver disease and death	Stable disease in some Progressive portal hypertension and/or chronic liver failure	Cholangitis Portal hypertension Biliary cirrhosis, portal hypertension and liver failure	Relapsing and remitting course Chronic pancreatitis Secondary biliary cirrhosis
Specific medical intervention	Early porto-enterostomy +/- subsequent liver transplantation	UDCA 13-15mg/kg/d	Consider UDCA but evidence lacking	Prednisone (20-40mg/d) largely successful with optimal treatment to be defined

Fig. 9.1 Overview of immune-mediated biliary diseases. Immune-mediated biliary diseases span a large range of processes. Generally clinical, serologic, radiologic, and histologic features are able to accurately diagnose patients

Box 9.1 Summary Criteria for Diagnosing Primary Biliary Cirrhosis, Primary Sclerosing Cholangitis, and Biliary Atresia

PBC: The AASLD [5] considers that a diagnosis of PBC should be suspected in the setting of chronic cholestasis after exclusion of other causes of liver disease. The diagnosis is suspected based on cholestatic serum liver tests and largely confirmed with tests for AMA. A liver biopsy can be used to further substantiate the diagnosis if needed. The diagnosis of PBC can be established when two of the following three criteria are met:

(a) Biochemical evidence of cholestasis based mainly on alkaline phosphatase elevation.
(b) The presence of AMA.
(c) Histologic evidence of nonsuppurative destructive cholangitis and destruction of interlobular bile ducts.

Patients with AMA-negative PBC refer to those who lack AMA but whose clinical presentation, liver histology, and natural history are nearly identical to patients with typical AMA-positive PBC. Nearly all of these patients have antinuclear and/or anti-smooth muscle antibodies. The diagnosis of AMA-negative PBC requires a liver biopsy that demonstrates the typical features of bile duct destruction seen in PBC. The diagnosis is more certain if granulomas are present. Large bile duct cholangiography should be normal in the setting of AMA-negative disease.

PSC: The AASLD [6] considers a diagnosis of PSC is made in patients with a cholestatic biochemical profile, when cholangiography (e.g., magnetic resonance cholangiography, endoscopic retrograde cholangiography, percutaneous transhepatic cholangiography) shows characteristic bile duct changes with multifocal strictures and segmental dilatations, and secondary causes of sclerosing cholangitis have been excluded. Patients who present with clinical, biochemical, and histological features compatible with PSC, but have a normal cholangiogram, are classified as small duct PSC.

AASLD recommendations include:

1. In patients with cholestatic biochemical profile, indirect (magnetic resonance imaging, MRI) or direct cholangiography (ERCP) for making the diagnosis of PSC.
2. Recommend against routine liver biopsy for the diagnosis of PSC in patients with typical cholangiographic findings.
3. In patients with a normal cholangiogram, recommend a liver biopsy to diagnose small duct PSC.
4. In patients with disproportionately elevated aminotransferases, recommend performing a liver biopsy to diagnose or exclude overlap syndrome.
5. In all patients with possible PSC, suggest measuring serum IgG4 levels to exclude IgG4-associated sclerosing cholangitis.

Biliary atresia: Benchimol et al. [9] concluded that total and direct bilirubin levels should be measured in any infant who is still jaundiced at 2–3 weeks of age. Cholestatic jaundice is indicated by direct reacting serum bilirubin levels >17 μmol/L (1.0 mg/dL); direct reacting bilirubin >20 % of the total serum bilirubin concentration, if total bilirubin is >85 μmol/L (5.0 mg/dL). Diagnostic investigations appropriate [10] for the further evaluation of suspected biliary atresia include:

(a) Ultrasound (the "triangular cord sign" at the porta hepatis has been reported in studies to have 73–100 % sensitivity and 98–100 % specificity for biliary atresia).
(b) Hepatobiliary scintigraphy (uptake of the tracker by hepatocytes should be followed by its excretion in bile into the intestine within 24 h; absent excretion has been demonstrated in two studies to have a sensitivity of 83 % and 100 % for biliary atresia, but much lower specificity, 33–80 %).
(c) Liver biopsy (evidence of extrahepatic biliary obstruction; a common diagnostic method).
(d) Intraoperative cholangiogram.

AASLD: American association for the study of liver disease.

in nature, and arises because of environmental and genetic risks interacting to generate loss of tolerance and subsequent chronic immune-mediated biliary damage. Although an organ-specific autoimmune disease, it is tightly associated with the presence of circulating antibodies to mitochondrial antigens (AMA; anti-mitochondrial antibody), and clinically associated with systemic autoimmunity in subjects and their families. A pediatric presentation is essentially not recognized albeit there are reports of AMA reactivity in children, and a handful of reports of teenagers with PBC [11].

PSC is similarly autoimmune in nature, but in contrast to PBC, is a large bile duct fibrosing and sclerosing disorder that has a tight association with IBD, is male predominant, and to date lacks a characteristic serologic reactivity. An associated, albeit rare, autoimmune biliary disease that can mimic the histologic and clinical appearances of PSC is autoimmune pancreatitis/IgG4-associated sclerosing cholangitis.

This cryptic disease is male predominant, usually associated with elevated serum or tissue IgG4 values, and in contrast to classic PSC, the biliary changes if identified early enough are steroid-responsive; this systemic autoimmune syndrome is not associated with IBD in the same way [12].

A large but less well defined group of biliary disorders, in which immune damage contributes to disease, are those classified as *drug-induced liver injury* [13–15]. Whilst an accurate history is usually the most relevant way to diagnose drug injuries, it is increasingly clear that for some groups of drugs that are associated with hepatotoxicity, there is genetic association with the HLA system, highlighting that immune presentation of drug or drug-conjugates is very pertinent to pathobiologic mechanisms.

Immune-mediated biliary injury can also be seen to accompany a variety of rare but specific settings, usually clinically overt, that include liver transplant rejection (e.g., bile duct loss in chronic rejection), graft-versus-host disease of the liver, sarcoidosis, and para-neoplastic syndromes.

Clinical Epidemiology and Risk

Biliary atresia presents either as a biliary atresia-splenic malformation syndrome (10–20 % of cases) associated with congenital malformations such as asplenia, cardiovascular defects, situs inversus, or more commonly as a predominant perinatal/postnatal, nonsyndromic form [10]. Estimates suggest an incidence of in 1 in 5,000 to 1 in 18,000 live births with clinical characteristics including persistent and progressive jaundice developing within weeks of birth, secondary to progressive fibro-obliterative obstruction of extrahepatic and intrahepatic bile ducts. Early diagnosis is essential as hepatoportoenterostomy (the Kasai procedure), if performed ideally within the first 45 days of life, can restore bile flow, and help prevent worsening of liver disease, albeit more than 70 % of children still eventually develop cirrhosis and require transplantation. Epidemiologic risk factors are poorly appreciated but ancestry effects and viral infections are recurrent themes of note, in predisposing or triggering disease.

In contrast the first clinical report of PBC originated in 1851 when Addison and Gull reported an adult woman with diverse clinical symptoms including symptomatic jaundice. With the ability to diagnose disease more widely arising because of easy access to anti-mitochondrial antibody testing, it became evident that PBC was the most common autoimmune liver disease. Estimated PBC incidence rates range from 0.33 to 5.8 per 100,000 inhabitants/year and prevalence rates range from 1.91 to 40.2 per 100,000 inhabitants. Upwards of 95 % of patients are AMA-positive and epidemiologic risk factors for disease include family history of disease, smoking, urinary tract infections, and autoimmune conditions in subjects and their family members. Geographical and temporal trends strongly support environmental toxins/challenges as being relevant to PBC, and this concurs with strong laboratory evidence for the importance of xenobiotics in disease.

PSC incidence and prevalence rates range from 0 to 1.3 per 100,000 inhabitants/year and 0–16.2 per 100,000 inhabitants, respectively. Although seen in both genders and across all ages, more than 60 % of patients are men and the median age at onset is 30–40 years. Patients are most likely nonsmokers and 80 % of Northern European populations have a clear association with IBD, as compared to Southern Europe and Asia where only ~30–50 % have IBD. For this reason PSC is commonly seen as a hepatobiliary manifestation of IBD. Autoimmune pancreatitis is much rarer, and accurate population epidemiology estimates are lacking.

Antibiotics are the most commonly implicated agents associated with DILI, but there remain an enormous number of herbal and dietary supplements, implicated in toxic liver injury [16]. The incidence of DILI due to an individual agent is not well defined but population-based studies suggest that the overall incidence of DILI may be as high as 10–15 cases per 100,000 patient years. Acute hepatocellular injury (~50 %) is more common than mixed or cholestatic liver injury but jaundiced patients have a ~10 % risk of short-term mortality. Registry studies continue to refine the role of genetic, environmental, and immunological factors in DILI.

Diagnostic Strategies

Patients are diagnosed based on a composite of history, physical examination, and investigations (laboratory, radiologic, histologic). The history and physical do provide some clues to diagnosis, but usually it is the presence and absence of specific investigative findings that allows a final confirmatory diagnosis to be reached. In some patients it may be necessary to repeat investigations over time before a clear diagnosis is reached, particularly if they are identified by simple screening liver biochemical changes, at a very early point in their disease course.

Patient history is important for setting the "scene," and calculating pretest probabilities of diagnosis. Whilst demographics, initial pattern of simple liver biochemistry, patterns of symptoms, drug history, and personal/family history of autoimmunity are not diagnostic in their own right, the woman in her 50s, who is a smoker with a family history of celiac disease, is much more likely to have PBC, than the man in his 30s, who doesn't smoke who presents with diarrhea and abnormal liver biochemistry (for which PSC is most likely); Table 9.1. Similarly biliary atresia is such an important and frequent diagnosis in a neonate with persistent conjugated hyperbilirubinemia, pale stools, or dark urine, that

Table 9.1 Common features of primary biliary cirrhosis and primary sclerosing cholangitis

	PBC	PSC
Age at diagnosis	Middle age (>45)	All ages (usually ~40 years)
Gender predominance	Female >Male (9:1)	Male >Female (7:3)
Serology		
ANA	~30–50 % PBC-specific ANA also exists	Nonspecific ANA exist in 70–80 %
ASMA	May be present	Up to 83 %
Anti-SLA/LP	May be present but not characteristic	May be present but not characteristic
pANCA		26–94 %
AMA	~95 % (anti-PDC-E2 highly specific)	Coincidental if present
Specific autoantigen	PDC-E2 (and E2 components of other OADC proteins) specific for AMA	Not identified
Immunoglobulins	IgM elevated in most	IgG elevated in 61 % IgM elevated in 45 %
MRCP	Normal	Diagnostic: multifocal stricturing throughout the hepatobiliary tree
Histology		
Interface hepatitis	Variably present	Variably present
Portal Inflammation	Lymphocytic infiltrate	Lymphocytic infiltrate
Biliary changes	Inflammatory duct lesion	Classically: onion-skin periductal fibrosis
Granulomas	Characteristic (but only present in a few cases)	Atypical (<10 %)
Coexisting IBD	Not characteristic	~80 % (geographically variable)
Response to immunosuppression	No	No

clinicians are duty-bound to exclude it. A conjugated bilirubin that is greater than 20 % of an elevated total serum bilirubin is diagnostic of cholestasis. Clinical features of Alagille syndrome may be overt and infectious and metabolic causes of cholestasis need to be considered and treated. Those patients for whom there is already a diagnosis of sarcoidosis, recent stem cell allogeneic transplantation, or liver transplantation are of course immediately apparent, and if as part of their primary process they present with biliary disease pretest probabilities predict the diagnosis as being related to their primary concern, rather than a new disease process.

Clinically cholestasis is non-specifically associated with hepatomegaly, and similarly other features of end-stage liver disease such as splenomegaly, jaundice, or ascites are not discriminatory. The presence of xanthelasma, skin pigmentation, or excoriations from chronic itch, again whilst mechanistically related to cholestasis, does not allow a specific diagnosis to be reached.

Repeatedly, particularly with drug-induced liver injury, consideration of alternate diagnoses is essential and investigations to exclude nonalcoholic fatty liver disease, viral/infectious hepatitis, inherited metabolic diseases such as alpha-1-antitrypsin, or simple gallstones, remain part of any basic screen. Malignancy can masquerade initially as undiagnosed biliary disease (i.e., idiopathic ductopenia) but usually the progressive nature of an underlying mitotic process becomes apparent, if not clear from imaging studies at the outset.

Serology: AMA was first detected using an immunofluorescence test in 1965, and the subsequent cloning of a major mitochondrial autoantigen was the next true milestone in PBC (and indeed understanding of autoimmunity more generally) [17, 18]. The target is the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2), an enzyme within the mitochondrial respiratory chain. In addition to PDC-E2, other 2-oxoacid dehydrogenases may also be targeted: 2-oxo glutarate dehydrogenase (OADC-E2), branched-chain 2-oxoacid dehydrogenase (BCOADC-E2), the E3 binding protein (E3BP), and PDC-E1α. The immunodominant epitope of PDC-E2 is the lipoylated domain. The lipoic acid residue attached to AMA epitopes is necessary for autoantibody binding. The autoantibodies targeting mitochondrial enzymes are not only of the IgG and IgM but also of the IgA isotype. In addition to the serum, AMA IgA can be found in other body fluids, such as the bile, saliva, and urine.

Anti-mitochondrial antibodies remain the serological hallmark of PBC because they are detected in ~95 % of patients, presumably as loss of tolerance is intimately entwined with disease mechanistically. They can be found in healthy blood donors, in autoimmune hepatitis (AIH), and also in acute liver injury, reiterating the need to test for AMA in the correct context, if its diagnostic utility is to be maximized, and to understand the laboratory modality used for testing (e.g., nonspecific immunofluorescence vs. specific ELISA or immunoblotting).

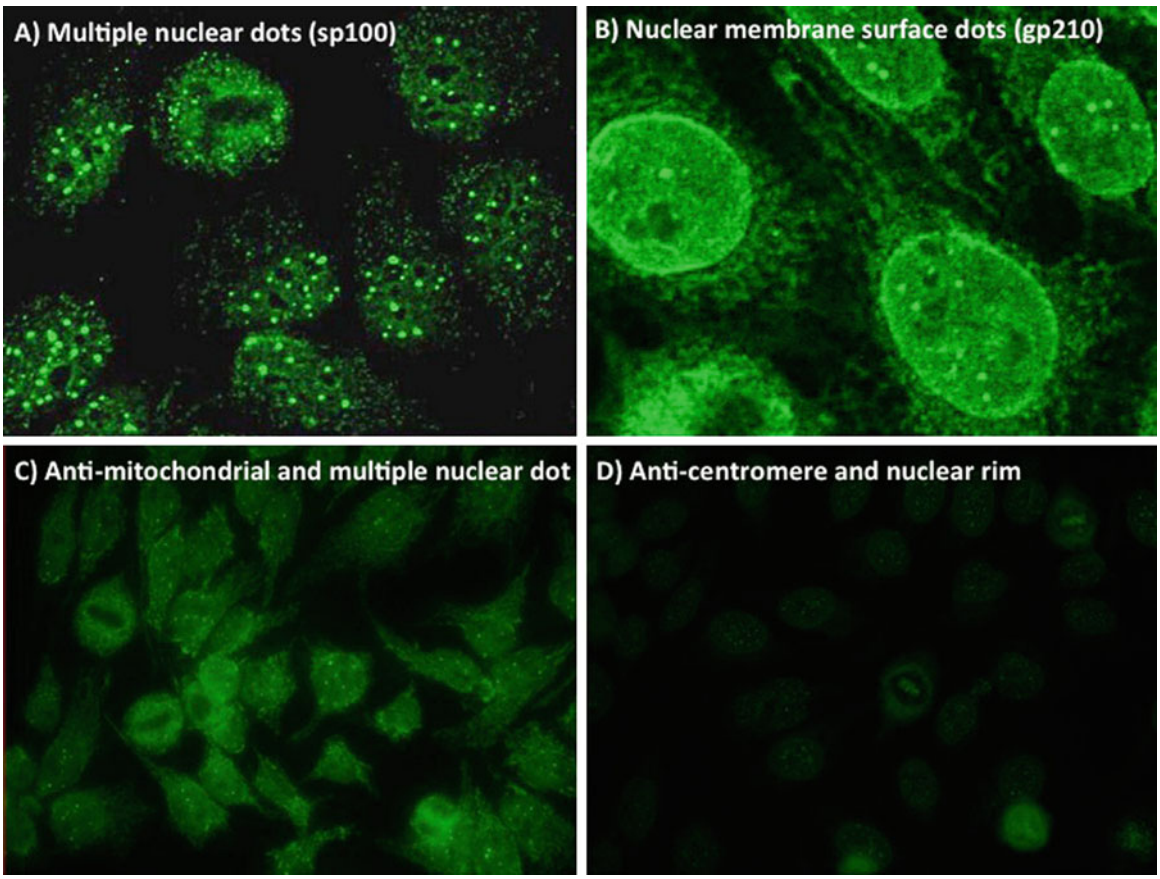


Fig. 9.2 Serologic patterns in autoimmune biliary disease. Serologic reactivity on Hep-G2 cells is shown for (**a**) multiple nuclear dots (sp100); (**b**) nuclear membrane surface dots (gp210); (**c**) anti-mitochondrial and multiple nuclear dot; and (**d**) anti-centromere and nuclear rim. The best approach to using serology for diagnostic purposes is to ensure a good relationship with the immunology testing laboratory and to interact closely with them for best advice on interpreting results

Positive antinuclear antibody-titres are also found in 30–50 % of patients with PBC, but in this setting ANA reactivity is, in contrast to AIH, often antigen-specific (anti-gp210 and anti-sp100) [19]. Patients with AIH histologically may also be AMA-positive (~10 %), generally behaving like typical AIH, albeit with rare instances of transition to PBC. ANA (8–77 %) and ASMA (up to 83 %) reactivity is also variably seen in PSC, although the identifiable autoantigen has not yet been recognized. Atypical, nonspecific antibodies to neutrophil cytoplasmic antigens (ANCA), distinct from those seen in microscopic polyangiitis or Wegener's granulomatosis, are detectable in up to 88 % of patients with PSC, ulcerative colitis (~87 %), and AIH (50–96 %). Although ANCA titres correlate with disease activity in the systemic vasculitides, this is not the case in PSC, and it has no role in making a diagnosis of PSC, or monitoring disease course.

Serologic reactivity should always be determined when investigating cholestasis (particularly in adults) but it is only for those with PBC, for which in the correct context, a diagnosis can be reached, given the high specificity and sensitivity. In the correct context, cholestasis with AMA is associated with a 95 % predictive probability of finding histologic evidence of PBC [20]. Overall, AMA testing is negative in 5 % of patients (the precise figure depending on the intensity of effort made to find AMA reactivity) who otherwise have all the features typical for PBC and an identical autoreactive T cell response to the autoantigen, PDC-E2. The pattern of serum immunoglobulin fractions in PBC is also characterized by an elevation of serum IgM (in contrast to AIH with its elevation in IgG) and this may correlate with an abnormal chronic B cell activation. IgM elevation however lacks specificity and is therefore used as supportive but not diagnostic evidence for disease.

Specific ANA directed against nuclear body or envelope proteins such as anti-Sp100, presenting as multiple [6–12] nuclear dots at indirect immunofluorescence staining and anti-gp210, presenting as perinuclear rims have shown a specificity of >95 % for PBC, although their sensitivity is low (Fig. 9.2). These specific ANA can be reasonably used as diagnostic markers for PBC in the absence of AMA, assuming as ever that the context of testing is appropriate. Anti-sp100 antibodies can be found in AIH, systemic lupus

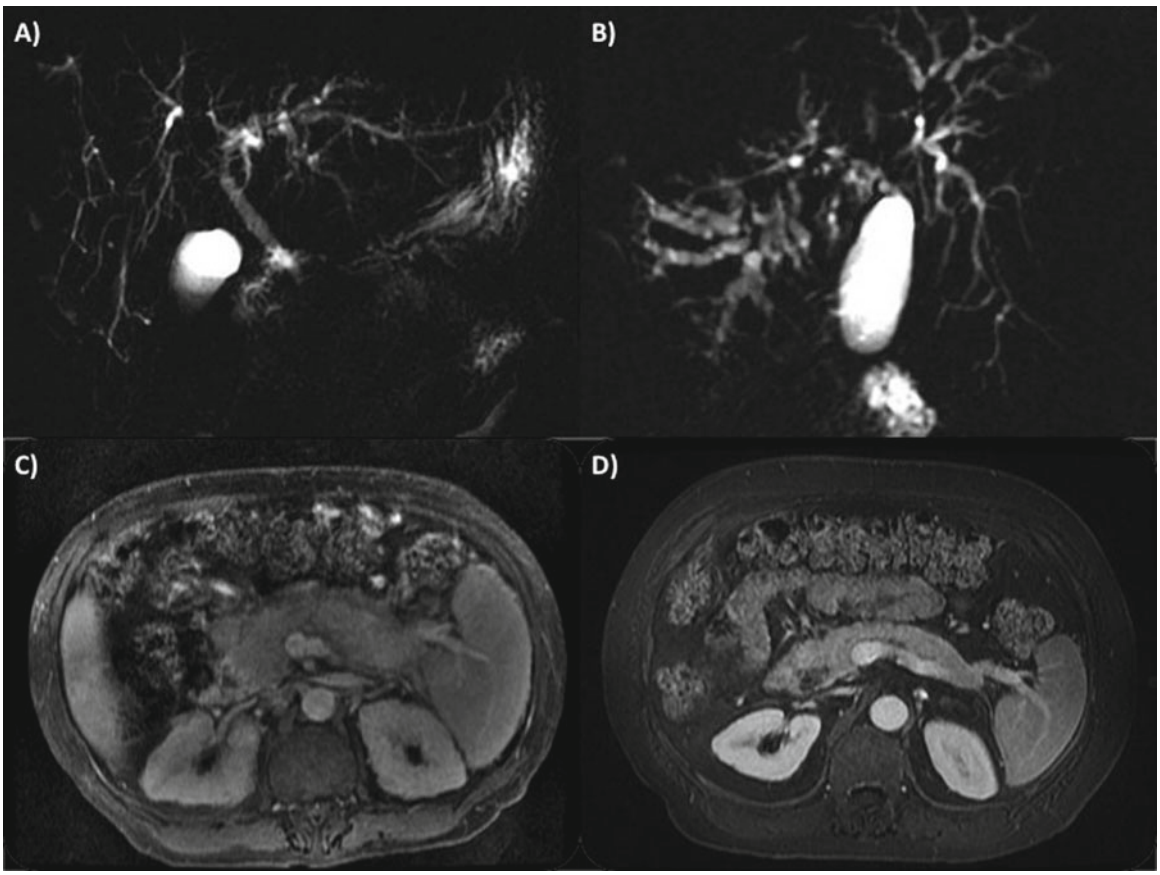


Fig. 9.3 Diagnostic cholangiography in sclerosing cholangitis. Panels (**a**, **b**) provide examples of abnormal cholangiograms as evident by MRI, that support the diagnosis of sclerosing cholangitis. Alternate etiologies need excluding before establishing the diagnosis as primary sclerosing cholangitis (PSC). Panels (**c**, **d**) show pre- and posttreatment findings for a patient who clinically and radiologically has type 1 autoimmune pancreatitis that resolves with steroid therapy

erythematosus, and primary Sjögren syndrome as well. Antibodies directed against centromeric proteins occur in PBC usually with co-occurring scleroderma. Other autoantibodies against nuclear constituents (e.g., dsDNA, scl-70, SSA-SSB, RNP, Jo-1) have also been detected in PBC, mostly in conjunction with coexisting rheumatologic disease. Whilst AMA titre is not clinically correlated with outcome, the titre may fall on treatment. There are however unexplained associations between antinuclear antibody reactivity for portal hypertension (anti-centromere), liver failure (anti-gp210), and HLA association (anti-sp100).

Most "secondary" etiologies for sclerosing cholangitis are identifiable by careful history taking but it is recommended to routinely measure IgG4 when evaluating for PSC. The diagnosis of autoimmune pancreatitis is not straightforward and cut-offs for interpreting in particular IgG4 levels not universally agreed. However, most but not all patients with biliary strictures will also have imaging evidence of autoimmune pancreatitis, and elevation of IgG4 will be marked. Low level elevation of IgG4 is found in over 10 % of patients with PSC in the absence of clear evidence of autoimmune pancreatitis, but may mark a subgroup of patients with a rapidly progressive disease phenotype [21].

Imaging: Ultrasound examination of the liver and biliary tree is obligatory in all cholestatic patients in order to differentiate intrahepatic from extrahepatic etiologies. When the biliary system appears normal and serum AMA is detected, no added radiologic investigation is necessary. Abdominal lymphadenopathy, particularly in the hilar region of the liver, is a frequent observation in any chronic liver disease, but particularly so in PBC and PSC, and shouldn't be mistaken for lymphoma. In drug-induced liver injury, imaging is performed to exclude alternate etiologies.

The diagnosis of PSC is most dependent on imaging and is reached in the presence of fibrotic strictures of the intra- and/or extrahepatic biliary system (Fig. 9.3). However, all chosen imaging modalities suffer with observer variability. Magnetic resonance cholangio-pancreatography is a noninvasive cholangiographic technique used in detection and characterization of bile duct abnormalities; invasive approaches, endoscopically or radiologically, are less frequently now

Table 9.2 Examples of diagnostic criteria for type 1 autoimmune pancreatitis/IgG4 sclerosing cholangitis

Diagnostic factors	Mayo clinic HISORt (2006)	Japan pancreas society (2006)	Korean criteria (2007)
II. Labs/IgG4	IgG4	– GGT, IgG, IgG4 – Autoantibody	– IgG or IgG4 – Autoantibody
III. Histology	– LPSP – IgG4+ cells	– LPSP	– LPSP – IgG4+ cells
IV. Other organ involvement	– Renal, RP, LN, lung, etc. – Response to steroid	– Not included	– Renal, RP, LN, lung, etc. – Response to steroid
V. Steroid response	– Pancreatic lesion – Extrapancreatic lesion	– Not included	– Pancreatic lesion – Extrapancreatic lesion
Definite diagnosis	III I + II Ia + II + V Ia + IV + V	I + II Ia + II I + III Ia + III	I + II, Ia + II I + III, Ia + III I + IV, Ia + IV I + V, Ia + V

LPSP lymphoplasmacytic sclerosing pancreatitis, *RP* retroperitoneal, *LN* lymphadenopathy

used for diagnosis, being more generally reserved for treatment. MRI images are evaluated for increased bile duct visualization, bile duct irregularities, bile duct strictures, and bile duct dilatation. Sclerosing cholangitis is usually defined by the presence of multiple and diffuse intrahepatic and/or extrahepatic bile duct strictures with or without associated biliary dilatation in the absence of apparent cholangiocarcinoma. A normal cholangiogram is one with no apparent stricture, irregularity, and bile duct dilatation. Evaluation of liver MRI abnormalities should include changes in morphologic features (segmental enlargement or atrophy, irregularity of the liver contour), the presence of confluent fibrosis, and existence of portal hypertension (splenomegaly and ascites or portosystemic collateral vessels), to ensure that cholangiopathy secondary to cirrhotic morphology is not mistaken for primary disease.

MRI is effective and safe making it the diagnostic test of choice for PSC, alongside a baseline ultrasound to exclude secondary etiologies, and to evaluate the gallbladder. Meta-analysis found that MRI as the first line investigation for diagnosis of PSC had a sensitivity of 0.86 and specificity of 0.94 [22]. Strictures, dilatations, and pruning of bile ducts are found in both intra- and extrahepatic bile ducts in 75 % patients whereas in 5 % disease is confined to extrahepatic bile ducts. Notably a diagnosis of PSC depends on exclusion of secondary causes of cholangitis, or radiologic mimics of sclerosing cholangitis, including biliary calculi, cholangiocarcinoma, biliary tract surgery, Caroli's disease, chronic biliary infection, biliary toxin exposure, chronic portal vein thrombosis, ischemic stricturing, and alternative liver diseases that can cause biliary injury (e.g., cholestatic drug-induced liver injury).

It is particularly important to consider IgG4-associated autoimmune pancreatitis/sclerosing cholangitis in the differential diagnosis given its high rate of steroid responsiveness. This is reported as more typically occurring in men (male:female ratio of 5:1) with an average age at presentation over 60. Presenting as a multisystem fibro-inflammatory condition there are various distinctive clinical, radiological, serological, and pathological features, which point towards the diagnosis, particularly since no single uniform presentation is predominate (Table 9.2). Frequently the diagnosis is reached in patients with painless obstructive jaundice secondary to an inflammatory pancreatic mass with biliary involvement. Abdominal pain and weight loss may also be present alongside exocrine or endocrine pancreatic insufficiency, whilst others present with extrapancreatic disease: sclerosing cholecystitis, retroperitoneal fibrosis, sclerosing sialadenitis, sclerosing dacryoadenitis, interstitial nephritis, pulmonary interstitial fibrosis, lymphadenopathy, and pseudotumors. Extrapancreatic disease is now recognized in 40–90 % of patients with AIP and can be synchronous or metachronous. The exquisite sensitivity of AIP to steroid therapy is a key feature in differentiating AIP from alternative processes, with clear clinical response to steroids usually striking, but disease relapse not infrequent upon steroid-withdrawal. For this latter reason, radiologists are key to raising the potential for diagnosis, and MRI an optimal modality for evaluating the biliary tree, pancreas, and abdomen more generally.

Neonatally ultrasonography is used to exclude choledochal cysts and evaluate for congenital anomalies associated with BA such as polysplenia or portal vein abnormalities. The triangular cord sign and gall bladder length can be helpful but findings are operator-dependent and therefore lack reliability. Although hepatic scintigraphy showing definite biliary excretion excludes BA, the absence of excretion has poor predictive value because any severe cholestatic syndrome can result in this. Liver biopsy (see below) can correctly predict extrahepatic biliary obstruction in more than

90 % of cases, directing evaluation towards cholangiography (usually operative), which should allow definitive diagnosis of BA: cholangiography will fail to find a patent biliary tree.

Liver Biopsy/Histology

Broadly speaking when cholestasis is persistent, and serologic or radiologic investigations do not give a clear answer, histology needs to be sought (Figs. 9.4 and 9.5). Inevitably liver biopsy is limited by sample size and disease heterogeneity across the whole liver, but nevertheless important disease features can be recognized and staged [23]. It is crucial to have a sufficient size of specimen to minimize error. There should be a minimum of ~10–12 portal tracts visualized before a confident diagnosis of bile duct loss can be established. Correlation with clinical findings is important, and appreciation that early disease can be nonspecific and mild is important, as patients need to be aware of the "risk" that histology does not provide absolute diagnostic certainty.

PBC: Histological staging of PBC (the so-called stages 1–4) is determined by the degree of (peri)portal inflammation, bile duct damage and proliferation, and the presence of fibrosis/cirrhosis. Broadly speaking stage 1 disease is characterized by portal inflammation with granulomatous destruction of the bile ducts, although granulomas are often not seen. Stage 2 is characterized by periportal hepatitis and bile duct proliferation. The presence of fibrous septa or bridging necrosis is defined as stage 3 and cirrhosis as stage 4. Florid duct lesions as defined by focal duct obliteration and granuloma formation are regarded as typical for PBC. The liver is not uniformly involved, and features of all four stages of PBC can be found in one specimen. Biliary pathology can be qualitatively hard to ascertain and interpret, and pathologists without significant experience in this area may find it helpful to review findings with an experienced tertiary center hepatic histopathologist.

PSC: The defining progressive and chronic injury involves small-, medium-, and large-sized bile ducts with an inflammatory and obliterative concentric periductal fibrosis

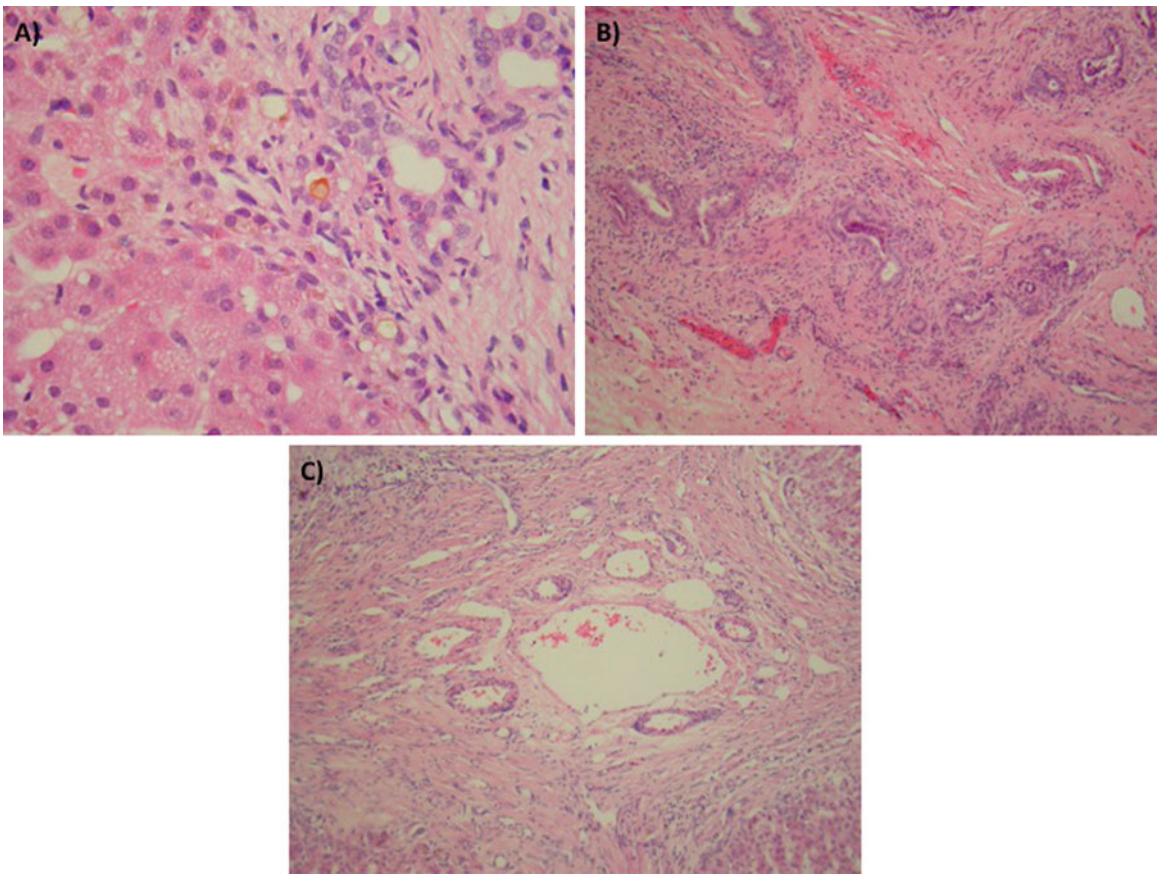


Fig. 9.4 Biliary atresia: histologic presentation. Histologically there are no diagnostic features for biliary atresia but observations include (**a**) the manifestations of large duct obstruction, (**b**) some inflammatory changes, and (**c**) bile duct paucity; vascular structures but no bile duct and no scar are seen

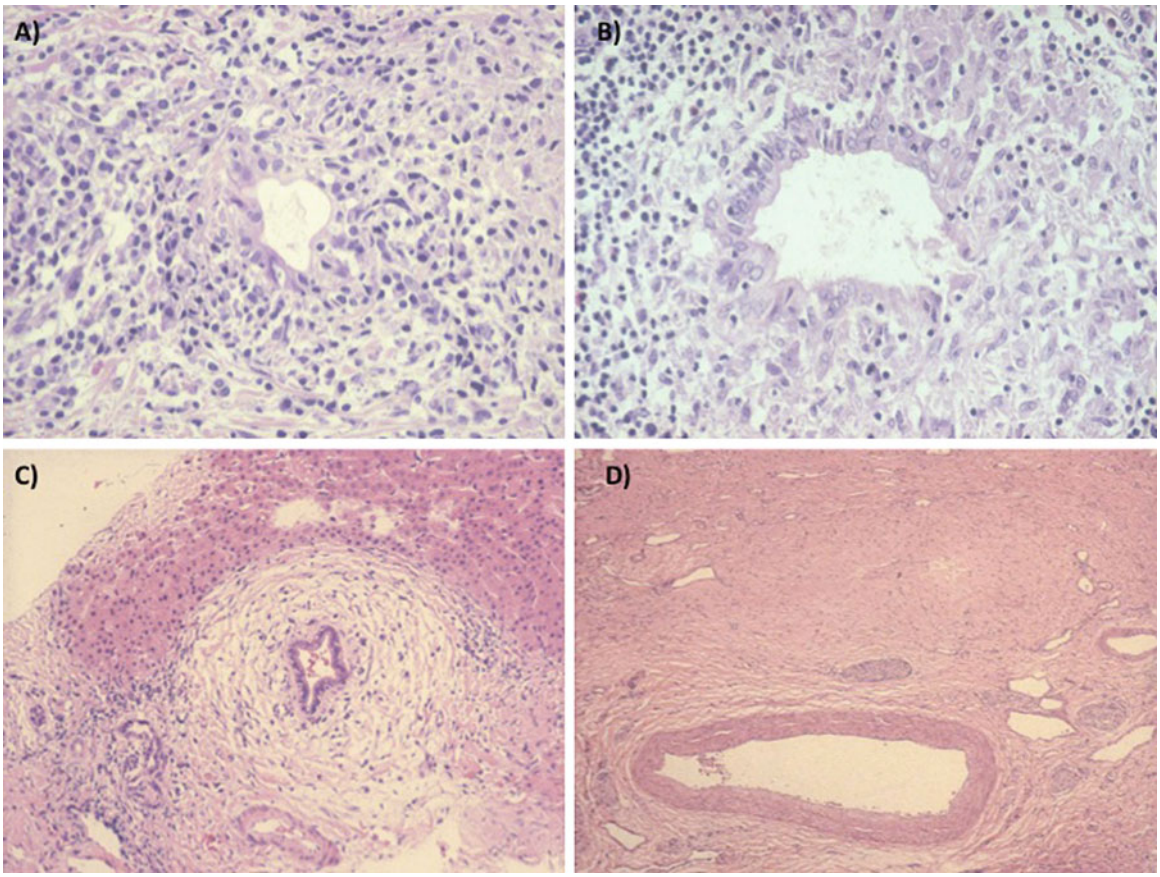


Fig. 9.5 Histologic findings in primary biliary cirrhosis and primary sclerosing cholangitis. Classic features of PBC: (**a**) lymphocytic bile duct lesion, (**b**) granulomatous bile duct lesion and PSC, (**c**) periductal fibrosis (early), and (**d**) fibro-obliterative duct lesion in a septal duct

("onion-skinning") leading to biliary stricturing. In early disease the changes are confined to portal tracts, with a diffuse mixed inflammatory cell infiltrate of lymphocytes, plasma cells, and neutrophils, usually more intense around the bile ducts. Cholangiocytes, which are normally mitotically dormant unless injured, demonstrate increased expression of adhesion molecules, inflammatory and pro-fibrogenic cytokines, and chemokines all of which contribute to a local inflammatory and fibrotic response. The combination of persistent portal inflammation, bile duct destruction, and periportal fibrosis leads to loss of bile ducts, disorganized ductular proliferation, and ultimately cirrhosis. Histologically inflammation and fibrogenesis may not parallel each other, with apparent inflammation not reflecting resulting fibrosis severity.

Biliary atresia: Portal findings in biliary atresia are broadly similar to that seen in large bile duct obstruction of any cause. Typically ductular reaction is prominent with proliferation of small, inter-anastomosing ductules located at the periphery of the portal tracts. Bile plugs are frequently seen within dilated lumens of ductules and are diagnostically helpful. Lymphocytic inflammation is usually present within portal tracts in biliary atresia but is generally mild. Other inflammatory cells, including eosinophils, plasma cells, and macrophages, are also present. Nonobstructive processes, e.g., α1-antitrypsin deficiency and total parenteral nutrition-associated liver disease, may at times show features that closely mimic an obstructive pattern, but are easily identifiable clinically and serologically.

Drug-induced liver injury: This can resemble almost any form of acute or chronic liver disease. In some cases drugs cause a recognizable presentation; e.g., anabolic steroids usually induce a bland cholestasis, whilst estrogenic steroids cause cholestasis with mild hepatocellular injury. Generally however there are no unique histological features that unequivocally confirm the diagnosis. Features favoring a drug reaction include disproportionately severe bilirubinostasis, with only mild inflammation, sharply circumscribed areas of centrilobular necrosis, eosinophils, and granulomas. When ductopenia is seen (chronic), the portal inflammation and fibrosis tend to be less prominent in drug-induced chronic cholestasis.

Overlap Syndromes

The imprecision of immune-mediated liver injury means that "overlap features," be they biochemical, serologic, histologic, or radiologic, are frequently observed across the classic autoimmune liver diseases. The term "overlap syndrome" is applied to describe poorly defined instances where either concurrently or consecutively there exists a coexistence of AIH, as well as clear features of either PBC or PSC [24, 25]. The challenge remains that no autoimmune liver disease has an absolute diagnostic test, all being diagnosed based on the presence and relative absence of various markers of biochemical, serological, radiological, and histological disease, with some clearly being less categorical and objective than others. This appraisal must be performed longitudinally rather than at a single point in time. Overlap syndromes therefore likely represent rather than distinct processes, the inherent distribution of clinical features across patient populations; the more extreme the distribution, the more distinct the apparent overlap. The prevalence of overlap features is hard to ascertain because of publication bias, challenges in definitions (serological overlap is arguably not of the same significance as histological or radiological overlap), and limitations in test interpretation (e.g., there are insufficiently reproducible ways to grade interface hepatitis, and interface hepatitis itself is likely a common mechanism of liver injury across diseases). Overlap designations therefore tend to be arbitrary and imprecise, and the clinical phenotypes of patients with the same overlap designation exhibit considerable heterogeneity. Presentations that raise the spectre of overlap therefore span (a) an immunoserological overlap: e.g., positive ANA/ASMA-titres and elevated IgG in conjunction with AMA-positive PBC; or AMA positivity in AIH; (b) a biochemical overlap: AST/ALT >5×ULN in patients with PBC or PSC; or ALP >3×ULN in patients with AIH (or γGT >5×ULN in children); (c) a radiological overlap: clinical features of AIH with cholangiographic abnormalities indicative of an inflammatory cholangiopathy; (d) a histological overlap: lymphoplasmacytic infiltrate and interface hepatitis on liver biopsy with bile duct lesions indicative of either PBC or PSC; (e) varying combinations of the above including temporally, i.e., consecutive vs. sequential presentations.

With no codified diagnostic approach, reported prevalence figures are variable, with some clinicians identifying an overlap as infrequently as 5 %, whilst others see patients with overlap syndromes as often as 20 % of the time. Younger patients with AIH tend to have a higher chance of having overlapping biliary features, and clinically this should always be borne in mind; reports suggest, for example, that upwards of 50 % of children with AIH have cholangiopathy, and the so-called "autoimmune sclerosing cholangitis."

Overlap syndromes should be diagnosed conservatively and robustly, and clinical investigations must be interpreted cautiously, with a good quality liver biopsy or cholangiogram presenting the strongest means to diagnose overlap. Clinically overlap should be considered in the differential when a patient deviates from the normal clinical course and expected response to therapy, but it is not necessary to overdiagnose or over-treat.

Conclusion

Immune-mediated biliary disease represents an important and ongoing clinical concern with disease spanning a number of broad disease processes. Current classification remains relatively crude and does not address the significant clinical heterogeneity encountered amongst patients. Future efforts are likely to apply "omics" platforms to better classify patients using biosignatures derived from serum, DNA, or urine, the goal being to define patients more closely to their own disease course. In this way it will ultimately prove possible to tailor therapy more appropriately, and to more clearly stratify risk of adverse outcome for patients individually.

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Bacterial Infections in Liver

10

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
APC	Antigen presenting cell
CDC	Center for Diseases Control and Prevention
DC	Dendritic cell
GGT	Gamma-glutamyl transferase
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HSC	Hepatic stellate cell
IFN	Interferon
IL	Interleukin
InlA	Internalin A/B
KC	Kupffer cell
LFT	Liver function test
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
mDC	Myeloid DC
MHC	Major histocompatibility complex
NK	Natural killer
NKT	Natural killer T cell
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PD	Programmed death
pDC	Plasmacytoid DC
PD-L1	Programmed death ligand 1
PPR	Pattern recognition patterns
PSC	Primary sclerosing cholangitis
Th	T-helper
TLR	Toll-like receptor

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Key Points

- Bacterial infections may affect the liver via direct infection, or as sequelae from extrahepatic infections.
- Immunocompromised patients, and especially those with HIV-AIDS, are by far the most at-risk group.
- Organisms commonly cultured in jaundice of sepsis include *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, *Salmonella*, *Bacterioides*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.
- Direct hepatic bacterial infections include *Mycobacteria*, *Listeria monocytogenes*, *Brucella* species, *Legionella pneumophilia*, *Burkholderia psuedomallei*, *Francisella tularensis*, *Treponema pallidum*, and *Neiserria gonorrhoae*.
- *Mycobacteria* implicated in liver disease include *Mycobacterium tuberculosis*, *bovis*, *kansasii*, *gordonae*, *leprae*, and *avium-intracellulare.*

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- Histopathological analysis of granulomas in liver biopsies may narrow down a causative infectious agent.
- The mechanisms enabling invasion, replication, and survival within the liver as well as the factors determining the efficiency of innate and adaptive immune responses are poorly understood.

Introduction

Bacterial infections may cause liver dysfunction through direct infection or as a result of inflammatory mediators from bacterial infections in other body sites. This is not surprising given the extent of the hepatic vascular supply, as well as significant venous drainage from the gastrointestinal system. Abnormal liver functions tests (LFTs) may occur in a variety of septic conditions not directly involving the liver, such as in community-acquired pneumonia. Neonates and infants under 1 year are especially susceptible to liver dysfunction in septic states, due to low bile salt-independent bile flow. Signs and symptoms include jaundice with fever, rigors, and confusion. Abnormalities in LFTs often appear 24–48 h after the onset of initial symptoms, and include mild elevations in transaminases and alkaline phosphatase, with significant hyperbilirubinemia. Canalicular cholestasis, focal hepatocyte fat droplets, and periportal cell infiltrates are commonly encountered histological findings. Sinusoidal leukostasis and adherence to hepatic endothelial cells result from release of TNF-alpha, IL-1, IL-8, and activation of C5a.

Differential diagnosis in these cases includes but is not limited to cholestasis of sepsis/pneumonia/bacteremia, acute respiratory distress syndrome, drug hepatotoxicity, biliary obstruction, ischemic hepatopathy, hepatosplenic candidiasis, de novo liver disease, and chronic liver disease. Investigations should include an abdominal ultrasound, cultures of blood, sputum and urine, in addition to stool samples testing if indicated. Early therapy with broad-spectrum antibiotics should be initiated while awaiting culture results. Organisms commonly cultured in jaundice of sepsis include *Escherichia coli*, *Klebsiella*, *Pseudomonas aeruginosa*, *Salmonella*, *Bacterioides*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.

When direct hepatic bacterial infection occurs, the organisms commonly isolated include *Mycobacteria*, *Listeria monocytogenes*, *Brucella melitensis/abortus/suis*, *Legionella pneumophilia*, *Burkholderia psuedomallei*, *Francisella tularensis*, *Treponema pallidum*, and *Neiserria gonorrhoae*. Liver damage may result from direct cytotoxicity of infected parenchymal cells and/or Kupffer cells or indirectly by bystander damage mainly due to infectious-induced cytokines. In several cases, liver damage is caused by both direct cytotoxicity and cytokine-induced hepatocyte destruction.

This chapter focuses on the immunology of these bacteria and its relevance to immune-mediated destruction of the liver.

Several liver histopathologists consider bacterial infections of the liver based on their ability to cause granulomatous disease [1]. Histopathological examination assesses the morphological characteristics of the granulomas and their location, the presence or absence of organisms or foreign material within the granulomas, the phenotype of the cellular infiltrates, and the histopathological features of the liver biopsy specimen [2].

The morphology of some granulomas is diagnostically helpful and can provide clues for the infectious cause of the disease (Table 10.1) [3]. Several hepatic granulomas are due to noninfectious disorders (Table 10.2). Histopathological features of hepatic granulomas due to infectious and noninfectious causes are illustrated in Fig. 10.1. Several classifications have been made to address the types of granulomatous lesions of the liver [3].

- Epithelioid granulomas and in particular those showing necrosis (necrotizing) frequently relate to infectious agents.
- Fibrin ring granuloma is a characteristic form of hepatic granuloma consisting of an epithelioid granuloma with a central lipid vacuole surrounded by a fibrin ring. These granulomas were typically associated with Q fever, caused by *Coxiella burnetti*, but can also be seen in infectious and noninfectious diseases such as leishmaniasis, *Mycobacterium avium-intracellulare* (MAI) infection, typhoid fever, Boutonneuse fever, toxoplasmosis, cytomegalovirus infection, mononucleosis, Hodgkin disease, and drug-induced reaction (allopurinol).
- Foamy macrophage aggregates can be seen in infectious diseases and immunosuppressed patients, and their characteristic feature is the lack of significant inflammatory infiltrates.
- Lipogranulomas contain lipid and are associated with mineral oils in foods.
- Microgranulomas are often mixed with inflammatory cell subpopulations and apoptotic hepatocytes, and do not correspond to a specific etiologic factor.
- Granulomatous inflammation indicates poorly formed granulomas, frequently admixed with inflammatory cells. Suppurative inflammation may be the predominant feature, and this can be caused by certain infectious agents.
- Stellate abscesses with associated granulomatous inflammation. This pattern is also usually associated with infectious etiologies (such as infection with *Bartonella henselae*).

Based on the ability to identify the infectious cause of granuloma formation, three sub-categories are noted: (a) those

Table 10.1 Major infectious causes of hepatic granulomas

Type of granuloma cause	Epithelioid granuloma	Fibrin granulomas	Stellate microabscesses with granulomatous inflammation	Microgranulomas	Lipo-granuloma	Foamy macrophage aggregates	Predominantly suppurative ± granulomatous inflammation
Mycobacterium tuberculosis	Yes (usually caseating)						
Tuberculoid leprosy	Yes						
Lepromatous leprosy						Yes	
Mycobacterium avium-intracellulare	Yes (immune-competent)					Yes (immunosuppressed)	
Brucellosis	Yes						
Tertiary syphilis (*Treponema pallidum*)	Yes						
Chlamydia	Yes						
Schistosomiasis	Yes						
Q fever (*Coxiella burnetii*)		Yes					
Toxoplasmosis		Yes					
Salmonella		Yes					
Leishmaniasis		Yes				Yes	
Listeriosis				Yes (rarely)			Yes
Actinomycosis			Yes				
Nocardia			Yes				
Bartonella			Yes				
Tularemia			Yes				Yes
Candida/other fungi			Yes				
Histoplasmosis						Yes	
Melioidosis (*Burkholderia pseudomallei*)							Yes
Cytomegalovirus		Yes					
Epstein-Barr virus		Yes					

Table 10.2 Major noninfectious causes of hepatic granulomas

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

due to well recognized organisms; (b) those due to organisms which are detected by highly sensitive molecular techniques but not by conventional microbiological techniques; and (c) those due to pathogens which have not been identified, but which are suspected [4].

The prevalence of hepatic granulomas in liver biopsy specimen varies amongst studies, and largely depends on the cohorts under investigation and the study design (Table 10.3) [5].

The prevailing notion for the mechanisms leading to granuloma formation is that granulomas develop when humoral and cellular immunity does not succeed to eradicate the offending stimuli (infectious or noninfectious). These immune responses are mainly of the delayed hypersensitivity type, and their main tasks are first to isolate, and second to deactivate/neutralize the persistent effect of the stimulus. The granulomatous reactivity targets immunologically inert constituents such as the large foreign bodies, or reaction to immunologically active antigenic compounds. Granulomas are largely caused by pathogens that require a macrophage-based machinery for infectious clearance. Specific bacterial infections such as mycobacterial infections or infections due to brucella, bartonella, and rickettsia are mainly associated with granulomatous inflammation, while others such as listeria and tularemia induce or relate with a combination of suppurative and granulomatous inflammation.

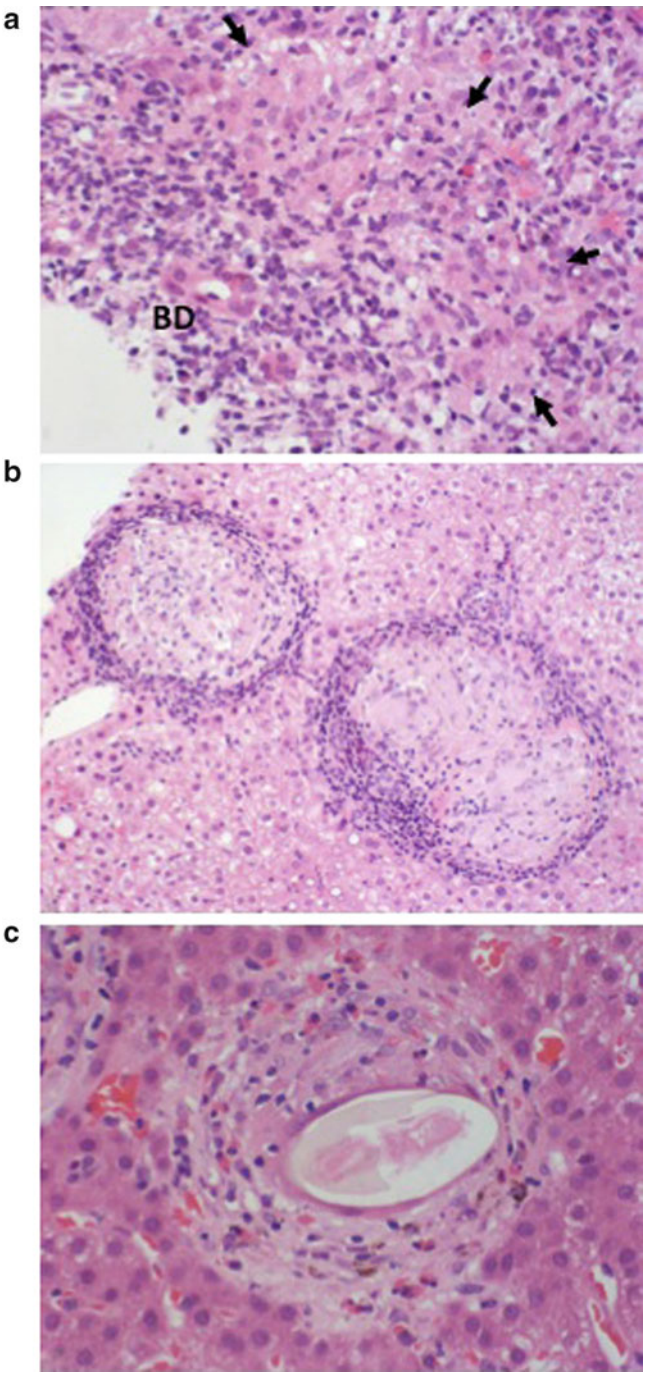

Fig. 10.1 Histological features of epithelioid granuloma due to primary biliary cirrhosis (**a**), sarcoidosis (**b**), and schistosomiasis (kindly provided by Dr. Yoh Zen, King's College Hospital, London)

Mycobacteria

Background and epidemiology: *Mycobacteria* implicated in liver disease include *Mycobacterium tuberculosis*, *bovis*, *kansasii*, *gordonae*, *leprae*, and *avium-intracellulare* [1, 6–8]. An increased incidence of infection with these organisms has

Table 10.3 Prevalence of granulomas in patients with liver diseases varies amongst studies

Study	Origin	Total number of liver biopsies	Total number of liver biopsies with granulomas *n* (%)
Drebber et al. [92]	Germany	12,161	442 (3.6)
McCluggage and Sloan [93]	Northern Ireland	4,075	163 (4)
Dourakis et al. [94]	Greece	1,768	66 (3.7)
Gaya et al. [95]	UK	1,662	63 (3.8)
Satti et al. [96]	Saudi Arabia	404	59 (14.6)

A summary of data from representative studies investigating 404–12,161 liver biopsies is given

been correlated with an increased incidence of HIV-AIDS, especially in regard to *Mycobacterium avium-intracellulare* [9]. At-risk groups include HIV positive individuals, IV drug users, alcohol abusers, patients with diabetes mellitus, and those on immunosuppressive therapy. Approximately 10 % of cases are as a result of miliary tuberculosis, with primary Mycobacterial liver infection being extremely rare. Approximately 50–80 % of patients with terminal pulmonary *Mycobacterium tuberculosis* have been noted to have hepatic involvement, with up to 91 % having hepatic involvement at autopsy [10]. Pathological features include granuloma formation, microvesicular steatosis, reactive hepatitis, and peliosis hepatitis.

The most common scenarios of hepatic mycobacterial involvement include miliary tuberculosis, primary hepatic infection (although rare as noted above), or a nodular tuberloma/abscess with no other organ involvement. Malignancy may be initially suspected in the nodular form, due to its radiological appearance. Patients with hepatic mycobacterial infection may present with hepatomegaly, fever, and pyrexia, with mild increases in ALP and GGT, hypoproteinemia, and hyperglobulinemia. Aminotransferases are usually normal. Encephalopathy and hypoglycemia may occur in rare instances. Weight loss and evidence of involvement in other organ systems (lungs, lymph nodes, genitourinary tract, and gastrointestinal tract) are also often present. Investigations in these patients should aim to rule out viral hepatitis, as well as pharmacological drug toxicity such as highly active antiretroviral therapy in HIV infected patients.

Diagnosis: Histological staining of acid-fast bacilli is the mainstay of diagnosis, with tissue being obtained via ultrasound or guided CT, as well as laparoscopic biopsy. Nucleic acid amplification tests for *Mycobacterium tuberculosis* now show 80 % positive predictive value and 90 % negative predictive rate.

Treatment: Treatment of confirmed cases should include rifampicin, isoniazid, pyrazinamide, and ethambutol for 8 weeks, followed by isoniazid and rifampicin for another 16 weeks or more [11]. Monitoring for related drug toxicity should also be preformed. *Mycobacterium avium-intracellulare* is treated with erythromycin or clarithromycin, with ethambutol. Other agents which may be added include clofazimine, rifabutin, rifampin, siprofloxacin, and amikacin. Patients with advanced HIV infection or AIDS should be prophylactically treated with clarithromycin or azithromycin.

The pathophysiological features of tuberculosis have been extensively studied and reviewed elsewhere [11, 12]. The mechanisms responsible for the formation of granulomas due to mycobacterial infections are extensively studied [13]. Due to space constraints, this chapter will not discuss data related to the pathogenicity of these infections and their relevance to the liver, but will rather discuss the immunobiological features of other infectious agents that are not usually presented comprehensively in the form of a chapter in other books.

Listeria monocytogenes

Background and epidemiology: *Listeria monocytogenes*, an ubiquitous saprophytic Gram-positive bacterium, is the causative agent of human listeriosis. The pathogen was first described by E.G.D. Murray in 1926, who reported the sudden death of six young rabbits characterized by a marked increase in the number of peripheral monocytes. In 1927, Harvey Pirie isolated the same organism from the liver of gerbils, and has renamed the bacterium from *Bacterium monocytogenes* to *Listerella hepatolytica* in tribute to Lister. From 1940 and onwards the name *Listeria monocytogenes* was finally adopted. In the early 1960s, the pioneer work of G.B. Mackaness [14] and subsequent studies thereafter demonstrated the important role of innate and adaptive immunity required for the clearance of *Listeria monocytogenes* [15]. The nonself-/self-immune interaction involving *Listeria monocytogenes* is one of the best studied so far, and has been used to understand basic aspects of how the immune system works.

The source for infection is almost exclusively ingestion of *Listeria monocytogenes*-contaminated dairy and meat products, such as soft cheeses, raw and cooked meat, frankfurters, pâtés, raw and smoked fish, milk, coleslaw, and vegetables.

Disease features: The clinical manifestations of listeriosis vary in severity, and can range from self-limited acute, febrile gastroenteritis in healthy persons to severe septicemia and fatal meningoencephalitis in immunocompromised individuals [16–18]. Placental infection in pregnant women may be the cause of abortion, stillbirth, or meningoencephalitis. The incidence of listeriosis has been declining over the years

(around three cases per million population in USA and Northern Europe), but recent outbreaks have been noted in North America. Also, increasing rates of listeriosis have been reported in several European countries. This has raised significant concerns globally, also in view of its fatal outcome in 30 % of the affected cases [17, 19, 20]. Patients with liver involvement (hepatic listeriosis) are usually under immunosuppressive treatment, and have hematological malignancies, underlying cirrhosis or diabetes. Histopathologic assessment reveals scattered microabscesses with or without small granulomas of the microgranulomatous type [21, 22]. Laboratory features often demonstrate raised transaminases, leukocytosis with neutrophilia, and low glucose levels.

Diagnosis: The diagnosis of central nervous listeriosis is based on the isolation of the bacteria in the cerebrospinal fluid. *Listeria monocytogenes* culturing in the CSF requires usually 24–72 h. The specificity of the assay is very good but the sensitivity is extremely low due to the low number of bacteria within the CSF, especially in the case of previous inadequate treatment by antibiotics. Serologic testing based on antibody detection against listeriolysin O is also used. Nucleic acid amplification testing by real-time PCR assay can assist the molecular detection of the pathogen. Blood culture is the most important test for the diagnosis of hepatic listeriosis.

Treatment: Patients with *Listeria* infections who do not spontaneously clear the infection or those at increased risk, such as pregnant women, usually require intravenous antibiotic treatment to prevent, control, or slowdown more severe disease. An early administration of antibiotics of pregnant females can be lifesaving for the fetus. Meningitis requires 3 weeks treatment, and brain abscesses are treated for 6 weeks. The first line of treatment includes ampicillin, although trimethoprim-sulfamethoxazole is also used successfully.

Immunobiology: The survival of *Listeria monocytogenes* within macrophages enables invasion of nonphagocytic cells and the replication of the pathogen [15, 23]. Internalization of the bacterium involves attachment of *Listeria monocytogenes* to host cells, and interaction of the bacterial surface molecule internalin A (InlA) and InlB with their cellular receptors, the adhesion molecule E-cadherin and the hepatocyte growth factor receptor Met, respectively [24]. The decisive role of InlA and/or InlB locus for the entry of *Listeria monocytogenes* into hepatocytes has been known for many years [25]. Also, InlB expression and the production of InlB advance the escape from phagocytic vacuole and the replication of the pathogen within the cytoplasm of mouse hepatocytes.

The interaction between InlA/E-cadherin and InlB/Met promotes the recruitment of endocytic effectors, and initiates a series of events including escape of internalized bacteria trapped in the phagocytic vacuole through expression of listeriolysin O-mediated lysis of the phagosomal membrane, locomotion in the cytosol of the invaded cell via actA-dependent actin assembly and remodeling [26], bacterial engulfment and subsequent protrusion into neighboring cells, and non-lytic spreading [23]. This immune evasion strategy allows the pathogen to multiply by avoiding neutralization from microbial-specific antibodies.

Listeria monocytogenes crosses the intestinal epithelium and spreads though the lymphatic system and bloodstream to the liver, spleen, and other organs [27]. The former two are major sites of *Listeria* replication during systemic infection.

Multiplication of the pathogen within hepatocytes results in abscess and granuloma formation (see below). In recent years, comparative genomic and molecular investigations focusing on the pathogenic *Listeria monocytogenes* and the nonpathogenic *Listeria innocua* have delineated several bacterial factors essential for infectivity. These include the bile salt hydrolase encoded by *Listeria monocytogenes*, a virulence factor which participates in the intestinal and hepatic phases of listeriosis. This bile salt hydrolase counteracts the antibacterial effects of bile acids within the intestine and in hepatocytes [28].

Autophagy is a multifaceted machinery used in the recycling of cellular components through lysosomal-mediated degradation, but also describes a mechanism of defense against external pathogens and a regulator of host immune responses to microbial and autoantigenic targets. The role of autophagy in *Listeria monocytogenes* infection remains elusive [29]. Data provided so far suggests that *Listeria monocytogenes* replicates better in autophagy-deficient cells, and mutants lacking ActA are efficient targets of the autophagy machinery, a finding which underlines the important role played by this molecule. Conflicting data suggesting that *Listeria monocytogenes* induces autophagy have also been obtained. However, the master regulator of antimicrobial activity appears to be listeriolysin O, through various mechanisms including the induction of autophagy depending on the cell type, the modulation of specific cellular signaling pathways, and gene expression.

Innate immunity is very import for the eradication of *Listeria monocytogenes*. Various cell subsets including macrophages, neutrophils, natural killer, dendritic, and mast cells participate in the first line of defense against this pathogen. Neutrophils attracted by chemokines secreted from infected hepatocytes migrate towards the site of inflammation, and their role is more important in defending against *Listeria monocytogenes* infection of hepatocytes rather than the spleen. Mice deficient in neutrophils display increased susceptibility to *Listeria monocytogenes*. Also, Kupffer cells infected with *Listeria monocytogenes* secrete tumor necrosis factor-α (TNFα) and IL-12 which triggers NK activation and

IFNγ secretion. Subsequent production of ROS and NOS from activated Kupffer cells and neutrophils kills *Listeria monocytogenes*. Furthermore, infection with this pathogen promotes the recruitment of a DC subset in the spleen, the so-called TNF/iNOS-producing (Tip)-DC that is lacking in CCR2-deficient mice [30]. The differentiation of IFNγ-producing Th1 CD4+ T cells is promoted by the secretion of IL-12. Conventional DCs can also prime CD8+ T cells to proliferate and differentiate into cytotoxic CD8+ T cells. Subsequent infections with *Listeria monocytogenes* are controlled by CD8+ T-cell proliferation, which is mediated by CD4+ regulatory T cells. Mice inoculated with *Listeria monocytogenes* by intraperitoneal injections demonstrate IL-17A production by γδ T cells. IL-17A is a chemoattractant of neutrophils within liver, which are important for bacterial clearance but are also essential for the formation of small, hepatic granulomata. Studies of *Listeria monocytogenes infected* IL-23p19 knockout mice demonstrated that IL-23 regulates the production of IL-17A and IL-17F from γδ T cells, but not from NK CD4+, or CD8+ T cells. This results in optimal liver neutrophil recruitment and enhanced clearance of *Listeria*. The decisive role of type I IFN in the control of the infection is underlined by the fact that the knockout mice for type I IFN receptor are more resistant to *Listeria monocytogenes* infection.

Bacteria are found preferentially within the cytosol of macrophages and hepatocytes, as demonstrated by in vivo studies [31]. A multi-specific antigen-specific CD8+ T cell response is required for the clearance of the bacterium [15], and data obtained so far have demonstrated that listeriolysin O-derived MHC I-restricted peptides are processed, followed by presentation on MHC class I. It also appears that cross-priming-deficient mice cannot facilitate the generation of antigen-specific CD8+ T cells to stimulate MHC I-restricted CTL responses following infection with *Listeria*, suggesting that dendritic cell cross-priming may play an important role in generating *Listeria*-specific CD8 T-cell responses.

Brucella melitensis, abortus, suis

Background and epidemiology: *Brucella* spp. belong to the $\alpha 2$ subdivision of the proteobacteria (as are bartonella and rickettsia) and are small, Gram-negative, nonmotile, nonspore-forming coccobacilli that cause brucellosis [32]. Humans become infected through occupational exposure and by ingesting contaminated food. Pathogens resembling *Brucella*e have been detected in carbonized cheese as far back as the Roman era. Since it's identification in the nineteenth century, disease caused by these bacteria has been given several names, including rock fever, undulant fever, Malta fever, Crimean fever, Gibraltar fever, Mediterranean fever, and Bang's disease. The pathogen was named *Brucella* to honour David Bruce, who in 1887 was the first to recognize the bacterium as the causative agent of the disease. In 1897, Bernhand Bang was able to isolate *Brucella abortus*, which is known to induce abortion in cattle, and of brucellosis in human beings, while in 1905, Sir Themistocles Zammit, a Maltese doctor and archaeologist, identified that a major source of the pathogen was unpasteurized milk. With half a million new cases per year, Brucellosis is the most common human zoonosis. However, Brucellosis remains underdiagnosed, likely due to it's complex serodiagnosis, slow growth in blood culture, as well as a nonspecific symptomatology. Multiple pathogens have been identified over the years, with *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella bovis*, *Brucella canis*, and *Brucella neotomae* having been identified in the past, while two new species (*Brucella cataceae* and *Brucella pinnipediae*) have been added recently. *Brucella melitensis* arises from cattle and goats in the Mediterranean basin, whereas *abortus* and *suis* arise from cattle and pig in North America. All represent a significant public health concern [33].

Disease features: Brucellosis is rarely fatal, but its complications can lead to significant debilitation. These include peripheral arthritis, spondylitis, sacrolitis, epididymoorchitis, and even central nervous system disorders (neurobrucellosis). Infection with these bacteria has been traditionally divided into acute, subacute, and chronic phases. This classification is widely used but is rather objective and is of limited clinical use. Symptoms in the acute phase predominantly include fever and rigors, constitutional symptoms such as malaise and arthralgias, as well as hepatosplenomegaly and to a lesser extent lymphadenopathy. The chronic phase is also characterized by recurrent pyrexia over a 2-week period, fatigue, malaise, and hepatosplenomegaly. Approximately 30 % of patients will have raised aminotransferases and cholestatic enzymes. Microscopically, the disease is characterized by granulomas, the extent of which is dependent on the host's immune response at early stages of the infection. Granulomas can be small and poorly formed or discrete and epithelioid. Giant cells may also be present. Portal tract infiltration and fibrosis is also commonly seen.

Diagnosis: Clinical history greatly assists in making the diagnosis. The pathogens are difficult to culture, and are rarely seen on special stains. The serum agglutination test—developed by David Bruce—remains the most widely used test for the diagnosis of brucellosis. ELISA testing is also used. Positive cultures are obtained in 15–70 % of the cases. Cultures of bone marrow material are easier due to the high concentration of the pathogen in the reticuloendothelial system, and this is considered the gold standard for the diagnosis of brucellosis. The elimination of the infectious agent from the bone marrow is also a reliable indication of the eradication of the pathogen. Molecular detection of the

pathogen by PCR is also assisting diagnosis, especially in those clinically suspected cases, who are negative by other diagnostic testing

Treatment: The rationale behind effective treatment in patients with brucellosis is the administration of antibiotics that can enter macrophages and can exert their action in the acidic intracellular environment. The choice of treatment and its duration largely depends on the clinical phenotypes of the disease [34]. The guidelines followed by most physicians worldwide include doxycycline for a period of 6 weeks, combined with streptomycin for 2–3 weeks or rifampin for 6 weeks. Other combinations using aminoglycosides such as gentamicin and netilmicin or treatment based on trimethoprim-sulfamethoxazole are also used. Neurobrucellosis is adequately treated with standard triple regimen combination, while rifampin is the treatment of choice in pregnant women with brucellosis.

Immunobiology: *Brucella* can be parasitic within human antigen presenting cells, manipulating the antibacterial defense machinery of the immune system (i.e., phagocytosis, phagolysosome fusion, antigen presentation, cytokine secretion, and apoptosis). *Brucella*'s subversion of innate immunity leads to malfunctioned CD4+ T cell responses and T-cell anergy in chronically infected patients. Anti-*Brucella* spp.-specific immune responses involve both arms of innate and adaptive immunity, including CD4+ and CD8+ T cells, as well as activated macrophages and pro-inflammatory IFNγ and TNFα cytokine production [35]. Brucella/host interactions are illustrated in Fig. 10.2.

Brucella resists elimination through the inhibition of programmed cell death of the infected cells, and does not bear classic virulence factors. The noncanonical lipopolysaccharide (LPS) structure of *Brucella* reduces its agonist activity for toll-like receptor (TLR)4 and leads to insufficient production of bactericidal nitrogen and oxygen intermediates, as well as pro-inflammatory cytokines with antibacterial potential [36]. Compared to other Gram-negative bacteria, the lipid A moiety of Brucella's LPS is structured in such a way that elicits a diminished antibacterial response [37]. A charge reduction in *Brucella* LPS explains its resistance to bactericidal peptides. Also, *Brucella*'s LPS O-antigen regulates the attachment of the pathogen to cell surface receptors in such a way that the pathogen persists because of minimal macrophage activity [38]. Finally, the O-antigen down-modulates T-cell activation through its interaction with MHC class II molecules, that leads to the formation of complexes which influence the ability of infected macrophages for antigen presentation [39]. Interestingly, MHC-II expression on antigen presenting cells is down-regulated by HKBA or *Brucella* lipoproteins, a phenomenon which is dependent on TLR2 and mediated by IL-6 [40]. However, TLR2 does not appear to play a significant role in the control of *Brucella abortus* infection in vivo, contrary to the significance of TLR9 which is required for clearance of this bacterium in infected mice.

The survival of *Brucella* is largely achieved by the formation of the gradually evolving Brucella-containing vacuole. Though most Brucellae are eliminated by phagolysosome fusion, some 15–30 % of them survive in these vacuoles that migrate from the endocytic compartment to the endoplasmic reticulum, where the bacterium proliferates. The preference of *Brucella* for endoplasmic reticulum has been recognized previously, and accounts for clinical signs of hepatomegaly, splenomegaly, and peripheral lymphadenopathy. Liver involvement is not surprising, given that the liver is the largest organ of the reticuloendothelial system in the human body [41, 42]. Data from animal studies have demonstrated a rapid localization of *Brucella* in the lysosomal and mitochondrial fraction of Kupffer cells [43, 44].

Autophagy promotes *Brucella melitensis* strain 16M survival in murine macrophages [45], as infection with this strain favors autophagosome formation, augments autophagy flux, and leads to the overexpression of LC3-II, an autophagy marker, while pharmacologically induced inhibition of autophagy abrogates efficiency for *Brucella* replication [45]. Also, recent data indicates that *Brucella abortus* can "hitch a ride" with autophagy, selectively subverting autophagy machinery to ensure cell-to-cell spreading [46].

Legionella pneumophilia

Background and epidemiology: *Legionella pneumophila*, the causative agent of legionellosis, is an anerobic, flagellated, nonspore-forming, Gram-negative coccobacillus of the genus *Legionella* [47]. An outbreak of legionellosis, also known as Legionnaire' disease or legion fever, was first noted in 1976 in members of the American Legion met for their annual convention at a hotel in Philadelphia, Pennsylvania. One year later investigators from CDC isolated the causative pathogen of the disease. Unclassified agents isolated in 1947 and 1959 were found to be similar to *Legionella*. Approximately 10,000–18,000 cases of Legionnaires' disease are recorded each year in the USA.

Legionella is well known as a contaminant of water cooling systems, as well as water supplies, with numerous outbreaks reported in the past. Inhalation of contaminated aerosols is the most common mode of infection. Temperature affects the survival of *Legionella*, the ideal growth ranging between 35 and 46 °C (95–115 °F). The pathogen can survive at temperatures below 20 °C (68 °F) but is inactive. *Legionella pneumophila* can travel airborne at 6–10 km or more from its source.

Disease features: This pathogen is a common cause community-acquired and nosocomial pneumonia.

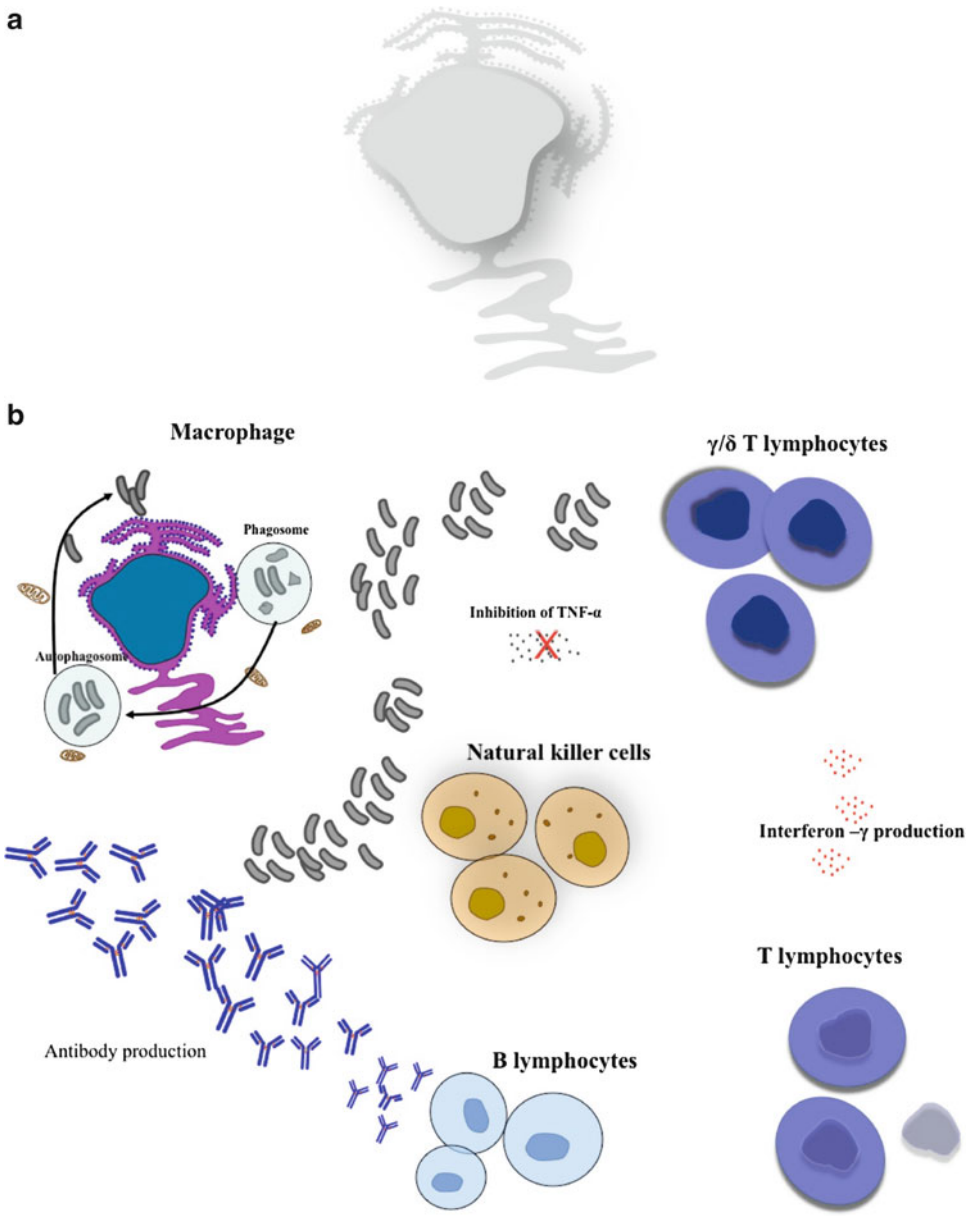

Fig. 10.2 Host–pathogen interactions in the pathogenesis of brucellosis. The pathogen enters the macrophages and multiplies in the endoplasmic reticulum. (**a**) Brucella enter the macrophages, where the minority of the bacteria survive in specialized evolving compartments. (**b**) Close interplay of bacteria, macrophages, lymphocyte populations, and inflammatory mediators in brucellosis. Inhibition of pro-inflammatory mediators like that of tumor necrosis factor (TNF)**α** abrogates the ability of NK cells to eliminate the pathogen. The production of interferon (IFN)**γ** provokes a bactericidal effect by natural killer cells and T lymphocytes directly and through macrophage induction. T-cell-dependent B-cell antibody production by B lymphocytes is also induced but plays a minor role in the immune response. Cellular immune responses include both effector and suppressor cells (modified according to [32])

Extrapulmonary legionellosis is uncommon. Affected patients tend to be over 40 years of age, and males are affected more than females. Smokers, alcoholics, and those with chronic underlying lung disease are especially susceptible to infection. The heart is a known site of extrapulmonary infection. There is often widespread dissemination to other organs, as well as a mild portal infiltrate and sinusoidal neutrophils. *Legionella* sp. strains have been involved in sporadic cases of sinusitis, cellulitis, pancreatitis, peritonitis, and pyelonephritis. Patients often present with a chest infection and jaundice is not overly common being present in approximately 10 % of patients. Aminotransferases and ALP are raised in approximately half of patients.

Diagnosis: Laboratory evidence of the infection is largely sought by the *Legionella* urinary antigen. This test is very

sensitive for *Legionella pneumophila* serogroup. The mainstay test is that of culture, which requires up to 7–10 days to obtain a positive result.

Treatment: Erythromycin and clarithromycin are the antibiotics of choice.

Immunobiology: While defense of primary infection with *Legionella pneumophila* largely depends on innate immunity, efficient bacterial clearance and protection from reinfection rests on the activation of adaptive immunity. This bacterial pathogen has evolved virulence mechanisms that allow it to replicate within monocytes and alveolar macrophages. The survival of *Legionella pneumophila* following its internalization largely depends on an organelle trafficking/intracellular multiplication (Dot/Icm) type IV secretion system that translocates to the host cytosol a large number of effector proteins and bacterial PAMP proteins, such as flagellin, nucleic acids, or peptidoglycan fragments, which modulate host innate defense such as the NF-κB pathway and apoptosis. Manipulated *Legionella pneumophila* lacking the specialized Dot/Icm system is recognized by TLRs. 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 lysosome.

Previous data demonstrating that the host's responses to *Legionella pneumophila* are modulated through activation of molecules related to TLRs or cytosolic pattern recognition receptors are now complemented by new findings suggesting that the immunity to this pathogen also depends on inhibition of host protein synthesis [48]. Innate immune responses act in concert with adaptive immune cells against *Legionella pneumophila*. In the absence of cytoplasmic pattern recognition, MyD88 appears important for T cell priming. In the presence of cytoplasmic pattern recognition, MyD88-mediated signals involving *L. pneumophila*-derived flagellin and in the host side the inflammasome/IL-1 axis are essential for Th17 differentiation [49]. Lack of MyD88-dependent TLR signaling abolishes Th17 development and promotes the induction of simultaneous Th1/Th2 responses that do not depend on the host's inflammasome–IL-1 axis [49].

Thus, it has become apparent that the unique host transcriptional response to virulent *Legionella pneumophila* is due to the activity of secreted bacterial proteins that inhibit host translation. Intriguingly, various bacterial toxins or chemical reagents that can inhibit host translation can coordinate the induction of unique transcriptional responses in close interaction with host sensors of microbial molecules but without bacterial infection being an absolute requirement [48].

The extent by which *Legionella pneumophila* manipulates the host process of autophagy is under investigation [50]. Knockdown of the autophagy-related Atg5 gene in mice promotes *Legionella pneumophila* replication [51]. As well, *Legionella pneumophila* mutants deficient in genes lacking macroautophagy pathway replicate better compared to those not deficient to such contents [52].

Legionella pneumophila infection induces rapid apoptotic DCs death mediated by caspase-3. DCs deficient in the pro-apoptotic proteins Bax and Bak which are essential for the initiation of the apoptosis pathway can restore *Legionella pneumophila* replication in DCs [53]. Overproduction of the antiapoptotic protein Bcl-2 has also the same effect [53].

Bartonella

Background and epidemiology: *Bartonella* spp. are Gram-negative facultative intracellular aerobic bacteria of the alpha-2 subgroup of proteobacteria, that can cause infections in humans. In 1909, L.A. Barton described erythrocyte-adherent organisms that were named after him in 1913. Recently a similar group, *Rochalimaea* was combined with *Bartonella*. Accumulating evidence suggest that blood-sucking arthropods act as vectors of *Bartonella* spp. and the actual transmission is mostly mediated by flea feces and superficial scratching.

Disease features: Though at least a dozen of species belong to the genus of Bartonella, few are responsible for disease in humans [54]. The most commonly encountered pathogens for humans are *Bartonella bacilliformis* causing Oroya fever and verruga peruana. *Bartonella henselae* is the cause of cat scratch disease and peliosis of the liver (often called bacillary peliosis), while *Bartonella quintana* causes trench fever. The most frequently encountered clinical features include a history of a typical cat scratch, prolonged fever and hepatosplenic disease, while ocular, neurological, dermatological, hematological, orthopedic, and cardiological manifestations are uncommon.

Diagnosis: Cat scratch disease is diagnosed on the basis of clinical manifestation and exposure history. Serological tests are used to confirm the diagnosis, but false cross-reactive reactions limit the interpretations of the results. *Bartonella* DNA is detectable by PCR or culture of pus or lymph node aspirates. Isolation of *B. quintana* from blood cultures and serological tests assist the diagnosis of trench fever, which can be diagnosed by incubation at 37 °C. Trench fever can also be diagnosed by serology. Direct observation of the pathogen in peripheral blood smears is possible during the acute phase of infection (Oroya fever).

Treatment: The use of certain antibiotics such as azithromycin can significantly decrease the lymph node volume. Penicillins, tetracyclines, cephalosporins, aminoglycosides, and fluoroquinolones have also been used.

Immunobiology: In recent years, our knowledge on the pathogenetic mechanisms of *Bartonella* infection in humans has evolved mostly in relation to immune evasion of *Bartonella* spp., their interactions with erythrocytes and endothelial cells, and induction of neoangiogenesis [55]. The virulence factors of Bartonella have been studied extensively (Table 10.4). The various phases of *Bartonella* spp. bacteremia in a mammalian reservoir host are illustrated in Fig. 10.3 [56].

Table 10.4 Summary of the major pathogenicity factors of *Bartonella* spp.

Factor(s)	Function
Major	
LPS	Lipopolysaccharide, detoxified
Sub-major	
Angiogenic factor	Stimulates endothelial cell proliferation
Deformin	Deformation of erythrocyte membranes
Flagella	Motility, binding to and invasion of erythrocytes
Hemolysin	Contact-dependent hemolysis
Hbp/Pap 31	Omp family, hemin-binding proteins
IalA-B	Putative invasins of erythrocytes
Iba	Autotransporter, putative adhesins
Omp43	Putative adhesin for endothelial cells
pili	Type IV-like poli, twitching motility, cell adhesion
Trw	T4SS, establishment of intraerythrocytic infection
VirB-D4-Bep	T4SS, supervision of endothelial cell function

Early studies suggesting that the virulence factors of pathogens such as Bartonella spp. are relatively few have been followed by recent data indicating that the number of virulence factors is rather large and involves distinctive factors. This is illustrated rather well in the case of Bartonella [55]

Infection of the mammalian host causes chronic intraerythrocytic bacteremia resulting in a wide spectrum of symptoms from asymptomatic self-limited disease to more severe and life-threatening infections depending on the immune status of the infected individual.

In immunocompetent individuals, the response is granulomatous and suppurative as typically seen in biopsied lymph nodes in patients with cat scratch disease. In immunocompromised patients (e.g., AIDS), *Bartonella henselae* infections can result in tumorous proliferations of endothelial cells in the skin or inner organs, which are called bacillary angiomatosis or peliosis hepatitis, respectively [56].

It has been demonstrated that *Bartonella* spp. are able to establish infection through evasion of the immune system, using a variety of evolved mechanisms Phagocytes and dendritic cells are the first line of defense against infectious agents. TLRs on professional phagocytes, such as tissue-resident macrophages, can recognize LPS, which results in secretion of pro-inflammatory cytokines and subsequent recruitment of other inflammatory cells to the site of pathogen entry.

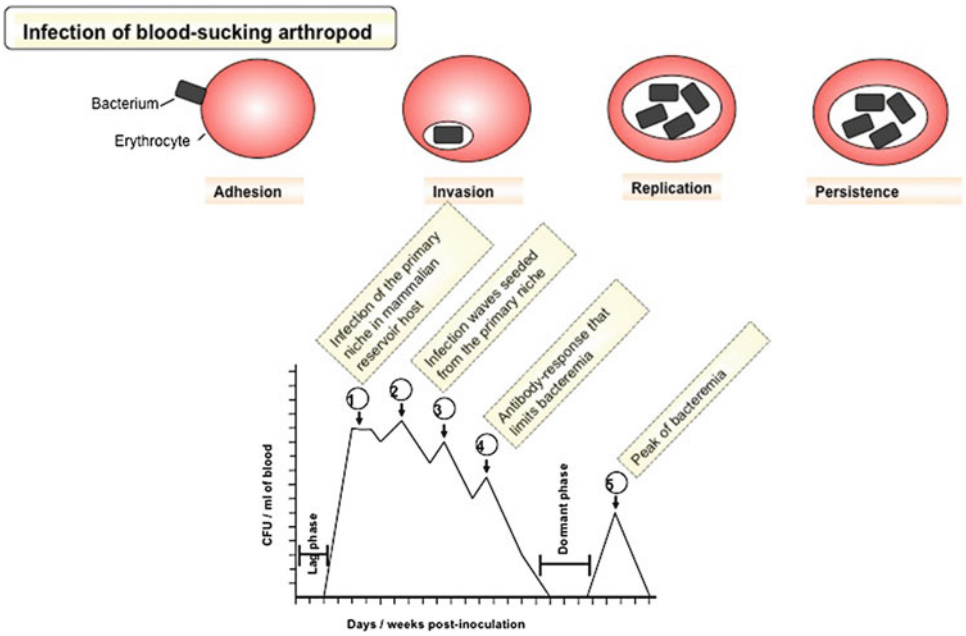

Fig. 10.3 Intraerythrocytic bacteremia of *Bartonella* spp. in a mammalian reservoir host over time. The initial inoculation of pathogens in arthropod feces that enter the skin through tiny cuts is followed by the persistence of the bacteria in the primary niche (lag phase). A rapid increase of bacteria numbers leads to bacteremia in the bloodstream (*arrow 1*). Invasion of bacteria to erythrocytes is followed by their replication in a steady pace, which is maintained for the remaining life span of the infected erythrocytes. *Arrows 2–4* illustrate additional erythrocyte infection waves at regular intervals, up to a point that humoral immunity clears the infection by blocking the erythrocyte invasion. In *arrow 5*, bacteremia reaches its peak over a period of weeks to months (dormant phase). During the long-lasting intraerythrocytic bacteremia, pathogens are transmitted to other susceptible hosts via mediated by blood-sucking arthropods (with slight modifications from [56])

Table 10.5 Main characteristics of lipid A of LPS in *Bartonella henselae*, *Escherichia coli*, *and Legionella pneumophila*

	Lipid A of LPS		
Pathogen	*Bartonella henselae*	*Escherichia coli*	*Legionella pneumophila*
Endotoxicity	Very low	High	Low
Acylation	Penta-acylation	Hexa-acetylation	Hexa-acylation
Long chain fatty acid	One long chain fatty acid	No long chain fatty acids	One long fatty acid

LPS from the outer wall of *Bartonella* has a unique structure, consisting of Lipid A and long chain fatty acids. Table 10.5 summarizes the main characteristics of *Bartonella*'s LPS compared to those of *Escherichia coli* and *Legionella pneumophila*. The unique surface structure of *Bartonella*'s LPS has been shown to avoid recognition of the bacteria from TLR4 on dendritic cells, which is an essential step of innate immunity. Accordingly, it is suggested the LPS of *Bartonella quintana* has an antagonistic role to TLR4, as shown by reduced production of almost all cytokines by TLR4 in response to LPS [57]. These data suggest that evading TLR4 responses might contribute to the establishment of long-lasting bacteremia with *Bartonella* spp. without symptoms of septic shock.

Additionally, *Bartonella henselae* can avoid lysosomal fusion and acidification after the bacteria invades phagocytes such as endothelial cells and macrophages.

Some *Bartonella* spp. express flagella, a rod-like structure that is important for bacterial motility and serves as recognition site for TLR5. The flagellin of *Bartonella bacilliformis*, which is the main constituent of flagella, has been reported to evade TLR5 recognition, and is believed to be a TLR5 agonist.

Experimental data report unusual trafficking and delayed lysosomal destruction of *Bartonella henselae* after entering the macrophages in a unique vacuolar compartment. Various proteins expressed on the surface of different *Bartonella* spp. may serve as virulence factors, though their detailed molecular functions in relation to the impaired effector functions of professional phagocytes need to be elucidated further. In line with this, *B. henselae* have been shown to produce a *Bartonella* adhesin, which is a potent inhibitor of phagocytic uptake of *Bartonella* by mouse macrophages. *Bartonella* adhesin's expression varies amongst strains of *Bartonella henselae* due to unknown regulatory mechanisms.

One additional survival strategy for *Bartonella* spp., as shown in one report, is their ability to inhibit the production of oxidative bursts in polymorphonuclear leukocytes, which is one of the most important antimicrobial effector mechanisms of polymorphonuclear leukocytes.

Th1 immune responses have been implicated in the pathogenesis of *Bartonella* infection, as shown in clinical and in vitro studies [58]. As shown in vitro, mice splenocytes produced increased levels of IFN-γ and IL-12 in response to *B. henselae* compared to controls. In acutely infected immunocompetent patients with cat scratch disease, pro-inflammatory cytokines such as IL-2 and IL-6 and immunoregulatory cytokines such as IL-10 are upregulated. In patients with low CD4 count, high levels of IL-10 production have been demonstrated during the acute illness [58]. Even though such differences of the host immune response may explain the difficulty in limiting the infection in the immunosuppressed patient, this assumption has not been validated in patients with HIV infection.

Existing data indicate that the ability of *Bartonella* spp. to bind to extracellular matrix may play an important role in the early stages of the disease. Bartonella adhesion A binds to vitronectin, laminin, hyaluronic acid, fibronectin, and collagens I, II, and IV. Also, *Bartonella quintana* and *Bartonella bacilliformis* variably express different conserved adhesins, even though not all have been functionally characterized.

In line with this, *Bartonella* spp. are also able to attach to and invade both human fibroblast and epithelial cells, suggesting a possible role of integrins in the bacterial uptake. These cells may offer a transient and immunologically privileged niche for *Bartonella* spp. after entry through the skin.

The ability of *Bartonella* spp. to cause the angiogenic lesions represents a fascinating aspect of the pathogenesis of these bacteria. Mechanisms most likely implicated in angiogenesis induced by *B. henselae* include NF-κB-dependent pro-inflammatory gene activation, direct promotion of endothelial cell proliferation, inhibition of endothelial cell apoptosis, and upregulation of angiogenic growth factors from peripheral cells. Part of these features has been shown to be IL-8-dependent.

Burkholderia pseudomallei

Background and epidemiology: *Burkholderia pseudomallei* is a Gram-negative, motile, nonspore-forming, saprophytic aerobe which causes melioidosis, an emerging tropical disease also known as pseudoglanders, Vietnamese time bomb, or Whitmore's disease [59]. In April 1911, A. Whitmore has described a glanders-like disease in a 40-year-old Burmese in Rangoon. The disease is endemic primarily to Southeast Asia and Northern Australia. Cases occur mainly during periods of heavy rain. Meliodosis cases have been noted in Africa, the Middle East, China, India, and South America. According to the U.S. CDC, confirmed cases of melioidosis range from none to five each year and the affected individuals are travelers or immigrants.

The pathogen is found in contaminated soil and water. It typically gains entry into the host via skin wounds or rarely through inhalation. Whether melioidosis can spread from person to person is a matter of debate. The bacterium has

been considered a potential agent for biological warfare and biological terrorism. The severity of the disease caused by this pathogen, its aerosol infectivity, and worldwide availability have led to the insertion of *Burkholderia pseudomallei* in the list of potential agents of biological warfare or bioterrorism.

Disease features: The disease commonly presents as a fulminant septicemia, which is often associated with acute pneumonia. Asymptomatic infections also occur, and may become symptomatic several years after the initial exposure. The longest incubation period reported in the literature was that of 62 years. At-risk groups include those with chronic underlying liver disease, renal failure, diabetes mellitus, as well as the immunosuppressed individuals. Patients present with fever, rigors, cough, and chest infection, but may also present with meningitis and hepatosplenomegaly. Approximately 40 % of patients are jaundiced.

The pathology of this bacterium is induced by the release of exotoxins/proteases, causing inflammation and necrosis. Epitheloid and giant cell granulomas are often encountered [60].

Diagnosis: Clinical suspected cases are diagnosed on the basis of blood cultures, as well as via aspiration and culture of pus from abscesses. Urine, sputum, and skin-lesion samples are also analyzed. Apart from classical microbiological procedures (microscopy, culture, and biochemical identification) efforts have been made to identify *Burkholderia pseudomallei* using specific antibodies and PCR-based molecular analysis. The current "gold standard" species-specific assay for *Burkholderia pseudomallei* is based on amplification of *orf2* of the type 3 secretion system-1 cluster, which is only found in *Burkholderia pseudomallei*. The direct antigen detection is done within hours and can confirm the diagnosis before proof stemming from cultures. ELISA, immunoblotting, indirect hemagglutination, and immunofluorescence serological tests are used in serology.

Treatment: Depending on the isolate of the pathogen, there is sensitivity to imipenem, piperacillin, amoxycillin-clavulanic acid, doxycycline, ceftazidime, aztreonam, and chloramphenicol. Resistance to colistin and gentamicin has been noted.

Immunobiology: Following its invasion, *Burkholderia pseudomallei* escapes from the endocytotic vesicle of macrophages into the host cytosol (Fig. 10.4). This escape largely depends on a functional type 3 secretion system-3 [61]. Spreading of *Burkholderia pseudomallei* into neighboring cells is achieved by the induction of cell fusion and actin-associated membrane protrusion. The *Burkholderia pseudomallei-induced* cell fusion and the formation of multinucleated giant cells may represent a central mechanism for intercellular spread and plaque formation of this pathogen [62]. The fine specificity of immunity specific for recovery from melioidosis is poorly defined. Work on murine models of melioidosis has indicated that humoral and cellular immunity play a protective role [63]. IFNγ-producing NK and T cells appear to be capable of controlling the infection [64]. Various *Burkholderia pseudomallei* antigens can induce cytokine production by lymphocyte populations isolated from seropositive healthy individuals living in endemic areas, or from individuals who have recovered from melioidosis. The magnitude of these responses is proportional to *Burkholderia pseudomallei*-specific antibody titers [65].

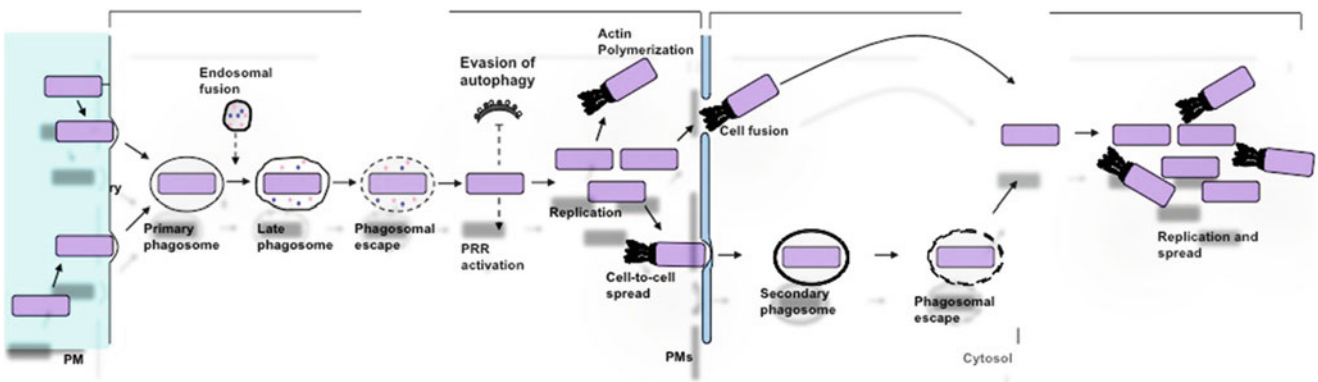

Fig. 10.4 Intracellular life of *Burkholderia pseudomallei*. The uptake of the pathogen from the host cells is followed by entry of bacteria into the primary phagosomes. Secretion of Bsa T3SS (type III secretion system) takes place during the process of phagosomal maturation. Various effectors induce the disruption of vacuolar membranes, and ease the escape of bacteria into the host cytosol. The free bacteria are able to activate pathogen recognition receptors (PRRs) and BimA-dependent actin-based motility, thus evading the killing by host autophagy. T6SS (type VI secretion system) effectors can influence bacteria's ability for replication. They also promote polymerization of actin, as well as plasma membrane (PM) fusion. The entry of pathogens to adjacent cells, initiates a similar cascade of events which facilitates escape from phagosomes, leading to the replication of bacteria and their spreading (modified from [90])

IFNγ production eliminates intracellular *Burkholderia pseudomallei* in macrophages, but the role of the Kupffer cells in the clearance of this pathogen remains poorly understood. Murine studies have shown that *Burkholderia pseudomallei*-containing phagosomes in hepatocytes fuse with lysosomes, causing bacterial degradation [66].

Recent data based on HepG2 cells show that in vitro invasion and intracellular replication of *Burkholderia pseudomallei* in human hepatocytes involves type 3 and type 6 secretion systems [67]. It also appears that IL-1β and TNFα augment the maximal antibacterial activity of hepatocytes, while IFNγ contributes to growth restriction of *B. pseudomallei*. The effects mediated by IFNγ are independent of NO and ROS production, suggesting the existence of NO- and ROS-independent mechanisms which participate in the intracellular growth of the pathogen in activated hepatocytes [67].

Leptospira interrogans

Background and epidemiology: *Leptospira interrogans* is the causative agent of human leptospirosis, a water-borne zoonosis [68, 69]. The disease is also known as muf fever, autumn fever, 7-day fever, swineherd fever, Canicola fever, Fort Bragg fever, and hemorrhagic jaundice. The most severe form of the disease is known as Weil disease (named by Adolf Weil, who was the first to describe it). The pathogen is an aerobic, tightly coiled spirochete. Its morphological view under the microscope, similar to a question mark, has given the species its name. Leptospirosis remains a significant threat in tropical and subtropical countries. Wild rats serve as reservoirs when sewage disposal is poor. The serovars *Leptospira icterohaemorraghiae* is responsible for approximately 50 % of cases with Weil disease, but in most infected individuals causes anicteric illness. Rats are the most common host for *Leptospira icterohaemorraghiae*, but has also been found in dogs, cattle, and swine. Infection in humans occurs by direct contact with contaminated urine or water. The pathogen enters the human body through tiny cuts. Outbreaks have been noted amongst those exposed to river/lake contaminated by urine of animals.

Disease features: Most cases run subclinical or mild disease courses, and may present with fever (every 3 days), headache, malaise, myalgias, and a transient rash. Symptoms last for 7 days. Weil syndrome, also known as icteric leptospirosis, is seen in less than 10 % of the infected individuals and patients present with jaundice, hepatomegaly, vomiting, fever and chills, myalgias, mental disturbances, and possibly multi-organ failure. Mortality rates range between 5 and 15 %.

Diagnosis: Laboratory diagnosis of leptospirosis is generally straightforward. Blood, spinal fluid, and urine cultures are used to confirm the presence of the pathogen. The culture may take as long as a month to show a growth, and this limits the applicability of cultures for diagnostic purposes. In the second phase of the disease, the pathogen is seen in the urine by dark field microscopy. The microagglutination test, as well as other immunoassays, is used for diagnostic purposes.

Treatment: Leptospirosis is treated with doxycycline or penicillin, which should be given early in the course of the disease; 100 mg of doxycycline twice a day reduces duration and severity of symptoms in anicteric patients, while 200 mg once per week is recommended as short-term prophylaxis for travelers. Early administration of penicillin may be beneficial for the reduction of symptoms/disease duration.

Immunobiology: Leptospires can induce hepatitis in humans characterized by swelling of parenchymal cells, disruption of the liver cord, enlargement of Kupffer cells, and bile stasis in biliary canaliculi [70]. Early electron microscopy studies showed in experimentally infected mice that leptospires are almost entirely found within Kupffer cells. Subsequent data have indicated that the high motility and thinness of these spirochetes is probably responsible for their ability to escape efficient uptake from reticuloendothelial cells. This gives them the opportunity to penetrate the endothelial lining of the liver sinusoids, being able to reach the spaces between liver parenchymal cells [71]. More recent data suggest that spirochetes are associated with Kupffer and endothelial cells, further suggesting their ability to penetrate the endothelial lining of the liver sinusoids and to reach biliary canaliculi [72]. Direct proof of these findings is needed.

Francisella tularensis

Background: *Francisella tularensis subspecies tularensis* is the causative agent of tularemia. Tularemia, a potentially fatal disease, is endemic in North America, Russia, and Europe. Transmission is via bites from ticks and deer flies, with reservoirs being present in squirrels, hairs, and musk rats. Infection typically occurs in the summer months. The elderly and those with chronic diseases are most susceptible to infection. These pathogens are extremely infective, and exposure to as few as ten organisms can lead to 30–35 % mortality, in untreated individuals, who are infected [73].

Disease features: The most common presentations include fever, rash, ulcer at the site of tick or fly bite, regional lymphadenopathy, and occasional lung, eye, and oropharyngeal involvement. The histopathology is characterized by coagulative necrosis and surrounding inflammatory cell infiltrate, and occasionally, abscess formation [74]. Abnormal LFTs

(in the form of raised aminotransferases) only occur in 10 % of patients.

Diagnosis: The disease is diagnosed by blood cultures or serological tests. Serum testing of antibodies against the bacterium is diagnostically relevant, and tests based on *Francisella* LPS as antigenic source have been developed. Six to ten days after the onset of symptoms (i.e., 2 weeks after infection), antibody tests become seropositive and reach their peak 4–8 weeks or months after infection.

Treatment: Antibiotic treatment is with streptomycin, gentamicin, and tetracyclines.

Immunobiology: Arthropod vectors allow *Francisella tularensis* to enter the host through inhalation, ingestion, abrasion, and transmission. The pathogen is able to survive and replicate within macrophages, and can infect various organs including the liver, lungs, and spleen [75]. The important role for complement, lipid rafts, and caveolin-1 for effective internalization of *Francisella tularensis* in macrophages has been repeatedly demonstrated. Upon its entry into macrophages, the pathogen escapes from the phagosomal compartment and finds its way to replicate within the macrophage cytosol. The pathogen manipulates the immune response of the host, leading to significantly diminished pro-inflammatory cytokine expression at the early stages of infection [76]. The infected cells are unable to respond to TLR-dependent secondary stimuli [77]. Recent data have shown that *Francisella tularensis* is able to significantly impair the apoptosis of neutrophils, to prolong their existence, and to provoke persistent inflammation [78]. These events appear important for granuloma formation, and subsequent cell destruction and tissue damage.

The secretion system of *Francisella tularensis* is very similar to type VI secretion systems of other intracellular pathogens. Most of the genes important for intracellular growth and virulence of the bacterium are largely found on a region known as the *Francisella* Pathogenicity Island, which includes a cluster of 17 genes [79].

Investigation of the role of human γδ T cells in controlling *Francisella tularensis* infection has led to the appreciation that in the presence of human γδ T cells bacterial numbers are markedly reduced and that IFN-γ neutralization increases the survival of the pathogen [80].

γδ T cells are increased in liver-resident lymphocyte populations. Ongoing work on two closely related *F. tularensis* subspecies, the *F. tularensis* subspecies *holarctica* live vaccine strain (*Francisella* LVS), and the *Francisella tularensis* subspecies *novicida*, has provided novel insights regarding the ability of this pathogen to invade hepatocytes. *Francisella* LVS is an attenuated strain, which can infect both human and murine cells, while *Francisella tularensis* subspecies *novicida* can only infect mice and does not normally cause disease in healthy humans. These pathogens have common virulence factors and colonize to the same sites during in vivo murine infections. They are also able to infect phagocytic and nonphagocytic cells. These bacteria show a high level of colonization in murine hepatocytes in vivo and can be used as infection models of hepatocyte. Accumulating data have shown that *Francisella species* such as *Francisella novicida* uses various mechanisms for efficient internalization into murine hepatocytes. Effective internalization implicates clathrin-mediated endocytosis and cholesterol-rich microdomains [81].

Rickettsia

Background: Rickettsial species are transmitted by ticks, fleas, and mites. Most rickettsial infections can affect the liver, and the 12 main species of Rickettsia can induce abnormal LFTs, as well as jaundice and hepatosplenomegaly. In most cases, however, liver involvement is subclinical. *Rickettsia rickettsia* is the causative organism of Rocky Mountain Spotted Fever, and is transmitted via tick bites [82].

Disease features: Although rash, headache, and fever are considered classical presentations of Rocky Mountain Spotted Fever, gastrointestinal symptoms may precede rash by up to 3 days, and include abdominal pain, vomiting, diarrhea, jaundice, and hepatosplenomegaly. Up to 60 % of patients may have elevated aminotransferases.

Diagnosis: The diagnosis of Rocky Mountain Spotted Fever is largely clinical, as serological testing is not reliable early in the disease course. Thrombocytopenia, hyponatremia, and elevated transaminases are not present in all patients. *Rickettsia rickettsii* infectivity is largely limited to endothelial cells, and for this reason, blood cultures and molecular analysis by PCR frequently produce negative results unless the disease is at an advanced stage. Culture of *Rickettsia rickettsii* is performed at specialized laboratories. Skin biopsy specimen can be subjected to PCR or immunohistochemical analysis in patients with a rash. PCR, culture, and immunohistochemistry are also helpful in liver, spleen, and kidney specimen collected in deceased patients undergoing autopsy. Antibodies against *Rickettsia rickettsii* are detected 7–10 days after the initiation of symptoms. The serological test of choice is the indirect immunofluorescence assay for the detection of *Rickettsia rickettsii*. Past exposure to the pathogen largely explains why approximately 10 % of healthy individuals previously exposed to *R. rickettsii* may have elevated antibody titers.

Treatment: The treatment of choice is doxycycline for both adults and children. Prevention of fatality requires doxycycline

to be started in the first 5 days of symptoms. Early initiation of treatment leads to fever decline within 24–72 h.

Other Rickettsial species: Epidemic typhus, caused by *Rickettsia prowazeckii*, is transmitted by lice. Epidemic typhus is seen in cases of poverty, poor hygiene, and in natural disasters such as earthquakes and floods. The clinical presentation includes fever, headache, and myalgia with rash. Other forms of typhus include *Rickettsia typhi* (worldwide but mostly in the subtropics) and scrub typhus caused by Orientia tsutsugamuchi (mostly present in a triangular geographical region bordered by Japan, Australia, and India). These three forms of typhus may cause liver dysfunction, indicated by jaundice and elevated aminotransferases. Often, the clinical presentation of these may be confused with viral hepatitis. The diagnosis of typhus is based on a rise in convalescent antibody titers. Treatment is with doxycycline.

Rickettsia conorii, the causative agent of Boutonneuse fever and South African tick bite fever, can cause granulomatous liver disease.

Pathogen detection is not an easy task in the rickettsial illnesses, but serological testing based on immunoassays and immunofluorescence can be helpful. The pathophysiology of infections with these pathogens and their interactions with the host have been detailed elsewhere [82, 83].

Coxiella burnetii

Background and epidemiology: *Coxiella burnetii* is a highly pleomorphic coccobacillus with a Gram-negative cell wall, that causes Q fever, a zoonosis with worldwide distribution [84]. Primary reservoirs are mainly cattle, sheep, and goats and transmission to humans occurs either via inhalation of contaminated aerosols, or via ingestion of unpasteurized milk or dairy products, as well as via tick bites. In rare cases, human-to-human transmission has been reported.

Q fever is a systemic illness known to cause severe illness, and is presently only known to affect humans [85]. The disease was named "q fever" in 1937 by Edward Derrick for query, "until fuller knowledge should allow a better name."

Initially, *Coxiella burnetii* was classified as *Rickettsia*e, due to similarities with those species, such as being an obligate intracellular organism and having a tick reservoir. Classification of *Coxiella burnetti* has changed based on phylogenetic analysis of its genome and is now included in the gamma subgroup of the proteobacteria, in the Legionellales order and Coxiellaceae family.

In the life cycle, two variants are distinguishable by electron microscopy: small-cell variants (SCV), which are resistant to heat, pressure, and chemicals that survive well in the environment, and large-cell variants (LCV) that multiply in the host monocyte and macrophage. After entering the host cell, *Coxiella burnetti* changes from SCV to LCV.

In terms of its antigenic state, *Coxiella burnetii* undergoes phase variation, which relates to variation of LPS chemical composition. Phase I is the natural form, occurring in infected animals and humans and is characterized by high infectivity. Phase II is not very infectious and occurs in laboratory conditions after passages in cell lines or chicken embryos. Phase I microorganisms are virulent, while phase II microorganisms are avirulent in immune-competent hosts.

Diagnosis: During acute infection the antibody response is directed primarily against phase II antigen, whereas in chronic infections the predominant response is directed against phase I. Diagnosis of acute Q fever relies on the detection by indirect immunofluorescence of IgM titer of ≥1:50 and phase II antigen IgG titer of ≥1:200, while phase I antigen IgG of 1:800 or low or absent phase II antigen IgM are compatible for chronic infection.

Disease features: One bacterium is capable of initiating infection, rendering the bacterium highly infectious. Clinically, Q fever can cause acute and chronic illness, while chronic sequels of the disease, including chronic fatigue syndrome have been recognized as a distinct category. There are data to suggest that the disease course in humans relates to the infecting strain of *Coxiella burnetti*, while controversy exist on whether strains causing acute and chronic infection are genetically different.

Acute infection is in the majority of cases a self-limiting illness which resolves spontaneously in 2–14 days. More severe illness includes pneumonia, hepatitis, endocarditis, osteomyelitis, neurological manifestations, while pregnancy, preexisting valvular heart disease, and immunosuppression are risk factors for evolution to chronic disease.

Endocarditis is the most common manifestation of chronic Q fever and occurs in the majority of the cases with preexisting valvular heart disease. Liver involvement in Q fever can manifest either as acute hepatitis, or as granulomatous lesions in patients with fever of unknown origin or even as incidental finding in patient with acute Q fever pneumonia.

Hepatitis is a common manifestation of infection, occurring in 60 % of patients infected in the USA. ALP is elevated compared to aminotransferases, and jaundice may be present in 30 % of patients. Liver involvement may manifest with histological changes, including fibrin ring granulomas, which are fat vacuoles surrounded by fibrinoid necrosis, histiocytes, and lymphocytes. Histological assessment of patients with Q fever can reveal intermediate hepatic granulomas between epithelioid and fibrin ring types. Hepatic granulomas can also be seen in patients with Boutonneuse fever and South African tick bite fever caused by *Rickettsia conorii*.

Treatment: The gold standard for the treatment of acute Q fever is doxycycline, with clarithromycin being an alternative

option. Chronic Q fever treatment involves a combination of drugs, preferably doxycycline plus hydroxychloroquine.

Immunobiology: The pathogenesis of Q fever in humans is not clear [86]. As evident by animal models, after entry into the host, *C. burnetii* is engulfed by resident macrophages and transported to individual organs, such as the lungs, liver, and spleen causing specific symptoms and histopathological changes. The spleen, liver, and other tissues of the reticuloendothelial system are typically the most seriously infected organs.

The phase II state of the pathogen enters the host cell via the phagolysosomal pathway and the CR3 receptor, while phase I cells engage different receptors on monocytes and macrophages resulting in differences in uptake and intracellular replication between the two phases.

Acidic conditions within the phagolysosome permit the bacteria to grow and to proliferate. This subsequently leads to rupture of the host cell and infection of the surrounding cell population of the host.

Accumulating data suggest that *Coxiella burnetti* uses ways to evade immune responses. Phase I *Coxiella burnetti* has been shown not to be able to activate host macrophage responses via TLR4, suggesting the pathogen to be a TLR4 antagonist.

In terms of recognition of *Coxiella burnetti* by dendritic cells, significant differences between phase I and phase II bacteria have been reported, that may contribute to the establishment of persistent infection. Phase I bacteria can infect and grow inside dendritic cells without subsequent induction of maturation and cytokine production by these cells, as determined by IL-12p70 production or p38 mitogen-activated protein kinase phosphorylation. This is in contrast to phase II bacteria, which can elicit significant cytokine production.

Cellular immunity is clearly important for the control of *Coxiella burnetti* infection, as it is evident by IFN-γ production by T cells in convalescent and those who have been vaccinated [87]. The subsequent production of reactive oxygen species plays an important role in controlling intracellular replication of bacteria. In accordance with this, in a mouse model of acute Q fever, T cell-deficient and IFN-γ k/o mice showed greatly increased susceptibility to *Coxiella burnetti* infection.

IL-10, a pleiotropic cytokine with pro-inflammatory and anti-inflammatory properties, has an established role in the evolution of acute Q fever to chronic infection, as is evident by increased IL-10 levels in patients with chronic Q fever. In an animal model of chronic Q fever, transgenic mice overproducing IL-10 by macrophages were more prone to establish a more robust infection.

In accordance with this, a positive correlation between IL-10 levels during acute Q fever and the risk for development of chronic infection in the future has been demonstrated. The overexpression of IL-10 in macrophages prevents the competence of the host to mount effective immune responses, including the ability to elicit granulomatous response, as evident by a reduced granuloma formation compared to acute Q fever.

Clostridia Infections

Background and epidemiology: *Clostridium* species are gram-positive anaerobic spore-forming, rod-shaped bacilli. These pathogens most commonly live in an oxygen-sensitive vegetative form or in a heat-stable spore form, capable of surviving in harsh conditions. Transmission occurs from person to person by the fecal–oral route, as well as from medical devices and instrument to patient.

Clostridium difficile has emerged as a major cause of antibiotic-associated (mainly clindamycin, cephalosporins, carbapenems, and fluoroquinolones) diarrheal episodes in hospitalized patients. Its name comes from the Greek κλωστήρ (kloster, spindle) and the Latin *difficile* (difficult). Researchers in the UK were the first to report in 1978 the close association of *Clostridium difficile* infection and pseudomembranous colitis. The epidemiology of *Clostridium difficile* infections has changed radically over the last decade, partly due to the wide-spread use of broad-spectrum antibiotics, which destroys the normal gut flora and allows for the colonization of this pathogen and the subsequent release of its toxins. The incidence and severity of *Clostridium difficile* infections dramatically increased in the USA, Canada, and Europe, largely due to a new hypervirulent and epidemic strain of *Clostridium difficile*. Several outbreaks have been reported worldwide. In fact, *Clostridium difficile* is the leading cause of diarrheic episodes noted in hospitalized patients and the fourth most common nosocomial infectious agent. Approximately 15,000 people die every year in USA due to *Clostridium difficile* infection and its severe complications.

The disease spectrum ranges from mild self-limiting diarrhea to moderately severe diarrhea due to colitis without pseudomembrane formation, or pseudomembranous colitis. Up to 3 % of the affected patients suffer from fulminant colitis, characterized by paralytic ileus, colonic perforation, and toxic megacolon, which is fatal in approximately one-third of affected patients. Latent symptoms of *C. difficile* infection can mimic colitis flares like those noted in patients with inflammatory bowel disease and flu-like symptoms.

Diagnosis: Confirmation of the infection in patients with clinical suspicion of the infection is based on laboratory confirmation. No single test is sensitive, specific, and robust. *Clostridium difficile* toxin detection along with culture and isolation of the pathogen strains is considered to be the most accurate approach for the diagnosis of the infection. The most frequent test is that of a fecal cytotoxin assay which identifies *Clostridium difficile* toxin B in cell culture. The

Table 10.6 Guidelines for the antibiotic treatment of *Clostridium difficile* infection according to the severity of the disease, issued by the Infectious Diseases Society of America (IDSA) and the Society for Health Care Epidemiology of America (SHEA)

Severity of *Clostridium difficile-associated disease*	Recommended antibiotic treatment
Mild/moderate disease	Metronidazole 500 mg×3 orally (10–14 days)
Severe disease	Vancomycin 125 mg×4 orally (10–14 days)
Complicated disease (paralytic ileus-toxic megacolon)	Metronidazole 500 mg×3 Intravenously and vancomycin 500 mg×4 orally

test shows a sensitivity of 67–100 % and specificity of 85–100 %. An ELISA detecting toxin A or both toxins A and B is also commonly used. This immunoassay has a 75–85 % sensitivity and 95–100 % specificity. Anaerobic stool culture is considered to be the most sensitive method to detect the pathogen, but has the limitation that can also isolate non-toxigenic strains. Epidemiological studies are based on molecular assays such as PCR ribotyping, pulsed field gel electrophoresis (PFGE), multilocus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), and an RFLP-PCR-based toxinotyping method.

Treatment: The management of *Clostridium difficile* infection includes fluid replacement, restoration of electrolyte balance, and discontinuation of the inciting antibiotic. Metronidazole is the fist-line antibiotic treatment for patients with mild disease (Table 10.6), while vancomycin is used for severe disease. For patients with complicated disease (paralytic ileus, toxic megacolon), a combination of oral vancomycin and intravenously metronidazole is recommended. Recurrences are noted in up to 20 % of the cases. Other therapeutic agents used for the management of the infection include nitazoxanide, daxomicin, ramoplanin, rifamixin, tigecycline, rifalazil, as well as passive immunotherapy and probiotics to maintain the homeostasis of the gut flora.

Infections with other *Clostridia* species (such as *Clostridium perfringens*, *C. novyi*, *C. welchii*, *C. histolyticum*, *C. septicum*, *C. tertium*, and *C. sordellii*) can be the cause of necrotizing soft tissue disorders. Infections of internal organs are infrequent. The cause of cellular destruction and tissue damage is the release of exotoxins from the pathogens.

Clostridium difficile toxins A and B are the major virulence factors of this pathogen. The main clinical symptoms and signs of diseases caused by *Clostridium difficile* include diarrheal episodes and mucosal inflammation of variable severity.

Clostridium *infection and necrotizing cholangiohepatitis*: *Clostridium* infection is recognized as an infrequent cause of necrotizing cholangiohepatitis in patients who underwent liver transplantation. In 2010, Richard Howard reviewed the literature and reported a total of 21 cases of necrotizing infections of the liver, 13 (62 %) of which were caused by *Clostridial* species [88]. Amongst those 13 cases, ten died shortly after becoming symptomatic. The most common cause of the infection was *Clostridium perfringens* (ten cases), the remaining three being due to *Clostridium sordellii* (one case), *Clostridium clostridiiforme* (one case), and the third due to undetermined clostridium species. *Candida*, *Enterobacter cloacae*, *Streptococcus*, *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Bacteroides*, and *Enterococcus faecium* were also found responsible for acute necrotizing cholangiohepatitis in transplanted livers. The former data indicate *Clostridium perfringens*, one of the most common causes of food poisoning in the USA and UK, as a potential cause of necrotizing cholangiohepatitis in patients receiving liver transplantation.

More recent data suggest that the prevalence of *Clostridium* infection in transplanted livers may not be as rare as it was originally believed, and implicate *Clostridium difficile* as a causative agent of liver damage.

A review of patient charts over 2 years in a single Liver Transplantation Centre in the USA revealed 24 cases of *Clostridium difficile* infections, 14 of whom developed hyperbilirubinemia. Amongst those 14, seven progressed to liver failure, including five cases with a fatal outcome [89]. It should be noted that although gangrene due to *Clostridia* species can infect other organs of seemingly healthy individuals, it has not yet been recognized as a cause of infection in heart, kidney, or lung-transplanted patients.

Clostridial species can be found in bile isolated from healthy individuals who undergo cholecystectomy. The exact mechanisms that lead to the induction of liver injury remain unclear (Fig. 10.5). Exotoxin-induced Kupffer cell activation may lead to the production of mediators that induce hepatocyte destruction and necrotizing cholangiohepatitis, but there is no solid evidence in support of this. In such a scenario, more cases with liver disease would be expected due to the increased prevalence of *Clostridium* infections in recent years.

Treponema pallidum and *Neisseria gonococcus*

Disease features and liver involvement: *Treponema pallidum* is the causative agent of syphilis. Liver involvement during infection with this pathogen has been known for some time. Hepatitis may occur in primary, secondary, or tertiary syphilis. Although 40 % of patients with secondary syphilis will have abnormal LFTs, only 10 % show liver manifestations, and this may lead to suspicion of viral hepatitis alone. Indeed, 1–12 % of patients with syphilis will present with jaundice. Hepatitis B and C virus infection, as well as HIV, should also

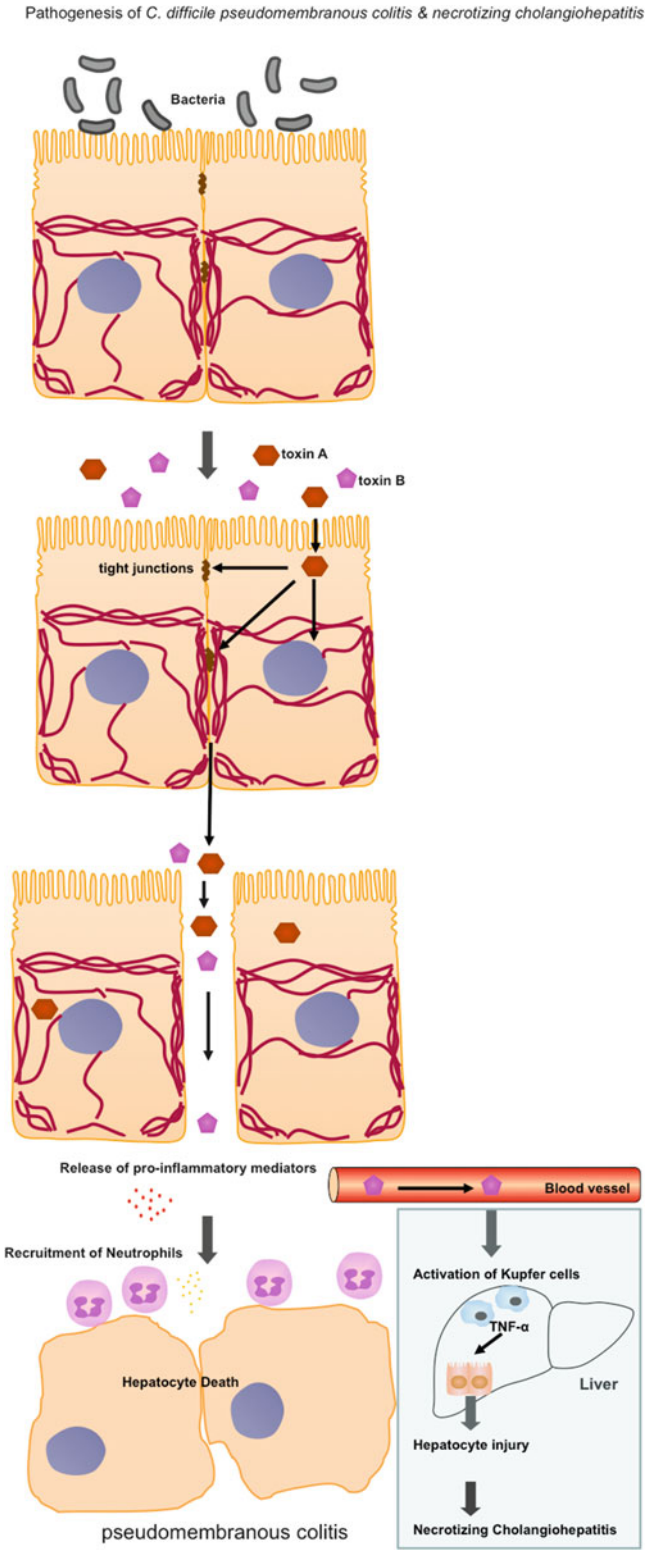

Fig. 10.5 Pathogenesis of *Clostridium difficile*-induced intestinal and liver injury. *C. difficile* colonizes the intestine, with bacterial cells attaching to host cells. Toxigenic strains of the pathogen produce toxins A and B (TcdA and TcdB) that bind to the surface of the cell. Internalization of the toxins leads to the disruption of tight junctions and loosening of the epithelial barrier, provoking cell death. The induction of pro-inflammatory mediators stimulates neutrophil accumulation, local inflammation, and cellular destruction leading to the development of pseudomembranous colitis [91]. In a hypothetical scenario, *Clostridium difficile* exotoxin may cross the intestinal barrier to enter the bloodstream, where it may interact with the Kupffer cells leading to their activation. Activation of Kupffer cell activation can lead to the generation of superoxide and TNF**α**, leading to acute liver injury in susceptible individuals infected with *Clostridium difficile*

be considered in patients with *Treponema pallidum* infections. Signs pointing towards a diagnosis of *Treponema pallidum* infection include maculopapular eruptions on the palms and soles, fever, arthralgias, and diffuse adenopathy. Primary lesions (chancres) are not always reported, as they are not always visible to the patient and are painless.

The pathogenic processes underlying liver involvement are not well defined, but may include portal lymph node involvement with biliary obstruction, autoantibody-mediated liver destruction, or portal pyemia.

Diagnosis: Histologically, inflammation and epitheloid granulomas with patchy necrosis may be observed, although it is often the case that the liver is histologically normal, especially in early stages of the disease. A liver ultrasound may show multiple hypoechoic lesions. White cell counts are usually normal, aminotransferase levels are either normal or elevated, and ALP levels are significantly raised compared to aminotransferases. TPHA and fluorescent treponemal antibody absorption (FTA-ABS) tests would be positive. It should be noted that false positive results may occur in autoimmune disease, hepatitis, and HIV, and false negatives can occur during early testing within the primary phase.

Treatment: Management of the disease includes a single intramuscular injection of penicillin G or a single dose of oral azithromycin. Doxycycline and tetracycline are also used as alternative treatment options. Physicians should be aware that patients with hepatitis being treated for syphilis demonstrate a high incidence of Jarish-Herxheimer reaction when treatment is initiated. This involves myalgia, fevers, and chills upon therapy initiation. Although the cause of this is not well defined, it is believed to be initiated by the release of pyogenes from spirochetes being killed by antibiotic therapy. Although this reaction is often self-limiting, NSAIDs and steroids have been used as a pretreatment.

Neisseria gonococcus

A well known manifestation of *Neisseria gonococcus* is the Fitz-Hugh–Curtis syndrome, which involves liver capsule inflammation, with ensuing fibrosis between the liver capsule and the parietal peritoneum. Liver involvement more commonly affects females, with cervical involvement being

present in only 1 % of patients. It is believed that the bacteria enter the peritoneal cavity via the fallopian tubes, resulting in a peritonitis and perihepatitis. Symptoms include right upper quadrant pain, tenderness, and fever. White cell counts are often elevated, and the hallmark "violin string adhesions" may be seen on laparoscopy. Organisms may be cultured from peritoneal washings.

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The Diagnosis and Classification of Parasitic Diseases of the Liver

11

Shyamapada Mandal and Manisha Mandal

Key Points

- The recovering of trophozoites and cysts *of Entamoeba histolytica* in feces of patients with hepatic amebiasis strengthens the hypothesis of amebic etiology.
- A semisynthetic derivative of a novel morphinan alkaloid, tazopsine, isolated from *Strychnopsis thouarsii* stem bark, has been found active against *P. falciparum* hepatic stages.
- Using bone marrow aspirate direct examination and indirect immunofluorescence antibody test, confirmation of visceral leishmaniasis might be possible in nearly 100 % of cases.
- *Toxoplasma gondii* is an obligate intracellular parasite of the phylum apicomplexa. *T. gondii* infection with liver cirrhosis has been reported in an epidemiological study.
- Fine-needle aspiration cytology (FNAC) from the liver abscess showed the presence of fertilized eggs of *Ascaris lumbricoides*; liver abscess due to ascariasis is indeed a rare, though a known entity, constituting about 1 % of total cases of hepatobiliary ascariasis.
- *Capillaria hepatica* (*C. hepatica*) is a parasitic nematode causing hepatic capillariasis in numerous mammals. *C. hepatica* might lead to serious liver disorders; relevant clinical reports are rare, because of the nonspecific nature of clinical symptoms, leading to misdiagnosis.
- Liver infection with *E. granulosus* results in the development of one or several unilocular hydatid cysts, while *E. multilocularis* metacestodes develop as a series of small, interconnected cysts, growing as a metastasising lesion almost exclusively in the liver.
- The trematode parasites causing liver diseases include *C. sinensis* (causing clonorchiasis), *O. viverrini* and *O. felineus* (causing opisthorchiasis), and *F. hepatica* and *F. gigantica* (causing fascioliasis) and are known as liver flukes, and from the public health point of view these are food-borne parasites.
- Infectious liver diseases can be accurately evaluated with ultrasonography (US), computed tomography (CT), and magnetic resonance (MR) imaging.

Introduction

Parasitic infections are endemic and represent a major public health problem in developing countries. The protozoan pathogens have become a major threat to human health; the helminthic infestation is exceedingly common on a global scale, and the liver is frequently the primary organ involved.

The protozoan diseases of humans such as toxoplasmosis and amebiasis, caused respectively by the infection of *Toxoplasma gondii* (*T. gondii*) and *Entamoeba histolytica* (*E. histolytica*), are contracted from contaminated food and/or water, while leishmaniasis (visceral form) and malaria are caused by vector-borne parasites *Leishmania donovani* and *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae,* and *P. knowlesi*), respectively.

Helminths (parasitic worms) that infect the liver and hepatobiliary system include nematodes (roundworms), cestodes (tapeworms), and trematodes (flatworms or flukes).

Among the parasitic liver diseases of humans caused by the nematode infection, toxocariasis results from zoonotic transmission of the round worms, *Toxocara canis* (*T. canis*) of dogs and *Toxocara cati* (*T. cati*) of cats. The pathogenesis of disease can be attributed to physical obstruction of the intestine or hepatobiliary tract in *Ascaris lumbricoides* (*A. lumbricoides*) infection caused by the ingestion of fecally excreted embryonated *A. lumbricoides* eggs. *Capillaria hepatica* infection is a known cause of human liver disease.

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The most serious human disease caused by larval cestode (phylum Platyhelminthes), involving the liver, is echinococcosis—a parasitic zoonosis, resulting from accidental infection with larval stages of the canid tapeworm, *Echinococcus granulosus* (*E. granulosus*), which frequently occurs as an adult in dogs and as a larval cyst in wild and domesticated animals including sheep.

Several species of flukes (phylum Platyhelminthes and class Trematoda) infect humans through food consumption, and only that cause liver infection are considered here. Clonorchiasis and opisthorchiasis are trematodiases caused by the infestation of liver flukes (family Opisthorchiidae): *Clonorchis sinensis* (*C. sinensis*), *Opisthorchis viverrini* (*O. viverrini*), and *Opisthorchis felineus* (*O. felineus*); these are closely related trematodes and have similar life cycles and the same pathophysiology and disease manifestations. *Fasciola hepatica* (*F. hepatica*) and *Fasciola gigantica* (*F. gigantica*), belonging to the family Fasciolidae, cause fascioliasis in sheep and cattle; humans are accidental hosts.

The parasitic infection to humans is the cause of major public health problem in the globe, and hence the present chapter focuses on updated findings on clinical, diagnostic, and treatment aspects and parasitic diseases of liver, which can be applied to current protocols in endemic areas.

Protozoan Infection

The early and proper diagnosis of protozoan parasitic infection causing liver disorder help treat the patients effectively with chemotherapeutic regimen as shown in Table 11.1; the protozoan infection involving liver are discussed below.

Amebic Liver Abscess

Etiology

The amebic liver abscess (ALA; an inflammatory space-occupying lesion of the liver), is the extra-intestinal form of amebiasis, the etiological agent of which is *E. histolytica* that exists in two forms: cyst (infective stage) and trophozoite (invasive stage). Infection occurs through the ingestion of cysts in contaminated water (and food). Excystation in the colon result in the formation of trophozoite that can penetrate and invade the colonic mucosal barrier, leading to symptomatic amebiasis. The trophozoites can spread hematogenously via the portal circulation to the liver, and then to other organs like lungs and brain. The extra-intestinal infection by *E. histolytica* mostly involves liver; pleuro-pulmonary involvement, known as the second most common extra-intestinal pattern of infection, is frequently associated with ALA.

Clinical Features

ALA is the most common inflammatory space-occupying lesion of the liver due to *E. histolytica*, for which colon is the initial site of infection. Most of the patients present with an acute illness and duration of symptoms <2 weeks; the main presenting features include abdominal pain (which is usually moderate and restricted to the right upper quadrant or to the epigastrium), fever (moderate in most instances), and anorexia. Cough with or without expectoration and pleuritic chest pain has also been reported in ALA. The acute phase of amebiasis (amebic hepatitis) causes tender hepatomegaly, which is more common in chronic carriers, and an amebic abscess may develop. Early and correct diagnosis of ALA is imperative, because delayed diagnosis and treatment leads to complication including rupture of abscesses [1].

Diagnosis

The liver abscesses can be detected by abdominal ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI). The typical appearance is a nonhomogeneous, hypoechoic, round or oval mass with well-defined borders; still these cannot distinguish amebic from pyogenic abscesses. Shenoy et al. [2] reported a case of hepatomegaly (liver span: 20.5 cm) by abdominal US with abscess in the right lobe; CT abdomen showed right liver abscess with multiple septations, but the proper diagnosis was due to sputum microscopy (revealing the presence of *E. histolytica* trophozoites) and by serology. However, percutaneous diagnostic needle aspiration may sometimes be required to differentiate between amebic and pyogenic liver abscess. The identification of *E. histolytica* can be supported by the signs and symptoms presented by the patients and the detection of erythrophagocytic trophozoites. The recommended serological test like ELISA demonstrates the

Table 11.1 Chemotherapeutic regimen for diseases caused by protozoan parasites involving human liver infection. ALA; amebic liver abscess, VL; visceral leishmaniasis

Protozoan infection	Parasite involved	Treatment protocol	Reference
ALA	*E. histolytica*	Metronidazole (750 mg orally 3 times a day × 7–10 days) *Tinidazole* (800 mg orally 3 times a day × 5 days)	Haque et al. [78]
Malaria hepatic stage	*P. vivax* *P. ovale*	*Primaquine* (30 mg base orally per day × 14 days) for radical cure of *P. vivax* and *P. ovale* (to eliminate hypnozoites)	Griffith et al. [74]
VL	*L. donovani* complex	*Liposomal amphotericin* B (intravenously 1–3 mg per kg per day × 5 days) *Miltefosine* (oral dose of 2.5 mg per kg per day × 28 days)	Murray et al. [75]

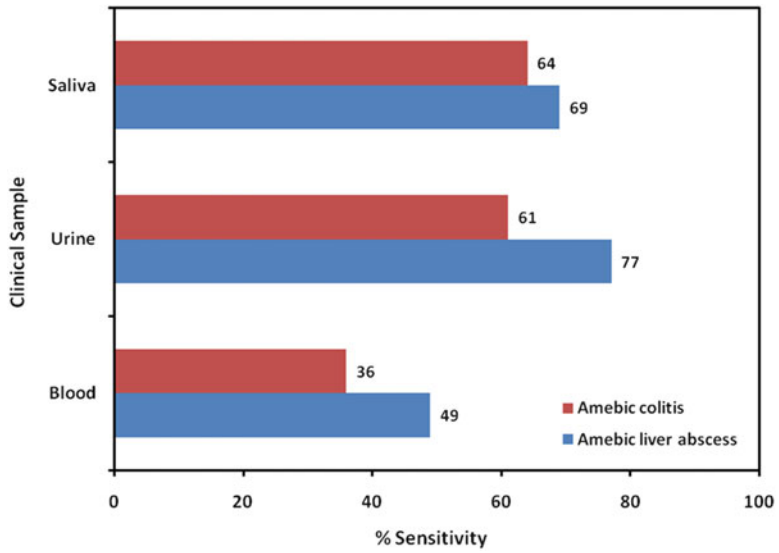

Fig. 11.1 Percent sensitivity of *E. histolytica* DNA detection by real-time PCR in different clinical samples from cases of amebic liver abscess and amebic colitis (text data converted [4])

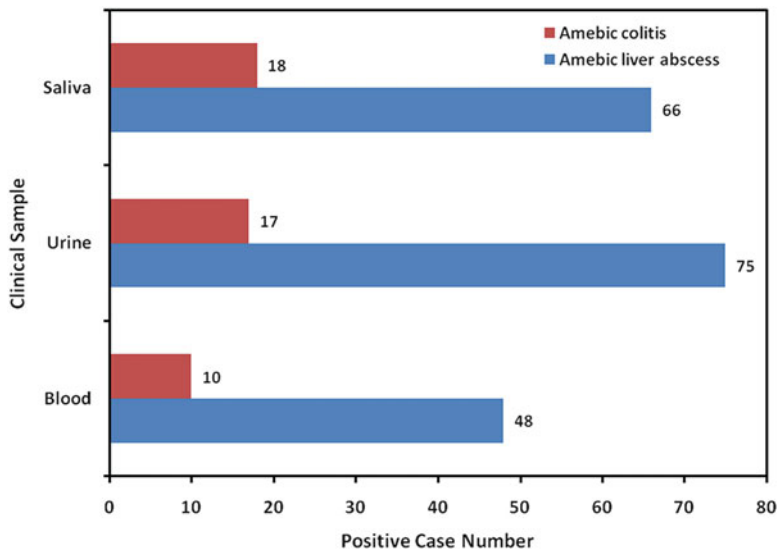

Fig. 11.2 Parasitic load in different clinical samples from cases of amebic liver abscess and amebic colitis (data table partly converted [4])

presence of serum anti-lectin antibodies, which is nearly 100 % sensitive, and thus promising in diagnosis of patients with ALA and asymptomatic *E. histolytica* infection [3]. It has been shown that urine and saliva are more suitable specimens than blood for detection of *E. histolytica* DNA in ALA patients [4]; the sensitivity of real-time PCR using different clinical samples, with percent detection and parasitic load of ALA cases, are depicted in Figs. 11.1 and 11.2.

Treatment

The amebicides such as emetine and dehydroemetine act in the liver and intestinal wall, and chloroquine acts only in the liver; oral or intravenous metronidazole or tinidazole leads to rapid clinical improvement of ALA. However, proper and timely treatment of luminal amebiasis with duodohydroxyquin, diloxanide furoate, and paromomycin help protect to form ALA. The aspiration of liver abscess has been indicated in lack of clinical improvement in 48–72 h, left lobe abscess, thin rim of liver tissue around the abscess (<10 mm), and seronegative abscesses [6], while open surgical drainage may be required in cases of a large abscesses with a poor yield on needle aspiration. Having treatment with metronidazole or tinidazole for invasive amebiasis, luminal agents (diloxanide furoate, paromomycin, and iodoquinol) can be given to disrupt intestinal colonization, and thus asymptomatic patients with confirmed *E. histolytica* infection should be treated with luminal agents in order to prevent the development of invasive amebiasis including ALA [7].

A number of ameba proteins have been tested as possible vaccine candidates, some of which are found effective in the animal model, and the two most promising candidates include the 25-kDa serine-rich *E. histolytica* protein (SREHP) and the 260-kDa galactose/N-acetyl galactosamine-inhibitable ameba lectin (Gal/GalNAc) [8].

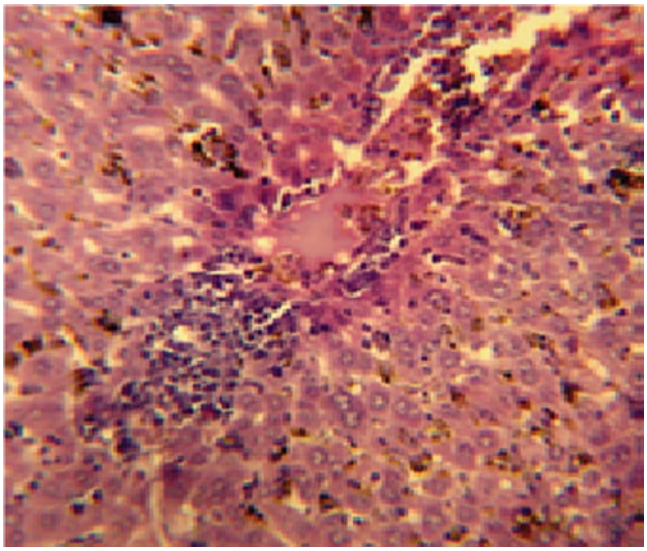

Fig. 11.3 Control (infected without treatment) liver: showing severe hepatic necrosis with Kupffer cell hyperplasia and hemosiderosis (adapted from Soniran et al. [9])

Malaria (Asymptomatic Liver Stage)

Etiology

After transmission by an infected mosquito, malaria sporozoites enter a blood vessel at the bite site and travel via the bloodstream to the liver, their initial site of replication in the mammalian host. *Plasmodium* parasites undergo a clinically silent and obligatory developmental phase in the host liver cells before infecting erythrocytes to cause malaria symptoms. The liver serves as the reservoir for hypnozoites (formed in case of *P. vivax* and *P. ovale* infection)—the dormant parasitic forms, which in activation may lead to relapses long after the initial blood infection.

Clinical Features

Soniran et al. [9] noted hepatic necrosis (liver cell death), hemosiderosis (accumulation of iron) in the liver, and Kupffer cell hyperplasia in mice with established *Plasmodium berghei* infection (Fig. 11.3). Sporozoites can subsequently pass through a number of hepatocytes, which die by necrosis, before settling in a hepatocyte for further liver stage development; however, hepatic malaria remains asymptomatic; in the *Plasmodium* life cycle, the asexual blood stages (rings, trophozoites, schizonts) are responsible for the symptoms of malaria, and thus are the main target of chemotherapy.

Diagnosis

Microscopy is the standard method for parasitological diagnosis of malaria and is performed by examining a stained thick or thin blood smear for the presence of malaria parasites; thick film is recommended for parasite detection and thin film is recommended for species identification. The clinical diagnosis of malaria is imprecise and unreliable. The confirmatory tests, to detect the presence of malaria parasites, also include rapid diagnostic tests (RDTs) that detect histidine-rich protein 2 (HRP2) specific for *P. falciparum*, and parasite lactate dehydrogenase (pLDH) or aldolase, which have the ability to differentiate between falciparum and non-falciparum malaria (vivax, malariae, and ovale). The infection can be diagnosed through the detection of antibodies to malaria parasites and through PCR-based detection of parasite DNA.

Treatment

There is the necessity of two classes of drugs: one for the treatment of acute malaria and the other for the elimination of liver stages to avoid subsequent relapse (which is an acute blood-stage infection originating from a hypnozoite). The primary tissue schizontocide acting on schizont in liver includes primaquine, which is also known as hypnozoitocide killing the dormant hypnozoites in liver. A semisynthetic derivative of a novel morphinan alkaloid, tazopsine, isolated from *Strychnopsis thouarsii* stem bark, has shown to be specifically active against *P. falciparum* hepatic stages [10]. da Cruz et al. [11] demonstrated that decoquinate emerged as the strongest inhibitor of *Plasmodium* liver stages in vitro and showed that the oral administration of a single dose of the drug can effectively prevent the appearance of disease, warranting its exploitation as a potent antimalarial compound.

Visceral Leishmaniasis

Etiology

Visceral leishmaniasis (VL), also known as Kala-azar, is mainly caused by two species of the *Leishmania* parasite: *L. donovani*, prevalent in South Asia and East Africa, and *L. infantum*, prevalent in the Mediterranean region and in Latin America. *L. donovani* and *L. infantum* spread systemically to propagate in macrophage of internal organs, primarily the liver. The female phlebotomine sandflies transmit the disease, by inoculation of the promastigote form into the skin. The parasites disseminate through the lymphatic and vascular systems and infect other monocytes and macrophages in the reticulo-endothelial system, resulting in infiltration of the bone marrow, hepatosplenomegaly, and sometimes enlarged lymph nodes (lymphadenopathy).

Clinical Features

The expression infection varies from none (subclinical), to oligosymptomatic, to fully established (kala azar). The disease is characterized by prolonged fever, enlarged spleen and liver, substantial weight loss, and progressive anemia. As the disease advances, splenomegaly can increase, causing

abdominal distension and pain, which is sometimes increased by concomitant hepatomegaly [12].

Diagnosis

Because that the current drugs used in the treatment of VL are toxic and as the clinical presentation of the disease lacks specificity, confirmatory tests are required. Laboratory confirmation of the diagnosis is achieved by detecting *Leishmania* parasites or DNA in infected tissue (such as in bone marrow, liver, lymph node, or blood), through light-microscopic examination of stained specimens, culture techniques, or molecular methods. Serologic testing can provide supportive evidence for the diagnosis. The 200 kDa *L. donovani* amastigote antigenic fraction was found 96.6 % sensitive and 100 % specific [13]. The detection of parasites in the blood or organs by culture or by using molecular techniques such as PCR is more sensitive than microscopic examination.

Treatment

Miltefosine, paromomycin, and liposomal amphotericin B are gradually replacing pentavalent antimonials and conventional amphotericin B as the preferred treatments in some regions. Liposomal amphotericin B is a very safe and highly effective treatment for primary VL in *L. infantum* endemic areas and in the *L. donovani* South Asian focus (India, Bangladesh, Nepal), where it was recently recommended as first-line treatment by the WHO expert committee on the control of leishmaniasis [14, 15]. The 17-day SSG and paromomycin combination treatment had a good safety profile and was similar in efficacy to the standard 30-day sodium stibogluconate treatment, suggesting suitability for VL treatment in East Africa [16].

Toxoplasmosis

Etiology

An apicomplexan parasite *Toxoplasma gondii* (*T. gondii*) infects humans worldwide causing toxoplasmosis, which is an important zoonotic disease in humans. Major routes of infection with *T. gondii* include eating undercooked or raw meat (of sheep, goats, pigs) containing tissue cysts, and ingesting food or water contaminated with oocysts (oval in outline measuring 10–15 μm long and 8–12 μm wide) shed by cats [17]. Alvarado-Esquivel et al. [18] reported that the seropositivity to *T. gondii* was comparable among liver disease patients and controls; however, more studies with larger sample sizes are needed to elucidate the association of *T. gondii* with liver disease.

Clinical Features

The parasite mainly affect the central nervous system, and various other organs (lymph nodes, eyes, and heart) in human body, and can be associated with liver disease, in which a number of pathological changes like hepatomegaly, granuloma, hepatitis, and necrosis are seen to occur; *T. gondii* infection with liver cirrhosis has been reported in an epidemiological study [19].

Diagnosis

Sabin-Feldman dye test is highly sensitive and specific to detect *T. gondii* infection, with no evidence for false results in humans, IgM-ELISA tests have proved useful for screening programs; detection of *T. gondii* DNA from a single tachyzoite using the B1 gene in PCR has proven very useful in the diagnosis of clinical toxoplasmosis [20]. Su et al. [21] documented some widely used molecular methods and proposed an integrated approach for the detection and identification of *T. gondii*.

Treatment

As the treatment regimen sulfonamides found effective against *T. gondii*, pyrimethamine found synergistic with sulfonamides against dividing tachyzoites, folic acid, and yeast improves activity of sulfadiazine and pyrimethamine; spiramycin and clindamycin found to have anti-toxoplasmic activity [20]. Kavitha et al. [22] reported that fractions from *Eurycoma longifolia* root are likely the sources of new compounds that could be used to treat *T. gondii* infections.

Nematode Infection

The nematode infection to human liver includes the roundworms such as *A. lumbricoides*, *C. hepatica*, *T. canis,* and *T. cati* that require chemotherapeutics for effective treatment (Table 11.2).

Ascariasis

Etiology

A. lumbricoides, the human intestinal nematode, causes ascariasis, which is the most widespread helminthiasis worldwide. Biliary ascariasis is the most common extra-intestinal complication of *A. lumbricoides* infestation, and the liver abscess as a part of complication of biliary ascariasis is seen but uncommon. This parasitic infection causes ascariasis of the liver, which can be caused by adult worms and eggs located in the bile ducts or in the liver parenchyma and it may be due to larvae remaining in the hepatic parenchyma during their life cycle.

Clinical Features

The patients infected with *Ascaris* can present with biliary colic, tender hepatomegaly, acute cholangitis, acalculous

Table 11.2 Chemotherapeutic regimen for nematodiases involving human liver infection

Nematodiasis	Parasite involved	Treatment protocol	Reference
Ascariasis	*A. lumbricoides*	*Albendazole* (single-dose 400 mg)	Pockros and Capozza [24]
		Mebendazole (single-dose 500 mg or 100 mg twice daily × 3 days)	
Hepatic capillariasis	*C. hepatica*	*Disophenol* (intramuscular single-dose of 7.5 mg/kg body weight)	Li et al. [30]
		Pyrantel tartrate (single oral dose of 30 mg/kg body weight)	
Toxocariasis	*T. canis*	*Albendazole* (400 mg twice daily × 5 days) or mebendazole (100–200 mg twice daily × 5 days)	Pockros and Capozza [24]
	T. cati	*Diethylcarbamazine* (3 mg/kg 3 times daily × 14–21 days alternatively)	Treska et al. [76]

cholecystitis, acute pancreatitis, and hepatic abscess. The female penetrates deeply into the bile ducts, lays eggs that are carried into the liver parenchyma causing granuloma, known as granulomatous hepatitis. Another possibility is the production of a liver abscess when the adult parasite dies inside the liver, giving rise to a necrotic focus. Hepatobiliary and pancreatic disease is most commonly caused by direct mechanical obstruction of the pancreatobiliary system from adult worms.

Diagnosis

The diagnosis of ascariasis can be done with the identification of an adult worm, larva, or egg from a patient. It has been reported that the diagnosis of biliary ascariasis with liver abscess is made by ultrasonogram of the abdomen, and after ERCP (endoscopic retrograde cholangiopancreatography) multiple round worms have been extracted from the common bile duct [23]. The specific diagnosis of liver ascariasis has been established by the observation of eggs; most of the cases reported have been diagnosed during surgery or during autopsy. Transabdominal ultrasound, CT scan, or MRI can be useful in diagnosing hepatobiliary ascariasis.

Treatment

The current treatment of choice is with one of the two benzimidazole compounds: albendazole or mebendazole. Single-dose therapy with albendazole and mebendazole are effective; piperazine citrate has been recommended in pregnancy. Fine-needle aspiration cytology (FNAC) from the liver abscess showed the presence of fertilized eggs of *A. lumbricoides* [5].

Toxocariasis

Etiology

Toxocariasis is a zoonotic disease caused by the infection with larvae of ascarid nematode *Toxocara canis* and *T. cati*, the adults of which reside in the digestive tract of their definitive host, the dogs and cats, respectively. Once the embryonated *Toxocara* eggs are accidentally ingested by humans, the larvae hatch in the small intestine and migrate mainly through liver, lungs, and central nervous system.

Clinical Features

The major clinical consequences of prolonged migration of *T. canis* larvae in humans are visceral larva migrans (VLM) and ocular larva migrans (OLM). VLM occurs most commonly in young children and results in chronic eosinophilia, malaise, fever, hepatomegaly, and upper abdominal discomfort; some patients may also have nausea and vomiting. Patients can develop granulomatous hepatitis, hepatic abscesses, and/or tender hepatosplenomegaly [24].

Diagnosis

Serology, using *T. canis* excretory–secretory (TES) products of the larvae, is an effective laboratory-based option for diagnosis of hepatic toxocariasis, and has been considered a useful predictor of *T. canis* infection when coupled to relevant clinical data. In patients with hypereosinophilia and hepatic parenchymal nodules on CT and sonography, ELISA with *Toxocara* excretory/secretory antigen should be performed; in patients with sustained hypereosinophilia showing multiple small ill-defined, low-attenuating, or hypoechoic nodular lesions in the liver on CT and sonography, VLM of *T. canis* should be considered [25]. The enzyme immunoassay (EIA) using TES antigens from infective-stage larvae is the most useful diagnostic test for toxocaral VLM; for VLM and some forms of covert toxocariasis, the sensitivity and specificity of the *Toxocara* EIA has been estimated at 78 % and 92 %, respectively [26]. A duplex quantitative real-time PCR (2qPCR) targeting the ribosomal RNA gene internal transcribed spacer (ITS2) appears to be a very promising tool for rapid and specific identification of *T. canis* and *T. cati* eggs in fecal and soil samples [27]. Human stool microscopy is of no benefit because *Toxocara* species do not complete their life cycle in humans.

Treatment

The drugs potentially effective in toxocariasis include benzimidazoles (albendazole, mebendazole, and thiabendazole) and diethylcarbamazine [28]. In severe infestation, systemic corticosteroids have been advocated to reduce inflammatory complications. A patient with confirmed hepatic toxocariasis treated with albendazole (400 mg twice daily for 5 days) continued to have fever, and hence given mebendazole

(200 mg twice daily for 5 days); following 1 month of treatment the patient's symptoms had resolved [29].

Hepatic Capillariasis

Etiology

The etiological agent of hepatic capillariasis, a serious liver disorder of mammals including humans, is a nematode parasite, *C. hepatica* (order Trichurida, family Trichinellidae); the adults colonize the liver of the hosts. The parasite could accidentally be transmitted to humans by ingestion of the embryonated eggs. The female measures 53–78×0.11–0.20 mm, but males are 24–37×0.07–0.10 mm; the *C. hepatica* egg measures 48–66×28–36 mm, and numerous minipores are seen in the outer shell [30]. Soon after egg-laying the adults die and disintegrate, causing focal necro-inflammatory lesions.

Clinical Features

The adults, and the eggs laid by the females can cause chronic inflammation in the liver; the inflammatory infiltration may persist until encapsulation, or calcification of dead worms. The experimental findings of Gomes et al. [31], suggested that focal lesions and septal fibrosis are by *C. hepatica* infection. Infection of hepatic tissue with *C. hepatica* causes clinical symptoms similar to acute viral hepatitis and that the classical triad (fever, hepatomegaly, and eosinophilia) may also be present [30, 32].

Diagnosis

Huang et al. [33] reported that the diagnosis of hepatica capillariasis is possible by ELISA, with high sensitivity and specificity, against *C. hepatica* infection. The liver biopsy is a precise and quick method of confirming *C. hepatica* infection [30], but serological testing by indirect immunofluorescence assay is also recommended for diagnostic and screening purpose [34].

Treatment

The medication with albendazole has been reported effective against *C. hepatica*; [35] the infection can be treated with prednisone, disophenol, and pyrantel tartrate. It has been demonstrated that the adults can be killed, and the egg-laying activity can be prevented with the administration of disophenol intramuscularly with a single dose (7.5 mg/kg body weight) and with pyrantel tartrate orally (30 mg/kg body weight) [30].

Cestode Infection (Cestodiasis)

The helminthic tapeworms, the cestodes, causing human liver infection include *Echinococcus multilocularis* (*E. multilocularis*) and *Echinococcus granulosus* (*E. granulosus*).

Echinococcosis

Etiology

Echinococcosis is a zoonosis caused by the infection of larval stages of taeniid cestodes belonging to the genus *Echinococcus*. The disease can be differentiated into cystic echinococcosis (CE) and alveolar echinococcosis (AE), associated with *E. multilocularis* (dog tapeworm) and *E. granulosus* (tapeworm of fox) infection, respectively. When dispersed eggs are taken by humans, oncospheres that are released into the duodenum penetrate into the intestinal wall and enter the vessels of the portal vein to reach to the liver—the major site for cyst development (about 75 % of cases) both in the alveolar and in the cystic forms. The *E. granulosus* young cysts are spherical, unilocular vesicles, consisting of an internal germinal layer and an outer acellular layer, while the *E. multilocularis* has a tumor like, infiltrative behavior, which is responsible for tissue destruction and finally for liver failure.

Clinical Features

The liver disease in echinococcosis results from the significant destruction of the hepatic parenchyma by the parasitic cysts; usually the right lobe of the liver represents the site for metacestode establishment. Liver infection with *E. granulosus* results in the development of one or several unilocular hydatid cysts, while *E. multilocularis* metacestodes develop as a series of small, interconnected cysts, growing as a metastasising lesion almost exclusively in the liver [35]. *E. multilocularis* produces multilocular alveolar cysts (1–10 mm in diameter) that resemble alveoli; [36] the number and size of different types of lesions are depicted in Table 11.3 and Fig. 11.4. The cyst types on US from cystic echinococcosis patients are described by Brunetti et al. [37]; Table 11.4 shows the cyst characteristics. The disease is usually asymptomatic for a long period of time, because cyst growth is commonly slow; the most frequent symptoms include fatigue and abdominal pain; patients may present jaundice, hepatomegaly, or anaphylaxis, due to cyst leakage or rupture. The AE of the liver may cause Budd-Chiari syndrome-related hepatic encephalopathy [38].

Table 11.3 The magnetic resonance imaging (MRI) characteristics of alveolar echinococcosis in the liver

Lesion type	Characteristics
Type 1	Presence of numerous small round cysts without any solid component
Type 2	Presence of multiple small round cysts with a solid component
Type 3	Presence of a solid component around large irregular cysts with many small round cysts
Type 4	Presence of a solid component without cysts
Type 6	Presence of a large cyst without solid component

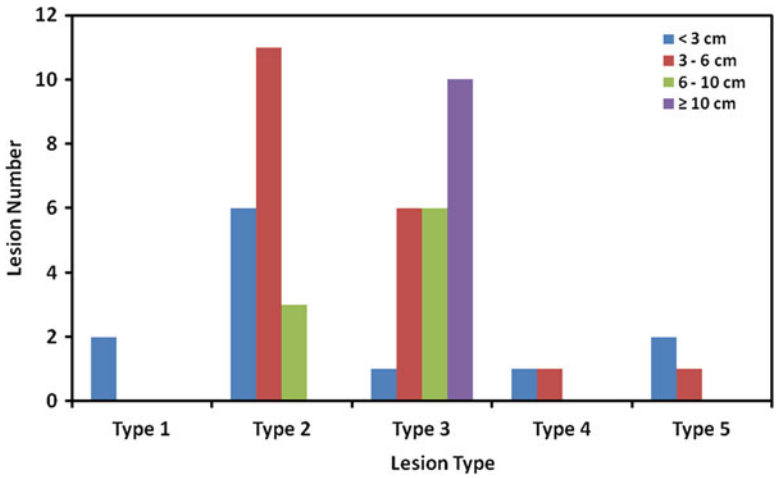

Fig. 11.4 Type, number, and size of alveolar echinococcal lesions on MRI of liver (data table partly converted [36])

Table 11.4 Ultrasound classification of hepatic echinococcal cysts from *E. granulosus* infection (Brunetti et al. [37])

Lesion type	Characteristics
CE 1	Presence of unilocular unechoic cystic lesion with double line sign
CE 2	Presence of multiseptated honeycomb cyst
CE 3A	Presence of cyst with detached membranes
CE 3B	Presence of cyst with daughter cysts in solid matrix
CE 4	Presence of cyst with heterogenous hypoechoic/hyperechoic
	Contents without daughter cysts
CE 5	Presence of solid and calcified wall

On CT scan a pedunculated hydatid cyst of 45 × 35 × 20 cm from the left lobe of liver, occupying the whole peritoneal cavity, has currently been reported; [39] the largest hydatid cyst recorded earlier was of 37 × 14.88 × 15 cm; [40] the two cases were cured by partial cystectomy and PAIR (puncture, aspiration, injection, and re-aspiration), respectively.

Diagnosis

The diagnosis of hepatic echinococcosis (CE and AE) is based on clinical and epidemiologic findings, imaging techniques, nucleic acid detection, and serology. Demonstration of scolices, hooklets, or protoscolices in aspirated fluid by microscopy is very specific, yet aspiration of hydatid fluid for diagnosis is not usually recommended because of the risk of an anaphylactic reaction.

The noninvasive imaging techniques like CT scans, MRI, and US imaging are used in detecting hydatid cysts in infected organs including the liver [41]. At CT, the *E. granulosus* cyst usually appears as a sharply defined, hypoattenuating lesion with a thick wall, while MR imaging demonstrates the pericyst, matrix or hydatid sand, and the daughter cysts; a solid component is rarely seen [36, 42]. The CT and MR images typically display multiple irregular, ill-defined lesions scattered throughout the involved liver that are generally hypoattenuating at CT and hyperintense at MR imaging [42]. The MR findings of AE in the liver are multiple small round cysts with a weakly enhanced solid component, which can be a large and/or irregular lesion [36].

Combining US and serological data, it is possible to classify seropositive patients into three groups: with active hepatic lesions, with calcified lesions, and with no evidence of hepatic lesions [43]. On the basis of the radiographic findings, the suspected diagnosis of echinococcal disease was found positive with a serologic test (such as ELISA) for *E. multilocularis* infection [38]. A rapid dot immunogold filtration assay for serodiagnosis of human CE and AE has been developed using four native antigen prepared from cyst fluid extracts of *E. granulosus* (EgCF and AgB), *E. granulosus* protoscolex extract (EgP), and *E. multilocularis* metacestode antigen (Em2) [44].

Detecting the presence of specific microsatellite sequences and mitochondrial 12S rDNA the AE can clearly be distinguished from CE [45].

Treatment

The current methods of treatment include surgery, and percutaneous drainage consisting of PAIR; these methods are used principally for liver cysts. Treatment with benzimidazole (albendazole and mebendazole) demonstrated efficacy in the management of liver infection [46]. Long-term treatment with mebendazole (50 mg/kg/day) or albendazole (15 mg/kg/day) inhibits growth of larval *E. multilocularis*, and both albendazole (10–15 mg/kg/day) and mebendazole (40–50 mg/kg/day) have demonstrated efficacy against CE [47]. The combination treatment with albendazole and praziquantel has been used successfully in the treatment of hydatid disease; percutaneous treatment of liver cysts combined with albendazole has been recorded superior to surgical cystectomy. Beside this, repetitive surgical operations and accompanying disorders may increase postoperative mortality [48].

Trematode Infection (Trematodiasis)

The trematode parasites causing liver diseases include *C. sinensis* (causing clonorchiasis), *O. viverrini* and *O. felineus* (causing opisthorchiasis), and *F. hepatica* and *F. gigantica* (causing fascioliasis) and are known as liver flukes, and from the public health point of view these are food-borne parasites. *C. sinensis* and *O. viverrini* induce cholelithiasis, cholestasis, cholangitis, cholecystitis, biliary and liver abscess and cirrhosis, pancreatitis, hepatitis, and cholangiocarcinoma, while the patients with *O. felineus* and *Fasciola* exhibit same pathological changes and clinical manifestations as *C. sinensis* and *O. viverrini*, except the carcinogenic potentiality [49]. The adult *C. sinensis* can be distinguished from *O. viverrini* and *O. felineus* by the presence of branched testes in tandem position and the continuously distributed vitelline glands; [50] the adult morphology of *O. viverrini* and *C. sinensis* are presented in Fig. 11.5. The anti-trematodial drugs are depicted in Table 11.5.

Fig. 11.5 Adult flukes: (**a**) *O. viverrini* and (**b**) *C. sinensis* (adapted from Sripa et al. [73])

Fascioliasis

Etiology

Liver flukes belonging to the genus *Fasciola* are among the causes of food-borne diseases of parasitic etiology. The disease fascioliasis is caused by the infection of *F. hepatica* (in temperate and subtropical areas) or *F. gigantica* (in tropical and subtropical zones). Ingestion of freshwater plants such as watercress contaminated with infective metacercariae is known to constitute the fascioliasis infection in humans. Mature flukes measure 20–40 mm long and 8–12 mm wide.

Clinical Features

The infection with *F. hepatica* comprises two clinical phases: the hepatic (acute) phase occurs when the worm enters the liver and begins to migrate through the parenchyma, and the clinical features of this stage include fever, abdominal pain, nausea, vomiting, eosinophilia, hepatomegaly, weight loss, elevated liver enzymes, and hypergammaglobulinemia, while the biliary (chronic) stage manifests as intermittent right upper quadrant pain, cholangitis, bile duct stones, and biliary obstruction [51]. Hepatic fascioliasis manifests as clusters of microabscesses arranged in a characteristic tract-like fashion, usually in the subcapsular regions; a large cyst-like necrotic lesion may also be seen [52]. The infection may be characterized with eosinophilia, leucocytosis, fever, anorexia, and weight loss; [53] the distribution of the symptoms is depicted in Fig. 11.6.

Diagnosis

Diagnosis mainly relied on egg finding, followed by serology, intradermal reaction, surgery, and erratic fluke observation [53]. Detection of eggs in the feces, bile, or duodenal aspirate is the definitive test. The *F. hepatica* eggs (ellipsoidal and light yellow-brown in color) measure 130–150×63–90 μm

Table 11.5 Chemotherapeutic regimen for trematodiases involving human liver infection

Trematodiasis	Parasite involved	Treatment protocol	Reference
Fascioliasis	*F. hepatica*	*Triclabendazole* (10–12 mg/kg/day) *Biothionol* (alternative therapy: 30–50 mg/kg/day × 10–15 days)	Pockros and Capozza [24]
	F. gigantica	*Dehydroemetine* (alternative intramuscular 1 mg/kg/day × 14 days)	
Clonorchiasis	*C. sinensis*	*Praziquantel* (25 mg/kg 3 times/day × 1 day)	Marcos et al. [77]
Opisthorchiasis	*O. felineus*	*Praziquantel* (75 mg/kg in 3 divided doses over 1 day, or 40–50 mg/kg single dose)	Pockros and Capozza [24]; Mairiang and Mairiang [62]; Marcos et al. [77]
	O. viverrini	*Albendazole* (alternatively 10 mg/kg/day × 7 days)	
Schistosomiasis	*S. mansoni*	Praziquantel (40 mg/kg/day in 2 divided doses over 1 day for *S. mansoni*, and 60 mg/kg/day in three divided doses over 1 day for *S. japonicum*)	Pockros and Capozza [24]
	S. japonicum		

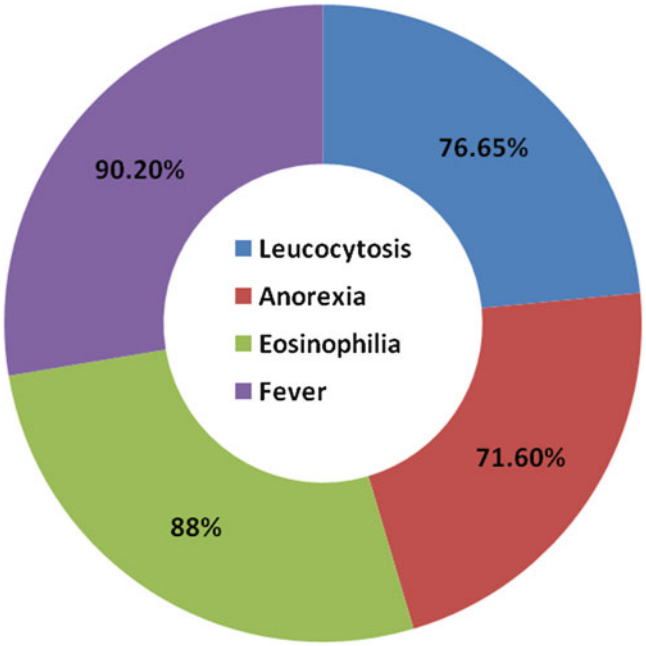

Fig. 11.6 Various symptoms in human infection with *F. hepatica*

and have an indistinct operculum [51]. The ELISA-based tests are highly sensitive (100 %) and specific (97 %) in diagnosing the infection [53]. The ELISA testing is sensitive for both hepatic and biliary stages, while stool tests for *F. hepatica* eggs are positive only in the biliary stage. In any patient with peripheral eosinophilia, abdominal pain, and elevated liver enzymes, especially when CT reveals tubular and nodular hypodense lesions particularly in subcapsular area, *F. hepatica* infection should be considered [54]. Hepatic US demonstrates adult flukes in bile ducts and hyperechoic lesions caused due to migrating larvae in the organ. *F. gigantica* was found to be different from *F. hepatica* at five nucleotide positions in the first internal transcribed spacers (ITS-1) of rDNA sequences [55].

Treatment

Emetine appears as the drug mostly used against *F. hepatica* infection. The Centers for Disease Control and Prevention recommended triclabendazole as the first-line agent for the treatment of *F. hepatica*, and bithionol has been reported as an alternative drug [54]. In biliary fascioliasis, ERCP and sphincterotomy have been used for diagnosis and therapy to extract the parasites from the biliary tree [51]. Surgery reports are numerous.

Clonorchiasis

Etiology

The oriental liver fluke *C. sinensis*, a member of the family Opisthorchiidae, is the etiological agent of a substantial subclinical or clinical disease, called clonorchiasis, which is zoonotic; [56] humans contract the disease through the ingestion of freshwater fish (raw or undercooked) bearing infective metacercariae (round or oval, measuring 0.13–0.14 × 0.09–0.10 mm). Dogs and cats are the most important animal reservoirs for human infection with *C. sinensis*. On ingestion, the metacercariae excyst, travel to the small intestine and liver, feed upon the bile and mature. In humans, the adult *C. sinensis* resides within the biliary tract, mainly inside the intra-hepatic bile ducts.

Clinical Features

The *C. sinensis* infection is characterized by hyperplasia of the intra-hepatic bile duct, followed by periductal fibrosis in chronic cases; the clinical signs include abdominal discomfort, diarrhea, peripheral eosinophilia, fever, acute pain in the right upper quadrant, and in chronic cases, portal hepatomegaly can be seen [56]. Jang et al. [57] reported, on CT findings, of an unusual case of hepatic parasitic abscess with intra-hepatic bile duct dilation due to *C. sinensis* infection.

Diagnosis

The diagnosis of clonorchiasis is done by microscopic findings of fecal eggs [58], which are oval (measuring 27–35 × 12–20 μm), with an operculum at the slender end and prominent shoulders; broad abopercular end with small spine-like structure [56]. However, an accurate and feasible diagnostic method for clonorchiasis is US that characterize clonorchiasis as diffuse, mild, uniform dilatation of the small peripheral intra-hepatic bile ducts without a focal obstructing lesion; the CT imaging of clonorchiasis is same as those observed by US [59]. Li et al. [58] reported that the recombinant Cs26GST and Cs28GST proteins are specific serodiagnostic antigens for human clonorchiasis, because of their non-cross-reactivity to the sera of paragonimiasis, schistosomasis, or cysticercosis; thus, a mixed antigen of recombinant 28 and 26 kDa glutathion S-transferases, Cs28GST and Cs26GST (producing 76 % sensitivity and 95 % specificity), can be considered an useful serodiagnostic reagent for human clonorchiasis. The *C. sinensis* adults are flat, leaf-like, 8–15 mm long and 1.5–4 mm wide and differ from *O. felineus* and *O. viverrini* in having branched testes. A PCR-based molecular identification method, multiplex ligation-dependent probe amplification (MLPA), as has been evaluated by Sun et al. [60], allowed rapid and specific detection of single nucleotide differences between *C. sinensis*, *O. viverrini,* and *O. felineus*, and thus MLPA was found as a potential tool for specific identification of infections by opisthorchid liver flukes in endemic areas.

Treatment

Praziquantel and albendazole are effectively used in the treatment of clonorchiasis, and the cure rates are reported as 98–100 % and 90–100 %, respectively [56].

Opisthorchiasis

Etiology

The etiological agent of opisthorchiasis is *Opisthorchis viverrini* (*O viverrini*) (commonly known as carcinogenic human liver fluke), a food-borne trematode (monoecious), and is a member of the family Opisthorchiidae. Humans are infected by eating raw or undercooked cyprinoid fishes harboring the *O. viverrini* infective metacercariae. In human (definitive host of the parasite), *O viverrini* inhabits mainly in the intra- and extra-hepatic bile ducts and, rarely, in the gallbladder and pancreatic duct. The *O viverrini* infection can induce several pathologic changes in the liver, gallbladder, and extra-hepatic bile ducts. *O. viverrini* is morphologically similar to *C. sinensis* but it is slightly smaller in size (5.4–10.2×0.8–1.9 mm), the main difference from *C. sinensis*.

Clinical Features

The acute symptoms of *O. felineus* infection consist of fever, anorexia, diarrhea or constipation, pain and discomfort in the upper right quadrant of the abdomen and urticarial skin rash, while chronic complications include suppurative cholangitis, liver abscess, and cholangiocarcinoma [61, 62]. In opisthorchiasis, enlargement of the left hepatic lobe and the gallbladder occurs, and the laboratory findings include eosinophilia and increased liver enzymes. Microscopically, the intrahepatic lesions of opisthorchiasis are confined to the biliary tree, particularly to the large- and medium-sized bile ducts where the flukes reside.

Diagnosis

The classic method for the diagnosis of human opisthorchiasis is by microscopic examination of fecal samples for *Opisthorchis* eggs; however, determining correctly the species of the causative parasite on the basis of the presence of eggs is difficult since the eggs of *O. viverrini*, *O. felineus*, and *C. sinensis* are morphologically similar. *O. viverrini* can be distinguished from *O. felineus* in having deeper lobulation and a more posterior location of the testes [50]. On US of the liver, the combination of cystic or mulberry-like dilations of intra-hepatic bile ducts is pathognomonic of opisthorchiasis. Currently, a rapid, specific, and sensitive real-time FRET (fluorescence resonance energy transfer) PCR study has been developed for detection of *O. viverrini* in human stool samples, and the method is considered as a powerful tool for diagnosis of human opisthorchiasis [63].

Treatment

A single dose (40 and 50 mg/kg) of praziquantel treatment provides an *O. viverrini* eradication rate of 91 % and 97 %, respectively, and thus the drug has been used for an opisthorchiasis control program in endemic regions. Mebendazole and albendazole are also effective for the eradication of opisthorchiasis [62].

Schistosomal Hepatitis

Etiology

Three species of the genus *Schistosoma* (*S. mansoni*, *S. japonicum,* and *S. haematobium*) are responsible for causing schistosomiasis in humans [64]. As the worms develop in the liver, it is a focal point of pathogenic insult and subsequent pathological damage in schistosomiasis [65].

The signs and symptoms of schistosomiasis are due to the host immune response to schistosome eggs trapped in the tissues. The eggs secrete antigens that excite eosinophilic inflammatory and liver granuloma formation that progress to fibrosis [66], leading to interruption of normal blood flow in the venous system to the sinusoids resulting in portal hypertension and hepatosplenomegaly.

Clinical Features

In acute schistosomiasis, the main symptoms are fever, headache, myalgia, right upper quadrant pain, and bloody diarrhea; hepatomegaly is usually seen, while in chronic schistosomiasis, granulomas develop at the site of accumulation of eggs, such as liver in case of *S. mansoni* and *S. japonicum*. The eggs of *S. mansoni* and *S. japonicum* embolize to the liver, where granulomatous inflammation as well as fibrosis occurs. The combination of chronic schistosomiasis caused by *S. mansoni* and hepatitis-B virus infection may result in a higher risk of hepatocellular carcinoma. Chronic infection with *S. japonicum* or *S mansoni* results in the formation of cirrhosis and the risk of development of hepatocellular carcinoma [64].

Diagnosis

The diagnosis is based on epidemiologic data, clinical manifestations, eosinophilia, the presence of living eggs at stool examination, or positive serologic findings for *Schistosoma* infection. Demonstration of parasite eggs in stool is the most common method of diagnosis of schistosomiasis and species identification. The seropositive individuals with a history of current contact with cercariae-infested water were examined by US, and those with typical pathological features were defined as cases with chronic schistosomiasis; [67] periportal fibrosis can be seen on US, CT, and MRI and is characteristic of schistosomiasis. PCR-based techniques are capable of detecting DNA released from *S. mansoni*, *S. haematobium,* and *S. japonicum* [68].

Treatment

Praziquantel, which works exclusively against adult worms, is the mainstay of treatment and a critical part of community-based schistosomiasis control programs. Oxamniquine is the only alternative to praziquantel for *S. mansoni* infection [69]. Artemether, which is well known for its antimalarial activity, does kill schistosomula during the first 21 days in the body; combining artemether with praziquantel appears to produce

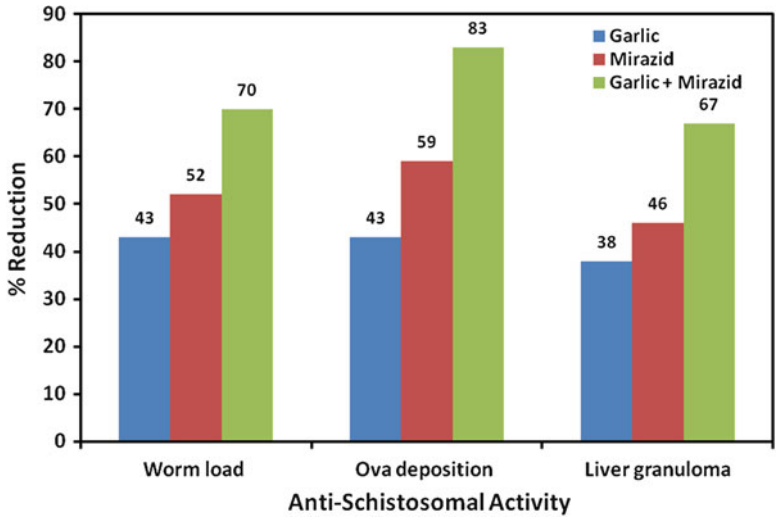

Fig. 11.7 Anti-schistosomal activity of garlic and mirazid alone and in combination in mice-model (data table partly converted [71])

a synergistic killing of adult worms [70]. El-kott et al. [71] reported that *Allium sativum* (garlic), *Commiphora molmol* derivative (mirazid), and a combination of both had anti-schistosomal activity in mice; the reduction in worm load, ova deposition, and liver granuloma (size and number) by the components alone and in combination are depicted in Fig. 11.7.

Conclusion

The parasites infecting the liver include those that may be transmitted by vectors, by food consumption, or by direct transmission from the environment. Many such infections are preventable by simple measures of improved health and sanitation conditions, through better hygiene, proper handling, and preparation of foods that minimize the risk of infection from food-borne zoonoses, while a concerted control of vectors is mandatory to prevent vector-borne infection.

The characteristic imaging features from US, CT, and MRI are useful to demonstrate the flukes, liver abscesses or cysts, for the accurate and specific detection of hepatic infections; the techniques are useful too, in the follow-up of patients with parasitic diseases involving the liver [42]. The global spread of multidrug-resistant parasites has led to an urgent need for new chemotherapeutic agents, and thus, more simple and accurate diagnostic methods and more effective treatment measures need to be further developed. The sensitivity of antibody detection in the serum, saliva, and urine samples has been reported as 72 %, 56 %, and 84 %, respectively, while the corresponding specificity was 76 % for all the samples, and if antigen detection is combined with antibody detection, the sensitivity for serum, saliva, and urine samples rises to 72 %, 68 %, and 88 %, respectively [72].

The discovery of molecules with action against the hepatic phase of the involved parasites pave the ways to reevaluate causal prophylaxis as a tool that can be incorporated in control strategies and enhance global efforts to reduce the major burden exerted by the parasites causing liver diseases [10]. Also, the scientists are moving forward with vaccine preventive strategies in order to decrease the morbidity and mortality from parasitic infection in the globe [8].

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Viral Diseases of the Liver

12

Gadi Lalazar and Yaron Ilan

Abbreviations

CMV	Cytomegalovirus
EBV	Epstein–Barr virus
HHV	Human herpes virus
HSV	Herpes simplex virus
IM	Infectious mononucleosis
PCR	Polymerase chain reaction
PTLD	Post-transplant lymphoproliferative disorder
VZV	Varicella zoster virus
XLP	X-linked lymphoproliferative disorder

Key Points (6–12)

1. A variety of viruses in addition to the classic hepatitis viruses A to E can affect the liver. These include Epstein–Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), human herpes viruses 6, 7, and 8, human parvovirus B19, adenoviruses, and others.
2. 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 liver failure.
3. Both the innate and adaptive arms of the immune system play a role in the pathogenesis of virally mediated target organ involvement.
4. In most immune-competent patients an asymptomatic or mild disease occurs, while immune-suppressed patients and organ transplant recipients are at high risk for the development of severe systemic infection.
5. Antiviral agents have a role in the treatment of immune-compromised patients and in immune-competent patients who present with severe life-threatening disease.
6. EBV may be associated with increased risk of malignancy and post-transplant lymphoproliferative disorders (PTLDs).

Introduction

Viruses other than the classic hepatotropic viruses, hepatitis A through E, may cause hepatic injury [1]. Among these are Epstein–Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), human herpes viruses (HHV) 6, 7, and 8, human parvovirus B19, and adenoviruses (Table 12.1). The clinical presentation of infections with these viruses may be indistinguishable from that associated with infection with classic hepatotropic viruses. The presentation ranges from a mild and transient elevation of aminotransferases to acute hepatitis and liver liver failure [1]. These viruses should be considered as possible etiologic agents in patients who have acute liver injury and whose serologic markers for the classic hepatotropic viruses are not indicative of an active infection [1]. In the present chapter, we review the clinical manifestations and the potential for immune-mediated liver injury associated with several of these viruses (see summary Table 12.2).

Epstein–Barr Virus

EBV Infection

EBV is a double-stranded DNA virus that is a member of the gamma herpes virus family [1]. Its genome consists of a linear DNA molecule that encodes nearly 100 viral proteins. Expression of different combinations of these proteins allows

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Table 12.1 Non-hepatotropic viruses that may affect the liver

Herpes viruses: Cytomegalovirus, Epstein–Barr virus, varicella zoster virus, human herpes virus 6, human herpes virus 7, and human herpes virus 8
Adenoviruses
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
Erythrovirus: Parvovirus B19
Filoviruses: Ebola virus and Marburg virus
Flaviviruses: Dengue, Lujo virus, Kyasanur Forest disease virus, Omsk hemorrhagic fever virus, and yellow fever virus
Orthomyxoviruses: Influenza
Picornaviruses: Echovirus
Reovirus: Colorado tick fever virus, Reovirus 3

the virus to establish different forms of infection [2]. Cell entry and translocation of EBV particles to the nucleus is confirmed by detection of the EBV genome in isolated nuclei [3]. While B cells in the oropharynx may be the primary site of infection, resting memory B cells are thought to be the site of persistence of EBV throughout the body. 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 consequence in the majority of individuals [4]. After infecting B lymphocytes, the linear EBV genome becomes circular, forming an episome, which usually remains latent in these B cells. Only ten of the viral proteins are expressed in latently infected B cells in vitro. Limited gene expression during latency ensures successful escape from cytotoxic T lymphocyte (CTL) recognition [2]. EBV shares the tendency of establishing latency in the host with other herpes viruses [2]. Viral replication is spontaneously activated in only a small percentage of latently infected B cells [5].

EBV infection is a common and lifelong infection affecting over 90 % of humans worldwide. The virus replicates in nasopharyngeal epithelial cells, and seropositive persons actively shed the virus in saliva [1, 6]. 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, as well as 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 heterophil antibodies is sensitive but not specific. In the vast majority of cases, there is no indication for liver biopsy, but when performed there may be portal and sinusoidal mononuclear cell infiltration with focal hepatic necrosis or fatty infiltration [1, 7]. Specifically, the diagnosis of EBV hepatitis is established based on the combination of elevated aminotransferases, serology compatible with active EBV infection, typical findings on liver biopsy, and demonstration of the presence of the viral genome in liver tissue by various molecular methods.

The Role of the Immune System in EBV Infection

Imbalances in the equilibrium between the virus and the host's immune system lead to the development of liver damage in EBV-infected patients. EBV can also be involved in the development of tumors such as lymphoproliferative disorders, Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma [4]. The demonstration that immunotherapeutic approaches are effective for some of these cancer patients further supports a role for the immune system in disease pathogenesis [4]. In the context of EBV-related tumors, the expression of viral antigens by malignant cells makes them suitable targets for immune therapy. Infusion of EBV-specific CTLs has proved to be safe and effective and induces protective antiviral immunity, which is lacking in EBV-associated malignancy [4].

Both the innate and the adaptive arms of the immune system play a role in anti-EBV immunity [4, 8]. EBV interacts with NK cells, neutrophils, monocytes, and macrophages, as well as with epithelial cells that are relevant to viral resistance [4]. The tonsils are the primary site for EBV infection. EBV triggers monocyte TLRs, inducing maturation of DCs, which activate CD16–CD56 bright NK cells via IL12. NK cells hamper pathogen entry at mucosal sites, thus restricting EBV infection until the adaptive immunity establishes viral immune control [9]. IFN-γ secreted by DC-activated NK cells is associated with delayed latent EBV antigen expression. It inhibits B-cell transformation, decreasing their proliferation during the first week following infection [4, 10]. IFN-γ also promotes an EBV-specific adaptive immune response by favoring a Th1-polarization.

Early after primary viral infection, NK cells are thought to limit the viral burden until virus-specific T cells are able to eliminate the infection or maintain viral titers at low levels. Innate immunity uses several "pattern recognition" receptors to sense pathogen-associated molecular patterns (PAMPs) [4]. Toll-like receptor (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 [11]. Following viral entry into cells, viral DNA is

Table 12.2 Clinical features, diagnosis, and treatment summary table

Virus	Population at risk	Clinical and laboratory features	Acute liver failure	Unique complications	Diagnostic tests	Treatment	Effective antiviral medication
EBV	IC	Lymphocytosis	Rare	Splenic rupture	Monospot	ICP: only if severe complications	Ganciclovir
	Age >30 (esp. >60)	Monocytosis	More common in immunosuppressed patients (60 % in patients with XLP)	PTLD	EBV VCA IgM	Steroids	Valganciclovir
	XLP	Splenomegaly		HLH	PCR (blood and tissue)	Antivirals (if steroid refractory)	Valacyclovir
	Solid organ transplant recipients (especially pediatric)	Gradually rising liver enzymes		AIH exac.	Liver biopsy—rarely needed (portal and sinusoidal mononuclear cell infiltration with focal hepatic necrosis or fatty infiltration)	IC: anti-EBV CTLs antivirals	Famiclovir Foscarent
CMV	IC	Hepatosplenomegaly	Rare	Graft rejection and loss encephalitis, pneumonitis, hepatitis, uveitis, retinitis, colitis	CMV IgM	ICP—only in severe end organ disease	Ganciclovir
	Solid organ transplant recipients	Aminotransferases lower than in "classic viral hepatitis"	More common in IC		PCR (blood and tissue)	IC—antivirals ± IVIG	Valganciclovir
	Neonates (congenital CMV)	Leukopenia			Liver biopsy—important (giant multinucleated cell reaction with an inflammatory response, multifocal necrosis, biliary stasis. Large nuclear inclusions in hepatocytes or bile duct epithelium)	Organ transplant recipients—prophylactic vs. preemptive treatment	Foscarnet
	IBD	Thrombocytopenia			Immuno-histochemistry		Cidofovir CMV hyperimmune globulin Leflunomide
HSV	IC	Leukopenia, thrombocytopenia, relatively mild elevation in bilirubin	Rare	Esophagitis	HSV PCR (blood and tissue) Liver biopsy—essential (focal—extensive, hemorrhagic, or coagulative hepatocyte necrosis, limited inflammatory response). Typical intranuclear inclusions (Cowdry type A) at the margins of the foci of necrosis	Early high-dose acyclovir	Acyclovir
	Pregnancy (third trimester)	Mucocutaneous lesions (50 %)	More common in pregnancy, IC, and neonates	Pneumonitis			
	Neonates						
VZV	Adults	Cutaneous rash	Rare	Graft loss	Viral isolation from skin lesions	Early therapy with acyclovir in severe disease or IC patients	Acyclovir
	IC		More common in immunocompromised individuals		HSV PCR (blood and tissue)		
	Liver transplant recipients				Liver biopsy (foci of coagulative necrosis and intranuclear inclusions with an inflammatory response)		

IC immunocompromised, *ICP* immunocompetent, *XLP* X-linked lymphoproliferative disorder, *PTLD* post-transplant lymphoproliferative disorder, *HLH* hemophagocytic lymphohistiocytosis, *AIH* autoimmune hepatitis, *CTLs* cytotoxic T lymphocytes

recognized by TLR9. Dual interactions through TLR2 on the cell membrane and intracellular TLR9 lead to a rapid production of IL-8, initiating an effective antiviral immunity.

Innate lymphocytes also play a role in resistance to EBV-associated malignancies. Mutations in SAP (signaling-lymphocyte activation-molecule-(SLAM)-associated protein) are associated with loss of EBV-specific immune control [4]. 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) [4]. EBV-transformed B lymphocytes express high levels of EBI3 protein, which has immunosuppressive activity [12].

The EBV genome is also detected in non-B cells, including phagocytes. Monocytes and macrophages are involved in the uptake of small vesicles called exosomes that contain viral mRNA. Exosomes play a role during the early phases of EBV infection and also involve innate immunity-related cell types that are not targeted by the virus [4]. 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 [13]. Infected neutrophils rapidly die by apoptosis [14]. 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, while upregulation of IL-1R and induction of apoptosis in neutrophils inhibit anti-EBV immune responses [12].

Episodes of monocytopenia are observed during the acute phase of IM [4]. Patients with EBV-associated malignancy show a deficiency in monocyte-mediated ADCC, suggesting that monocyte functions are affected during the course of EBV infection. This is also demonstrated by the reduced phagocytic activity observed in EBV-infected monocytes [3]. EBV infection inhibits the functional ability of macrophages to respond to bacterial challenge by reducing their phagocytic potential [15]. 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 access of the virus to the 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 [3, 4].

CTLs are major determinants in the control of acute EBV infection and are directed against both lytic and latent antigens [16]. About half of the total CD8+ T cells in acute infection are specific for a single lytic EBV epitope, and most of these epitope-specific cells have an activated/memory phenotype. In the late stages of infection, the frequency of epitope-specific CD8+ T cells directed against latent EBV proteins selectively increases, confirming that CTLs are the most important cells for limiting infection in the convalescent phase of virus infection.

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 [16]. In latent infection, the virus expresses fewer proteins, does not replicate, and is able to persist within the host cell. EBV has developed the ability to rapidly promote the expression of its own genes while simultaneously shutting down the transcriptional program of its host cell [4]. TNF-α levels are increased in IM patients, indicating its importance in ongoing antiviral response. However, the entire virus inhibits TNF-α secretion by monocytes and macrophages [3]. EBV downregulates TNF-α mRNA transcripts via suppressive action at the transcriptional level [4]. EBV proteins can also modulate IFN signaling. This effect promotes viral persistence and may also contribute to tumor development [4, 17].

EBV reactivation associated with increased specific CTL-response to a lytic EBV epitope can lead to EBV-associated chronic hepatitis [18]. EBV reactivation in these patients is based on an increased percentage of terminally differentiated CD28-CD27-CD8+ T cells, suggestive of chronic antigen stimulation [18]. Diminished expression of the co-stimulatory molecules CD28 and CD27 compromises CD8+ reactivation, making cells more resistant to apoptosis [19]. A T-cell pool with low expression of CD28 and CD27 has low ability to control reactivation of virus and is a typical finding in an elderly group. Similar changes were found in younger patients under chronic CMV and EBV antigen stimulation [2, 20].

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 [21]. EBV stimulates strong humoral responses to lytic cycle proteins. IgM and developing IgG responses to nucleocapsid and envelope proteins are detectable in primary EBV infection [4]. IgG responses to immediate-early and early lytic cycle proteins and to the latent proteins EBNA1 and 2 are also detectable, together with neutralizing antibodies directed against gp350 [21].

Clinical Manifestations of Acute Liver Involvement in EBV Infection

Various clinical conditions have been associated with EBV, including infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, peripheral T-cell lymphoma, and post-transplant lymphoproliferative disorder (PTLD) [22, 23].

Primary EBV infection takes place in the oropharyngeal region, to which the virus is conveyed by saliva droplets from infected individuals. Primary infection leads to transient viremia followed by a strong T-cell adaptive immune response that holds the infection latent in immunocompetent individuals [22, 24]. If infection is delayed to 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 exclusively found in B blasts that proliferate under the influence of latent genes [4]. 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 entirely repressed, a process described as "true latency." Short episodes of spontaneous reactivation and consequent viral replication normally occur in healthy individuals [24]. Manifestations of liver involvement 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 up to 90 % of patients, but symptomatic hepatitis is rare [23]. 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 is suggested by the presence of a lymphocytosis and/or splenomegaly [23].

Compared with IM, which usually affects young patients, EBV hepatitis usually affects an older age group. In a recent review of nearly 2,000 cases in England, 10/17 patients (59 %) were aged >30, and 7/17 (41 %) were ≥60 years [23]. While 88 % had clinical or biochemical jaundice, 100 % had lymphocytosis, and 88 % had splenomegaly, only 12 % had the classic symptoms of IM. Symptoms lasted for a median of 8 weeks, and only 3/17 patients required a brief hospitalization. Severe cholestatic jaundice and right upper quadrant abdominal pain, which could be mistaken for bile duct obstruction, may occur in elderly patients [25]. 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 EBV liver involvement include posttransfusion hepatitis, granulomatous hepatitis, and fatal fulminant hepatitis [1, 26]. EBV superinfection may occur in patients with preexisting autoimmune hepatitis, resulting in severe hepatic decompensation [27]. Cases of liver failure were described both in immunocompromised and immunocompetent hosts [26, 28, 29].

Viral replication may cause significant clinical entities and severe complications in patients with diminished cell-mediated immunity [2, 30].

EBV-Mediated Chronic Liver Damage

Chronic EBV hepatitis in immune-competent patients was suggested in several studies [31]. However, EBV was not detected in human hepatocytes [2]. Specific latent antigens, as well as EBER transcripts, were detected in infiltrating CD8+ CTLs, implying that hepatocytes suffer from "collateral" damage [2]. Chronic hepatitis might also be induced by a 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 [32–34]. It is suggested that reactivation leading to liver damage can occur whether the infected lymphocytes are incidentally or intentionally in the liver.

Chronic active EBV infection (CAEBV) may result from a disturbance in the host–virus balance and Th1/Th2 misbalance, and may be associated with an aggressive clinical course. CAEBV is defined by chronic severe illness, which begins as a primary EBV infection associated with elevated transaminases, abnormal EBV serology, suggestive histopathological features, and detection of viral genome in the liver tissue. Evidence of recurrent EBV reactivations, increased circulating EBV-specific CTLs, and increased CD38 B-cell expression, along with increased LDH levels, mild splenomegaly, and thrombocytopenia, can support the diagnosis [2, 31]. CAEBV may also progress to a chronic or recurrent IM-like disease [35]. In Western countries, CAEBV is milder than in Asian countries [2]. The mild form is characterized by intact immune control of B cells, relatively low viremia, and EBV-specific CTL expansion comparable to those of seropositive donors. 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 [36], chronic granulomatous hepatitis [37], and vanishing bile duct syndrome [38]. While the existence of acute mononuclear hepatitis during primary EBV infection is accepted, skepticism has been expressed as to the hypothesis that EBV causes chronic liver disease in immune-competent patients. EBV in this setting may be referred to as an "incidental virus," reflecting a co-infection with other hepatotropic viruses that are a more likely cause of chronic liver disease or amplification of the EBV genome in circulating B cells that turn up in the liver [2].

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, compared to EBV patients without reactivation [2]. 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 high sequence homology with IL-10, and exogenous IL-10 enhances HCV replication. EBNA1 can promote HCV replication. IFN-γ inhibits HBV replication in the absence of cell necrosis. T-cell cross-activation may also explain HBV or HCV reactivation [2].

Post-transplant Lymphoproliferative Disorder

PTLD is a spectrum of lymphoproliferative diseases occurring in the post-transplantation setting. EBV infection is the main cause of PTLD. The incidence of PTLD ranges from 0.5 to 30 % [72, 73]. Risk factors for the development of PTLD 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 immune suppression (specifically anti-T-cell immunosuppression) [39]. PTLD complicates up to 10 % of pediatric liver graft recipients, with a mortality of up to 50 %. In the pediatric population, post-transplant primary infection within 3 months of OLT was associated with sustained EBV detection and increased the risk of the late occurrence of PTLD [40].

PTLD emerges as either of 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 lymphoma. Conversely, solid organ transplant patients develop PTLD of recipient origin when EBV released from the transplanted organ infects the recipient's B cells [4, 39].

The spectrum of PTLD ranges from polymorphic lymphocyte proliferation to high-grade life-threatening monoclonal lymphoma [39]. The interplay between the EBV life cycle and latency and non-viral factors determines the histology and clinical presentation of the disease. The majority of PTLD is of B-cell origin. EBV's in vitro transforming abilities, distinctive latency, and clonality within the malignant cells determine the biology of the disease [39]. Measurement of viral load by quantitative polymerase chain reaction (PCR) can assist in the surveillance and diagnosis of PTLD, although its specificity for the diagnosis is only 50 % [39]. Post-transplantation patients should be monitored by EBV PCR levels in the peripheral blood with the purpose of detecting active EBV infection early and instituting preemptive therapy prior to the development of overt PTLD.

Management options for PTLD include reduction of immune suppression, biological therapy with anti-B cell antibodies, combination chemotherapy, and adoptive immunotherapy using EBV-specific CTLs [41]. 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 [39]. These patients should be monitored closely for acute allograft rejection. Newer immunosuppressants, including mycophenolate mofetil and sirolimus, appear to be associated with fewer post-transplant malignancies.

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 be comprised of treatment with anti-CD20 antibodies in combination with antivirals (acyclovir or ganciclovir), IVIG, or steroids.

EBV-Mediated Liver Cancer

EBV has been considered a major factor in the development of a wide range of cancers both in immunocompetent and immunocompromised individuals [2]. 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 greater amount of EBV DNA was reported in HCV-positive HCC compared to HBV-associated HCC. In some studies, up to 30 % of liver cancers were found to harbor EBV DNA [42]. This finding, however, was not confirmed in other studies. A possible source of detected EBV DNA might be the infiltrating lymphocytes [2]. The weak positivity of EBV DNA in some liver tissues was explained by others as possible amplification of EBV DNA in the lymphoid infiltrate or blood, reflecting a high EBV DNA load in these patients.

Treatment of EBV Hepatitis

Primary EBV infection is subclinical in the majority of immunocompetent individuals; it may lead to IM in adolescents and adults. It is generally self-limiting; therefore, in immunocompetent individuals, symptomatic treatment alone is recommended. This includes rest, adequate hydration and nutrition, and analgesics or antipyretics as needed. In patients suffering from IM, avoidance of exertion and participation in sports is recommended for at least 3 weeks due to the rare risk of splenic rupture. Rare patients suffering from severe complications of acute EBV are usually treated with corticosteroids even though there is little evidence to support their

use [43, 44]. The dose used varies in different reports. 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 [45], especially in cases of refractory disease [46]. 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 [47]. 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) [46]. 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 [48]. Patients with immunodeficiencies are at increased risk of liver failure and the development of lethal lymphoproliferative diseases. The major pathogenic causes thought to be important in the development of lymphoproliferative disorders/lymphomas are primary immunodeficiency (XLP, ataxia telangiectasia, 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 effective antiviral medication. In seronegative patients with XLP, monthly prophylaxis with IVIG is recommended. Patients who have developed EBV-associated lymphoproliferative disease may benefit from chemotherapy, radiation therapy, or biological therapy with monoclonal antibodies or EBV-specific CTLs. Hematopoetic stem cell transplantation is the only potentially curative therapy for many patients but is usually recommended only in children [49].

Cytomegalovirus

CMV Infection and Diagnosis

Human CMV is a double-stranded DNA virus that is the largest member of the beta herpesviridae family. The cellular response to CMV infection is characterized by cytomegaly and a spectrum of prominent clinical syndromes. The spectrum of clinical syndromes associated with CMV disease ranges from asymptomatic infection to life-threatening congenital CMV syndrome in neonates to infectious mononucleosis syndrome in young adults to severe pulmonary, retinal, neurological, gastrointestinal, and hepatic diseases in immunocompromised hosts [1]. Infection can be acquired either in the perinatal period and infancy or in adulthood through sexual contact, blood transfusions, or organ transplantation [1].

Serologic studies of CMV-IgM antibodies are helpful for the diagnosis of primary infections. Viral culture techniques use the "shell vial" assay and CMV early antigens. Molecular techniques to detect CMV early antigen or CMV DNA increase sensitivity for detecting CMV infection in blood and end organ tissue. To clearly establish the diagnosis of active CMV infection, it is necessary to have histological evidence of cellular injury associated with infection. Distinct pathologic findings on liver biopsy are important for the diagnosis of CMV hepatitis, especially in immunocompromised hosts. Giant multinucleated cell reaction with an inflammatory response, multifocal necrosis, and biliary stasis are common. Large nuclear inclusion-bearing cells, the so-called owl's eye inclusions, are detected in hepatocytes or in bile duct epithelium.

CMV Infection in the Immunocompetent Host

The seroprevalence for CMV worldwide ranges from 60 to 100 % [50]. Most primary CMV infections in immunocompetent adults are asymptomatic or associated with a mild IM syndrome. Symptomatic CMV infection in non-immunocompromised hosts has traditionally been considered to display a benign self-limited course of a disease that resembles EBV-IM syndrome. Similar to other herpes viruses, all primary infections resolve and enter into lifelong latency in which live viruses are sequestered in a non-replicative state. Persons with latent infection and intact immune systems have no symptoms but exhibit antibodies to CMV. Circulating lymphocytes, monocytes, and polymorphonuclear leukocytes may serve as the reservoir site of viral latency. The risk for intermittent reactivation is increased with immunosuppression [1].

Liver dysfunction is commonly associated with CMV mononucleosis. It is usually mild and rarely symptomatic in the immunocompetent patient. Hepatosplenomegaly and laboratory evidence of mild to moderate elevation of liver enzymes are the predominant features, with increased aminotransferases and alkaline phosphatase in the majority of cases, but the levels of these are lower than are encountered in acute hepatitis due to "classic" hepatitis viruses [1, 51]. Rare manifestations of CMV hepatitis include tender hepatomegaly, granulomatous hepatitis, anicteric or icteric cholestatic hepatitis, and acute hepatitis with massive necrosis [88].

The morbidity and mortality that CMV infection may cause in immunocompetent hosts were recently reviewed in 290 patients [52]. Severe CMV infections affected almost every system. The gastrointestinal tract (gastroenteritis, duodenitis, ileitis, colitis, proctitis) and the central nervous system (meningitis, encephalitis, transverse myelitis, nerve

palsies, myeloradiculopathy) were the most frequent sites [52, 53]. In addition, hematological manifestations (hemolytic anemia and thrombocytopenia), ocular (uveitis, retinitis), liver (hepatitis), pulmonary (pneumonitis), and thrombosis of the arterial and venous system (deep venous thrombosis, portal vein thrombosis, pulmonary embolism) have been described [52, 54]. Several cases were treated with ganciclovir or with valganciclovir, some with fatal outcome despite therapy.

A special population afflicted by CMV disease consists of patients with preexisting inflammatory bowel disease [55]. TNF-α and IFN-γ are frequently elevated in these patients and may promote reactivation of a latent CMV infection, which further promotes additional cytokine release, particularly of 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, colovesical fistula, perforation, and peritonitis.

CMV Infection in the Immunocompromised Host

In immunocompromised patients, CMV disease results either from a primary infection or, more commonly, from reactivation of a latent infection [1, 52]. Disseminated CMV infections in immunocompromised patients with impaired cell-mediated immunity, 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. However, the incidence and severity of CMV disease closely parallels the degree of cellular immune dysfunction, characterized by decreased numbers of CTLs and natural killer cells [56]. The 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 complications such as microcephaly, mental retardation, spastic paralysis, hepatosplenomegaly, anemia, thrombocytopenia, deafness, and optic nerve atrophy leading to blindness [52].

CMV is the most common opportunistic viral infection in AIDS patients, causing retinitis, central nervous system infections, esophagitis, and colitis. CMV may also invade the hepatobiliary tract in AIDS patients, causing hepatitis, pancreatitis, and acute acalculous cholecystitis [57]. The presence of CMV retinitis, gastrointestinal disease, or viremia in AIDS patients increases the risk for a cholestatic syndrome caused by papillary stenosis and sclerosing cholangitis (AIDS cholangiopathy), which does not usually respond to antiviral therapy. Hepatitis is the most frequent organ-specific complication of CMV infection after liver transplantation, affecting 10 % of recipients and with a higher incidence among seronegative recipients than seropositive patients (26 % vs. 9 %, respectively). In these cases, infection occurs as a consequence of reactivation rather than primary infection [1].

Treatment of CMV Infection

The current opinion is that CMV infection in immunocompetent patients does not require treatment [52]. 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 a treatment effect [45].

For severe cases, particularly in patients with impaired cell-mediated immunity, therapy can be life-saving [1]. Drugs approved for treatment of CMV disease include ganciclovir, valganciclovir, foscarnet, and cidofovir. Ganciclovir is considered 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 become alternative antiviral agents [58]. Valganciclovir has recently been evaluated among liver transplant recipients with CMV disease [1, 56]. 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. Long-term administration of these agents may lead to the emergence of resistant viral strains [45].

CMV in Liver Transplant Recipients

CMV infection is a common complication following liver transplantation and contributes to morbidity and mortality in these patients [56]. CMV evades the immune system resulting in a state of latency in several types of 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 [56].

Overall, 18–29 % of liver transplant recipients will develop CMV disease [59]. A lack of 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 recipients, CMV R+) [60]. The incidence is reduced in liver transplant recipients who receive antiviral prophylaxis with valganciclovir 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 [59, 61]. A recent randomized control trial showed that 200 days of prophylaxis are more effective than 100 days of therapy in high-risk (D+/R−) patients; however, this has yet to become a standard recommendation due to safety and cost [62]. 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 [56].

The use of highly potent pharmacologic immune suppression 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 [60]. The severity of immune dysfunction is strongest with lymphocyte-depleting drugs such as anti-CD3 and antithymocyte globulin [56].

Defects in innate 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. In a study of 92 liver transplant recipients, a genetic polymorphism in the TLR-2 gene was associated with a higher degree of CMV replication and a higher incidence of CMV disease. This polymorphism decreased the 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.

CMV disease in liver recipients manifests with fever, bone marrow suppression, and organ-invasive disease. 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 (CMV gastritis, esophagitis, enteritis, and colitis), although any organ may be involved. CMV hepatitis is common in liver transplant recipients compared to other solid organ transplant recipients and manifests with symptoms indistinguishable from acute allograft rejection [56]. The availability of sensitive tests for the rapid detection of CMV in the blood may obviate the need for a liver biopsy to differentiate CMV infection from 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 [56]. CMV is known to be a potent up-regulator of alloantigens, thereby increasing the risk of acute rejection and chronic allograft dysfunction. CMV is associated with vanishing bile duct syndrome and ductopenic rejection, leading to chronic cholestasis and allograft failure and with a higher incidence of hepatic artery thrombosis. The immunomodulatory effects of CMV predispose to other opportunistic infections including fungi, other viruses, and bacteria such as *Nocardia*. CMV-infected transplant recipients are more likely to develop EBV-associated PTLD or to develop coinfections with other viruses such as human herpes virus HHV6 and HHV7 [63]. An association between CMV and an accelerated course of HCV recurrence was described [64]. Forty-eight percent of HCV-transplanted patients who developed CMV disease had allograft loss or died within 3 years of transplantation, compared to 35 % of patients with asymptomatic CMV infection and 17 % of those who did not develop CMV infection [64].

CMV infection is an independent predictor of mortality after solid organ transplantation. The use of anti-CMV drugs, either through antiviral prophylaxis or preemptive therapy, led to reduction in the overall 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 fivefold increased risk of all-cause mortality and an 11-fold increased risk of infection-related mortality [65].

Allograft rejection can promote CMV reactivation and is a significant risk factor for CMV disease following liver transplantation [56]. 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. The risk of CMV disease after liver transplantation is associated in direct proportion with the degree of CMV replication, which is partly a function of "over-immunosuppression" [66].

There are two strategies for CMV disease prevention after liver transplantation: preemptive therapy and antiviral prophylaxis [56]. 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 [67]. Preemptive therapy with oral ganciclovir or intravenous ganciclovir or valganciclovir resulted in reduction of CMV disease by 70 % [68], and, unlike antiviral prophylaxis, was not associated with late-onset CMV disease. Valganciclovir is currently the most commonly used drug for preemptive therapy.

Preemptive 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 [66].

For antiviral prophylaxis, antiviral drugs such as ganciclovir and valganciclovir are administered to patients at risk of CMV disease after liver transplantation [61, 69–73]. While there is no clear consensus regarding antiviral prophylaxis, it is administered by the majority of transplant centers for prevention of primary CMV disease in high-risk CMV D+/R− transplant recipients [74]. Prophylaxis is recommended in all CMV D+/R− liver recipients [75]. Several clinical trials have demonstrated its effectiveness in preventing the direct and indirect effects of CMV after liver transplantation [68]. Compared to placebo, patients who received antiviral prophylaxis had a 58–80 % reduction in CMV disease and a 40 % reduction in CMV infection [68]. The use of acyclovir as anti-CMV prophylaxis after liver transplantation has been supplanted by ganciclovir and valganciclovir because of their superior efficacy [71, 76, 77]. Prophylactic versus preemptive therapy for intermediate- and low-risk groups (D+/R+, D−/R+, and D−/R−, respectively) 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. 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 post-transplant CMV disease [69]. 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 [78]. Valganciclovir is a possible oral treatment for mild to moderate disease [78]. In cases of ganciclovir-resistant CMV disease, treatment options include foscarnet, cidofovir, CMV hyperimmune globulins, or leflunomide [69]. Compartmentalized CMV disease refers to clinical syndromes wherein the virus is detected in the affected tissues but is minimally detectable or undetectable in the blood [56, 69]. In the gastrointestinal system, "compartmentalized" CMV disease in the form of gastritis, esophagitis, enteritis, or colitis constitutes the vast majority of tissue-invasive conditions [60].

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 site involved and patient's age and immune status [1]. HSV viremia results in visceral involvement, affecting mainly the esophagus, lungs, and liver. Liver involvement occurs in neonatal infections, pregnancy, and immunocompromised hosts, where it is frequently a fulminant disease [1].

HSV is an uncommon cause of hepatitis in immunocompetent patients. A mild asymptomatic elevation of aminotransferase levels can be detected in 14 % of healthy adults with genital infection [79]. Fulminant hepatitis with more than 100-fold rise in aminotransferases was reported and associated with a favorable outcome after antiviral therapy [80]. The incidence of HSV hepatitis was reported to be up to 6 % of fulminant hepatitis cases.

In immunocompromised hosts, HSV hepatitis has occurred 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 essential to establish the diagnosis of HSV hepatitis. It shows focal, 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].

In neonates, hepatitis occurs with multi-organ involvement and carries a high mortality rate. In pregnant women, it is observed in the context of disseminated primary infection during the third trimester and presenting as fulminant hepatitis. Mucocutaneous lesions are present in only half of cases; thus, many cases are not diagnosed until autopsy. Early diagnosis and treatment with antiviral therapy may reverse an otherwise fatal process [1].

The treatment of choice in these patients is early high-dose acyclovir [81, 82]. Recurrence was not observed, suggesting that disseminated HSV infection should not be an absolute contraindication for transplantation in certain clinical settings [1, 83, 84].

The importance of additional human herpes viruses (HHV6 and 7) has been debated in recent years. According to some series, HHV6-infected patients have higher rates of acute and chronic allograft rejection, bacterial and opportunistic infections, a higher risk for CMV disease, and shorter graft survival [85]. While HHV6 reactivation is common after solid organ transplantation, clinical disease is rare, manifesting as fever, myelosuppression, and end organ disease including encephalitis and hepatitis. Treatment is indicated for end organ disease and includes foscarnet, ganciclovir, and cidofovir [86].

Varicella Zoster Virus

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 immune-competent adults. In transplanted patients, primary

infection can present with an aggressive liver disease [1]. Such infection 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 [87]. 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 the setting of VZV hepatitis, especially in immunocompromised patients [1, 88].

Parvovirus (B19)

Parvovirus (B19), a small DNA virus, is a member of the parvoviridae family. Its clinical manifestations include erythema infectiosum, hydrops fetalis and fetal death in children, and arthritis in adults. Leucopenia, 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. Infection is usually benign and self-limiting, and symptomatic therapy alone is recommended [1].

Adenoviruses

There are 50 different serotypes of adenoviruses that cause acute infections of the respiratory system, conjunctivae, and gastrointestinal tract and occasionally hemorrhagic cystitis, infantile diarrhea, intussusception, and central nervous system infections [1]. Multi-organ involvement has been reported in immunocompromised, and rarely in immunocompetent, patients, associated with increased mortality [89]. Fatal cases of adenovirus infection with fulminant hepatitis were reported in immunosuppressed adults [90]. No specific therapy for adenovirus hepatitis is currently available, and cidofovir has been recently suggested as an optional treatment [1].

Additional Viruses That May Cause Hepatitis

Several viruses may involve the liver as a part of an acute viral infection (Table 12.1). This infection may manifest as mild hepatitis and rarely as severe hepatitis and liver failure, along with other severe manifestations such as hemorrhagic fever. Therapy is supportive with anecdotal reports supporting antiviral therapy. Patients with liver failure should be considered for urgent liver transplantation; however, this may be hindered by concomitant damage to other organs.

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Hepatitis A: Immune Response and Virus Evolution

13

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Key Points

- The hepatitis A virus (HAV) is an atypical Picornaviridae that causes hepatitis A (HA), one of the most common preventable infectious diseases worldwide. HAV is mainly transmitted through the fecal–oral route and induces self-limited acute hepatitis in primates without chronic sequela. The reduction in the incidence of HAV infections in developed and developing countries has led to a diminished prevalence of natural protective immunity among adults.
- Safe and effective inactivated HAV vaccines have been recommended as a universal childhood immunization and for high-risk groups, such as travelers to endemic regions and men having sex with men. The need to complete the vaccination course and increase the number of doses in immunosuppressed individuals is critical for maintaining vaccine-induced protection.
- HAV presents unique features within the Picornaviridae family at the structural, molecular, and genomic levels. Cryo-electron microscopy images of the HAV capsid revealed an extremely smooth surface and the absence of a pit or canyon. The 2A protein is required during morphogenesis for pentamer formation and is removed from the capsid by a cellular protease(s). The nucleotide diversity in the capsid-coding region is similar to that of other picornaviruses, but the amino acid variability is much lower. HAV exists as a single serotype, but antigenic variants emerged in immunosuppressed individuals who received an incomplete schedule of vaccinations.
- The HAV genome contains a type III internal ribosome entry site (IRES) that is highly inefficient in translation. HAV requires an intact eIFG4 factor for translation, which is not cleaved by $3C^{pro}$, the only virus-encoded protease. HAV does not induce the shut-off of cellular protein synthesis and competes poorly for cellular resources, such as tRNAs.
- The genomic composition of HAV presents a remarkable CpG bias. The HAV codon usage is highly deoptimized compared with the cellular codon usage. Fine-tuning translation selection and mutation bias play a critical role in shaping the codon usage in the capsid region, which is required to slow translation and increase the folding precision essential for the biological properties of the capsid.
- HAV replicates in a quiescent mode due to an inefficient IRES and highly deoptimized codon usage. These characteristics contribute to the ability of HAV to evade innate and acquired immunity, particularly during the long incubation period, when this virus replicates in the liver.
- Interaction of HAV with its cellular receptor, HAVCR1, at the cell surface of regulatory T-cells (Treg) blocks T-cell receptor activation and shuts-off Treg function, thereby preventing the production of TGF-β. The transitory shutoff of Treg function is a unique feature of HAV infection that allows the virus to evade and suppress the host immune response in early infection.
- HAV induces a strong humoral immune response in the acute phase, which plays a significant role in viral clearance and protection, and a weak cellular immunity response that plays an uncertain role in viral clearance.
- HAV infection exerts a protective effect against autoimmune and allergic diseases, likely due to the transitory shut-off of Treg function.
- HAV infection serves as a model for the development of therapeutic strategies to prevent chronic hepatitis, cancer, transplant rejection, and autoimmune and allergic diseases.

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Introduction

Hepatitis A Epidemiology

Hepatitis A (HA) is the most common form of acute hepatitis worldwide [1]. The HA etiological agent is the hepatitis A virus (HAV), an enteric virus primarily transmitted through the fecal–oral route [2]. The distribution of HA in different geographical areas of the world has been associated with socioeconomic development and access to clean water and sanitation [1–3]. The incidence of HAV infection is high in developing regions and low in developed regions [2]. In developing countries, most individuals are exposed to HAV during early childhood, when the infection is primarily asymptomatic. In developed countries, infection typically occurs at an older age associated with more severe clinical symptoms. Because HAV infection induces life-long immunity, severe HA is rare in adults of highly endemic regions. In contrast, HA in low endemic areas primarily occurs in immunologically naïve adults who come in contact with the virus while travelling to endemic regions [4, 5], have risky sexual practices [6, 7], or consume contaminated water or food [8–10].

In recent decades, improved sanitation and living standards have resulted in epidemiological shifts in the acquisition of HAV infection, from childhood to adulthood, and in a lower prevalence of the disease, in many parts of the world, including countries in Mediterranean Europe [11–13], Eastern Europe [14, 15], Asia, and America [16–18].

Hepatitis A Vaccines

HAV infection induces life-long immunity [2], and natural protective immunity to HAV approaches 100 % in the adult population of developing countries. However, in developed countries, the immunologically HAV naïve population is continuously growing [19] and at risk for developing a more severe course of the disease due to increased age and/or preexisting liver disease [3]. Although typically mild, HA has been associated with prolonged convalescence and can be a serious and even fatal disease [3]. In addition, there is no specific antiviral therapy. Consequently, the HA disease burden is substantial and justifies the implementation of vaccination campaigns.

Highly effective inactivated HAV vaccines are available (for a review, see [20]). The monovalent HAV inactivated vaccines licensed in the Western hemisphere are HAVRIX® from Glaxo-Smith-Kline, Avaxim® from Sanofi-Pasteur, Epaxal® from Swiss Serum Institute, and VAQTA® from Merck. Combination vaccines, including the HAV and HBV antigens (Twinrix® and Ambirix®) or HAV and typhoid antigens (HEPATRIX®, VIATIM®, and VIVAXIM®), are also available. In addition to these inactivated vaccines, two live attenuated vaccines based on the H2 and L-A-1 HAV strains are used in China. These vaccines provide long-lasting immunity through the induction of high titers of specific and neutralizing antibodies that persist for at least 15 years [21]. HAV exists in a single serotype and the vaccines are highly efficacious against all genotypes of the virus [21, 22]. The inactivated vaccines are based on attenuated strains of HAV grown in cell culture (strains HM175, GBM, RG-SB, and CR326F), purified, inactivated with formalin, and adsorbed to alum adjuvant. The high cost of these vaccines, which reflects the poor growth of HAV in cell culture and the significant scale-up required to produce sufficient antigen for immunization, is the primary argument against universal vaccination campaigns. However, evidence of the effectiveness of pediatric mass vaccination programs in reducing the incidence of HA has been shown in several countries [12, 23]. As a general rule, HAV vaccination should be recommended in low and intermediate endemic regions for at least high-risk groups, including travelers to high endemic areas, men having sex with men (MSM), drug users, and patients receiving blood products. In addition, HAV vaccines are particularly recommended for mass vaccination programs in countries receiving high numbers of immigrants from endemic areas.

Hepatitis A Virus Biology

Capsid and Antigenic Structure

HAV has an icosahedral protein capsid comprising 60 copies of each of three major structural proteins, VP1, VP2, and VP3. The X-ray crystallographic structure is not available manly due to the low viral yields obtained in cell culture and the intrinsic characteristics of the virus that tends to precipitate at high concentration. However, preliminary 3D images of HAV generated using cryo-electron microscopy (Holland Cheng, unpublished results reviewed in [19]) revealed the lack of a well-defined canyon around the fivefold axis of symmetry, which contains the receptor binding residues in other picornaviruses and plays an important biological role. While the HAV receptor-binding region has not been mapped in detail, it has recently been suggested that HAV cellular receptor 1 (HAVCR1) binds to the immunodominant antigenic site of HAV [24], which is formed by VP1 and VP3 residues (see below) that bridge the five- and threefold axes of symmetry [19]. The region of the HAV capsid that interacts with glycophorin A on human erythrocytes has been identified in an area that typically contains a canyon in other picornaviruses [25]. The HAV–glycophorin A interaction is favored under acidic conditions and impaired under neutral biological conditions, suggesting the occurrence of acid-dependent "breathings" around the fivefold axis of symmetry.

Three main sites define the antigenic structure of HAV (Table 13.1). The immunodominant site comprises closely

Table 13.1 The antigenic structure of HAV capsid

Antigenic sites	Residues replaced in in vitro isolated mAb-resistant mutants[a]	Residues replaced/ deleted in naturally isolated antigenic mutants[b]
Immunodominant site or multiple mAb binding site	*VP3*: P65S, D70A, D70H, D70N, D70Y S71P, Q74R	*VP3*: V72I
	VP1: S102L, N104D, K105R, V171E, A176D, Q232E	*VP1*: V166G, V171A, Y181S, R189T, A280V, A280E, deletion of S102, N104 and K105
Glycophorin A binding site or H7C27 mAb binding site	*VP1*: G217D, K221E, K221M	None
4E7 mAb binding site	None	None

Three epitopes have been described: the immunodominant site, which is defined by most of the existing mAbs against HAV, the glycophorin A binding site, which is defined by mAb H7C27, and a third uncharacterized site defined by mAb 4E7. Neutralization escape mutants are frequently selected in vitro using mAbs against the immunodominant antigenic site but are rarely isolated using mAb H2C27 directed against the glycophorin A binding site. Natural variants of the immunodominant but not glycophorin A binding site have been isolated from MSM HIV-positive patients. Mutants that are resistant to neutralization with mAb 4E7 have not been isolated, suggesting a critical role for this unmapped site in the biology of HAV

[a]From [25–27]

[b]From [9, 62, 153]

clustered epitopes defined by two major groups of escape mutations that include residues 70, 71, and 74 of VP3 and residues 102, 171, and 176 of VP1 [26, 27]. A second epitope is the glycophorin A binding site, which is represented by mutations near residue 221 of VP1 [25, 26]. A third antigenic site was defined using neutralizing monoclonal antibody (mAb) 4E7 [26], which neutralizes mutants at the other two antigenic sites. However, this third antigenic site remains unmapped because it has not been possible to isolate mutants that escape neutralization with mAb 4E7.

Genome Structure and Replication Cycle

The HAV genome is composed of a 7,500 nucleotide RNA molecule that includes a 5′ noncoding region (5′ NCR) segment of approximately 734 bases, followed by a long open reading frame (ORF) encoding a single polyprotein of 2,227 amino acids and a short 3′ noncoding region (3′ NCR) that terminates in a 3′ polyadenylic acid tract. A small, genome-linked protein (VPg) is covalently attached to the 5′ end of the virion RNA (Fig. 13.1).

The primary target for HAV replication are hepatocytes, although other cells, such as the crypt cells of the intestine and the Kupffer cells of the liver, have also been shown to contain HAV antigen [2].

The replicative cycle of HAV is similar to other picornaviruses [28]. HAV binds to the cell surface receptor, which

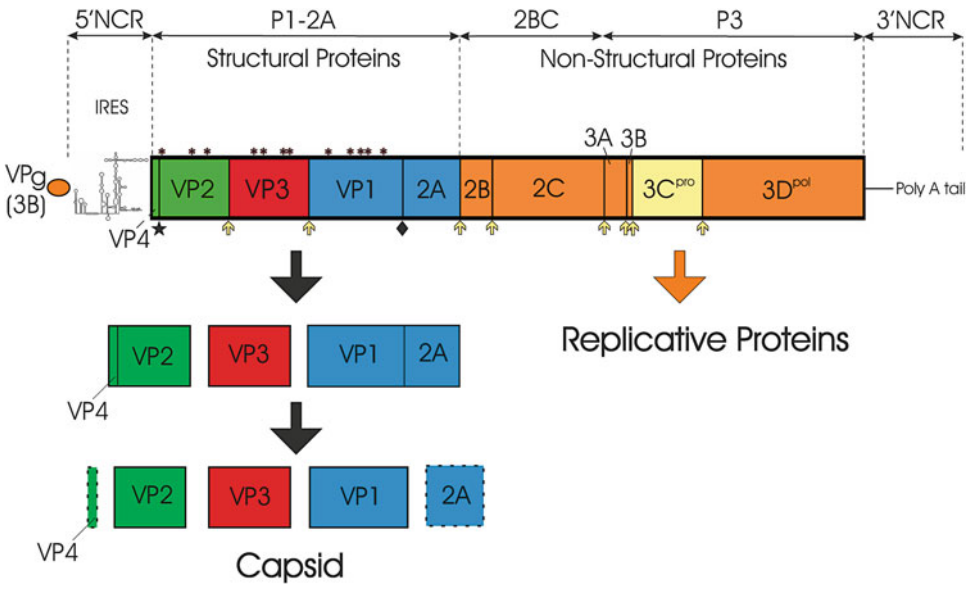

Fig. 13.1 Hepatitis A virus genomic organization and expression of viral proteins. The hepatitis A virus genome is composed of a 7.5 kb positive-strand RNA molecule containing a single open reading frame encoding a polyprotein. The polyprotein is autoprocessed by the viral protease $3C^{pro}$ (*yellow box*) at all cleavage sites (*yellow arrows*), with two exceptions: the VP1-2A cleavage is performed by a yet-to-be-identified cellular protease (*diamond*) that releases the 2A fragment from the capsid, and a possible morphogenic processing activity (*star*) that cleaves VP0 into VP2 and VP4. Strategically located clusters of rare codons in the capsid-coding region (*asterisks*) are likely to play an essential role in capsid folding through the regulation of the translation rate. *IRES* internal ribosome entry site, *NCR* noncoding region, *VPg* small viral protein 3B linked to the 5'end of the viral genome

has been identified in primate cells as the HAVCR1 [29, 30]. Binding of HAV to the immunoglobulin-like domain of HAVCR1 [31] triggers alterations of the virion and uncoating of the viral genome [32]. Havcr1, the mouse ortholog of HAVCR1, which was unfortunately rebranded as Tim-1, a name previously used for other genes, does not function as an HAV receptor [33]. It has recently been shown that the interaction of HAV with HAVCR1 plays a significant role in the pathogenesis of HAV [34]. Antibody-dependent enhancement of HAV infection [35, 36] has been observed in viral particles coated with IgA via interactions with the asialoglycoprotein receptor, a receptor that binds and internalizes IgA molecules [37]. However, there is no evidence that IgA or the asialoglycoprotein receptor alter the HAV particle or uncoat the viral genome. Additionally, it has been shown that the IgAλ is a specific ligand of HAVCR1, which binds to HAV particles and synergistically enhances the interaction of HAV with HAVCR1 [38]. The uncoating of the HAV positive-sense RNA viral genome contained in the capsid occurs slowly and can take several hours compared with the 30 min observed with most picornaviruses [39]. Once the virus reaches the cytoplasm, the HAV RNA is translated into a polyprotein (Fig. 13.1). An IRES within the 5′ NCR drives HAV translation (Fig. 13.1). The synthesized polyprotein is first autoprocessed in *cis* by the virally encoded cysteine protease, $3C^{pro}$, generating the P1-2A capsid and P2-P3 non-structural precursors. The P1-2A capsid precursor is further processed *in trans* by $3C^{Pro}$ at VP0-VP3 and VP3-VP1 cleavage sites. Important differences exist between HAV and other picornaviruses in the function and maturation of VP1-2A, also known as PX, and VP0. The 2A protein in PX is required for pentamer formation [19], and it is removed from the mature viral particles by an unknown host cell protease that cleaves PX, at the VP1-2A junction [40, 41]. Currently, there is some evidence that 2A is required for the assembly of pentamers into capsids [42]. VP0 is most likely cleaved at the VP4-VP2 junction by autocatalytic processing during capsid maturation, which results in the loss of a small VP4 peptide of 21–23 amino acids from the viral capsid.

The P2-P3 precursor is also processed by $3C^{Pro}$ into different intermediates and the mature proteins 2B, 2C, 3A, $3B^{VPg}$ (a small protein also known as VPg that serves as the protein primer for RNA synthesis), $3C^{Pro}$ (the viral protease), and $3D^{Pol}$ (the viral RNA-dependent RNA polymerase RdRp). The mature proteins and precursors, such as 2BC, 3AB, and 3CD, participate in the replication process. The precursors have different activities compared with the mature proteins, which increases the availability of viral proteins with different functions, a feature that has been observed in other small genomes.

The 3CD precursor, which has protease activity, $3C^{Pro}$, $3D^{Pol}$, and several membrane-associated viral proteins (2BC, 2C, and 3AB) interact with the 3′ end of the genomic RNA to initiate the synthesis of the negative-strand viral RNA. This negative-strand copy of the genome is used as template for the synthesis of multiple new copies of positive-strand RNA, which is subsequently recycled for further RNA synthesis or translated into new proteins. The positive-strand RNA molecules are packaged into the viral capsids, and the newly synthesized virions are secreted across the apical membrane of hepatocytes into the bile canaliculi, where they are transported with the bile to the small intestine.

Despite having a genomic structure and replicative cycle similar to other Picornaviridae, HAV possesses unique features that separate this virus from all the other members of the family. For instance, the type III IRES of HAV shows lower translation efficiency than the IRES of other family members [43]. HAV possesses a complex internal stem-loop near the 5′ end of the polymerase-coding sequence that function as a *cis*-acting replication element (CRE), which is longer and contains a larger top stem loop than the CRE of other picornaviruses [44]. HAV has only one virally encoded protease, $3C^{pro}$. The additional proteases found in other picornaviruses play a crucial role in the primary cleavage of the viral polyprotein and mediate the shut-off of cap-dependent cellular protein synthesis. This shut-off is advantageous for the virus replication strategy because the cellular translation machinery is utilized almost exclusively for the production of viral proteins. The cellular translation initiation factor eIF4G, which is required for the formation of the translation initiation complex, is cleaved by these additional picornavirus proteases, an early event required for the shut-off of host cell protein synthesis [45, 46]. An immediate consequence of the lack of these additional proteases is the inability of HAV to induce cellular protein synthesis shut-off, which is a hallmark of this virus that generally does not induce cytopathic effect (CPE).

Genomic Composition

The genomic sequences of all organisms present several layers of biases, i.e., the preference or avoidance for certain codons, codon-pairs, and dinucleotides. There are at least four mechanisms underlying these biases that in combination drive the evolutionary force acting within viral genomes. First, mutational bias and the specific nucleotide composition determine the primary genomic sequence. Second, translation selection, which is based on the optimal codon adaptation to the tRNA pool, permits highly efficient and accurate translation. Third, fine-tuning translation kinetics selection, which results in the right combination of codons that facilitate an adequate ribosome-traffic rate for the separation of protein-folding events to ensure "beneficial" and avoid "unwanted" interactions within the growing peptide. Fourth, evasion mechanisms that are selected to escape antiviral cell responses that could limit or prevent viral replication.

Remarkably, HAV shows significant codon usage and dinucleotide bias but not codon-pair bias. These biases are obviously but not exclusively associated with genomic compositional constraints [47]. The highly inefficient HAV IRES and lack of a mechanism to shut off cellular protein synthesis suggest that the virus requires a strategy to compete for the cellular translational machinery. To do so, HAV employs a highly biased codon usage, achieving a high degree of deoptimization compared with other picornaviral and cellular codon usages [48, 49]. HAV uses a high proportion of rare codons, which are defined as codons with frequencies of less than 30 % of the most abundant synonyms in the HAV genome. HAV indeed maintains a unique highly deoptimized codon usage and rarely uses cellular abundant codons, which paradoxically become "viral rare codons." The Relative Codon Deoptimization Index (RCDI) measures the adaptation of the virus codon usage to that of the host. An RCDI value of 1 indicates that the virus follows the codon usage of the host cell, while progressively higher values indicate an increasing deviation from the host codon usage. The HAV RCDI value of 1.70 confirms a highly deoptimized codon usage compared with other picornaviruses [50]. In the HAV P1 genomic region, rare codons are strategically clustered at the carboxyl end of the highly structured protein elements. These clusters of rare codons are highly conserved in HAV strains, indicating that they play a significant role in the biology of HAV. It has been hypothesized that clusters of rare codons control the speed of translation by transiently stalling translation complexes to identify suitable tRNAs present at low concentrations in the tRNA pool. This ribosome stalling would assure the proper folding of the nascent protein [51, 52], which has also been postulated for HAV [49]. The critical role of HAV codon usage and particularly the clusters of rare codons in the capsid region is evident in HAV variants adapted to grow in conditions of artificially induced shut-off of cellular protein synthesis [50]. This study showed that the initial fitness loss during the adaptation was followed by a re-deoptimization of the cellular codon usage, particularly affecting the rare codon clusters located in the capsid [50]. Consequently, it can be postulated that translation kinetics, i.e., the right combination of codons (common and rare), which facilitates an adequate ribosome traffic rate to ensure proper protein folding, is the selective force driving codon usage in the HAV capsid region. It is likely that HAV evolved this mechanism to ensure the proper folding of a capsid that is uniquely resistant to high temperatures, acid pH, and detergents. The HAV capsid is highly resistant in the environment, enabling transmission of the virus by contaminated food and water. In summary, HAV utilizes codon deoptimization to (a) avoid competition for cellular tRNAs in the absence of the shut-off of cellular protein synthesis, (b) provide sufficient time for the proper folding of a highly resistant capsid, and (c) replicate inefficiently due to the low protein synthesis rate of the polyprotein, which prevents CPE and helps the virus evade the immune system.

Pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) during infection form part of the innate immune system. Toll-like receptors (TLRs) are PRRs activated by PAMPs including the genomes of pathogens with unmethylated CpG. However, CpG-mediated innate immune recognition has been shown in DNA pathogens [53] although evidences are scarce in RNA pathogens. The genome of HAV has a markedly low occurrence of CpG dinucleotides [48, 54]. This low CpG content (0.36 %) cannot be explained by an overall low G+C content in the HAV genome (37 %) because the GpC dinucleotide content is much higher (2.96 %), suggesting that HAV evolved a dinucleotide bias to avoid CpG RNA motifs that could also activate PRRs.

Quasispecies Dynamics

Mutation, recombination, and genome segment reassortment are universal mechanisms that drive the genetic variability of viruses. Because these mechanisms are replication-dependent and viruses replicate at exceptionally high rates, virus populations become extremely variable. This variability is particularly critical in RNA viruses, such as hepatitis A, that use error-prone polymerases lacking proofreading activity, leading to complex mutant genome populations or quasispecies. Viral quasispecies act as a unit of selection and are dynamic distributions of nonidentical but closely related viral genomes subjected to a continuous process of genetic variation, competition, and selection [55]. RNA viruses have the capacity to quickly explore the plasticity of large segments of the sequence space, as a result of their high mutation rates, which are in the range of 10^{-3}–10^{-5} substitutions per copied nucleotide. However, viral diversity is limited by genomic size and diverse selective constraints. The equilibrium between these forces shapes the actual genetic and antigenic diversity of viruses and particularly of HAV. Indeed, HAV occurs as a swarm of mutants or quasispecies [56] with nucleotide diversity similar to other picornaviruses [49]. This diversity facilitates the classification of HAV into several genotypes and subgenotypes. Six genotypes have been defined based on a genetic distance of more than 15 % nucleotide variation in the highly variable VP1-2A region [2]. Three of these six genotypes (I, II, and III) are of human origin, while the others (IV, V, and VI) are of simian origin. Genotypes I, II, and III contain additional subgenotypes defined by a nucleotide divergence of 7–7.5 %. The genetic diversity of HAV in nature has been demonstrated by the emergence and reemergence of new subgenotypes [5, 57]. Genotypic characterization has been used to trace the origin of outbreaks and is also a predictor of the outcome of the

disease, which seems to be more severe in patients infected with certain subgenotypes, such as IIIA [58], and associated with fulminant hepatitis in patients infected with subgenotype IB [59].

In HAV, the diversity at the amino acid level is limited compared with that at the nucleotide level. Although HAV has several genotypes, there is a single serotype [2], and only a few antigenic variants have been isolated in nature, suggesting that strong capsid constraints limit antigenic variability. Interestingly, codon usage contributes to the low antigenic variability of the HAV capsid [60]. A total of 15 % of the surface capsid residues are encoded by rare codons, which are highly conserved among the different HAV strains, and many of these residues are located near or at epitope regions. The substitution of these rare codons is negatively selected, even under specific immune pressure [60]. The need to maintain these rare codons reflects a requirement to maintain proper capsid folding (see above). It is unlikely that a nucleotide substitution would generate a new codon of similar rarity and compatible amino acid. Additional biological constraints have also been suggested to play a critical role in the low antigenic variability of HAV [61]. Antigenic variants with changes in the immunodominant site but not in the glycophorin A binding site (Table 13.1) have been isolated from patients. However, the fitness of in vitro isolated mutants containing changes in the immunodominant site is significantly lower than that of the glycophorin A binding site [60]. Conformational changes at this site could increase binding to erythrocytes, which is likely to have an adverse effect in pathogenesis due to viral clearance and the availability of free virus to infect cells. Consequently, HAV minimizes the interactions with glycophorin A constraining changes in this capsid region.

Emergence of Antigenic Variants

Several HAV natural antigenic variants containing mutations in the immunodominant site have recently emerged in MSM HIV-positive patients [62]. These natural variants were isolated in an outbreak of HA, in which only 4 % of the patients were previously vaccinated and from those 62 % were HIV-positive. The majority of the vaccinated patients (88 %) received only one of the two doses required to complete the recommended vaccination schedule, constituting the optimal condition for the selection of variants that are able to escape antibody neutralization despite a lower fitness. The immunocompromised population has an impaired immunological response to HAV vaccines, resulting in lower concentrations of anti-HAV IgG compared with healthy individuals. In fact, HIV-positive individuals require additional vaccine doses to achieve adequate protection levels [63, 64]. In addition, MSM HIV-positive patients shed high titers of HAV in feces, reaching more than 10^{11} particles/g during the prodromal phase of the infection. This high HAV titer increases the chance of transmission to seronegative and perhaps "partially vaccinated" close contacts. A high virus input combined with a low concentration of specific IgG could allow a fraction of the viruses to replicate and generate a swarm of mutants resistant to the effects of the vaccine. When the viral input is low, such as in food-borne transmission, low concentrations of IgG neutralize the virus infection. However, it is unknown whether the vaccine could protect MSM close contacts against infection with a high dose of virus.

In vitro isolated mAb-resistant mutants (MAR) that contain similar amino acid changes found in the natural variants isolated from the MSM patients showed resistance to neutralization with the sera from vacinees. In addition, these MAR mutants exhibited lower fitness than parental viruses in the absence of antibodies and higher fitness in the presence of antibodies. The expansion of these newly emerged strains could pose a public health risk. Efforts to complete the HAV recommended vaccination schedules, particularly for HIV-positive MSM, could prevent the potential emergence of other antigenic variants. The emergence of a new serotype requires extensive substitutions in the capsid, which is unlikely to occur in HAV due to severe genomic, structural, and biological constraints. However, forcing HAV through bottleneck conditions, such as immune selective pressures, could result in the emergence of new variants of unknown pathogenic consequences.

Hepatitis A Infection

Clinical and Histological Features of the Infection

HAV infects humans and non-human primates (NHPs), causing acute HA, a self-limiting disease that resolves spontaneously without inducing chronic sequela ([65] and references therein). HAV is primarily transmitted through the fecal–oral route and rarely by blood and blood products, but injection drug-users have a higher incidence of HAV infection. Consumption of contaminated food or water and direct contact with an infected person are the most common forms of HAV transmission [66]. Although HAV is highly contagious, oral infection is 3,200-fold less effective than intravenous inoculation in NHPs [67], indicating that the natural fecal–oral route of infection is less efficient than the parenteral route. The diseases caused by oral and parenteral infection are indistinguishable, with the latter having a shorter incubation period and seroconversion time [67]. It is unknown why HA is an age-dependent disease. The majority of children younger than 6 years old develop unapparent infections. An increased incidence of fulminant HA has been described

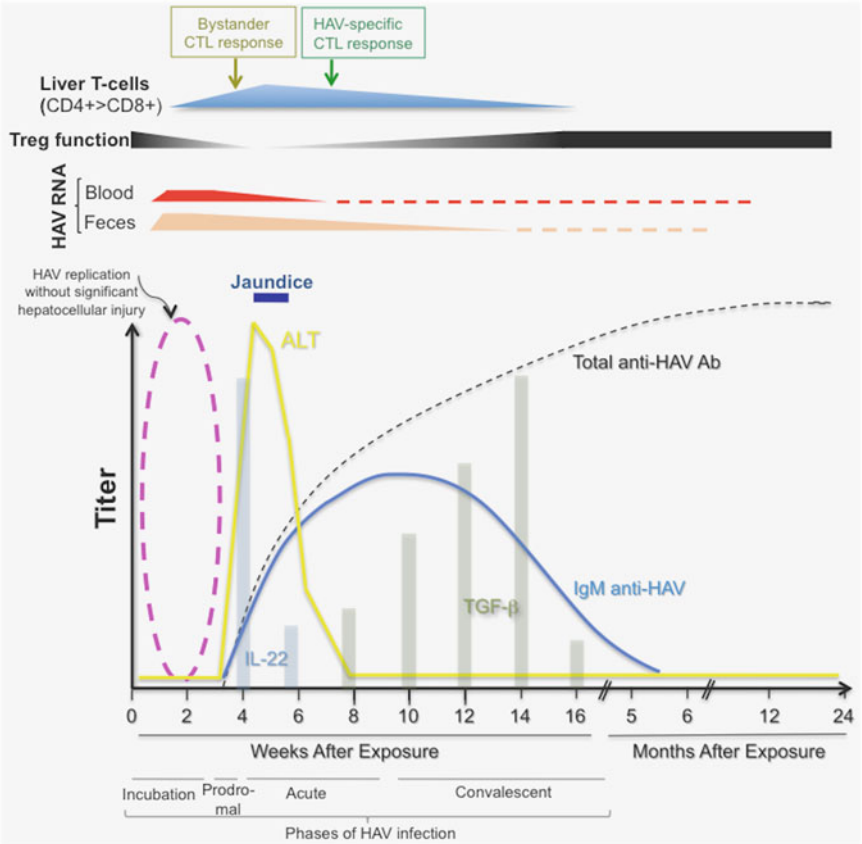

Fig. 13.2 Natural history of HAV in humans. Schematic representation of biomarkers from patients with a normal course of acute HAV infection. HAV reaches peak viremia and is shed in stools during the incubation period and prodromal phases. HAV infection blocks Treg function early in infection. Bystander and HAV-specific CTLs are activated at different times postinfection. Increased levels of alanine aminotransferase (ALT) elevation in the blood indicate hepatocellular injury. Interleukins IL-22 and TGF-β (histograms) are produced at different times postinfection. Anti-HAV IgM and IgG antibodies appear in the blood. Modified from Kaplan et al. [65]

in Argentina, India, and other developing countries [68, 69]. The cause(s) for this higher incidence of fulminant HA in young children from developing countries compared with the USA is unknown but could reflect co-infections, treatments, the underlying health conditions of the population, and the management of severe hepatitis cases. Symptomatic HA increases with age and 53 % of adults ≥60 years of age develop severe HA, which requires hospitalization. HA-associated fatalities range from 0.1 % in patients ≤15 years of age to 1.8 % in patients ≥50 years of age, and fatalities are higher in individuals with underlying liver disease.

During the normal course of infection (Fig. 13.2), HAV is ingested and reaches the liver from the gastrointestinal tract (GI) through an unknown mechanism. HAV replicates extensively in the liver during the *incubation period* of 15–50 days [70, 71] without causing cell damage, as assessed by normal levels of liver enzymes in the blood. The virus grows in the infected hepatocytes and is primarily secreted into the bile, which reaches the intestinal content and is shed with the stools. HAV produced in the hepatocytes also reaches the blood causing viremia. A variable amount of virus is produced during infection that can reach $10^{7–11}$ genome equivalents (Geq) per gram of feces and $10^{4–7}$ Geq/mL of plasma. The peak secretion of virus in stools and to a lesser extent in the blood occurs simultaneously in the absence of significant liver damage [72], which is a hallmark of HAV infection. Patients are most infectious during the 1–4 weeks of incubation, particularly at the peak of viral production. Following the incubation period, there is a short pre-icteric *prodromal phase* that lasts days to weeks, marked by the appearance of dark urine containing elevated levels of bilirubin caused by a reduction in liver function. During this phase, patients suffer nonspecific symptoms, including malaise, flu-like symptoms, anorexia, and fever, but there is limited hepatocellular damage (low ALT elevation). Additional symptoms could include myalgia, arthralgia, cough, pharyngitis, diarrhea, pruritus, and urticaria. After the prodromal phase, an icteric *acute phase* ensues, which typically lasts 2–4 weeks but can range from 7 to 87 days. Yellowing of the skin and eyes (jaundice), transient elevation of total IgM in blood, and hepatomegaly and hepatic tenderness in 50 % of the patients are characteristics of the HA acute phase. The prodromal nonspecific symptoms tend to diminish during jaundice, and bilirubin levels rarely exceed 170 μmol/L. Hepatocellular injury during the acute phase results in the rapid elevation of serum transaminases that reach peak levels of 500–2,000 U/L. The appearance of anti-HAV IgM and IgA, which can last 3–6 months, followed by anti-HAV IgG, which can persist for life, marks the rapid decline in the levels of virus in stools and blood, reaching basal levels in 2–3 weeks. In some

instances, HAV RNA in blood and liver can be detected for more than 1 year using sensitive PCR methods, but it is unclear whether this represents residual viral nucleic acids that form stable double-stranded materials or infectious virus. The virus is cleared in the *convalescent phase*, and the patient recovers. Humoral immunity plays a significant role in virus clearance and protection, and passive immunity is effective in pre- and postexposure prophylactics. It is unclear what is the role of HAV-specific cellular immunity in hepatocellular damage and the final clearance of the virus [73]. HAV infection is typically resolved within 3–6 months after infection without the establishment of chronic infection.

The majority of HA cases follow a normal course of infection. However, a significant proportion of HA patients present complications that are briefly described below (for reviews, see [2, 3, 74, 75]). After apparent recovery, 6–10 % of patients develop *relapsing HA*, with HAV shedding in stools and increase levels of bilirubin and aminotransferases in the blood. Patients with relapsing HAV have a good prognosis and recover completely; however, multiple relapses of HA occur, and the disease lasts 16–40 weeks. The basis for relapsing HA is not understood, but it is possible that a limited cellular immune response allows several HAV-infection cycles before achieving complete viral clearance. HAV infection can cause *prolonged HA*, a rare form of the disease in which patients develop jaundice that lasts approximately 120 days and recover completely in approximately 6 months. In *cholestatic HA*, which has a low incidence in HA patients, bile ducts are disrupted, and there is an accumulation of bile. Bilirubin levels exceed twice the levels observed during the normal course of HA, whereas aminotransferases decline to normal levels. Patients presenting cholestatic HA develop periodic pruritus, anorexia, and diarrhea. Corticosteroids help resolve the cholestasis, and patients recover completely. *Fulminant HA* is a rare form of HA characterized by severe jaundice, coagulopathy, encephalopathy, multiple organ failure, coma, and death in 70–95 % of patients. Liver transplantation can increase the survival rate to 65 % or greater. The fatality rate of fulminant HA has been estimated to be <1.5 % of hospitalized cases, with higher frequency in adult cases [76]; fatality can also occur in young children from developing countries [69, 77]. Although the cause(s) of fulminant HA is not understood, association in developed countries with rapid viral clearance and familial cases suggest a genetic predisposition ([59] and references therein). *Fatal HA* can also be caused by underlying liver disease from HBV, HCV, or other infections, and occurs at an approximately rate of 0.2 % and increases with age. HAV infection can trigger *autoimmune hepatitis* in individuals with genetic predisposition (relatives with type 1 autoimmune hepatitis) who have T-cell suppressor-inducer defects associated with the presence of autoantibodies to asialoglycoprotein receptor expressed on hepatocytes [78]. A low frequency of various *extrahepatic manifestations* of HA has been previously reported, including transient lupus-like disease, vasculitis, cryoglobulinemia, skin rashes, arthritis, neurological complications, pancreatitis, and glomerulonephritis, but the etiology of this complication is not fully understood.

After infection, HAV antigens and particles can be detected in the cytoplasm of hepatocytes and liver-resident macrophages (Kupffer cells) in HA patients ([71] and references therein) and experimentally infected NHPs [70, 79, 80]. The presence of HAV antigen and particles in Kupffer cells suggests that these cells support virus replication or phagocyte infected cells and immune complexes. In serial liver biopsies of experimentally infected NHPs, HAV antigens are first detected as diffuse and fine granular fluorescence in hepatocytes and Kupffer cells approximately 2 weeks after infection before or concomitant with virus shedding in the stools. HAV antigens accumulate in focal areas in these cells before becoming undetectable at 4–6 weeks after infection [70, 81]. The histopathological analysis of liver biopsies from acute-phase serologically confirmed cases revealed slight to moderate parenchymal changes characterized by focal necrosis, ballooning (large degenerated cells with pale-staining vacuolated or reticulated cytoplasm), acidophilic degeneration (apoptotic cells), and Kupffer cell proliferation in conjunction with various degrees of portal and lobular inflammation. Only 10 % of the patients develop steatosis (fatty degeneration). Follow-up liver biopsies obtained at 1–5 months after acute HA showed that 2/3 of the patients had normal and nonspecific reactive changes, and the remaining patients continued to present acute HA characteristics. Biopsies at 1 year after acute HA were normal, indicating the absence of chronic sequela. Because cells containing HAV antigens are scattered through the liver lobules, the direct killing of HAV-infected cells cannot explain the characteristic acute phase portal tract mononuclear cell infiltrate and periportal hepatocyte necrosis, which is likely to result from a bystander effect [81–84]. During the first 2 weeks of infection in NHPs, HAV antigen accumulates in hepatocytes concomitant with a limited leukocyte infiltration, primarily polymorphonuclear (PMN) cells, that distribute through the liver lobules without inducing portal inflammation [81]. The increased infiltration of lymphocytes, enlarged macrophages, and lymphoblasts results in portal inflammation that is mild at week 3 and significant at week 4 postinfection [81, 85]. This immune response is consistent with an immuno-silent early infection of 2–3 weeks, followed by an acute inflammatory response, resulting in hepatocellular injury (Fig. 13.2). Inflammation is reduced in the convalescent phase, and the liver tissue is regenerated via mitosis.

Innate Immunity

HAV induces a limited innate immune response compared with HCV [85]. This weak innate immune response that

cannot prevent HAV growth during the incubation period and early acute infection resembles the innate immunity response in HBV infection (for a review, see [86]). HAV prevents the induction of IFN-β ([87] and references therein) in infected cells, but it is currently unknown how HAV evades other innate immunity mechanisms, such as the effect of IFN-α and IFN-γ, the activation of 2′–5′ oligoadenylate synthetases (OAS)/RNase L system and protein kinase R (PKR) by RNA replication complexes, and the activation of innate immunity effector cells, such as NK, NKT, and DCs.

Type I IFNs, primarily IFN-α and -β, are an intricate component of innate immunity that act as an intracellular defense mechanism against viruses and induce the activation and expansion of lymphocytes that control intracellular infections. IFN-α and -β signal through the same receptor, IFNAR, but use different adaptor molecules for transcription activation. HAV-infected cell cultures treated for several weeks with a high dose of IFN-α/-β were cured from the virus infection [88–90], indicating that a robust treatment with type I IFN clears the HAV infection in vitro. However, the role of type I interferon in HA viral clearance and immunomodulation is highly controversial [91–93] because patients and experimentally infected chimpanzees [85] clear the HAV infection under low or basal levels of type I IFN. Leukocytes and plasmacytoid dendritic cells produce IFN-α in some acute HA patients [92], but this cytokine does not interfere with the growth of HAV in the liver. In infected cells, HAV blocks IFN-β production [94, 95], preventing the positive feedback loop responsible for the *autocrine* and *paracrine activation* of antiviral cell functions. To do so, HAV targets the activation of IFN regulatory factor 3 (IRF3) that prevents the transcription of IFN-β. The 2B protein of HAV interferes with the activation of IRF3 [96]. The expression of the 3ABC intermediate in cell culture results in the cleavage of mitochondrial antiviral signaling protein (MAVS) [97], an adaptor molecule required for the ssRNA/dsRNA-mediated activation of IRF3. Similarly, the expression of the 3CD intermediate in cell culture results in the cleavage of TIR domain-containing adaptor-inducing IFN-β (TRIF) and prevents the dsRNA toll-like receptor 3 (TLR3)-mediated activation of IRF3 [87]. The strong and multistage targeting of INF-β production suggests that HAV needs to block production of this cytokine to lower the overall level of type I IFN, which could block infection as shown in in vitro experiments using infected cell cultures (see above). Taken together, these data indicate that although HAV blocks IFN-β production in infected hepatocytes, the type I IFN produced by cells that are not susceptible to HAV infection, such as leukocytes and DCs, is not required to clear the virus infection and suggest that other immune non-cytolytic mechanism(s) are involved in this process.

Similar to other RNA viruses, HAV replicates via dsRNA intermediates that could activate OAS/RNase L and PKR pathways, resulting in the induction of an intracellular antiviral defense mechanisms that lead to apoptosis and prevent viral growth [98]. HAV infection typically does not cause cytopathic effect, cellular RNA degradation, or shut-off of host protein synthesis and can inhibit apoptosis induced by dsRNA [94]. However, some attenuated HAV variants highly adapted to grow in cell culture cause CPE through the induction of apoptosis [99] via the activation of the OAS/RNase L system [100]. Taken together, these data suggest that most HAV strains block the activation of the OAS/RNase L and PKR pathway through unknown mechanism(s).

There is no evidence that innate effector cells that reside in the liver, such as NK, NKT, and Kupffer cells, play any role in preventing or limiting the spread of HAV during the long incubation and acute phases. In vitro, NK cells from HAV seropositive and seronegative donors and patients with acute HA preferentially kill HAV-infected compared with uninfected cell culture cells [101–103]. The activity of NK cells was similar in the acute and convalescent phases of HAV infection [73]. In vitro, HAV suppresses the monocyte-to-macrophage maturation [104]. These data suggest that HAV limits innate effector cells from targeting HAV-infected hepatocytes through unknown mechanisms.

Humoral Immune Responses

HAV replicates in the liver for 2–3 weeks without inducing a humoral response. The appearance of anti-HAV IgM concomitant with IgA occurs at approximately 4 weeks postinfection at the peak of HAV viremia and virus shedding in the stools, followed by the appearance of IgG antibodies. In the acute phase, infiltrating plasma cells produce IgM and IgA antibodies, and in the convalescent phase, these cells produce IgG, IgA, and to a lower extent, IgM [105]. The anti-HAV IgM antibodies can be detected for 6 months after infection and in some cases, persist for several years [106], whereas IgG antibodies persist for life. The role of anti-HAV IgA antibodies is unclear, but these antibodies can be detected in sera for several years [107]. Anti-HAV secretory IgA has been detected in saliva and feces in samples from some patients, but it is of a transitory nature [108–110] and typically does not neutralize HAV [111]. Consequently, gastrointestinal immunity does not play a significant role in protection against HAV infection. In addition to antibodies specific to HAV, HAV infection elicits the transitory elevation of autoantibodies [78, 112, 113] and the production of high levels of HAV nonspecific IgM antibodies [114] against, for example, bacteria present in the intestinal flora [115]. The production of nonspecific antibodies reflects the transitory impairment of regulatory T-cells (Treg) [116] induced by the interaction of HAV with HAVCR1 at the cell surface of Treg, which shuts off Treg function [34].

HAV infection elicits an antibody response directed mainly to the immunodominant site in the viral capsid [117],

but some antibodies also target nonstructural proteins [118]. Pre- and postexposure prophylaxis with pooled immune serum globulin (ISG) preparations containing anti-HAV antibodies from convalescent individuals is highly efficacious in preventing acute HA (for a review, see [119]). A significant number of ISG recipients develop anicteric HA and have abnormal liver function [120, 121], indicating that passive immunity does not confer sterilizing immunity. The mechanism underlying antibody protection against HAV infection is not fully understood. Neutralizing antibodies reduce primary and secondary infections in liver cells. Complement-dependent cytolytic antibodies were not detected in the sera from acute and convalescent patients, confirming that the antibody-mediated lysis of infected cells does not play a role in controlling HAV infection [122, 123]. The antibody-mediated non-cytolytic clearance of infected cells plays a significant role in the clearance of some viruses [124] but has not been investigated in HA.

Cellular Immune Responses

HAV replicates extensively in the liver for 3–4 weeks after infection, reaching a peak in viremia and virus shedding in stools before liver enzyme elevation and the onset of symptoms [125, 126]. In vitro, most cell culture-adapted HAV strains [127] and wild-type HAV [90] do not cause CPE in cell culture. Furthermore, HAV infection does not induce cytolytic antibodies that induce hepatocellular damage [122]. Consequently, hepatocellular damage in HA is induced through a self-limiting immune-mediated necroinflammatory process characterized by a weak innate immunity response, which fails to prevent HAV replication in the early stages of infection, and a cellular immunity response activated late in infection, after the virus reaches peak growth. The unique cellular immunity mechanisms involved in infection and clearance of HAV are under investigation. In contrast with HBV and HCV, in which CD8+ T-cells are a key factor in clearing infection (see corresponding chapters in this book), the role of T-cells in clearing the virus in HA is poorly understood. In the 1980s, the immunohistochemical analysis of liver biopsies from patients with acute HA symptoms revealed that the predominant lymphocytes infiltrating the liver are CD4+ T helper cells and B-cells and not CD8+ T-cells, as shown for HBV and HCV infections [105, 128]. A recent analysis of liver transcriptome and immune response in chimpanzees infected with HAV [85, 129] further supports these initial findings. These recent studies showed that HA is characterized by a strong B-cell activation in the liver and a predominant CD4+ T helper cell response in the liver and peripheral blood. This strong B-cell and CD4+ T helper cell activation in the liver precedes a weak CD8+ T-cell response that contracts rapidly before the clearance of viral RNA from infected hepatocytes. Interestingly, the HAV-specific CD4+ T -cell response in the chimpanzees contracted very slowly within 8–12 months, mirroring the clearance of the residual HAV genomes in infected hepatocytes [129].

The pioneering work mainly from the Vallbracht and Flehmig groups in Germany during the 1980s and 1990s suggested that CD8+ cytotoxic T lymphocytes (CTLs) played a major role in acute HA and the clearance of the virus [73, 130–132]. However, a model based only on CTLs killing infected cells and clearing the virus, which does not occur in the pathogenesis of HBV and HCV, is also unlikely in the pathogenic process of HAV. Interestingly, HAV-specific CTLs were isolated from the peripheral blood of late acute and early convalescent patients but not early in infection [73]. The recent mapping of HAV-specific MHC class I epitopes in humans [133] and chimpanzees [129] and the use of labeled class I tetramers loaded with HAV peptides further confirmed the presence of HAV-specific CD8+ T-cells in the peripheral blood of late acute and early convalescent individuals. The isolation of T-cells from biopsies confirmed the presence of infiltrating HAV-specific CTLs in the liver of patients with acute HA [131, 132]. However, the contribution of infiltrating HAV-specific CTLs to liver damage and virus clearance is uncertain due to the lack of temporal association between HAV-specific CTL function, which peaks weeks after the onset of jaundice, and the clearance of the virus, which starts before the onset of symptoms at approximately 4 weeks after infection [73, 129]. Further evidence against a role for HAV-specific CTLs in hepatocellular damage and viral clearance was provided by recent data, which (a) suggested that hepatocellular damage in acute HAV primarily reflects the bystander activation of CD8+ T-cells [84] and (b) showed that CD8+ T-cell effector functions during viremia were absent or restricted to IFN-γ production in HAV-infected chimpanzees [129]. Because a significant number of liver cells are already infected with HAV at the time of symptoms, an uncontrolled CTL response could lead to massive liver damage, as observed in fulminant hepatitis. Therefore, the lack of an HAV-specific CTL response at the onset of symptoms is consistent with the low rates of fulminant HA, suggesting non-cytolytic mechanisms for the clearance of virus infection.

CD4+ regulatory T-cells (Tregs), which express CD25+ and the FoxP3 transcription factor, play a significant role in the suppression of autoimmune disease [134] and the immune response to bacteria, viruses, parasites, and fungi [135]. Pathogens have evolved strategies to activate Tregs to limit immune responses and prevent tissue damage, which is advantageous for the expansion and survival of obligate intracellular parasites [135]. For example, the increased frequency and function of Tregs has been associated with HCV chronicity [136]. However, HAV infection temporarily blocks the function of Tregs, which cannot suppress T effector

cells during acute HAV infection [116]. It has recently been shown that Tregs express the HAVCR1 at the cell surface and binding of HAV to HAVCR1 blocks T-cells receptor activation and shuts off Treg function [34]. Although HAV does not replicate in T-cells [34], the HAV–HAVCR1 interaction on Tregs and perhaps other immune cells significantly affects the modulation of immune responses, which could account for the suppression of innate and cellular responses observed during HAV infection.

The analysis of cytokines in blood from acute HAV patients [34, 137], chimpanzees [129], and monkeys [138] revealed that only a few cytokines are produced during HAV infection. IL-22, a cytokine that protects liver cells from damage, was detected in human blood during the early acute phase. TGF-β, a pleiotropic cytokine that is needed to mount effective T-cell responses, was detected in human blood during the late acute and convalescent phases. The lack of TGF-β during the HA incubation period and acute phase is consistent with the limited CD8+ T-cell response observed during the acute phase. IFN-α, a cytokine primarily produced by plasmacytoid DCs, is required to induce CD8+ T-cell responses and has been detected in some patients with acute HA but did not have a significant impact on the outcome of the disease [137]. HAV-infected chimpanzees produce INF-γ, TNF-α, IL-2, and IL-21 [129], reflecting a stronger CD4+ T-cell response, that limits the severity of HA compared with humans.

In summary, HAV infection induces a late and limited cellular immunity response, which plays an uncertain role in hepatocellular injury and virus clearance.

HAV Infection and the Permanent Modulation of Immune Responses

Epidemiological studies showed an inverse association between HAV infection and the development of autoimmune [139] and allergic diseases [140, 141]. These studies tested the hygiene hypothesis [142], which states that the lack of exposure to microbes during childhood increases susceptibility to allergic diseases due to the suppression of the development of the immune system, and highlighted HAV infection as a permanent modulator of the immune response. The mechanism by which HAV modulates immune responses is not well understood but involves the interaction with its cellular receptor HAVCR1 [29, 30, 143]. It has been shown that HAVCR1 is associated with susceptibility to rheumatoid arthritis [144–146], suggesting a role for this receptor in autoimmune diseases, and that HAVCR1 alleles are protective against the development of allergies [147]. The recent finding that Treg function is impaired in patients with acute HA [116] due to the interaction of HAV with HAVCR1 expressed on Treg [34], implicated these cells in the permanent modulation of immune responses due to HAV infection. Shutting off the suppression function of Treg through the HAV–HAVCR1 interaction could induce the uncontrolled expansion of allergic and autoimmune effector cells, resulting in activation-induced cell death (AICD) [148] and the cleansing of pathogenic effector cells. The AICD of T effector cells that contribute to allergy and autoimmunity due to HAV infection is consistent with the hygiene hypothesis and provides a mechanism that might explain the inverse association between HAV infection and development of autoimmune and allergy diseases.

Pathogenesis and Liver Damage

The pathogenesis of HAV is poorly understood, primarily due to the lack of a small animal model. It is unknown how HAV reaches the liver after ingestion and whether there is an extrahepatic site of replication in the GI. Small amounts of HAV have been detected in saliva and throat swabs in chimpanzees [149], the crypt cells of the small intestine in orally infected owl monkeys [81], and the salivary glands of intravenously infected cynomolgus monkeys [150]. However, it is unclear whether HAV replicates in the GI, the detected antigen is derived from the inoculum, or the virus is produced in the liver and transported through the blood to the GI. If HAV replicates in the GI, infected cells could secrete the virus to the blood compartment. Alternatively, HAV could reach the blood stream through transcytosis or bound to scavenger cells that sample the lumen of the GI. Once in the bloodstream, HAV is distributed through the body, reaching the liver, its target site of replication. Unfortunately, the determinants of HAV hepatotropism and hepatovirulence are currently unknown.

We are beginning to understand the mechanism of pathogenesis of HAV and the intricate pathways that this unique picornavirus has evolved to evade immune surveillance. HAV grows unchecked by the immune system for several weeks without inducing hepatocellular injury while reaching a peak in shedding and viremia, which precedes the complete clearance of the virus in the context of a self-limiting liver disease. A model of HAV pathogenesis involving the shut-off of Treg function has recently been proposed [65] and is updated herein (Fig. 13.3). Once this virus reaches the liver, HAV replicates extensively in hepatocytes (A) during the 3–4 week incubation period, evading mechanisms of innate and acquired immunity. In infected hepatocytes, HAV prevents the activation of intracellular antiviral pathways triggered by dsRNA replication intermediates through unknown mechanisms(s) and blocks the production of IFN-β. The virus is shed in feces and reaches the blood, inducing a viremic stage (B). HAV binds to HAVCR1 expressed at the cell surface of Tregs, temporarily shutting-off their function

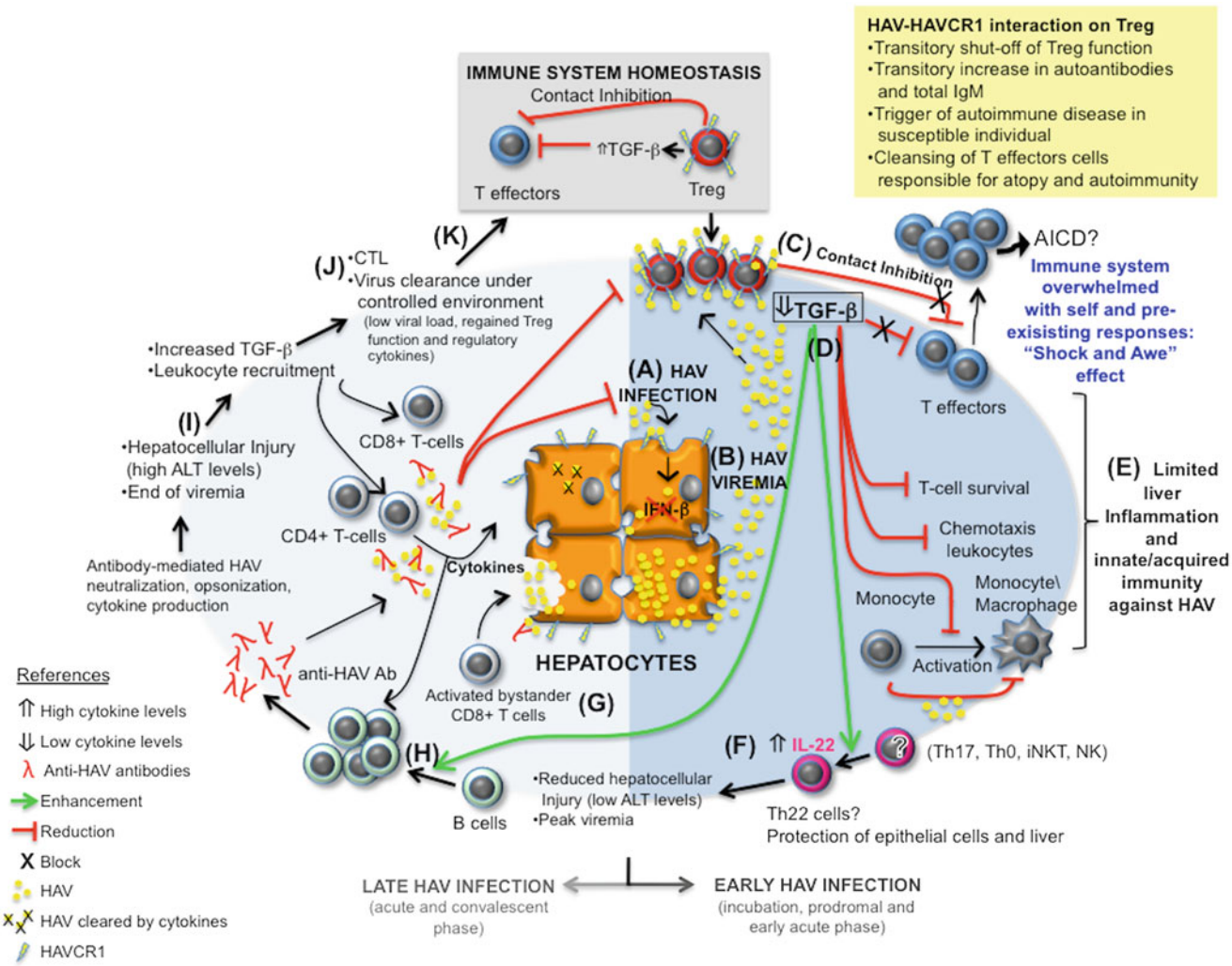

Fig. 13.3 Model of the pathogenesis of HAV involving the shut-off of Treg function. Schematic representation of the cycle of immune events leading to a typical course of HAV infection. The HA immunopathogenesis is characterized by the evasion of innate and cellular immune responses and a strong humoral immune response. HAV infection induces limited inflammation process that culminates in the complete clearance of the virus. The HAV pathogenic process is described as a cycle. (A–F) The distinct immune milieu induced by HAV infection during early (incubation, prodromal, and early acute phase) facilitates virus replication in the liver, evading immune recognition without triggering hepatocellular injury. (G–K) A strong humoral response and limited cellular response results in the clearance of HAV through non-cytolytic and cytolytic mechanisms in the acute and convalescent phases. (A) HAV replicates in hepatocytes, blocking the production of IFN-β and other intracellular antiviral systems activated by dsRNA replication intermediates. (B) The virus is secreted into the blood, inducing the viremic stage. (C) HAV binds to HAVCR1 on Treg, causing a transitory block of Treg function and thereby facilitating the activation of anti-self and preexisting responses that overwhelm the immune system, inducing a "Shock and Awe" effect that limits anti-HAV de novo responses. The clonal expansion of T-cells might result in AICD and the deletion of pathogenic T-cells. (D) HAV blocks Treg from producing TGF-β, a pleiotropic cytokine required for initiating and maintaining cellular immune responses. (E) The HAV infection limits liver inflammation and innate and acquired immunity against the virus. (F) HAV induces the production of IL-22, which protects the liver and epithelial cells from injury. (G) The lack of Treg function favors the activation of bystander CD8+ T-cells that mediate the hepatocellular damage during the early acute phase. (H) Low levels of TGF-β favor the development of a strong anti-HAV antibody response that reduces viremia. (I) The gradual restoration of Treg function and production of TGF-β facilitate the recruitment of leukocytes to the liver and contributes to the inflammatory process. (J) The activation of HAV-specific CTLs in the early convalescence phase contributes to the final clearance of the virus. (K) The immune system returns to homeostasis. Modified from Kaplan et al. [65]

and preventing the suppression of T effector cells (C). This transitory shut-off of Treg function facilitates the activation of anti-self and preexisting responses, inducing a "Shock and Awe" effect that overwhelms the immune system and limits de novo anti-HAV immune responses. This effect has been well documented in HA patients who exhibit a transitory elevation of autoantibodies [78, 112, 113] and nonspecific IgM antibodies [114, 115], indicating that HAV infection

disrupts the homeostasis of the immune system. The shut-off of Treg function allows the clonal expansion of T-cells, resulting in AICD [151] and the deletion of pathogenic T-cells. This "cleansing mechanism" could be responsible for the protective effect of HAV infection in the development of allergy and autoimmunity. HAV infection blocks Treg from producing TGF-β (D), which affects T-cell survival, the chemotaxis of leukocytes to the site of infection, and activation of monocytes limiting liver inflammation and innate and acquired immunity against HAV (E). The increase in the production of IL-22 (F) during the viremic phase might compensate for the lack of Treg function by preventing collateral tissue damage because IL-22 protects the liver and epithelial cells from injury [152]. This particular immune milieu during early HAV infection (incubation, prodromal, and early acute phase) allows the replication of HAV in the liver with limited or no inflammation and hepatocellular injury. The lack of Treg function favors the activation of bystander CD8+ T-cells (G), which mediate hepatocellular damage in the early acute phase. The low levels of TGF-β favor the development of a strong anti-HAV antibody response (H), which reduces viremia and prevents the HAV–HAVCR1 interaction. A gradual restoration of Treg function and the production of TGF-β allows the recruitment of leukocytes (I) to the liver, which contributes to the inflammatory process in the late acute and convalescent phases. These effects are consistent with recent findings in HAV-infected chimpanzees [129], in which a peak in HAV-specific CD4+ T helper cell function occurs after hepatocellular injury and contracts very slowly. A peak in HAV-specific CD8+ T-cells with limited effector function, primarily, restricted to the production of IFN-γ, follows the of CD4+ T-cell peak. The HAV-specific CD8+ T-cells contract in a matter of days, which is consistent with a restricted cytokine milieu and the induction of AICD. The production of cytokines by the infiltrating leukocytes in the convalescent phase promotes viral clearance through cytolytic and non-cytolytic mechanisms. The activation of HAV-specific CTLs, which peaks at 3–4 weeks after jaundice in the convalescence phase (J), and the strong humoral response, which neutralizes the virus and contributes to the non-cytolytic clearance of the virus from infected cells, is likely to complete the clearance of HAV. The contraction of the T-cell response and restoration of the Treg function normalize biomarkers and return the immune system to homeostasis (K). It is likely that genetic and environmental factors could affect this typical course of HAV infection and result in the aggressive and early activation of CTLs. Such a scenario could reduce virus yield but increases hepatocellular damage, resulting in severe HA and perhaps fulminant hepatitis. In addition, a lack or weak CTL response in the convalescent phase may prevent the clearance of the virus and result in recurring HA. This unique pathogenic process allows the extensive growth of HAV in the liver during a long incubation period evading the immune response, results in the clearance of the virus without chronic sequela, induces life-long immunity against HAV, and confers protection against autoimmune and allergic diseases.

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Immunopathogenesis of Chronic Hepatitis B and the Clinical Events That Shape its Natural History

14

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Key Points

- Innate immunity to hepatitis B virus (HBV) limits viral spreading and involves appropriate activation of natural killer cells, induction of α/β and γ interferons, and release of proinflammatory cytokines. An appropriate innate immune response is important for a healthy adaptive immune response.
- HBV may act as a stealth virus, remaining undetected by the host innate immune response in the early phases of acute infection. This allows more efficient spread and can delay and/or alter the subsequent adaptive immune response.
- CD4 cells contribute indirectly to the control of HBV infection by facilitating the induction and maintenance of virus-specific B cell and CD8 T cell responses. An early CD4 cell response to HBV infection is required to induce the CD8 response that clears the infection.
- A vigorous polyclonal, multi-specific CD8+ T cell response directed towards HBV core, envelope, and polymerase epitopes is critical to establishing viral control. Viral clearance is achieved through both cytolytic and noncytolytic functions of the adaptive immune system.
- Conversely, development of chronic HBV infection is associated with a weak or absent polyclonal, multi-specific CD8+ response. The ability to suppress HBV during chronic infection is limited by the fact that HBV-specific CD8+ reactivity is narrowly focused and spontaneous mutations in immunogenic viral epitopes can result in diminished T cell recognition of viral antigens and the emergence of escape mutants.
- The origin of the immune-tolerant phase of chronic infection is incompletely understood and probably results from several factors including the immunotolerogenic effect of HBeAg, the inhibitory effects of regulatory T cells, T cell depletion associated with high levels of HBsAg, inhibitory effects of the HBV X and polymerase proteins, and impaired dendritic cell function.
- The natural history of chronic HBV infection is characterized by an ongoing state of inflammation and hepatocyte injury resulting from continuous cell-mediated responses to HBV and the destruction of infected hepatocytes.
- Mutant variants of HBV may be selected under immunologic pressure during longstanding chronic infection, and this may affect host immune responses. Examples of this may be seen in the selection of HBV mutants that disrupt HBeAg production and core gene mutations that down regulate the immunologic efficiency of CD8+ CTLs.
- HBV reactivation during chronic infection can occur when immunosuppressive drug therapy is given. The risk for reactivation not only correlates with the potency of immune suppressive therapy, but also with the serologic status of the host. This suggests that the preexisting gradient of immune control over viral replication may be an important determining factor.

Introduction

It is estimated that more than two billion people worldwide have been exposed to hepatitis B virus (HBV) and as many as 350 million people have chronic infection [1, 2]. HBV is a major cause of chronic liver disease, liver failure, and hepatocellular carcinoma (HCC). Chronic HBV infection is currently the most common risk factor for HCC worldwide, accounting for more than half of cases and resulting in significant morbidity and mortality [3, 4]. The natural history of HBV infection involves a complex interplay between viral

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factors and immunologic determinants of spontaneous resolution, chronic infection, and progression of chronic liver disease within the host. In this chapter, we will primarily review the current knowledge about the immunological interface between host response and HBV in addition to the many clinical correlates supporting the assertion that these are critical to viral persistence, disease progression, and response to antiviral therapy.

The Liver Microenvironment

Broad cellular mixture and microorganization of the liver has profound implications for its immune function [5]. Hepatocytes or parenchymal cells constitute approximately 70 % of the total cell population of the liver. Endothelial cells, lymphocytes, macrophages, and dendritic cells each have important immunologic functions and collectively constitute 90 % of the remaining cells. Even hepatocytes are capable of functioning as antigen-presenting cells under the right physiologic conditions. Collectively, this makes the liver a unique immunological site as antigen-rich blood from the gastrointestinal tract and circulation is pressed through a network of sinusoids and scanned by antigen-presenting cells and effector lymphocytes.

The liver sinusoids are lined by a fenestrated monolayer of sinusoidal endothelial cells (Fig. 14.1). Liver sinusoidal endothelial cells (LSECs) have a different cellular morphology from endothelial cells in other organs and they express molecules that promote antigen uptake and promote antigen presentation, including major histocompatibility complex (MHC) class I and II antigens. LSECs are in close proximity to two other types of resident antigen-presenting cells that traverse the vascular spaces (Kupffer cells and dendritic cells). Kupffer cells are resident macrophages that line the hepatic sinusoids. They are in close proximity to passing lymphocytes and pass through the space of Disse where they make direct contact with hepatocytes and phagocytize apoptotic hepatocytes. Dendritic cells are antigen-presenting cells that are derived from the bone marrow. They are typically located around the central vein and portal tracts where they are capable of inhibiting proliferation and proinflammatory cytokine production by activated lymphocytes.

The average human liver contains approximately 10^{10} lymphocytes including subpopulations of the innate (NK and NKT) and adaptive immune systems (T and B cells). Lymphocytes are found scattered throughout the liver parenchyma as well as in the portal tracts. Conventional T cells include CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T cells which recognize antigens in the context of MHC

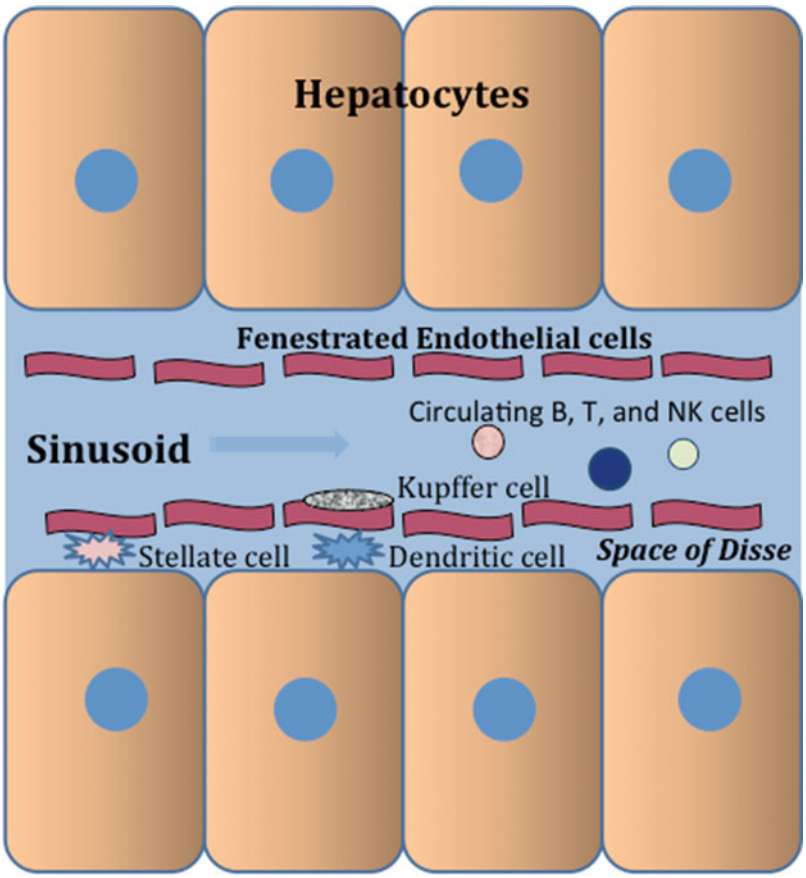

Fig. 14.1 The liver microenvironment. Immune cells in the healthy liver are depicted (see text for further details). Kupffer cells, liver sinusoidal endothelial cells, stellate cells, and dendritic cells are all in close proximity and their cellular functions interact to affect viral antigen presentation. Regulatory T cells are presumed not to be present or activated

class I and II molecules, respectively. Unconventional T cells comprise various cell types that are categorized into two subpopulations, natural killer (NK) cells which bear NK cell markers and those that do not (NKT cells). NKT cells are vital for the innate immune response and can constitute up to 30 % of the intrahepatic lymphocyte population. NKT cell migration to and expansion within the liver is controlled by NK cells. The latter cells have potent cytolytic activity against virus-infected or tumor cells.

The Hepatitis B Virus

Genomic Organization and Viral Transcripts

HBV is an enveloped, partially double-stranded DNA virus of the Hepadnaviridae family [6]. The hepatitis B virion is approximately 42 nm in size. The virion comprises an inner nucleocapsid of hepatitis B core antigen (HBcAg), which encloses a copy of double-stranded circular HBV DNA and the HBV DNA polymerase, and an outer lipid bilayer envelope containing viral glycoproteins including the hepatitis B surface antigen (HBsAg) (Fig. 14.2). The double-stranded circular HBV DNA genome is approximately 3.2 kb and encodes four major RNA transcripts of 3.5, 2.4, 2.1, and 0.7 kb in length. The largest transcript (3.5 kb) functions as both messenger RNA and as pre-genomic RNA. Translation of HBV RNA into proteins involves four overlapping open reading frames (ORF): S (surface envelope), C (core), P (polymerase), and the X protein. Both the S and C ORFs have in-frame initiation codons that facilitate translation of different proteins within the RNA transcript. As a result, the C ORF encodes for the pre-core and core (HBcAg) proteins, while the S ORF encodes for a large, middle, and small HBsAg protein. The pre-core protein undergoes proteolysis within the endoplasmic reticulum and becomes the hepatitis B e antigen (HBeAg). The P ORF encodes the HBV polymerase, an enzyme responsible for DNA synthesis, reverse transcription, and degradation of pre-genomic RNA. The X ORF encodes the X protein (HBxAg), which is required for viral replication and is thought to be involved in several functions such as signal transduction, cell cycle regulation, transcriptional activation, and DNA repair [7, 8].

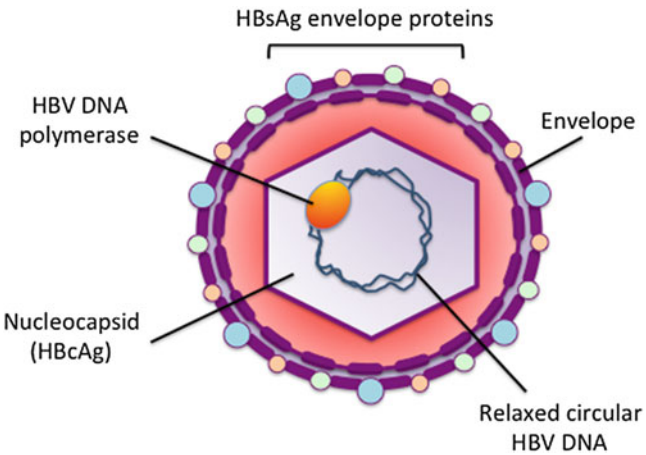

Fig. 14.2 Hepatitis B virus and major viral transcripts. The envelope protein (HBsAg) and nucleocapsid protein (HBcAg) have important interactions with the host immune system. Mutations in the HBV DNA polymerase protein can lead to resistance to antiviral therapy. Also, due to the overlapping nature of the DNA polymerase gene with the HBsAg gene, mutations in the former can be associated with configurational changes in the "a" epitope that result in impaired binding to neutralizing antibody (anti-HBs). The X gene transcript (not shown here) may have a negative impact on innate immunity by its effects on cell signaling. See text for further details

Life Cycle

The HBV life cycle begins with binding of the hepatitis B virion to hepatocytes through interactions between cell surface receptors and viral envelope proteins, including HBsAg (Fig. 14.3) [6]. The virion is allowed entry into hepatocytes through endocytosis and the nucleocapsid is then released into the cytoplasm. The nucleocapsid, which is uncoated at the nuclear membrane, releases its relaxed circular HBV DNA (rcDNA) into the nucleus. The rcDNA is converted into a covalently closed circular double-stranded DNA (cccDNA) molecule within the nucleus, involving a process of DNA repair and covalent ligation of both strands of circular DNA [9, 10]. The newly formed HBV cccDNA then serves as a template for subsequent transcription of viral RNA mediated by the host cellular RNA polymerase II. HBV cccDNA is characterized by a high degree of stability, as it remains in the nucleus for the lifetime of the hepatocyte and appears to be resistant to eradication even in the setting of spontaneous resolution of acute infection or successful antiviral therapy [11–13]. Assembly of the HBV virion occurs within the cytoplasm. Pre-genomic RNA produced in the hepatocyte nucleus is enclosed within nucleocapsids and becomes a template for reverse transcription and synthesis of new HBV DNA. Pre-genomic RNA within the nucleocapsid is converted into viral rcDNA through reverse transcription mediated by the HBV DNA polymerase. Final assembly of hepatitis B virions takes place at the endoplasmic reticulum and mature virions are excreted through a process of budding and vesicular transport into the extracellular space where the virus is free to infect other hepatocytes. As HBV infection is limited to only humans and chimpanzees, several animal models of hepadnaviral infection including woodchuck and avian hepatitis have provided a greater understanding of the molecular biology of HBV, its life cycle, the development of chronic hepatitis, and the role of host immunity in disease pathogenesis; however, one major limitation of animal and tissue culture systems is that they do not reflect the

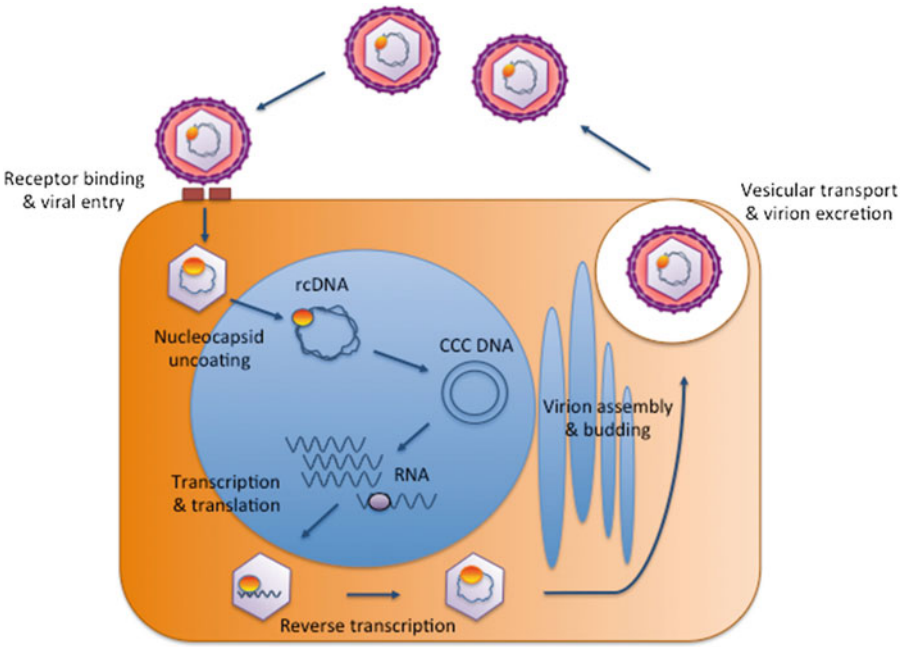

Fig. 14.3 The hepatitis B virus life cycle. During the process of viral replication, unincorporated relaxed circular HBV DNA enters the nucleus where it assumes the covalently closed circular DNA (cccDNA) configuration. All viral transcripts are encoded from the HBV cccDNA which ensures that virion production and antigenic stimulation by core antigen and its derivatory peptides continues for the lifetime of the hepatocyte. Apoptosis of hepatocytes and immune-mediated hepatocytolysis (spontaneous or antiviral induced) can both reduce the amount of cccDNA in hepatic tissue. See text for further details

complex environment of host–viral interactions that occur within humans.

HBV replicates at a very high rate, producing approximately 10^{11} virions per day within the infected host [14]. As the HBV DNA polymerase does not have a proofreading function, the high rates of replication in HBV infection are associated with a high frequency of spontaneous mutations. The rate of naturally occurring mutations in HBV is estimated to be as high as 3.2×10^{-5} nucleotide substitutions per site per year, up to tenfold greater than rates reported in other DNA viruses [15]. Consequently, HBV may exist in the form of multiple quasispecies within the host, in which some variants may predominate as a result of selection pressures including host immunity, replication fitness of specific mutations, and antiviral therapy [16].

Acute Hepatitis B

Acute exposure to HBV is associated with an incubation phase of low viral replication followed by a progressive rise in viremia with a peak level of serum HBV DNA at approximately 8–10 weeks following exposure [17, 18]. At that time, it is estimated that up to 100 % of hepatocytes can be infected with HBV [18–20]. Various factors involving both the virus and the host play a role in determining whether an individual will achieve spontaneous resolution of the infection or whether the virus will persist in the form of a chronic infection. Longitudinal studies in cohorts following single-source outbreaks of HBV infection have noted NK cell activity coinciding with peak viremia following acute exposure [17]. These and other human studies have suggested that a vigorous polyclonal, multi-specific CD8+ T cell-mediated response directed towards HBV core, envelope, and polymerase epitopes is critical to establishing control of HBV and defines cases of spontaneous resolution in the setting of acute infection [17, 21–23]. Reports also suggest that clonal expansion of CD8+ T cells directed against HBV core epitopes predominate and may be particularly important in achieving spontaneous resolution [17, 23]. B cells are not essential for clearance of HBV following acute infection, although the humoral adaptive response plays a role in establishing long-term immunity and immune surveillance following resolution [24]. Although these studies provide key insight into the host immune response to HBV, they have largely been limited to peripheral blood assessment in the human model.

Chimpanzee studies have provided even greater insight into the host immune response in both the peripheral blood and in the liver that characterize the ability to achieve viral control following acute exposure to HBV. Both cytolytic and noncytolytic functions of the adaptive immune system enable the host to achieve clearance of HBV during acute infection. Studies in acutely infected chimpanzees found that a vigorous and multi-specific CD8+ T cell-mediated response in the liver and peripheral blood directed towards HBV is critical in the achievement of viral clearance [18]. Although the CD4+ T cell response was observed early following acute exposure, HBV DNA levels did not decline until the emergence of a multi-specific CD8+ cytotoxic response directed towards HBV envelope, core, and polymerase proteins. The CD8+ T cell-mediated clearance of HBV in these studies was not

Table 14.1 Innate and adaptive immunity in acute and chronic hepatitis B

Phase of immunologic response	Timing	Key effector cells	Major cytokines produced	Effect of normal response or hyper-responsiveness	Effect of hypo-responsiveness
Innate	Early (days to weeks after exposure)	NK, NKT CD4+Th1, CD8+ CTLs[a]	α/β and γ IFN, TNF-α	Viral clearance or fulminant hepatitis	Viral persistence and lack of cytokine symptoms
Adaptive	Later (weeks to months after exposure or long-term)	CD8+ CTLs	Proinflammatory cytokines (e.g., IL-1, 12, 23), TGF-β		Gradient of necroinflammatory changes in liver

[a]Evidence from transgenic mice experiments indicate that these cells deliver an apoptotic signal to infected hepatocytes [29]

altered by depletion of CD4+ T cells; however, CD8+ depletion resulted in prolongation of HBV viremia until CD8+ subsets were restored [18]. The observed CD8+ response in this setting coincided with elevations in ALT suggestive of necroinflammatory activity as well as a significant increase in intrahepatic expression of interferon (IFN)-γ and IFN-γ-induced genes [18, 25]. These and other studies suggest that the host immunity is capable of suppressing HBV replication noncytolytically through the activity of IFN-γ and TNF-α, as HBV clearance can be achieved without destruction of infected hepatocytes. The noncytolytic mechanism is driven by an effective CD8+ response within the liver and appears to be a primary route of eliminating virus in the acute setting [19, 26–29], possibly through disruption of HBV nucleocapsid assembly [30, 31].

The size of the HBV inoculum can have an impact on the outcome of infection, particularly in relation to both the CD4+ and CD8+ T cell response. Although CD8+ T cells have been recognized as the primary cell group responsible for achieving clearance of HBV, experiments in chimpanzees have demonstrated that an early CD4+ response is critical to orchestrating a successful well-coordinated influx of CD8+ T cells in liver tissue [20, 24]. In these studies, chimpanzees inoculated with very high or very low doses of HBV demonstrated a prolonged course of CD8+ T cell-mediated clearance associated with a late appearance and extended duration of hepatocellular necroinflammatory activity and elevated liver enzymes. In these cases, a CD4+ response was elicited only after viral spread had occurred. In the chimpanzees with persistent infection due to low dose inoculation, the CD4+ response was absent, while those receiving an intermediate dose demonstrated early CD4+ activity before viral spread and a subsequent rapid CD8+-induced clearance of HBV [20]. A possible clinical correlation to this phenomenon was observed in human subjects inoculated with varying concentrations of HBsAg-positive serum. In these experiments the time to appearance of serum HBsAg and time to evidence of clinical hepatitis were inversely related to the dose of inocula [32]. One may speculate that a low-dose inoculum was not sufficient to trigger an early CD4+ response needed to coordinate the rapid and effective clearance of HBV by CD8+ T cells.

The host innate immune system appears to contribute to achieving resolution of acute infection through activation of NK, NKT cells, and Kupffer cells, resulting in further cytokine production and direct cytotoxicity mediated by these cell types (Table 14.1). The innate immune system is also important in limiting the spread of new infection and recruiting the adaptive immune system to initiate a targeted cytotoxic response. Although it appears that HBV is capable of suppressing the innate immune system in the setting of acute infection through mechanisms such as downregulation of toll-like receptor (TLR) expression and signaling [33–37], the presence of NK and NKT cell activity has been observed early in the course of acute infection with HBV [17, 38, 39]; however, their role appears to be supportive in the process of achieving viral clearance [24].

Chronic Infection

A failure to develop an adequate adaptive immune response following acute exposure to HBV may result in persistence of viremia and subsequent development of chronic infection (Table 14.1). In studies comparing individuals with chronic infection vs. spontaneous resolution, it has been well established that those with chronic infection consistently demonstrate very weak or absent polyclonal and multi-specific CD8+ responses to the HBeAg, polymerase, core, or surface antigens based on peripheral blood assessment [21, 22, 40, 41]. The weak cytotoxic response seen in those with persistent HBV infection is likely the primary determinant of chronicity and does not appear to result from the emergence of escape mutations [42]. However, the ability to suppress HBV during chronic infection is variable and some individuals maintain persistently low levels of viremia. Cases of low level viremia have been associated with the presence of peripheral and liver-infiltrating multi-specific CD8+ T cells, particularly directed towards HBV core epitopes [43, 44]. In addition, these CD8+ T cells demonstrate the capacity for expansion following core antigenic stimulation in contrast with individuals with high viral loads [44]. Despite the presence of an ongoing immune response to HBV in some

Table 14.2 Extrahepatic manifestations of chronic hepatitis B infection

Polyarteritis nodosa
Palpable purpura
Membranoproliferative glomerulonephritis
Membranous glomerulonephritis
Mesangial proliferative glomerulonephritis
Essential mixed cryoglobulinemia

individuals with chronic infection, the CD8+ T cell reactivity directed towards HBV antigens appears to be narrowly focused, in which spontaneous mutations corresponding with specific epitopes can result in diminished T cell recognition of viral antigens, the emergence of escape mutants, and subsequent persistence of infection [45].

In the presence of viral persistence, the host cell-mediated immunity may continue with a course of ongoing cellular activation and CD8+ T cell cytotoxicity within the liver; however, this response is incapable of achieving control of HBV infection (Table 14.1) [46]. Consequently, a state of chronic inflammation and hepatocyte injury persists within the liver resulting from long-term cell-mediated immunity against HBV and the destruction of infected hepatocytes. This is associated with persistent elevations in serum aminotransferases and risk of progressive hepatic fibrosis, cirrhosis, and HCC. In addition, extrahepatic manifestations of chronic HBV infection can arise including vasculitis, renal disease, and arthritis. The extrahepatic features of chronic HBV infection are typically associated with circulating immune complexes within the host which are capable of activating the serum complement pathway (Table 14.2) [47].

Viral Evasion or Downregulation of the Host Immune Response

Viral Interference with Host Immunity

In order to promote the establishment of chronic infection following acute exposure, HBV may have developed several means of exerting interference over the host immune system, thereby limiting host recognition of HBV during the early phases of acute infection. Mechanisms employed by the virus may include immunoregulation by viral transcripts such as the HBeAg to establish a tolerogenic environment, limiting pattern recognition and surveillance by the innate immune system, altering proinflammatory cytokine and chemokine expression, and fostering mutational escape.

Observations in acutely infected individuals have revealed an absence of significant proinflammatory cytokine production following exposure to HBV, including type I interferons (α and β), interleukin (IL)-15, and IFN-λ1 [38]. In addition, peak viremia following acute exposure may coincide with reduced peripheral blood NK cell activation and a diminished capacity to secrete IFN-γ or TNF-α, possibly mediated by increased IL-10 production. These findings are supported by chimpanzee studies, in which HBV does not appear to induce intrahepatic expression of any immune response genes during the periods of viral entry and expansion within acutely infected chimpanzees [25]. These findings support the view that HBV acts as a stealth virus, in which it is able to undergo active replication in the early phases of acute infection virtually unnoticed by the host innate immune system. However, more recent data have suggested that rather than going entirely unnoticed by the host, an innate immune response may in fact occur early during acute exposure to HBV. Although data are limited in humans, early innate immune responses have been reported, in which an induction of NK and NKT cell cytotoxicity can be measured within 2 weeks of HBsAg detection following acute exposure to HBV [39]. Other in vitro studies have reported the presence of an early innate response to acute infection including the induction of a type 1 interferon response in cell culture and recognition of HBV by Kupffer cells with subsequent activation of nuclear factor kappa B (NF-κB) and IL-6 release [48, 49]. Despite this initial innate response, the virus may through the mechanisms described above induce a state of partial immune tolerance and delay the subsequent adaptive immunity required to achieve control of infection and eventual viral clearance [50].

The HBeAg may have an immunoregulatory role and promote the induction of immune tolerance early during HBV infection. Ultimately, HBeAg may also facilitate the development of chronic infection. In contrast with adults acutely exposed to HBV in which progression to chronic infection occurs in only 10 % of cases, vertical transmission of HBV is associated with a high rate of chronicity of up to 90 %. Studies utilizing a transgenic mouse model suggest HBeAg could be transported across the placenta, leading to immunotolerance of HBV characterized by a reduction in T cell proliferative responses to both HBeAg and HBcAg which are cross-reactive at the T cell level [51]. Further studies have found that HBeAg may elicit a tolerogenic state in which the presence of HBeAg regulates humoral and cell-mediated immunity against the HBcAg by inhibition of anti-HBc antibody production and reduction in both proliferative and secretory responses in HBeAg- and HBcAg-specific T cells [52]. Proposed mechanisms by which HBeAg could elicit tolerance in this setting include HBeAg- and HBcAg-specific T cell clonal deletion, induction of T cell anergy, and downregulation of cellular gene expression involving products associated with cell cycle regulation, transcription, signal transduction, intracellular trafficking, and cell surface proteins [53, 54].

HBV may also exert immunotolerance to the host innate immune system through interactions between the HBeAg

Table 14.3 Proposed mechanisms for downregulation of innate and adaptive immune responses by HBV

HBeAg-mediated inhibition of HBcAg- and HBeAg-specific immunity
T cell clonal deletion
Induction of T cell anergy
Downregulation of cellular gene expression and function
Suppression of TLR-mediated immune response, expression, and signaling pathways
Core gene mutational escape within CTL epitopes
HBsAg-mediated disruption of innate and adaptive immunity
Inhibition of TLR-9-mediated IFN-α production by plasmacytoid dendritic cells
CD8+ T cell dysfunction
Inhibition of type 1 interferon transcriptional response via disruption of STAT-1 methylation and nuclear translocation
HBV polymerase and X protein inhibition of innate immunity
Disruption of IRF signaling and IFN-β production[a]
MAVS protein downregulation[b]
Diminished proteasome activity[b]

TLR toll-like receptor, *STAT-1* signal transducer and activator of transcription 1, *IRF* interferon regulatory factor, *MAVS* mitochondrial antiviral signaling
[a]Associated with both HBV polymerase and X proteins
[b]Associated with HBV X protein

and TLRs on hepatocytes as well as non-parenchymal liver cells. TLRs are cell surface pathogen recognition receptors involved in the early innate immune response that recognize pathogen-associated molecular patterns (PAMPs). Activation of TLRs by PAMPs occurs immediately following exposure to infectious agents, leading to a cascade of signal transduction and cellular expression of pro-inflammatory cytokines and the subsequent recruitment of the adaptive immune system. There is evidence that HBV itself may directly contribute to tolerance because single-stranded HBV RNA replicative intermediates and HBeAg can suppress TLR-mediated immune responses. Overall, patients with chronic HBV appear to have a reduced expression of various TLRs compared with controls [33]. In patients with HBeAg-positive chronic HBV infection, there is decreased expression of TLR-2 on hepatocytes, Kupffer cells, and peripheral monocytes, resulting in a reduction in TNF-α production compared with HBeAg-negative individuals [34]. More recent data have revealed that HBeAg inhibits TLR signaling pathways and suppresses NF-κB and IFN-β activation via interaction with TLR-2 and Toll/IL-1 receptor (TIR) adapter molecules including Mal, TRAM, and MyD88 [35].

Other mechanisms by which HBV could evade the early host immune response include mutational escape and inhibitory effects mediated by HBV proteins in addition to the HBeAg (Table 14.3). Naturally occurring HBV mutations that occur within CTL epitopes may permit viral escape through disruption of MHC binding or T cell receptor peptide recognition. Studies assessing the effect of naturally occurring variant HBV peptides on cytotoxic T cell activity found that mutations within the HBV core region (amino acid sequence 18–27) were associated with impairment of viral peptide recognition, cytotoxic activity, and IFN-γ secretory response [55]. The HBsAg may promote viral persistence through effects on both the adaptive and innate immune systems. In the setting of high concentrations of virus, increased levels of HBsAg may contribute to CD8+ T cell dysfunction, characterized by altered HBsAg-specific HLA/peptide tetramer binding [56]. HBV may disrupt the innate immune response through downregulation and direct inhibition of TLR-9-mediated IFN-α production by circulating plasmacytoid dendritic cells, possibly mediated by HBsAg [36, 37]. Both the HBV polymerase and X protein may also play a role in inhibition of innate immunity through disruption of IFN-β production via blockade of interferon regulatory factor (IRF) signaling [57–59]. Additionally, the HBV X protein may have an inhibitory effect on the host innate immunity, possibly through disruption of proteasome activity and downregulation of the mitochondrial antiviral signaling (MAVS) protein, which could also diminish IFN-β production [60–62]. HBV may also have an inhibitory effect on the intracellular transcriptional response to type 1 interferons through disruption of methylation and nuclear translocation of the transcription factor, signal transducer, and activator of transcription 1 (STAT-1), which could impact virologic response in the setting of interferon alfa therapy [63, 64].

Viral Variation and Immunologic Response

Spontaneous mutations involving the HBV genome occur most frequently within the pre-core and core promoter regions, leading to a loss or significantly diminished expression of HBeAg. The most common mutation occurring in the pre-core region, G1896A, results in the formation of a stop codon leading to a loss of pre-core protein and HBeAg production. In contrast, mutations occurring within the core promoter region lead to downregulation of pre-core protein expression such that HBeAg can still be produced, but at very low levels. Core promoter variants are most frequently characterized by the dual mutation A1762T and G1764A.

The frequencies of pre-core and core promoter mutations vary by HBV genotype, which accounts for differences in the geographic distribution and prevalence of mutations within various populations [16]. The G1896A pre-core mutation is most frequently encountered in HBV genotype D infection while the core promoter mutations A1762T and G1764A are most commonly associated with genotype C. Although the pre-core mutation does not appear to have an impact on risk of disease severity or progression, it may play a role in maintaining chronicity of HBV infection within the host. Studies suggest that the emergence of G1896A may

occur at the time of HBeAg seroconversion in which the mutant variant that halts HBeAg production could be selected under immunologic pressure as a means of evasion and viral persistence [16]. Longitudinal data in patients with chronic infection have noted HBeAg seroconversion and the emergence of pre-core mutations coinciding with the development of core mutations within helper T cell epitopes, also suggesting that this may occur as a result of immune pressure exerted by the host [65]. In contrast with pre-core mutations, the presence of core promoter mutations has been associated with more advanced liver disease and an increased risk of HCC. Overall, spontaneous alterations of the core protein epitope, possibly occurring under selection pressure mediated by the host HBV-specific immune response, could limit cytotoxic T cell recognition of HBV and contribute to viral escape and persistence of chronic infection.

Mutations involving the pre-S and S regions of the HBV genome can alter encoding of the HBsAg, allow for viral escape from host immunity, and be a source of occult HBV infection. As the S gene (surface envelope) and the P gene (polymerase) are overlapped, mutations that occur in either gene may result in changes to the HBsAg. In some cases, HBV DNA may exist in the liver or serum, yet HBsAg remains negative based on available assays. This phenomenon is known as occult HBV infection and is characterized by typically low-level viremia (<200 IU/mL); however, the virus is capable of replication, transmission, and can lead to liver disease [66]. The observation that higher frequencies of mutations involving specific CD8+ T cell and B cell epitopes within the HBsAg protein occur in the setting of occult HBV suggest that these variants arise from immune pressure exerted by the host [67, 68]. Key areas of the HBsAg protein altered by this process include the "a" determinant of the major hydrophilic region (MHR), which is important in the binding of HBsAg to neutralizing antibodies (HBsAb). Mutations affecting the MHR as well as surface promoters in the pre-S2 and S regions may also lead to decreased HBsAg production, further contributing to difficulties with detection of HBs antigenemia [69, 70]. Vaccine and hepatitis B immunoglobulin (HBIG) escape mutations have also been described in infants born to HBsAg carrier mothers and in liver transplant recipients with chronic HBV. In this setting, S gene mutations lead to failure of HBsAg neutralization and appear to occur as a result of selection pressure exerted by the host immunity, the HBV vaccine, or administration of HBIG [71–73].

Natural History of Chronic Hepatitis B

The natural history of chronic HBV infection is highly variable between individuals, in which periods of immune tolerance and immune activation can alter the course of infection from an immunological as well as clinical standpoint. Chronic infection with HBV is clinically defined by four different phases of infection which are determined by the interplay between viral and host factors. These phases include what are known as the immune-tolerant phase, immune clearance, the inactive HBsAg carrier state, and reactivation phase. Chronic infection is a dynamic process in which an individual may transition between clinical phases at different points during the course of infection.

Immune-Tolerant Phase

The immune-tolerant phase is most frequently associated with younger individuals who likely acquired HBV infection perinatally through vertical or horizontal transmission. Various factors, including the role of HBeAg as a toleragen in the setting of exposure to HBV early in life, have been considered to be integral in its pathogenesis [51–54]. This phase is characterized by very high serum HBV DNA levels, a positive serum HBeAg, normal serum aminotransferases, and minimal histologic activity on liver biopsy (Fig. 14.4). The immune-tolerant phase has been regarded as one in which the host immune system is essentially devoid of awareness or recognition of HBV as a consequence of a tolerogenic state induced by the virus. However, recent studies have demonstrated that the host immunity in this setting is indeed responsive to HBV antigens. Compared with healthy controls, individuals known to be in the immune-tolerant phase have been shown to have increased baseline production of pro-inflammatory cytokines in peripheral mononuclear cells and increased responsiveness to TLR ligands that results in cytokine and chemokine production involving IL-6, CCL3, and CXCL10 [74]. In fact, immune-tolerant individuals appear to have a similar cytokine profile to others with chronic HBV infection without evidence of T cell exhaustion as measured in peripheral mononuclear cells at baseline and following antigenic stimulation by multiple HBV peptides [75].

Regulatory T cells (Tregs) may play a role in establishing tolerance within the liver in patients with chronic HBV infection. Tregs are inhibitory CD4+ T cells expressing the forkhead transcription factor 3 (Foxp3) and CD25 that are found at increased levels in peripheral blood and liver in the setting of chronic infection but not in individuals who undergo spontaneous resolution [76]. Although Tregs can be cytoprotective in the setting of acute infection in which they may act to limit pro-inflammatory cytokine production, cell-mediated cytotoxicity, and activation of the innate immune system, they may also promote viral persistence in chronic infection [77]. Increasing peripheral and intrahepatic Tregs lead to inhibition of HBV-specific T cell proliferation and IFN-γ production in chronically infected individuals. Some studies have noted that increased proportions of circulating and liver-infiltrating Tregs correlate with elevated

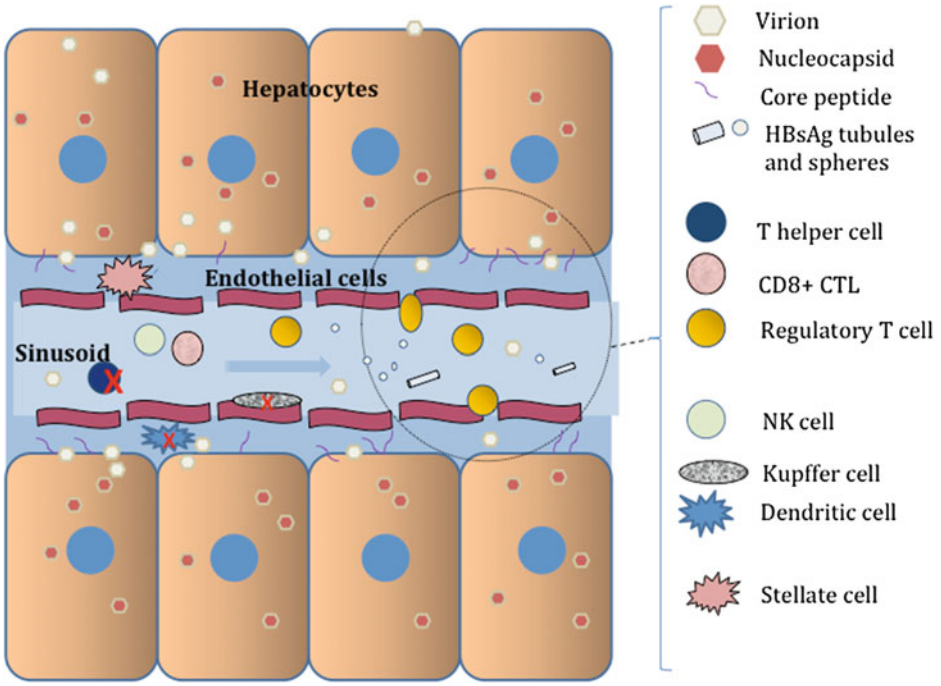

Fig. 14.4 Schematic of immunotolerant stage of chronic hepatitis B. This phase of chronic hepatitis B is characterized by inordinately high levels of viral replication in the absence of biochemical or histologic evidence of liver damage. Immunotolerance occurs primarily in individuals with early life exposure to HBV and is likely to be multifactorial in origin. One possible mechanism is that regulatory T cell (Treg) activity is enhanced (depicted by increased number of Tregs in *orange*) leading to impaired activation of adaptive cellular immune responses. As HBeAg has been shown to be an immunotolerogen in perinatal infection (being cross-reactive with HBcAg at the T cell level), it might also promote immunotolerance. High concentrations of circulating HBsAg (depicted as gray tubules and spheres) may impair the immunologic function of antigen-specific T cells leading to their depletion. See text for further details

Table 14.4 Factors postulated to be important in immunotolerance with chronic hepatitis B infection

Activated T regulatory cells in liver tissue
Exhaustion of CD8+ CTLs and T helper cells by excessive HBsAg or high level HBeAg
Inefficient expression of HLA class I molecule-core peptide sequences
Impairment of dendritic cell function
Immunotolerogenic effect of HBeAg[a]

[a]HBeAg is cross-reactive with HBcAg at the T cell level and this may reduce efficient T cell responses to core epitopes

HBV DNA levels and decreased serum aminotransferases [78, 79]. In particular, Tregs expressing CD39 have a strong positive correlation with HBV DNA levels and are increased in asymptomatic carriers with normal ALT levels [79].

While Tregs are probably an important contributing factor to the immunotolerant state, this is almost undoubtedly an oversimplification (Table 14.4). It is worth pointing out that an increased frequency of Tregs in patients with chronic hepatitis B has been reported in some but not in other studies. Furthermore, most studies have been based on peripheral cell evaluations and a detailed analysis of the intrahepatic frequency and function of these cells is likely necessary to reveal their role. Other factors that are likely to be relevant in the immunotolerant phase of chronic hepatitis B, and for which partial data exist, are T cell depletion in the face of high levels of HBsAg, the immunotolerogenic effect of HBeAg as mentioned above, the effects of HBV X and polymerase proteins on adaptive immune responses, impaired dendritic cell function, or any factors that impede migration of effector cells to infected hepatocytes.

Immune Clearance (or Active) Phase

The immune clearance phase is characterized by elevated ALT levels and significant HBV-associated disease on liver biopsy, which are due to the effects of continuing CD8+ CTL activity and proinflammatory cytokines (Fig. 14.5). In this phase, HBeAg is initially detectable for a period of several years to decades and HBV DNA levels are typically elevated. A narrowly focused range of specificity against HBV characterizes the host cell-mediated immunity in this phase, such that the virus persists in the face of ongoing cytotoxic activity within the liver. In some individuals, increasing immunologic pressure targeting the core protein and HBeAg may precipitate a transition in which HBeAg seroconversion occurs. This event is frequently associated with mutations

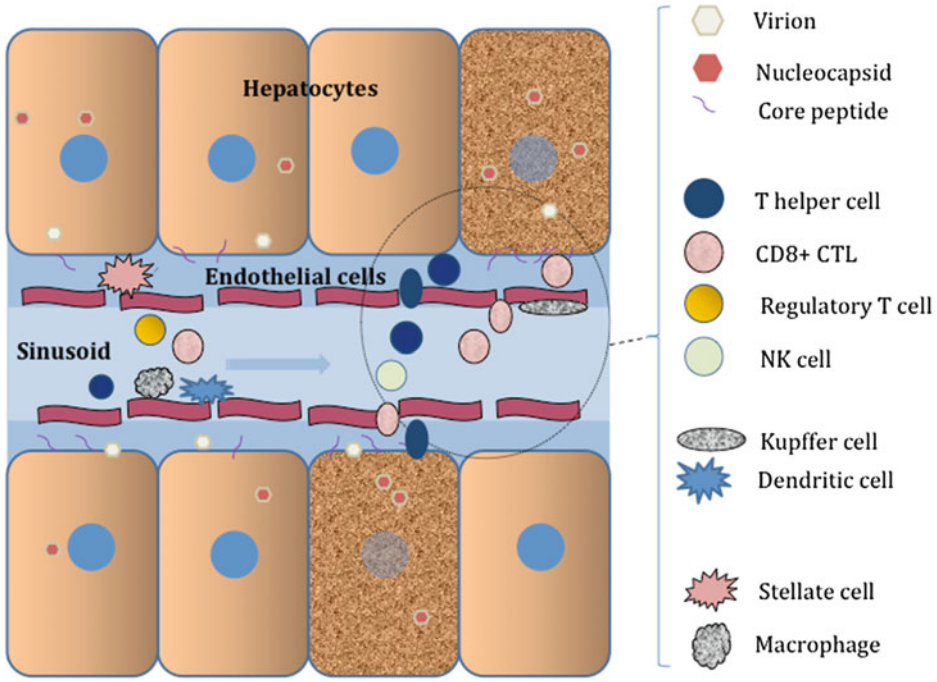

Fig. 14.5 Schematic of the immune clearance or immune active phase of chronic hepatitis B. CD8+ CTLs (see in *orange color*) are the main effector cells for viral clearance and disease pathogenesis. CTLs are attracted to immunoactivating HBcAg epitopes on core peptides (in *purple color*) that are expressed on the surface of infected hepatocytes in conjunction with HLA class I antigens. The CTLs release perforin, granzyme, and other chemical mediators that result in damage and eventual elimination of the core peptide expressing hepatocytes (see stippled hepatocytes). The NK, NK-T, Kupffer cells, and dendritic cells play an active role in processing viral antigens and lead to the release of proinflammatory cytokines during the immune active phase. See text for further details

within the pre-core region that result in a stop codon so that HBeAg cannot be produced. Although prevalence varies by HBV genotype, over 25 % of individuals with chronic HBV infection in mixed-genotype populations such as the United States are positive for pre-core variants [16]. In the setting of HBeAg loss, the virus may continue to replicate causing chronic necroinflammation. Anti-HBe-positive chronic hepatitis B is often associated with significant disease on liver biopsy and an increased frequency of cirrhosis. HBV DNA is typically not as elevated as in HBeAg-positive hepatitis B, and most patients have fluctuations in both ALT and HBV DNA [80]. The immunologic events associated with this pattern of periodic exacerbation have remained unclear. Recent sequencing experiments have demonstrated, however, that these acute exacerbations may be associated with the emergence and selection of mutations in B and T cell antigenic epitopes in the core gene as well as other areas of the HBV genome involved with regulation of viral transcription. It has been postulated that the accumulation of these variants may impair immunologic responses, which permits poorer control of viral replication [81, 82].

Inactive Carrier State

The inactive HBsAg carrier state is notable for persistently detectable serum HBsAg, yet normal ALT levels, low or undetectable HBV DNA, and typically minimal disease on liver biopsy (Fig. 14.6). This phase is also associated with HBeAg seroconversion. Despite the apparent lack of overt cytotoxic activity within the liver, these individuals maintain strong peripheral blood and intrahepatic HBV-specific CD8+ T cell-mediated immunity [44]. These data suggest that inactive carriers maintain a long-term state of virologic control, although viral replication also exists at a low level.

Clinical and Laboratory Correlates of the Immune Response

Immune Control

Strong multi-specific polyclonal CD8+ T cell responses persist after resolution of acute HBV infection [22]. In addition, the humoral immune response plays a key role in maintaining immune control of acute infection as well as prevention of reactivation through the action of neutralizing antibodies. The emergence of detectable neutralizing antibodies in the serum directed towards multiple HBV peptides, including core protein, HBeAg, and the envelope protein (HBsAg) indicate resolution of acute infection. The persistence of HBV-specific cell-mediated and humoral immunity are essential to maintaining long-term immunologic control of HBV.

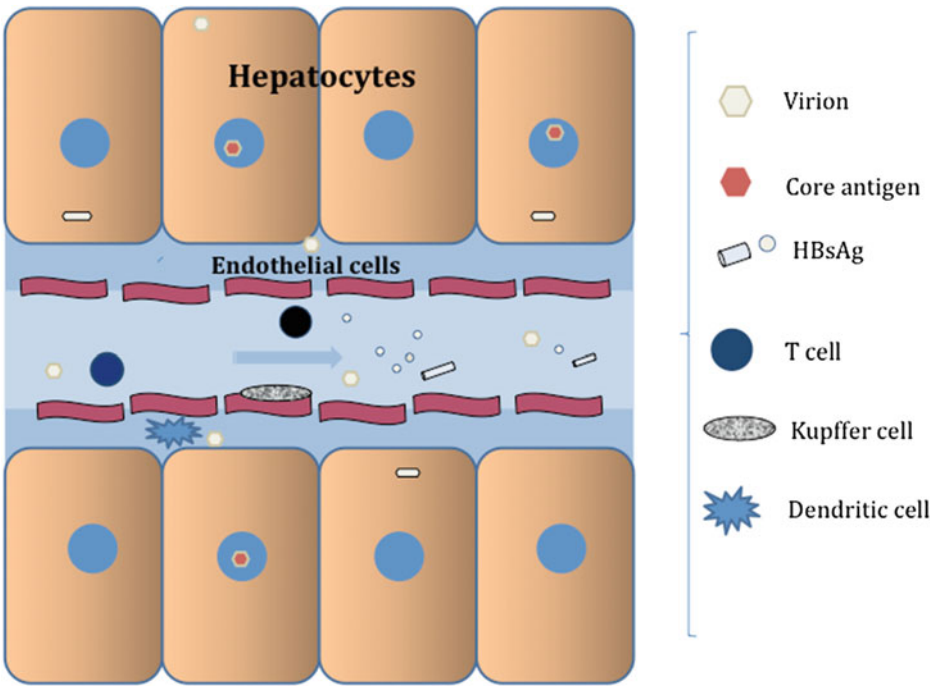

Fig. 14.6 Schematic of the inactive carrier phase of chronic hepatitis B. These patients have minimal if any evidence of histologic injury with the possible exception of minor degrees of fibrosis from past disease activity. Immunohistochemical staining for HBcAg and HBsAg (see legend) generally demonstrates very sparse staining within the nucleus and cytoplasm, respectively. Minute amounts of covalently closed circular HBV DNA are detected in hepatic tissue at concentrations that are several orders of magnitude less than that observed in the immune clearance phase (see ref. [12]). This observation is consistent with better immunologic control over viral replication

Factors Affecting HBV Reactivation

HBV reactivation is clinically characterized by a sudden increase in viral replication that is often associated with a recurrence of necroinflammatory liver disease. This may occur spontaneously but is particularly common when immunosuppressive events occur such as untreated HIV infection or cancer chemotherapy [83].

A biological gradient of cccDNA concentration exists in hepatic tissue of patients with chronic hepatitis B when examined during the active, inactive, and resolved phases of chronic infection. The highest levels have been observed in patients in the immune active phase (viremia with aminotransferase elevations) with progressively lower concentrations in the inactive HBsAg carrier and recovered phases of infection, respectively [11, 12]. The highest amounts of cccDNA in liver tissue have been detected in HBeAg-positive chronic hepatitis B [12]. This is likely to contribute to the high rate of reactivation when these patients are given immunosuppressive drugs. Conversely, the minute amounts of cccDNA detected in patients with resolved infection (HBsAg-negative) probably explain the significantly lower rate of HBV reactivation when the same immunosuppressive therapy is given.

The sequence of events in HBV reactivation due to immunosuppressive therapy is proposed to be an initial phase of enhanced viral replication during drug therapy followed by a later phase of immunologic restitution after drug withdrawal and an robust immunologic response to increased target antigens. The immunologic events that are associated with cancer chemotherapy remain unclear. One small study demonstrated a significant increase in HBV-specific CD8+ T cells and lower numbers of Tregs in patients with reactivated hepatitis B after cancer chemotherapy when compared to individuals with chronic hepatitis B and inactive HBsAg carriers [84].

A corticosteroid-responsive element has been shown to exist in the HBV genome, which when stimulated, results in increased viral replication and transcription [85]. It has also been demonstrated that increases in viral replication (and a presumed secondary accumulation of intrahepatic viral antigens) occur with a variety of other immunosuppressive medications and most notably with cancer chemotherapy. Immunologic reconstitution after discontinuation of immunosuppressive medications is an important factor in the pathogenesis of liver injury associated with cancer chemotherapy and abrupt corticosteroid withdrawal [83]. This does not explain observed cases of reactivation during maintenance treatment with TNF-α and other cytokine inhibitors. Of some relevance here are adoptive transfer experiments in transgenic mice which have shown that CTL secretion of TNF-α has a virocidal effect on HBV [26].

The frequency of reactivation after immunosuppressive drug therapy correlates with the serologic status of the host. Reactivation occurs more commonly in patients with preexisting viral replication and inactive HBsAg carriers than in those with resolved infection. Patients with anti-HBc alone appear to be more likely to demonstrate reactivation after immunosuppressive drug therapy than those with the more

complete immunologic recovery implied by the detection of both neutralizing anti-HBs and anti-HBc. The importance of anti-HBs in the prevention of HBV reactivation can also be seen in solid organ donation, particularly liver transplantation. In this instance, it has been shown that anti-HBs/anti-HBc-positive recipients of anti-HBc-positive organs are less likely to undergo reactivation without antiviral therapy [86].

Spontaneous Reactivation

Pathogenetic events for spontaneous reactivation are even less well understood. In theory, this may be explained by the emergence and selection of replication-fit viral variants and secondary effects on the immune system as described above. Alternatively, spontaneous reactivation may be due to primary changes in the immune response to HBV. For example, it is possible that a dysregulation of Tregs may contribute to spontaneous HBV reactivation. A recent study of CD4+ Foxp3+ Tregs has indicated distinct populations of resting Tregs, activated Tregs (aTregs), and cells that are cytokine-secreting and non-immunosuppressive (non-regs) [87]. The frequency of aTregs was found to be selectively elevated in patients with HBV-associated active disease but not in inactive HBsAg carriers [78]. The aTreg frequency was shown to be strongly correlated with HBV DNA levels whereas the reverse was true for cytokine producing non-regulatory T cells that expressed Foxp3 antigen [78].

Necroinflammatory Flares

Necroinflammatory flares that are associated with an acute rise in serum aminotransferase levels can occur spontaneously during chronic HBV infection and during immunologic reconstitution due to highly active antiretroviral therapy (HAART) or adoptive transfer immunotherapy.

Spontaneous Hepatitis Flares

Spontaneous flares can occur at the time of HBeAg seroconversion as a heralding manifestation of lasting immune suppression. In flares that occur prior to HBeAg seroconversion, an increased frequency of circulating core-specific CD8+ T cells as well as increased core- and HBeAg-specific T cell proliferative responses have been reported [43, 88]. One investigation compared Th1/Th2 cytokine expression during and after spontaneous hepatitis flares in patients chronically infected with genotypes B and C (mean ALT during flares in excess of 200 U/L for both groups). In this study patients with genotype B were found to have a greater Th1 phenotype (enhanced IFN-γ and lower IL-10 levels) after stimulation with HBV core antigen. This was felt to possibly contribute to the higher rate of spontaneous HBeAg seroconversion in the genotype B patients during follow up [89].

Immunologic Reconstitution

Flares can also occur in the setting of immunologic reconstitution following HAART therapy of HIV 1 infection particularly in patients with low CD4 counts prior to treatment [83]. Such flares may be serious but are not apt to occur when HAART therapy includes tenofovir.

Immunologic reconstitution also occurs with successful bone marrow engraftment. Host cell-mediated responses directed towards HBV core antigen appear to be particularly important in cases of HBsAg carriers who received bone marrow transplants from HBV-immune donors [90]. The bone marrow recipients adopted donor HBV-specific immunity and all developed hepatitis flares following engraftment at which time core-specific CD4+ and CD8+ T cells predominated over HBsAg-specific T cells.

Immunologic Changes During Antiviral Therapy

Antiviral therapy may modify the immune response to HBV. ALT flares in association with declining serum HBV. DNA levels have occurred during treatment with both interferon alfa and nucleoside analogue therapy. While the data with nucleoside analogues created much interest initially, greater clinical interest continues to be given to the immune enhancing potential of interferon because of its known broad immunologic interactions.

Interferon Alfa

Interferon alfa is an approved therapy for chronic HBV infection and the only agent currently available that is immunomodulatory. Type 1 interferons have several immune-mediated associations. They play a role in the noncytopathic clearance of HBV from infected hepatocytes in those who demonstrate spontaneous resolution [19, 26, 30, 31]. Genomic variation of HBV within a host may influence virologic response to interferon therapy. It has been recognized that HBV genotype A may be more sensitive to interferon-based therapy [91–94]. Further evaluation of this reveals that genotype A infection is associated with the least variation within the core gene compared with other genotypes [95]. Earlier studies support these findings, as naturally occurring mutations within the HBV core protein resulting in inhibition of HLA-A2 restricted cytotoxic T cell function have been associated with a poor virologic response in patients treated with interferon alfa [55, 96].

During interferon therapy, the development of a severe ALT flare is among the strongest predictors of a successful virologic response [97]. Presumably, the occurrence of a

flare during interferon alfa therapy may coincide with an increase in host CTL activity directed towards HBcAg or other viral epitopes. Other predictive factors of a successful response include elevated baseline ALT, low HBV DNA, active necroinflammatory disease, and low quantitative HBeAg levels [94, 98–100]. One way of interpreting these associations is that each of the predictors reflects a higher state of immunologic activation at baseline. The degree of the hepatitis flare has been shown to correlate with a virologic response to interferon therapy in patients with high serum HBV DNA levels [97].

Nucleoside and Nucleotide Analogues

The nucleoside analogues, lamivudine, telbivudine, and entecavir, and the nucleotide analogues, adefovir and tenofovir, are approved treatments for HBV. Similar to interferon alfa therapy, higher baseline serum ALT levels have been associated with a higher rate of HBeAg-seroconversion attesting to the importance of the baseline immune response to HBV [101].

These agents target the HBV polymerase and do not have any specific immunologic enhancing effects. Early studies involving lamivudine revealed that nucleoside analogues are capable of restoring CD4+ and CD8+ T cell responsiveness against multiple HBV epitopes in patients with chronic HBV infection undergoing antiviral therapy [102–104]. As HBV is lymphotropic as well as hepatotropic, it has been hypothesized that this may be due to reduction of viral burden within lymphocytes and enhancement of their function. This was first observed ex vivo in lamivudine-treated patients and appears to be a transient phenomenon [102, 103]. Similar relationships have been observed with other nucleoside analogues such as adefovir and telbivudine [105].

Necroinflammatory flares can also occur during antiviral therapy with low genetic barrier nucleoside analogues that are due to HBV-resistant drug mutants [83]. Hepatitis flares after sudden withdrawal of nucleoside analogue therapy that are due to rapid resurgence of wild type HBV replication have been well described as well [106].

Conclusions

Much has been learned about the immunology of acute and chronic hepatitis B over the past decade. While our knowledge remains incomplete, one thing is very clear: efficient control of HBV infection requires the coordinated action of both innate and adaptive immune responses. The innate response is not only important in noncytolytic killing of virus, but is also essential for adaptive immune responses that terminate disease activity quickly and provide long-term immunologic control over the virus. Evidence has begun to emerge that the immune response to HBV is not only determined by the host, but also by viral adaptation and change. Therefore, gaining more knowledge in the future will require coordinative efforts of both liver immunologists and molecular virologists.

The reader should be reminded that nucleoside inhibitors used to treat hepatitis B have little if any effect on the immune system and predominantly work by potent inhibition of viral replication. Greatly needed are immunotherapies that are more effective than interferon alfa and safe enough to be given to a wide range of patients. This would provide a major leap forward in treating this condition.

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Hepatitis C

15

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Key Points

- Hepatitis C virus (HCV) is a single-stranded RNA virus which belongs to the *Flaviviridae* family. It has a highly heterogeneous genome of about 9,600 nucleotides with at least seven distinct genotypes and about 100 subtypes. Sequence heterogeneity accounts in part for the variability in pathogenic potential and for sensitivity to interferon (IFN)-α therapy.
- Approximately 2.5 % of the world population is chronically infected with HCV. There are some hyperendemic areas, like Egypt, where the prevalence rate exceeds 15 %.
- More than 60 % of the infected patients develops chronic infection, a slow and indolently progressive disease leading to cirrhosis in up to 30 % of patients after 30 years. HCV-related cirrhosis may then evolve toward liver failure or hepatocellular carcinoma (HCC).
- Early innate defence mechanisms are triggered immediately after infection; however, HCV has evolved a number of strategies to block intracellular signalling pathways, including NS3/4A and core mediated inhibition of interferon-stimulated gene (ISG) expression.
- NK cell phenotype is skewed toward activation in chronic HCV infection and a functional dichotomy characterized by a polarization toward cytotoxicity and reduced IFN-γ production has been consistently observed. Intrahepatic NK cells show instead impaired cytotoxic function and an exhausted phenotype.
- Antibody responses to structural and non-structural proteins appear several weeks after acute HCV infection; however, the role of neutralizing antibodies in preventing HCV infection remains controversial. Antibodies specific for virus receptors, such as Claudin-1 and scavenger receptor BI may efficiently prevent HCV infection and are able to inhibit infection by escape variants selected by neutralizing antibodies.
- HCV-specific T-cell responses are barely detectable in the peripheral blood in chronic hepatitis C, but there is evidence of a greater frequency in the intrahepatic compartment.

  A functional impairment of HCV-specific CD4 and CD8 cells has been reported by several groups ex vivo in chronic HCV infection which can only partially be restored upon prolonged cytokine exposure. In particular, HCV-specific CD4 cell function is deeply impaired, probably due to defective IL-2 production.
- Patients with self-limiting infection develop more vigorous and broadly specific CD8 responses compared with those developing persistent infection. Moreover, the inefficiency of CD4 T-cell responses during chronic HCV infection does not support a full maturation of HCV-specific CD8 cells which may remain dysfunctional expressing PD-1 and remaining CD127 negative. Spontaneous control of HCV infection is instead associated with successful maturation of CD8 T-cell memory, indicated by the acquisition of a CD127+/CCR7+ phenotype.
- A more broadly reactive and more vigorous HCV-specific CD4 T-cell responses are present in patients who eventually recover from acute hepatitis C self-limited infection compared with patients evolving toward chronic infection. Broadly directed HCV-specific CD4+ T-cell responses are usually generated in acute HCV infection, but rapid exhaustion and deletion of these cells occurs in

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the majority of patients. Th1 cytokines (IFN-γ and IL-2) usually prevail in patients who succeed in clearing HCV spontaneously whereas patients evolving toward chronic hepatitis are characterized by a predominant type 2 cytokine environment.

- The evolution of acute HCV infection seems to be relatively independent of immunosuppression although recurring hepatitis C in the transplanted liver and coinfection with HIV may run a rather aggressive clinical course.
- The current standard of care for chronic hepatitis C is based on a combination of pegylated IFN-α and ribavirin. Patients with HCV genotype 1 can receive, in addition, an inhibitor of the viral NS3/4A serine protease, such as telaprevir and boceprevir. With such regimes, sustained virological response rates can be expected to be around 70 % in patients infected with genotype 1. SVR rates with traditional dual treatment are about 75–90 % in those infected with genotypes 2 and 3.
- Novel direct-acting antivirals (DAA) may target several viral proteins, such as the NS3/4A serine protease, the NS4B, the NS5A or still the viral polymerase, encoded by the NS5B region. Some pangenotypic DAA combinations to be used in effective IFN-α-free combinations, have now entered phase III of development, and are expected to be marketed in a few years from now.

Introduction

Hepatitis C virus (HCV) is the prototype human pathogen having been discovered purely by molecular biology rather than by traditional virological tools. Since the early 1970s, the existence of a virus responsible for the majority of parenterally transmitted non-A, non-B hepatitis had been suspected, but it was not until 1989 that Michael Houghton and coworkers, at Chiron Corporation, identified and cloned HCV. It took another 10 years to develop an efficient system to study HCV replication in vitro, thus paving the way to large-scale screening of small molecules with direct antiviral activity. Meanwhile, the details of the HCV transmission, pathogenesis and natural history were unravelled, leading to a full appreciation of the global health burden associated with this infection. HCV affects about 2.4 % of the world population, and molecular biology advances may again contribute substantially—through the development of safe and efficacious drugs—to its eradication in the near future. The main focus of this chapter is to provide an overview of the innate and adaptive immune responses against HCV. In addition, we will describe the latest developments in the treatment of acute and chronic hepatitis C.

Epidemiology

An estimated 170 million individuals, i.e. 2.4 % of the world population, are infected with HCV [1]. In Europe, between 7.3 and 8.8 million persons are infected with HCV, with prevalences ranging from 0.4 to 3.5 %, the highest rates being found in the South and the East [2].

Outside Europe, hyperendemicity areas include some African countries like Egypt, where the prevalence rate exceeds 15 %.

In developed countries, the main routes of HCV transmission until the 1990s were blood transfusion, unsafe medical practices and intravenous drug use. After the implementation of sensitive screening assays on all blood products, transfusion-associated hepatitis C has been virtually eliminated. Similarly, safer medical procedures have dramatically reduced the iatrogenic transmission of HCV, while effective harm reduction programs have only partially impacted the spread of HCV among the drug user community. Thus, sharing paraphernalia connected with the parenteral illicit drug use nowadays accounts for the vast majority (i.e. up to 85 %) of new HCV infections in developed countries. HCV is rather inefficiently transmitted via sexual intercourse among partners in monogamous relationships, while the risk of perinatal transmission of HCV is lower than 5 %. On the other hand, unprotected sex in the male homosexual community has become an important route of transmission of HCV [3]. Contrary to developed countries, in resource-poor areas of the world, limited awareness and ongoing adoption of unsafe medical procedures still account for the majority of HCV incident cases [4], with a recent, ominous increase of viral spread associated with illicit drug use.

Natural History of HCV Infection

Acute HCV infection is asymptomatic in the majority of cases. Thus, its diagnosis may be difficult, leading to an underestimation of its true incidence. Since there are no specific markers of acute HCV infection, diagnosis is usually based on a documented seroconversion to anti-HCV in a person who was previously anti-HCV-negative, often accompanied by an increase in the alanine aminotransferase (ALT) levels. HCV RNA may be detected in the serum as early as 3–7 days after exposure, but seroconversion to anti-HCV occurs several weeks to months thereafter. The most common symptoms, when present, are fatigue, flu-like symptoms, dyspepsia, jaundice, and abdominal pain, and may appear from 2 to 12 weeks after infection. Acute liver failure due to HCV is rare if at all attributable to it. Since most symptoms are not specific and may be mild or absent, patients often fail

Table 15.1 Clinical and demographic factors associated with faster or slower HCV-induced liver disease

Slower progression	Faster progression
Normal ALT	High intrahepatic inflammation
Female gender	Male gender
Young age at infection	Older age at infection
	High alcohol consumption
	Steatosis and/or insulin resistance
	Coinfections (HIV, HBV)

to report and their infection is detected later in life after progression to chronicity, which occurs in the majority of cases (up to 85 %). Factors associated with increased chances of spontaneous eradication at the time of acute hepatitis are the presence of symptoms, (especially jaundice), female sex, young age, clearance of serum HCV RNA within 4 weeks after the onset of clinical symptoms, the host genetic background (in particular, genetic polymorphisms upstream of *IFNλ 3*) [5], the vigour of the cellular immune response and the absence of human immunodeficiency virus (HIV) coinfection. The source of infection, size of inoculum, age and sex do not seem to influence the risk of chronicity. Importantly, eradication after acute hepatitis C does not confer permanent protection, and patients (especially when at risk of a novel exposure) should be counselled about HCV reinfection.

Chronic infection with HCV is defined by viremia persisting for more than 6 months and is associated in most cases with chronic intrahepatic necroinflammation. HCV is considered a non-cytopathic virus, although it may widely interfere with the host cell physiology, especially as far as lipid and glucose metabolism is concerned. Chronic hepatitis C is a slowly but relentlessly progressing disease leading to cirrhosis in up to 30 % of patients after 30 years. HCV-related cirrhosis may then evolve toward liver failure or hepatocellular carcinoma (HCC) (~2–5 % yearly). Progression to cirrhosis and HCC accounts for the morbidity and mortality associated with chronic hepatitis C. Notably, the variability of hepatitis C progression over time is influenced by several cofactors related to the virus, to the host and to the environment (Table 15.1). The most important factor influencing hepatitis C progression is the extent of intrahepatic inflammation elicited by the viral infection itself, suggesting that the quality and vigour of the immune response is a critical factor in determining fibrogenesis, which should be considered as a typical wound-healing process. Consistent with this, patients with chronic hepatitis C and persistently normal ALT present a slower liver disease progression. Other factors influencing hepatitis progression include male sex, age at infection, some host genetic polymorphisms, the pattern of alcohol consumption, the metabolic syndrome, some coinfections (e.g. with HBV and *Schistosoma mansoni*), the occurrence of immunosuppression, such as after transplantation or in poorly controlled HIV infection. Viral factors do not seem to have a strong impact on hepatitis C progression, although there is some data suggesting that the HCV genotype 3 may be associated with accelerated fibrosis progression rate [6]. At the stage of cirrhosis, however, the risk of developing HCC has been reported to be increased in patients infected with genotype 1 compared to other genotypes [7].

Hepatitis C Virus

HCV is a member of the Hepacivirus genus within the *Flaviviridae* family, which includes also the Pestivirus and the Flavivirus genera. It has a positive sense, single-stranded RNA genome of about 9,600 nucleotides and highly heterogeneous: sequence variability accounts for its classification in at least seven distinct genotypes and about 100 subtypes. This sequence heterogeneity accounts in part for the variability in pathogenic potential, as genotype 1b has been associated with an increased risk of HCC development [8], genotype 3a with steatosis [9] and possibly accelerated fibrosis progression rate [6] and genotype 2 with more frequent hepatitis flares [10]. Genotypes show variable sensitivity to IFN-α therapy and genotype 1 subtypes (1a > 1b) pose differential risk to select for protease inhibitor-resistant variants [11].

HCV is an enveloped virus, about 30–60 nm in diameter. A lipid-containing envelope, comprising the two viral glycoproteins E1 and E2, surrounds a nucleocapsid containing the core protein and the genomic RNA. The viral attachment to the hepatocyte surface is a complex, multistep process involving viral and cell factors. Several proteins play an essential role in viral entry: tetraspanin CD81, scavenger receptor B1 and the tight junction proteins claudin-1 and occludin are all involved [12] In addition, also the LDL receptor is implicated in viral attachment to the cell, in keeping with the peculiar structure of HCV virions, that circulate in the form of lipid-enriched, low-density particles, the so-called lipoviroparticles [13]. Upon release in the cell cytoplasm, the viral genome is translated in its own proteins. To this aim, the single open reading frame contained in the genome directs the synthesis of a polyprotein of about 3,000 aminoacids. This is in turn processed by cell and viral proteases into ten structural (core, E1 and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins via an internal ribosome entry site (IRES) located in the 5′ non-translated region of the genome [14]. The genetic organization of HCV and the function of its proteins are illustrated in Fig. 15.1. p7 is a membrane-associated oligomeric protein

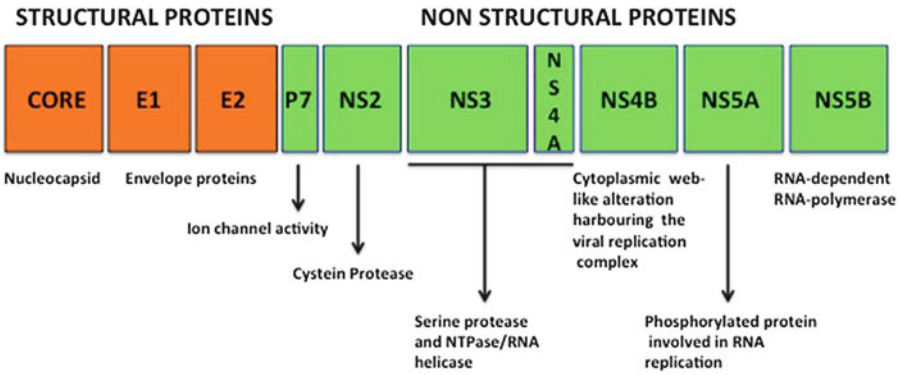

Fig. 15.1 Genetic organization of HCV

with ion channel activity, essential for assembly and release of infectious HCV particles [15] and, as such, a potential and attractive target for antiviral therapy. The NS2 protein is a zinc-dependent cysteine protease found in the form of homodimers: it is an integral membrane protein that cleaves at the NS2/NS3 junction and is essential for infectious virion assembly [16]. The non-structural protein 3-4A (NS3-4A) is a complex composed of NS3 and its cofactor NS4A. It is characterized by a serine and an NTPase/RNA helicase activities. The serine protease activity is responsible for cleaving all viral non-structural proteins downstream of NS3. Thus, the NS3-4A complex is essential for the viral polyprotein processing, replication and virion formation: as such, two specific inhibitors of this complex (telaprevir and boceprevir) have been the first-in-class antivirals to be successfully added to the current standard of care. Interestingly, the NS3-4A protease can also target some host proteins involved in the innate immune response and some growth factor signalling, thus contributing to the HCV pathogenesis as discussed below [17] The NS4B protein is highly hydrophobic protein with several transmembrane domains involved in the initiation of cytoplasmic web-like alterations ("membranous web") in proximity of endoplasmic reticulum and harbouring the viral replication complex [18]. The NS5A is a phosphorylated protein involved in RNA replication: potent inhibitors of this protein have been developed. Finally, the NS5B is an RNA-dependent RNA-polymerase responsible for replicating the viral genome. Nucleotide inhibitors of NS5B are among the most promising direct-acting antivirals (DAA) being developed, as no resistance conferring viral variants have been reported in clinical trials so far. The virion assembly proceeds through largely unknown steps, which are tightly linked with the host cell lipid synthesis, in particular with the cytoplasmic lipid droplets, which appear to function as virion assembly platform. The viral egress, finally, is exploiting the hepatocyte very low-density lipoprotein secretion pathway, resulting in release of the above mentioned, triglyceride-rich lipoviroparticles [19].

Therapy for Acute and Chronic Hepatitis C

Acute Hepatitis C

Patients with acute hepatitis C should be considered for antiviral therapy in order to prevent progression to chronic hepatitis. High SVR rates (>90 %) have been reported with pegylated IFN-α monotherapy, irrespectively of HCV genotype. Adding ribavirin does not increase the SVR rate in such patients, and may only be considered in patients with slow virological response or other negative predictors of response [20]. The ideal time point for starting therapy has not been conclusively established. It has been suggested to follow acute hepatitis C patients with sequential determinations of serum HCV RNA and to treat only those who are still viremic 12 weeks from onset [21]. Alternatively, only unfavourable *IFNλ* polymorphisms (i.e. rs12979860 TT homozygotes or CT heterozygotes) may be considered for early treatment and adopt a watchful approach only in those carrying the CC alleles. Treatment of acute hepatitis C should be based on pegylated IFN-α monotherapy for 24 weeks. There is currently no indication for administering IFN-α as post-exposure prophylaxis.

Chronic Infection

The current standard of care for chronic hepatitis C is based on a combination of pegylated IFN-α and ribavirin. While IFN-α is acting essentially as an antiviral, the mechanism of action of ribavirin is multifaceted [22]. Patients with HCV genotype 1 can receive, in addition, an inhibitor of the viral NS3/4A serine protease, such as telaprevir and boceprevir. With such regimes, sustained virological response rates (defined as undetectable HCV RNA in serum 6 months after the end of therapy) can be expected to be around 70 % in patients infected with genotype 1 [23] SVR rates with traditional dual treatment are about 75–90 % in those infected

with genotypes 2 and 3, respectively, and somehow less in genotype 4, where different subtypes and host cofactors may account for a more variable treatment outcome [24–26]. Treatment duration varies according to the infecting genotypes, some host and disease baseline features—such as the presence of advanced fibrosis—and the virological response pattern during the treatment itself. Thus, therapy may last between 12 and 72 weeks, and is characterized by several side effects, which can be occasionally serious, if not life-threatening. This translates into significant direct and indirect costs, both in financial and human terms, which, although offset by the saved years of life, still add to the barriers against an optimal treatment effectiveness. Thus, it is easy to justify the major efforts that have been devoted to identify novel DAA to be used in effective IFN-α-free combinations. DAA may target several viral proteins, such as the NS3/4A serine protease, the NS4B, the NS5A or still the viral polymerase, encoded by the NS5B region [27]. Other approaches are aimed at blocking host factors that are involved in the life cycle, such as one of its receptors, or replication cofactors. Some IFN-α-free, and possibly ribavirin-free, pangenotypic DAA combinations have now entered phase III of development, and are expected to enter the market in a few years from now.

Immune Responses to HCV

Innate Immunity

Viruses, particularly those responsible for chronic infection, have a remarkable ability to become adapted to many different environments. In the case of RNA viruses, such as HCV, this is in part mediated by their high mutational rates, allowing for the rapid selection of variants that overcome hostile environments. These are initially provided by early innate defence mechanisms which are triggered immediately after infection and have the function to limit the extent of microbial spread [28]. Recognition of pathogens occurs through a series of receptors that sense regular patterns of molecular structure shared by many micro-organisms but are not present on the host's own cells. These patterns are called pathogen-associated molecular patterns (PAMP) and the receptors involved in their recognition pathogen recognition receptors (PRR). The innate signalling receptors consist of a tetrad of PRR relevant to viruses: (1) Toll-like receptors (TLRs) which sense all microbes; (2) retinoic acid-inducible gene I (RIG-I)-like RNA helicases (RLH); (3) melanoma differentiation-associated 5 (MDA-5) both of which predominantly sense viruses; (4) nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) which sense bacteria and viruses [29]. TLR are the earliest receptors involved in defence against infections in multicellular organisms. Humans express ten TLR genes and each is devoted to recognizing a distinct set of PAMP. Cell surface TLRs (TLR-2, TLR-1, TLR-4, TLR-5 and TLR-6) usually recognize microbial proteins expressed on extracellular pathogens, whereas intracellular TLRs (TL-3, TLR-7, TLR-8 and TLR-9) are located in endosomal wall and sense nucleic acid fragments generated following microbial processing. Cytoplasmic viral RNAs resulting from viral replication are instead sensed by RLH or MDA-5 which recognize viral ssRNA and dsRNA, respectively. They both differ from intracellular TLRs, which interact primarily with RNA or DNA entering the endocytic pathway. After virus sensing, TLRs, RIG-I and MDA5 pathways induce type I and type III interferons (IFNs). Indeed, a hallmark of the innate immune responses of mammalian hosts to viral infections is the rapid induction of IFNs and other cytokines. IFNs inhibit viral replication in infected cells and establish an antiviral state in uninfected neighbouring cells by inducing the expression of interferon-stimulated genes (ISGs) with broad antiviral activities. IFNs also play an important role in activation of various immune effector cells, thereby linking innate and adaptive immunity [30]. There are three types of IFNs that are defined by the receptors they utilize. The type I IFNs comprise IFN-β and a number of IFN-α subtypes. These can be produced by most cell types in the body and act through an equally broadly expressed receptor. Type II IFN includes only a single molecule, IFN-γ, and its production is confined to NK cells and activated T cells. Type III IFNs, the most recently identified group of IFNs, include three members, IFNλ 1 (*IL29*), IFNλ 2 (*IL28A*) and IFNλ 3 (*IL28B*) [31]. IFNλs can be produced by many but not all cell types and target a receptor that has primarily an epithelial distribution (that includes hepatocytes). The IFNs activate downstream signalling pathways known as Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling, by binding to and activating their class-specific cell surface receptors [30].

Prompt HCV sensing occurs via TLRs and RLH. Indeed, the core protein activates the TLR-2-MyD88 signalling cascade when complexed with co-receptors (TLR-1 and TLR-6) and there is evidence that both core and NS3/4A use the heterodimeric TLR-2/TLR-6 complex [32] to elicit an inflammatory cytokine production [33]. Among other TLRs, TLR-3 sensing dsRNA has recently been shown to be responsible for induced production of IFNλ by a rare subset of myeloid dendritic cells, mDC2, providing important mechanistic insights into the role of type III IFNs in HCV infection [34]. Moreover, recent data indicates that type III IFNs and in particular IFNλ 1 are vigorously induced following infection of primary human hepatocytes in vitro and mediate stronger antiviral activity than type I IFNs [35]. Collectively, these findings lend support to the concept that IFNλ s play a major role in the pathogenesis of HCV infection and that innate immunity exert significant control in this setting. To this

regard, a recently recognized important host genetic factor associated with spontaneous [5] and treatment-induced [36] HCV viral clearance is *IFNλ 3* polymorphism. As discussed above, *IFNλ 3* gene encodes for IFNλ 3 [37] and members of IFNλ family have been implicated in the killing of tumour target cells [38, 39]. Interestingly, although the cellular receptors of IFN-α and IFNλ are different [39, 40], they share the intracellular JAK-STAT signal pathway, suggesting a pathogenetic role for this molecule. However, unfavourable *IFNλ 3* single nucleotide polymorphisms (snp) do not seem to be clearly associated with specific defects of innate immune responses, although in one study rs12979860 *IFNλ 3* TT homozygosis was associated with increased expression of the natural killer (NK) cell NKG2A inhibitory receptor and reduced expression of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) on $CD56^{dim}$ NK cells [41] suggesting a possible role of *IFNλ 3* in inhibiting NK cell responses to HCV.

Microarray analysis has demonstrated that HCV infection is generally associated with induction of a strong ISG response in the liver in vivo [42, 43]. In addition, the expression of intrahepatic chemokines such as the CCR5 ligands regulated and normal T cell expressed and secreted (RANTES or CCL5), macrophage inflammatory protein (MIP)-1α (or CCL4), and MIP-1β, and the CXCR3 ligands, interferon gamma-induced protein 10 (IP-10 or CXCL10), interferon-inducible T-cell α chemoattractant (I-Tac or CXCL11), and monokine induced by IFN-γ (MIG or CXCL9), is often elevated in hepatitis C, and the levels of some of these correlate with the outcome of HCV infection or severity of liver inflammation [44–46]. IP-10 is the most studied CXC-protein during HCV infection, since high peripheral IP-10 pretreatment level has been associated with lack of SVR to IFN-α and ribavirin treatment [47–51]. Moreover, contrary to SVR, IP-10 levels do not decrease during therapy in non-responder patients [47–53].

Another large family of cytoplasmic microbial sensors are the NLRs which share with TLR the same ability to activate nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) to start the inflammatory response. Some NLRs have a pyrin domain and are known as NLRPs (NLRP1 to NLRP14) [54]. In stressed cells such as those exposed to infection, they assemble with an adapter protein and caspase-1 to form large multiprotein complexes called inflammasome [55]. Inflammasome activation results in cleavage of pro-IL-1β and pro-IL-18. The mature form of the former is a proinflammatory cytokine, and a central regulator of inflammation, whereas the latter activates NK cells to produce IFN-γ. There is evidence that IL-1β levels are increased in patients with chronic HCV infection and these levels are higher in those with extrahepatic manifestations such as cryoglobulinaemia [56] and that serum IL-1β and caspase-1 levels are decreased in responders to antiviral therapy [57]. Patients with cirrhosis have high intrahepatic levels of IL-1β and preliminary evidence suggests that HCV uptake by macrophages or Kupffer cells triggers the production of IL-1β and a proinflammatory response, which mediates hepatic inflammation and promote liver disease during HCV infection [58]. NLRP3 inflammasome activation and IL-1β production were also reported in HCVcc-infected Huh-7 cells [59] indicating that inflammasome upregulation may be a component of HCV immunopathology.

Innate immune defence against viruses may also include autophagy, an essential catabolic process of eukaryotic cells, as a possible defence mechanism [60]. Indeed several viruses, including HCV, were reported to depend upon, but at the same time be controlled by autophagic processes [61]. It is known that HCV replication triggers the unfolded protein response [62] which in turn leads to the induction of autophagy [63, 64]. Interestingly, it was shown that induction of autophagy by HCV might be directly involved in the suppression of type I IFN production, as RLH stimulation in cells with a knock-down of key regulators of autophagy yielded significantly higher IFN-β induction rates [65, 66].

HCV Strategies to Avoid Control by Innate Immune Sensors

Innate immune responses to HCV have been the subject of intense investigation over the past few years inasmuch as it may be stated that most of the progress in our understanding of immunopathogenesis of this condition has been attained in this particular field of immunology. Studies on HCV sensing and escape from the innate immune system have been largely performed using in vitro cell culture systems and in vivo in experimentally infected chimpanzees, as they can be studied from the onset of infection through the course of the disease, with the caveat that the primate model may not be entirely representative of the human setting. Contrary to hepatitis B virus (HBV) infection in which no appreciable changes in innate immune response genes are detected in the liver of HBV infected chimpanzees in the first weeks of infection, HCV seems to be able to efficiently induce IFN--α/β-response genes and is sensitive to IFNs in vitro [67]. Yet, HCV seems to ignore early innate defence mechanisms, as it replicates almost immediately after penetration into target cells, suggesting that the virus has developed strategies to modulate the antiviral function of the innate immunity by blocking intracellular signalling pathways to attenuate the IFN antiviral effect [68–70]. Mechanisms of HCV protein interference with IFN signalling pathways are shown in Fig. 15.2. One of the TLR pathways that appears to be

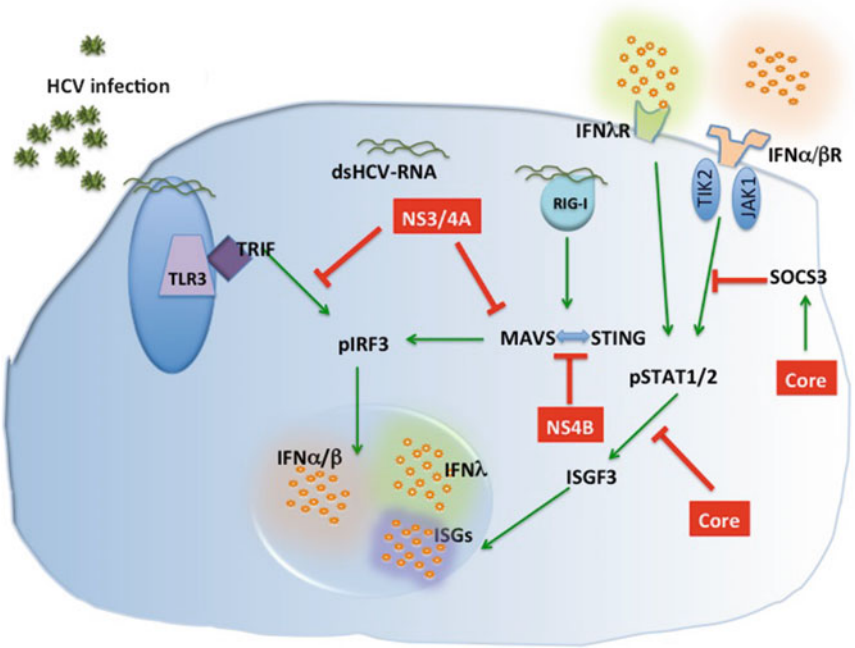

Fig. 15.2 HCV strategies to avoid innate immune responses. Different HCV proteins are able to inhibit pathways leading to ISG expression and IFN production. NS3/4A cleaves TRIF adapter protein and MAVS resulting in failure to activate interferon regulatory factor 3 (IRF3) with consequent impaired activation of downstream target genes, including IFN-β. HCV core protein-mediated SOCS-3 upregulation reduces expression of ISG. HCV core protein can directly bind to STAT1, blocking STAT1/STAT2 heterodimerization and thus inhibiting IFN signal transduction. HCV-NS4B blocks RIG-I-mediated activation of IFN-β production signalling through binding of stimulator of interferon genes (STING) and blocking STING–MAVS interaction

directly impaired by HCV proteins is that of TLR3 which proceeds through the adapter protein, TIR-domain-containing adapter-inducing IFN-β (TRIF) [71]. It has been shown that the NS3/4A HCV serine protease can cleave adapter proteins such as TRIF in the endosome-borne TLR3 pathway [72] and the mitochondrial antiviral signalling (MAVS) protein in the cytosolic RLH pathway, thus disturbing binding of RIG-I to MAVS disrupting PRR signalling [73]. This, in turn, results in failure to activate interferon regulatory factor 3 (IRF3) with consequent impaired activation of downstream target genes, including IFN-β [74, 75]. The observation that MAVS is cleaved in HCV-infected Huh-7 cells by the viral protease has more recently been confirmed in humans [76]. Of note IRF-3 blockade was found to be essential for HCV replication efficiency and it is interesting to note that forced stimulation of the RLH pathway in HCV permissive cells, results in marked reduction of viral replication [77]. Moreover, the HCV core protein can inhibit the STAT 1 pathway and IFN signalling via HCV core-mediated upregulation of suppressor of cytokine signalling (SOCS) 3 [78] or interferon-stimulated gene factor 3 (ISGF3) blockade [79] resulting in reduced expression of ISG. Interestingly, a recent study addressing the antiviral activity of more than 300 ISGs against several viruses including HCV, identified broadly active key regulators of antiviral signalling as the most potent antiviral factors, such as RIG-I, MDA-5, IRF1, IRF2 and IRF7, beside a few virus-specific ISGs, which upon overexpression trigger transcription of numerous target genes strongly supporting the notion that there is not a single antiviral factor responsible for IFN-mediated inhibition of viral replication [80]. Although several lines of evidence suggest that HCV infection disrupts IFN responses at multiple levels as described above, it is clear that ISGs are often prominently expressed in the liver in both acute and chronic infections. Interestingly, persistent induction of ISG is associated with poor response to IFN-α-based treatment most likely as a result of refractoriness of IFN-α signal transduction pathway [81–83], whereas patients who eventually develop sustained virological response have low pre-therapy expression of ISGs within the liver, but demonstrate impressive upregulation of these genes when treated. Why this occurs remains obscure.

Thus, HCV appears to have evolved several strategies to elude control by cellular sensors. Impaired sensing allows viral replication to outpace host's immune responses. The presumably reduced production of key cytokines involved in priming the cellular arm of innate immunity, the most notable of which are NK cells, may be responsible for inadequate development of efficient adaptive immunity, ultimately resulting in virus persistence.

NK Cells in HCV Infection

NK cells develop in the bone marrow from the same progenitors as T and B lymphocytes and circulate in the blood. They are also named large granular lymphocytes because of their size larger than T and B cells and of their distinctive cytotoxic granules containing granzymes and the pore-forming protein perforin. NK cells lack the CD3 T-cell co-receptor and typically express CD56. They are important antiviral effectors of innate immunity either via direct killing of infected cells or cytokine (namely IFN-γ and TNF-α) production [84]. These two functions are apparently mediated by different NK subpopulations, with cytotoxicity being generally performed by $CD56^{dim}$ NK cells, the major population of peripheral blood NK cells, whereas $CD56^{bright}$ NK cells are mainly responsible for cytokine secretion. This reportedly rigid distribution of tasks has recently been challenged as $CD56^{dim}$ can mediate both functions, being able to produce large amount of IFN-γ during the first hours after stimulation [85]. NK cells are activated in response to IFNs or certain macrophage-derived cytokines (e.g. IL-12, IL-15, IL-18), and are controlled by a complex network of signals which interact with membrane-expressed, germ line-encoded, inhibitory and activating receptors [86]. The latter allows recognition of altered self via binding to ligands expressed by stressed cells, effectively functioning as danger signals [87]. Upregulation of these ligands, such as NKG2D, NKG2C and the natural cytotoxicity receptors (NCRs: NKp30, NKp46, NKp44, NKp80), may tip the balance of NK cells from inhibition to activation ("induced self" recognition). Inhibitory receptors act to prevent NK cells from killing normal host cells and fall into two large families: one is characterized by immunoglobulin-like domains hence their name killer cell immunoglobulin-like receptors (KIRs) which recognize HLA-B and -C, and the other consists of the C-type lectin-like proteins, the most notable of which is NKG2A which binds to HLA-E [88]. Certain KIRs have been associated with the evolution of HCV infection toward persistence or recovery. Indeed, in a large immunogenetic study, preferential expression of the inhibitory receptor KIR2DL3 on NK cells has been reported in patients with a self-limited outcome of acute HCV infection acquired by a low-dose exposure [89]. Since KIR2DL3 has a lower affinity for its HLA-C ligand than other KIRs, KIR2DL3-mediated inhibition of NK cells is inherently weak; this may predispose NK cells from these individuals to be more easily activated by viral infection allowing them to control it more efficiently. The real impact of these mechanisms on NK cell function in vivo is however unknown because functional correlates for these observations have not been defined. Interestingly, NK cell responses are readily generated during acute HCV infection. While one study showed decreased expression of the NKG2A inhibitory receptor in spontaneously resolving acute hepatitis C [90], another one reported significantly increased cytotoxicity and IFN-γ production in patients with acute hepatitis C compared with healthy donors, irrespectively of clinical outcome [91]. Therefore, NK cell activation appeared to be a by-product of IFN-α induction by HCV rather than playing a direct role in viral clearance, a task performed by T cells rather than NK cells [92]. Because of the widespread availability of patients with chronic hepatitis C and the interest to broaden our understanding of the role of NK cells in this context, several studies focused in this area. Despite the availability of standardized reagents, simple questions such as quantifying the number of circulating NK cells, examining their phenotype and correlating those parameters to NK cell function yielded in many cases diverging data in chronic HCV infection, with some ex vivo studies suggesting that reduced NK cell frequencies did not affect spontaneous or cytokine-induced cytolytic effector function [93–97], while others showed instead deficient NK cytolytic activity [98]. Evidence in support of this latter data would come from early observations showing that the interaction of the E2 protein of HCV with CD81 on NK cells inhibits their activation [99, 100] and that NK cell function can be deficient in individuals chronically infected with HCV [101] suggesting that HCV proteins may actually contribute to impair NK cell responses. Indeed, upregulation of the inhibitory receptor CD94/NKG2A leading to altered NK/dendritic cell crosstalk has been reported in chronic HCV infection [102]. The reasons for such controversial findings are not immediately apparent, and the quality of some studies could be biased by a small sample size. In a more comprehensive study involving a sizeable number of patients with chronic HCV infection [95], essentially confirmed by others [96, 97], increased frequencies of NK cells expressing the activating receptors NKG2D and NKG2C, associated with decreased frequency of NK cells expressing KIR3DL1, were found in HCV-infected patients, supporting the concept of a phenotype skewed toward activation in this setting. In line with phenotypic data, NK cells from HCV-positive patients responded well to cytokine stimulation displaying normal or increased cytolytic activity. However, there was a major functional defect characterized by deficient IFN-γ and TNF-α production, suggesting the existence of a functional dichotomy, featuring enhanced or normal cytolytic activity and reduced cytokine production (Fig. 15.3). Of note, NK functional defects are usually combined so that impaired cytotoxicity is virtually always associated with deficient cytokine production; however, the existence of different regulatory pathways allows single functional alterations of one of the two. To this end, mechanistic insights into the cause of a reduced IFN-γ secretion by NK cells in this setting came from recent studies

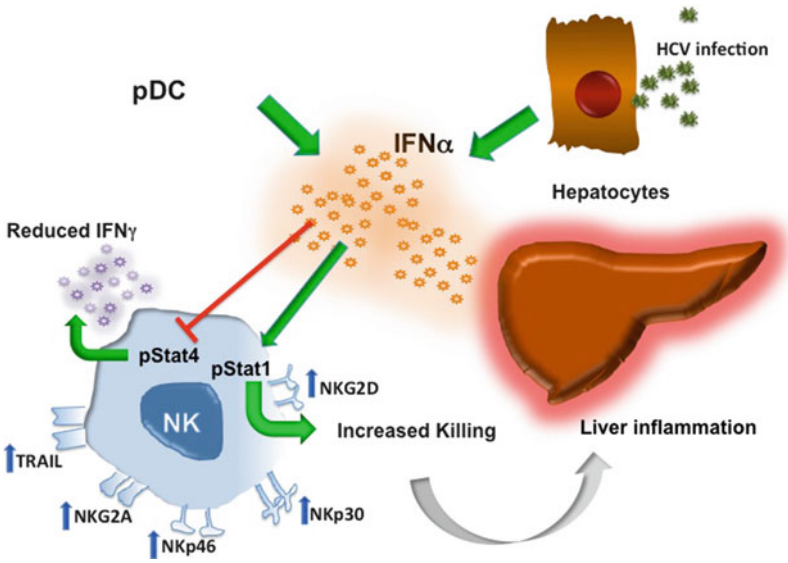

Fig. 15.3 NK cell functional dichotomy in chronic HCV infection. Hepatocytes and pDC release substantial amounts of IFN-α as a consequence of chronic HCV infection which preferentially stimulates STAT-1 rather than STAT-4 phosphorylation, resulting in reduced IFN-γ synthesis and secretion, upregulation of several NK cell activating receptors and polarizing NK cells toward cytolytic activity. This functional dichotomy would eventually result in the inability to eliminate HCV while maintaining continuous liver inflammation. Blue arrows indicate NK receptor upregulation

indicating altered IFN-α signalling resulting from increased IFN-α-stimulated STAT1 phosphorylation, which polarizes NK cells toward cytotoxicity, and a concomitantly reduced IFN-α-induced STAT4 phosphorylation yielding reduced NK cell IFN-γ mRNA levels [103, 104]. Because the antiviral effect produced by cytokines is more efficient than single target cell lysis, the dysfunctional cytokine secretion shown here may be an important mechanism contributing to virus persistence. The fundamental importance of IFN-γ in the control of viral infections has indeed been shown in several studies which demonstrated it to be a powerful non-cytolytic mechanism of viral clearance from infected hepatocytes [105, 106]. In line with this, the functional NK cell defect described above for chronic hepatitis C has been interpreted as a consequence of chronic exposure to HCV-induced IFN-α leading to chronic liver inflammation via cytotoxic mechanisms but not to viral clearance because of insufficient IFN-γ production [97, 107]. Whether the findings obtained with peripheral blood (PB) NK cells are relevant to the liver compartment where immune-mediated chronic necroinflammation actually takes place remains to be elucidated.

Liver-Infiltrating NK Cells in Chronic HCV Infection

The presence of intrahepatic (IH) NK cells in humans has been a matter of intense discussion over the past several years. Are there really resident lymphoid cells in the healthy adult human liver or are the mononuclear cells extracted from the liver simply originated from the blood flowing through the sinusoids? In any case, current evidence suggests that NK cells are uniquely enriched in the healthy liver, their percentage being approximately threefold higher than that in the peripheral blood [108, 109]. Several studies in chronic HCV infection emphasized differences between the IH and PB compartments [95, 97, 110]. In some studies, a larger proportion of IH NK cells express activation molecules and TRAIL compared with the PB compartment and this led many to advocate it as a proof of a pathogenetic role for NK in liver necroinflammation [99]. However, the vast majority of studies in humans lack functional evaluation of IH NK cells and, therefore, it is impossible to know whether phenotypic changes actually mirror alterations in IH NK cell cytolytic potential or cytokine production. More importantly, until recently no appropriate controls were used to validate the quality of ex vivo data so that most phenotypic and functional alterations described may not be specific to HCV infection. Recent data using IH NK cells from patients with no parenchymal liver disease undergoing elective surgery for gallstones showed instead that ex vivo isolated IH NK cells from patients with chronic HCV infection were less in numbers and displayed reduced degranulation ability compared with controls with apparently conserved NKG2D-mediated IFN-γ production [111]. These findings are in contrast with those of peripheral blood and it is still unclear why the cytolytic NK defect is apparently restricted to the intrahepatic compartment. It may be that the peculiar liver environment plays an important role in this process. Indeed, selected NK cell populations can accumulate inside the

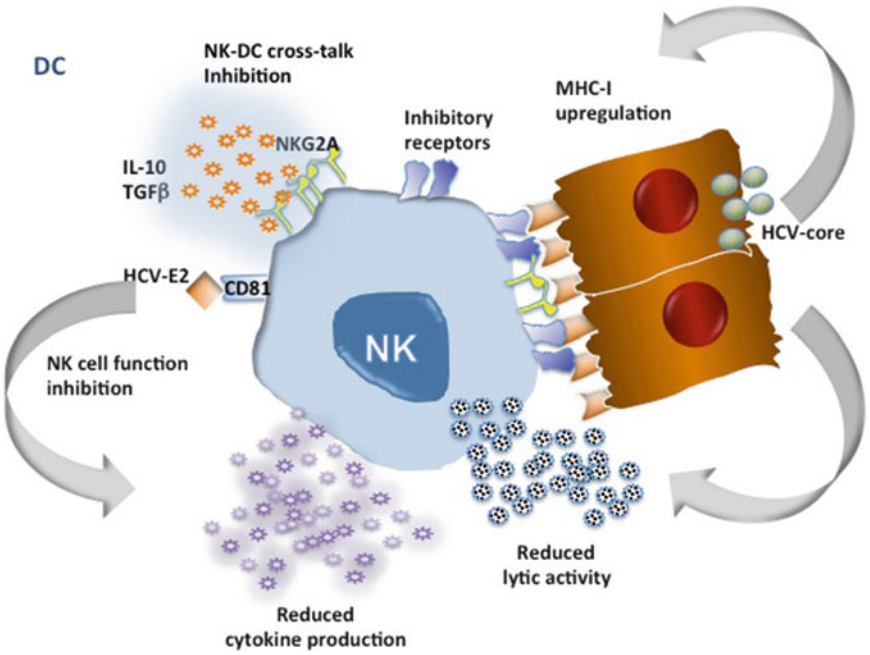

Fig. 15.4 Inhibition of intrahepatic NK cell responses in HCV infection. HCV core protein mediated MHC-I upregulation results in NK cell inhibition via binding to inhibitory receptors. Upregulation of NKG2A leads to altered NK-dendritic cell cross-talk. E2 binding to CD81 induces inhibition of NK cell function. The IL-10- and TGF-β-rich intrahepatic environment would contribute to NK immunosuppression

liver, as it has recently been shown [112] which can display a unique functionality. Moreover, the relatively impaired IH NK cytotoxic function detected in our study may have different explanations related to the liver compartmentalization of the virus which may have a direct inhibitory effect on NK cell function (Fig. 15.4). For instance, it is known that HCV is able to inhibit NK cells by interaction between the E2 protein and CD81 [99, 113] and that the HCV core protein induces upregulation of major histocompatibility complex (MHC)-I on hepatocytes [114] and the HCV peptide 35–44 stabilizes the expression of HLA-E on liver cells inhibiting NKG2A-mediated cytolysis [115]. In addition, it has been shown that intrahepatic levels of IL-10 determine an immunosuppressive environment both in mice [116] and humans [117] and, in agreement with the aforementioned, it has been reported that IH, HCV-specific IL-10-producing, non-classical regulatory CD8+ T cells may inhibit liver damage during chronic infection [117, 118]. This, coupled to exhaustion induced by continuous receptor engagement [119, 120] would eventually lead to defective cytolytic function. The functional cytotoxic defect observed was mirrored by a unique phenotype characterized by increased expression of activating (NKp46, NKG2D) receptors in the face of reduced TRAIL and CD107a expression, compared with controls [111]. These findings indicate dysfunctional IH NK cell cytotoxicity associated with TRAIL downregulation in chronic HCV infection, which may contribute to virus persistence and possibly to fibrosis progression, since it is known that NK cells can inhibit liver fibrogenesis [121]. Interestingly, several recent studies provided evidence in support of NK cells controlling liver fibrosis in patients with chronic hepatitis C most likely through a cytotoxic effect on hepatic stellate cells (HSCs), an essential component in liver fibrogenesis. To this end, it is interesting to note that the ability of NK cells to induce HSC apoptosis correlate inversely with the stage of liver fibrosis [122]. Moreover, a peculiar NKp46^{hi} NK cell subset endowed with strong cytolytic activity against HSCs and stronger IFN-γ secretion in vitro [112] has been recently shown to attenuate liver fibrosis [123]. Thus NKp46 expression would be linked to antiviral as well as antifibrotic activity. The role of NK cells and other innate immune cells in hepatic fibrogenesis is reviewed elsewhere in this book (Bin Gao, Chap. 10).

What is the role of HCV in modulation of NK cell responses? Interestingly, contrary to healthy controls, PBMC NK cells from HCV-infected patients fail to upregulate TRAIL and CD107a when exposed to culture-derived HCV (HCVcc), suggesting an accessory cell-dependent, direct effect of the virus on TRAIL-mediated cytotoxicity [111]. The importance of the role of TRAIL in chronic HCV infection is further emphasized by evidence that TRAIL is upregulated at the gene level in patients who have successfully responded to IFN-α treatment [124] and by data showing upregulation of this molecule on NK cells from healthy donors following IFN-α exposure in vitro [97]. These previously unappreciated findings are compatible, on the one hand, with the inability to clear HCV from the liver and on the other with occasional resistance to IFN-α-based therapies.

Other Cells Involved in Innate Immune Responses to HCV

γδ T cells appear to play an important role in viral infections, but contrary to the better known αβ T cells do not generally recognize antigen as peptides presented by MHC molecules; instead, they seem to recognize their target antigens directly which allows prompt responses to molecules expressed by different cell types [125]. Candidate ligands are heat-shock proteins, MHC class Ib molecules, unorthodox nucleotides and phospholipids [126]. One study showed that Vγ9/Vδ2 T cells activated by non-peptidic antigens robustly inhibit HCV replication in cell lines harbouring subgenomic HCV replicons [127] providing evidence for a possible role of these cells in HCV control.

The role of NKT in HCV infection is less clear, at least in humans. These cells are able to recognize glycolipid antigens (namely alpha-galactosylceramide, α-GalCer) presented to them by the MHC-like molecule CD1d [128]. They rapidly secrete a variety of cytokines including IL-4, IL-10, IL-13, TNF-α and IFN-γ and are thought to have a primarily regulatory function. The CD1d-dependent NKT cells can be grouped into two types of cells: type I NKT cells, also called classical or invariant NKT (iNKT) cells because they express an invariant T-cell receptor α (TCR-α) chain, comprise 95 % of liver NKT cells, whereas type II NKT cells express diverse TCRs and make up less than 5 % of liver NKT cells. Although activation of iNKT cells by the exogenous lipid antigen α-GalCer has been extensively investigated, the endogenous ligands that activate NKT cells remain largely unknown. Available evidence indicates that CD1d-reactive iNKT are apparently rare in HCV-infected livers [129], even though they may play an important role in fibrosis progression [130].

Adaptive Immunity and HCV

Prompt and efficient innate immune responses are mandatory to prime naïve T or B lymphocytes that will then eliminate, and permanently remember the pathogens encountered, via specific recognition of microbial epitopes. Successful effector responses and memory establishment by CD4 T helper cells are dependent on the presence of a wide array of stimulatory signals during priming: those provided by professional APCs (e.g. DCs), duration of antigenic stimulus, the cytokine milieu, etc. Priming of protective (cytotoxic) CD8 T-cell responses essentially requires the same conditions, but the long-lasting CD8 T-cell memory seems to be conditioned by the constant presence of memory CD4 T cells [131]. These mechanisms guarantee the prompt emergence of high frequencies of competent effector T cells that are essential for recovery. Upon resolution of infection, effector cells disappear, whereas memory cells remain numerically constant because of the expression of receptors specific for the homeostatic (IL-7 and IL-15) cytokines [132]. The homeostatic proliferation of memory cells in the absence of antigen is thus critical for the prompt differentiation into effector cells, should they re-encounter the original infecting pathogen.

Following exposure to HCV, host adaptive immune responses largely determine whether the virus is spontaneously eradicated or persist [92] akin to most viral infections, although some key factors in immunopathogenesis still remain elusive. Below we shall analyse in detail the different components of adaptive immunity to HCV and the kinetics of these responses in relation to infection outcome.

B Cell Responses

The lifelong intrinsic capacity of B lymphocytes to edit their B-cell receptor (BCR) in order to cope with many different pathogens usually results in a huge repertoire of antibodies, among which neutralizing antibodies are meant to cooperate with T cells to eradicate pathogens, and to control the continuous emergence of microbial mutants. The significance of humoral immunity has been extensively investigated in HCV infection, even though the role of antibodies in controlling the virus is far from being clarified. Antibody responses to structural and non-structural proteins appear several weeks after acute HCV infection and their presence appears to correlate better with ongoing rather than past infection, since their vigour and breadth progressively diminish as a function of time after spontaneous recovery [133]. Antibody seroconversion is a key element for the diagnosis of acute infection, since chronic infection can reactivate mimicking acute hepatitis [10]. However, several studies failed to demonstrate correlations between specific patterns of antibody specificities and clinical outcome of acute infection, initially supporting the concept that efficient neutralizing antibody responses were not elicited at all during HCV infection. Evidence in favour of this hypothesis came from experimental transmission studies in chimpanzees which showed lack of protective humoral immunity from homologous or heterologous virus challenge [134, 135], akin to observations in multiply exposed intravenous drug users [136]. The finding that agammaglobulinaemic patients can resolve acute HCV infection upon IFN-α treatment, lends support to the hypothesis that HCV-specific T cells may compensate for the lack of neutralizing antibodies to achieve HCV clearance [137]. However, it is fair to say that there are data reporting neutralization of HCV-containing inocula by homologous but not heterologous plasma [138] at least suggesting the existence of isolate-specific neutralizing antibodies.

Infectious retrovirus-HCV pseudoparticles produced by assembling HCV envelope glycoproteins on a retroviral core have been used to compare neutralizing antibody responses in patients with resolving and chronically evolving acute hepatitis C [139]. Overall, detection of neutralizing antibodies, which were broadly cross-reactive across genotypes, did not correlate with viral clearance. The reasons for the overall lack of effect of neutralizing antibodies are poorly understood although available data suggest that neutralizing antibody responses lag behind the rapidly evolving HCV sequences present within the viral quasispecies population [140]. In addition, development of neutralizing antibodies may be detrimental in certain conditions such as recurring hepatitis C after liver transplantation, since they may be responsible for selection of viral variants contributing to the complexity and diversity of the circulating viral quasispecies in this setting [141].

Beside these considerations on the controversial role of neutralizing antibodies in preventing HCV infection, there is evidence that antibodies specific for virus receptors, such as Claudin 1 [142] and scavenger receptor B I may efficiently prevent HCV infection in vitro [143] and in vivo [144] and are able to inhibit infection by escape variants selected by neutralizing antibodies [142]. This suggests that such reagents may be successfully employed in the prophylaxis of HCV reinfection following liver transplantation, thus paving the way to new developments in the field.

Role of B Cells in Extrahepatic Manifestations of HCV Infection

Although antibodies have been rather extensively studied in the context of HCV infection, there is growing evidence that B cells are chronically activated in persistent HCV infection and that this phenomenon may have important pathogenetic implications for the extrahepatic manifestations, particularly lymphoproliferative disorders, arising as an indirect process from chronic antigenic stimulation. Recent findings point to the possible role of HCV E2 envelope protein–CD81 interaction [145] which may reduce the activation threshold of B cells potentially leading to polyclonal and, eventually, oligo- and monoclonal expansion of B lymphocytes. Polyclonal B cell activation is a typical feature of chronic HCV infection and is associated with upregulation of B-cell activation molecules [145, 146]. However, there is controversy over the type of B cells that is activated since in one study it was found to be the naive (CD27−) subset [145] whereas in another it was the memory (CD27+) subset [146] which also showed increased propensity to differentiate into immunoglobulin-producing cells, thus providing a plausible pathogenetic basis for B-cell lymphoproliferative disorders and autoimmunity observed in chronic HCV infection. Another recent study reported skewing of B cell subsets resulting in increased frequencies of immature transitional and mature activated B cell subset frequencies in HCV-infected subjects [147]. Interestingly, mature activated B cells were less prone to proliferate, and were more intrinsically resistant to apoptosis, suggesting that these cells tended to be terminally differentiated into antibody-secreting cells as shown in a previous study [148]. Taken together, these results indicate that in the setting of chronic HCV infection, a state of activation results in B cell subset skewing which, contrary to HIV infection where B cells show signs of exhaustion and consequent functional impairment [149], in HCV infection maintains an overall intact or enhanced B cell response. This data, however, does not provide definitive mechanistic insights into the pathogenesis of mixed cryoglobulinaemia (MC) and other lymphoproliferative disorders. Patients with symptomatic MC have clonal expansions of hypermutated, rheumatoid factor-bearing marginal zone-like IgM+/CD27+ peripheral B cells using the *VH1-69* gene [150], have a global transcriptional profile suggestive of anergy and apoptosis, and a large proportion of them have immunophenotypic features of anergy, suggesting that a significant proportion of this clonal population may be exhausted and, therefore, refractory to ongoing antigenic stimulation [151]. This data would be compatible with more recent findings suggesting that infection with HCV induces apoptosis of naïve mature B-cells resulting in reduction in the size of the naïve B-cell subset leading to a compensatory increased size of the immature B-cell subset egressing from the bone marrow, particularly immature transitional B cells, and that this process is accelerated in the presence of mixed cryoglobulinaemia [152]. It has been postulated that this mechanism would be responsible for maintaining B-cell homeostasis by replenishing the naïve B-cell pool in HCV-induced cryoglobulinaemia, although this does not fully contribute to explain why this occurs only in chronic HCV infection and not in other persistent viral infections.

T-Cell Responses: CD8

Although information about the early kinetics of the T-cell response in natural HCV infection is limited, available data indicate that despite the rapid onset of HCV replication, HCV-specific T-cell responses are induced after a long time following virus exposure, compared to other virus infections, being detected in the peripheral blood of infected patients only several weeks after infection [153–160]. As soon as they become detectable, HCV-specific CD8 cells seem to be only partially able to express their effector function since they can be inefficient for some additional weeks after their induction in producing IFN-γ and IL-2 upon ex vivo peptide stimulation [154–156, 158, 160, 161]. This early CD8

dysfunction is generally detectable regardless of the subsequent outcome to virus control or persistence. While CD8 appear to recognize several HCV peptide epitopes [156–159, 162], patients with self-limiting infection reportedly develop a more vigorous and broadly specific CD8 responses compared with those developing persistent infection, although this view is not shared by all investigators [157–159, 162–166]. Therefore, the available evidence points to an overall impaired antiviral function of CD8 T cells and, in agreement with this hypothesis, a large population of effector memory CCR7– T cells in patients with acute hepatitis C display poor effector function ex vivo which may be responsible for incomplete effector CD8 T-cell differentiation shown in patients with acute HCV infection, which can be rescued by IL-2 stimulation in vitro, suggesting that such defect is reversible and not pervasive [160]. However, this tendency to impaired development of CD8 in this condition may be one of the many factors responsible for the inability of the host adaptive immunity to efficiently eradicate HCV infection. This problem may be further compounded by the inefficiency of CD4 T-cell responses during chronic HCV infection which may be unable to support a full maturation of HCV-specific CD8 cells which may remain dysfunctional [158, 166, 167], expressing PD-1 and remaining CD127 negative [167]. Spontaneous control of HCV infection is instead associated with successful maturation of CD8 memory, indicated by the acquisition of a CD127+/CCR7+ phenotype [158, 168–170].

Another recently described possible mechanism of viral persistence is the emergence of a large population of mixed polyfunctional (type-1, -2, -17) CD8+ T-cell effector responses specific for apoptotic T-cell-associated self-epitopes rather than dysfunctional virus-specific CD8+ T cells [171], providing a plausible explanation as to why the enormous expansion of activated T cells, during persisting viral infections such as HCV, consists predominantly of virus non-specific CD8 T cells. Chronic evolution was associated with the selection of autoreactive CD8+ T cells with higher TCR avidity, whereas those with lower avidity underwent prompt contraction, as seen in patients undergoing infection resolution. Consistent with this data, several models of chronic viral infection demonstrated that virus-specific CD4+ or CD8+ T cells producing elevated levels of IL-17 correlate with viral persistence [172, 173]; however, no definite experimental data is yet available on the role of IL-17 producing T cells in HCV infection.

T-Cell Responses: CD4

There is general consensus that patients who eventually recover from acute hepatitis C self-limited infection display more broadly reactive and more vigorous ex vivo HCV-specific CD4 T-cell responses compared with patients evolving toward chronic infection [158, 159, 162, 166, 174–176], in whom weaker and more narrowly focused responses are generally demonstrable. Moreover, it has recently been shown that viral clearance is associated with reversal of HCV-specific T-cell exhaustion, as evidenced by reduced PD-1 expression and improved T-cell function [177]. Surprisingly, in a recent comprehensive analysis of CD4 T-cell responses in acute hepatitis C, broadly directed HCV-specific CD4+ T-cell responses were universally detectable at early stages of infection, regardless of clinical outcome, challenging the paradigm that HCV persistence is the result of a failure to prime HCV-specific CD4+ T cells [178]. However, persistent viremia was associated with early proliferative defects of the HCV-specific CD4+ T cells. These findings suggest that broadly directed HCV-specific CD4+ T-cell responses are usually generated in acute HCV infection, but rapid exhaustion and deletion of these cells occurs in the majority of patients. Th1 cytokines (IFN-γ and IL-2) usually prevail on the scene in patients who succeed in clearing HCV spontaneously whereas patients evolving toward chronic hepatitis are instead characterized by a predominant type 2 cytokine environment [158]. Thus, human studies are consistent with the interpretation that the efficiency of the CD4 responses is determinant in dictating the fate of infection by directly contributing to virus control or persistence.

Mechanisms Responsible for Impaired T-Cell Responses in Chronic HCV Infection

HCV-specific T-cell responses are barely detectable in the peripheral blood in chronic hepatitis C [179, 180] and there is evidence of a greater frequency in the intrahepatic compartment, although most of the liver infiltrating T cells are not antigen-specific [179, 181–183]. A functional impairment of HCV-specific CD4 and CD8 cells has been reported by several groups ex vivo in chronic HCV infection which can only partially be restored upon prolonged cytokine exposure [67, 117, 118, 153, 180, 184–186]. In particular, HCV-specific CD4 cell function is deeply impaired [176], and it has been suggested that this is due to defective IL-2 production [186].

Different mechanisms have been proposed to explain how the virus can successfully evade T-cell surveillance following initial infection inducing a progressive deterioration of the T-cell function. These include the direct inhibitory effect of viral proteins on T-cell responses and the mutational escape from T-cell surveillance with the emergence of poorly immunogenic variant epitopes. Moreover, viral proteins can influence directly or indirectly the efficiency of T-cell responses by interfering with T-cell function or via impairment of innate immune responses resulting in poor T-cell

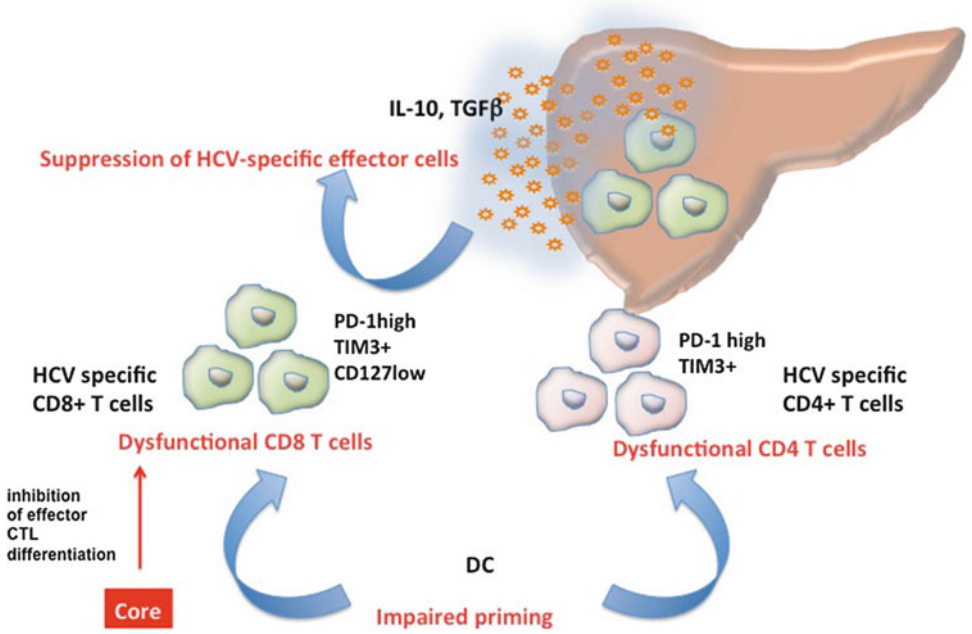

Fig. 15.5 HCV strategies to inhibit T-cell responses. Impaired T cell priming by dendritic cells and the immunosuppressive environment caused by IL-10 and TGF-β produced by Treg would result in impaired CD4 and CD8 function. A balance between Teff and Treg would be regulated by upregulation of exhaustion markers, such as PD1, Tim-3 and CTLA-4, on exhausted CD8 T cells leading to inhibitory signalling after PD-L1 engagement. A balance between naturally occurring (CD4+/CD25+/FoxP3+) Treg and Teff would insure survival of both host and pathogen by maintaining low-level chronic liver inflammation. Moreover, HCV proteins such as core may inhibit HCV-specific CD8 T-cell differentiation toward an effector phenotype

priming and maturation. Rapid HCV replication may in turn outpace host adaptive immunity thus contributing to delayed responses and T-cell exhaustion induced by persistent exposure to viral antigens (Fig. 15.5).

Viral Escape

The continuous generation of escape variants selected under the pressure of adaptive immunity is responsible of shaping the viral quasispecies generated by the error-prone viral RNA-dependent RNA polymerase. Sequential emergence of viral mutations allowing HCV to escape from T and B cell recognition has been described [187, 188]. The reported coexistence in individual patients of HCV-specific CD8 populations expressing variable degrees of functional impairment [189] and the lack of adequate CD4 help consistently observed in patients evolving into persistent infection [158, 167] support the possibility that escape mutations can actually emerge in HCV infection despite the multispecificity of the HCV-specific CD8 response. This is also suggested by chimpanzees' studies where the absence of adequate CD4 help can predispose to the accumulation of escape mutations in HLA class I restricted epitopes [190]. Even though several studies provided clear evidence of HCV escape in patients with acute HCV infection by the identification of mutations within multiple CTL epitopes, direct proof of virus evasion from CD8 surveillance by escape mutations in human HCV infection has been more difficult to obtain [159, 189, 191, 192]. Mutations emerged despite the multispecific nature of the CD8 response and frequently occurred within multiple epitopes in patients evolving to chronic infection. In contrast, amino acid substitutions were not detected in patients who resolved infection spontaneously, including one of the two patients infected from a single source who developed divergent outcomes of infection [159] and recognition of the mutated epitopes in the different studies was either reduced or totally abrogated providing evidence of viral escape. This may occur through mutations of anchor residues required for HLA binding or residues acting as TCR contact sites [193]. Substitutions of TCR binding residues can also generate antagonist peptides able to downregulate wild type-specific T-cell responses [193]. In addition, amino acid substitutions in flanking regions of HLA class I restricted epitopes can alter proteasomal processing impairing generation of the epitope [194, 195].

Although immune escape driven by selection pressure from virus-specific CD8 T cells has been demonstrated in both chimpanzees and humans infected with HCV, only a minority of CD4 T-cell epitopes show amino acid changes,

suggesting that CD4 T cells rarely exert selection pressure against the HCV genome, at least in the chimpanzee model [196]. This indicates that other mechanisms for silencing CD4 T cells may be operative in persistent HCV infection.

In addition to mutant selection, impaired T-cell function may also occur via several inhibitory pathways. These include hyperexpression of negative costimulatory molecules involved in T-cell exhaustion due to exposure to high antigen concentrations, inhibitory cytokines, such as IL-10 and TGF-β, regulatory T cells (Treg), such as CD4+CD25+FoxP3+ cells. Antiviral T-cell responses can also be modulated by HCV gene products, either by directly suppressing T-cell function or by interfering with NK and dendritic cell activity, making these cells unable to adequately support priming and activation of virus-specific adaptive responses. Finally, defective accessory cell (e.g. DC) function and antigen presentation may affect priming and differentiation of HCV-specific T-cell responses.

T-Cell Exhaustion and Role of Treg in Viral Persistence

Functional exhaustion of virus-specific T cells caused by persistent exposure to high antigen loads is an important mechanism of T-cell dysfunction in most chronic viral infections. This has been convincingly shown by the possibility to restore in vivo the CD8 function by blockade of the programmed cell-death 1(PD-1)/PD-ligand 1 (PD-L1) pathway. A similar mechanism of CD8 T-cell inhibition has been shown in chronic HCV infection where HCV-specific CD8 cells express a typically exhausted phenotype, i.e. high PD-1 and low CD127 [169, 197, 198]. Interestingly, a combination of stimulation of CD137 signalling via CD137L and PD-1 antibody blockade was not able to restore intrahepatic T-cell function in chronic HCV infection whereas this met with success in patients with chronic HBV infection [199]. Curiously peripheral blood HCV-specific T cells were sensitive to these reagents, suggesting that the intrahepatic environment may play a major modulatory role in this process. Restoration of exhausted CD8 T-cell function has also been recently studied in detail in the peripheral blood of patients who spontaneously recovered from acute hepatitis C and in those who developed sustained virological response (SVR) after successful antiviral treatment [200]. Of note, SVR did not lead to full maturation of a functional memory CD8 T-cell response in patients with either acute or chronic HCV but it was associated with persistence of a deeper level of T-cell dysfunction compared with resolvers from acute infection. In the group of treated chronic infections, exhaustion was more successfully overcome following PD-1/PDL-1 blockade, but it is worrisome that HCV can induce a long-lasting impairment of CD8 responses even after successful antiviral therapy.

Another important regulatory T-cell molecule is the T-cell Ig and mucin domain protein-3 (Tim-3) which has raised the possibility that a therapeutic strategy targeting these inhibitory pathways might be of clinical benefit in patients with HCV infection. Interestingly, similarly to PD-1 [201], Tim-3 has been shown to negatively regulate CD4+CD25+Foxp3+ Treg during HCV infection [202]. Tim-3 expression was significantly higher on Treg cells which tended to accumulate in the peripheral blood, whereas PD-1+ Treg are enriched in the liver. Moreover, blockade of Tim-3 on CD4+CD25+ T cells promoted expansion of effector T cells (Teff) more substantially than Treg through improving STAT-5 signalling, thus correcting the imbalance of Foxp3+ Tregs/Foxp3− Teffs that was induced by HCV infection, in a manner similar to PD-L1 blockade that upregulates STAT-5 phosphorylation in Treg ex vivo [201]. In addition, in human HCV infection another non-classical population of Treg is the HCV-specific CD8 Treg subset that suppresses T-cell responses by production of IL-10 or TGF-β and is highly enriched in the liver [118, 119] and the peripheral blood [206] of patients with chronic hepatitis C. However, further studies are needed to fully understand their role in this clinical setting.

Thus the fine balance between effector and regulatory T cells would be advantageous for both the pathogen and the host allowing, on the one hand, persistent survival of the pathogen and at the same time prevention of rapidly progressive necroinflammation of the host's liver leading eventually to the development of severe disease and cirrhosis.

T-Cell Inhibition by HCV Proteins

The HCV core and envelope proteins have been shown to modulate a variety of adaptive immune responses in vitro. Notably, HCV core has been reported to block differentiation of HCV-specific T cells toward an effector phenotype by downregulation of intracytoplasmic IL-2 and phosphorylated ERK1/2 MAP kinase [203] A specific sequence of the core protein can bind the globular domain of the complement C1q receptor, also expressed on T cells, inhibiting proliferation and cytokine secretion [204, 205]. In addition, it has been shown that the HCV-E2 protein can bind to CD81, a major HCV receptor, which results in a costimulatory effect on T cells [206, 207]. However, it must be emphasized that the majority of studies investigated the effect of single HCV proteins on immune cells and it is still unclear whether these observations can be extended to the pathophysiology of HCV infection in the context of a fully replicating virus system.

Impaired or intact professional antigen-presenting cell function? Dendritic cells act as a link between innate and adaptive immunity. Signals delivered by the innate immune

system (type I IFN production, interactions with NK cells) lead to the proper maturation of DCs, which are critical for triggering antigen-specific immune responses [208]. However, despite extensive investigation, there is no general consensus regarding the effects of HCV on DC function. Current evidence indicates that DC, particularly myeloid DC, migrate to the liver in response to HCV infection and chemokines such as RANTES, MIP-1a and MIP-1β [209]. Patients with chronic HCV infection express high levels of CCR5 but not CCR7, a phenotype of immature DC as normally mature DC downregulate CCR5 and upregulate CCR7. Interestingly, the HCV E2 protein renders CCR7-expressing DC unresponsive to CCL21, the natural ligand of CCR7, and this may attenuate antiviral T-cell responses as a cause of the inability of DC to home to the lymph nodes [210]. Regardless of their distribution (i.e. liver vs. peripheral blood), there is controversial data indicating either normal or altered pDC and mDC function in patients with chronic hepatitis C. The alleged changes include decreased IFN-α and IL-12 secretion, and increased IL-10 production, which polarize T cells toward a Th-2 phenotype [211]. Several core, NS3, NS4 and NS5 proteins have been implicated in impaired DC function by diminishing the HLA and costimulatory molecule expression, reducing cytokine production, inhibiting TLR signalling, and decreasing allostimulatory activity. However, an impaired DC function would be difficult to reconcile with the evidence that T-cell dysfunction is HCV-specific and that chronically infected patients are not globally immunocompromised. Failure to confirm DC dysfunction would depend on a number of factors including the heterogeneity of the patient populations studied in different laboratories, the often small sample size and the fact that most human studies were conducted with DC generated in vitro from CD14+ monocyte precursors present in the peripheral blood.

HCV and Immunosuppression

It is impossible to comprehensively analyse the role of immunosuppression in the pathogenesis of hepatitis C due to obvious space constraints. The most common clinical settings in which immune responses to HCV may be affected by chronic immunosuppression are liver transplantation (OLT), HIV coinfection, pharmacological immunosuppression for concomitant autoimmune diseases and immunosuppressive treatment for lymphoproliferative disorders. Graft reinfection occurs immediately after OLT for end-stage liver disease caused by chronic HCV infection; however, disease progression may rapidly develop and is often unpredictable. Indeed, allograft cirrhosis occurs in up to 30 % of liver transplant recipients in the 5 years following surgery, with ensuing graft failure and need for re-transplantation [212]. The causes for such a rapid progression are largely unknown, although it may be inferred that accumulation of highly pathogenic HCV variants associated with dysfunctional host immune responses secondary to immune suppression may be responsible for this phenomenon. Innate immune cells such as NK and NKT cells have been examined in HCV reinfection in the OLT setting and peculiar NK phenotypic changes were described, including an association between decreased frequencies of CD56+ NK/NKT cells and the severity of liver diseases post-OLT [213] and increased homing of NK cells expressing activating receptor to the liver which correlated with serum ALT values [214]. However, T-cell responses appear to play a more relevant role in disease progression and may also contribute to viral clearance during antiviral treatment [215, 216].

Coinfection with HIV-1 is rather common in individuals exposed to multiple inocula such as intravenous drug users and sexually promiscuous persons. The effects of HIV-1 on the pathogenesis of HCV infection are deleterious and include a higher rate of viral persistence, increased viral loads, a faster rate of fibrosis progression, and higher rates of hepatic decompensation [217]. Moreover, HIV-1/HCV–coinfected individuals have worse treatment outcomes following IFN-α-based therapies compared with their HCV-mono-infected counterparts [218]. Beside the many similarities between these viruses that replicate at very high levels and are particularly prone to mutate and generate a complex and diverse quasispecies, there are profound differences in terms of prevalence of virus-specific circulating T cells, those specific for HIV being more abundant compared with HCV [219], and virus tropism for target cells. More importantly, HCV RNA replication is confined to the cytoplasm and there is a good chance for HCV infection to be completely eradicated whereas this still remains a chimera for HIV, despite the availability of highly effective drugs capable of completely suppressing HIV replication. HIV-coinfected patients have higher HCV viral loads when compared with HCV-monoinfected individuals [220], suggesting that impaired HCV-specific CD4 and CD8 T-cell responses may be responsible for failure to control HCV replication in this setting as reported in the literature [221, 222]. Restoration of CD4 T cell count by effective antiretroviral therapy may recover efficient HCV-specific T-cell function which may be responsible for transiently increased necroinflammation (“reconstitution hepatitis”), on the one hand, and better long-term control of HCV replication and disease progression on the other [223]. In view of the realistic possibility of eradicating HCV in the vast majority of patients in the not-too-distant future, thanks to the forthcoming potent DAAs, it will be still controversial whether it may be preferable to initiate HCV or HIV treatment first, taking into account that potentially severe side effects may occur in the coinfected patient treated with antiretroviral and DAAs simultaneously.

Prospects for a Prophylactic HCV Vaccine

The efforts to obtain a vaccine that will be protective against all HCV strains have met with insurmountable difficulties essentially related to the extreme complexity and diversity of viral variants and the relative inefficiency of neutralizing antibody responses. This is exemplified by evidence of multiple hepatitis episodes after recovery in intravenous drug addicts [224]. For these reasons, an alternative approach has been followed which used recombinant adenoviral vectors encoding for the HCV non-structural region to boost cell-mediated responses [225, 226]. Using this approach, in chimpanzees vaccine-induced T cells displayed higher levels of CD127, a marker of memory precursors, and lower levels of PD-1 than infection-induced T cells. Vaccine-induced, but not infection-induced, T cells were polyfunctional, being able to secrete a wide array of cytokines [227].

However, a recent meta-analysis on HCV-vaccinated chimpanzees showed that vaccines that contained only structural proteins had clearance rates that were significantly higher than vaccines that contained non-structural components [228]. Along these lines, cross-neutralization activity was recently tested using infectious recombinant HCV (HCVcc) expressing structural proteins of heterologous HCV strains (genotypes 1–7) in Huh-7.5 human hepatoma cell cultures [229–231]. Compared with pre-immunization sera, post-immunization sera of many vaccine recipients exhibited measurable neutralizing activities against all HCV genotypes tested, shedding hope on the feasibility of a traditional, protein-derived global vaccine against hepatitis C since immunization with a single strain of HCV elicited broadly cross-neutralizing antibodies against isolates of all major genotypes of HCV. Future characterization of these cross-neutralizing antibodies and conserved epitope(s) will, however, be critical for rational vaccine design. Indeed, despite this encouraging data, it must be emphasized that most in vivo efficacy data for prophylactic vaccines were obtained in chimpanzees [232] and, therefore, the ideal objective of attaining sterilizing protective immunity still remains elusive, whereas it should at least be possible to prevent viral persistence.

Concluding Remarks

Factors influencing the fate of HCV infection are sketchily summarized in Fig. 15.6. The evolution of acute HCV infection seems to be relatively independent of immunosuppression although recurring hepatitis C in the transplanted liver and coinfection with HIV may run a rather aggressive clinical course. Host immunogenetics plays an important role in viral clearance with *IFNλ 3* polymorphism being an important predictor of spontaneous or drug-induced recovery from infection. The virus appears to ignore the age factor which determines the fate of HBV infection and induces persistent infection largely as a result of failure to contain the infection by innate immune responses. Adaptive immunity does develop but it is ephemeral, rapidly exhausted and quickly outpaced by this rapidly replicating RNA virus which is prone to transcriptional errors and evolves into a swarm of highly homologous, yet antigenically diverse, variants

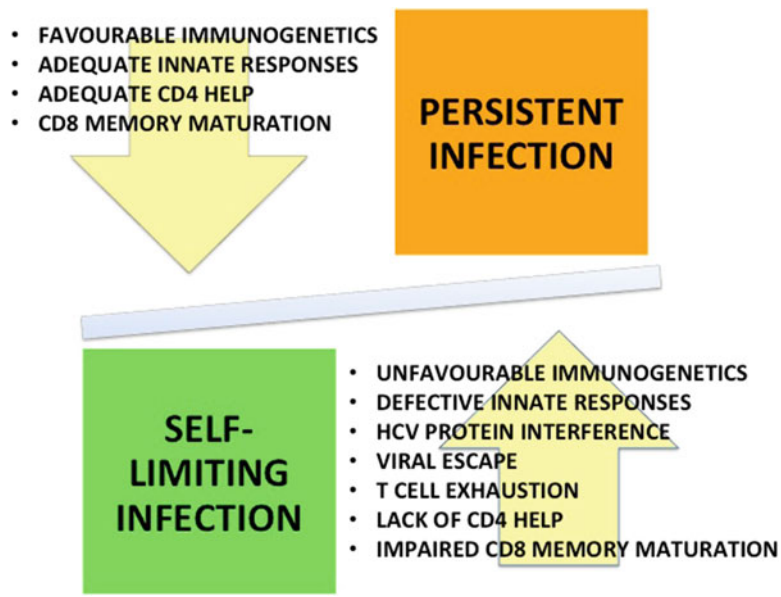

Fig. 15.6 Immunological factors associated with self-limiting vs. chronic HCV infection

contributing to the quasispecies distribution of the virus population. This situation is deleterious for efficient development of neutralizing antibody responses which can be detected with similar frequency in acute and chronic infection and are by and large irrelevant with respect to clinical outcome. Virus-specific T-cell responses are more vigorous and broadly reactive in self-limiting than chronically evolving infections and the scarcity of IL-2 in the micro-environment, possibly consumed by Treg, may also contribute to T-cell dysfunction. Exhaustion by persistent exposure to high antigen concentrations, defective development of central memory T cells and the inhibitory effect of certain viral proteins (particularly the core protein) on T-cell function have also been advocated as important factors involved in failure to eradicate HCV. Finally, crippled T-cell functionality is only rarely restored following spontaneous or treatment-induced recovery, suggesting that this, rather than the inefficiency of antibody-mediated neutralization, may be responsible for lack of protective immunity.

Second-generation DAAs appear to possess an extraordinary potent antiviral effect leading to unexpectedly high SVR rates. It will be interesting to see whether a potentially curable chronic viral infection such as HCV could additionally benefit in the future from immunomodulating agents particularly in difficult-to-treat patients.

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Immunopathogenesis of Hepatitis D

16

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Key Points

- The hepatitis delta virus (HDV) is a noncytopathic circular, single-stranded RNA virus; it is the only member of the delta virus genus. An estimated 15–20 million people are infected worldwide.
- Chronic hepatitis delta is the most severe form of viral hepatitis; the development of fibrosis and the progression towards cirrhosis are faster than in HBV monoinfected patients.
- The only available treatment option is peg-interferon-alpha, reaching SVR rates from 17 to 43 %. The nucleoside or nucleotide analogues used to treat other hepatitis viruses show no antiviral effect on HDV.
- Migrant populations and special risk groups show particular high HDV prevalences.
- Hepatitis delta is a dynamic disease, with possible viral interactions and contribution by both HBV and HDV to the progression of the disease.
- The clinical manifestation of hepatitis delta differs between regions and has changed during the last 3 decades.
- The HDV genotype is important for the clinical course of the disease.
- Innate immunity in hepatitis delta is not well studied. HDV can interfere with IFN-α signaling, and NK cells have been implicated in pathogenesis and as a predictor of treatment response.
- Adaptive cellular immune responses against HDV are detectable but weak.

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Introduction

Infection with the hepatitis D virus (HDV) causes hepatitis delta, which is considered to be the most severe form of viral hepatitis. HDV is a defective virus, using the hepatitis B virus (HBV) surface antigen (HBsAg) as its envelope protein; thus, hepatitis delta affects only carriers of the HBsAg. Of the 350 million people infected with HBV worldwide, 15–20 million are estimated to be coinfected with HDV; thus, hepatitis delta infection represents a global health burden. Immune responses to HDV are less well studied than for HCV and HBV, but in recent years, advances have been made to gain more knowledge on the interaction of HDV and the immune system. Since HDV is noncytopathic, immune responses play a key role not only in the control of the infection but also in the pathogenesis of liver disease.

History of Hepatitis Delta

The hepatitis delta virus was first described by Mario Rizzetto in 1977. He detected a previously unknown antigen-antibody system in liver biopsies of HBV-infected patients with severe disease, which was then believed to be an unidentified antigen of the hepatitis B virus and was termed delta antigen [1]. Studies with HBV-infected chimpanzees later proved the infectivity of the delta antigen and led to the discovery of the novel hepatitis virus [2]. In 1986, the group of Michael Houghton unravelled characteristics of the HDV genome, being composed of circular, single-stranded RNA [3], shortly after the size and structure of the virion of HDV were described by Bonino et al. [4]. The chimpanzee remained an important model during the early virological research of HDV infection, for instance, the development of persistent infection was shown in chimpanzees [5]. Another animal model to study hepatitis delta virology is the eastern woodchuck. Woodchucks can also be infected with a hepatitis virus, the woodchuck hepatitis virus (WHV), which has a surface antigen similar to that of HBV [6]. Experimental

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infection of WHV-positive animals with HDV in vivo was first successfully performed in 1984 [7], followed by the analysis of in vitro replication of HDV in primary woodchuck hepatocytes [8]. Only recently, primary human hepatocytes could be infected with hepatitis delta virions assembled with WHV surface antigen [9]. In 2012 a humanized mice model was developed to study the effects of HDV infection [10].

Virology and Life Cycle of HDV

The HDV is the smallest virus known to infect man, with an RNA genome of only 1679 base pairs [11]. Lacking any relatives, it is the only member of the genus delta virus. The genome is circular and single-stranded and contains one open reading frame (ORF) which encodes for a single protein, the hepatitis delta antigen (HDAg). Hepatitis D viral particles are approximately 35 nm in diameter, covered by HBsAg, and contain HDV RNA and HDAg [4]. Two isoforms of HDAg exist, the large HDAg of 214 amino acids with a molecular weight of 27 kDa, also termed L-HDAg, and the small HDAg of 195 amino acids and 24 kDa, accordingly termed S-HDAg. By posttranscriptional modification, the stop codon at the end of the sequence encoding the S-HDAg is altered, and the L-HDAg is translated from the same ORF [12]. This editing takes place on the antigenomic RNA strand, an intermediate variant of HDV RNA during the virus' life cycle, and is performed by the enzyme adenosine deaminase acting on RNA (ADAR1) [13]. The two proteins of HDV have different functions. The small HD antigen, which is translated first, is relocated to the nucleus and acts as a positive regulator of viral replication by inhibiting transcription of host templates via RNA polymerase [14], possibly by replacing the cellular factor NELF which is a negative regulator of RNA polymerase activity [15]. The large HD antigen inhibits genome replication [16] but is essential for virion assembly [17].

The mechanism by which HDV enters its target cells, human hepatocytes, is not yet identified. Presumably it will utilize the same unidentified purinergic receptor that HBV uses, as it is coated with HBsAg particles [18]. HDV uses the host RNA polymerase II for genome replication, which takes place in a rolling circle mechanism, similar to the replication of bacterial plasmids [19]. The circular genome is replicated into a linear, multimeric molecule which is later self-cleaved by autocatalytic activity through the formation of the so-called ribozymes [20]. These self-catalytic RNA structures are abundant in nature [21]. The crystal structure of the HDV ribozyme was described in 1998 [22], and the search for similar ribozymes led to the discovery of an HDV-like sequence in the human genome, the cytoplasmic polyadenylation element-binding protein 3, CPEB3 [23]. This indicates that HDV might have developed from the human transcriptome. On the other hand, HDV does not have similarities with other viruses infecting man or animals, but rather shares some features with plant-pathogenic viroids. Plant viroids are smaller than HDV and do not contain ORFs [24]. Ultimately, the origin of HDV remains unknown so far.

Among the different posttranslational modifications of HDAg during the HDV life cycle, the prenylation of a C-terminal cysteine of L-HDAg is noteworthy, as this modification is crucial for binding of HBsAg and thus virus assembly [25]. S-HDAg is being phosphorylated to regulate antigenomic RNA replication [26], becomes sumoylated to improve genomic RNA and mRNA synthesis [27], and undergoes methylation which controls subcellular localization of S-HDAg [28]. Assembly of the viral particles starts in the nucleus of the host cell, where large and small HDAg associate with HDV RNA molecules. After nuclear export, 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 [29]. A schematic overview of the important steps of the viral replication of HDV is given in Fig. 16.1.

Geographical Distribution and Epidemiology of Hepatitis Delta

Eight different genotypes of hepatitis delta virus have been described so far [30]. Between genotypes, the sequence similarity is 60–70 %, whereas differences can be as much as 15 % between subspecies of the same genotype. Each genotype has a distinct geographical distribution (Fig. 16.2). Genotype I can be found in most parts of the world and is the most prevalent one in Central Europe, Northern America, and Central Asia [31]. Genotype II is the most important HDV genotype in East Asian countries and was initially discovered in Japan [32], while genotype III is exclusively found in Central and South America [33], where it is responsible for the outbreaks of fulminant hepatitis [34, 35]. Genotype IV is the second most prevalent genotype in Asia [36], and genotypes V to VIII are usually only prevalent in African countries [30, 37], though recently HDV-VIII was detected in two Brazilian patients [38].

Of the 350 million people worldwide that are infected with hepatitis B virus, 15–20 million are believed to be coinfected with HDV [39], and HDV infection must therefore be considered a global health problem. While systematic vaccination against HBV has also led to a decline in HDV prevalence over the last 20 years in Italy [40] or Taiwan [41], a continuation of this downwards trend could not be observed after 1999 at Hannover Medical School, as 8–12 % of HBsAg carriers tested positive for anti-HDV

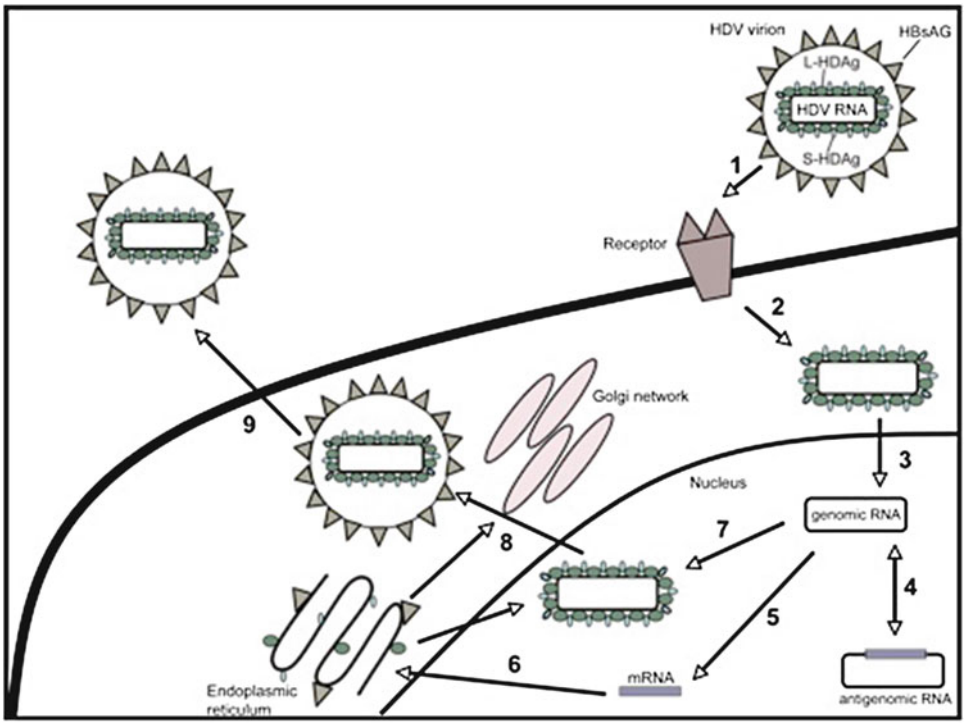

Fig. 16.1 Replication and morphogenesis of hepatitis D virus. (1) Cell entry and uncoating. (2) Nuclear import mediated by the nuclear localization signal. (3) Replication of the HDV genome. (4) Transcription of the mRNA. (5) Translation of the mRNA to small and large HDAg particles at the endoplasmatic reticulum (ER). (6) Association of HDAg particles and RNA. (7) Final viral morphogenesis in the Golgi apparatus. (8) Secretion of new viral particles

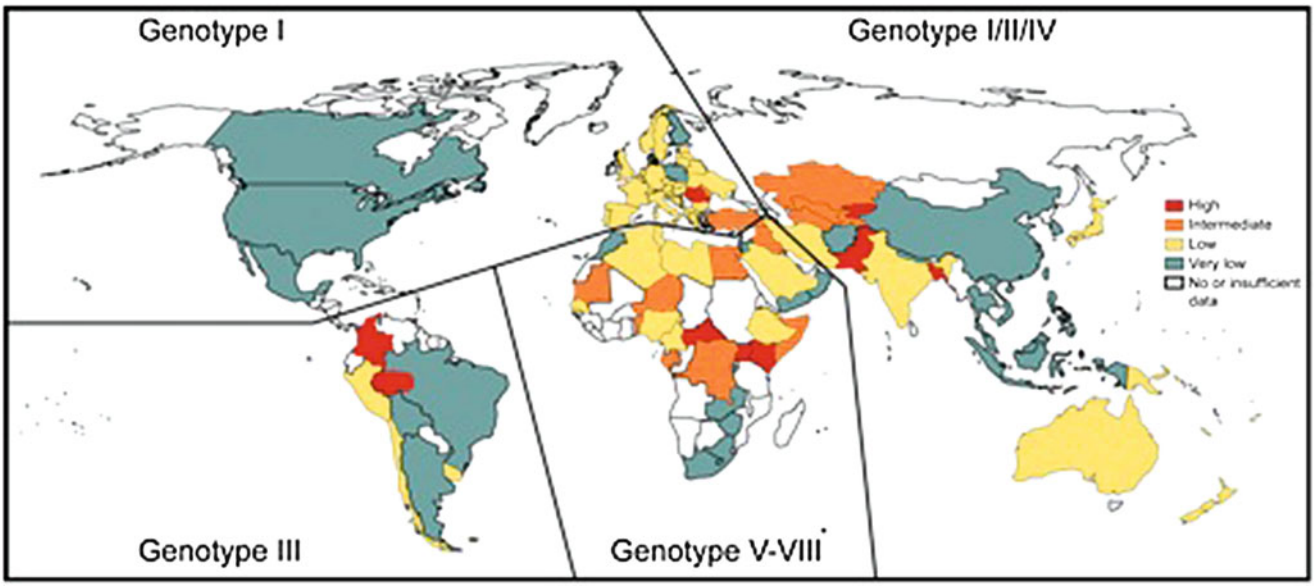

Fig. 16.2 Global prevalence of hepatitis delta infection and distribution of HDV genotypes

between 1999 and 2006 [42]. Also other European centers have reported high HDV prevalences. In France the majority of patients are immigrants from African countries [43]. Similarly, in Germany the vast majority of HDV patients are immigrants, though mainly from Turkey or former Soviet Union states [44]. A study from London also identified immigrants as the main HDV-infected patient group, followed by intravenous drug users (IVDU) [45]. Thus, IVDU

represent another risk group in Central Europe as well as in the United States. A recent paper from Baltimore, Maryland, described an HDV prevalence of up to 50 % in HBsAg-positive IVDU [46]. The high prevalence of HDV infection in IVDU as well as in immigrants makes hepatitis delta not only a global health problem but also a local problem in Central Europe and Germany.

The modes of transmission of HDV are not completely understood in all details. HDV transmission depends on the presence of HBsAG. It is therefore paramount to state that an HBV vaccination will protect against infection with HDV. Intrafamiliar transmissions via vertical and sexual contact as well as infection during childbirth or early during childhood have been reported [47]. Medical treatment with contaminated blood transfusions or unsterile conditions are other possible ways of transmission. IVDU, dialysis patients, hemophiliacs, and HIV carriers represent additional risk groups [48].

Diagnosis of HDV Infection

The first step is testing for HDV antibodies; anti-HDV-positive patients then need to be tested for HDV RNA to confirm HDV replication. A big problem in HDV diagnostics represents the lack of an international WHO HDV standard. Different assays show a large variability in assay performance. In particular HDV genotypes 5–7 are not appropriately covered by many in-house assays. Few assays have been developed on automatic platforms [49]. HDV RNA quantification is required to monitor treatment response to interferon-alpha-based therapies.

Clinical Course of HDV Infection

The hepatitis delta virus is causing the most severe form of disease among all hepatitis viruses. Dependent on the type of infection, there are two possible courses of the disease. Simultaneous coinfection with both HBV and HDV might cause fulminant hepatitis but results in self-limitation and recovery in 95 % of cases [50]. On the contrary, the disease course will usually be more serious and progress to chronicity in most cases when a patient with chronic HBV infection becomes superinfected with HDV [51]. Chronic hepatitis delta is characterized by more severe liver pathogenesis than chronic HBV monoinfection alone, with faster progression to fibrosis and earlier development of cirrhosis. Interestingly and in contrast to previous reports [52], hepatic decompensation, and not necessarily hepatocellular carcinoma, was the most frequent clinical event in a longitudinal study recently performed at Hannover Medical School [53]. This finding was also confirmed by an Italian longitudinal study [54]. A recent article investigating HDV coinfection in European HIV-infected patients reported an HDV prevalence of 14.5 % in 422 HBsAg-positive HIV carriers. The authors state that HDV coinfection increases the risk of liver-related deaths and overall mortality in HIV patients without a direct influence on progression to AIDS [55]. Hepatitis delta virus is not believed to be directly cytopathic but liver damage that occurs is rather an immune-mediated effect. The activity of liver disease in hepatitis delta is independent of HDV viremia [56], but seems to be influenced by HDV genotype, as infection with genotype II was shown to have a milder course than infection with genotype I [57], whereas genotype III infection accounts for severe outbreaks of fulminant hepatitis among the indigenous population in the Amazonas region of South America [34, 35, 58]. Regardless of the genotype, severity of the disease as well as prognosis is worse in HDV than in HBV or hepatitis C virus (HCV) [52, 53]. HBeAg positivity seems not to be associated with the outcome of hepatitis delta [59].

Another striking feature of HDV infection is the change in clinical presentation during the last 3 decades. When first discovered, most patients suffering from hepatitis D were rather young and had acquired the disease in their local area. Patients presented either with severed acute hepatitis or advanced chronic liver disease. The incidence of acute hepatitis delta subsequently decreased [40], and many patients with severe chronic disease died during the 1980s and 1990s. Thus, the clinical picture of hepatitis delta patients changed, and mainly patients with mild chronic liver disease survived. However, many patients progressed meanwhile to liver cirrhosis, and at present the proportion of patients with advanced cirrhosis has increased again. This change in patient profile has nicely been described in a study from Barcelona [60]. A schematic depiction of this evolution is shown in Fig. 16.3.

Treatment of HDV Infection

The only available treatment option for hepatitis delta is the injection of interferon-alpha (IFN-α) [61]. Recombinant interferon (IFN) has been used since the 1980s for the treatment of hepatitis delta [62], whereas nowadays pegylated interferon (peg-IFN) is being used. Addition of polyethylene glycol improves bioavailability; thus, half-life and duration of the effect of the interferon are prolonged, which allows weekly administration instead of daily [63]. Rates of sustained virological response (SVR), defined by undetectable HDV RNA in serum 24 weeks after the end of treatment, of 17 % [64] to 43 % [65] have been reported. Both aforementioned studies confirmed the efficacy and safety of the use of peg-IFN for the treatment of hepatitis delta. The so far longest prospective and randomized trial investigating peg-IFN-alpha

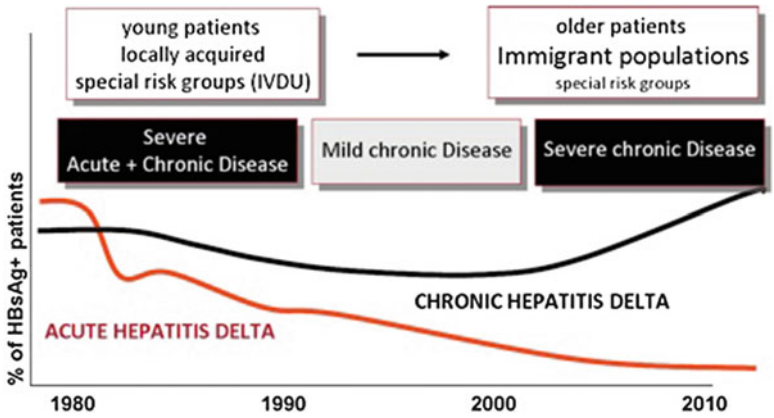

Fig. 16.3 Evolution of the clinical presentation of HDV patients

Table 16.1 Overview of the most important interferon treatment studies performed in HDV infection

Reference	Substance	Number of patients	Summary
Rizzetto et al. [62]	Recombinant interferon-alpha	6	First evaluation of safety and efficacy of treatment with recombinant IFN. Virological and biochemical response in 5 of 6 patients
Erhardt et al. [64]	Pegylated interferon-alpha	12	Pilot study for the use of peg-IFN in HDV treatment. Virological response in 17 % of patients, strong ALT reduction in both responder and nonresponder patients
Castelnau et al. [65]	Pegylated interferon-alpha	14	Evaluation of safety and efficacy of peg-IFN for HDV treatment. Virological response in 43 % of patients and biochemical response in 57 %
Wolters et al. [70]	Interferon plus lamivudine	8	Combination therapy with interferon and lamivudine. No positive effect of lamivudine
Niro et al. [68]	Interferon plus ribavirin	38	Combination therapy of HDV infection with interferon and ribavirin. No additional effect of ribavirin
Wedemeyer et al. [66]	Interferon plus adefovir	90	Comparison of peg-IFN plus adefovir versus either drug alone. No effect on HDV replication of adefovir alone, no additional effect in combination with interferon
Yurdaydin et al. [74]	Interferon plus tenofovir	120	Comparison of peg-IFN plus tenofovir versus peg-IFN alone

treatment of HDV infection showed SVR rates concerning HDV in 28–31 % of patients [66].

Nucleotide or nucleoside analogues, which are commonly used for the treatment of HBV, HCV, or hepatitis E virus (HEV) infection, have no antiviral effect on HDV, due to the lack of viral enzymatic activity: ribavirin alone proved to be ineffective against HDV already in 1994 [67] and also in combination with interferon in 2006 [68]. Similarly, lamivudine was not beneficial neither as monotherapy [69] nor in combination with interferon [70]. Famciclovir demonstrated also no effect against HDV [71]. Furthermore, a recently published international study testing adefovir-peg-interferon combination therapy versus either drug alone did not find adefovir alone to be effective against HDV in terms of viral response, while reduction of serum HBsAg levels was improved by combination therapy [66]. As HDV is dependent on HBsAg for assembly of viral particles, HBsAg negativation can be considered one endpoint of HDV treatment as well. In one treatment study in the woodchuck animal model, the nucleoside analogue clevudine was able to reduce WHV surface antigen levels accompanied by lower HDV RNA levels [72]. However, a small pilot study evaluating the treatment of HDV infection with clevudine in humans could not reproduce this finding [73] (Table 16.1).

Most recent interim data from the HIDIT2 study comparing tenofovir alone versus tenofovir in combination with peg-IFN showed that after 48 weeks of therapy, combination treatment and PEG-IFN-a-2a alone showed a similar efficacy concerning HDV RNA suppression and HBsAg reduction [74].

Different steps in the life cycle of the HDV could be exploited as novel treatment targets. Inhibition of prenylation of the HDAg prevents the formation of infective viral particles in vitro [75] as well as in vivo in a mouse model of HDV infection [76]. First treatment trials investigating prenylation inhibitors in humans have recently started (http://www.clinicaltrials.gov).

De novo infection with HDV of human hepatocytes in humanized mice could be prevented by the use of the novel HBV entry inhibitor Myrcludex-B [10], which is in preclinical development [77]. Despite these promising new drugs, currently the choice of treatment options for HDV infection

Table 16.2 Selected studies on HDV immunology

Reference	System/organism	Main finding
Rizzetto et al. [86]	HDV-infected patients	Presence of antibodies against HDAg in serum of HDV patients
Negro et al. [87]	Chimpanzees	Antibodies do not mount protective immunity against reinfection with HDV
Guilhot et al. [79]	Mice	HDAg expressed in transgenic mice does not have a cytopathic effect
Nisini et al. [89]	HDV-infected patients	Discovery of four MHC class II-restricted epitopes of HDAg
Accapezzato et al. [90]	Human T cell clones and autologous B cell lines from an HDV patient	Intracellular processing is not necessary for the generation of one of the CD4-specific epitopes of HDAg
Huang et al. [93]	Mice	Induction of CD4+ T cell responses by a DNA vaccine
Fiedler et al. [88]	Woodchucks	DNA vaccine could induce antibodies production as well as T cell responses, but no protective immunity
Mauch et al. [94]	Mice	Induction of CD8+ T cell responses by DNA vaccine
Huang et al. [92]	In silico, mice and HDV-resolved patients	Identification of two HLA-A2-restricted epitopes of HDAg
Huang et al.[93]		
Aslan et al. [91]	HDV-infected patients	High frequency of perforin-positive CD4 T cells in the blood of HDV-infected patients
Pugnale et al. [80]	Human hepatoma cells	Hepatitis delta antigen inhibits interferon signaling
Grabowski et al. [95]	HDV-infected patients	HDV-specific T cell cytokine responses correlate with response to therapy

is very limited, and response rates are poor. A better understanding of immunological processes during HDV infection could therefore help to improve current treatment strategies.

Immunology of Hepatitis Delta

Immunology of HDV infection is far less studied than that of HBV or HCV infection. An overview of the major immunological studies in hepatitis delta is given in Table 16.2. Like HCV and HBV [78], HDV is not believed to have a direct cytopathic effect [79]. Little is known about the early activation of the immune system in hepatitis delta. While no comprehensive studies on ISG activation have been performed, there is evidence that HDV interferes with the innate immunity, as the hepatitis delta virus has been shown to inhibit IFN-a signaling in vitro. This occurs via blocking the activation of the molecule Tyk2, which is part of the JAK-STAT pathway [80]. Preventing the early interferon response may account for the successful establishment of persistent infection. Furthermore this could be an explanation for the weak response rates to treatment with exogenous interferon-alpha.

The role of NK cells during infection with the HDV is largely unknown, and only few studies were done, all before 1990. These early reports indicated a role for this cell type in liver cell injury [81] and using NK cell responses as a predicator of treatment response [82]. It has been shown by numerous groups that NK cells play an important role in the control of HCV infection, and they have been implicated in the pathogenesis of liver disease [83]. Expression of certain receptors on NK cells correlates with higher chance or lower chance of clearance of acute infection [84, 85]. Preliminary results from our own lab suggest a distinct phenotypical pattern for the expression of certain receptors on the surface of NK cells in chronic hepatitis delta and reduced cytotoxicity and cytokine production (Fig. 16.4).

Antibodies against the HDAg can be detected in the blood of patients with both acute and chronic infection [86]. They do not provide protective immunity though, as previously infected chimpanzees could be reinfected with HDV despite the presence of antibodies [87]. Antibodies induced by DNA vaccination in woodchucks do not also protect the animals from hepatitis delta virus infection [88].

Studies on the role of cellular immunology in HDV infection are scarce. Knowledge of immunodominant epitopes is crucial to analyze virus-specific immune responses. Still, as of today, only two groups have identified T cell epitopes of the HDAg. Four different MHC class II-restricted epitopes were discovered in a screening of T helper cells from eight HDV-infected patients by Nisini et al. [89]. The same group later revealed that the origin of these epitopes is extracellular processing. The impact of this finding on the immunopathology of HDV was not clarified though [90]. Another study regarding the role of T helper cells in HDV infection has focused on cytotoxic CD4+ lymphocytes in viral hepatitis [91]. The frequency of perforin-positive CD4 T cells was higher in HDV-infected patients than in individuals with HBV or HCV and correlated with elevated levels of aspartate transaminase (AST) and decreased platelet numbers, which can serve as a marker for strength of liver disease. While cytotoxic CD8+ lymphocytes play a pivotal role in the clearance of viral infections, including HBV and HCV, no comprehensive studies on the role of CTLs in HDV infection have been performed so far. By in silico epitope prediction, a Taiwanese group identified two HLA-A2-restricted epitopes of the HDAg [92]. CD8+ T cells specific for the predicted peptides could be detected in HLA-A2-transgenic mice after DNA vaccination with a plasmid encoding the HDAg. In two out of four HLA-A2-positive patients with resolved HDV

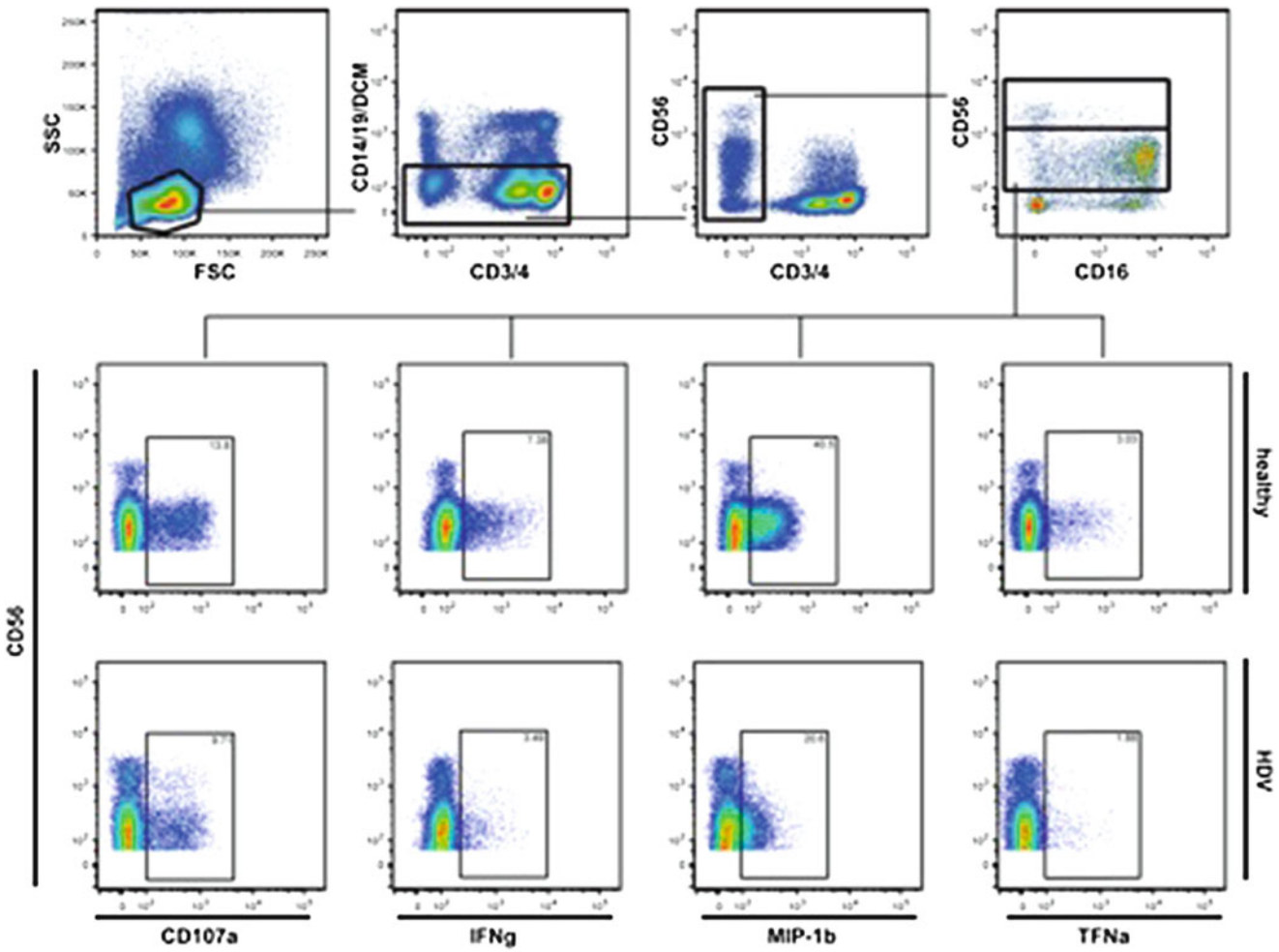

Fig. 16.4 Natural killer cells have a decreased functionality in HDV infection (own unpublished data). The *upper* panel is showing the gating strategy employed to identify NK cells. The *lower* panel compares different parameters of functionality of NK cells (cytotoxicity as indicated by CD107a expression and cytokine production) in a healthy individual and a representative HDV-infected patient. PBMCs were cocultred with K562 cells for 6 h and then analysed by flow-cytometry

infection, epitope-specific and functional CTLs could also be detected. The authors proposed the possible application of the immunogenic epitopes as a therapeutic vaccine to boost immune control of the infection. However, no therapeutic or protective vaccine against HDV has been developed so far. One study performed in woodchucks evaluated a DNA-based vaccine against HDV that was able to induce anti-HDV antibody production as well as T cell proliferation in response to stimulation of peripheral blood mononuclear cells (PBMC) with HDAg peptides. Nevertheless, the vaccinated animals did not show protective immunity when challenged with HDV [88]. Further vaccination studies have been performed in mice. Though mice are not susceptible for HBV or HCV infection of the liver in the natural way, the animals can be inoculated with a DNA plasmid encoding for the sequence of the HDAg. Through DNA vaccination, both CD4+ [93] and CD8+ T cell responses [94] have been induced. A recent study investigated HDV-specific cytokine responses of T cells in patients with hepatitis delta prior due and during peg-interferon-alpha-based therapy. PBMC were stimulated with overlapping 15mer peptides covering the entire HDV proteins. HDV-specific interferon-gamma and IL-2 responses were more pronounced in individuals with lower HDV viremia suggesting a contribution of virus-specific T cell responses to control of HDV replication. Cytokine responses changed during interferon therapy and showed some correlation with responses to therapy [95]. Still, more studies are needed to define in detail the specificity and strength of HDV-specific T cell responses in acute and chronic hepatitis delta. More T cell epitopes need to be defined. Moreover the role of functional exhaustion, T cell escape of the HDV virion, and various other cell types such as immunoregulatory T cells require further investigation (Fig. 16.5).

It is important to consider that HDV-infected patients are always coinfected with HBV; thus, the immune system is dealing with two infections at the same time. While HDV has

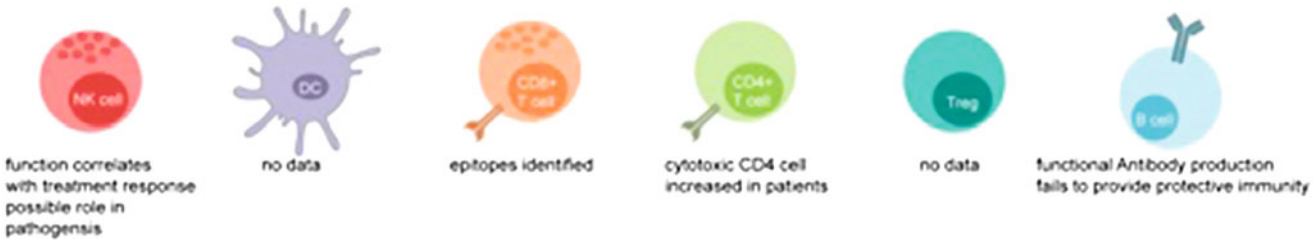

Fig. 16.5 Schematic depiction of the main immune cell populations and there role and function during HDV infection

been shown to be virologically dominant and able to suppress HCV replication in HCV-HBV-HDV triple-infected patients [96, 97], the virological interplay between HBV and HDV [98] and the resulting dominance patterns seem to be rather complex [99]. Interestingly the suppressive effects of HDV on HBV replication seem to be independent from the phase of HBV infection as HBV DNA frequently tests negative even in HBeAg-positive hepatitis delta patients [59]. The role of HBV in HDV pathogenesis should not be underestimated [100], and the same probably holds true for the immunology of hepatitis delta.

Concluding Remarks

Some progress has been made in recent years to understand the pathogenesis of hepatitis delta. Still, various questions are unresolved: how this unique virus escapes from a sufficient immune control and why HDV infection is causing a particular severe course of chronic hepatitis. Very limited knowledge on the interaction of HDV with the immune system is available, which, however, would be crucial for the development of novel immunotherapies. As HDV does not have any viral enzyme which could be targeted by direct antiviral approaches, it will be crucial for future therapies to enhance anti-HDV immunity. With the invention of new animal models, better understanding of the virus and the human immune system, we will hopefully be able to unravel these mysteries.

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Hepatitis E

17

Hiroki Takahashi and Mikio Zeniya

Hepatitis E virus (HEV) was first discovered in developing countries, and acute hepatitis E became popular acute hepatitis in developing countries. However, recent reports revealed that acute hepatitis E was not a rare disease in developed countries.

- From clinical immunological point of view, the following facts are very important points:
  - Though most HEV infection course acute hepatitis, chronic hepatitis occurs in immunocompromised individuals including recipients of organ transplants.
  - Though most HEV infection course mild and self-limited hepatitis, it became more severe and often leading to fulminant hepatic failure (FHF) and death frequently in pregnant women.
  - Development of efficacious vaccine with the results of immunological analysis is needed.
- From basic immunological point of view, the following facts are very interesting points:
  - Abnormality of NK and NKT cell in HEV-infected patients was reported. However, the detail of innate immune responses in HEV-infected patients is not enough analyzed.
  - Extensive analyses of acquired immunity to HEV especially T cell response to HEV revealed that HEV-specific immune response participated in the pathogenesis.
  - The immunological mechanisms which relate to chronic infection in immunocompromized patients and severe hepatitis in pregnant women are not fully understood.

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Introduction

Hepatitis E caused by the infection of HEV had thought to be limited to residents of developing countries. However, recent studies revealed that hepatitis E had a wider geographic and host species distribution [1–3]. Currently HEV is recognized as the most common cause of acute viral hepatitis in the world, and it has been estimated that HEV infection causes three million symptomatic cases of acute hepatitis E each year, resulting in approximately 70,000 deaths worldwide [4].

Since HEV itself appears noncytopathic against hepatocyte like other hepatitis virus such as hepatitis B virus and hepatitis C virus, immunological interactions between host and HEV are supposed to be involved in the hepatocyte injury and pathogenesis of hepatitis E. Immunological abnormalities in innate and acquired immune response against HEV in the patients may also relate to the pathogenesis of hepatitis E. In addition, chronic infection of HEV, which was believed to be less common as the clinical feature of HEV infection, became to be observed in immunocompromised individuals including recipients of organ transplants [5]. Another characteristic clinical feature of hepatitis E is to progress more severe and often leading to FHF and death in pregnant women [6]. Immunological abnormalities in recipients of organ transplants and pregnant women are thought to relate to the pathogenesis of those clinical features.

Particular therapy of hepatitis E is not required because generally hepatitis E is acute and self-limiting. However, the prevention of sporadic and epidemic HEV infection is a very important social problem especially in developing countries. The best way to avoid HEV infection is to develop efficacious vaccine of HEV. This is the matter of immunological interest as well.

We would like to review and summarize the most resent knowledge about the immunological aspects of the pathogenesis of hepatitis E in this chapter.

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General Aspect of HEV Virology

HEV was first recognized during a waterborne epidemic of unexplained hepatitis which occurred in Kashmir Valley, India, at 1978 [7]. The new virus named as HEV, which belongs to genus Hepevirus in the Hepeviridae family, was detected by Balayan et al. [8] by electron microscopy in the stool of the patients who were infected during the outbreak which occurred in a Soviet military camp located in Afghanistan [8].

Reyes et al. cloned and sequenced HEV genome at 1990 [9]. They discovered that HEV was a small, with a size of 27–34 nm, non-enveloped virus with a positive-sense single-stranded RNA genome. The genome of HEV consists of three open reading frames (ORF) [10]. ORF1 codes for non-structural proteins including methyl transferase, papain-like cysteine protease, proline-rich hypervariable region, RNA helicase, and RNA-dependent RNA polymerase which are required for replication and protein processing. ORF2 codes for capsid proteins that are responsible for virion assembly, interaction with target cells, and immunogenicity. Truncated versions of the ORF2 protein expressed in insect cell or bacterial systems assemble into empty viruslike particles (VLPs), which have been used as candidate vaccines [11]. ORF3 codes for a small multifunctional protein which participates in virion morphogenesis and release in the host and regulates the cellular environment. The ORF2 and ORF3 proteins are translated from a single bicistronic mRNA and overlap each other, but neither overlaps ORF1 (Fig. 17.1).

HEV has four genotypes and only one serotype. Genotypes 1 and 2 exclusively infect humans and transmitted via contaminated water in developing countries [12]. Genotype 1 occurs mainly in Asia and genotype 2 in Africa and Mexico. Genotypes 3 and 4 infect human beings, pigs [13], and several other mammalian species [14, 15] and are responsible for sporadic cases of autochthonous hepatitis E in both developing and developed countries.

Several reports from India revealed the association of gene polymorphisms and susceptibility to HEV infection recently. The association of TNF-α-308AA genotype with susceptibility to HEV and that of TNF-α-1031CC and IFN-γ+874TT and TA with clinical disease, irrespective of the outcome, were revealed [16]. The other report showed a significant association of heterozygous genotypes (CA)12/(CA)14 and (CA)12/(CA)16 in intron 1 of the IFN-γ gene to acute HEV infection [17].

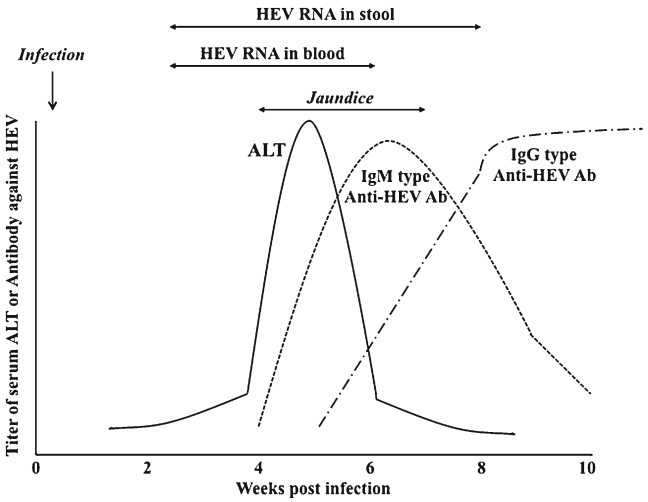

Fig. 17.1 HEV genome and its proteins

Clinical Property of HEV Infection

As mentioned above, acute hepatitis E was first recognized in India, and subsequently endemic hepatitis E has been identified in many countries with poor sanitation in south Asia, Southeast Asia, and Africa as the most common cause of acute viral hepatitis. Thus, hepatitis E was initially thought to occur only in developing countries. However, recent reports have revealed that HEV infection also occurs in developed countries as acute, self-limiting hepatitis [1–3]. In recent years, autochthonous genotype 3 infections have been reported in Europe, New Zealand, and North America. Both genotype 3 and genotype 4 are present in Japan. Nowadays, HEV is recognized as the most common cause of acute viral hepatitis in the world. On the other hand, several studies reported that anti-HEV seroprevalence rates were less than 5 % in developed countries.

The most popular transmission way of HEV is feco-oral, usually through contaminated drinking water. Person to person spread is thought to be uncommon. However, HEV infection can occur by blood transfusion. IgM- and IgG-type anti-HEV antibodies were detected in recipients of blood transfusions in India [18], Hong Kong [19], and Japan [20]. The cases of HEV transmissions by blood transfusion were also described in France [21] and the UK [22] recently.

Hepatitis E is usually acute and self-limiting hepatitis that lasts 4–6 weeks. Acute hepatitis E is mainly caused by genotype 1 and genotype 2 in developing countries and by genotype 3 and genotype 4 in developed countries. Most genotype 3 infections in developed countries seem to be asymptomatic.

However, chronic hepatitis E and severe hepatitis or FHF caused by HEV become to be recognized. Chronic infection of HEV is observed in immunocompromised individuals such as recipients of organ transplants. FHF is observed in specific high-risk groups such as elderly men with coexisting illnesses including chronic liver diseases [23] and pregnant women [24]. Though mortality rates in epidemics are very low ranging from 0.2 to 4.0 %, it reaches 10–25 % in pregnant women.

Another interesting clinical feature of HEV infection is a zoonosis. Several animal species especially domestic swine, wild boar, wild deer, pigs, and rodents were found to be reservoirs of HEV genotypes 3 and 4 [13]. Human infections occur through intake of uncooked or undercooked meat of the infected animals and pig livers or sausages made from these livers and sold in supermarkets [25]. Interestingly, the seropositivity rates of anti-HEV antibody range from 2 to 3 % in blood donors to 20 % in people exposed to animal reservoir [26]. The best way to prevent food-borne transmitted hepatitis E is to avoid eating uncooked meat because the risk for food-borne HEV transmission can be reduced by cooking meat. HEV can be inactivated by temperatures over 70 °C.

Serology of HEV Infection: HEV-Specific Antibody

Exposure and recent infection of HEV are diagnosed by serological tests using IgM- and IgG-type anti-HEV antibody. After an incubation period of 2–6 weeks, IgM-type anti-HEV antibody, which can be detected specifically in >95 % of acute hepatitis E patients who were defined by the detection of the HEV genome in serum, appears and wanes off in 4–6 months. Class-switched IgG-type anti-HEV antibody persists for at least 1 year in many patients. The quantization of IgM-type anti-HEV antibody and its ratio to total Ig provides insight into infection timing and prior immunity. Although four genotypes of HEV are recognized, they elicit very similar antibody responses and appear to represent a single serotype (Fig. 17.2).

The clinical problem is the existence of acute hepatitis E patients who are negative for IgM-type anti-HEV antibody in acute period. Zhang et al. evaluated the utility of IgA-type anti-HEV antibody and reported that the positive rate of IgA-type anti-HEV antibody, IgM-type anti-HEV antibody, and IgG-type anti-HEV antibody in 84 samples positive for HEV RNA was 96.3 %, 97.6 %, and 88.1 %, respectively, and no sample was negative for IgA-type anti-HEV antibody and IgM-type anti-HEV antibody simultaneously [27]. It means that detection of IgA-type anti-HEV antibody can be a useful supplement for the diagnosis of acute HEV infection especially in patients negative for IgM-type anti-HEV antibody. On the other hand, false-positive results for IgM-type anti-HEV antibody occur sometimes. To avoid this inconvenience, Pan et al. developed the new ELISA by using the covering amino acids 459–607 in ORF2 which formed the immunodominant B cell epitopes and revealed that poor reactivity of a truncated ORF2 polypeptide can be used to exclude nonspecific binding in the detection of IgM-type anti-HEV antibody [28].

Very interestingly, anti-HEV antibodies were detected in 13 % of the patients with autoimmune hepatitis, but not of primary biliary cirrhosis. This result indicated that HEV related the pathogenesis in some cases of autoimmune hepatitis [29].

Innate Immune Response in HEV-Infected Patients

The importance of innate immune response against virus as antiviral protective immunity has been revealed in cellular and molecular level in the last decade. The molecules which participate in recognizing the bacteria or virus such as Toll-like receptors were identified, and it became clear that such pattern recognition receptors participated in the pathogenesis of virus infection. Besides, several kinds of cells which belong to innate cell such as natural killer (NK) cell, natural killer T (NKT) cells, and newly identified natural helper cell are

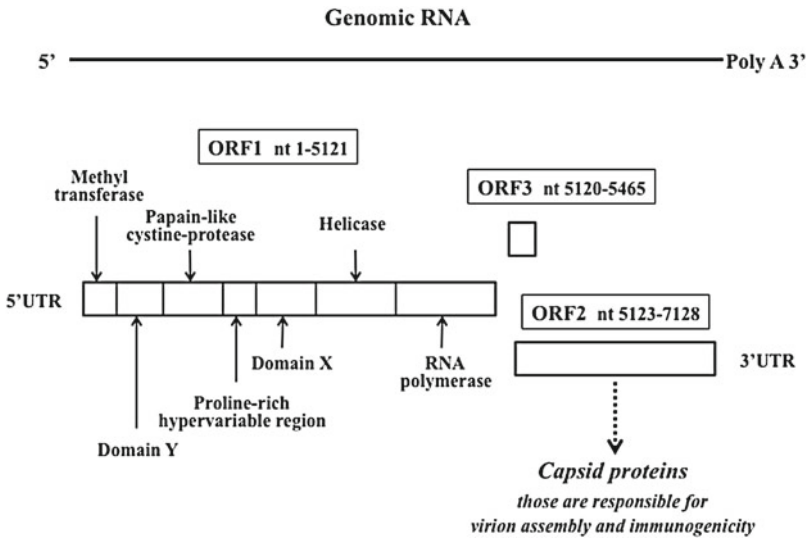

Fig. 17.2 Clinical course of acute hepatitis E

Table 17.1 Abnormality of innate immune response in acute hepatitis E

• Decreased number of NK, NKT cell in peripheral blood
– Their number is normalized during the period of recovery
• Activation status and function are not attenuated

Table 17.2 HEV-specific acquired immune response in acute hepatitis E

• Memory T cell response to HEV antigen exists among seronegative healthy residents of an endemic area
• Dominant intrahepatic infiltration of CD8-positive cytotoxic cell is observed in acute hepatitis E patients
• Higher reactivity of CD4-positive T cell to ORF proteins of HEV is observed in acute hepatitis E patients

also revealed in the pathogenesis of virus infection. Such molecules and cells should be related to the pathogenesis of acute hepatitis E. However, only a few studies which analyze innate immune response in acute hepatitis E were reported.

Srivastava et al. assessed the frequency and activation status of NK and NKT cells and cytotoxic activity of NK cells in the peripheral blood mononuclear cells obtained from acute hepatitis E patients [30]. They revealed that patients had fewer NK cells and NKT cells than controls though the activation status of NK cells and NKT cells and NK cell cytotoxicity was similar to controls. All the parameters normalized during period of recovery. They concluded that reversible alterations in NK and NKT cell number and activation status during acute hepatitis E suggested a role of these cells in the pathogenesis of acute hepatitis E.

Recent study made clear that significantly high level of circulating IL-1α and sIL-2Rα during the acute phase of HEV infection [31]. The study also showed the lack of robust HEV ORF2-specific CTL response in the peripheral blood of acute hepatitis E patients during the acute and recovered phases of the disease, suggesting the involvement of innate immune cells/localization of the immune events which relate to acquired immune response.

Since innate immune response participates in the induction of acquired immune response as this report indicated, pathogenesis of HEV infection should be analyzed from innate immune point of view comprehensively in near feature (Table 17.1).

HEV-Specific Acquired Immune Response in HEV-Infected Patients (Table 17.2)

As other hepatitis virus, HEV itself appears noncytopathic to hepatocyte. It means that the liver injury during HEV infection may be mediated by the host immune response. Recently, the existence of memory T cell response to HEV antigen among seronegative healthy residents of an endemic area was reported [32]. In addition, persistent HEV infections have been described in organ transplant recipients receiving immunosuppressive medications [33]. These reports suggest that HEV is controlled by adaptive immune responses.

At first, intrahepatic infiltration of CD8-positive T cell was observed in acute hepatitis E. The histological and immunohistochemical analysis of acute hepatitis E revealed the dominant intrahepatic infiltration of CD8-positive cytotoxic cell population [34]. Another immunohistochemical study demonstrated a significant infiltration of activated CD8-positive T cells containing granzymes [35]. These reports showed the important role of CD8-positive T cell in HEV-induced liver injury.

Then the reactivity of lymphocytes against HEV-related proteins was investigated by using peripheral blood lymphocytes obtained from acute hepatitis E patients. Peripheral blood lymphocytes obtained from acute hepatitis E patients showed higher reactivity when stimulated by seven peptides with amino acid sequences corresponding to ORF2 and 3 proteins of HEV compared with control [36]. Reactivity to one peptide corresponding to ORF2 was more frequent in patients than in controls, indicating that lymphocytes of patients with acute hepatitis E showed sensitization to HEV peptides.

The following analysis was performed to identify the immunogenic peptide in ORF proteins in acute hepatitis E. The lymphocyte proliferation assays of CD4-positive T cell using overlapping peptide libraries of recombinant ORF2 protein or pools of overlapping ORF2/ORF3 peptides, which were performed to define T cell epitopes in HEV proteins, revealed that amino acids 73–156, 289–372, 361–444, and 505–588 of ORF2 protein were associated with significant proliferation though ORF3 peptide pools did not induce proliferative responses. Lymphocyte proliferation in response to the peptide pool corresponding to amino acids 289–372 of ORF2 protein was associated with the presence of HLA-DRB1 allele 010X [37]. Another study with ELISPOT assay in acute hepatitis E patients revealed that amino acids 181–249 and 301–489 of ORF2 protein were the immunodominant regions for IFN-γ-producing cells [38–40]. These data may prove useful for designing HEV vaccines and for studying the immunopathogenesis of acute hepatitis E.

The studies which tried to reveal the relation between HEV-specific T cell response and disease condition were also performed. ELISPOT assay which is stimulated by ORF2 amino acids 368–606 revealed that HEV-specific T cell response decreased along with the decreasing IgM-type anti-HEV antibody titer and normalization of liver function, indicating that HEV-specific T cell response may play a role in the pathogenesis of acute hepatitis E and its recovery [39]. The study which assessed viral load, anti-HEV antibody titers, recombinant ORF2 protein-induced Th1/Th2 cytokines levels, and cellular immune responses in self-limiting

acute hepatitis E and FHF with HEV infection made clear that significantly higher levels of both Th1 (IFN-γ, IL-2, and TNF-α) and Th2 (IL-10) cytokine and higher titers of IgM- and IgG-type anti-HEV antibody were recorded in FHF patients compared to acute hepatitis E patients though all FHF patients were negative for HEV RNA [40, 41]. Thus, higher HEV-specific acquired immune responses may participate in the pathogenesis of FHF with HEV infection. On the other hand, the study which tried to distinguish immunopathogenesis of symptomatic and asymptomatic HEV infections by using ORF2 protein-stimulated IFN-γ ELISPOT assay revealed that asymptomatic acute hepatitis E patients are likely to have stronger immune responses compared to symptomatic patients [41, 42]. Another study which aimed to distinguish immunopathogenesis of the patients with HEV infection of varying disease severity such as asymptomatic infection, uncomplicated acute viral hepatitis, and FHF was performed by evaluating cytokine-secreting CD4-positive T cells and antibody-producing B cells specific for HEV with intracellular cytokine staining and ELISPOT assay [42, 43]. The results showed that patients with FHF had a less marked expansion of HEV-specific IFN-γ- or TNF-α-secreting CD4-positive T cells and a more marked expansion of B cells that can secrete IgG-type anti-HEV antibody than patients with uncomplicated acute infection and control patients.

Very recent study which performed transcriptional profiling analysis using GeneChip DNA microarrays to identify the genes that were differentially expressed in acute hepatitis E, resolving phase of HEV infection and controls revealed that increased activation of the genes involved in pro-inflammatory responses in CD4-positive T cells during acute hepatitis E [43, 44]. Additional RT-PCR analysis confirmed that in cells from acute hepatitis E patients, there is an increased expression of CCR5, CCR9, CXCR3, CXCR4, STAT1, IRF-9, IFN-α, and TNF-α, together with a down-regulation of IL-2, SOCS3, and IL-10, with respect to cells from resolving phase patients. In addition, higher frequencies of CD8-positive and activated CD38+ CD69+ T cells were observed in acute hepatitis E patients than in resolving phase patients, who in turn exhibited higher CCR9 expression than cells from patients in active phase. The CD11a high subpopulation within CD4+ CD45RA+ cells was increased in both acute hepatitis E patients and resolving phase patients. These findings suggest the involvement of a circulating CD45RA+ CD11a high population with CCR5 expression in the pathogenesis processes of acute hepatitis E and the importance of CCR9-positive cells in resolution phase (Table 17.3).

The study which revealed the importance of atypical effector T cell was also performed. The assay which evaluates T cell reactivity to ORF2 proteins by measuring secreting IFN-γ and TNF-α and cytokine mRNA level revealed that IFN-γ levels in the supernatants and IFN-γ mRNA transcripts in cells were elevated in ORF2-stimulated CD4+/CD69+ and CD8+/CD69+ cells in acute hepatitis E patients [38–44]. These data indicated that CD4-positive IFN-γ-secreting cells, which do not belong either to the helper Th1 or Th2 phenotype, as is the case with NKT cells, may be involved in the pathogenesis of acute hepatitis E.

Table 17.3 Difference of HEV-specific acquired immune response according to disease condition of acute hepatitis E

• Asymptomatic acute hepatitis E patients have stronger HEV-specific T cell responses compared to symptomatic patients
• HEV-specific T cell response is decreased in the recovery phase compared to the active phase
• Significantly higher levels of Th1 (IFN-γ, IL-2, TNF-α) and Th2 (IL-10) cytokine and higher titers of IgM- and IgG-type anti-HEV antibody are recorded in fulminant hepatitis compared to acute hepatitis E

How about the role of regulatory T cells (T reg) which has been extensively studied in HBV or HCV infection in HEV infection? Flow cytometry analysis of Foxp3-positive T reg showed the significant elevation of T reg population in overall hepatitis E patients compared to controls [45]. Comparisons of HEV-specific cytokines/chemokines production by T reg revealed that the levels of IL-10 were elevated in acute hepatitis E versus recovered individuals and controls. These results suggest that T reg cells might be playing a pivotal role in HEV infection.

Chronic HEV Infection

In recent years, cases of chronic HEV infection, which were associated with progressive liver disease which rapidly progresses to liver cirrhosis and end-stage liver disease, have been described in several cohorts of immunocompromised individuals including recipients of organ transplants and patients with hematological disorders receiving chemotherapy (Fig. 17.3). All reports of chronic hepatitis E have involved genotype 3 but not genotype 1 or 2. Thus, screening for HEV RNA should be part of the diagnostic work-up of elevated liver transaminases in organ transplantation recipients or immunocompromised individuals. Since human immunodeficiency virus (HIV)-infected patients are in immunocompromised condition as well, persistent HEV infection may occur in HEV/HIV coinfected patients. However, it has not been clear how frequently does chronic hepatitis E develop among HIV-positive patients. Patients with chronic hepatitis E should be considered for treatment with pegylated interferon alpha-2a/alpha-2b [46] or ribavirin [47] for 3–12 months because prolonged viremia has been associated with the development of liver cirrhosis and hepatic failure.

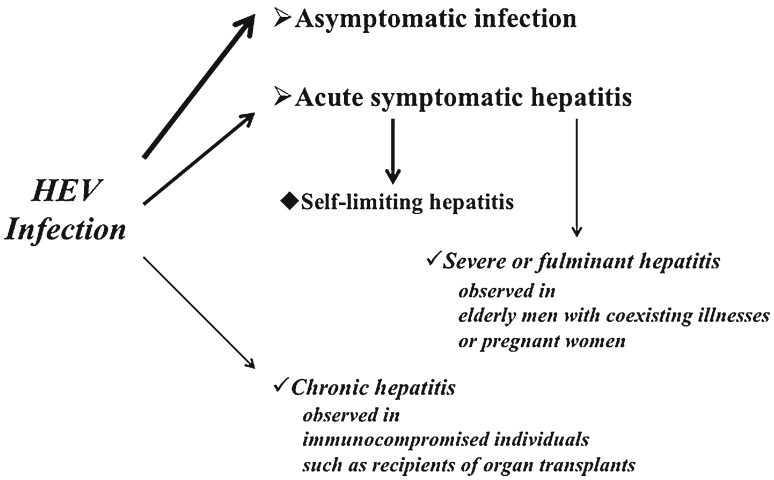

Fig. 17.3 Clinical features of HEV infection

§Organ Transplant Recipients

Organ transplant recipients have a risk to become a chronic hepatitis E when they are infected with HEV because they have been receiving immunosuppressive therapy which prevents appropriate immune response against HEV. Since it has been reported that acute hepatitis E can progress to chronicity in up to 66 % of solid organ transplant recipients in a recent study that summarized retrospective data collected from 17 centers on hepatitis E in transplant recipients [48], proper diagnosis is therefore important, as reducing immunosuppressive therapy can allow clearance of the virus. Immunosuppressed individuals should be tested for HEV RNA because antibody tests might not be sensitive enough for these patients.

Halleux et al. reported a case of chronic hepatitis E in a renal transplant recipient that went undiagnosed for many years [49]. Aggarwal et al. identified 14 cases of acute hepatitis E in three patients receiving liver transplants, nine receiving kidney transplants, and two receiving kidney and pancreas transplants [5]. All patients were positive for serum HEV RNA, and chronic hepatitis developed in eight patients, as confirmed by persistently elevated aminotransferase levels, serum HEV RNA, and histologic features of chronic hepatitis. The time from transplantation to diagnosis was significantly shorter, and the total counts of lymphocytes and of CD2, CD3, and CD4 T cells were significantly lower in patients in whom chronic disease developed.

The study which investigated T cell responses against HEV in anti-HEV antibody-positive but HEV RNA-negative organ transplant recipients and transplant recipients with chronic hepatitis E revealed that strong and multispecific HEV-specific T cell responses were absent in patients with chronic HEV infection but become detectable after viral clearance [50]. Interestingly, HEV-specific T cell responses can be restored in vitro by blocking the PD-1 or CTLA-4 pathways though combination of PD-1 and CTLA-4 blockade had no synergistic effects. Since PD-1-positive exhausted T cells, which were thought to participate in the persistent infection of hepatitis C virus, were observed in chronic hepatitis C, the same immunological abnormalities which allow chronic viral infection may exist in chronic hepatitis E as well. These results suggested that chronic hepatitis E was associated with impaired HEV-specific T cell responses and enhancing adaptive cellular immunity against HEV might prevent persistent HEV infections (Table 17.4).

Table 17.4 Immunological abnormalities in chronic hepatitis E

• Total counts of lymphocytes and of CD2, CD3, and CD4 T cells are significantly lower
• Strong and multispecific HEV-specific T cell responses are absent
• HEV-specific T cell responses can be restored in vitro by blocking the PD-1 or CTLA-4 pathways

§HEV/HIV Coinfection

Though the incidence of HEV infection in patients with HIV is low in general, HIV-infected individuals more frequently have anti-HEV antibody compared to individuals without HIV infection [51]. Up to now only 14 PCR-proven cases have been documented worldwide, and ten had acute hepatitis E, four had chronic hepatitis E, and one had histologically proven cirrhosis [52]. Reports from Europe could not identify persistent HEV infections in HIV-infected cohorts. Therefore, persistent HEV infection is rarely observed in HIV-infected patients. The patients who developed chronic hepatitis E had low CD4 counts and high level of HIV [52], suggesting only subjects with strong immune impairments seem to be at risk for chronic hepatitis E.

Immunological Aspect of HEV Infection in Pregnant Women

Though HEV infection is usually self-limited and has a case-fatality rate of less than <0.1 % in men and nonpregnant women, it became more severe and often leading to FHF and death frequently in pregnant women (Fig. 17.3). It has been reported that mortality rates reach 15–20 % [53]. This is a very serious clinical problem especially in developing countries. The mechanisms of this phenomenon are not well understood yet. However, several research works to explain this phenomenon from immunological point of view have been reported.

Pal et al. reported that peripheral blood mononuclear cells from pregnant women with acute hepatitis E had lower lymphocyte proliferation response to phytohemagglutinin (PHA) than those in the healthy pregnant woman and nonpregnant woman [54]. Though a positive lymphocyte proliferation response to mixture of HEV antigen proteins was similar among pregnant women with acute hepatitis E, healthy pregnant woman, and nonpregnant woman, cytokine production by peripheral blood mononuclear cells in response to PHA and HEV antigen proteins showed a reduction in the production of Th1 cytokines and an increase in that of Th2 cytokines in the pregnant women with acute hepatitis E. These results indicated the existence of a Th2 bias in pregnant women with acute hepatitis E, and such bias may participate in the greater severity of hepatitis E among pregnant women.

The works which tried to make clear the role of hormones including pregnant-related hormones such as progesterone in this phenomenon were performed. Jilani et al. revealed that diminished cellular immunity (indicated by a decrease in CD4, an increase in CD8 cell counts, and lowered CD4/CD8 cell ratio) and a high level of steroid hormones that influence viral replication/expression during pregnancy appear to be the plausible reasons for the severity of hepatitis E [55]. Bose et al. reported that expression of progesterone receptor and progesterone-induced blocking factor in the placenta was reduced in both mRNA and protein level in HEV-infected pregnant women with FHF compared to pregnant women with acute hepatitis E and healthy pregnant women [56]. Variants in the gene that encodes the progesterone receptor variants can alter its expression level. The higher serum IL-12/IL-10 ratio observed in women with FHF compared to other groups correlated with fetal mortality in acute hepatitis E and FHF. These results indicated that reduced expression of progesterone receptor and progesterone-induced blocking factor and a higher IL-12/IL-10 ratio resulted in poor pregnancy outcome in HEV-infected pregnant women. In addition, pregnant-related hormones such as estrogens might impair cellular immunity by triggering adapter protein (ORF3) which could facilitate viral replication and lead to the release of cytokines and liver cell apoptosis. Further studies are needed to make clear the detailed mechanisms of the worse prognosis of HEV infection in pregnant women (Table 17.5).

Table 17.5 Immunological abnormalities in HEV-infected pregnant women

• PBMC from pregnant women with acute hepatitis E has lower lymphocyte proliferation response to PHA than those in the healthy pregnant woman and nonpregnant woman
• Th2 bias of cytokine production in response to PHA and HEV proteins exists in pregnant women with acute hepatitis E
• Reduced expression of progesterone receptor and progesterone-induced blocking factor, a higher IL-12/IL-10 ratio exists in pregnant women with fulminant hepatitis E compared to pregnant women with acute hepatitis E

Vaccine: The Immunological Way for Prevention of HEV Infection

From the clinical point of view, the most important matter to prevent sporadic and epidemic hepatitis E is the development of efficacious vaccine of HEV. A huge number of researches had performed to develop the HEV vaccine. Since HEV does not replicate efficiently in cell culture, the vaccine has been developed based on recombinant proteins derived from the capsid gene of HEV. Though some vaccine candidates are evaluated in phase 2/3 trials, at this time no approved vaccine against HEV is commercially available. Another approach to develop the HEV vaccine is DNA vaccine. Immunization with plasmid containing ORF2 gene has been tried.

Identification of linear B cell epitopes encoded by the HEV protein which was recognized by antibodies from acute hepatitis E patients is necessary to develop an efficacious vaccine using recombinant proteins. A lot of works which tried to identify B cell epitopes had been reported.

At first Kaur et al. identified linear B cell epitopes in three ORFs of HEV [57]. Epitopes were identified throughout the polyprotein encoded by ORF1, but they appeared to be particularly concentrated in the region of the RNA-dependent RNA polymerase. Distinct epitopes were identified in the presumed structural protein encoded by ORF2, and one epitope was identified close to the carboxyl terminus of the protein encoded by ORF3. Their work revealed that antibody response is directed against the RNA-dependent RNA polymerase. Coursaget et al. showed that two overlapping synthetic peptides corresponding to overlapping DNA sequences of the ORF 3 were immunoreactive [58]. Khudyakov et al. identified two immunodominant regions at positions aa 394–470 and 546–580 of the ORF2 protein [59]. Riddell et al. reported that sequences spanning aa 394–457 of the ORF2 protein participate in the formation of strongly immunodominant epitopes

on the surface of HEV particles [60]. Zhang et al. tried to identify antigenic epitopes of the ORF2 protein from newly identified Chinese strain of genotype 4 and revealed that position aa 464–629 was an immunodominant epitope [61]. They also showed that monoclonal antibody against this protein neutralized HEV genotype 4.

These reports revealed that ORF2 was the most potent candidate of B cell epitope of HEV. However, antigenic epitopes are different according to reports. With such situation, recombinant vaccine against HEV which contain recombinant HEV capsid protein (56 and 53 kDa) expressed in insect cells was developed. These HEV capsid proteins consist of amino aa 112–607 and 112–578 and proved to protect Rhesus monkeys from hepatitis E when challenged with a high intravenous dose of homologous or heterologous HEV. In a phase 2 trial in Nepal, male army recruits with undetectable anti-HEV were randomized to receive 56 kDa vaccine (898 participants) or placebo (896 participants) at 0, 1, and 6 months and were followed up for an average of 804 days. The vaccine was well tolerated and highly immunogenic, with 95.5 % (95 % CI 85.6–98.6) efficacy against hepatitis E [11].

Recently, another type of bacterially expressed particulate HEV vaccine HEV239 was developed. The vaccine peptide has a 26 amino acid extension from the N terminal of another peptide E2 in ORF2 protein which has been shown to protect monkeys against HEV infection previously. However, HEV239 is over 200 times more immunogenic than E2, and it became clear that HEV239 can efficiently evoke a vigorous and predominant T cell response and induce significant antibody response as well in athymic mice. A randomized controlled phase 2 clinical trial showed HEV239 was safe and immunogenic for humans with an efficacy of 83 % [62]. In a phase 3 trial in 11 townships in eastern China, participants were randomly assigned to receive either three intramuscular injections of HEV 239 at 0, 1, and 6 months or hepatitis B vaccine as a placebo and were followed up for occurrence of acute hepatitis to month 19. The vaccine was well tolerated and protected against hepatitis E, with an efficacy of 100 % (95 % CI 72.1–100.0) [63].

The efficacy of HEV DNA vaccine in animal model is also reported. Injection of an expression vector pJHEV containing ORF2 gene generates a strong antibody response in BALB/c mice that can bind to and agglutinate HEV [64]. The plasmid pcHEV23 containing fragments of HEV ORF2 and ORF3 chimeric gene also can successfully induce HEV-specific humoral and cellular immune response in BALB/c mice [65].

Conclusion

Though HEV infection used to be thought to induce self-limited acute hepatitis mainly in developing countries, recent studies revealed that HEV infection may be geographically more widespread than was previously believed. In addition, it became obvious that hepatitis E progresses to chronic hepatitis in the immunocompromised patients and develops to severe disease in pregnant women or patients with coexisting chronic liver diseases. Doctors not only in developing countries but also in developed countries should keep in mind about HEV when they diagnose the reason for unexplained hepatitis especially in those who are pregnant or with organ transplant. From the immunological point of view, it is interesting and important to analyze how innate and acquired immune responses participate in the pathogenesis of both acute and chronic hepatitis E more in detail.

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Primary Biliary Cirrhosis

18

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Abbreviations

2-OADC	2-oxo acid dehydrogenase complex
ALP	Alkaline phosphatase
AMAs	Antimitochondrial autoantibodies
ANAs	Antinuclear antibodies
AST	Aspartate transaminase
α-GalCer	α-Galactosylceramide
BCOADC	Branched-chain 2-oxo acid dehydrogenase complex
BECs	Biliary epithelial cells
CA	Cholangitis activity
CTLA-4	Cytotoxic T lymphocyte antigen-4
E3BP	Dihydrolipoamide dehydrogenase (E3)-binding protein
ELISA	Enzyme-linked immunosorbent assay
GWAS	Genome-wide association studies
HA	Hepatitis activity
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
IP	Intraperitoneally
IRAK-M	Interleukin-1 receptor-associated kinase M
LFA-3	Lymphocyte-associated antigen 3
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
NF-kB	NF kappa B
NKT	Natural killer T
OGDC	Oxoglutarate dehydrogenase complex
OLT	Orthotopic liver transplantation
PBC	Primary biliary cirrhosis
PD	Programmed death
PDC	Pyruvate dehydrogenase complex
QSAR	Quantitative structure–activity relationship
SNPs	Single-nucleotide polymorphisms
STAT4	Signal transducer and activator of transcription 4
TGF	Transforming growth factor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
UDCA	Ursodeoxycholic acid
VCAM-1	Vascular cell adhesion molecule-1

Key Points

- Primary biliary cirrhosis (PBC) is characterized by immune-mediated destruction of small intrahepatic bile ducts with portal inflammation.
- PBC includes a striking female predominance and high-titer serum autoantibodies to mitochondrial antigens (AMA).
- The most common symptoms accompanying PBC at diagnosis in precirrhotic stages are defined as fatigue and pruritus although we are witnessing a dramatic change in patient presentation patterns.
- The presence of serum AMA and autoreactive T and B cells in conjunction with the co-occurrence of other autoimmune diseases, implies an autoimmune pathogenesis for PBC.
- The diagnosis of PBC is based on three criteria: detectable serum AMA increased plasma cholestasis enzymes (alkaline phosphatase [ALP]) for longer than 6 months, and a diagnostic liver histology with portal infiltration of lymphocytes.
- The etiology of PBC remains unclear but it has been proposed that a common theme includes a complex genetic background and one or more environmental triggers.

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- In most cases PBC progresses slowly over years.
- Several medical treatments have been studied in patients with PBC. Among these ursodeoxycholic acid (UDCA) is most commonly used. Liver transplantation is the only definitive treatment, although recurrences are common.
- The use of immunosuppressants is (at present) not encouraged in PBC.

Introduction

PBC is a chronic cholestatic liver disease characterized by high-titer serum antimitochondrial autoantibodies (AMAs) and autoimmune-mediated destruction of small- and medium-sized intrahepatic bile ducts [1–3]. The first description of biliary cirrhosis, albeit possibly secondary, can be traced back to the work of the Italian pathologist Giovanni Battista Morgagni from Padua in 1761; the first report of nonobstructive biliary cirrhosis was by Addison and Gull in 1851. Subsequently, the term PBC was accepted in the medical literature [4], and in 1959 Dame Sheila Sherlock described the first series of patients affected by PBC who had been followed over the previous decade and noted that patients presented with pruritus as well as the signs and symptoms of end-stage liver disease including jaundice [5].

From a clinical standpoint, PBC is a peculiar, yet representative, autoimmune disease. PBC affects women more frequently than men, with a female to male ratio of 9:1, with middle-age onset [6]. Epidemiological data indicate a geographical pattern of PBC prevalence and incidence rates, being more prevalent in Northern Europe and North America [7, 8]. The diagnosis of PBC is made when two of the three criteria are fulfilled, i.e., presence of serum AMA, increased enzymes indicating cholestasis (i.e., ALP) for longer than 6 months, and a compatible or diagnostic liver histology [2]. Clinical symptoms include fatigue, pruritus, and jaundice. The progression of PBC varies widely for unknown reasons, as represented by certain patients remaining asymptomatic and others reaching liver failure at young ages [9, 10]. Several clinical and experimental findings strongly imply an autoimmune pathogenesis for PBC, whereas the disease onset recognizes two necessary components in a permissive genetic background and an environmental trigger [11–13]. UDCA is the only licensed therapy for PBC. However, there are still many unknowns about UDCA. UDCA is believed to slow progression to cirrhosis, but response is inadequate or absent in about 30 % of patients, with nonresponders facing a fivefold increased risk of death or need for orthotopic liver transplant (OLT) [14].

The association between serum AMA and PBC was first recognized as specific in 1965 by Walker and colleagues [15]. The AMA antigens were cloned and identified as the E2 subunits of 2-oxo acid dehydrogenase complex, with the E2 subunit of pyruvate dehydrogenase complex (PDC-E2) as the major mitochondrial autoantigen [6, 16, 17]. This discovery led to the development of more sensitive assays for the determination of AMA, although indirect immunofluorescence (IIF) remains the method of routine testing in most clinical centers [18, 19].

Clinical and Pathological Features

Diagnosis

In 2009, both the practice guidelines of the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver recommended that the diagnosis of PBC be established when two of the following three criteria are met: (a) biochemical evidence of cholestasis based mainly on ALP elevation, (b) presence of AMA (titer >1:40), and (c) histological evidence of nonsuppurative destructive cholangitis and destruction of interlobular bile ducts [2, 20].

Patients lacking detectable AMA but otherwise presenting signs of PBC should be regarded as "AMA-negative PBC," and they appear to follow a similar natural history compared with their AMA-positive counterparts [21]. Antinuclear antibodies (ANAs) directed against nuclear body or envelope proteins such as anti-Sp100 and anti-gp210 show a high specificity for PBC (>95 %) and can be used in AMA-negative patients [22, 23]. Individuals positive for PBC-specific autoantibodies with normal serum liver tests should be followed with annual reassessment of biochemical markers of cholestasis. A liver biopsy is needed for the diagnosis of PBC in the absence of PBC-specific antibodies, and it may also be helpful when an additional or alternative process is suspected (i.e., autoimmune hepatitis [AIH]).

Clinical Features

PBC is generally a slowly progressive disease with a possibility to lead to cirrhosis and liver failure. However, patients with PBC often suffer from a variety of symptoms long before the development of cirrhosis (Table 18.1). Although the quality of life of PBC patients is generally well preserved, PBC patients suffer from a variety of symptoms that are beyond the immediate impact of liver failure and affect their lifestyle, personal relationships, and work activities [10, 24].

Fatigue. Fatigue is an incompletely defined, nonspecific symptom that is believed to affect up to 70 % of patients with PBC while often being overlooked by patients and physicians. It is still unclear whether the severity of fatigue is

Table 18.1 Clinical features of PBC

Symptoms
Fatigue
Pruritus
Other clinical features
Portal hypertension
Bone density reduction
Hyperlipidemia
Steatorrhea
Malabsorption of fat-soluble vitamins
Association with other disorders
Autoimmune disorders: Raynaud's and Sjögren's syndrome are most frequent
PBC at the stage of cirrhosis can be complicated by the occurrence of hepatocellular carcinoma

dependent on the stage of PBC or its other features (pruritus or severe cholestasis) [25, 26]. More importantly, the specificity of fatigue is still debated, as well-controlled studies are lacking to define the importance of chronic liver disease per se [26]. Quality of life questionnaires, named PBC-40 and PBC-27, have been developed to monitor fatigue in PBC patients [27, 28]. Morphological abnormalities of the central nervous system owing to the accumulation of manganese have been postulated but not proven as a putative cause of fatigue in PBC [29]. No medical treatment has been shown to be effective, although fatigue has never been included as an end point in any of the large controlled clinical trials. The degree of daytime somnolence is correlated strongly with the magnitude of fatigue in PBC patients [30]. The CNS-acting drug modafinil therapy is associated, where tolerated by patients, with improvement in excessive daytime somnolence and associated fatigue in PBC. Further study in placebo-controlled trials is needed [31]. The presence of fatigue in PBC is independently associated with a significantly increased risk of death in general, and cardiac death in particular. Factors underpinning fatigue in PBC, and the mechanisms whereby fatigue is associated with increased mortality, warrant further study [32].

Pruritus. Pruritus is considered the second most common presenting symptom of PBC. Longitudinal data demonstrate that the vast majority of patients will experience this symptom during the progression of disease, and its appearance most commonly precedes jaundice by months or years. Pruritus can be localized or diffuse, but at the time of onset, it more frequently worsens at night, following contact with certain fabrics (wool) or in warm climates. The bases of PBC-associated pruritus are not clear, and two hypotheses have been proposed, i.e., serum bile acid retention secondary to chronic cholestasis or, alternatively but not exclusively, an amplified release of endogenous opioids [33]. Recent studies demonstrate that lysophospholipase, autotaxin, and its product, lysophosphatidic acid, as potential mediators of cholestatic pruritus [34]. Serum autotaxin activity is specifically increased in patients with cholestatic, but not other forms of systemic, pruritus and closely correlates with the effectiveness of therapeutic interventions. The beneficial antipruritic action of rifampicin may be explained, at least partly, by the PXR-dependent transcriptional inhibition of autotaxin expression [35].

Finding an effective medical treatment for pruritus in PBC is often challenging. Trials of antihistamines or phenobarbital for treatment have proven that these medications are ineffective, whereas the use of cholestyramine (4 g before and after the first meal) ameliorates pruritus [10]. In selected cases poorly responsive to resins, rifampicin has been used to achieve rapid symptom relief; its prolonged use, however, is not recommended. Oral opiate antagonists can be used as third-line agents [36]. However, problems have been reported with an opiate withdrawal-like reaction on initiation (which can be ameliorated, to some extent, by the use of an i.v. naloxone induction phase in which the dose is rapidly escalated to a level at which conversion to the lowest dose oral opiate antagonist preparation can be instituted [37]) and ongoing problems resulting from pain and confusion. The use of sertraline is encouraged by promising preliminary data but warrants further evaluation. More recently, a study demonstrated that ultraviolet B (UVB) phototherapy appears to be a promising and well-tolerated treatment for cholestasis-associated pruritus [38]. In addition, albumin dialysis using MARS may be an effective procedure for managing resistant pruritus in most patients with chronic cholestasis [39]. Eventually, in patients with intractable pruritus, liver transplantation is the ultimate therapeutic option.

Portal Hypertension. Portal hypertension is a common finding in patients with PBC, but significantly fewer patients now present with acute digestive bleeding or other signs of portal hypertension, compared with the first reported series of affected individuals. Interestingly, portal hypertension in PBC does not imply the presence of liver cirrhosis. Esophageal varices can occur in a minority of early-stage PBC patients. Male sex, low albumin, elevated bilirubin, and/or prolonged prothrombin time was proposed to be used as a model to noninvasively predict esophageal varices [40]. High alkaline phosphate values and low platelet counts at diagnosis and decreased platelet counts during follow-up are useful predictors of esophageal varices in patients with early PBC [41]. Longitudinal studies indicate that about 58 % of untreated patients will eventually develop endoscopic signs of portal hypertension over a 4-year follow-up [42]. The prevention and treatment of PBC-associated portal hypertension is not different from other chronic liver diseases and is based mostly on the use of β-blockers.

Reduction in Bone Density. A metabolic bone disease is found in PBC, with accelerated bone loss owing to reduced bone deposition being noted in patients compared with sex- and age-matched healthy individuals. These findings are still somewhat contentious, and conflicting data have been reported. A mild reduction in bone density (osteopenia) is present in about 30 % of patients, and frank osteoporosis is diagnosed in 10 % of patients. There were approximately twofold relative increases in the risk of any fracture, hip fracture, and ulna/radius fracture in the PBC cohort compared with the general population [43]. In a recent study, the prevalence of vertebral, non-vertebral, and overall fractures was 11.2 %, 12.2 %, 20.8 %, respectively, in patients with PBC. Osteoporosis was associated with age, weight, height, histological stage, severity, and duration of liver damage; fractures were associated with osteoporosis, menopause, age, and height but not with severity of PBC. Fractures, particularly vertebral fractures, are associated with osteoporosis, osteopenia, and T scores less than −1.5, whereas osteoporosis and osteopenia are associated with the severity of liver damage. Patients with T scores less than −1.5 might require additional monitoring and be considered for therapy to prevent fractures [44]. The bone loss can, moreover, worsen after liver transplantation, possibly owing to the administration of specific immunosuppressive drugs and steroids.

The mechanisms leading to metabolic bone alterations are not completely understood, as no significant changes in the metabolism of calcium and vitamin D can be found in patients with PBC. The current treatment of bone loss in PBC, similar to non-PBC cases, includes oral calcium supplementation, weight-bearing activity, and oral vitamin D replacement (if a deficiency is present). Postmenopausal hormone replacement therapy should be considered as effective but as prone to cause long-term side effects in women with PBC as in the general population. However, as estrogens have been associated with worsening of the cholestatic pattern, jaundice and signs of liver failure should be monitored closely, particularly during the first months of treatment. A large improvement in the femoral bone mineral density of patients treated with alendronate has been observed. Bone mineral density changes were independent of concomitant estrogen therapy, and oral alendronate appears to be well tolerated [45]. Larger studies are needed to evaluate formally the safety and efficacy of other proposed treatments.

Hyperlipidemia. Alteration in the blood lipid profile is a common finding in PBC (up to 85 % of patients present with hyperlipidemia) and often precedes the diagnosis. Both serum cholesterol and serum triglyceride levels can be raised as the result of chronic cholestasis, but it seems that these patients are not exposed to greater cardiovascular risk; in fact, these alterations do not correlate with increased incidence of cardiovascular events or early atherosclerotic lesions [46]. PBC was also not associated with an increased risk of myocardial infarction, stroke, and transient ischemic attack. Therefore, strategies for the prevention of vascular events in PBC patients should be similar to those in the general population [47]. Reduced LDL oxidation may be associated with no significant increase in atherosclerosis in patients with PBC, even in those with severe hypercholesterolemia [48]. Treatment with UDCA may reduce blood lipid levels via unknown mechanisms, and the use of statins is still debated. Statin therapy effectively reduces serum cholesterol levels and, however, does not improve cholestasis in PBC with an incomplete biochemical response to UDCA [49].

Steatorrhea and Malabsorption. Long-standing cholestasis leads to steatorrhea by inducing bacterial overgrowth syndrome in the gut. The mechanism is mediated by the impaired flow of bile acids to the small intestine and is commonly found in advanced stages of PBC [50]. Oral replacement of medium-chain triglycerides for long-chain compounds, along with an overall reduction of fat in the diet, can be offered as treatment for symptoms. Pancreatic enzyme replacement medications can also improve the symptoms when pancreatic insufficiency is suspected. Empirical antibiotic regimens can treat the bacterial overgrowth, but their use, particularly when prolonged, should be carefully evaluated. Malabsorption of fat-soluble vitamins is commonly found in advanced stages of PBC [51]. The most common deficiency, involving vitamin A, although almost always symptomless, is present in 20 % of cases. Oral replacement therapy can overcome impaired absorption, and monitoring of serum concentrations is recommended after 6–12 months to avoid potential hepatotoxicity or overcorrection. In less common deficiencies such as vitamin E (potentially leading to ataxia), vitamin K (influencing coagulation), and vitamin D (see reduction in bone density), oral or parenteral supplementations are safe and effective.

Associated Conditions. Various disorders, particularly other autoimmune syndromes, have been reported to be associated with PBC. According to our most recent data, as many as 33 % of patients with PBC will present with another autoimmune disease [52]. Among the autoimmune conditions found in PBC, Raynaud's (12 %) and Sjögren's syndrome (10 %) are most frequently observed, but scleroderma comorbidity is not uncommon. Both PBC and Sjögren's syndrome are characterized by inflammation of target epithelial elements. Both diseases can be considered on the basis of a number of other related clinical aspects, including the proposed unique apoptotic features of the target tissue, the role of secretory IgA, and the frequency with which both diseases overlap with each other. Indeed, PBC may be considered as Sjögren's syndrome of the liver, whereas Sjögren's syndrome can be equally discussed as PBC of the salivary glands [53].

Malignancies. Like other chronic liver conditions that lead to cirrhosis, PBC at the stage of cirrhosis can be complicated by the occurrence of hepatocellular carcinoma (HCC), and patients should be periodically monitored [54]. The prevalence of HCC in Barcelona and Padova is 3.3 % and 3.4 %, respectively, and the incidence was 0.4 and 0.4 per 100 patient-years, respectively. Advanced histological stage is the only factor associated with the development of HCC [55]. In a national survey from Japan, the HCC incidence of PBC patients was 2.4 %; males are at risk of developing HCC at any histological stage of PBC [56]. Most recently, a systemic analysis showed that PBC patients have a significantly higher risk of overall cancer (pooled rate ratio [RR], 1.55) and HCC (pooled RR, 18.80) when compared with the general population [57]. From a clinical perspective, this implies that in PBC patients with cirrhosis, screening for HCC should be performed using ultrasonography (and computed tomography in selected cases) twice a year to estimate the prognosis and to choose among therapeutic alternatives, particularly when OLT is being evaluated. Surveillance for HCC in patients with PBC is very important since patients who were diagnosed with HCC during a surveillance program are more likely to undergo therapy and have a significantly better survival independent of disease severity than those not included in a surveillance program [58]. Apart from liver cirrhosis, there do not seem to be any PBC-specific risk factors for the development of HCC. The treatment of HCC in PBC should follow the same guidelines as in other chronic liver diseases. No association between PBC and cholangiocellular carcinoma or breast cancer is found.

Natural History

The progression of PBC varies widely, as represented by patients remaining asymptomatic for decades and others reaching liver failure at relatively young ages [10]. The factors influencing the severity and progression of the disease remain unknown, although data seem to indicate that genetic factors other than those inducing the disease ("second hit") might play a role. In general terms, the natural history of the disease can be divided into 3 time periods preceding liver failure, i.e., asymptomatic, symptomatic, and pre-liver failure. The duration of these periods can vary significantly, but we note that the first stage might last for decades and the third is usually very rapid. The diagnosis of PBC is currently most commonly made within the first stage; patients presenting with symptoms or advanced disease are significantly less frequent compared with older reports. Interestingly, asymptomatic patients are commonly older than symptomatic ones, which possibly implies differences in the progression of PBC in these two groups [59].

Having symptoms at presentation is considered a major factor determining survival rates of patients with PBC. In fact, asymptomatic PBC is accompanied by 10-year survival rates similar to those of the general population. On the other hand, 67 % of precirrhotic patients will develop liver cirrhosis over a 7-year observation period, whereas 70 % of asymptomatic patients will develop symptoms. Accordingly, more recent regression models indicate that asymptomatic patients with PBC have significantly lower survival than the general population. Based on the somewhat conflicting data, it has been hypothesized that survival rates of asymptomatic patients with PBC are shorter than those of the general population if symptoms develop during follow-up [60]. An additional confounding factor is provided by the rate of non-liver-related deaths that appears to cause the reduced survival of asymptomatic patients [61]. In a longitudinal prospective study of a geographically defined complete cohort of PBC patients in North-East England, survival in PBC patients is substantially reduced compared with case-matched community controls. In a recent study from Canada, survival in PBC patients was significantly lower than that of the age-/sex-matched Canadian population; male sex and an older age at diagnosis were independent predictors of mortality [62]. Further studies on large populations and longer follow-up periods are warranted.

Patients with symptomatic PBC show a more rapid progression to late-stage disease and a worse prognosis than their asymptomatic counterparts; survival time among symptomatic subjects is 6–10 years. Older age at diagnosis and signs of advanced disease (clinical, histological, or biochemical) are also associated with a worse prognosis. The establishment of accurate prognostic models to predict survival in patients with PBC is of obvious importance in clinical practice. The model based on the Mayo score is the only validated one and also the most widely utilized [63]; it is based on clinical (age, presence of ascites) and biochemical variables, as represented by cholestasis (bilirubin levels) and liver function (prothrombin time, albumin). We submit that this model is a static representation of a dynamic entity and has a lower accuracy for patients with noncirrhotic disease. Recently, it has been reported that PBC-specific serum ANAs, albeit found in a minority of patients, can predict a more aggressive disease, as indicated by longitudinal data on long follow-up periods [64].

Liver stiffness measurement using transient elastography has indeed been shown as a simple, reliable surrogate marker of liver fibrosis in various chronic liver diseases. A recent study confirms that transient elastography is of high performance for the diagnosis of severe fibrosis or cirrhosis and is one, if not the best, current surrogate marker of liver fibrosis in PBC [65]. Over an average follow-up of 3 years, on-treatment liver stiffness appears mostly stable in noncirrhotic patients with PBC, whereas it significantly increases in

patients with compensated cirrhosis. Furthermore, it indicates that the absolute value of liver stiffness and, even more significantly, its increase over time are major predictive factors of poor outcome in PBC [65].

Similar to other autoimmune diseases, PBC is characterized by a striking female predominance, with a female to male ratio estimated as 10 to 1 [66–68]. So far, the reason for this observation remains unknown, but a role of sex hormones or X chromosome defects (see Sex Chromosomes) has been proposed. In an attempt to explain the female preponderance, the prevailing view is that this gender difference may involve the effects of sex hormones on the immune system. Sex hormones are believed to influence the onset and severity of autoimmune disease by modulating lymphocytes at various stages in life [69]. Although specific studies are lacking on the influence that sex hormones have on the occurrence of PBC in either sex, such studies have been conducted for other autoimmune conditions, mostly in animal models. In humans, several case reports have shown an exacerbation of systemic lupus erythematosus and rheumatic diseases with the administration of oral contraceptives. In PBC, we reported an increased liver expression of estrogen receptors [70] and a beneficial effect of tamoxifen, an antiestrogenic agent, in PBC patients [71].

Asymptomatic Versus Symptomatic PBC

The number of asymptomatic patients at the time of diagnosis has been steadily increasing [4, 10]. The increasing number of symptomless patients most likely also represents the growing awareness of the syndrome as well as, perhaps more importantly, the availability of more sensitive noninvasive tests. However, we cannot rule out at present that higher prevalence rates are in fact secondary to prolonged survival of affected individuals. At present, in the absence of symptoms, the diagnosis of PBC is established primarily based on liver condition or cholestasis in the vast majority of cases [72]. The diagnosis of asymptomatic/early-stage patients is critically important since these patients will have good response to UDCA therapy and good prognosis. A recent study showed that early-stage patients with ALP and AST levels $\leq$1.5 ULN and normal bilirubin level after 1 year of UDCA treatment appear to be at very low or no risk of liver failure or progression to cirrhosis [73].

We note, however, that during extended clinical follow-up, most AMA-positive patients will eventually develop PBC-associated symptoms [74]. The most common symptoms accompanying PBC are fatigue and pruritus; classically described physical findings may include skin hyperpigmentation, hepatosplenomegaly, and (rarely) xanthelasmas (caused by deposition of cholesterol) [10]. End-stage symptoms are those common to all liver etiologies of cirrhosis and include jaundice, ascites, encephalopathy, and upper digestive bleeding. Importantly, endoscopic signs of portal hypertension, such as esophageal varices or portal hypertensive gastropathy, can be encountered at histologically proven early-stage PBC, i.e., without evidence of liver cirrhosis, and are thought to be secondary to presinusoidal fibrosis and inflammation induced by granulomas [75].

Histological Features

According to Ludwig's classification [76], histology identifies four PBC stages (Table 18.2). Stage I is characterized by portal tract inflammation with predominantly lymphoplasmacytic infiltrates, resulting in vanishing septal and interlobular bile ducts (diameter less than 100 μm). At this stage, bile duct obliteration and granulomas (possibly found at all stages) are strongly suggestive of PBC. In stage II, a periportal inflammatory infiltrate is observed, and signs of cholangitis, granulomas, and florid proliferation of ductules are typical. Stage III is characterized by septal or bridging fibrosis, with ductopenia (over half of the visible interlobular bile ducts having vanished), and copper deposition in periportal and paraseptal hepatocytes can be seen. Stage IV corresponds to frank cirrhosis. Recently, a new staging and grading system for PBC that takes into account necroinflammatory activity and histological heterogeneity is proposed [77]. Scores for fibrosis, bile duct loss, and chronic cholestasis were combined for staging. Cholangitis activity (CA) and hepatitis activity (HA) were graded as CA0–3, and HA0–3, respectively. The diagnostic value of this new staging and grading system for PBC needs to be validated in the future [77]. Finally, the possibility of a sampling error should be considered when one is evaluating histology in PBC; in the case of variable staging within one biopsy, the highest stage should be accepted. Figure 18.1 illustrates the histological findings in two representative cases of PBC.

A peculiar characteristic of PBC that can be found at any histological stage is epithelioid granulomas with no signs of caseous necrosis. A large retrospective study has demonstrated that 23.8 % of cases of granulomas encountered in unselected

Table 18.2 Histological stages of PBC

Stage	Histological features
I	Portal tract infiltration with predominantly lymphoplasmacytic infiltrates, vanishing septal and interlobular bile ducts, granuloma
II	Periportal infiltration of lymphocytes, granuloma, biliary ductule proliferation
III	Septal or bridging fibrosis, ductopenia, copper deposition in periportal and paraseptal hepatocytes
IV	Cirrhosis

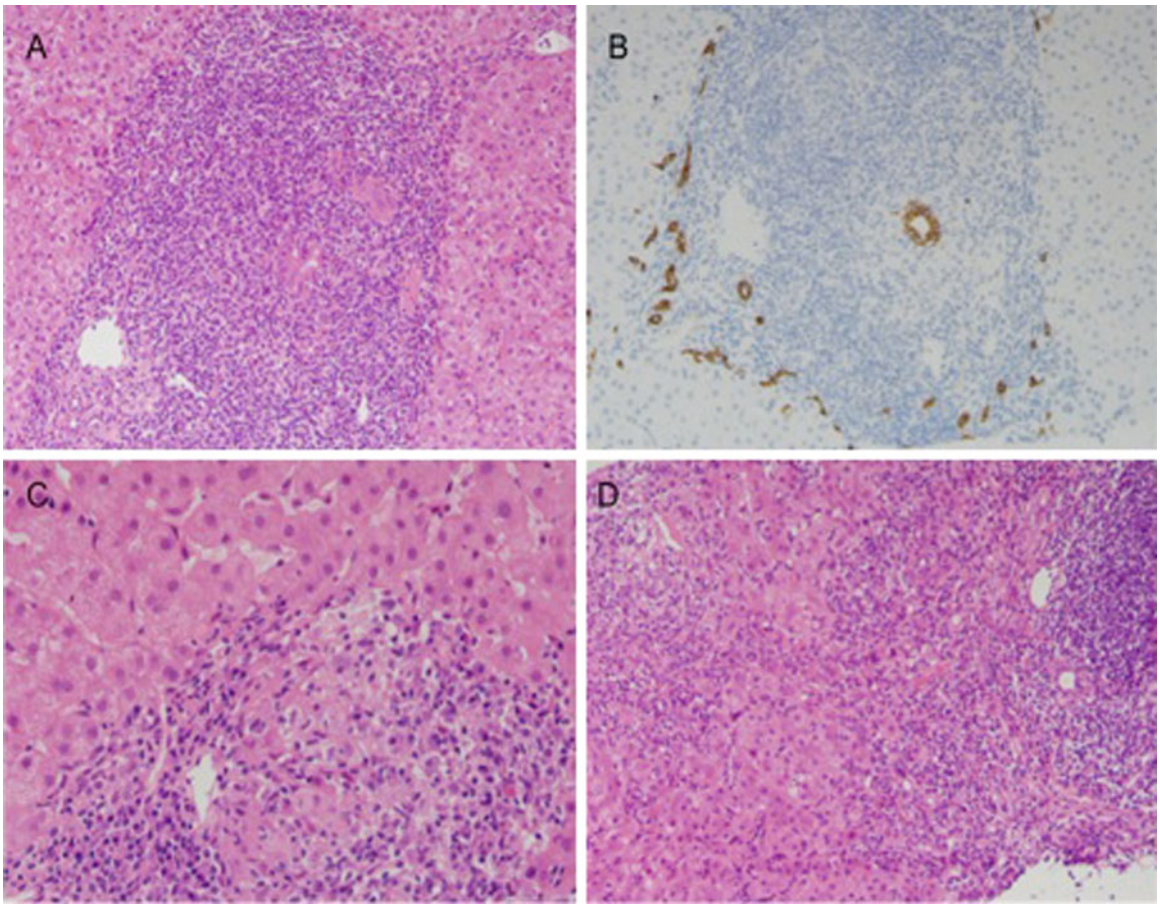

Fig. 18.1 The characteristic histological features of primary biliary cirrhosis. (**a**) Florid cholangitis (×200); (**b**) CK7 staining for bile ducts (×200); (**c**) epithelioid granuloma in portal tract (×400); (**d**) cholangitis with interface hepatitis (×200) ((**a**, **b**) pictures are from a same patient. (**c**, **d**) are from another patient)

liver biopsies could be attributed to PBC. The mechanisms leading to granuloma formation are still largely unknown, although experimental findings suggest that Gram-positive bacteria through lipoteichoic acid might initiate the process [78], and osteopontin might also mediate the recruitment of mononuclear cells [79]. Recent work has demonstrated that the dendritic cell marker CD11c is a sensitive tool to identify liver granulomas in PBC. Furthermore, immature DCs are important to the mechanisms leading to granuloma formation in PBC liver [80].

In addition, the presence of eosinophils in the portal tract is a specific finding in PBC histology [81], although its significance, along with a peripheral hypereosinophilia, is currently poorly understood [82]. Recently, it was reported that the loss of the canals of Hering reflected by CK19 immunostaining is an early feature in PBC; this "minimal change" feature may support a clinical diagnosis of PBC even in the absence of characteristic, granulomatous, duct destructive lesions [83].

Epidemiology

Epidemiology is expected to provide important clues to our understanding of the enigmatic etiopathogenesis of PBC. First, a systematic review of population-based studies indicates a wide range in the yearly incidence (0.33–5.8/100.000) and point prevalence (1.91–40.2/100.000) rates [62, 74, 84–98] (Fig. 18.2). Though different ethnic representations may also contribute, it is likely that methodological issues, based on the retrospective survey of diagnosed cases, and time trend play a major role, also in view of the prolonged asymptomatic period of the disease. Of note, the highest prevalence rates (35–40/100.000) were found in areas characterized by high medical awareness and easier access to healthcare. The steep increase in PBC prevalence observed in data collected over time and the wide geographical variations have been the object of discussion [99]. In some studies similar methodology led to widely variable data in different geographical areas or in groups with different ethnic backgrounds in a

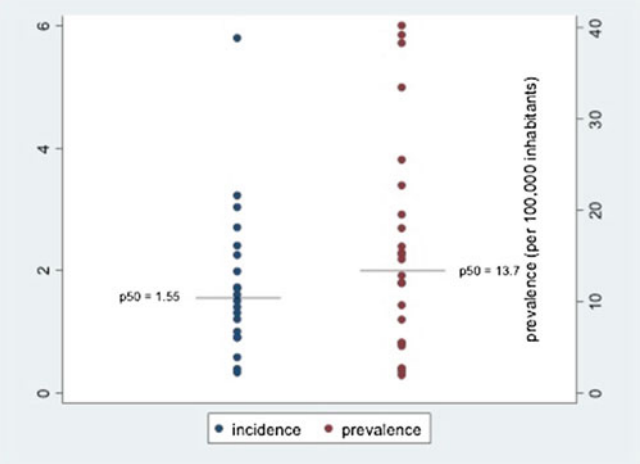

Fig. 18.2 PBC incidence and prevalence rates [62, 74, 84–98]

Table 18.3 Features of PBC for and against autoimmune pathogenesis

In support of autoimmunity
Antigen-specific serum autoantibodies
Autoreactive T cells
Adaptive transfer of cholangitis using $CD8^+$ T cells (in murine models)
Functional T regulatory defects
Female predominance
Genetic predisposition
Autoimmune comorbidity
Against autoimmunity
Absence of disease after autoantibody transfer (in mice)
Absence of correlation between titer of antimitochondrial antibodies and disease severity
Failure to respond to immunosuppressive agents (based on limited data)

phenomenon coined geoepidemiology [8], thus leaving the crucial questions unanswered and warranting this critical reappraisal of epidemiology papers. Second, the search for serum AMA in unselected population sera may identify the largest possible number of patients who have or will develop the disease. Indeed, a surprisingly high AMA prevalence rate, ranging between 0.43 and 1 %, appears likely in the general population despite the lack of adequate work-up in most studies [23]. Third, the median female to male ratio for PBC is classically accepted as 9–10:1 but is significantly lower for AMA prevalence (2.5:1), death certificates for PBC (4.3:1), and liver transplantation (6:1), thus suggesting that PBC in men may be underdiagnosed in early stages or manifest a more severe progression [67]. Lastly, studies of both PBC and serum AMA prevalence among family members and monozygotic twins strongly support the role played by genetic factors in the etiopathogenesis of the disease. In conclusion, PBC epidemiology is far from being a closed case, and the numerous open issues will be solved through a collaborative effort and powerful data mining tools.

Autoimmune Features

Several clinical and experimental findings strongly imply an autoimmune pathogenesis for PBC, being both a model and a paradox for autoimmune conditions (Table 18.3). The former is indicated by the characteristics of PBC that are common to other conditions, such as the female predominance, the genetic predisposition, or the presence of specific autoantibodies in the vast majority of cases. Such autoantibodies, however, in the case of PBC also constitute the basis for the disease being a paradox, as their direct pathogenetic role is still being defined [100].

PBC is characterized by the presence of detectable AMA in over 90 % of affected individuals, although we note that patients lacking AMA can present with a similar disease picture and progression as found in AMA-positive subjects, seemingly arguing against a pathogenic role for these autoantibodies. Autoreactive T cells, both CD4 and CD8, have been identified in AMA-negative PBC, and such lymphocytes and AMA recognize overlapping epitopes within the mitochondrial antigens [11, 101]. Second, autoantibodies should interact with the target antigen, the passive transfer of autoantibodies should reproduce the clinical features, and experimental immunization with the antigen should produce a model disease. An intriguing feature of PBC, and of certain other autoimmune diseases, is that the immunological offense is organ specific but the autoantigen is not tissue specific. As noted, no direct proof has yet been provided for a direct pathogenic role of AMA in the bile duct injury observed in PBC. Third, in autoimmune diseases the reduction in autoantibody levels should ameliorate the disease; this criterion is poorly fulfilled in PBC, in which there is no correlation between the pattern or titer of AMA and progression or severity of disease [3]. Finally, it is well established that most autoimmune diseases are responsive to immunosuppressive therapy. In PBC, all classic immunosuppressive agents have thus far proved relatively ineffective [14].

Autoantibodies

Antimitochondrial Antibodies (*AMAs*). Serum AMAs are highly specific for PBC and can be detected in nearly 95 % of patients. In most clinical settings, however, immunofluorescence techniques are used for initial screening of cases (Fig. 18.3). When AMAs are determined with more recently developed techniques, based on the use of recombinant mitochondrial antigens, e.g., immunoblotting, the specificity of the test is significantly higher [18, 19, 102] (Fig. 18.4).

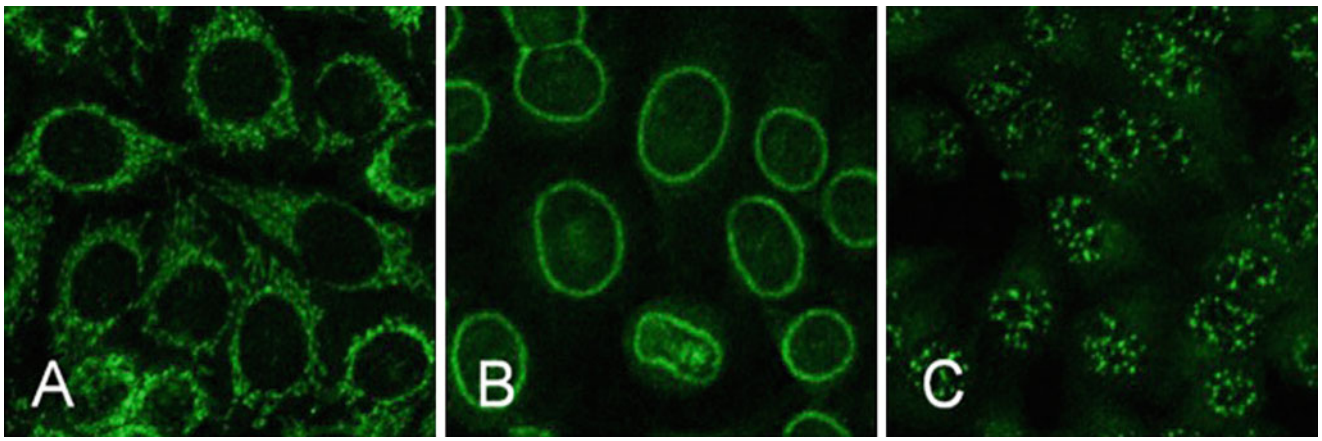

Fig. 18.3 Representative immunofluorscence pattern of PBC sera on HEP-2 cells. Sera samples from patients with PBC were diluted at 1:80 and analyzed for reactivity on HEP-2 cells by immunofluorescence. (**a**) Cytoplasmic pattern of AMA staining; (**b**) ring pattern of nuclear pore staining; (**c**) nuclear dot pattern

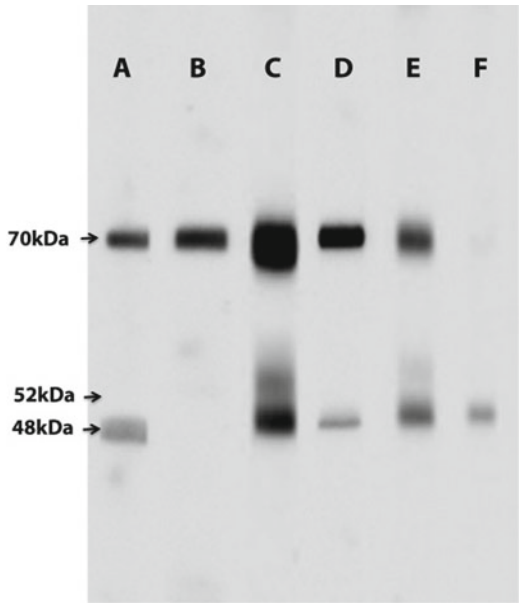

Fig. 18.4 Immunoblot of PBC sera against mitochondrial preparations. Sera from patients can react with one or more mitochondrial proteins. Sera samples from patients with PBC were diluted at 1:500 and analyzed for Ig reactivity against mammalian mitochondrial preparation by immunonlotting. Note the various pattern of AMA reactivity against PDC-E2 at 70 KDa, BCOADC-E2 at 52 KDa and OGDC-E2 at 48 KDa. Sera react to PDC-E2 and OGDC-E2 (lane A), PDC-E2 only (lane B). PDC-E2, BCOADC-E2 and OGDC-E2 (lane C), PDC-E2 and OGDC-E2 only (lane D and E), OGDC-E2 only (lane F)

Table 18.4 Major autoantigens in patients with PBC

Mitochondrial autoantigens	
E2 subunits of 2-OADC	PDC-E2
	OGDC-E2
	BCOADC-E2
Pyruvate dehydrogenase complex	E3BP
	PDC E1α
Nuclear autoantigens	
Multiple nuclear dots	Sp100
	PML
Nuclear membranes	gp210
	Nucleoporin p62
Centromeres	CENP A, B, and C

2-OADC 2-oxo acid dehydrogenase complex, *PDC* pyruvate dehydrogenase complex, *OGDC* oxoglutarate dehydrogenase complex, *BCOADC* branched-chain 2-oxo acid dehydrogenase complex, *E3BP* dihydrolipoamide dehydrogenase (E3)-binding protein

The epitopes of AMA are localized within the lipoyl domains within the E2 components of the 2-oxo acid dehydrogenase (OADC) family of enzymes, particularly the dihydrolipoamide acetyltransferase (E2 component) of the pyruvate dehydrogenase complex (PDC). Less frequent autoantigens are the E2 components of 2-oxoglutarate dehydrogenase (OADC-E2) and branched-chain 2-oxo acid dehydrogenase (BCOADC-E2) complexes, the E3-binding protein (E3BP), and the E1α subunit of the pyruvate dehydrogenase complex (PDC-E1α) [23] (Table 18.4). In a recent study, individual bead assays were done with the three mitochondrial autoantigens, PDC-E2, BCOADC-E2, and OGDC-E2; 20 % of the rigorously defined AMA-negative patient group had antibodies to one or more of the mitochondrial autoantigens. Furthermore, 100 % of these newly detected AMA-positive patients were ANA positive [19]. Although extremely useful as diagnostic marker, AMAs are not clinically helpful during follow-up as several studies demonstrate that they do not correlate with stage [23]. It is also noted that AMAs are often detectable for several years before the onset of overt clinical disease [103].

Antinuclear Antibody (*ANA*). As many as 50 % of patients with PBC have detectable serum ANA, most commonly

producing "nuclear rim" or "multiple nuclear dots" patterns by immunofluorescence (Fig. 18.3), based on recognition by the autoantibodies of gp210 and nucleoporin 62 (within the nuclear pore complex [NPC]) as well as Sp100 and polymorphonuclear leukocytes (PMLs) (possibly also cross-reacting with small ubiquitin-like modifiers [SUMOs]), respectively [22, 23]. Rim-like ANAs, on the other hand, react against proteins of the NPCs, supramolecular structures that include gp210 (a 210-kDa transmembrane glycoprotein involved in the attachment of NPC constituents within the nuclear membrane), p62 (a nuclear pore glycoprotein), and the inner nuclear membrane protein lamin B receptor (LBR). Both the perinuclear and nuclear dot ANA patterns are very specific for PBC [104] (Table 19.2), while anticentromere autoantibodies (ACA) are not specific and found in only 10 % of PBC patients [105], similar to other autoantibodies [106]. Serum anti-gp210 is detected in about 25 % (10–40 %) of AMA-positive and up to 50 % of AMA-negative patients (in both cases with high specificity). Autoantibodies reacting with p62 or LBR are found in about 13 % and 1 % of patients with PBC, respectively. Of interest, the ANA specificities have been found more frequently in patients with severe disease in cross-sectional studies [104, 105, 107–110] and, even more interestingly, the presence of anti-NPC is associated with worst prognosis [64, 111] in longitudinal observations. Interestingly, positive-anti-gp210 antibodies often represent a hepatic failure type of progression in PBC, while positive-anticentromere antibodies often reflect a portal hypertension type of progression [111]. These data have obvious relevant implications for the clinical management of PBC since anti-NPC and ACA testing are important for identifying asymptomatic patients with an unfavorable disease outcome and warranting early therapy [23]. Unfortunately, the pathogenic role of these antibodies has been poorly investigated and remains unknown.

An IgG/IgA dual isotype ELISA detecting the three major mitochondrial and nuclear (gp210 and sp100) antigens is an appropriate first-line test for the diagnosis of PBC, including for patients negative for markers assessed using conventional methods [18]. Recently, Sp140 has been identified as a new and highly specific autoantigen in PBC [112]. The very frequent coexistence of anti-Sp140, anti-Sp100, and anti-PML antibodies suggests that the nuclear body is a multiantigenic complex in PBC and enhances the diagnostic significance of these reactivities, which are particularly useful in AMA-negative cases [112]. In addition, an increased prevalence of ANA (targeting dsDNA, Sm, chromatin, ribosomal-P, RNP, SmRNP, SSA, SSB, and centromere) and thrombophilia-associated autoantibodies (i.e., anti-beta2GPI, phosphatydilserine, prothrombin) in PBC sera was demonstrated. Furthermore, there is an association between thrombophilia-associated autoantibodies and PBC stage [113].

Genetic Features

It is currently believed that the development of PBC, as well as of most of complex diseases, requires that an environmental factor, particularly a drug or an infection, initiates an autoimmune reaction in a genetically predisposed individual [114]. However, although strongly implicated by family and twin studies, no specific/reliable genetic factors involved in susceptibility to PBC have been identified and recapitulated.

Familial PBC and Genetic Predisposition. In the past, a scenario named "familial PBC" was proposed based on a number of studies reporting an increased risk of developing PBC within family members of affected individuals [114]. The main part of these studies as well as population-based epidemiological reports was performed in Great Britain, where the prevalence rates of familial PBC were reported to be 6.4 % [115]. A number of studies from North America, Europe, and Japan reported a similar figure ranging between 3.8 and 9.0 %. Also the sibling relative risk, another estimate of the familial prevalence of PBC, was found to be increased [115]. Finally, a recent large-scale US study showed an increased risk of disease (odds ratio at 10.7) in first-degree relative with PBC [52]. Of course, these findings might be explained by some shared environmental factors by family members. Also the coexistence with other autoimmune diseases in more than one-third of patients with PBC strongly suggests a role for genetic factor in this disease [52]. Similarly, the prevalence of AMA in first-degree relatives of patients with PBC is high as 13.1 %, while the control is 1 %. The identification and follow-up of these relatives may lead to earlier disease diagnosis and treatment [99]. Again, by evaluating 8 monozygotic and 8 dizygotic twin pairs in which at least one subject was affected by PBC, a concordance rate of 63 % was found, the highest among autoimmune diseases [116]. Finally, a role for genetics in PBC is also suggested by animal models of this disease [114].

Case–Control Association Studies and Clues from Genome-Wide Association Studies (*GWAS*). A large number of classical case–control studies have attempted to identify genes with a role in disease susceptibility and progression by evaluating one or few single-nucleotide polymorphisms (SNPs) [114]. Because of the autoimmune nature of PBC, most of these genes were already implicated in other autoimmune disorders and/or code for immune-related molecules, such as cytotoxic T lymphocyte antigen-4 (CTLA-4); tumor necrosis factor (TNF); vitamin D receptor; caspase 8; Toll-like receptors (TLRs); interleukins (IL) 1, 2, and 10; and numerous cytokine and chemokine receptors [114]. However, such approaches have led to very few insights into the genetic basis of PBC, mainly due to small sample size and lack

Table 18.5 Loci found to be associated with PBC by GWAS

Gene loci	Pre-GWAS	Canada/United States [117]	Italy–Canada/United States (meta-analysis) [118]	United Kingdom [119]	Japan [120]
HLA	Yes	Yes	Yes	Yes	Yes
IL12A	–	Yes	Yes	Yes	–
IL12RB2	–	Yes	Yes	Yes	–
IRF5/TNPO3	–	Yes	Yes	Yes	–
ORMDL3/IKZF3	–	Yes	–	Yes	Yes
MMEL1	–	Yes	–	Yes	–
SPIB	–	Yes	Yes	Yes	–
DENND1B	–	–	Yes	Yes	–
CTLA-4		Yes	–	–	–
STAT4	–	Yes	–	Yes	–
CD80	–	–	–	Yes	Yes
NFKB1	–	–	–	Yes	–
IL7R	–	–	–	Yes	Yes
CXCR5	–	–	–	Yes	–
TNFRSF1A	–	–	–	Yes	–
TNFSF15	–	–	–	–	Yes
POU2AF1	–	–	–	–	Yes

of replication. The data related to CTLA-4 gene association studies provide an example of a long list of studies performed with a long story of contrasting evidence, and no clear answer. Classical candidate gene studies with appropriate size and replication should only focus on investigating variant frequencies in different geographical areas, on dissecting interaction between risk loci, and on risk loci influencing outcomes, symptoms, and treatment response.

Since the recent completion of the human genome sequence, and thanks to impressive advances in molecular technology, the field of human genetics has changed, and we are now witnessing an explosion of new information about the allelic architecture of PBC as well as of many other human complex diseases [114]. The four GWAS in PBC have identified a number of non-Human leukocyte antigen (HLA) loci, with plausible candidate genes that indicate the involvement of the innate and adaptive immune systems in the etiopathogenesis of PBC [117–120] (Table 18.5). The first GWAS was performed in cases from North America [117], then more solid data were provided by combining datasets from the North American GWAS with a separate Italian GWAS [118], and finally a UK GWAS [119]. Taking together, these findings support a role for the TNF, TLR, and NF-kB pathways and, among the associations consistently reported, are those with the IL12A and IL12RB2 loci, the gene encoding interferon regulatory factor 5 (IRF5), the gene encoding the SPi-B transcription factor (SPIB), as well as two other loci, the gene encoding the IKAROS family zinc finger 3 (IKZF3) and that encoding ORM1-like 2 (ORMDL3), also implicated as a risk for other autoimmune diseases. Suggestive associations were also observed between PBC and DENND1B and the signal transducer and activator of transcription 4 (STAT4), two other loci associated with other autoimmune conditions. The list of associate genes is still growing, and the most recent UK GWAS identified novel associations between PBC and loci, such as NFKB1, IL7R, CD80, CXCR5, and TNFAIP2 [114]. This suggests caution and the need to focus future research studies on rare variants or on copy number variants or gene expression. A second observation is the impressive consistency among the findings of these three GWAS, thus indicating the presence of a common genetic pattern for PBC. The fourth GWAS in PBC was performed in Japanese populations and identified two significant susceptibility loci, TNFSF15 and POU2AF1, in addition to the HLA region [120]. Among 21 non-HLA susceptibility loci for PBC identified in the GWAS of individuals of European descent, only three loci (IL7R, IKZF3, and CD80) demonstrated significant associations in the Japanese population. Indeed, another study from Japan failed to confirm some genetic variants found in previous GWAS [121]. These observations indicate the existence of ethnic differences in genetic susceptibility loci to PBC and the importance of TNF signaling and B-cell differentiation for the development of PBC in individuals of European descent and Japanese individuals. In the future it will be important to replicate the reported associations in additional non-European populations. After the first GWAS evaluating association with common genetic variants, a second wave of GWAS has been initiated to demonstrate how data from dense fine-mapping arrays coupled with functional genomic data can be used to identify candidate causal variants for functional follow-up [122–124].

Human Leukocyte Antigen (*HLA*) *Associations*. The HLA, located in the major histocompatibility complex (MHC), is one of the most widely studied regions in the human genome because it contains important genetic information of many complex genetic diseases [125]. The role of HLA genes has yet to be fully dissected, but it is known that HLA genes encode cell-surface molecules that, by means of peptide presentation, mediate immunological events, such as cellular immune responses to tumors and pathogens, and of course self-tolerance. Similar to other genetically complex diseases, HLA has been extensively studied in PBC, but for a long time data have suggested only a low risk conferred by the HLA DRB1*08 allele; this was likely because early studies manifest limitations such as insufficient statistical power, lack of careful matching between cases and controls, and because multiple replications have rarely been carried out.

Only recently has the story begun to change when, in order to overcome these flaws, HLA variants in the largest PBC series have been evaluated [126], showing that PBC susceptibility is associated not only with the HLA DRB1*08 allele but also with protective DRB1*11 and DRB1*13 alleles, a finding later confirmed in other geographical areas [127]. Interestingly, because these protective alleles influence the penetrance of a number of infectious agents, these data support an infectious theory for PBC origin. The interest in HLA genes in PBC arising from these studies has been amplified by the three recent GWAS in PBC which identified the HLA region as the strongest associations [117–120]. Even more interesting, a recent study was able to better define the association of PBC with HLA, by genotyping 676 Italian cases and 1,440 controls with dense SNPs for which classical HLA alleles and amino acids were imputed [128]. Interestingly, not only has it been demonstrated that the HLA signals can be attributed to classical DRB1 and DPB1 genes but it has also been provided evidences by a conditional analyses supporting a predominant role of DRB1 (mostly *08, *11, and *14) and the independent association of DPB1 [128].

Role of Sex Chromosome Defects. It has been proposed that the presence of sex chromosome defects might explain both the genetic predisposition to the disease and the female preponderance in PBC [66]. Indeed, an age-dependent enhanced monosomy X in the peripheral white blood cells of women with PBC has been reported [129]; later that one X chromosome is preferentially lost [130], and finally that epigenetic factors influencing PBC onset are more complex than methylation differences at X-linked promoters [131]. More recently, our group demonstrated that the Y chromosome is lost more frequently in PBC males compared to healthy controls, and this phenomenon increases with aging [132]. We were, thus, able to confirm the existence of an analogous mechanism in the male population to previously identified X haploinsufficiency in female patients with organ-specific autoimmune disease, and we propose that this commonality might represent a relevant feature in the etiopathogenesis of autoimmune diseases that should be further investigated.

Environmental Influences

Although data on familial clustering of PBC and twin studies provide evidence for a genetic basis underlying PBC [133, 134], clusters of nonrelated individuals suggest that environmental factors also play a role in the development of the disease [135]. Environmental components including infectious agents and chemical xenobiotics [13, 136–139] have been implicated in initiating PBC.

Infectious Agents. The ability of infectious agents, particularly bacteria, to induce autoimmune responses in experimental settings has been documented, and molecular mimicry is the most widely studied mechanism explaining these observations [140]. This paradigm suggests that microbes present peptides sharing different degrees of homology with self-proteins, thus leading to a promiscuous antibody and cell-mediated immune response capable of reacting with both microbial and self-epitopes. T-cell activation produces cross-reacting T cells, leading to self-tissue destruction and thus perpetuating the autoimmune response, possibly through degeneracy of the TCR and cross-priming. Of the bacterial strains suggested to lead to PBC through molecular mimicry [141], the greatest amount of evidence has been reported for *Escherichia coli*, mostly based on the reports of an increased prevalence of urinary tract infections in patients with PBC [52].

Based on serum cross-reactivity, several infectious agents have been proposed for the initiation of PBC, including *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus aureus*, *Salmonella minnesota*, *Mycobacterium gordonae*, *Neisseria meningitidis*, and *Trypanosoma brucei* [138, 141]. More recently, the common commensal yeast *Saccharomyces cerevisiae* has also been investigated in PBC, based on the expression of AMA antigens in extramitochondrial sites, but serological studies have indicated that the reactivity of anti-S. *cerevisiae* antibodies was not specific for the disease [142]. Interestingly, contrasting evidence has been collected on the role of *Chlamydia pneumoniae* in the pathogenesis of PBC [143, 144]. Finally, our group has recently provided serological data suggesting that a ubiquitous xenobiotic metabolizing the Gram-negative bacterium *Novosphingobium aromaticivorans* is a candidate yet for the induction of PBC, as it elicits a specific antibody reaction (estimated to be 100- to 1,000-fold higher than that against *E. coli*) and its 16S rRNA-specific sequences were detected in human fecal samples [145].

For completeness, we also note that a human retrovirus has been proposed by one group as being involved in the

pathogenesis of PBC [146]. However, our laboratory failed to confirm such a hypothesis using a different molecular and immunological approach in a large series of patients and controls [147, 148], therefore discouraging the idea of the usefulness of any antiretroviral therapy in PBC and suggesting that the original data was flawed.

Xenobiotics. Xenobiotics are foreign compounds that may either alter or complex to defined self- or non-self-proteins, inducing a change in the molecular structure of the native protein sufficient to induce an immune response [139]. Such immune responses may then result in cross-recognition of the self form, which could in turn perpetuate the immune response, thus leading to chronic autoimmunity.

The human liver is at risk for chemical-induced injury because many environmental chemicals are metabolized primarily by hepatocytes [149]; such liver injuries are initiated by the metabolic conversion of xenobiotics into reactive intermediate species, such as electrophilic compounds or free radicals, which can readily alter the structure and function of cellular macromolecules to form neo-antigens. Furthermore, reactive intermediate species could lead to oxidative stress, deregulation of cell signaling pathways, dysfunction of biomolecules, organelle malfunction, and eventual cell death [150]. Although it is not clear how xenobiotics or the modified cellular proteins initiate autoimmunity in PBC, the analysis of serum samples from subjects with acute liver failure indicates that severe liver oxidant injury could lead to AMA production [151].

Compelling experimental evidence demonstrated that specific organic structures attached to the mitochondrial antigens were recognized by sera from PBC patients with a higher affinity than native forms of such antigens [137]. Such findings indicated for the first time that an organic compound may serve as a mimotope for an autoantigen, thus further providing evidence for a potential mechanism by which environmental organic compounds may cause PBC. One halogenated compound was able to induce AMA production in animal models [152].

We believe that the ability of lipoic acid to rotate by means of its "swinging arms" with respect to the bulk of the entire PDC-E2 molecule makes the dithiolane ring vulnerable to xenobiotic modification [153]. Recent quantitative structure–activity relationship (QSAR) analysis on a focused panel of lipoic acid mimic in which the lipoyl disulfide bond is modified suggests that direct alteration of the lipoyl ring—i.e., disruption of the S–S linkage—renders the lipoic acid "activated" and receptive for xenobiotic modification and subsequent AMA recognition [154, 155]. Data from immunological characterization of antigen and Ig isotype specificities against one such lipoyl acid mimic and rPDC-E2 strongly support a xenobiotic etiology in PBC. This observation is of significance in light of the high frequency of AMAs in patients with acute liver failure. In particular, we note that AMA with the same antigen and epitope specificity as in patients with PBC was found in almost 35 % of acetaminophen-poisoning subjects, suggesting that the PDC-E2 lipoyl domain is likely a target of acetaminophen-induced reactive oxygen species. The generation of highly reactive electrophilic metabolites such as NAPQI, which also deplete the intracellular glutathione pool, could render PDC-E2 vulnerable to further modification by electrophiles. Such mechanisms of in vivo generation of xenobiotic-modified self-proteins could lead to the breaking of tolerance to native proteins through molecular mimicry and antigen spreading in genetically susceptible individuals [13].

Risk Factors. Although genetics should be regarded as the major determinant in susceptibility to PBC, several other factors have been proposed. Our epidemiological study has demonstrated that a high risk of developing PBC is associated with a positive family history for PBC, a history of urinary or vaginal infections, comorbidity with other autoimmune diseases, lifestyle factors such as smoking, and previous pregnancies. Furthermore, we observed that the frequent use of nail polish also slightly increased the risk of having PBC [52]. There is a significant association between a lifetime tobacco consumption of > or =10 pack-years and advanced histological disease at presentation (OR = 13.3), suggesting that smoking may accelerate the progression of PBC. This could be induced by exposure to chemicals in cigarette smoke [156]. It was confirmed by a recent study in which smoking and the use of some cosmetics as well as urinary infections appear important for PBC among environmental risk factors. Among possible genetic risk factors, a family history of PBC is a strong association and that a previous history of obstetric cholestasis as another putative "genetic" risk [157]. Another study confirms some of the previously reported risk factors for PBC, namely, family history of disease and individual history of smoking, urinary tract infections, and autoimmune conditions, and interestingly identifies the use of oral contraceptives as a putative protective factor [158]. Significant clusters of PBC were identified surrounding toxic waste sites, suggesting that toxin exposure may be a risk factor influencing the clustering of PBC cases [136].

Animal Models

Several murine models that manifest characteristic clinical features of human PBC have been reported within the past few years (Table 18.6). Here, we discuss the current data on three spontaneous and one induced murine model of PBC. The spontaneous models are (a) NOD.c3c4, (b) dominant-negative TGF-β receptor II (dnTGFβRII), and (c) IL-2Rα$^{-/-}$

Table 18.6 Comparison of immunological and histological features of human PBC and animal models of autoimmune cholangitis

	Human	Spontaneous models			Induced models		
	PBC patients	NOD.c3c4	dnTGFβRII mice	IL-2Ra$^{-/-}$ mice	2-OA-BSA immunization	2-OA-BSA + alpha glyceramide immunization	*Novosphingobium aromaticivorans* immunization
Background/strain	N/A	NOD.c3c4	C57BL/6 Overexpression of a dn form of TGFβRII under CD4 promoter	C57BL/6	C57BL/6	C57BL/6	NOD 1101
B-cell immunity							
AMA	90–95 %	50–60 %	100 %	100 %	100 %	100 %	100 %
Dominant AMA target protein	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2
Dominant epitope	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain
Liver histology							
Portal lymphoid infiltrates	+++	+++	+++	+++	+	++	+
CD4 cell	+	++	+	+	+	+	+
CD8 cell	++	+	++	++	++	+++	+
B cell	+	–	+	+	+	+	+
Bile duct destruction	+–+++	+	++	+++	+	++	+
Granuloma	+–++	+	–	–	+	+	-

mouse line models. The major induced model is a xenobiotic-immunized mouse. Each of these animal models resembles the AMA profile that is specific to human PBC and contains lymphocyte infiltration with biliary epithelial cell (BEC) pathology (Table 18.1). The availability of these animal models has greatly facilitated the understanding of the immunological, genetic, and environmental components in the development of PBC.

NOD.c3c4 Mice Line

Nonobese diabetic (NOD) is a well-known mouse model that exhibits susceptibility to the spontaneous development of autoimmune insulin-dependent diabetes mellitus (IDDM) [159]. In addition to IDDM, NOD mice are also prone to develop other autoimmune syndromes. In 2006, Irie et al. [160] reported that NOD.c3c4 mice developed liver lesions and AMAs similar to that of patients with PBC. NOD.c3c4 mice have large chromosome 3 and 4 B6-/B10-derived regions of their genome introgressed onto the NOD background. NOD.c3c4 mice exhibit liver histopathological abnormalities including liver infiltrates, hepatic lesions on the portal tracts, epithelial granuloma-like formation, and early fibrosis. Fifty to sixty percent spontaneously develop autoantibodies to PDC-E2 at a relatively young age of 9–10 weeks [160], and 80–90 % develop antinuclear antibodies [160]. Histochemical analysis demonstrated that affected areas of the biliary epithelium are infiltrated with CD3$^+$, CD4$^+$, and CD8$^+$ T cells. The treatment of NOD.c3c4 mice with anti-CD3 protects them from autoimmune biliary disease. Furthermore, NOD.c3c4-scid mice develop disease after adoptive transfer of splenocytes or CD4$^+$ T cells, demonstrating a central role for T cells in the pathogenesis in this model. However, unlike human PBC, the mice also develop common bile duct dilation and proliferative biliary epithelium.

Dominant-Negative TGF-β Receptor II (dnTGFβRII) Mice

In 2006, Oertelt et al. reported the presence of PBC-like liver pathology and AMAs in the dnTGFβRII mice [161]. The dnTGFβII mice have an overexpression of a dominant-negative form of TGF-β receptor type II under the control of the CD4 promoter [162]. TGF-β receptor II is critical for signal transduction of TGF-β, which regulates the activation of lymphocytes. Deficiency in TGF-β results in various pleiotropic immunological abnormalities including colitis and early death [163, 164].

dnTGFβRII mice exhibit major serological and histological characteristics of human PBC, suggesting that the dnTGFβRII pathway is important in the pathogenesis of PBC [12, 161]. They are 100 % AMA positive with autoantibodies directed against PDC-E2, BDOADC-E2, and OGDC-E2, the major mitochondrial autoantigens in human PBC. The liver and serum cytokine levels reflect a Th1 profile. The liver histology of dnTGFβRII mice manifests lymphoid cell infiltration in the portal tracts of 100 % of the mice including CD4-, CD8-, and CD19-positive cells as seen in

human PBC. This is accompanied by bile duct injury in 25–50 % of mice up to 22 weeks of age, which is also seen in human PBC [161].

Similar to patients with PBC, there is an elevated level of CD8/CD4 T cells in the livers of dnTGFβRII mice [161]. To understand the role of $CD4^+$ and $CD8^+$ T cells in liver pathology in these mice, we performed adoptive transfer studies. The transfer of dnTGFβRII-derived $CD8^+$ T cells into $Rag1^{-/-}$ recipients resulted in liver-specific autoimmunity, whereas $CD4^+$ T-cell transfer led to colitis, indicating that $CD8^+$ T cells are the primary contributors for bile duct destruction in this model [12].

We also examined the role of AMAs in disease pathology in the dnTGFβRII mice model of PBC. Briefly, dnTGFβRII mice were crossed with B-cell-deficient mice ($IgM^{-/-}$) and were evaluated for the development of liver inflammation, as well as the severity of accompanying colitis. $IgM^{-/-}$ dnTGFβRII mice developed a more severe cholangitis and colitis compared to dnTGFβRII mice, indicating a suppressive effect of B cells on the inflammatory response in the dnTGFβRII mice [165]. To further determine the role of B cells in tissue pathology in dnTGFβRII mice, we examined the effects of therapeutic B-cell depletion using anti-mouse CD20 monoclonal antibody. Young (4–6 weeks) and old (20–22 weeks) dnTGFβRII mice were injected intraperitoneally with anti-CD20 every 2 weeks, and then the disease phenotype was compared with that of the control Ab-treated mice [166]. The treatment of young mice demonstrated a fully depleted serum AMA, a lower incidence of liver inflammation, and a fewer number of activated hepatic $CD8^+$ T cells, whereas colon inflammation was significantly exacerbated. In contrast, anti-CD20 treatment of animals with established disease was ineffective.

Previous studies have shown that CD1d expression and the frequency of CD1d-restricted NKT cells were increased in the livers of patients with PBC [167]. To examine the role of CD1d-restricted NKT cells in the pathogenesis of PBC, we generated $CD1d^{-/-}$ dnTGFβRII mice and showed that these mice had decreased mononuclear cell infiltration in liver and lower INF-γ serum levels, which ultimately ameliorated liver injury compared to that of dnTGFβRII mice [168]. Data from this work suggests that CD1d-restricted NKT cells have a primarily proinflammatory phenotype with a Th1 cytokine bias and promote deprivation of TGFβ signaling.

In addition to INF-γ, IL-12 has also been implicated in autoimmune inflammatory diseases. Interestingly, deletion of IL-12p40 in dnTGFβRII mice resulted in lower levels of inflammatory cytokines, immune infiltrates, and bile duct damage but does not alter AMA levels, whereas deletion of INF-γ has no effect on autoimmune cholangitis in the dnTGFβRII mice [169].

Although the dnTGFβRII mice exhibit features resembling that seen in PBC, it is also important to note that there are some differences from human PBC, such as the lack of a female bias, eosinophilic infiltration, and granuloma formation [170]. Nevertheless, the association with a decrease in peripheral Tregs in PBC patients [171, 172] and the role of TGF-β in immunomodulation makes the dnTGFβRII mice an interesting model for PBC.

IL-2Rα$^{-/-}$ Mice

IL-2 is critical for the development and peripheral expansion of $CD4^+$ $CD25^+$ Tregs, which promote self-tolerance by suppressing T-cell responses in vivo [173]. Previously, it was reported that a child with a genetic deficiency of IL-2Rα developed clinical manifestations similar to PBC [174]. Interestingly, C57BL/6J IL-2Rα$^{-/-}$ mice had serological and pathological characteristics resembling those of chronic nonsuppurative destructive cholangitis, which is also seen in human PBC. Serologically, IL-2Rα$^{-/-}$ mice are 100 % PDC-E2 positive and 80 % ANA positive. Their portal tracts exhibited an abundance of $CD4^+$ and $CD8^+$ infiltrates when compared with control mice and yield a higher percentage of $CD8^+$ than $CD4^+$ T cells. There is also evidence of decreased frequency of $CD4^+Fox3^+$Treg cells in the blood of IL-2Rα$^{-/-}$ mice, suggesting that the inflammatory lesions in the liver are due to a reduction in Tregs [175].

The role of T cells in autoimmune cholangitis is demonstrated through a series of experiments. This IL-2Rα$^{-/-}$ model reflects exacerbated intrahepatic biliary ductular destruction but had diminished colitis in IL-2Rα$^{-/-}$–$CD4^{-/-}$ mice and lacked biliary pathology in IL-2Rα$^{-/-}$–$CD8^{-/-}$ mice [176]. These observations are in agreement with adoptive transfer studies in dnTGFβRII mice, where $CD8^+$, but not $CD4^+$ T cells, was found to be the major T-cell player responsible for bile duct destruction. It should be noted that IL-2Rα$^{-/-}$ mice exhibit autoimmune cholangitis, concomitant inflammatory bowel disease, and that 25–50 % of IL-2Rα$^{-/-}$ mice die from severe hemolytic anemia between 8 and 20 weeks of age.

2-Octynoic Acid-BSA-Immunized Mice

Based on the premises of QSAR analysis that detectable levels of immunoreactivity of PBC sera against extensive panels of protein microarrays mimic the inner lipoyl domain of PDC-E2 [177], we hypothesized that xenobiotic modification of the native lipoyl moiety of the major mitochondrial autoantigen PDC-E2 may lead to the breach of tolerance in PBC. Previous studies demonstrated that rabbits immunized with one such xenobiotic, 6-bromohexanoate conjugated to bovine serum albumin, produced AMAs to PDC-E2, BCOADC-E2, and OGDC-E2, but without any PBC-like liver pathology [152]. In 2008, Wakabayashi et al. reported

that murine immunization with 2-octynoic acid (2-OA) coupled to bovine serum albumin (BSA) intraperitoneally (IP) induces antimitochondrial antibodies and cholangitis [178]. When using 2-OA-BSA as an immunogen in B6 mice and NOD1101 mice, it led to high-titer AMAs, portal inflammation, and autoimmune cholangitis similar to human PBC [179]. Using this mouse model, we investigated the role of B cells in PBC by depleting B cells using two different monoclonal antibodies, CD20 and CD79. The results of the experiment revealed that B-cell depletion led to exacerbated cholangitis, with higher T-cell infiltrates and inflammatory cytokines, indicating a protective role of B cells in PBC [166].

Taking advantage of our experience in this xenobiotic-induced model of PBC, we have investigated the role of innate immunity and natural killer T (NKT) cells on modulating disease activity in this xenobiotic-induced mouse model. Briefly, we immunized mice with and without the addition of α-galactosylceramide (α-GalCer), an invariant NKT-cell activator. 2-OA-BSA-immunized mice exposed to α-GalCer developed a profound exacerbation of their autoimmune cholangitis, including significant increases in $CD8^+$ T-cell infiltrates, portal inflammation, granuloma formation, and bile duct damage. More excitingly, these mice produced increased levels of AMA and have evidence of fibrosis, a feature not previously reported in any other murine models of PBC [180]. These results are critical and emphasize the role of innate immunity in the natural history of PBC. Furthermore, the data also provides clues to the mechanisms by which biliary disease becomes perpetuated in humans as well as explaining the recurrence of PBC following liver transplantation in the absence of MHC compatibility. Thus, in the absence of MHC restriction, disease reoccurrence would depend on a nonadaptive cellular mechanism, i.e., innate immunity, suggesting that BECs are more than simply an innocent victim of an immune attack. Rather, they attract immune attack by virtue of the unique biochemical mechanisms by which they process handle PDC-E2 during apoptosis [181, 182]. Our data would also explain the relative failure of immunosuppressive drugs to alter PBC, because such agents are ineffective against innate mechanisms.

Pathogenic Mechanisms

There have been significant advances in our understanding of the immunobiology of PBC and, in particular, a rigorous dissection of not only the serological abnormalities, including AMA but also the definition of autoreactive CD4 and CD8 cells [183]. One of the major open questions in PBC is the selective destruction of small bile ducts in PBC despite the presence of mitochondrial antigens in virtually all nucleated cells. Of relevance, Odin and colleagues demonstrated that PDC-E2 remains immunologically intact in BECs following apoptosis and it is still recognizable as such by AMA [184]. It is reasoned that the absence of glutathiolation [184, 185] may contribute to this unique feature of the BEC. Moreover, it has been reported that PDC-E2 is preserved in apoptotic bodies of BEC, but no other epithelial cells, during apoptosis, constituting an apotope [186], which is able to induce proinflammatory cytokine secretion from mature monocyte-derived macrophages from patients with PBC in the presence of AMA, including high levels of IL-12 [118, 181, 186]. It has been postulated that the unique apoptotic features of BECs allow the exposure of a potent intracellular autoantigen to the PBC-associated multi-lineage autoimmune response that leads to the tissue-specific autoimmune injury. This scenario justifies the biliary specificity of PBC, its recurrence following OLT [187], the therapeutic failure of immunosuppressive agents [3, 188], as well as the effects of UDCA in PBC, a drug that has anti-apoptotic properties [189].

Adaptive Immunity. The involvement of cellular immune mechanisms in the biliary damage is clearly suggested by the presence of high number of helper ($CD4^+$) TCR $\alpha\beta^+$ and $CD8^+$ T cells in the portal tracts from patients with PBC [190–194]. Autoreactive PDC-E2-specific CD4 T cells have been reported in both peripheral blood and liver tissue of patients with PBC but not in healthy and disease controls. In support of their role in the liver damage, a 150-fold increase in number of CD4 T cells specifically targeting PDC-E2 was found in the peri-hepatic lymph nodes and liver compared with blood of patients with PBC. Our group also characterized the antigen specificity of these cells and demonstrated that in HLA DR4*0101-positive patients, autoreactive CD4 T cells recognized a single epitope of 163–176 aa sequence which encompass the lipoic acid-binding residue of the inner lipoyl domain of PDC-E2 which is shared by serum AMA. Furthermore, our group demonstrated that these cells are of proinflammatory nature only in PBC patients but not in controls, based on the production of proinflammatory cytokines such as IFN-γ [193]. Autoreactive T cells are believed to be involved in the pathogenesis of PBC, and infiltration of T cells is believed to be one of the major immunological features of the disease [193, 195, 196] including in AMA-negative cases [197, 198]. However, the findings point to a predominant role for the $CD8^+$ T subpopulation in PBC [12, 176]. Notably, the HLA class I-restricted epitope for $CD8^+$ T cells, i.e., 159–167 amino acid residues, maps closely to the epitopes recognized by serum AMA as well as by CD4 T cells, that is, the autoepitope for both CD4 and CD8 T cells overlaps with the B-cell (AMA) epitope. As for autoreactive $CD4^+$ T cells, there is approximately a tenfold higher frequency of PDC-E2159–167-specific CD8 T cells within the liver compared to blood of PBC patients. Functionally, it has been shown that autoreactive CD8 T cells in this disease

have the ability to produce IFN-γ rather than IL-4/IL-10 cytokines [199].

Regulatory T Cells. Recent studies have pointed out the critical role of $CD4^+$/CD25high regulatory T cells (Tregs) in the prevention of autoimmune disease [200–203]. An important role for $CD4^+$/CD25high Tregs in the prevention of autoimmunity and maintenance of self-tolerance has also been hypothesized. Some studies have demonstrated that the transfer of T cells lacking the $CD4^+$/CD25high Treg subset into athymic nude mice results in the development of various T-cell-mediated autoimmune diseases. Experimental data demonstrate that PBC patients display significantly lower frequencies of $CD4^+$/CD25high Tregs as percentages of total TCR-α^+/$CD4^+$ T cells, which may contribute to the breakdown of tolerance in PBC [172]. Similarly, the CD8 Treg subset as a special regulatory T-cell subpopulation has significant phenotypic alterations, including increased expression of CD127 and reduced CD39 in patients with PBC. Furthermore, in vitro induction of CD8 Tregs by incubation with IL-10 is significantly reduced in PBC patients [204].

Th17 Cells. Interleukin-17 has been recently identified as a key cytokine involved in numerous autoimmune processes. $CD4^+$ T cells are a major source of IL-17, which compose a distinct T helper subset (Th17 cells). The frequency of IL-17^+ lymphocytic infiltration in liver tissues from PBC patients and those with other liver dysfunctions is increased as compared to healthy livers [205]. IL-2 receptor alpha knockout mice as murine model of human PBC also demonstrate marked aggregations of IL-17-positive cells within portal tracts and increased frequencies of Th17 cells in liver compared to the periphery. Furthermore, the liver microenvironment plays a role in Th17 induction in cases of liver autoimmunity and other liver inflammatory diseases [205].

Innate Immunity. The role of the innate response in PBC has been overlooked until recently when several studies have shed promising light and suggest a role of innate immunity in the onset and perpetuation of autoimmune cholangitis [180, 206–208]. Indeed, genome-wide case–control association studies in PBC have identified a significant association with genes of the innate immune system, i.e., IL-12A interleukin-12 receptor, beta2 subunit (IL-12RB2), and STAT4 polymorphisms [117–119]. The most important target cells of IL-12 are T cells, NK cells, and NKT cells, for which IL-12 induces proliferation, differentiation, enhancement of cytotoxicity, and the production of cytokines, particularly IFN-γ, and B cells, for which IL-12, directly or through the effects of IFN-γ, enhances the activation and production of Th1-associated classes of immunoglobulin. Further, IL-12 through STAT4 activation stabilizes t-bet, which itself drives Th1 differentiation [209]. The IL-12 heterodimer signals through the cell-surface IL-12 receptor, which is composed of two chains, IL-12Rβ1 and IL-12Rβ2. IL-12R is expressed mainly by activated T cells and NK cells but has been shown also on other cell types, such as DCs and B-cell lines. The specific cellular effects of IL-12 are due mainly to its ability to induce the activation of the transcription factor STAT4 [206].

Data demonstrated that not only are peripheral monocytes increased in frequency and absolute number in PBC patients compared to controls but that they also produce significantly increased levels of proinflammatory cytokines (IL-1, IL-6, and TNF-α) when cultured and challenged with different ligands for TLR2, TLR3, TLR4, TLR5, and TLR9 [210]. Moreover, BECs produce monocyte chemotactic protein-1 (MCP-1/CCL2) as a result of the innate immune response, and bile ductules play a role in hepatic fibrosis caused by hepatic stellate cells. Also, biliary innate immune responses induce the production of two chemokines, fractalkine and macrophage inflammatory protein-3α (MIP-3α), causing the migration of inflammatory cells and a population of antigen-presenting cell (APC) found in epithelium, Langerhans cell, and involve chronic cholangitis associated with biliary epithelium-specific innate and acquired immunity in PBC [211].

The innate immunity in PBC patients is characterized by an increased response to pathogen-associated stimuli, as indicated by higher levels of proinflammatory cytokines secreted by monocytes from patients with PBC after exposition to microorganism patterns [210]. It has been demonstrated that there is a marked increase in the frequency and absolute number of blood and liver NK cells in PBC patients. Moreover, in the same study the cytotoxic activity and perforin expression by isolated NK cells were significantly increased in PBC patients associated with increased levels of plasma IL-8 and the expression of IL-8 receptor on NK cells. In contrast, the levels of IFN-γ, IL-6, and IL-8 synthesized by NK cells were significantly decreased in PBC as compared to controls [167]. Whereas the innate immune system hyperresponsiveness, in its sole entity, is probably not sufficient for the breakdown of tolerance, these alterations might ultimately play a role in the initiation and/or perpetuation of the autoimmune adaptive response.

The Immunological Role of Biliary Epithelial Cells

Small BECs, which line the intrahepatic biliary epithelium, are the target cells of the immune destruction in PBC (Table 18.7). Although they represent a small proportion (3–5 %) of the cells of the liver [212], due to the exposure of the biliary tract to foreign antigens, they are equipped to respond through various immunological pathways [213].

Table 18.7 The immunological role of biliary epithelial cells

Cholangiocyte expression	Function	Mechanism
TLRs 2, 3, 4, and 5	Recognize pathogens	
IRAK-M	Maintain tolerance	Negative regulator of TLR signaling
PD ligands, TRAIL	Limit immune response	Induce apoptosis of leukocytes
IL-6, IL-8, and MCP-1	Chemotactic	Recruitment of immune cells to protect against infection
Defensins, cathelicidin	Antimicrobial, chemotactic	Disrupt microbial membranes; recruit CD4+ T cells and immature dendritic cells
ICAM-1, LFA-3, VCAM-1	Cholangiocyte–leukocyte interaction	Leukocyte migration to inflammatory sites
HLA class II molecules	Antigen presentation	
CD80, CD86, CD40	Costimulation of T cells	

TLRs Toll-like receptors, *IRAK-M* interleukin-1 receptor-associated kinase M, *PD* programmed death, *TRAIL* TNF-related apoptosis-inducing ligand, *MCP-1* monocyte chemotactic protein-1, *ICAM-1* intercellular adhesion molecule 1, *LFA-3* lymphocyte-associated antigen 3, *VCAM-1* vascular cell adhesion molecule-1, *HLA* human leukocyte antigen

Indeed, BECs participate in immune responses; protect against pathogens by expressing TLR and antimicrobial peptides [214]; act as APCs by expressing HLA molecules and costimulatory molecules [215]; recruit leukocytes to the target site by expressing adhesion molecules [216], cytokines, and chemokines [217]; and induce apoptosis of leukocytes to limit the immune responses [218]. PBC is a mucosal disease, and a balance between inflammatory responses and tolerance is a key in mucosal environments. The immunopathological characteristics of BEC may strongly contribute to their unique vulnerability and thus the biliary specificity of PBC. However, recent studies regarding apoptosis in BECs suggest that this is not the only factor involved.

Apoptosis in PBC

Apoptosis is essential in maintaining immune cell populations [219, 220]. Several reports suggest a correlation between apoptosis and autoimmunity through an impairment of apoptosis or an ineffective removal of apoptotic bodies, leading to the release of intracellular components that are a potential source of autoantigenic stimulation [221–225] and autoimmunity onset [226–228]. The presence of intact autoantigens within apoptotic bodies [229], their participation in the processes involved in autoantigen presentation [230], and the activation of innate immunity through macrophage cytokine secretion in concert [231] are likely links between apoptosis and autoimmunity [220]. Several studies have investigated apoptosis of BECs specifically in PBC. There is an increased DNA fragmentation, implying increased apoptosis, in the BEC of patients with PBC when compared with normal controls [232, 233]. Fas, FasL, perforin, granzyme B, and TRAIL expressed significantly greater levels on BECs of patients with PBC [217, 218, 233]. In addition, the upregulation of WAF1 and p53 related to biliary apoptosis is found in BECs of PBC [234]. TdT-mediated deoxyuridine triphosphate nick-end labeling staining has also shown significantly greater apoptosis of BECs in PBC than in other chronic cholestatic diseases, even when controlled for similar degrees of inflammation [218, 233–235]. Notably, BEC apoptosis is of considerable importance for understanding PBC because there are qualitative differences between the metabolic processing of PDC-E2 during apoptosis of BECs compared with other epithelial cells [184, 185, 206, 227]. In addition, it has been demonstrated that PDC-E2 is immunologically intact during apoptosis in BECs and it localizes in the apoptotic bodies of BECs where it is accessible to AMA recognition [186], although the mechanism by which PDC-E2 translocates to the cell membrane has not been elucidated; PDC-E2 was not detected in apoptotic blebs from a number of other epithelial cell lines [186, 236], whereas seven mitochondrial and four nuclear proteins were present in naive, untreated cultures of BECs and epithelial controls [236]. Finally, recent data show that there is an intense inflammatory cytokine production in the presence of the unique triad of BEC apotopes, macrophages from PBC, and AMAs [181]. These latter findings provide a mechanism to understand the biliary specificity of PBC, the recurrence of disease following liver transplantation, the success of ursodiol in treating PBC, and emphasize a critical role of the innate immune system in the perpetuation of this autoimmune disease.

Treatment

Several medical treatments have been investigated in patients with PBC (Table 18.8). Currently, UDCA is the only accepted and licensed therapy for PBC.

UDCA. UDCA accounts for 4 % of the bile acid pool in human bile. Compared with other bile acids, such as chenodeoxycholic and deoxycholic acids, UDCA is more hydrophilic. Its absorption (30–60 % following an oral dose) occurs mainly in the small intestine, and its presence decreases cholesterol secretion into bile, possibly lowering

Table 18.8 Efficacy and toxicity of the principal drugs investigated for the medical treatment of PBC

Drugs	Efficacy	Toxicity
D-penicillamine	–	+
Cyclosporine	+/–	+
Chlorambucil	+/–	+
Azathioprine	+/–	+
Methotrexate	+/–	+
Glucocorticoids	+/–	+/–
Colchicine	+/–	–
Ursodeoxycholic acid	+	–
Fibrates	+	–

its conversion to bile acids. The mechanism of action of UDCA in PBC is incompletely understood, but it has been hypothesized that it is based on different factors, including modification of the bile acid pool, reduction in proinflammatory cytokines, effects on apoptosis and on vasoactive mediators, and modification of the bile acid pool. However, since UDCA's anti-inflammatory effects are found only in bile ducts, it has been assumed that its effect is mediated by modification of the bile acid pool. Recently, it was shown that there is a strong correlation of biliary and trough plasma enrichment of UDCA in PBC and healthy patients, enhanced taurine conjugation of biliary chenodeoxycholic acid, a putatively protective mechanism, in PBC, and reveals stabilization of intestinal detoxification by UDCA via posttranscriptional upregulation of key duodenal export pumps BCRP and P-glycoprotein [237]. Doses ranging from 13 to 15 mg/kg of UDCA are currently used and lead to optimum bile enrichment. Accordingly, a meta-analysis demonstrated that increased survival is obtained only when a dose greater than 13 mg/kg is prescribed [238], even though a complete biochemical response to UDCA (normalization of serum liver tests in the absence of cirrhosis) is achieved in approx 40 % of treated patients [239]. Pares et al. have recently demonstrated that biochemical response to UDCA after 1 year is associated with a survival similar to that of the matched control population, supporting the favorable effects of this treatment in PBC [240]. Similarly, Kuiper et al. have shown that prognosis for UDCA-treated patients with early PBC is comparable to that of the general population. Survival of those with advanced PBC with biochemical response to UDCA is significantly better than for nonresponders. Thus, UDCA may be of benefit irrespective of the stage of disease [241]. Other criteria have been proposed to define UDCA response and prognosis during UDCA therapy [2, 20]. Most recently, among UK-PBC cohort of patients with PBC, response to UDCA treatment and symptoms are related to sex and age at presentation, with the lowest response rates and highest levels of symptoms in women presenting at <50 years of age [242]. However, a systematic review and meta-analysis of 16 randomized clinical trials on 1,447 patients was performed to evaluate the efficacy UDCA versus placebo or no intervention. The study showed that UDCA did not provide any benefits in mortality and mortality or liver transplantation in patients with PBC. UDCA did not improve pruritus, fatigue, autoimmune conditions, liver histology, or portal pressure. Although UDCA appears to improve certain biochemical variables, such as serum bilirubin, and ascites and jaundice, the findings were based on limited data [243, 244]. Similar observations were also reported in an independent study [245].

Other Medical Treatments. Based on the success rates observed in other autoimmune diseases, the use of immunosuppressive drugs has been attempted in PBC, but efficacy has been poor. Immunosuppressive drugs used in PBC have included corticosteroids, azathioprine, cyclosporine, methotrexate, penicillamine, and colchicines (Table 18.8). Their use is currently encouraged only in combination with UDCA in selected cases. In the event of an unsatisfactory response to UDCA alone, these drugs are still considered, but the lack of efficacy and the risk of serious side effects make their use highly debatable. Definitive data are still awaited on the efficacy of UDCA plus, mycophenolate mofetil [246], methotrexate [188], budesonide [247, 248], and tamoxifen [71, 249]. Among these, fibrate is a widely used hypolipidemic agent and is well known as a ligand of the peroxisome proliferator-activated receptors. Recently, this agent has come to be recognized as a potential anti-cholestatic drug for the treatment of PBC that does not respond sufficiently to UDCA therapy [250]. Most recently, a study from Japan has demonstrated that bezafibrate is a dual PPAR/PXR agonist with potent anti-cholestatic efficacy in early-stage PBC patients with an incomplete biochemical response to UDCA therapy [251]. A number of novel drugs, such as an FXR agonist molecule and an anti-IL-12 monoclonal antibody, are currently under evaluation.

To examine if the development of PBC can be inhibited by blocking T-cell activation and hence the development of autoimmune cholangitis in our xenobiotic-induced model of PBC, we administered CTLA-4 Ig one day before xenobiotic immunization and monitored the animals for AMA and liver histology. CTLA-4 Ig completely inhibited the manifestations of cholangitis, including AMA production, intrahepatic T-cell infiltrates, and bile duct damage. More importantly, treatment with CTLA-4 Ig after the development of autoimmune cholangitis in xenobiotic-immunized mice also resulted in significant therapeutic benefit, including reduced intrahepatic T-cell infiltrate xenobiotic cell damage, although AMA levels were not altered. These data suggest that an optimized regimen with CTLA-4 Ig has the potential to serve as an investigative therapeutic tool in patients with PBC [252].

With the development of well-characterized monoclonal antibodies specific for the B-cell populations, anti-CD20 and

anti-CD79, we have taken advantage of our well-defined xenobiotic-induced model of autoimmune cholangitis to examine the effect of B-cell-specific depletion in the pathogenesis of murine PBC. Our results showed that in vivo depletion of B cells using either anti-CD20 or anti-CD79, prior to the induction of disease, resulted in the development of a more severe form of cholangitis than in the isotype-matched control monoclonal antibody control mice. In fact, anti-CD20-/CD79-treated mice had increased liver T-cell infiltrates and higher levels of proinflammatory cytokines.

On the other hand, we conducted a clinical study to determine the safety and potential efficacy of B-cell depletion with the anti-CD20 monoclonal antibody rituximab in patients with PBC and an incomplete response to UDCA. This open-label study enrolled six patients with PBC and incomplete responses to UDC to be treated with 2 doses of 1,000 mg rituximab separated by 2 weeks and followed for 52 weeks. The primary end points were safety and changes in B-cell function. Four out of six patients completed the study with notable beneficial effects including decrease in total serum IgG, IgM, and IgA as well as AMAs by 16 weeks and thereafter returned to baseline levels by 36 weeks. Transient decreases in memory B-cell and T-cell frequencies and an increase in CD25(high) CD4(+) T cells were observed after treatment. These changes were associated with significant increases in mRNA levels of FoxP3 and TGF-beta and a decrease in TNF-alpha in $CD4^+$ T cells. Notably, serum ALP levels were significantly reduced up to 36 weeks following rituximab treatment. These data, which differ from the xenobiotic-induced mouse model, suggest that anti-CD20 monoclonal is a potential mechanism for the treatment of patients with PBC with an incomplete response to UDCA [253].

Liver Transplantation. Liver transplantation is the ultimate treatment for end-stage PBC, with survival rates of 92 and 85 % at 1 and 5 years after transplant, respectively [254]. Interestingly, the frequency of OLT for PBC in a large series from the United Kingdom was reported to have decreased over the past decade, along with increased age at the time of transplantation. Cumulatively, such data could once again indicate that the natural history of PBC might be influenced by earlier diagnosis or medical treatment. The use of UDCA in transplanted patients is currently considered safe, and no contraindications have been identified so far. In a recent study, 81 patients who underwent living donor liver transplantation for PBC were followed up for 6.2 years. The 5-year patient survival rate was 80 % with 1 % recurrence. The nonrelated or blood-related donor factor and number of HLA matches did not correlate with prognosis [255]. Prevalence rates for recurrent PBC reported by individual liver transplantation programs range between 9 and 35 % [254]. Some of the risk factors of recurrent PBC may include recipient factors such as age, gender, HLA status, and immunosuppression, as well as donor factors such as age, gender, and ischemic time. Experimental data have demonstrated that in the early recurrent PBC after liver transplantation, there is a biliary epithelial–mesenchymal transition driven by TGF-β, which potentially explains BEC loss in the livers [256].

Overlap Syndromes

The International AIH Group recently proposed that patients with features of more than one autoimmune liver disease should be categorized according to whether they manifest predominantly as AIH, PBC, or PSC/small duct PSC, thus ruling out the possibility that the coexistence of the two conditions represents a nosological entity per se, as suggested for other autoimmune coexisting conditions [257]. AIH–PBC overlap syndrome is found in 10 % of adults with AIH or PBC. Besides overlaps, transitions are also possible in rare cases from PBC to AIH or AIH to PBC. Thus, the clinical management of overlap syndromes is based on single diseases, whereas medical treatment is empiric. Therefore, UDCA is used for chronic cholestasis, immunosuppressants (mainly steroids and azathioprine) are used for AIH, and liver transplantation is indicated for end-stage disease. A recent study has demonstrated that the combination of UDCA and immunosuppressors appears to be the best therapeutic option for strictly defined PBC–AIH overlap syndrome [258]. Most recently, our group found that plasma IgG $\geq 1.3 \times$ ULN had a sensitivity of only 60 % but a specificity of 97 % in identifying cases of corticosteroid-responsive PBC–AIH overlap syndrome, while the use of a higher threshold of $2.0 \times$ ULN reduced the sensitivity to 10 %. This is of particular significance since the Paris criteria include IgG of $2.0 \times$ ULN and modifying this criterion to a lower level may be helpful in identifying the corticosteroid-responsive PBC–AIH variant [259].

Concluding Remarks

PBC is considered a unique disease within the range of autoimmunity, and future efforts should be dedicated to overcoming some conceptual and logistic difficulties. First, only study of a very large number of patients will unravel the genetic basis of PBC. Given the relatively rare prevalence of the disease, only a worldwide effort will allow the collection of a population large enough to guarantee enough statistical power for a linkage analysis. Second, the role of xenobiotics and infectious agents in the onset of PBC should be further probed, particularly with respect to the development of animal models and the use of detailed epidemiological studies

to ascertain the exposure to specific environmental factors. Third, it is crucial to determine the pathogenic role of AMA in bile duct damage of PBC. Once again, the development of an animal model appears to be the only way to provide a clear demonstration of such a pathogenic mechanism. Finally, from a clinical standpoint, new clinical trials are needed to identify novel second-line therapies in the long-term treatment of PBC. It is indeed clear that there is a significant unmet need in PBC, with an important subgroup of patients failing to respond to current licensed therapy. Successful applications of second-line therapy will lengthen survival and, we would anticipate, improve the quality of life of patients. Together with the trend toward an earlier diagnosis of the disease, more effective medical treatment, possibly using specific monoclonal antibodies, will be the cornerstone in reducing the need for liver transplantation in patients affected by PBC.

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Autoimmune Hepatitis

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Key Points

- Autoimmune hepatitis (AIH) is characterised 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 antibodies (ANA) and/or anti-smooth muscle antibodies (SMA), 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 on a freshly prepared rodent substrate that includes the 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 immunoassays 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 radioimmunoassays, 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, while 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).
- 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, anovulatory amenorrhoea and profoundly elevated serum immunoglobulins [1]. The presence of lupus erythematosus cells and the detection of anti-nuclear 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 recognised in the early 1960s,

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resulted in the publication of three controlled clinical trials which incontrovertibly showed the life-saving value of corticosteroids in the treatment of "HBsAg-negative hepatitis" [3–5]. The recognition that "chronic active autoimmune hepatitis", as it was then known, 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, characterised 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], subsequently revised [8]. More recently, a simplified system, designed for use in clinical practice, has been proposed by the group [9].

Two types of AIH are recognised 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 characterised 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). Immunosuppressive therapy, which remains the mainstay of treatment, should be instituted as soon as the diagnosis is made and, generally, the response is good. If left untreated, AIH usually progresses to liver failure requiring transplantation. The aetiology of AIH 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 [10]. This chapter will review recent breakthroughs in our understanding of the pathogenesis of AIH, linking them to advances in clinical practice.

Epidemiology

The exact prevalence of AIH is unknown due to a lack of epidemiological studies performed after the introduction of the standardised diagnostic scoring system introduced by the IAIHG. 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 [11]. 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 [12], but these figures are biased by the fact that the study was hospital-based in a tertiary referral centre. Notably, the first study to utilise the IAIHG scoring system reports a much higher prevalence of definite AIH; 35.9 cases per 100,000 within the native Alaskan population [13]. More recently, another study using the IAIHG standardised 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 [14]. 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 [15]. 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 [16].

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 sevenfold 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 with AIH-1 and one-third with AIH-2.

AIH is characterised by strong female preponderance, with a female/male ratio of approximately 3.6:1 [17]. Although AIH was historically believed to be a disease of the young and middle aged, it can present from childhood to late adulthood and can affect all ethnicities [18].

Aetiology and Pathogenesis

Genetics

Several genetic factors interact to influence susceptibility to AIH, clinical manifestations, response to treatment and overall prognosis.

The strongest genetic associations are found within genes of the human leukocyte antigen (HLA) region (the human major histocompatibility complex, MHC)—located on the short arm of chromosome 6 (Table 19.1)—which are involved in the presentation of antigenic peptides to T cells, and are therefore implicated in the initiation of an adaptive immune response [10].

There are particularly strong associations within the HLA-DRB1 locus [19], with the HLA DR3 (*DRB1*0301*) and DR4 (*DRB1*0401*) molecules conferring susceptibility to AIH-1 in Europe and North America [20]. Both heterodimers contain a K (lysine) residue at position 71 of the *DRB1* polypeptide and the hexameric amino acid sequence L (leucin) L (leucin) E (glutamic acid) Q (glutamine) K (lysine) R (arginine) at position 67–72 [19]. The associations with

Table 19.1 Reported 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	
	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	
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)	

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–kidney microsomal antibody type 1 antibodies, *anti-LC-1* anti-liver cytosol type 1 antibodies, *ANA*, antinuclear antibodies

HLA DR3 and DR4 are considered strong enough to contribute to the diagnosis of AIH according to the revised diagnostic scoring system designed by the IAIHG [8]. These alleles have also been linked to clinical manifestations, response to treatment and overall prognosis. In white Northern Europeans, for example, *DRB1*0301* is especially frequent amongst those deteriorating despite corticosteroid treatment. In Japan, Argentina and Mexico, susceptibility to AIH is linked to *DRB1*0405* and *DRB1*0404* alleles, which encode R (arginine) rather than K (lysine) at position 71. These alleles share the LLEQ-R motif with *DRB1*0401* and *DRB1*0301* [21], suggesting that K or R at this location, in the context of LLEQ-R, may enhance binding of autoantigenic peptides, thus influencing susceptibility to AIH. However, there are two other potential models on the basis of data from Argentina and Brazil—where valine/glycine dimorphism at position 86 of the DR-β polypeptide has been proposed—and from Japan—where AIH-1 patients were found to have *DRB1* alleles encoding histidine at position 13 [19]. These three models indicate geographically/ethnically different genetic associations, suggesting that the peptides presented by MHC class II are distinct, potentially deriving from different antigens. Thus, these HLA associations are potentially indicative of the prevailing environmental insults triggering AIH-1 in different environments. In this regard, it is interesting to note that in South America, possession of HLA *DRB1*1301* allele, which predisposes to paediatric AIH-1 in this population, is also associated with persistent infection with the endemic hepatitis A virus [10].

Recently, the HLA-DQ2 locus has also been linked to susceptibility to AIH-1 in Latin America, while the possession of DR5 or DQ3 is associated with protection against AIH occurrence [22].

HLA DR7 (*DRB1*0701*) and DR3 (*DRB1*0301*) confer susceptibility to AIH-2. Patients positive for *DRB1*0701* have a more aggressive form of the disease with worse overall prognosis [23]. *HLA-DQB1*0201* has also been linked to the development of AIH-2, although this allele is in linkage disequilibrium with *DRB1*0701* and *DRB1*0301*, both associated with AIH-2 [24]. Interestingly, *DRB1*0301* is also associated with seropositivity for both anti-LKM-1 and anti-LC-1 antibodies, while *DRB1*0701* is more predominant

amongst patients positive for anti-LKM-1 only. Additionally, children positive for *DRB1*0701* develop a more restricted repertoire of anti-LKM-1 epitopes compared to those positive for *DRB1*0301* [25].

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 [26]. Additionally, a polymorphism at position 308 in the tumour necrosis factor alpha (TNF-α) gene promoter is particularly frequent in patients with AIH-1 from Europe and North America and is associated with a poorer response to steroids [27]. A FAS gene promoter polymorphism at position 670 also enhances susceptibility to AIH and influences progression to a more aggressive form characterised by the early development of cirrhosis [28]. Polymorphisms in the vitamin D receptor can also be predisposing factors to the development of autoimmune liver disease [29].

A form of AIH resembling AIH-2 has been described in some 20 % of patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Additionally, *AIRE1* mutations have been reported in three children with severe AIH-2 with extra-hepatic autoimmune manifestations, as well as in four children with AIH-1 with a family history of autoimmune disease [30].

Potential Triggers

One potential trigger for AIH in patients with increased genetic susceptibility is an immune response to exogenous pathogens that cross-reacts with structurally similar liver autoantigens. This phenomenon is 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 [31, 32]. 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 [33]. Within anti-LKM-1 positive chronic HCV patients, reactivity against a key autoantigenic target of anti-LKM-1, the epitope $CYP2D6_{193–212}$, 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 $HCV_{2985–2990}$) and cytomegalovirus (exon $CMV_{130–135}$) [34]. There is also sequence homology between $CYP2D6_{254–271}$ and amino acids present in the E1 HCV and the IE1 75 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 [10]. 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 [35]. 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's thyroiditis and inflammatory bowel disease (IBD) [36], had serum HCV antibody positivity, linking HCV infection with a breakdown of immune tolerance.

The antibiotics nitrofurantoin and minocycline [37] 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 [37].

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 autoaggressive cellular immune attack is the basis of this condition (Fig. 19.1). Over the past 3 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 [38]. 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 [38].

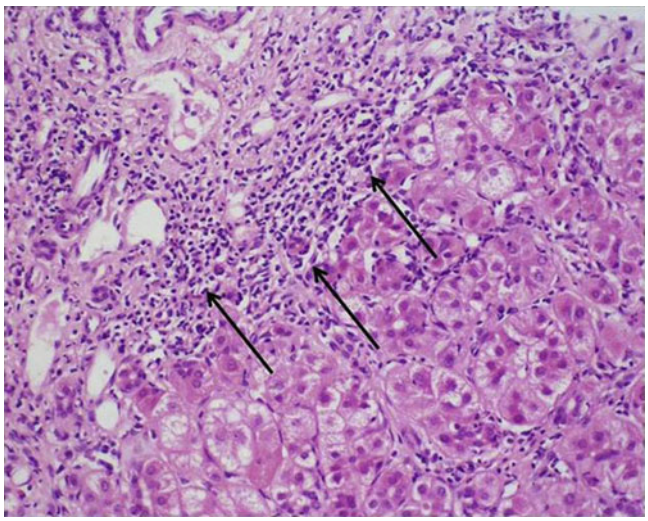

Fig. 19.1 Histology of autoimmune hepatitis (AIH). The portal and periportal inflammatory infiltrate characteristic of AIH (interface hepatitis, *arrows*) is composed of lymphocytes, monocytes/macrophages and plasma cells (picture kindly provided by Dr Yoh Zen, Institute of Liver Studies, King's College Hospital)

Regardless of the nature of the initial trigger, the immune response in AIH is believed to be orchestrated by naïve $CD4^{pos}$ lymphocytes recognising a self-antigenic peptide contained within a HLA class II molecule expressed by an antigen presenting cell (APC). In the presence of the appropriate co-stimulatory signals—provided by the interaction of CD28 expressed by the naïve $CD4^{pos}$ T cell and CD80/CD86 expressed by the APC the naïve cell differentiates into an effector cell subtype depending on the type of cytokines present in the microenvironment and the nature of the antigen. The liver is home to several specialised APC populations, and antigen presentation can occur in situ without the need to trafficking to the regional lymphoid tissue [39].

The effector T cell subsets are largely defined by the cytokines they produce; Th1 cells produce IL-2 as well as the main mediator of the tissue damage in AIH, IFN-γ. IFN-γ stimulates $CD8^{pos}$ T cells and enhances the expression of HLA class I molecules by hepatocytes while inducing the aberrant expression of HLA class II molecules and activating monocytes/macrophages, which in turn release IL-1 and TNF-α. On the other hand, Th2 cells produce IL-4, IL-10 and IL-13 cytokines that induce the maturation of B cells into plasma cells, with consequent production of autoantibodies. Autoantibodies themselves can contribute to liver damage by triggering antibody-mediated cellular cytotoxicity and complement activation. Th17 cells produce IL-17, IL-22 and TNF-α [10], while inducing hepatocytes to secrete IL6, which further enhances Th17 activation. Although Th17 cells have been shown to be elevated in the circulation and liver of AIH patients, their precise contribution to the pathogenesis of AIH is unknown and currently under investigation. Mechanisms leading to and/or perpetuating the autoimmune liver attack in AIH are depicted in Fig. 19.2.

Impairment of Regulatory T Cells

The development of autoimmune diseases is favoured by the breakdown of self-tolerance mechanisms that, in health, prevent the majority of autoreactive T cell clones from entering the periphery. As circulating autoreactive T cells are, however, present in health, there are both intrinsic and extrinsic peripheral tolerance mechanisms to limit autoimmune tissue damage. Key to this is the dominant form of immune suppression exerted by professional regulatory T cells (Tregs).

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 minimised by their exposure to therapeutic doses of steroids in vitro [40]. Such cells were a subpopulation of T lymphocytes able to control immune responses against a liver-specific membrane autoantigen [40]. 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 [41–46].

The most accepted definition of Tregs relies upon the expression of several markers, including CD4, the IL-2 receptor α chain (CD25) and the transcription factor FOXP3. 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 [41, 42, 45]. 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 [41, 42]. 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 [41]. Moreover, in AIH Tregs enhance the activation of monocytes, cells of the innate immune system abundantly present in the portal-periportal inflammatory infiltrate [47], and fail to create a regulatory milieu that would support and enhance their own function [43].

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 mucin-domain-containing molecule-3 (Tim-3), which upon ligation of galectin-9 expressed

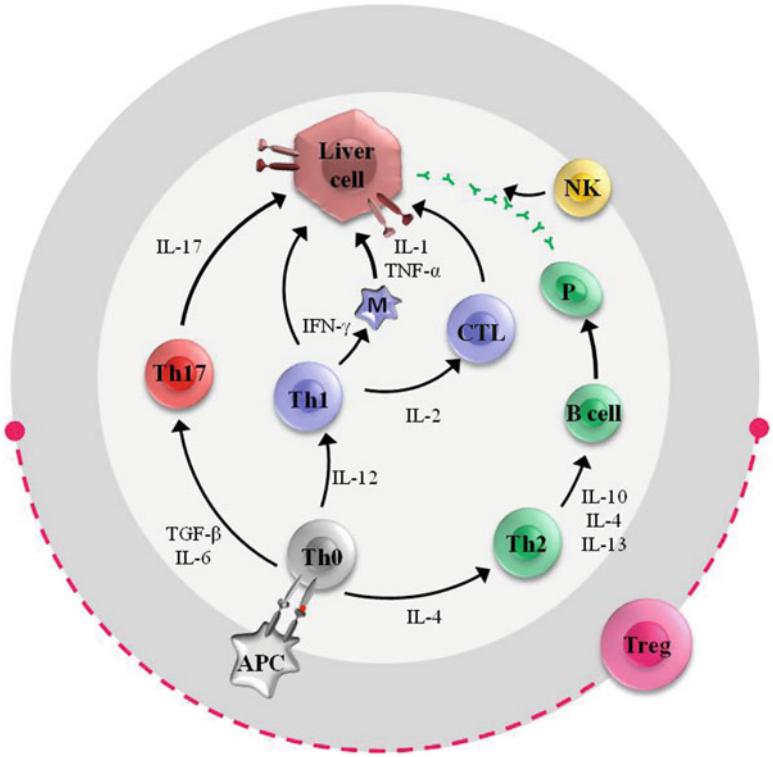

Fig. 19.2 Autoimmune attack to the liver cell. An autoantigenic peptide is presented to an uncommitted T helper (Th0) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC). Th0 cells become activated and, according to the cytokines present in the microenvironment and the nature of the antigen, differentiate into Th1, Th2 or Th17 cells, initiating a series of immune reactions determined by the cytokines they produce: Th2 secrete mainly IL-4, IL-10 and IL-13, and direct autoantibody production by B lymphocytes; Th1 secrete IL-2 and IFN-γ, which stimulate T cytotoxic lymphocytes (CTL), enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumour necrosis factor alpha (TNF-α). If regulatory T cells do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of CTL; cytokines released by Th1 and recruited macrophages; complement activation or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface. The role of the recently described Th17 cells, which arise in the presence of transforming growth factor beta (TGF-β) and IL-6, is under investigation

by Tregs, induces effector cell apoptosis [48]. The mechanisms that account for the impaired function of Tregs in AIH are depicted in Fig. 19.3.

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 [41, 42]. Using a polyclonal T cell stimulation strategy (that engages the T cell receptor (TCR) via CD3 and the co-stimulatory molecule CD28, while 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 [44]. Interestingly, expanded Tregs express higher levels of FOXP3 and are more effective suppressors compared to freshly isolated Tregs [44].

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 differ. Since de novo generation of Tregs relies on strong TCR signalling, the risk exists of effector Th17 cell expansion and contamination, which needs to be addressed when considering Treg therapy for AIH [10]. 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 [49].

The potential for successful Treg therapy is particularly strong in AIH-2, given that the antigenic regions ($CYP2D6_{217–260}$ and $CYP2D6_{305–348}$), targeted by B, CD4 and CD8 T cells, are well characterised [50]. Several lines of evidence demonstrate that autoantigen-specific Tregs suppress more efficiently

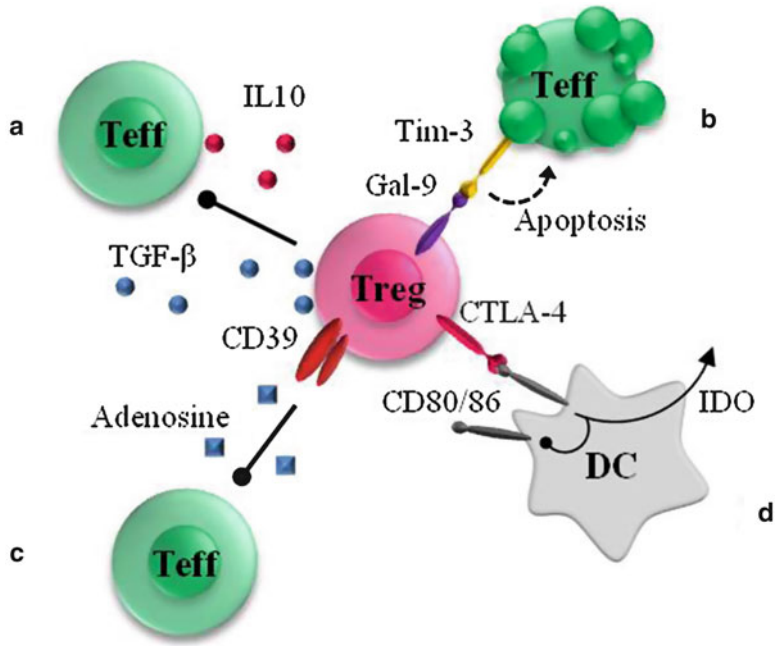

Fig. 19.3 The regulatory T cell in AIH: current understanding. Several mechanisms may determine the defective suppressive ability of regulatory T cells (Tregs) in AIH: (**a**) low Treg number and impaired ability to suppress proliferation of effector cells and to secrete the anti-inflammatory cytokines 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 (DC) with consequent reduction in the production of immunosuppressive indoleamine 2,3-dioxygenase (IDO)

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 autoreactive T cells has been achieved by Treg co-culture with semi-mature dendritic cells loaded with the CYP2D6 peptides [46].

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 [45]. In addition, NKT cells from AIH patients produce lower quantities of the regulatory cytokine IL-4 compared to healthy controls [45].

Animal Models

The ideal animal model of AIH should be characterised by a well-defined initiating event followed by chronic inflammation leading to fibrosis. The models generated to date do not reproduce the human condition faithfully, although they have contributed considerably to our understanding of AIH.

Models utilising the autoantigenic target of AIH-2 are particularly promising. One model is based on immunising C57BL/6 female mice with a plasmid-containing cDNA of the targets of anti-LKM-1 and anti-LC-1 autoantibodies—human CYP2D6 and formiminotransferase cyclodeaminase (FTCD)—alongside the end of the terminal region of murine CTLA-4 [51]. After three immunisations, and the inclusion of an IL-12 cDNA-encoding plasmid, the mice became autoantibody seropositive and a modest liver damage accompanied by portal and periportal CD4, CD8 T and B cell infiltration was observed. Interestingly, the same immunisation protocol used in different mouse strains failed to yield inflammatory changes, highlighting the importance of the genetic background [51]. Moreover, the severity of the liver disease depended on the age at the time of xeno-immunisation; 7-week-old C57BL/6 female mice developed more severe disease characterised by higher serum alanine aminotransferase and autoantibody titre compared to both younger and older individuals [52]. Interestingly, xeno-immunised C57BL/6 male mice developed minimal liver inflammation which was associated with elevated frequencies of $FOXP3^{pos}$ Tregs in the peripheral blood and liver compared to immunised

female mice. The same group has recently reported restoration of peripheral tolerance to liver autoantigens through the adoptive transfer of expanded Tregs in mice with AIH [53].

A second model uses CYP2D6 transgenic mice, breaking tolerance with an adenovirus-CYP2D6 vector [54]. While focal hepatocyte necrosis was seen both in mice treated with the adenovirus-CYP2D6 vector and control mice treated with adenovirus alone, only the former developed the chronic histological fibrotic changes reminiscent of AIH.

Another transgenic mouse model uses chicken ovalbumin (OVA) expressed on the surface of hepatocytes [55]. Repeated injections of OVA-specific T cells led to chronic hepatitis characterised by lobular and portal inflammation. Hepatic damage was mediated by OVA-specific cytotoxic T cells with help provided by OVA-specific $CD4^{pos}$ T cells [55].

A direct pathogenic involvement of $CD4^{pos}$ effector T cells has, in contrast, been proposed in a *Tgf-b*$^{-/-}$ model of AIH. In this case, $CD4^{pos}$ T cells were the most important source of IFN-γ, leading to the development of fulminant hepatitis [56]. Taken together, these results suggest that multiple immunological mechanisms determine diverse patterns of disease presentation and outcome.

In a bid to better understand the role of immune-regulatory mechanisms in the pathogenesis of AIH, Kido et al. recently developed a mouse model of spontaneous AIH by inducing the concurrent loss of $FOXP3^{pos}$ regulatory T cells and PD-1-mediated signalling. Neonatal thymectomy was used to dramatically reduce the number of circulating nTregs in PD-1-deficient mice, leading to fatal AIH characterised 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 autoreactive T cells and protective Tregs in this condition [57].

Clinical Presentation and Natural History

AIH can present with diverse clinical manifestations [58], although the majority of adult patients have an insidious onset characterised by progressive fatigue, relapsing jaundice, amenorrhoea, weight loss and occasionally arthralgia [59]. Presentation with gastrointestinal bleeding, hypersplenism or other complications of portal hypertension can sometimes occur [60]. Some 25 % of patients are completely asymptomatic and are diagnosed after incidental discovery of abnormal liver function tests. Finally, 30–40 % of patients present with symptoms and signs mimicking those of acute hepatitis due to other causes. AIH can also, rarely, manifest as fulminant hepatic failure [61]. 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. Indeed, advanced fibrosis or cirrhosis can often be found in patients presenting acutely [62].

AIH can develop occasionally during pregnancy [63]. Post-partum development of AIH and exacerbations of existing disease in patients whose condition improved during pregnancy has also been described [64].

Approximately 40 % of AIH patients have a family history of autoimmune disease and at least 20 % have concomitant autoimmune diseases or will develop them during follow-up [60].

The complications associated with AIH mirror those found in other progressive liver diseases. Thus, chronic hepatitis progresses to cirrhosis and ultimately to hepatocellular carcinoma (HCC) despite the use of immunosuppressive therapy. HCC is relatively rare in AIH [62]: Yeoman et al. [65] reported 15 cases of HCC, all with underlying cirrhosis, amongst 243 patients with AIH who were followed up for 16 years. Wong et al. reported six cases of HCC, also all with underlying cirrhosis, amongst 322 patients followed up for 10 years [66]. Surveillance for HCC is therefore warranted [17].

Diagnosis

Scoring Systems

Elevated transaminase and IgG levels, autoantibody seropositivity and histological evidence of interface hepatitis form the basis for the diagnosis of AIH (Table 19.2).

Diagnostic criteria for AIH were established and revised by the IAIHG [7, 8], mainly for use in the research setting. This system, which includes positive and negative scores, enables the researcher to grade clinical, laboratory and histological features of AIH, including response to treatment. The score has also proved useful in the clinical context when assessing patients with few or atypical features of the disease. The distinction between a definite and probable diagnosis of AIH predominantly relates to the magnitude of the elevation in serum gamma globulin/IgG or autoantibody titre, as well as exposure to alcohol, or hepatotoxic medication or infection. Cholestatic laboratory and histological changes carry a negative score. In rare cases, when conventional autoantibodies are absent, the presence of anti-asialoglycoprotein receptor (anti-ASGPR), anti-soluble liver antigen/liver–pancreas (anti-SLA/LP) and atypical perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) supports a probable diagnosis of AIH. The scoring system also incorporates response to steroids. A definite diagnosis before steroid treatment requires a score higher than 15, while a definite diagnosis after steroid treatment requires a score greater than 17 (Table 19.3) [8].

In children, lower autoantibody titre—1:20 for ANA and SMA and 1:10 for anti-LKM-1—contributes to the diagnoses of AIH because healthy children are very rarely autoantibody positive.

Table 19.2 Descriptive criteria for the diagnosis of autoimmune hepatitis

Feature	Definite AIH	Probable AIH
Serum biochemistry	Any abnormality in serum aminotransferase levels, especially if the serum alkaline phosphatase level is not elevated	Same as for definite AIH but in patients with abnormal serum concentrations of copper or ceruloplasmin, Wilson disease must be excluded by appropriate investigations
	Normal levels of α1-anti-trypsin and ceruloplasmin	
Serum immunoglobulins	Total serum globulins or γ-globulin or IgG concentrations >1.5 times upper limit of normal	Any elevation of serum globulin, γ-globulin or IgG over the upper limit of normal
Serum autoantibodies	ANA, SMA or anti-LKM-1 titres ≥1:80	Same as for definite but at titres ≥1:40 or presence of other autoantibodies (anti-SLA, atypical pANCA (also known as pANNA), anti-LC-1, anti-ASGPR)
	Lower titres (1:20 for ANA and SMA; 1:10 for anti-LKM-1) are significant in children	
	Negative AMA	
Liver histology	Interface hepatitis of moderate or severe activity with or without lobular hepatitis or bridging necrosis	Same as for definite AIH
	No biliary lesions, granulomas or other pre-eminent changes suggestive of a different aetiology	
Viral markers	Negativity for markers of current infection with hepatitis A, B, C and E viruses	Same as for definite AIH
Other aetiological markers	Average alcohol consumption <25 g/day	Average alcohol consumption <50 g/day and no recent use of hepatotoxic drugs
	No recent use of hepatotoxic drugs	Patients who have consumed large amounts of alcohol or who have recently taken potentially hepatotoxic drugs may be included, if there is clear evidence of continuing liver damage after abstinence from alcohol or withdrawal of the drug

Adapted from Alvarez F, Berg PA et al. J Hepatol 1999; 31: 929–938
AIH autoimmune hepatitis, *AMA* anti-mitochondrial antibodies, *ANA* anti-nuclear antibodies, *SMA* anti-smooth muscle antibodies, *anti-LKM-1* anti-liver–kidney microsomal type 1 antibodies, *anti-SLA* anti-soluble liver antigen, *pANCA* perinuclear anti-neutrophil cytoplasmic antibody, *pANNA* peripheral anti-nuclear neutrophil antibodies, *anti-LC-1* anti-liver cytosol type 1, *anti-ASGPR* anti-asialoglycoprotein receptor

A simplified scoring system, for use in clinical practice, has recently been proposed by the IAIHG [9]. This system is based on only four criteria: positivity for autoantibodies, elevated IgG levels, histological evidence of interface hepatitis and the exclusion of viral hepatitis (Table 19.4). Neither scoring system is immediately applicable to the diagnosis of the juvenile form of AIH.

Laboratory Abnormalities

Laboratory abnormalities associated with AIH include elevated levels of aspartate (AST) and alanine (ALT) aminotransferase, which are usually more striking than the increase in serum bilirubin or alkaline phosphatase (AP). Nonetheless, cholestasis is evident in some patients; in these cases, extrahepatic obstruction and cholestatic forms of viral hepatitis, drug-induced disease, primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and overlap syndromes must be taken into account in the differential diagnosis [58].

AIH is also often associated with a generalised elevation of serum globulins, particularly gamma globulins, mainly due to an increase in IgG. The serum autoantibodies typically present include ANA, SMA, anti-LKM-1 and anti-LC-1. Anti-mitochondrial antibodies (AMA), typically associated with PBC, are occasionally found in AIH. Importantly, though autoantibodies play an important role in the scoring systems mentioned above, their presence in isolation does not equate a diagnosis of AIH, as they can be found in other liver diseases.

Diagnostic Autoantibodies

Tests for the presence of autoantibodies to constituents of the nuclei, smooth muscle and liver–kidney microsome type 1 should be performed as soon as a diagnosis of AIH is suspected [67]. As well as assisting the diagnosis of AIH, autoantibody profiles enable the differentiation of AIH into type 1 and type 2. ANA and/or SMA, characterising AIH-1, and anti-LKM-1, defining AIH-2, are rarely detected together [67]; in those rare cases in which they are present simultaneously, the clinical course is similar to that of AIH-2. Antibodies to LC-1, SLA/LP and neutrophil cytoplasmic antigens may assist in diagnosing patients negative for the defining AIH autoantibodies (Tables 19.5 and 19.6); for this reason, they have been incorporated in the original and revised IAIHG diagnostic scoring systems [7, 8].

Table 19.3 International Autoimmune Hepatitis Group 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, pANNA	+2
Response to therapy	Remission	+2
	Relapse	+3

Adapted from Alvarez F, Berg PA et al. J Hepatol 1999; 31: 929:938
Pretreatment score >15, definite AIH; 10–15, probable AIH; post-treatment score >17, definite AIH; 12–17, probable AIH
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 (also known as atypical pANCA), *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

Recognition and interpretation of immunofluorescence patterns is not always straightforward; such an operator-dependent technique allied to the relatively rarity of AIH can lead to reporting errors. Problems exist between laboratory reporting and clinical interpretation of the results that are partly dependent on insufficient standardisation of the tests but also partly dependent on a degree of unfamiliarity of some clinicians with the disease spectrum of AIH. In regard to standardisation, the IAIHG has established a serology committee to help define guidelines and develop procedures and reference standards for more reliable testing [67].

Table 19.4 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]
Anti-LKM-1	≥1:40	
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

Adapted from Hennes EM, Zeniya M et al. Hepatology 2008; 48: 169–176
Score ≥6, probable AIH; ≥7, definite AIH
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
[a]Addition of points achieved for all autoantibodies cannot exceed a maximum of 2 points

Anti-Nuclear Antibodies

ANA stains rodent kidney, stomach and liver sections. Liver sections in particular can reveal homogeneous, coarsely or finely speckled patterns: in most cases of AIH the pattern is homogeneous (Fig. 19.4). To obtain much clearer definition of the nuclear pattern, HEp2 cells with prominent nuclei can be used. However, because nuclear reactivity is frequent in healthy subjects, these cells are not suitable for screening purposes [68]. Likely molecular targets for ANA are single- and double-stranded deoxyribonucleic acid (DNA), small nuclear ribonucleoproteins (snRNPs), centromeres, histones, chromatin, cyclin A and probably several others [59]. The advent of new techniques using recombinant nuclear antigens and immunoassays will enable a better definition of ANA target antigens while allowing an assessment of their specificity for diagnosis and potential pathogenic role in AIH-1.

Anti-Smooth Muscle Antibodies

SMA stains the artery wall of the kidney, stomach and liver sections and the muscularis mucosa and lamina propria of the stomach. Within the kidney, vessel (V), glomeruli (G) and tubule (T) patterns can be visualised (Fig. 19.5) [67]. The V pattern can be found also in non-autoimmune inflammatory liver disease, autoimmune diseases not affecting the liver and viral infections, but the VG and VGT patterns are more specific for AIH. When cultured fibroblasts are used as a substrate, the "F-actin" or microfilament (MF) pattern—corresponding to the VGT pattern on rodent substrate—can be observed. Though it has been suggested that the VGT-MF pattern is due to a specific antibody

Table 19.5 Autoantibodies and their antigens in autoimmune liver diseases

Autoantibody	Target antigen(s)	Liver disease	Value in AIH	Conventional method of detection	Molecular-based assays
ANA	Chromatin	AIH	Diagnostic of AIH-1	IIF	N/A
	Histones	PBC			
	Centromeres	PSC			
	Cyclin A	Drug-induced			
	Ribonucleoproteins	Chronic hepatitis C			
		Chronic hepatitis B			
		NAFLD			
SMA	Microfilaments (filamentous actin)	Same as ANA	Diagnostic of AIH-1	IIF	N/A
	Intermediate filaments (vimentin, desmin)				
Anti-LKM-1	Cytochrome P4502D6	AIH-2	Diagnostic of AIH-2	IIF	ELISA, IB, RIA, LIA
		Chronic hepatitis C			
Anti-LC-1	Formininotransferase cyclodeaminase	AIH-2	Diagnostic of AIH-2	IIF, DID, CIE	ELISA, RIA
		Chronic hepatitis C	Prognosis of severe disease		
SLA/LP	*O*-Phosphoseryl-tRNA/ selenocysteinyl-tRNA synthase (SepSecS)	AIH	Diagnostic of AIH	Inhibition ELISA	ELISA, IB, RIA, LIA
		Chronic hepatitis C	Prognostic of severe disease, relapse and treatment dependence		
pANNA	Nuclear lamina proteins	AIH	Point towards diagnosis of AIH	IIF	N/A
		PSC/ASC			
AMA	E2 subunits of 2-oxoacid dehydrogenase complexes, particularly PDC-E2	PBC	Against diagnosis of AIH	IIF	ELISA, IB, RIA

ANA anti-nuclear antibodies, *SMA* 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, *pANNA* peripheral anti-nuclear neutrophil antibodies (also known as atypical pANCA), *AMA* 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* counterimmunoelectrophoresis, *ELISA* enzyme-linked immunosorbent assay, *IB* immunoblot, *LIA* line-immunoassay, *RIA* radioimmunoprecipitation assay, *N/A* not applicable

Table 19.6 Diagnostic and prognostic significance of autoantibodies in autoimmune hepatitis

Autoantibody	Diagnostic significance	Prognostic significance
ANA	Diagnostic of AIH-1	Not associated with disease course or outcome
SMA	Diagnostic of AIH-1	Not associated with disease course or outcome
Anti-LKM-1	Diagnostic of AIH-2	Associated with younger age at presentation
		More frequently associated with fulminant hepatic failure
		More frequently associated with partial IgA deficiency
Anti-LC-1	Diagnostic of AIH-2	Associated with severe liver inflammation and rapid progression to cirrhosis
SLA/LP	Diagnostic of AIH	Associated with more severe disease course, treatment dependence, relapse after drug withdrawal and need for transplantation
	Present when other markers are absent	
pANNA	Points towards the diagnosis of AIH	Associated with development of AIH-sclerosing cholangitis overlap syndrome
Anti-actin	Points towards the diagnosis of AIH	Associated with younger age at disease onset
		Associated with treatment dependence in children
		Predicts progression to liver failure and need for transplantation
Anti-ASGPR	Points towards the diagnosis of AIH	Associated with more severe interface hepatitis
		Predicts relapse after drug withdrawal

ANA anti-nuclear antibodies, *SMA* 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, *pANNA* peripheral anti-nuclear neutrophil antibodies, *anti-ASGPR* anti-asialoglycoprotein receptor, *Ig* immunoglobulin, *AIH* autoimmune hepatitis

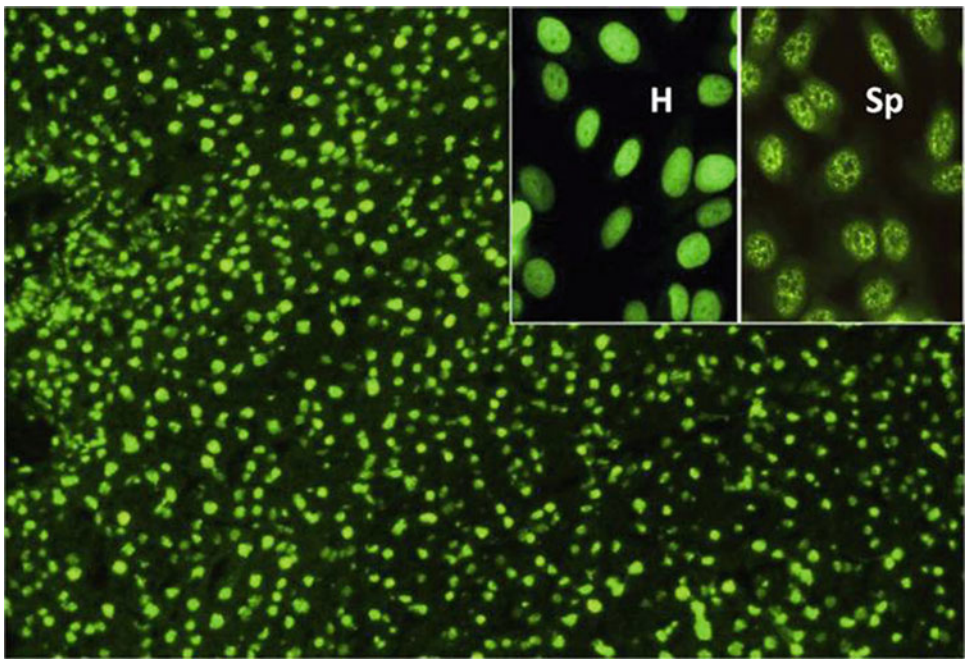

Fig. 19.4 Anti-nuclear antibody immunofluorescence pattern on rodent liver. The homogeneous pattern visible on rodent liver is the most common in AIH and is best visualised using human epithelial type 2 (HEp-2) cells (*inset*, H). The speckled pattern (*inset*, Sp) is less common in AIH, but more frequent in other conditions, such as chronic hepatitis C infection

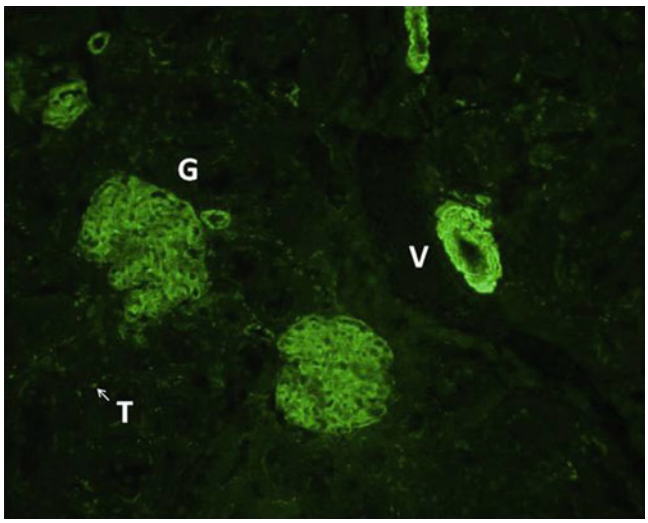

Fig. 19.5 Anti-smooth-muscle antibodies in AIH. Immunofluorescent pattern of anti-smooth-muscle antibodies (SMA) on a rodent renal section. SMA stains the smooth muscle within arterial vessels (V), glomeruli (G) and surrounding tubules (T) (picture kindly provided by Dr ET Davies, Department of Immunology, King's College Hospital)

uniquely found in AIH-1, it may just reflect high-titre SMA. Thus, neither the VGT nor the anti-MF patterns are entirely specific for the diagnosis of AIH-1. Some 20 % of SMA positive AIH-1 patients do not have the F-actin/VGT pattern [69], and, therefore, the absence of anti-actin SMA does not exclude the diagnosis of AIH. The molecular target of the microfilament reactivity remains to be conclusively identified.

Anti-Liver–Kidney Microsomal Type 1 Antibodies

Anti-LKM-1 antibodies strongly stain the liver cell cytoplasm and the P3 portion of the proximal renal tubules. Anti-LKM-1 are not uncommonly mistaken for AMA, since both react with the liver and kidney substrates. AMA stain the liver more faintly, the larger renal tubules more diffusely and the mitochondria-rich small distal tubules more strongly than anti-LKM-1 (Fig. 19.6). Anti-LKM-1 do not stain gastric parietal cells while AMA do. It is important to note that a small subset (3–5 %) of AIH patients are AMA positive, with [70] or without overlapping features of PBC [71, 72]. The identification of the molecular targets of anti-LKM-1 (CYP2D6) and AMA (enzymes of the 2-oxoacid dehydrogenase complex) has led to the development of immunoassays based on the use of the recombinant or purified antigens. Commercially available ELISAs, accurate for the detection of anti-LKM-1 and reasonably accurate for the detection of AMA, can be used to resolve doubts remaining after the examination of immunofluorescence patterns.

Variant Liver Microsomal Antibodies

Variant liver microsomal antibodies are most commonly directed against non-2D6 cytochrome-P450 isoforms. Anti-liver microsome (LM) antibodies only stain the liver cytoplasm, react with liver-specific cytochrome P4501A2 and occur in dihydralazine-induced hepatitis and in hepatitis associated with APECED [73]. Antibodies specific for P4502A6, which are found in APECED and occasionally hepatitis C patients, have an anti-LKM-1-like pattern on immunofluorescence. The term anti-LKM-2 was coined to describe anti-LKM-1-like microsomal antibodies directed against cytochrome P4502C9 that are produced during hepa-

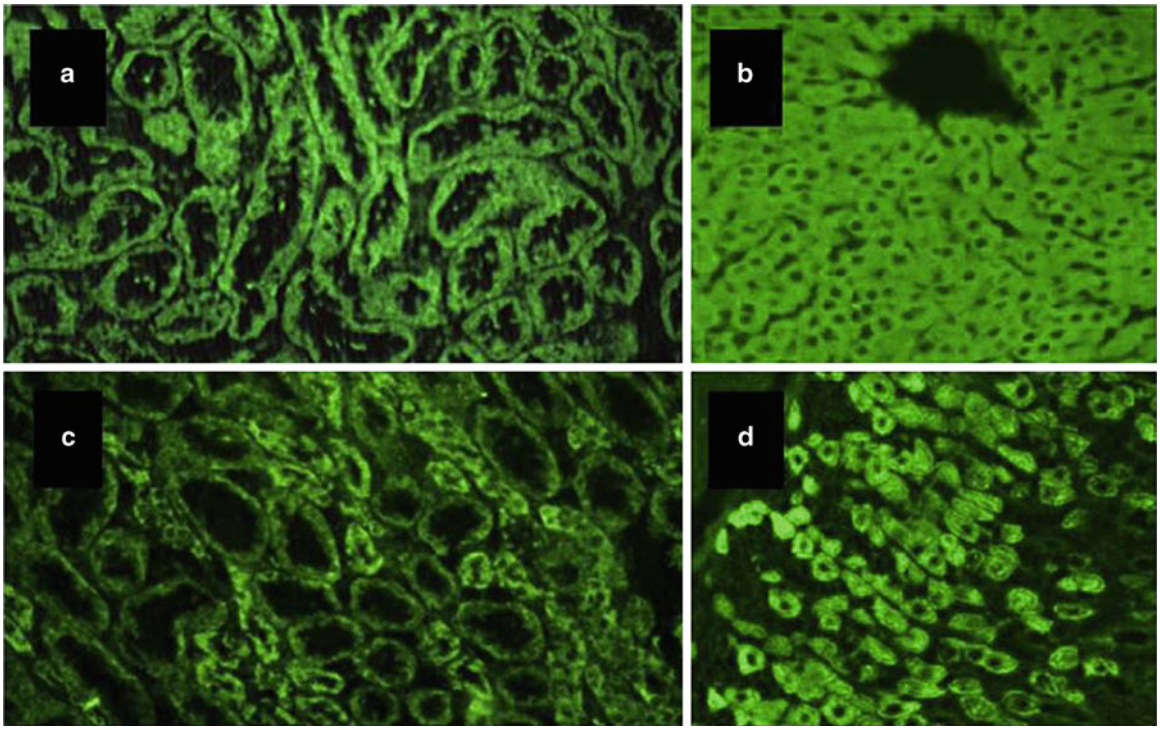

Fig. 19.6 Immunofluorescence pattern of anti-liver–kidney microsomal type 1 and anti-mitochondrial antibodies. Anti-liver–kidney microsomal type 1 (anti-LKM-1) antibodies (**a**, **b**) stain intensely the proximal tubules (**a**), while anti-mitochondrial (AMA) antibodies (**c**, **d**) stain the smaller, mitochondria-rich, distal tubules (**c**) of rodent kidney. Since these patterns are sometimes confused, the use of rodent liver (**b**) and stomach (**d**) sections is important to discriminate between the two reactivities, as AMA typically stain the gastric parietal cells (**d**) while anti-LKM-1 stain the liver (**b**) but not the stomach

titis induced by the anti-hypertensive tienilic acid, which is no longer marketed [74]. Anti-LKM-3, targeting members of the family 1 UDP glucuronosyltransferases (UGT), also gives an immunofluorescent pattern similar to anti-LKM-1, but this autoantibody is found mainly in hepatitis D (delta) [75].

Anti-Liver Cytosol Type 1 Antibodies

Anti-LC-1 seropositivity was initially described either alone or in combination with anti-LKM-1. In both instances the disease manifests as a clinical entity resembling AIH-2 [76]. Thus, the presence of anti-LC-1 in isolation scores positively towards AIH-2 diagnosis, enabling prompt initiation of treatment. However, anti-LC-1 has also infrequently been detected in association with the serological markers of AIH-1 and in patients with chronic hepatitis C virus infection [77]. Using the standard rodent liver, kidney and stomach sections, anti-LC-1 stains the cytoplasm of liver cells while sparing the centrilobular area (Fig. 19.7). Because the anti-LKM-1 immunofluorescence staining obscures that of anti-LC-1, anti-LC-1 must be sought using liver cytosol in double-dimension immunodiffusion, or counterimmunoelectrophoresis, with an appropriate positive reference serum. Anti-LC-1 reacts with a 58–60 kD protein in Western blot when the human liver cytosolic fraction is used as substrate. The molecular target of anti-LC-1 is FTCD [78]. The clinical relevance of anti-LC-1 is currently being assessed using molecularly based immunoassays.

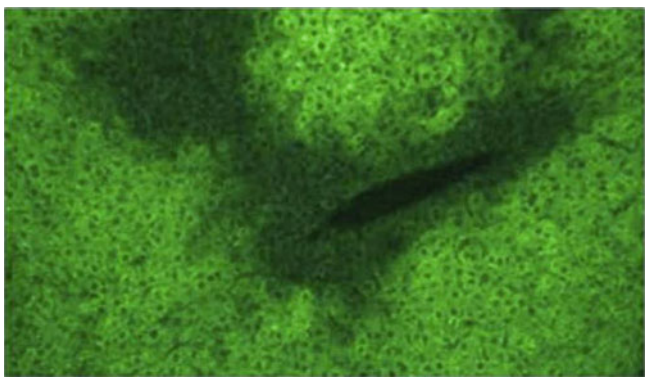

Fig. 19.7 Anti-liver cytosol type 1 antibodies. Immunofluorescent pattern of anti-liver cytosol type 1 (anti-LC-1) antibodies on a rodent liver section: they stain the cytoplasm of hepatocytes with a weakening of the stain around the central vein

Anti-Soluble Liver Antigen/Liver-Pancreas Antigen Antibodies

Previously described as two separate entities, anti-SLA and anti-LP target the same antigen and are therefore the same autoantibody [79]. Anti-SLA was once used to define a third

type of AIH negative for conventional autoantibodies [80]; however, early studies are limited by the use of a conventional autoantibody titre cut-off higher than that currently used for the diagnosis of AIH. Hence, several patients considered to have AIH-3 were probably positive for conventional autoantibodies. Nevertheless, anti-SLA is found occasionally in patients negative for ANA, SMA and anti-LKM-1 [81]. Anti-SLA appears to be highly specific for the diagnosis of AIH; it is frequently present in typical cases of type-1 and type-2 AIH, although it is also seen in the AIH/sclerosing cholangitis overlap syndrome. Patients seropositive for anti-SLA at the time of diagnosis have more severe disease and worse prognosis [81]. Screening of cDNA expression libraries using high-titre anti-SLA serum has led to the identification of its molecular target: UGA tRNA suppressor associated antigenic protein (tRNP(Ser)Sec), currently termed SepSecS [79]. Molecularly based diagnostic assays have become available, but their full evaluation is still under way.

Anti-Neutrophil Cytoplasmic Antibodies

ANCAs react with cytoplasmic components of neutrophils giving either a perinuclear (pANCA) or a cytoplasmic (cANCA) staining pattern. Akin to PSC and IBD, pANCAs are frequently detected in AIH-1, although the pattern is atypical, since they react with peripheral nuclear membrane components. The name "peripheral anti-nuclear neutrophil antibody" [pANNA] has therefore been suggested [82]. In contrast, pANNA is virtually absent in AIH-2. Positivity for pANNA can aid the diagnosis of AIH, particularly in the absence of other autoantibodies [67].

Anti-Asialoglycoprotein Receptor Antibodies

In an attempt to identify putative autoantigens 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 ASGPR [83], also known as hepatic lectin. ASGPR is the only known liver-specific autoantigen and is constitutively expressed on the hepatocellular membrane. Antibodies to ASGPR are found in 88 % of AIH patients, coexisting with ANA, SMA and anti-LKM-1 [84], and their titre correlates with inflammatory disease activity, providing an additional marker to monitor treatment efficacy [85]. Anti-ASGPR are, however, also found in PBC and viral and drug-induced hepatitis [86]. Moreover, commercial assays for the detection of anti-ASGPR await validation.

Histology

The extent of histological inflammatory activity and the occurrence of cirrhosis are not adequately reflected by serum transaminases and IgG levels. Thus, liver biopsy is mandatory in order to confirm the diagnosis of AIH and evaluate the severity of liver damage.

Hepatitis at the portal–parenchymal interface, known as interface hepatitis (Fig. 19.1), is characteristic, but not exclusive, of AIH [87]. Other typical histological lesions found in AIH are periportal lymphocytic or lymphoplasmacytic infiltration, hepatocyte swelling and/or pycnotic necrosis. Lymphocytes, plasma cells and histiocytes surround individual dying hepatocytes at the portal–parenchymal interface and in the lobule. Though plasma cells are usually abundant at the interface and throughout the lobule, their presence in low number does not preclude the diagnosis of AIH. In AIH presenting acutely or during episodes of relapse, panlobular hepatitis is often present, associated with bridging necrosis and, in the case of a fulminant presentation, massive necrosis. Though sampling variation is possible in needle biopsy specimens, especially when cirrhosis is present, the severity of the histological appearance is usually of prognostic value. Inflammatory changes surrounding the bile ducts are apparent in a small proportion of patients with AIH, suggesting an overlap with sclerosing cholangitis. This is, however, reported more frequently in the paediatric setting [88].

Treatment

The goal of treatment in AIH is to obtain early complete remission, to prevent disease progression and to maintain this long term on the lowest possible dose of medication [89]. With the exception of a fulminant presentation with encephalopathy, AIH responds satisfactorily to immunosuppressive treatment whatever the degree of liver impairment, with a reported rate of remission of approximately 80 % [62]. Although some authors have in the past defined remission as transaminase levels up to twice the upper limit of normal, a better outcome is achieved when normal transaminase levels are attained and maintained [17, 90].

Standard Treatment

The current immunosuppressive regimen in AIH is based upon three randomised clinical trials in adult AIH patients conducted in the early 1970s. These collectively demonstrated that treatment with prednisone improves liver function tests, ameliorates symptoms and prolongs survival [3–5]. Although azathioprine was not able to induce remission when used on its own, it did allow the maintenance of remission in association with a significantly lower dose of steroids. Initial treatment with prednisone (or prednisolone) with or without azathioprine should be instituted as soon as the diagnosis of AIH is made (Table 19.7) and not delayed for 6 months as once suggested [89].

Table 19.7 Immunosuppressive treatment regimens for adults and children with autoimmune hepatitis

Population	Initial regimen	Maintenance
Adults	Prednis(ol)one 60 mg/day or	Prednis(ol)one 10 mg/week reduction until 20 mg/day, followed by of 5 mg/week reduction until 10 mg/day and by 2.5 mg/week reduction to reach maintenance (5 mg/day)
	Prednis(ol)one 30–60 mg/day in combination with azathioprine 1–2 mg/kg/day	Azathioprine 1–2 mg/kg/day if in combination with prednis(ol)one or 2 mg/kg/day if alone
Children	Prednis(ol)one 2 mg/kg/daily (up to 60 mg/daily) decreased weekly if transaminase levels decrease. Azathioprine (1–2 mg/kg/day) added if transaminase levels plateau or increase	Prednis(ol)one tapered over 6–8 weeks to 0.1–0.2 mg/kg/day or 5 mg/day
	In the absence of jaundice, azathioprine can be started at the same time as prednisolone	Azathioprine 1–2 mg/kg/day if needed or added initially

Table 19.8 Side effects associated with standard treatment of autoimmune hepatitis

Prednisolone-related	Azathioprine-related
Common	
Facial rounding	Nausea
Dorsal hump striae	Emesis
Weight gain	Rash
Acne	Fever
Facial hirsutism	Cytopenia
Alopecia	
Uncommon	
Osteopenia	Pancreatitis
Vertebral compression	Opportunistic infections
Cataracts	Arthralgia
Diabetes (brittle)	Cholestatic liver injury
Emotional instability	
Hypertension (labile)	
Rare	
Pancreatitis	Malabsorption
Opportunistic infections	Malignancy
	Bone marrow failure
	Teratogenicity

The initial therapeutic approach depends partly upon histological findings [62]; the presence of interface hepatitis, with or without evidence of fibrosis or cirrhosis, warrants the initiation of standard treatment [17]. Serological elevations in transaminase or IgG levels do not correlate with histological damage and consequently provide limited help in respect of treatment initiation. In patients with mild portal inflammation only, institution of therapy is determined by AST and IgG levels, and/or by the presence of symptoms [58]. The therapeutic approach in patients with an asymptomatic or pauci-symptomatic form of the disease is less clear [89]. The benefit of therapy is undefined and may be so low that the risk of corticosteroid side effects might outweigh possible benefits, particularly when considering post-menopausal women and elderly patients [18].

Although some patients may remain in remission after withdrawal of treatment, most require long-term maintenance therapy. Despite the absence of firm guidelines, it is cautious not to attempt withdrawal of immunosuppression within 2 years of diagnosis [17]. 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 [89].

Adverse Side Effects

Side effects related to corticosteroid therapy must be considered and this may influence the decision to treat and the choice of medications (Table 19.8). The most common side effects of steroid treatment are cushingoid changes. Less common but severe side effects, requiring special precaution and monitoring, include osteoporosis, vertebral collapse, diabetes, cataract, hypertension and psychosis. These conditions are not necessarily contraindications for the use of corticosteroids, but in at least 13 % of patients, their development leads to dose reduction or even premature drug withdrawal. The most common reasons for treatment withdrawal are cosmetic changes or obesity, osteopenia with vertebral collapse and brittle diabetes [17].

Some 10–20 % of patients develop complications associated with azathioprine treatment. These include cholestatic hepatitis, veno-occlusive disease, pancreatitis, nausea and vomiting, rash and bone marrow suppression, which typically subside upon drug withdrawal [17]. The risk of developing the major side effect of azathioprine, cytopenia, is related to low erythrocyte concentration of thiopurine methyltransferase (TPMT) activity. The genes encoding TPMT are highly polymorphic, and TPMT deficiency is found in 0.3–0.5 % of the population, although not all patients with deficiency experience bone marrow failure. While azathioprine is contraindicated in homozygotes for TPMT deficiency, heterozygotes tolerate low doses well; the level of enzymatic activity may even increase with continued azathioprine administration. Enzyme activity should therefore be determined only in the presence of pretreatment cytopenia, cytopenia developing during therapy or administration of higher-than-conventional doses of azathioprine [89].

Continuous immunosuppressive therapy is associated with the development of malignancies [91]. The incidence of extra-hepatic neoplasm in treated AIH patients is 1 in 194 patient-years, and the probability of tumour occurrence is 3 % after 10 years [17, 91]. There is no predominant tumour cell type associated with the treatment of AIH, and the type of cancer is unrelated to age, sex, treatment regimen or cumulative duration of treatment [91]. Nevertheless, since the risk of malignancy in patients on chronic low dose azathioprine therapy is 1.4-fold higher than that of the age- and sex-matched normal population, the beneficial actions of this drug as a corticosteroid-sparing agent must be counterbalanced with this risk [91].

Alternative and New Treatments

In patients that fail to respond to standard therapy, or in those who are intolerant or non-compliant, alternative treatments—including methotrexate, cyclophosphamide, tacrolimus, ursodeoxycholic acid (UDCA), cyclosporine and mycophenolate mofetil (MMF)—have been proposed (Table 19.9) [92]. Decisions regarding the use of such regimens require careful assessment of the limited data available, which mainly include small series or case reports.

Although there are some encouraging results with alternative treatments, progress has been slow and none has yet been incorporated into a standard management algorithm [17].

MMF is a purine antagonist that selectively inhibits activated lymphocyte proliferation, without dependence on TPMT activity. In patients for whom standard immunosuppression fails to induce stable remission, or who are intolerant to azathioprine, MMF, together with prednisolone, is currently the treatment of choice [17]. This strategy is able to improve various AIH symptoms, although many patients do also experience drug intolerance [89].

The calcineurin inhibitors, cyclosporine and tacrolimus, have been used as a rescue treatment for difficult-to-treat cases of AIH. However, because a large study in this subgroup of patients is lacking, these should be used with caution [89].

Anti-TNF-α agents, such as infliximab, are commonly used to treat immune-mediated diseases such as rheumatoid arthritis, psoriasis and IBD. There is anecdotal evidence that infliximab is efficacious in the management of difficult-to-treat cases of AIH [93]. In the largest published retrospective series, treatment with infliximab led to a decrease in transaminase and IgG levels in 11 difficult-to-treat adult patients with AIH, but infectious complications occurred in seven of them [93]. Moreover, and worryingly, infliximab therapy for other diseases has been associated with the induction of severe de novo AIH [94]. Anecdotal evidence also suggests some benefit with the use of the anti-B cell monoclonal antibody rituximab in difficult-to-treat patients [95]. However, the occurrence of severe infections is an important risk factor associated with these biological treatments.

Table 19.9 Alternative treatments for autoimmune hepatitis

Agent	Pros	Cons
Mycophenolate mofetil	Favourable toxicity profile	Contradictory reports regarding its efficacy
	Experience as a transplant immunosuppressant	
Tacrolimus	Potent immunosuppressant	Renal toxicity
	Experience in the transplant setting	
Cyclosporine	Potent immunosuppressant	Renal toxicity
	Experience in the transplant setting	
Budesonide	High first-pass metabolism in the liver	Ineffective in cirrhotic patients
	Immunosuppressive action	Effective dose not established yet in children
Rituximab	Relatively favourable toxicity profile	Infections
		Efficacy yet to be demonstrated
Infliximab	Potent and directed immunomodulatory properties	Infections
		Paradoxical development of AIH
Cyclophosphamide	Efficacious in inducing remission in small uncontrolled series	Dependency on continuous therapy
		Haematological side effects
Methotrexate	Favourable toxicity profile	Efficacy yet to be demonstrated
Ursodeoxycholic acid	Putative immunomodulatory capacities	Efficacy yet to be demonstrated

Budesonide is a corticosteroid with very high affinity for the glucocorticoid receptor and high first-pass liver metabolism; hence, it is presently receiving considerable attention as an alternative to prednisone or prednisolone as primary treatment of AIH. Although initial reports were somewhat contradictory, a recent large European study found that a combination of budesonide and azathioprine could induce remission in 60 % of non-cirrhotic patients, while medium-dose standard steroids and azathioprine could only induce remission in 39 % of patients. The budesonide group had also fewer adverse effects [96]. It should be noted, however, that this reported rate of remission is much lower than that seen in both adults and children (~80 %) when a higher starting dose of prednisone is used. Moreover, because budesonide cannot be used in cirrhotic patients—representing at least a third of the AIH population—its clinical utility has limitations [97].

Table 19.10 Diagnosis of recurrent autoimmune hepatitis after liver transplantation

Liver transplant for autoimmune hepatitis
Elevation of transaminase levels
Interface hepatitis
Elevation of immunoglobulin G levels
Presence of autoantibodies (ANA, SMA and/or anti-LKM-1)
Corticosteroid dependency
Exclusion of other causes of graft dysfunction

Liver Transplantation

Liver transplantation (LT) is the ultimate treatment for AIH patients presenting with acute liver failure or developing end-stage chronic liver disease and for those with HCC that meet transplant criteria [98]. A combination of prednisolone and a calcineurin inhibitor is the most common immunosuppressive regimen used after LT. This leads to a very successful outcome with reported 5-year patient survival of 80–90 % and a 10-year patient survival of 75 % [17].

Recurrence of Autoimmune Hepatitis After Liver Transplantation

Although LT is a highly successful mode of treatment for AIH, primary disease reappears in some 30 % of cases [99]. Diagnosis of recurrent AIH is based on biochemical abnormalities, the presence of autoantibodies, interface hepatitis on liver histology and/or dependence on steroids (Table 19.10) [17]. Greater awareness and appropriate management has recently led to a decrease in the frequency of patients with recurrent AIH and, more importantly, has enabled better outcome for this condition [100]. Since corticosteroid discontinuation can increase the risk of recurrent disease, it is particularly important to exert caution when weaning immunosuppression in patients who underwent LT for AIH [100].

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 characterised by features identical to those of classical AIH, namely, hypergammaglobulinaemia, positivity for circulating autoantibodies and histological features of interface hepatitis [101]. Though reported amongst all age groups, this condition appears to be more prevalent in children.

Concluding Remarks

The diagnosis of AIH should be considered during the diagnostic work-up of any patient with increased liver enzyme levels. AIH is exquisitely responsive to immunosuppressive treatment, with symptom-free long-term survival for the majority of patients. For patients who do not respond to standard treatment, or who are difficult to treat, MMF and, in the absence of a response, calcineurin inhibitors should be tried in addition to steroids. There is evidence that environmental triggers—acting, for example, through molecular mimicry between micro-organisms and self—on a background of genetic susceptibility and lack of adequate immunoregulation are involved in the initiation and perpetuation of AIH. Our understanding of the pathogenic mechanisms leading to AIH will be enhanced once animal models more faithfully representing the human condition are developed. These will help in unravelling the contribution of innate and adaptive, effector and regulatory immune responses to the autoimmune liver attack. Current studies are paving the way for the development of novel treatments aimed at reconstituting self-tolerance by specific immunologic manoeuvres, such as adoptive transfer of autologous antigen-specific Tregs.

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Primary Sclerosing Cholangitis (PSC)

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Key Points

- Primary sclerosing cholangitis (PSC) is a rare chronic inflammatory liver disease of unknown etiology affecting the large and—to a lesser extent—the small bile ducts.
- Progressive destruction of bile ducts may lead to end-stage liver disease requiring liver transplantation.
- PSC is frequently associated with inflammatory bowel disease and carries a high risk for malignancy in the hepatobiliary tract and colorectum.
- The clinical picture is characterized by cholestasis in frequently asymptomatic patients and standardized magnetic resonance cholangiopancreaticography (MRCP) has become the diagnostic method of choice to visualize biliary strictures and dilatations. Endoscopic retrograde cholangiography (ERC) should be reserved for cases which require biliary intervention or biopsies for workup of unclear strictures.
- About 10 % of patients present with a normal cholangiogram of the large ducts. In these patients small duct PSC characterized by onion-skin-type fibrosis surrounding the small bile ducts can be diagnosed by liver biopsy.
- Despite recent advances in identifying prognostically and therapeutically important subgroups of PSC (e.g., with elevated IgG4), as well as the increasing availability of animal models, a clear picture of its pathogenesis is still lacking.
- Translocation of bacteria or bacterial products from the inflamed gut or homing of gut-primed memory T lymphocytes via aberrantly expressed adhesion molecules in genetically susceptible individuals may play a central role in PSC pathogenesis.
- Pharmacological treatment of patients with PSC still represents a major challenge, since therapy with ursodeoxycholic acid improves serum liver tests and surrogate parameter of prognosis without a proven survival benefit in PSC. New therapeutic strategies include new bile acid derivatives.
- Liver transplantation is recommended in end-stage liver disease and in patients with recurrent cholangitis or evidence of bile duct dysplasia.

Introduction

Primary sclerosing cholangitis (PSC) is a chronic progressive cholestatic liver disease of unknown etiology. It is characterized by diffuse inflammation and fibrosis of bile ducts leading to strictures primarily in large- and medium-sized ducts of the biliary tree (fibro-obliterative cholangiopathy) [1, 2]. The cause is presumably immune-mediated and it is frequently associated with inflammatory bowel disease (IBD) with peculiar features (PSC-IBD). The progressive destruction of bile ducts may lead to liver cirrhosis requiring liver transplantation. In addition, episodes of recurrent cholangitis and hepatobiliary/colorectal malignancies are frequently complicating the clinical course of PSC [3, 4]. Although the characterization and understanding of the disease has improved substantially since its first descriptions in the mid-nineteenth century [5], effective medical therapy is still lacking and this disorder still represents a potentially severe disease with poor prognosis. So far no established pharmacological therapy improving survival of PSC exists, and ursodeoxycholic acid (UDCA) in combination with endoscopic interventions of dominant strictures is presently widely applied in clinical practice. Liver transplantation is the only established treatment of PSC in patients with end-stage liver disease, recurrent cholangitis, or high-grade cholangiocyte dysplasia.

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Epidemiology of PSC

PSC is a rare disease affecting predominantly young to middle-aged male patients. An overall incidence rate of 0.77 per 100,000 person-years was shown in a meta-analysis, with considerable differences among the individual studies [6]. In population-based studies, slightly higher incidence rates were reported, with an overall increase during the time period examined [6–9]. Also the prevalence rates were reported to be higher in men compared to women with a range between 6 and 20 cases per 100,000. In addition, there is a geographic clustering with higher prevalence in Northern countries compared to Southern Europe and Asia [10–12]. First-degree relatives of PSC patients have an increased disease prevalence of 0.7 %. In siblings the prevalence even reaches 1.5 % [13, 14].

Pathogenesis of PSC

At present a conclusive pathogenetic concept for PSC is still lacking. PSC may represent a multifactorial disease and is increasingly recognized to split up into subtypes as identifiable causes emerge (e.g., IgG4-positive forms of cholangitis). Thus, the underlying causative mechanisms may vary considerably among the different clinical subtypes and there may not be one single etiology of PSC.

Genetic susceptibility: The familial/geographical clustering and association with human leukocyte antigen (HLA) and non-HLA haplotypes suggest that interplay of genetic and environmental factors may play a crucial role in the initiation and progression of PSC [15]. Strong evidence for genetic susceptibility comes from genome-wide association analyses (GWAs) showing associations with a subset of *HLA* and *non-HLA* genes involved in bile homeostasis and regulation of inflammatory pathways [16–18]. *HLA-DR3* (*DRB1*03*), *HLA-B8* (*HLA-B*08*), and *DRB1*13* (*DR6*) have been identified as susceptibility markers [19–23]. A positive association with three different HLA class II haplotypes: the (A) *DRB1*03*, *DQA1*0501*, *DQB1*02*, the (B) *DRB1*15*, *DQA1*0102*, *DQB1*0602*, and the (C) *DRB1*13*, *DQA1*0103*, *DQB1*0603* were reported in a large European study [22]. The *DRB1*03*, *DQA1*0501*, *DQB1*02* homozygous genotype was associated with the highest relative risk for PSC development [22]. A negative association was found for the *DRB1*04*, *DQA1*03*, *DQB1*0302* haplotype. In addition, the *HLA-A1* allele [24], the *HLA-C7* [25], the *major histocompatibility complex class I chain-related A* (*MICA*)**002* and *008/5.1* alleles [26, 27] as well as the *tumor necrosis factor alpha* (*TNFα*) *promoter −308 A* allele [28] were identified to play a role in PSC susceptibility. However, considerable heterogeneity in both HLA classes of different populations was found and may contribute to the observed differences.

Besides HLA genes, the G protein-coupled bile acid receptor 1 (GPBAR1) TGR5 has been implicated in PSC pathogenesis. Significant associations for one exonic single-nucleotide polymorphism of the *TGR5* gene were found for both PSC and UC [16, 29]. Although mice lacking the canalicular phospholipid export pump ABCB4 develop sclerosing cholangitis, current data do not support a major role of ABCB4 variants in human PSC pathogenesis. Nevertheless, *ABCB4* gene variants may still influence the natural course of the disease via altering bile composition and thereby increasing the aggressiveness of bile, which consequently could aggravate secondary response to any primary (immune-mediated or ischemic) bile duct injury [30].

Although a role of *CFTR* in development of PSC has been postulated based on the findings in animal models and the high prevalence of bile duct abnormalities resembling PSC in cystic fibrosis patients [31], so far no clear association of *CFTR* variants in PSC patients could be demonstrated [32]. Moreover, in a recent study investigating the influence of *CFTR* polymorphisms on the development and evolution of PSC, a protective role was demonstrated for the 1540G variant and the TG11-T7 haplotypes, particularly in subjects without IBD [33]. Other non-HLA genes which have been linked to bile formation/hepatobiliary homeostasis include the *multidrug resistance gene 1* (MDR1) due to its role in mediating membrane transport of a wide range of xenobiotics/toxins, and the *steroid and xenobiotic receptor* (*SXR*), which acts as a regulator of bile acid and cholesterol homeostasis, as well as drug transport and metabolism (e.g., cytochrome P450 3A4 (CYP3A4), MDR1 p-glycoprotein) [34].

Immunopathogenetic mechanisms: PSC is not a classical autoimmune disease (no specific autoantigen, male predominance, lack of response to immunosuppressive medication) [35]. Nevertheless, the association between PSC and IBD has been recognized for nearly 5 decades and this association suggests a common pathogenetic pathway of both diseases. Immunopathogenetic concepts, in which translocation of bacterial products from the inflamed gut or homing of gut-primed memory T lymphocytes via aberrantly expressed adhesion molecules plays a central role, have been implicated in PSC pathogenesis [36].

An increased permeability resulting from inflammation of the intestine may lead to translocation of bacteria or bacterial components and products which consecutively enter the portal-venous system thereby inducing an inflammatory reaction. This pathogenetic concept is frequently referred to as the "leaky gut or bacterial translocation hypothesis."

Leaky gut hypothesis: Bacteria may penetrate the inflamed gut mucosal layer, enter the liver and consequently stimulate release of chemokines/cytokines by Kupffer cells and macrophages leading to (peri)cholangitis and a consecutive wound healing process with concentric periductal fibrosis [35, 36].

This is supported from animal models which suggest that small intestinal bacterial overgrowth and infusion of bacterial antigens into the portal circulation can cause hepatic inflammation with at least some characteristic features of PSC [37, 38]. Small intestinal bacterial overgrowth in genetically susceptible rats induces macroscopic and microscopic features similar to human PSC [37]. Components of anaerobic bacteria, such as peptidoglycan-polysaccharides, have been addressed to be responsible for these morphological changes [34]. In addition, administration of a chemotactic peptide produced by *E. coli* into the colon of Wistar rats with acetate-induced colitis induced hepatic lesions reminiscent of PSC [34, 38]. Therefore it was hypothesized that in genetically susceptible individuals, bacterial antigens could function as molecular mimics to trigger the immune response for initiation of PSC. A study investigating explanted livers from PSC patients found evidence for increased bile duct bacterial isolates [34, 39]. However, a study from Scandinavia investigating intestinal permeability and small bowel bacterial flora in PSC patients identified bacterial overgrowth in only a minority of patients [40]. Moreover, indirect evidence against this concept may be drawn from negative studies on the use of antibiotics in PSC [41, 42]. Finally, portal bacteremia was shown to be uncommon in patients with ulcerative colitis [43]. Taken together, these data do not support a major role for bacterial overgrowth or translocation due to increased intestinal permeability in pathogenesis of PSC. Nevertheless, translocation of intestinal bacteria/bacterial products may be episodic and therefore difficult to detect. Future insights into PSC pathogenesis in this respect may come from the increasing interest in the gut microbiome.

Gut lymphocyte homing hypothesis: The fact that PSC may develop independent of IBD activity (e.g., after colectomy) led to the hypothesis that $CCR9^+$ $\alpha4\beta7^+$ memory T lymphocytes primed in the inflamed intestine may persist as long-lived memory cells, with the ability to trigger portal inflammation in PSC via aberrantly expressed adhesion molecules in the liver and gut [44]. This is currently referred to as the "gut lymphocyte homing hypothesis." In this respect, it was shown that intestinal vascular adhesion protein-1 (VAP-1) expression is increased in patients with IBD [45]. Furthermore, it could be demonstrated that under physiological conditions, gut-restricted adhesion molecule mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is expressed in PSC livers [46]. On the other hand, MAdCAM-1 staining in portal veins was also detected in other chronic liver diseases indicating that MAdCAM-1 expression could rather be a consequence than a cause of inflammation in PSC [47].

Cellular immune-mediated cholangiocyte damage: Histological findings indicate that the hepatic innate immune response is a primary event in the pathogenesis of PSC. A diffuse mixed inflammatory infiltrate consisting of lymphocytes, plasma cells, and neutrophils, which is most intense around the bile ducts, constitutes the early histological changes. Accordingly, PSC development might be an aberrant immune response to exogenous triggers such as bacteria or pathogen-associated molecular patterns (PAMPs) which enter the portal circulation via a permeable intestinal mucosa. Consequently, macrophages, dendritic cells (DCs), and NK cells are activated, secrete cytokines, and perpetuate inflammatory reaction by activation of NK cells through IL-12 and recruitment of lymphocytes via TNF-α, IL-1β, and CXCL8 [48]. In addition, biliary epithelial cells (BECs) seem to have an active role in propagating the proinflammatory and profibrotic response. In healthy livers, BECs express only HLA class I molecules, while in PSC, aberrant expression of HLA class II (HLA-DR, HLA-DQ, and HLA-DP) molecules by BECs was reported [35, 49]. This might initiate the immune response by binding antigens and presenting them to class II restricted T cells. In addition, BECs can transit into a phenotype with overexpression of adhesion molecules and show the ability to produce and secrete chemokines, proinflammatory cytokines, and growth factors, which further perpetuates and determines the inflammatory process [50].

The portal tract mononuclear cellular infiltrate in patients with PSC is predominantly composed of T lymphocytes. There are considerable differences in the constitution ($CD4^+$/$CD8^+$) of the T cell population within different studies [51, 52], which might partly be due to the focal nature of the disease. $CD4^+$ cells were shown to be more common in portal tracts, whereas $CD8^+$ cells predominate in areas of lobular hepatitis [53].

The presence of autoantibodies including perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) or antinuclear antibodies (ANA) in the sera of PSC patients further suggests an (auto)immune pathogenesis [54]. More than 80 % of PSC patients show atypical antineutrophil cytoplasmic antibodies (ANCA) [55]. Atypical p-ANCA appear to cross-react with human tubulin beta isoform 5 present in human neutrophils and the bacterial protein FtsZ that is present in bacteria of the intestinal microflora. This might present the basis for molecular mimicry in which autoantibodies triggered by a bacterial infection cross-react and alter normal immune cell function [56]. Additionally, autoantibodies directed against surface antigens of BECs were found more frequent in PSC compared with PBC, AIH, and control patients [57]. Binding of these antibodies to BECs induces upregulation of toll-like receptors (TLR), which further initiates the production of cytokines/chemokines by BECs. This cytokine and chemokine production initiates inflammation via recruitment of inflammatory cells which furthermore lead to perpetuation of the inflammation [34, 58].

Animal models: Presently, no ideal animal model resembling all characteristic hallmarks of human PSC exists [59]. The

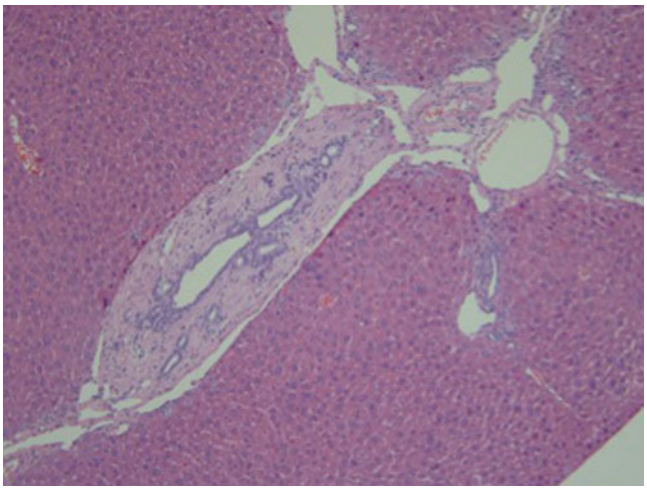

Fig. 20.1 Cholangitis and typical onion-skin-type periductal fibrosis in an *Abcb4*$^{-/-}$ mouse (H&E staining, ×10)

different animal models currently available can be classified into [34] (a) models of experimental biliary obstruction, (b) knockout mouse models, (c) cholangitis induced by infectious agents, (d) chemically induced cholangitis, (e) models using bacterial cell wall components, and (f) models of primary biliary epithelial and endothelial cell injury [59]. Chemically or genetically modified bile composition ("toxic bile concept") was shown to induce sclerosing cholangitis in a number of animal models [60–64]. *Mdr2* (*Abcb4*)$^{-/-}$ mice spontaneously develop cholangitis and typical onion-skin-type periductal fibrosis, resulting in some of the key phenotype features of human PSC [60, 61] (Fig. 20.1). This is due to a defective biliary phospholipid secretion resulting in increased concentration of free non-micellar bile acid. However, *Abcb4*$^{-/-}$ mice do not develop IBD or cholangiocellular (but hepatocellular) carcinoma, and the composition of bile in early-stage PSC patients was shown to be normal [65]. Furthermore, the role of human *MDR3* (*ABCB4*) variants in the pathogenesis of PSC is still unclear [65]. Since *Abcb4*$^{-/-}$ mice heave a very stable (P)SC phenotype which does not require prior manipulation, this model has been proven very useful to screen novel pharmacological approaches.

Another mouse model induces characteristic features of sclerosing cholangitis by feeding of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) [66]. The underlying mechanisms of DDC-induced cholangitis are unknown. An induction of a reactive cholangiocyte phenotype via toxic bile components or increased porphyrin secretion appears to be involved [34]. In addition impaired micelle formation and phospholipid secretion have been suggested to play a role in this model [67]. Feeding of lithocholic acid (LCA) to mice results in the development of periductal edema, bile infarcts, destructive cholangitis, and fibrosis [68]. These features are typically observed in early-stage PSC, and therefore this mouse model represents a valid short-term model to investigate early lesions in the development of sclerosing cholangitis [34].

A strong evidence for a potential role of vascular injury with ischemia of BECs in the development of sclerosing cholangitis comes from animal models of endothelial cell injury showing close morphological similarities with human PSC. In this respect, it is of particular interest that obliteration of the peribiliary capillary plexus is also a hallmark of bile duct injury in the *Abcb4*$^{-/-}$ model.

Clinical Presentation and Diagnosis of Primary Sclerosing Cholangitis

Clinical presentation: The disorder usually affects young to middle-aged (30–40 years) patients, with a male to female ratio of 2:1. Typical clinical symptoms include fatigue, intermittent jaundice, weight loss, right upper quadrant abdominal pain, and pruritus. However, up to 50 % of patients are oligo- to asymptomatic at the time of diagnosis and are diagnosed incidentally when persistently elevated serum alkaline phosphatase, usually in the setting of ulcerative colitis, is observed. The great majority (approximately 70 %) of cases with PSC suffer from concomitant ulcerative colitis. On the other hand, approximately 5 % of patients with ulcerative colitis develop PSC [69]. PSC can occur several years after colectomy; conversely, IBD may develop years after liver transplantation due to PSC [70–72].

Continued destruction of bile ducts in PSC leads to end-stage liver disease and portal hypertension, and therefore patients may also present with signs and symptoms of decompensated liver disease like ascites, peripheral edema, and variceal hemorrhage. Another frequently encountered clinical complication of PSC is episodic bacterial cholangitis with fever, chills, night sweats, and right abdominal pain, and patients may even develop biliary sepsis. Usually liver tests worsen during these episodes of bacterial cholangitis. PSC is associated with hepatobiliary and colorectal malignancies; therefore, attention has to be drawn to exclude malignant disease (PSC: Risk of Malignancy and Need for Surveillance).

Diagnosis of PSC: The diagnosis of PSC requires exclusion of secondary causes. These include ischemic bile duct lesions, viral or bacterial infections (e.g., cytomegalovirus or cryptosporidiosis), and chemical or surgical injury to bile ducts. In addition, sclerosing cholangitis can be mimicked by metastatic malignancies choledocholithiasis or choledochal cysts (Table 20.1).

Laboratory tests: Serum biochemical tests are usually unspecific and typically show a cholestatic pattern with elevation of serum alkaline phosphatase as the key finding. The finding of a cholestatic liver enzyme pattern in a patient with IBD should

Table 20.1 Causes of sclerosing cholangitis

Primary (PSC)	– Immune-mediated
Secondary (SSC)	– IgG4-associated cholangitis (IAC, AIP)
	– Recurrent bacterial cholangitis
	– Critically ill patients (SC-CIP), burns, trauma
	– Ischemic bile duct injury
	– Viral infections (HIV cholangiopathy, CMV)
	– Cryptosporidiosis
	– Surgical/mechanical trauma
	– Chemical injury
	– Portal biliopathy
	– Choledochal cysts
	– Malignancy/metastatic disease

therefore always raise the suspicion of PSC. Elevations of liver transaminases (usually 3–4 times upper limit of normal) and GGT are also frequently found, but in some cases an isolated elevation of AP can be seen. High levels of transaminases usually indicate acute biliary obstruction or might be due to an underlying overlap syndrome with autoimmune hepatitis. Serum alkaline phosphatase and bilirubin may take a fluctuating course during PSC; however, persistently elevated bilirubin might indicate advanced disease (especially with concomitant low albumin, impaired coagulation, and low platelet count) or significant stricturing. Autoantibodies—atypical p-ANCA—as indicated above (see Chap. 2: Pathogenesis of PSC) are unspecific and their presence does not correlate with stage of disease. Antimitochondrial antibodies are usually absent in PSC, and their presence in concomitance with a cholangiogram typically for PSC suggests a PBC-PSC overlap syndrome.

Imaging: Multifocal strictures and segmental dilatations of the biliary tree are the characteristic findings of PSC. High-quality, standardized magnetic resonance cholangiopancreaticography (MRCP) (Fig. 20.2a) shows excellent diagnostic performance and cost-effectiveness and has therefore replaced endoscopic retrograde cholangiopancreaticography (ERCP) (Fig. 20.2b) as the diagnostic method of choice [73, 74]. Due to its invasiveness and associated morbidity, ERCP is reserved for cases which require intervention for biliary obstructions or exclusion of malignancy or—in rare cases with a high degree of clinical suspicion—where high-quality MRCP was inconclusive and small duct PSC has been excluded by liver biopsy [75].

Liver biopsy: Unless small duct PSC or overlap syndrome is suspected, liver biopsy is not required for the diagnosis of PSC. About 5–15 % of patients present with small duct PSC characterized by onion-skin-type fibrosis surrounding the small bile ducts but show a normal cholangiography [76, 77]. This diagnosis can be made only by liver biopsy. Sampling variability due to the heterogenous involvement of liver is frequent, and thus typical onion-skin-type fibrosis might be absent on liver biopsy, which does not preclude the diagnosis of PSC. Liver

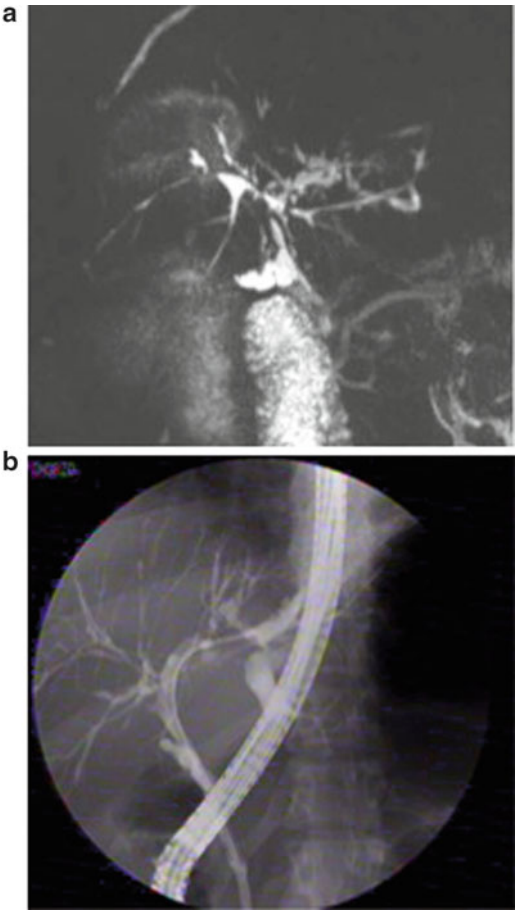

Fig. 20.2 (**a**) Magnetic resonance cholangiopancreaticography (MRCP) and (**b**) endoscopic retrograde cholangiography (ERC) showing bile duct irregularities in PSC with strictures and dilatations of large bile ducts

biopsy is a valuable tool in the diagnosis of overlap syndromes. PSC may overlap with AIH in about 8 % of cases; single cases of PBC-PSC overlap have been reported but are rare.

Colonoscopy: IBD associated with PSC shows peculiar features (PSC-IBD), including right-sided predominance and backwash ileitis [78]. PSC-IBD is usually oligo- to asymptomatic; therefore, all patients with a diagnosis of PSC require an initial colonoscopy with biopsies irrespective of symptoms to diagnose or exclude PSC-IBD. In most cases ulcerative colitis (UC) is present (about 80 %) although endoscopic apparent inflammation can differ from classical UC. In about 10 %, concomitant Crohn's disease (CD) is found (with a higher prevalence in small duct PSC), while the rest is indeterminate colitis. PSC-IBD has about a fivefold higher risk than the already increased colorectal cancer risk in UC. In addition, it is important that IBD may develop during the course of PSC (even after liver transplantation); thus, repetition of an initially negative screening colonoscopy is recommended every 5 years to detect late-onset PSC-IBD [79]. When IBD is present (bi)annual surveillance colonoscopies should be performed.

Subtypes of PSC

Small duct PSC: Patients with small duct PSC present with similar symptoms and similar laboratory abnormalities as classical PSC. However, small duct PSC has a favorable prognosis compared to classical disease, regarding survival and risk of developing malignancy [80, 81]. During the clinical course, a transition into the more classical phenotype is possible in up to 23 % after a median of 7.4 (interquartile range 5.1–14) years [80].

Overlap syndrome PSC-AIH: PSC may overlap with AIH in about 8 % of cases; single cases of PBC-PSC overlap have been reported but are rare. AIH-PSC overlap should be considered in patients with features of PSC and elevated transaminases in combination with positive autoantibodies and liver biopsy findings consistent with AIH. Patients with AIH-PSC overlap show a more progressive course of the disease but typically respond to corticosteroids.

IgG4-associated sclerosing cholangitis (*IAC*): IAC is characterized as a steroid-responsive systemic inflammatory disorder in which affected organs have a lymphoplasmacytic infiltrate rich in IgG4-positive cells and has recently become widely recognized as clinical entity distinct from classical PSC [82, 83]. Patients with IAC are less likely to have concomitant IBD and typically show a laboratory and clinical response to corticosteroids [82]. Moreover, relapse after withdrawal of therapy seems to be common.

Elevated serum IgG4 levels as well as positive staining for IgG4-positive plasma cells on liver biopsies, exclusion of malignancies, and response to steroid therapy are the typical diagnostic features of AIC. However, diagnosis can be challenging since serum IgG4 levels may be normal in 30–50 % of cases and elevated serum levels of IgG4 above the usual cutoff of 135–140 mg/dL may also be seen in many other liver diseases including liver cirrhosis and malignancy. Although in daily clinical practice a >2-fold increase in serum IgG4 is considered highly specific for autoimmune pancreatitis and IAC, the use of a twofold cutoff may not reliably distinguish IAC from cholangiocarcinoma (CCA). A cutoff of four times the upper limit of normal appears to be nearly 100 % specific for IAC [84].

Routine testing for IgG4 is recommended by recent guidelines for the management of PSC [85]. However, the prognostic and diagnostic relevance of elevated IgG4 levels in PSC is still unclear and the distinction from IAC is not always easy. About 10–20 % of PSC patients test positive for elevated serum IgG4 (mean around 240 mg/dL, range 216–357) [86] and may even have IgG4-positive plasma cells in their liver explant tissue [87]. It is currently unclear whether such patients may represent the following: (a) a subgroup of PSC with more rapid disease progression (shorter time to liver transplantation) and more advanced disease, (b) an overlap syndrome of PSC with IAC, or (c) could just be typical IAC which may have been misdiagnosed or misclassified as PSC. Importantly, not all patients showing elevated serum IgG4 levels will have classic autoimmune pancreatitis or IAC, but some of them may potentially benefit from immunosuppression, irrespective of their formal disease classification.

PSC: Risk of Malignancy and Need for Surveillance

In a national-based study including a large cohort of PSC patients, 44 % of deaths were due to malignancy [3]. The risk for hepatobiliary and colorectal cancer was 161-fold and 10-fold increased, respectively, in PSC patients compared to the general population. The frequency of CCA in PSC was 13 %, with an incidence rate of 1.5 % per year. The risk for HCC may be up to 2 % per year [88]. Concerning pancreatic cancer data are controversial. While one study claimed a 14-fold increased risk for pancreatic carcinoma [3], these data could not be confirmed by others. To date, malignancy has become the major cause of death in PSC [79, 89] and thorough cancer surveillance (Table 20.2) is essential [90, 91].

Cholangiocarcinoma (*CCA*): CCA has a very dismal prognosis with 5-year survival rates of less than 10 % [92]. In PSC, CCA has a lifetime prevalence of up to 15 % [93]. In contrast to CRC, the development of CCA is independent of disease duration, and about 50 % of bile duct malignancies present within the first year after diagnosis of PSC [94]. The diagnosis of CCA and tumor surveillance in PSC include serum and biliary tumor markers and noninvasive imaging modalities such as ultrasound, computed tomography, and magnetic resonance imaging. Further invasive strategies consist of ERCP combined with brush cytology, biopsy, cholangioscopy, and intraductal ultrasound which are not recommended as primary surveillance strategy due to their invasive nature. Therefore, for daily practice, pragmatic diagnostic algorithms including annual magnetic resonance imaging (MRCP) and serum CA 19-9 have been proposed (Table 20.2) [85, 89]. CA 19-9, however, has a rather low specificity, since it is also elevated in the presence of cholestasis. Thirty-two percent of patients with PSC have elevated serum CA 19-9 in the absence of CCA. When a dominant stricture and/or a CA 19-9 elevation above 129 U/mL is present, the patient should undergo endoscopic retrograde cholangiography (ERC) with brushings for conventional cytology or biopsies for histology [89]. A cytological fluorescence in situ hybridization (FISH) test for chromosomal abnormalities may improve diagnostic accuracy in selected patients [95]. In addition, proteomic profiling of bile seems promising for diagnosis of CCA by identification of the protein spermatogenesis associated 20 (SSP41), which can

Table 20.2 Cancer surveillance in PSC

Malignancy	Surveillance	Frequency
Cholangiocarcinoma	MRI/MRCP	Annually
	CA 19-9	
Gallbladder carcinoma	Ultrasound (MRI)	Annually
	Consider cholecystectomy, if lesions of any size are apparent	
Colorectal cancer	Colonoscopy from PSC diagnosis	Every 1–2 years (chromoendoscopy[a])
Hepatocellular carcinoma	Ultrasound/MRI	Annually
Pancreatic cancer (controversial, may represent distal CCA)	Ultrasound/MRI	Annually

MRI magnetic resonance imaging, *MRCP* magnetic resonance cholangiopancreaticography

[a]Data from IBD patients suggest a role of chromoendoscopy for cancer surveillance, however the benefit of chromoendoscopy in patients with PSC is not totally clear at the moment due to lack of data

also be measured in serum samples [96]. In the future, urinalysis for CCA proteomics may improve noninvasive diagnosis of CCA and provide distinction from benign lesions (peptide marker model: sensitivity 83 %, specificity 79 %) [97].

Several risk factors for CCA in PSC have been identified and help to select patients, who may benefit most from close tumor surveillance. These factors include older age at PSC diagnosis, alcohol, smoking, elevated bilirubin, long-standing IBD, presence of CRC or dysplasia in patients with UC, proctocolectomy, variceal bleeding, and polymorphisms of the NKG2D gene [98, 99]. On the other hand, improvement in serum alkaline phosphatase to below 1.5 ULN is associated with better outcome and reduced risk for CCA [100]. To date, surgical and systemic treatment options are still very limited. Surgical resection is possible only in patients with well-preserved liver function and just a small proportion of patients is eligible for surgical resection at the time of diagnosis and 5-year survival rates after curative resection are only about 30 % [101, 102]. These data are comparable to the outcome of a series of 223 patients with PSC undergoing orthotopic liver transplantation (OLT) between 1990 and 2001 [103]. Of these, in 31 patients, CCA was found in the explanted liver. One-, three-, and five-year survival rates were 65, 35, and 35 %. In a larger trial of 207 patients transplanted for CCA (Cincinnati registry), survival rates after 1, 2, and 5 years were 72, 48, and 23 % [104].

Recently, the Mayo protocol for liver transplantation in unresectable perihilar CCA without distant metastasis has been established, comprising of a neoadjuvant and adjuvant chemoradiotherapy as well as a staging laparotomy before liver transplantation [105]. Thereby, 5-year survival rates of 65 % can be achieved, while an 11.5 % dropout rate after start of therapy within the protocol was observed [106]. In highly selected patients with low tumor stages, 5-year survival rate of even 80 % comparable to other indication can be achieved [107]. Independent predictors of recurrence of CCA after OLT are elevated CA 19-9, portal vein encasement, and residual tumor on explant and occur in about 20 % of patients [108]. De novo onset of CCA after OLT with or without recurrent PSC is a very rare event [109, 110].

Gallbladder Carcinoma

The prevalence of gallbladder mass lesions in PSC patients is elevated in comparison to the general population (3–14 % vs. 0.35 %) [111]. Males are predominantly affected comprising 60 % of patients. Prognosis of gallbladder carcinoma (GBC) is similarly dismal as in CCA with 5-year survival rates of less than 10 % [112]. Since about 60 % of gallbladder mass lesions may harbor cancer or dysplasia, cholecystectomy can be considered for any gallbladder lesion in PSC patients with good liver function (Table 20.2). A polyp size of ≥0.8 cm was shown to predict the presence of GBC with a sensitivity of 100 % and a specificity of 70 % in PSC patients [113]. Notably, surgery-related morbidity and mortality in advanced liver disease in case of cholecystectomy must be taken into account.

Colorectal Cancer

Following CCA, CRC accounts for the second most frequent malignancy in PSC patients. The risk for development of CRC is 4.6-fold higher in patients with PSC and UC than in patients with UC alone [114], pertaining the proximal colon in 65 % of patients [115]. Its cumulative incidence in PSC patients with UC is 9 % after 10 years and 20–31 % after 20 years of disease duration [115]. Therefore, surveillance colonoscopy should be performed (bi)annually [85, 91]. Although the incidence of CRC might be increased after OLT, preemptive proctocolectomy prior to OLT did not improve survival after 5 years. However, the risk of progression from low- to high-grade dysplasia or even adenocarcinoma may be increased in PSC/UC patients.

Chemoprevention in PSC

The role of UDCA in chemoprevention of cancer is controversial. UDCA has been suggested to decrease prevalence of CRC in vitro and in vivo, which was also shown in patients with PSC and IBD in retrospective cohorts of a rather small sample size [4, 116]. On the contrary, high-dose UDCA (28–30 mg/kg/day) was even shown to be associated with an even higher risk of colorectal neoplasia, most of malignant tumors occurring after ≥2 years of UDCA treatment [117]. This may be mediated via secondary bile acids, such as LCA. Yet, the largest cohort so far treated with a dose of 17–23 mg/kg/day

of UDCA showed no difference in dysplasia-/cancer-free survival compared to placebo [118]. The use of 5-aminosalicylic acid (5-ASA) may also be supported by most studies in the setting of IBD and chemoprevention of CRC [119]. Nevertheless, data on 5-ASA in the setting of IBD and PSC are lacking.

Similarly, data on the prevention of CCA with the use of UDCA remain contradictory. The evidence for the efficiency in preventing CCA is limited. Retrospective studies suggest a potential beneficial effect for UDCA [120, 121].

Pharmacotherapy of PSC

The outcome of pharmacological treatment of patients with PSC is still unsatisfactory. Since the underlying pathogenesis of PSC is not completely understood, causal therapies are still lacking. Several therapeutic approaches including UDCA, antibiotics, immunosuppressants, as well as fatty acids and probiotics have been evaluated in clinical studies. To date, none of these strategies have proven to improve survival. Generally, it may be difficult to prove the efficacy of a given medication in PSC due to the fluctuations in the natural course and the slowly progressive course of the disease.

Ursodeoxycholic acid (*UDCA*): Similar to many other cholestatic disorders, UDCA improves liver biochemistry in PSC [122]. One of the first reported case series of PSC patients treated with UDCA described marked reduction in liver enzymes as well as reduction of pruritus and fatigue [123, 124]. Apart from changes in serum biochemistry, improvement of radiographic abnormalities encouraged the use of UDCA in PSC [125].

Although the initial placebo-controlled randomized studies showed that low- to medium-dose UDCA (13–15 mg/kg/day) has improved liver biochemistry and even histopathology [126], a long-term study failed to reduce death and disease progression [127]. Therefore, placebo-controlled trials with a higher dose and longer follow-up time were designed. An initial placebo-controlled study testing 20 mg/kg was encouraging, by showing not only improvement of liver biochemistry but also a reduction in disease progression [128]. Conversely, another large long-term trial using a UDCA dosage between 17 and 23 mg/kg showed a positive trend but was insufficiently powered to produce a statistically significant benefit in survival over 5 years of follow-up [120]. The use of high-dose UDCA (25–30 mg/kg/day) was beneficial in short-term use [129], but a long-term study was prematurely stopped due to higher rates of disease progression [118, 130].

In summary, the available data on UDCA (15–20 mg/kg/day) shows that it has beneficial effects on serum liver biochemistry, but a beneficial effect on survival could not be demonstrated so far (Table 20.3). Importantly, high-dose UDCA must be avoided in PSC patients. While current AASLD guidelines generally advise against the use of

Table 20.3 Therapeutic effect of UDCA in PSC

Study	Dose	Duration	Number of patients	Outcome
Chazouillères et al. [123]	750–1,250 mg/day	6 months	15	AP, ALT, GGT ↓
				Fatigue, pruritus ↓
O'Brien et al. [124]	10 mg/kg/day	6 + 18 months	12	Cholesterol ↓ liver enzyme ↓ bilirubin ↓
				Fatigue, pruritus ↓
Beuers et al. [126]	13–15 mg/kg/day	12 months	14 (6 UDCA and 8 placebo)	AP, ALT, GGT, bilirubin ↓
				Serum hydrophobic BA ↔
				Histopathological findings ↔
Lindor et al. [127]	13–15 mg/kg/day	26 months	105 (53 UDCA vs. 52 placebo)	Death, LTx, histological progression, decompensation of cirrhosis ↔
				AP, AST, GGT, bilirubin ↓
Mitchell et al. [128]	20 mg/kg/day	2 years	26 (13 UDCA vs. 13 placebo)	AP, GGT ↓
				Progression cholangiographic appearances ↓
				Liver fibrosis ↓
Olsson et al. [120]	17–23 mg/kg/day	5 years	219 (110 UDCA vs. 109 placebo)	Survival, cholangiocarcinoma ↔
				AP, ALT ↓
Harnois et al. [128]	25–30 mg/kg/day	1 year	30	AP, AST, bilirubin ↓
Lindor et al. [130]	28–30 mg/kg/day	Up to 5 years	150 (76 UDCA vs. 74 placebo)	AP, ALT ↓
Imam et al. [118]				Adverse events ↑

↑ increase, ↓ decrease, ↔ no change, *AP* alkaline phosphatase, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GGT* gamma-glutamyl transpeptidase, *BA* bile acids

UDCA in PSC [85], the EASL guidelines are more open for its use (especially in early stages), although emphasizing that the limited database does not yet allow a specific recommendation for the general use of UDCA in PSC [91]. However, as a result of limited therapeutic alternatives, most centers continue to treat PSC patients with standard dose (15–20 mg/kg/day) in the expectation to modify the disease course in a beneficial way when administered sufficiently early.

Combination treatment with UDCA: Combining metronidazole (see below) with UDCA in PSC improved serum biochemistry as well as Mayo risk score, but had no effect on disease progression [131]. Also combination therapy of UDCA with budesonide did not result in additional benefit [132].

Other bile acid-based strategies: Realizing the limitations of PSC therapy with UDCA, bile acid (BA) derivatives have been synthesized to potentiate the UDCA actions. As such, a 24-*nor*ursocdeoxycholic acid (*nor*UDCA) has shown to be superior to its mother compound in treating biliary fibrosis and cholangitis in the *Abcb4*$^{-/-}$ mouse model [133–135]. Anti-cholestatic, anti-fibrotic, and anti-inflammatory effects of *nor*UDCA were associated with induction of phase I and phase II BA detoxification enzymes with simultaneous induction of BA basolateral efflux systems by *nor*UDCA resulted in alternative renal excretion of more hydrophilic BA [133, 134]. Additionally, *nor*UDCA treatment resulted in the induction of bicarbonate-rich bile flow via cholehepatic shunting [134]. This probably main mechanism of action of *nor*UDCA is supported by the hypothesis that increased bicarbonate concentration in the bile protects the bile duct epithelial cells of toxic detergent effect of amphiphilic BA ("bicarbonate umbrella") [136–138]. Furthermore, *nor*UDCA has profound beneficial effects on lipoprotein composition and hepatic lipid metabolism [135, 139]. These preclinical studies make *nor*UDCA a very attractive therapeutic candidate for PSC. However, efficacy in humans has to be proven and a multicentric phase II study has been initiated.

Apart from UDCA and its derivatives, various BA-derived or non-BA-based activators of farnesoid X receptor (FXR) have been developed and could represent a novel treatment option for patients with PSC. Activation of this main nuclear BA receptor induces various genes protecting against toxic BA accumulation in cholestasis [140]. This involves reduction of intrahepatic BA concentration via downregulation of BA synthesis and increase in BA export, changing bile composition by inducing phospholipids and bicarbonate secretion as well as bile dilution via increased ductular secretion [141, 142]. Furthermore FXR activation shows direct anti-inflammatory effects in hepatocytes and non-parenchymal liver cells [143–145]. Of particular interest for PSC, where gut flora seems to play a crucial role in disease pathogenesis, is the pronounced effect of FXR in gut-liver axis (induction of FGF19, a suppressor of BA synthesis; reduction of bacterial overgrowth and intestinal permeability; anti-inflammatory effects in the intestine) [146–148]. The FXR ligand INT-747 (obeticholic acid) improved serum biochemistry as combination therapy with UDCA [149, 150] or as monotherapy in PBC [151]. In the *Abcb4*$^{-/-}$ mouse model INT-767, a dual FXR and TGR5 (G protein-coupled BA receptor) agonist (but not INT-747) ameliorated serum biochemistry, portal inflammation, and biliary fibrosis [152]. The mechanisms for this beneficial effect include FXR (but not TGR5)-dependent increase of biliary bicarbonate secretion and reduced BA synthesis and biliary output. Therefore, FXR agonists may also represent a potential therapeutic strategy for PSC and associated IBD.

Immunosuppressants: Attempts to treat PSC with mycophenolate mofetil, tacrolimus, corticosteroids, etanercept, cyclosporine, azathioprine, methotrexate, and infliximab have been largely unsuccessful, and therefore immunosuppressants are not recommended in the treatment of PSC. The use of corticosteroids and azathioprine is restricted to AIC and PSC-AIH overlap.

Antibiotics: Due to the proposed pathogenetic concept that enteric bacterial flora, portal bacteremia, or chronic bile duct infection may be important in the development and progression of PSC, the use of antibiotics was tested. In small studies, treatment with antibiotics such as vancomycin, metronidazole, and tetracycline showed biochemical improvement. Oral vancomycin profoundly improved liver enzymes in children with PSC [153]. However, the discontinuation of the treatment led to the recurrence of symptoms and abnormal laboratory findings. Metronidazole/UDCA combination therapy was shown to reduce serum alkaline phosphatase levels and Mayo risk score, but had no marked effect on disease progression [42]. Minocycline was tested in PSC patients in a pilot study for 1 year, and a reduction in AP levels as well as Mayo risk score was demonstrated [154].

Fatty acid supplementation: Docosahexaenoic acid (DHA) is a long-chain fatty acid known to be involved in inflammatory processes. Its anti-inflammatory effects are based at least in part on activation of peroxisome proliferator-activated receptor alpha (PPARα) involved in fatty acid metabolism with remarkable anti-inflammatory properties. Impaired PPARα activation is associated with bile duct injury in cystic fibrosis transmembrane conductance regulator knockout (Cftr$^{-/-}$) [155] and *Abcb4*$^{-/-}$ mice.

Due to the potential link between CFTR abnormalities and PSC, a pilot study tested DHA in PSC and improved AP levels after 1 year of treatment [156]. Notably, long-chain fatty acid has also been shown to be reduced in serum and liver of *Abcb4*$^{-/-}$ mice, and feeding high-fat and cholesterol diet led to improvement of biliary phenotype in these mice [135].

Endoscopic Treatment Options

When dominant strictures are present, underlying CCA has to be excluded. Dominant strictures are present in up to 50 % of cases [157, 158], and malignancy is present in 5–20 % of strictures. Endoscopic dilatation or dilation in combination with stent implantation improves biliary flow and subsequently symptoms. So far no controlled data are available as to which approach should be preferred [158]. The combination of stenting plus dilatation has been shown to be associated with increased risk of complications [158]. Thus, biliary endoprosthetic stent placement should be reserved when dilatation is unable to maintain lumen patency. Although symptoms can be improved substantially, endoscopic interventions have not been shown to improve disease progression or survival.

Orthotopic Liver Transplantation for PSC

OLT is the treatment of choice in patients with advanced liver disease due to PSC. In the United States and Europe, PSC is the fifth most common indication for OLT, whereas in the Scandinavian countries, it even represents the leading cause [159]. OLT for PSC achieves excellent outcomes superior to other indications. Five-year survival rates after OLT are as high as 85 % [160]. Quality of life among patients with OLT for PSC is comparable to other indications, while employment rates in PSC patients are even superior to patients transplanted for other indications [161]. Defining the most advantageous time point for liver transplantation is important but may be complicated due to difficult prediction of the disease course and the high risk of biliary tract cancer.

Timing of OLT: *prognostic models for PSC*: Appropriate timing for OLT is challenging since PSC patients often develop only mild abnormalities in coagulation parameters or albumin, and stable periods may be followed by rapid clinical deterioration in case of cholangitis. Several prognostic models have been developed to assist clinicians in predicting the natural history of PSC. The Mayo risk score first has been proposed by Wiesner et al. in 1992 [162] and was revised by Kim et al. in 2000. Including age, bilirubin, albumin, AST, and variceal bleeding, it may estimate survival up to 4 years of follow-up (see Table 20.4) [163]. Further scores have been proposed; however, MELD score has become the primary tool for predicting prognosis in PSC. A US study showed that patients with PSC have a lower risk of death prior to OLT or removal from the waiting list compared to other indications after the implementation of MELD for organ allocation [164]. In cases where MELD score does not properly reflect disease severity, additional MELD points might be gained. Exception rules resulting in additional MELD points include early hilar CCA and severe recurrent bacterial cholangitis by the United Network for Organ Sharing in the United States and Eurotransplant (country-specific).

Table 20.4 Mayo model for survival estimation in PSC adapted from Kim et al. [163]

Mayo risk score (R) = 0.03 (age [years]) + 0.54 $\log_e$ (bilirubin [mg/dL]) + 0.54 $\log_e$ (AST [IU/L]) + 1.24 (variceal bleeding [0/1]) − 0.84 (albumin [g/dL])	
Probability of survival $S_{(t)} = S_{0(t)}^{\exp(R-1.00)}$	
Time (years)	$S_{0(t)}$
1	0.963
2	0.919
3	0.873
4	0.833

$S_{0(t)}$ gives the estimated survival probabilities for a patient with a risk score of 1.00

Recurrence of Disease: Recurrence of PSC in the liver allograft may develop in about one fifth (range, 5.7–57.1 %) of patients [165, 166]. It leads to graft loss in a significant proportion of individuals and consecutively affects long-term survival [167, 168].

Several risk factors have been suggested to be responsible for recurrent PSC including HLA-DRB1*08 in either the recipient or the donor [169], absence of HLA-DR52 [170], recipient-donor gender mismatch [171], male recipient [172], related donor (living donor liver transplantation) [173, 174], use of OKT3 [175], extended donor criteria grafts [168], presence of CCA before transplantation [176], and concurrent CMV infection in the recipient. In addition, it has been found that patients with PSC are prone to ACR during the first weeks after OLT affecting up to 50 % of individuals [169]. A further strong association between recurrence of disease and IBD has been documented. Patients with UC are at a considerably higher risk for recurrent PSC after OLT [177], and a substantial reduction in the risk for recurrence is achieved by colectomy before or at the time of OLT [172]. The wide range of risk factors may reflect the difficulty of distinguishing recurrent PSC from SSC or histologic changes attributable to acute cellular allograft rejection. Non-anastomotic biliary strictures (NAS) develop in about 25 % of patients and may cause significant morbidity after OLT. The main risk factors for NAS are PSC and older donor age, while PSC, bilio-enteric anastomosis, and tacrolimus seem to be risk factors especially for late-onset NAS occurring later than 12 months post-OLT [178]. Differentiation of NAS due to recurrence of PSC from other causes should be based on a combination of radiological, histological, and biochemical investigations.

Table 20.5 Diagnostic criteria for recurrent PSC after OLT [160]

Confirmed diagnosis of PSC prior to liver transplantation
AND
Cholangiography
Intrahepatic and/or extrahepatic biliary stricturing, beading and irregularity >90 days after liver transplantation
OR
Histology
Fibrous cholangitis and/or fibro-obliterative lesions with or without ductopenia, biliary fibrosis, or biliary cirrhosis
Exclusion criteria
Hepatic artery thrombosis/stenosis
Established ductopenic rejection
Anastomotic strictures alone
Non-anastomotic strictures alone < post-transplantation day 90
AB0 incompatibility between donor and recipient

Although patients frequently present with cholestasis, clear laboratory marker profiles or specific symptoms are commonly lacking. Imaging may show characteristic multifocal strictures and segmental dilatations in recurrent PSC and may be confirmed by typical histological findings on liver biopsy. In 1999, Graziadei et al. have established diagnostic criteria for recurrent PSC (Table 20.5) [160].

Although no established pharmacological treatment of PSC exists, some centers use UDCA in this setting or recommend steroid discontinuation. No effect of immunosuppressive maintenance regimens has been identified [168, 177].

Impact of OLT on PSC-associated IBD: Since PSC-associated IBD is often quiescent, data on the disease course after OLT are contradictory. Clinical and histological inflammatory activity was found to improve in liver transplant recipients in some studies, whereas in other cohorts the activity of IBD worsened in the majority of patients and was even severe in 40 % of patients [179–181]. Cumulative risk for IBD after OLT was found to be 15 %, 39 %, and 54 % after 1, 5, and 10 years, respectively. Risk factors for recurrent IBD after OLT were symptoms at time of OLT, short interval of IBD before OLT, as well as use of tacrolimus, whereas 5-ASA was protective. CMV positive donor/negative recipient was a risk factor for de novo IBD after OLT [182]. However, data on CRC in patients with PSC suggest an increased risk after OLT as well as reduced survival [183–186]. Therefore, a careful surveillance by annual colonoscopy is mandatory. When high-grade dysplasia is present and consented between two independent pathologists, proctocolectomy is recommended. This may be avoided by chromoendoscopy and subsequent targeted/local endoscopic therapy (endoscopic mucosal resection) of suspicious lesions.

Summary and Conclusion

In summary, PSC should be considered a progressive chronic inflammatory condition affecting predominantly male patients with an increasing and geographical different overall incidence. Although progress in identifying underlying pathogenetic mechanisms of the disease has been made and several hypotheses have been created, further characterization of etiology and pathogenesis is required. The clinical presentation is characterized by a highly variable course and represents several challenges in diagnosis, surveillance, and treatment. Establishing the diagnosis requires exclusion of other treatable (secondary) causes and identification of emerging subentities. The development of tools for early diagnosis and the identification of markers indicating a progressive disease allowing a prediction of disease behavior in the individual patient remain a relevant challenge for future research. PSC is highly associated with IBD and carries a high risk of hepatobiliary and colorectal malignancies. Thus, surveillance strategies are crucial in patient management and have been proposed in current guidelines. Establishing new techniques and markers for identification and prediction of CCA therefore is also of magnitude importance for future research. Although lacking a proven survival benefit, UDCA (15–20 mg/kg/day) in combination with endoscopic therapy of dominant strictures is currently used in the treatment of PSC. Higher doses have been shown to be associated with negative outcome and should be avoided. Novel pharmacological therapies like *nor*UDCA are currently under evaluation and will hopefully add to the armamentarium of therapeutics which can exert a beneficial effect in this often fatal chronic liver disease.

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Overlap Syndromes

21

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Key Points

- Overlapping features between the classical autoimmune liver diseases (i.e., autoimmune hepatitis [AIH], primary sclerosing cholangitis [PSC], and primary biliary cirrhosis [PBC]) are not uncommon; however, the reported prevalence varies considerably due to the use of scoring systems designed for the diagnosis of the classical disorders.
- Patients with these features are usually designated with the term "overlap syndromes."
- The most common overlaps occur between AIH and either PBC or PSC.
- The International Autoimmune Hepatitis Group (IAIHG) scoring systems were the most widely applied, but are not recommended for this purpose.
- No universally accepted diagnostic criteria for these variants exist; diagnosis is usually arbitrary.
- The IAIHG suggests that patients should be classified according to the primary disorder and that those with overlapping features are not considered as being distinct diagnostic entities.
- Etiopathogenesis is unknown, but it is unlikely that the overlap conditions have a different pathogenesis from the classical disorders.
- Recognition of the overlap conditions is important due to potential therapeutic and prognostic implications.
- There are no evidence-based treatment strategies, but international guidelines recommend that combined therapy with UDCA and corticosteroids is considered in patients with PBC–AIH or PSC–AIH overlap.
- Therapy should be individualized and adjusted according to the response, with careful attention to side effects.

Introduction

A proportion of patients within the spectrum of autoimmune liver diseases may present with overlapping features of two classical disorders such as autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). These patients are often designated with the term "overlap syndromes" [1–4]. They usually represent a diagnostic challenge due to the lack of standardized diagnostic criteria. Nevertheless, recognizing this group of patients is important clinically since there may be associated therapeutic and prognostic implications. The etiopathogenesis of these "syndromes" remains elusive and whether they represent separate entities or variants of the classical disorders remains controversial. However, there is no evidence currently to support "overlap syndromes" as separate entities [5], and the International Autoimmune Hepatitis Group (IAIHG) suggests that these patients should be classified under a primary disorder, according to the predominating feature(s) [5].

The most commonly described overlaps occur between AIH and either PBC or PSC, whereas cases of PBC–PSC overlap are very rare [6]. In this chapter, we describe the various features that have been observed to overlap between the autoimmune liver disorders and discuss the clinical implications that the recognition of patients with such overlapping characteristics may have.

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Etiopathogenesis of "Overlap Syndromes"

Several explanations for the existence of overlapping features between the autoimmune liver diseases have been proposed (Table 21.1). The contention that "overlap syndromes" are the result of heterogeneous manifestations of a primary disorder is the most widely accepted, but further studies are required to clarify which explanation is correct [1–5, 7].

The exact pathogenesis of autoimmune liver diseases remains poorly understood. They are thought to occur in patients with a genetic susceptibility for self-tolerance breakdown on exposure to a triggering factor. The exact nature of the triggering and perpetuating factors is not known; however, environmental factors are likely involved [5, 8, 9]. This complex interaction culminates finally into an activation of both cellular and humoral mechanisms mediating liver injury. Despite this general mechanism, the localization of the injury is characteristically different with portal and periportal injury dominating the picture of AIH and biliary injury dominating the picture of PBC and PSC (Fig. 21.1).

Despite being strongly associated with the HLA region, the genetic susceptibility loci for the three classical disorders are generally different [8, 9]. Future genetic characterization of the autoimmune liver diseases and the subgroups with overlapping features may possibly contribute to clarify if there is a genetic basis for defining the overlaps as distinct entities.

Table 21.1 Proposed explanations for the occurrence of overlapping features between autoimmune liver diseases

Coexistence or sequential presentation of two independent diseases in a susceptible patient
"Overlap syndromes" represent distinct entities
"Overlap syndromes" are in the middle of a wide continuum of manifestations, ranging from pure hepatitic to pure cholestatic
"Overlap syndromes" are the result of heterogeneous manifestations of a primary disorder

Scoring Systems and the Diagnosis of "Overlap Syndromes"

The IAIHG primarily published an extensive scoring system for the diagnosis of AIH, then a revised version thereof, and subsequently a set of simplified diagnostic criteria [10–12]. These systems were designed basically to discriminate AIH from other disorders with similar features, rather than looking

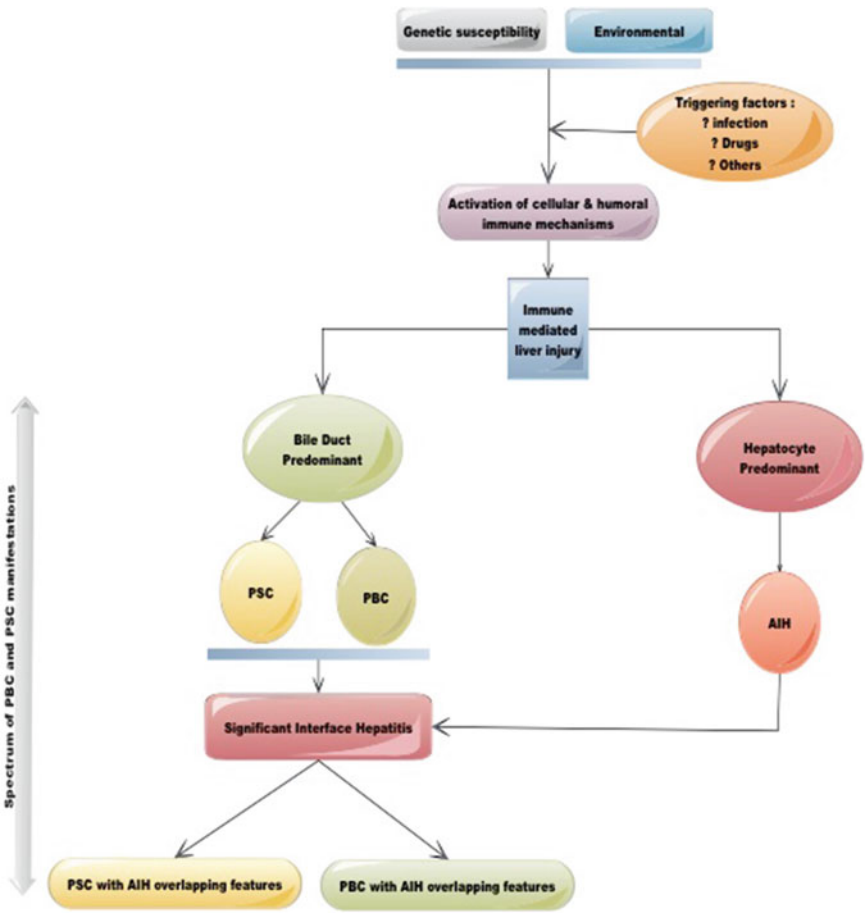

Fig. 21.1 A schematic representation of etiopathogenesis of autoimmune liver diseases illustrating the wide spectrum of possible manifestations

for similarities between disorders. The lack of internationally accepted diagnostic criteria for "overlap syndromes" has, however, led to an inappropriate use of these scoring systems in an attempt to subclassify variants with overlapping features between the autoimmune liver diseases.

The first scoring system [10] consisted of a set of descriptive criteria and a diagnostic scoring system enabling the classification into either "probable" or "definite" AIH. This system performed well in exclusion of AIH in patients with chronic HCV [13]; however, its performance for exclusion of "probable" AIH in patients with PBC and PSC was suboptimal with specificity ranging from 45 to 65 % [11, 14, 15]. A significant proportion of patients with PBC and PSC achieved sufficient scores for "probable" AIH leading to the overdiagnosis of "overlap syndromes." It was recognized subsequently that this was mainly due to the positive scoring for autoantibodies, mild to moderate elevations in serum immunoglobulins, low ratios of alkaline phosphatase (ALP) to aspartate (AST) or alanine (ALT) aminotransferases, concurrent immunological disorders and associated HLA markers which are commonly encountered in both PBC and PSC and to the inadequate scoring for histological evidence of biliary disease [11, 14]. Subsequently, a modified scoring system addressing these pitfalls was introduced [11]. Indeed, the application of the modified system reduced the prevalence of "probable" AIH in PSC patients from 33.3 % to 8.8 % and from 19 % to 6 %, respectively, in two major studies [11, 14, 16]. Recently, this modified scoring system was applied on 479 patients with autoimmune liver diseases from different countries [17]. Around 7 % of PBC patients and 14 % of PSC patients without clinical evidence of AIH overlapping features had scores of "probable" or "definite" AIH. On contrast, only 7 (29 %) patients out of 24 with clinically overlapping features (18 PBC–AIH, 6 PSC–AIH) achieved scores compatible with "probable" or "definite" AIH. The IAIHG scoring system was not able to define any specific differences between patients with overlapping features and patients with the classical disorders, reflecting its low sensitivity and specificity for its use in this context. Similar findings were reported by others [18].

A simplified scoring system [12] which eliminated several variables, including sex and other autoimmune diseases, from the modified scoring system, was subsequently introduced to simplify the diagnosis of AIH in the clinical daily setup. The performance of this scoring system in the setup of "overlap syndromes" is not well studied. It may have, theoretically, a better discriminative ability for these patients because it eliminates scores given to variables that are common for all autoimmune liver diseases. Indeed, one study applying the revised scoring system found a PBC–AIH overlapping feature prevalence of 19 % which dropped considerably to 4 % when scores for sex and other autoimmune diseases were eliminated [19], reflecting the impact of scoring for variables which are common to all autoimmune liver diseases. In another study that applied the simplified IAIHG scoring system to a group of 368 PBC patients, the proportion of patients classified as PBC–AIH overlaps was 6 %, a reduction from 12 % according to the revised IAIHG criteria [20].

In a recent report from the IAIHG, it was concluded that the IAIHG scoring systems should not be used to define subgroups of patients with overlapping features [5].

Other scoring criteria were used by some other investigators, however, few data exist about their clinical utility and they were limited to few studies compared to the more extensively used IAIHG scoring systems [21].

Characteristics of Autoimmune Liver Diseases

The scale of possible clinical manifestations of autoimmune liver diseases is wide (Table 21.2). None of these manifestations is pathognomonic; therefore, the finding of overlapping features should be interpreted carefully. The possibility of an alternative diagnosis like drug-induced hepatotoxicity, chronic viral hepatitis, or sarcoidosis should be kept in mind in patients with atypical presentations (Fig. 21.2).

Characteristics of AIH

The patient is typically a young or middle-aged female presenting with fatigue and malaise in combination with marked elevation of serum aminotransferases, hypergammaglobulinemia (typically IgG > 2 × upper limit of normal [ULN]), positive (≥1:40) antinuclear antibodies (ANA), and/or smooth muscle antibodies (SMA) [22, 23]. The diagnosis of definite AIH is established only with a liver biopsy showing the typical findings of interface hepatitis, lymphoplasmocytic infiltrates, rosetting of hepatocytes, and bridging necrosis. Other common biochemical abnormalities include mild to moderate ALP elevations and variable bilirubin elevations. Other autoantibodies associated with AIH include anti-liver–kidney microsomal antibodies (anti-LKM 1) which are seen in 3–4 % of AIH (classified as type 2) in association with positive anti-liver cytosol antigen 1 antibodies (anti-LC 1), but typically in the absence of ANAs and SMAs. Anti-soluble liver antigen/liver–pancreas antigen (anti-SLA/LP) antibodies are highly specific; however, they are detected only in 10–30 % of patients. They might be the only hint for the diagnosis in 20–30 % of patients who are negative for other autoantibodies. Perinuclear antineutrophil cytoplasmic antibodies (pANCA), often atypical, are present in 50–96 % of cases.

Table 21.2 Clinical features of AIH, PBC, and PSC

Feature	AIH	PBC	PSC
Females (%)	60–75	>90	30–35
Age	All age groups	Adults only	All age groups
	Median of 45 years	Typically 30–65 years	Typically 30–50 years
ALT and/or AST elevations	Marked (typically 3–10×ULN)	Normal or mild	Normal or mild
	May be mild or normal		
ALP elevation	May occur	Moderate to marked	Moderate to marked (typically 3×ULN). Variable, may be normal
Bilirubin elevation	Variable	Normal in majority but can be variable	Normal in majority but can be variable
Immunoglobulins	Hypergammaglobulinemia	IgM increased in most patients	IgG may be increased
	Typically elevated IgG (1.2–3.0×ULN)		IgM may be increased
Autoantibodies			
ANA	Significant titers (≥1:40) of ANA and/or SMA in 70–80 %	ANA in >30 % Anti-gp210 and/or anti-sp100 highly specific	8–77 %
SMA	May be positive in 33 % in isolation, 50 % together with positive ANAs	May be present	0–83 %
Anti-actin	May be more specific than SMA		
Anti-LKM	3–4 %		
Anti-SLA/LP	10–30 %	May be present	May be present
pANCA	50–96 %		26–94 %
	Often atypical pANCA		
AMA	Occasionally positive in low titers	90–95 %	Occasionally positive
	AMA anti-PDC-E2 rare	Anti-PDC-E2 very specific	
Liver histology	Definite AIH cannot be diagnosed without liver biopsy	Not required if AMA present	Not required for large duct PSC
Interface hepatitis	Typical	May be present	May be present
Portal inflammation	Plasma cell infiltrate	Lymphocytic infiltrate	Lymphocytic infiltrate
Biliary changes	May be present in 10 %	Typical (inflammatory biliary injury)	Typical (periductular fibrosis)
Granulomas	Atypical	Typical, invariably present	Atypical, but may be present
IBD	3–10 %	Rarely associated	Up to 80 %
	Exclude PSC		
Cholangiography	Normal (subtle changes can be present in advanced fibrosis)	Normal	Multifocal stricturing, normal in small duct PSC
Treatment	Immunosuppressive therapy	UDCA	No effective treatment

Adapted from Boberg et al. [5]

Characteristics of PBC

A PBC patient is usually a 30–65-year-old female presenting with pruritus and fatigue in combination with cholestatic biochemistry (mainly elevated ALP and gamma-glutamyl transpeptidase [GGT]) and high titers of anti-mitochondrial antibodies (AMA) directed against E2 subunit of the pyruvate dehydrogenase complex (anti-PDC-E2) [4, 24, 25]. These antibodies are detected in around 95 % of patients with high specificity (>90 %) for PBC. ANAs are detected in approximately one third of patients; however, they are antigen specific mainly in the form of anti-gp210 (nuclear pore membrane glycoprotein) and anti-Sp100 (nuclear protein Sp100) with very high specificity (>95 %) for PBC when present [24, 25]. IgM levels are usually elevated. The presence of a cholestatic biochemistry for at least 6 months with positive AMAs and/or anti-gp210/anti-Sp100 is enough to establish the diagnosis without a liver biopsy [4]. Liver biopsy is necessary to diagnose "AMA-negative PBC" which occurs in around 5 % of patients. This form was considered previously to be an "outlier syndrome" and was often called "autoimmune cholangitis"; however, several studies demonstrated that it is identical to AMA-positive PBC and should not be dealt with as a different entity [1, 2, 4]. The typical histology findings are a degenerating bile duct epithelium with focal bile duct obliteration and formation of granulomas, together constituting

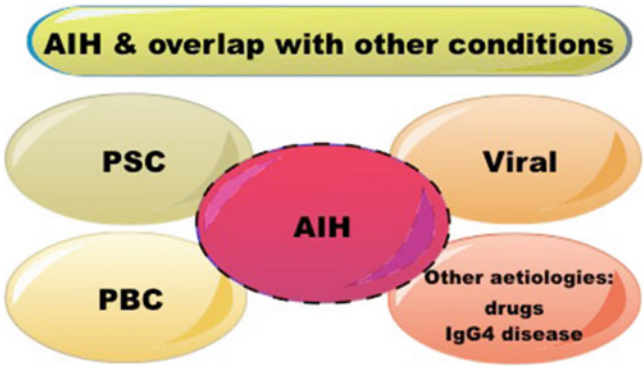

Fig. 21.2 The autoimmune liver diseases may have overlapping features including both symptoms, clinical and biochemical findings and immunological characteristics. Other possible etiologies in patients with atypical presentations must be kept in mind

what is known as "florid bile duct lesion" [26]. This typical picture is invariably present (only 32 % in one report).

Characteristics of PSC

The typical PSC patient is a 30–40-year-old male with inflammatory bowel disease (IBD), presenting with cholestatic biochemistry. ALP is moderately elevated in most patients and usually fluctuates. Mild elevations in transaminases are commonly seen. The diagnosis is established by either a high-quality magnetic resonance cholangiography (MRC) or an endoscopic retrograde cholangiography (ERC) demonstrating irregularities and stricturing of the intrahepatic and/or extrahepatic biliary tree [27]. Liver biopsy is not required for the diagnosis except in few patients with normal cholangiography to establish the diagnosis of "small duct PSC" [4]. The classical findings on histology include portal tract inflammation with infiltration of lymphocytes in the bile ducts and ductular proliferation. Periductal fibrosis is highly suggestive of PSC but is not always present.

Features Which May Overlap Between Autoimmune Liver Diseases

There is no pathognomonic feature for any of the three major autoimmune liver diseases. The diagnosis of these disorders is based mainly on a constellation of criteria, the presence of which in the absence of other confounders allows for appropriate diagnosis to be made. In addition, the criteria themselves are subject to variations inherent to the laboratory tests at one end and to the lack of reproducible assessment of descriptive criteria at the other end, for example, the assessment of the degree and significance of either interface hepatitis or biliary injury on a liver biopsy. Therefore, the knowledge of all the possible manifestations allows for a more accurate assessment to be made, particularly when a patient presents with overlapping features of two autoimmune liver diseases. It must be noted as well that the extent of the overlap is considerably variable ranging from overlap in few features to the fulfillment of the diagnostic criteria of two disorders.

Clinical Features Overlap

Despite being characteristic, none of the clinical features including age, gender, and symptoms has a discriminative ability for any particular disorder. The only absolute exception might be PBC which does not affect children. Otherwise all the three disorders can occur at any age or sex. "Overlap features" appear to be more likely in younger patients; however, no systematic studies have addressed this issue yet. The symptoms of autoimmune liver diseases are nonspecific. However, some symptoms are more likely to be seen in one disorder compared to the other. Pruritus is a common symptom of both PBC and PSC; however, it is reported to occur in AIH even in the absence of cholestasis. All the three disorders are frequently associated with other autoimmune diseases, either diagnosed before or after the diagnosis of an autoimmune liver disease, in up to 20 % of the patients. The strong association between PSC and IBD in patients of European origin is well known; however, IBD is reported to occur in around 3–10 % of AIH [5, 28] and has also been reported in PBC.

Biochemical Overlap

Around 10 % of AIH patients may present with predominantly cholestatic biochemistry. Abnormal ALP was reported in 81 % of patients with AIH (33 % >3×ULN, 10 % >4×ULN) [29]. Both PBC and PSC patients may have mildly elevated transaminase levels. Rarely, any of these disorders may have a normal biochemistry despite activity.

Immunoserological Overlap

Immunoserology is probably the most common area of overlap between autoimmune liver diseases. Compared to PSC, both AIH and PBC are usually associated with a characteristic serological reactivity pattern with autoantibodies which are highly specific that support the diagnosis. However, these serological patterns neither are pathognomonic nor have a prognostic value, and their absence does not preclude the

diagnosis of either AIH or PBC. Positive AMAs (even anti-PDC-E2-specific) are reported to occur in 8–10 % of AIH cases [30, 31] with some patients continuing to have persistently positive AMAs for many years with no other features of PBC, which signifies that this pattern of immunoreactivity also can be part of the spectrum of AIH. It must be remembered, however, that the presence of one specific characteristic feature of one of the disorders should initiate the search for another one to avoid missing patients with significant overlap who may benefit from a different therapeutic strategy. Non-organ-specific autoantibodies including ANAs and SMAs are reported to occur in large proportion of PSC patients (up to 77 % and 83 %, respectively) [5]. In addition, around 30–50 % of PBC patients are positive of ANAs and/or SMA [4], and if not specifically ordered, anti-Sp100 and anti-gp210 antibodies will be reported as positive ANAs. The pANCA is frequently detected in both PSC (26–94 %) and AIH (up to 92 %) and occasionally in PBC. Anti-SLA/LP antibodies which are highly specific for AIH have been reported in PBC and PSC [14, 21, 27]; however, their presence should alert the clinician for the possibility of AIH as differential diagnosis.

Elevated IgG and IgM levels are reported in up to 61 and 45 % [4, 14], respectively, of PSC patients, and variable proportions of PBC patients have elevated IgG. Interestingly, a unique serological profile in PBC–AIH overlaps with positive anti-dsDNA antibodies and AMAs was suggested by some investigators [32] with very high sensitivity and specificity; however, these findings need to be reproduced before a firm conclusion can be established.

Histological Overlap

The reported prevalence of histological overlap has been considerably variable, highlighting the inherent subjectivity of assessment and interpretation of the degree of various histological manifestations. Various degrees of bile duct injury including destructive cholangitis (up to 24 %) was demonstrated to occur in AIH by some studies [33] (Fig. 21.3a, b). These biliary changes are particularly common in children with AIH. Therefore, the presence of mild bile duct changes does not necessarily indicate an "overlap syndrome" and should be considered in the context of the clinical picture. It must be remembered, however, that the presence of florid bile duct lesions and granulomas is enough to preclude the diagnosis of definite AIH. In addition, a proper assessment with cholangiography is mandatory in the setup of significant bile duct injury to rule out the possibility of PSC [5]. On the other hand, interface hepatitis may occur as a part of the histological spectrum of PBC (up to 25–30 %) [19, 34] and PSC (up to 30 %) [14] (Fig. 21.3c–f). These findings should not be taken as an evidence for "overlap syndrome" outside the context of the other clinical findings.

Overlap Between PBC and AIH

Diagnosis

There are no agreed criteria for the diagnosis of the PBC–AIH "overlap syndrome" [5]. The diagnosis is based mainly on clinical judgment and can only be arbitrary. There are no clinical features that can distinguish this variant from either PBC or AIH. Most of the studies concluded that patients with overlapping features between PBC and AIH have significantly higher hepatitic markers (i.e., higher aminotransferases, higher IgG, higher frequencies of ANAs and SMAs, and higher scores for interface hepatitis) (Fig. 21.3) when compared to classical PBC and significantly more cholestatic markers (i.e., higher ALP, GGT, and IgM; positivity for AMAs; and higher bile duct injury scores) when compared to classical AIH [1, 19, 21, 35–37] (Table 21.3). The presence of these features in different combinations is described. The presence of one of the typical criteria of PBC in a patient with AIH should initiate the search for another criterion to support a potential case of a PBC–AIH overlap condition. Likewise, a suspicion of this overlap condition should be maintained in the PBC patient who presents with markedly elevated transaminases, high titers of SMA, positive SLA/LP antibodies, or unresponsiveness to UDCA [5]. It should be remembered that the overlap features can occur at the time of the initial presentation or can develop during the course of either PBC or AIH [35].

The presence of at least two out of three accepted criteria for each of AIH and PBC (also known as Paris criteria) (Table 21.4) was proposed by Chazouillères et al. to diagnose PBC–AIH overlap syndrome [1]. A recent study suggested a better sensitivity and specificity (92 % and 97 %, respectively) and a better performance compared to both the revised and the simplified IAIHG scoring systems of these criteria for the diagnosis of PBC–AIH "overlap syndrome" [38]. These criteria were incorporated in the recent European Association for the Study of the Liver diseases (EASL) practice guidelines for the management of cholestatic liver diseases [4]; however, they are not yet considered to be an international consensus [5]. The application of the IAIHG scoring systems on PBC patients to assess for AIH overlapping features is not recommended.

Prevalence

Cases with overlapping features between PBC and AIH were reported many years ago [39]. Thereafter, several studies examining the prevalence of this "overlap syndrome" were reported from different countries [1, 17, 19, 35, 36]. The reported prevalence has varied considerably depending on

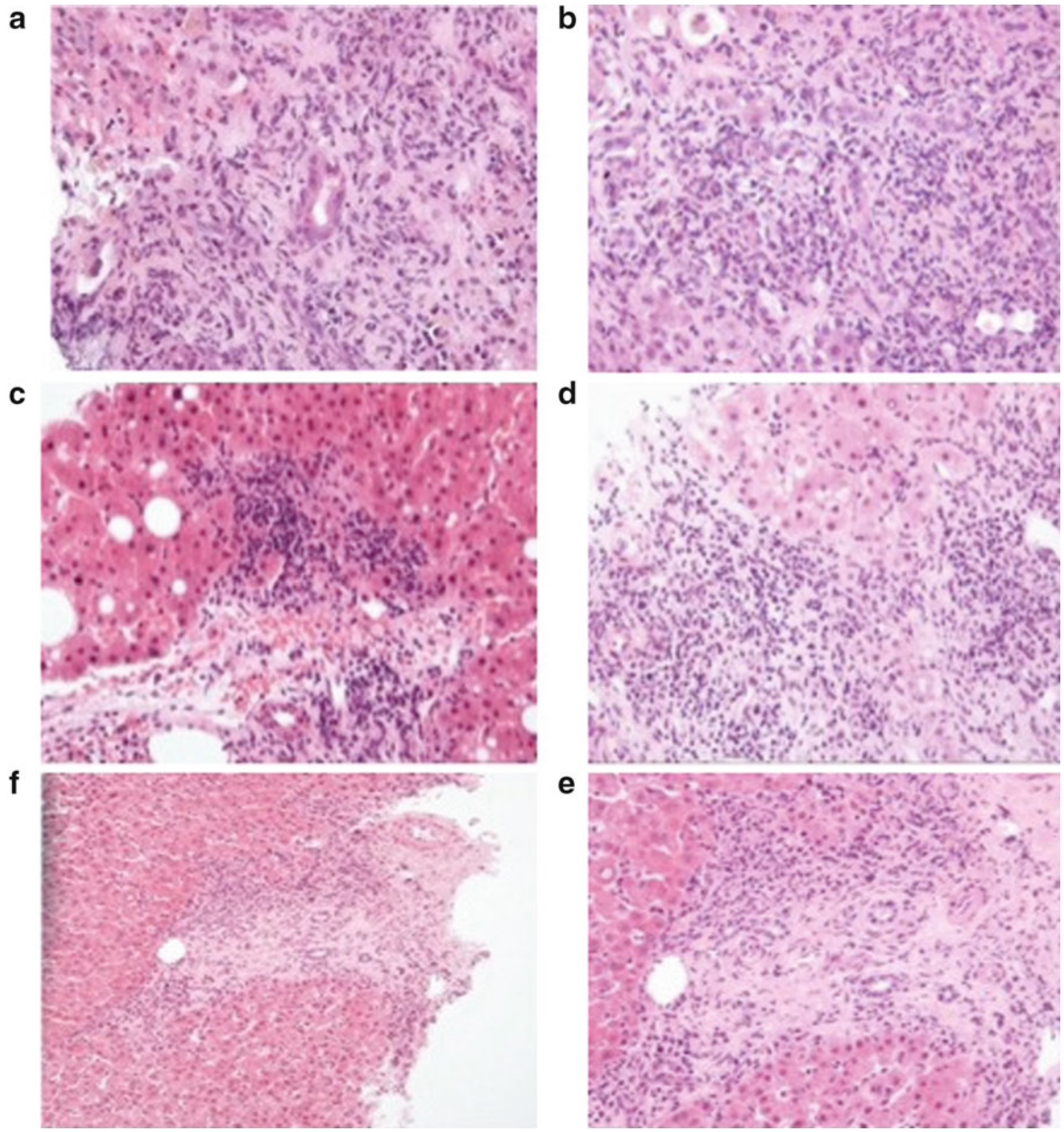

Fig. 21.3 Liver biopsy specimens from: (**a**, **b**) AIH patient with bile duct injury and no other evidence of PBC or PSC, (**c**, **d**) PBC patient with significant interface hepatitis but no other evidence of AIH and (**e**, **f**) PSC patient with significant interface hepatitis but no other evidence of AIH. This reflects the wide spectrum of manifestations which may occur on a liver biopsy in every single primary autoimmune liver disease (i.e., AIH, PBC, and PSC) (courtesy of Dr. Clare Verrill, Oxford University Hospitals)

the criteria used to define this overlap condition in every study. When the Paris criteria were applied on patients with the primary diagnosis of PBC, a prevalence of 4.8–9.2 % was reported in two large studies [1, 36]. More variable rates were reported by studies using the revised IAIHG scoring system (ranging from 2.1 to 19 %) [17, 19, 40]. The prevalence of this overlap appears to be lower in patients with the primary diagnosis of AIH. Only 8 (5 %) among 162 AIH type 1 patients in one study were classified as PBC–AIH "overlap syndrome" [35].

Treatment

Immunosuppressive therapy is the mainstay treatment for AIH [22, 23], while UDCA is the treatment of choice for PBC [4]. On contrast, the best management strategy for the patients with overlapping features between these two classical disorders remains a subject of debate. This is mainly due to the lack of controlled randomized clinical trials that have been impossible to perform due to the small numbers of patients with this variant. Therefore, most of the current

Table 21.3 Summary of clinical characteristics of PBC–AIH and PSC–AIH "overlap syndromes"

Feature	PBC–AIH "overlap syndrome"	PSC–AIH "overlap syndrome"
Age	Usually adults	Usually children, adolescents, and young patients
Females (%)	90	30 [2]
Prevalence	4.8–9.2 % (Paris criteria)	7–14 % (revised IAIHG scoring system)
	2.1–19 % (revised IAIHG scoring system)	
	6 % (simplified IAIHG scoring system)	
Transaminases	Higher than PBC, lower than AIH	Higher than PSC, lower than AIH
ALP	Higher than AIH	Higher than AIH
Bilirubin	Variable	Variable
AMA	Usually positive	Negative
ANA and/or SMA	Usually positive	Usually positive
pANCA	May be positive	Usually positive
IgG	Higher than PBC, lower than AIH	Higher than PSC, lower than AIH
IgM	Higher than AIH	Usually not elevated
Interface hepatitis	Usually present	Usually present
Bile duct injury	Usually present	Usually present
Cholangiography	Normal	Typical PSC findings (normal in small duct PSC–AIH "overlap")
IBD	Absent	Usually present
Response to UDCA monotherapy	Poor to good	Poor
Response to UDCA + corticosteroids	May respond	May respond

Table 21.4 Diagnostic criteria of PBC–AIH "overlap syndrome" of which at least 2 of 3 accepted criteria for PBC and AIH, respectively, should be present (proposed by Chazouillères et al. [1])

Diagnostic criteria of PBC–AIH overlap syndrome
PBC criteria
1. AP ≥2 × ULN or GGT ≥5 × ULN
2. AMA positive
3. Liver biopsy specimen showing florid bile duct lesions
AIH criteria
1. ALT ≥5 × ULN
2. IgG ≥2 × ULN or a positive test for SMA
3. Liver biopsy showing moderate or severe periportal or periseptal lymphocytic piecemeal necrosis

Histologic evidence of moderate to severe lymphocytic piecemeal necrosis (interface hepatitis) is considered mandatory for the diagnosis [4]

recommendations come mainly from personal experience and retrospective studies.

Some studies have suggested that the use of UDCA alone might be sufficient treatment for PBC–AIH "overlap syndrome." In a study comparing the effect of 24 months of UDCA on biochemical response and survival between 12 PBC patients with features of AIH and 159 pure PBC patients (7-year follow-up), no significant difference was observed [36]. In another study comparing 20 PBC–AIH overlap patients (16 treated with UDCA, 4 with UDCA + prednisolone) and 23 PBC patients (all treated with UDCA), no difference in response was demonstrated [41].

Immunosuppressive therapy alone was able to induce remission in 9 out of 12 PBC–AIH overlap patients in one study, a remission rate comparable to that seen with definite AIH patients followed up for similar period. The response was significantly associated with serum ALP below 2 × ULN before starting the treatment, and interestingly, patients with the "overlap syndrome" were less likely to progress to cirrhosis compared to patients with AIH [35].

On contrast, several studies demonstrated a better response to the combination of immunosuppressive therapy and UDCA. Among 11 patients diagnosed with PBC–AIH "overlap syndrome" according to Paris criteria, 5 patients were given UDCA monotherapy (13–15 mg/kg/day) and 6 patients were treated with prednisolone alone (0.5 mg/kg/day) [1]. Following a median period of 23 months, a significant improvement in ALP and GGT was observed with no significant improvement in ALT, IgG in the UDCA monotherapy group. Two patients normalized their ALP and ALT, and two patients had resolution of jaundice and pruritus. Interestingly, 3/5 patients had increased fibrosis. On the other hand, no patients in the steroid arm achieved complete response after a median treatment of 4 months. The nine nonresponding patients were then given a combination therapy of UDCA and prednisolone (with the addition of azathioprine in 5 patients) for a median of 18 months with achievement of near-complete response in all of them. Two patients were tapered of prednisolone later on and the remission was maintained with UDCA only. The conclusion from

this study was that the combination of UDCA and prednisolone is required in most patients with overlapping features to achieve a biochemical response; however, maintenance of remission could be achieved with UDCA monotherapy [1].

In another study from the same group, 17 patients with PBC–AIH overlap features were treated with either UDCA alone (11/17) or UDCA and immunosuppressive therapy combination (6/17) [47]. Only 3/11 patients in the UDCA arm achieved a biochemical response (defined as ALT < 2 ULN and IgG < 16 g/L) compared to 4/6 in the combination therapy group. Fibrosis progression in non-cirrhotic patients was observed more frequently in the UDCA monotherapy (4/8 nonresponders) compared to the combination therapy group ($p = 0.04$). Seven out of the eight nonresponders in the UDCA monotherapy group were then treated with the combination therapy with complete biochemical response and stable or decreased fibrosis achieved in 6 patients [42]. These findings were in keeping with those of another study examining the combination therapy in 16 out of 20 PBC–AIH "overlap syndrome" cases. All patients treated with this regimen had complete normalization of either ALT or AST with 14 achieving complete normalization of both. Thirteen patients had their ALP improved to below 1.5 × ULN1. These results strongly suggested that combined therapy (UDCA and corticosteroids) might be the best management strategy in most patients with well-defined PBC–AIH "overlap syndrome" [21]. A better response to the combination therapy was reported as well by several others. Interestingly, overlap patients may require lower doses of steroids and may indeed have higher response rates when compared to AIH [5].

The combination of UDCA and immunosuppressants for patients with overlapping features between PBC and AIH was recently recommended by the EASL practice guidelines for the management of cholestatic liver diseases, with the emphasis that this is not evidence-based. These guidelines suggested as well an alternative approach with starting UDCA alone as first-line therapy with the option of adding immunosuppressants in case of no response within the time frame of 3 months [4].

The role of other immunosuppressants like budesonide is yet to be clarified. Some data suggested a possible role of this drug as an alternative for conventional immunosuppressants, while others suggested no benefit [4]. The role of azathioprine in the long-term management of "overlap syndromes" is not well known. The successful experience with this drug in AIH makes its use in the setting of "overlap syndromes" as a steroid-sparing agent reasonable. Few case reports suggested a possible role for cyclosporine A in patients who failed the combination therapy [40]. Importantly, the treatment of patients with overlapping features between PBC and AIH should be individualized, and care must be taken to avoid deleterious side effects (like steroid-induced osteoporosis) if an appropriate beneficial effect is not obtained.

Prognosis

The natural history of PBC–AIH overlap conditions is not well characterized. There are reports of enhanced risk of progression to advanced fibrosis and portal hypertension with higher risk of dying of end-stage liver disease in patients not detected early in the course of their disease [43]. A histological analysis of liver biopsies from PBC patients 4 years before and after UDCA monotherapy linked the severity of lymphocytic interface hepatitis to the progression of fibrosis [44]. This study suggested that UDCA improves the bile duct injury but not the process leading to interface hepatitis. Similar findings were reported by another study [45]. The risk seems to be enhanced particularly in patients who fail to respond to the combination of UDCA and immunosuppressants. On the contrary, similar survival to PBC and AIH patients was reported by other investigators [46].

Overlap Between PSC and AIH

Diagnosis

In similarity with the PBC–AIH "overlap syndrome," no standard criteria are established to diagnose an "overlap syndrome" between PSC and AIH, a condition which affects mainly children, adolescents, and young patients [4, 5]. No specific clinical or laboratory features were reported to discriminate patients with the overlapping features from classical PSC or AIH. Higher IgG, hepatitic biochemistry, and scores for interface hepatitis were more frequent in these patients compared to classical PSC. On contrast, IBD, positive atypical pANCA, and lower transaminase levels are seen more frequently compared to AIH. The diagnosis is based mainly on the presence of characteristic biochemical, serological, and histological features of AIH in a patient who also has the cholangiographic features of PSC (Table 21.3). The sequential development of either AIH or PSC after the other condition can occur; however, the exact time course of such sequential development is not well characterized because cholangiography is not performed routinely in patients diagnosed initially with AIH. The development of AIH in a patient with a primary diagnosis of PSC seems to be rare [5]. Nevertheless, PSC patients should be observed for the development of hepatitic features and PSC should be ruled out in AIH patients who develop cholestatic biochemistry or become resistant to the standard treatment during the course of their disease. Patients who have had an initial diagnosis of AIH and subsequently undergo a cholangiographic examination which discloses features suggestive of PSC have often been classified as PSC–AIH "overlap syndromes"; however, it is the majority view to classify these patients as PSC because the cholangiography examination was not performed initially to rule out PSC [5].

Prevalence

The reported prevalence of patients with overlapping features between PSC and AIH has also been subject to the scoring systems and the methodology used in different studies. Early reports revealed a prevalence of "probable" AIH in PSC patients ranging between 19 and 33 % when the initial IAHG scoring system was used, dropping dramatically to 6–9 % when the revised scoring system was applied on the same cohorts [14, 16]. Interestingly, the prevalence of "definite" AIH seems to be stable (2 %) regardless of the version of the IAIHG scoring system used. In the largest cohorts of PSC patients evaluated by the revised scoring system till date, a prevalence of 7–14 % was reported [14, 16, 47]. A prevalence of 17 % was reported in a prospective analysis of 41 consecutive PSC patients from Italy [52]. The diagnosis of the "overlap syndromes" was based on the presence of (1) a revised AIH score >15, (2) ANA or SMA antibodies present in a titer of at least 1:40, and (3) liver histology with piecemeal necrosis, lymphocyte rosetting, and moderate or severe periportal or periseptal inflammation [48].

The prevalence of small duct PSC–AIH overlap is not well characterized and may be underestimated. It may represent a significant proportion of the overlap occasions [49].

PSC–AIH Overlap in Children (Autoimmune Sclerosing Cholangitis)

Children with PSC are more likely to have AIH features compared to adults with PSC [50]. The reported rates of a significant overlap have varied between 28 and 49 %. Among 55 children followed up prospectively, 27 were found to have abnormal cholangiograms and 52 % of them achieved the scores for definite AIH according to the IAIHG scoring system [51]. Interestingly, almost one-third did not have bile duct injury on histology and the diagnosis of PSC could have been overlooked if cholangiography had not been performed. The term "autoimmune sclerosing cholangitis" was used to describe this pediatric variant. IBD is strongly associated with PSC–AIH "overlap syndrome" in children compared to children with pure AIH. PSC should be considered in all children presenting with AIH features [52].

Treatment

PSC remains one of the few liver diseases with no effective treatment. UDCA has been tried in several studies with no significant effects on the natural history of this disease [27]. Furthermore, a possible detrimental effect with the use of higher doses of UDCA was recently reported [53]. Immunosuppressive therapies lack evidence for efficacy in PSC: nevertheless, a possible role for these agents in the PSC–AIH "overlap syndromes" has been suggested by some studies [54]. Similar to PBC–AIH "overlap syndrome," the performance of randomized controlled trials has been impossible due to the rarity of these cases, and all recommendations for treatment come from clinical experience and retrospective reports.

The clinical and biochemical response to immunosuppressive agents (mainly prednisolone and azathioprine) with or without UDCA has been described in several reports [55, 56]. However, the response rates were inconsistent among the different studies and seem to be less compared to patients with pure AIH. In addition, end points in the form of liver failure and the need of liver transplants were more likely to occur in patients with the overlap features in some studies [16, 35]. On contrast, a favorable biochemical response and an improved survival were reported by a few other studies. For example, a study using corticosteroids and azathioprine in 16 out of 24 patients with PSC–AIH overlap features demonstrated a good response based on the improvement of aminotransferases [49]. Another study demonstrated an initial response to immunosuppressive therapy in nine PSC–AIH overlap patients; however, only three maintained a long-term remission while another three underwent liver transplantation (after 4 months, 7 years, and 9 years, respectively) [47]. In the only prospective study, involving 7 patients with overlapping features of PSC–AIH, a favorable response and an improved survival were demonstrated with the combination therapy (corticosteroids + UDCA) compared to classical PSC patients [48].

The response to the combination of immunosuppressive therapy and UDCA appears to be much better in children with PSC–AIH overlap syndrome. Twenty-three out of 27 children with this overlap syndrome demonstrated a satisfactory response to the combination of immunosuppressants and UDCA, comparable to that observed in pure AIH [51].

Despite the less encouraging results reported compared to patients with PBC–AIH overlapping features, the use of the combination of immunosuppressives and UDCA seems to be the only option available currently for patients with PSC–AIH overlapping features. This strategy was incorporated in the recent EASL practice guideline for the management of cholestatic liver diseases with the emphasis that it is not evidence-based [4].

Prognosis

The prognosis of patients with overlapping features between PSC and AIH seems to be worse compared to classical AIH and the PBC–AIH "overlap syndrome," with more frequent progression to liver-related end points including death from end-stage liver disease and

requirement for liver transplantation [35, 46]. This could be explained partly by the lack of sufficient treatment for the PSC component of this condition.

Overlap Between PBC and PSC

Overlap between PBC and PSC has only been described in a few patients [6]. The two conditions can be clearly distinguished in the large majority of cases. However, liver histology may be similar, including the presence of granulomas, and AMAs may occasionally be positive in PSC.

Conclusions

Some patients present with overlapping features between the major autoimmune liver diseases (i.e., AIH, PBC, and PSC). These patients may be difficult to classify, and they are commonly designated with the term "overlap syndromes." The recognition of these overlapping conditions is important because there may be therapeutic and prognostic implications. The exact etiopathogenesis remains obscure, and despite the possible requirement for different management strategies, these overlaps do not have enough evidence to qualify them as separate entities. There are no clinical, biochemical, serological, or histological features with the ability to discriminate these variants from the classical disorders, and there are no standardized criteria to diagnose them. The IAIHG scoring systems were earlier inappropriately used to classify these "overlap syndromes"; however, this practice is not recommended due to the low sensitivity and specificity of the scoring systems in this context. The diagnosis can be only established on appropriate clinical assessment and can be described as arbitrary. Efforts should always be made to classify the primary disorder and to prescribe the appropriate therapy accordingly. The current therapeutic recommendations of the variant conditions are based mainly on personal experience and retrospective studies. The combination of UDCA and corticosteroids seems to be the best option for PBC–AIH "overlap syndrome" with lower efficacy in PSC–AIH "overlap syndromes." Therapy should in any case be individualized and not be prolonged if a beneficial effect cannot be documented. A suggested approach to the diagnosis and management of patients with overlap features is depicted in Fig. 21.4.

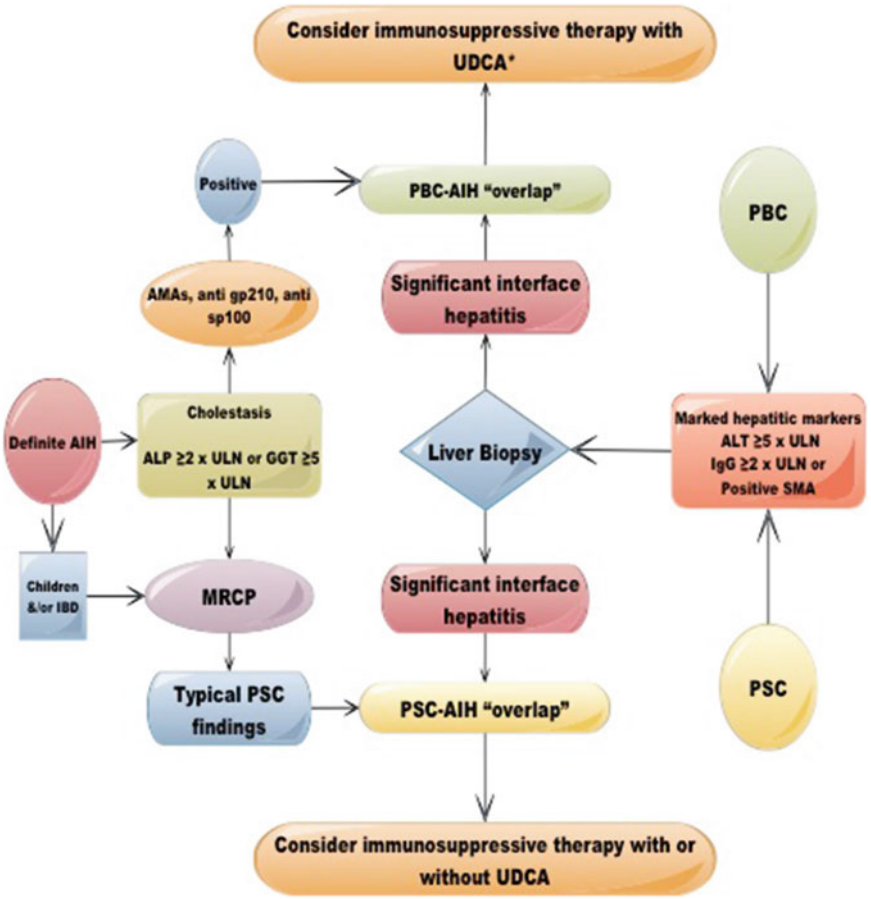

Fig. 21.4 A suggested algorithm for the diagnosis and management of patients with overlapping features of autoimmune liver diseases. Patients with PBC–AIH overlapping features might be treated initially with UDCA monotherapy, and if no response, combination therapy should be considered (*asterisks*)

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Alcoholic Liver Disease

22

Gyongyi Szabo

Key Points

- Alcoholic liver disease is the liver manifestation of the end-organ effects of chronic excessive alcohol intake.
- The effects of alcohol on gut integrity and the adipose tissue contribute to the development of ALD.
- Alcohol and its metabolites have some direct effects on the liver and reactive oxygen radicals generated during alcohol metabolism modulate functions of hepatocytes and other cell types in the liver.
- Activation of the innate immune system is a major component in the development and progression of alcoholic liver disease.
- Gut-derived and endogenous danger signals contribute to innate immune activation in ALD.
- Acute alcoholic hepatitis is mediated by pro-inflammatory cytokines.
- 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 alcoholic liver disease (ALD). Within the frame of the effects of alcohol on the liver and organ interactions, we discuss the cellular effects of alcohol and its metabolites, innate and adaptive immune responses, intracellular signaling pathways, and nuclear receptors. Current and emerging therapeutic approaches are discussed as potential translation of the basic findings in ALD to clinical applications.

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Clinical Characteristics of Alcoholic Liver Disease

Epidemiology and Natural History of ALD

It is estimated that there are 17.6 million alcoholic individuals in the USA and 140 million worldwide; while not all alcoholics develop symptomatic liver disease, about 12,109 deaths/year are attributed to ALD in the USA [1, 2]. The clinical spectrum of ALD includes liver steatosis, steatohepatitis, steatohepatitis with fibrosis, and cirrhosis that increases the risk for the development of hepatocellular cancer (HCC) [3]. Heavy alcohol consumption, including binge drinking, leads to liver steatosis in over 90 % of individuals, and fat deposition resolves after cessation of alcohol use in the absence of advanced liver disease (Fig. 22.1). Persistent heavy alcohol use leads to liver steatosis with inflammation and sets the stage for progressive liver disease. Inflammation triggers fibrosis, a deposition of extracellular matrix and collagen that over time leads to irreversible cirrhosis [1, 3, 4]. Continued alcohol intake is the most important risk factor for progression of ALD [2, 4, 5]. Cirrhosis, decompensated liver disease, and HCC can be life threatening, and liver transplantation is not typically offered to individuals with ongoing active alcohol use in most transport centers (in the USA) [6].

Clinical Findings and Diagnosis of ALD

Clinically, most patients with persistent alcohol use have nonspecific symptoms that may include nausea, vomiting, diarrhea, or hepatomegaly [2–4]. Typical laboratory findings in ALD often show increased transaminases (transaminases rarely increase above 300 mg/dL) with an aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio >1. Serum bilirubin and alkaline phosphatase are often elevated and indicate more severe forms of ALD. In patients with severe forms of ALD, impaired liver synthetic function is indicated by abnormal prothrombin time (PT/INR),

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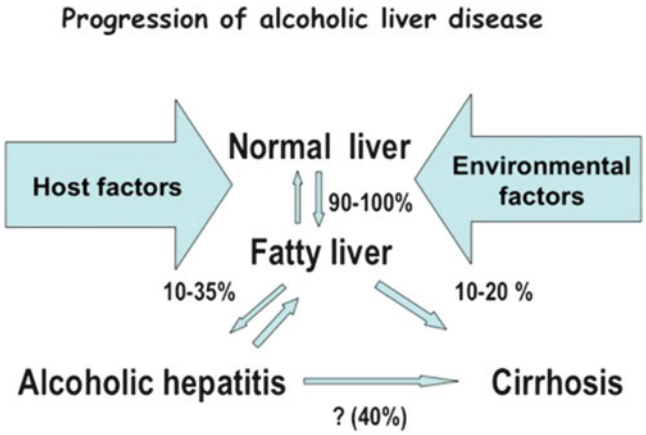

Fig. 22.1 Progression of alcoholic liver disease (ALD). Percentages represent the proportion of alcoholic individuals who will develop liver disease

decreased serum albumin, and thrombocytopenia [2–4]. Patients often have increased circulating white cell count (CBC). This does not necessarily indicate infection as it could be simply a manifestation of recruitment of immune cells from the bone marrow to the liver in response to the massive pro-inflammatory cytokine activation.

Different scoring systems are in use to establish the severity of ALD. The Maddrey discriminant factor >32 is the usual cutoff for defining severe alcoholic hepatitis. More recently, Model of end-stage liver disease (MELD) score >21 has been introduced as a cutoff for severe alcoholic hepatitis. The advantage of the MELD score is that it eliminates the variability of prothrombin time (PT) measurements that could vary between different diagnostic laboratories.

Acute Alcoholic Hepatitis

Acute alcoholic hepatitis (AAH) is the most severe form of ALD. It is a state of hepatic and systemic pro-inflammatory cascade activation with hepatocyte/liver dysfunction. Molecular mechanisms and biomarkers that trigger the development of AAH from stable ALD are yet to be delineated. Previous studies identified tumor necrosis factor (TNF) as a central mediator of ALD [7–11]. TNF-α was increased both in the serum and liver in human alcoholic hepatitis [7, 8, 12–14].

Patients with severe AAH have a high mortality and often develop jaundice, portal hypertension, and other signs of hepatic decompensation. While many cases of AAH 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 AAH is often complicated with upper GI bleeding, ascites, peripheral edema, and renal insufficiency. Systemic infections or SBP are other complicating factors often emerging in patients with ALD. Renal failure and hepatorenal syndrome in AAH carry high mortality [3]. Alcohol withdrawal and its physical and behavioral symptoms provide additional challenges in the clinical management of these patients.

Pathogenesis of ALD

Multiple key elements have been identified in the pathogenesis of ALD that include but are not limited to direct effects of alcohol and its metabolites on liver cells, alcohol-induced mitochondrial damage, production of reactive oxygen species (ROS), and induction of pro-inflammatory cytokines.

Organ Interactions in ALD

Alcohol affects virtually all organs in the body and it is increasingly evident that alcohol-induced changes in one organ can affect the function of other organs. Experimental evidence suggests a cross talk between the liver and intestine as well as the liver and adipose tissue in ALD [15, 16].

Gut–Liver Axis in ALD

Increasing evidence suggests that interactions between the liver and gut contribute to the development of ALD. In normal homeostasis, a balance is maintained between the gut microbiome, gut permeability, and translocation of gut-derived substances that reach the liver via the portal circulation summarized in [15, 17, 18]. The liver, as an immune organ, contains sensitive receptor systems on all of its cell types that 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 after excessive alcohol intake [17, 19, 20]. The central role of LPS has been demonstrated by several studies in animal models of ALD [19, 21–23]. 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 [24]. These effects of alcohol have been attributed to changes in intestinal permeability. Indeed, chronic alcohol exposure increases gut permeability by reducing epithelial cell barrier functions [20, 25]. Specifically, in vitro alcohol treatment of colonic epithelial cells decreases the expression of tight junction proteins such as zona occludin-1 (ZO-1) and the expression of the antimicrobial peptide, Reg3b [25]. Mechanistically, alcohol-induced ROS contributes to increased expression of microRNA-221 that in turn downregulates ZO-1 protein levels in intestinal epithelial cells [25].

In addition to the direct effects of alcohol on gut epithelium, alcohol consumption results in changes in the gut microbiome. Animal studies have revealed that there are quantitative and qualitative changes in the gut microbiome after prolonged alcohol feeding [26]. Specifically, there was a significant increase in the amount of bacteria in the cecum of alcohol-fed mice compared to controls [26]. Furthermore, the composition of the bacterial species has changed after alcohol treatment where the relative proportions of Firmicutes have increased at the expense of Bifidobacteria in alcohol-fed mice [26]. The specific role of these changes in the pathogenesis of ALD remains unclear; however, previous studies elegantly demonstrated that "sterilization" of the gut with nonabsorbable antibiotics has a significant protective effect on alcohol-induced steatosis and inflammation in animal models of ALD [21].

Liver and Adipose Interactions

The role of adipose tissue-derived adipokines, including adiponectin, has been highlighted in ALD [16]. Adiponectin contributes to the development of fatty liver and it also has pro-inflammatory effects. In animal models alcohol decreases gene expression and secretion of adiponectin in adipose tissues [27]. In vitro experiments revealed that alcohol decreases the activity of the mouse adiponectin promoter and decreases adiponectin secretion in differentiated adipocytes. Adiponectin exerts its biological effects through the adiponectin receptors 1 and 2. In mice AdipoR2 is downregulated in the human liver and decreased AdipoR1 was found in micropigs after chronic alcohol feeding [16].

Fat metabolism is also regulated by osteopontin, which is increased in the adipose tissue, liver, and serum of patients with fibrosis induced by chronic alcohol use [28]. Osteopontin has been suggested as a marker of liver disease progression [29–31].

The Effects of Alcohol, Metabolites, Reactive Oxygen Species, and Oxidative Stress

Alcohol Metabolism

Alcohol is metabolized by alcohol dehydrogenase (ADH) into acetaldehyde which is further metabolized into acetate by aldehyde dehydrogenase (ALDH) [32]. Acetaldehyde and acetate are short-lived and have high tissue toxicity; thus, many of the direct tissue effects of alcohol have been attributed to these metabolites (Fig. 22.2). Both of ADH and ALDH enzymes have limited capacity due to their low Michaelis constant. Thus, higher tissue concentration of alcohol is broken down by alternate enzyme systems including cytochrome P450 2E1 (CYP2E1) and microsomal enzymes that are upregulated in chronic alcohol use. Their by-products are ROS that contribute to direct cellular oxidative stress in hepatocytes and immune cells [33–35]. Alcohol metabolism results in increase in NADH/NAD+ ratio in the cytoplasm and mitochondria of hepatocytes [33, 36]. The increased NADP inhibits mitochondrial β oxidation and accumulation of lipids in hepatocytes [33].

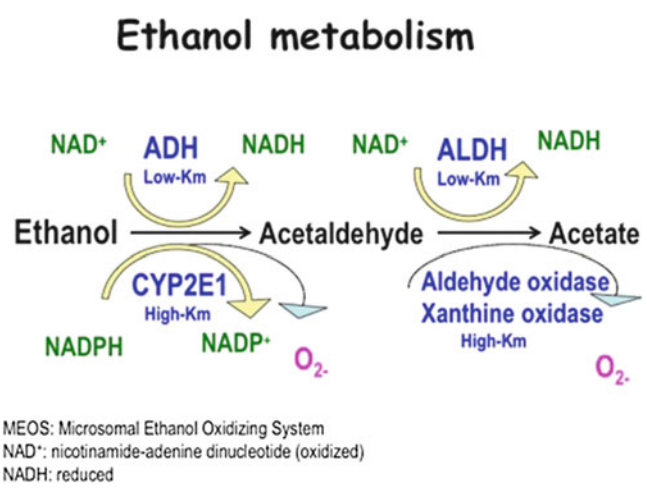

Fig. 22.2 Ethanol metabolism. The enzymes and intermediates of alcohol metabolism

CYP2E1 is an effective generator of ROS such as the superoxide anion radical and hydrogen peroxide and, in the presence of iron catalysts, produces powerful oxidants such as the hydroxyl radical. The role of CYP2E1 in hepatocyte damage in ALD has been established using elegant in vitro cell models and animal models [33, 37].

Reactive Oxygen Species and Mitochondrial Stress in ALD

In addition to ROS associated with direct alcohol metabolism, alcohol also increases mitochondrial oxidative stress [10]. Alcohol leads to alteration in mitochondrial membrane permeability and transition potential and contributes to apoptosis, release of cytochrome c, and caspase-3 activation [33, 38]. ROS also damages mitochondrial DNA and ribosomes.

The NADPH oxidase complex, involving various Nox proteins p47phox and p40, plays a role in ROS generation both in immune and parenchymal cells in the liver [39]. NADPH oxidases are activated in ALD in immune as well as in liver parenchymal cells [40, 41]. NADPH p47phox was shown to contribute to Kupffer cell activation in ALD [40, 42, 43].

Endoplasmic Reticulum (ER) Stress

The unfolded protein response also referred to as ER stress is a protective cellular mechanism that is disturbed by alcohol [44, 45]. Alcohol consumption results in increased expression of key components of the unfolded protein response including glucose regulatory proteins (GRP78, GRP 94, CHOP, and caspase-12) [46]. Intracellular glutathione levels

are depleted by chronic alcohol use and ER stress contributes to increased homocysteine levels [46, 47]. Upregulation of transcription factors SREBP-1c and SREBP-2 is associated with lipid accumulation.

Decreased Antioxidants

While alcohol increases ROS, it also reduces the availability of most antioxidant systems, thereby promoting oxidative stress and ROS-induced liver damage. Alcohol-fed mice had decreased expression of the antioxidant, superoxide dismutase (SOD) [44]. Glutathione sulfhydryl (GSH) and glutathione-S-transferase (GST) activity are also decreased in ALD [46].

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 evidence for recruitment of immune cells to the liver including cell populations of neutrophil leukocytes, monocytes, macrophages, T cells, and B cells [48, 49]. Other key aspects in the evaluation of immune responses in the liver are the interactions between the different immune cell types, including cross talk between liver parenchymal cells and immune cells. It is important to consider that the normal liver has an immunotolerant tissue environment that is profoundly changed in ALD where a state of pro-inflammatory cell and cytokine activation prevails and disturbs parenchymal cell functions in the liver [50]. The pathomechanism of ALD involves complex interactions between the effects of alcohol and its toxic metabolites on various cell types in the liver and gut, induction of ROS, and upregulation of the inflammatory cascade [8, 10, 35, 51–53]. Studies using antibiotics to "sterilize" the gut and experiments with elimination of Kupffer cells (KC) identified both gut-derived factors, such as LPS, and Kupffer cell activation as central components in ALD (Fig. 22.3) [15, 17, 20, 21, 25, 42, 54–56]. Chronic alcohol sensitizes macrophages to LPS-induced inflammatory cytokine production [57, 58].

Role of Innate Immunity

The innate immune system is the first line of defense in recognition and response to danger signals in the liver [52]. 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 of dying cells [59–63]. 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 between danger signals, immune cells, and parenchymal cells during the different states of ALD. Both soluble mediators and different cell types of the innate system contribute to the liver and systemic inflammation that characterizes ALD and particularly AAH. Overexpression of pro-inflammatory 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 represent dysregulation of innate immunity [23, 48, 51, 64, 65].

Soluble Mediators

Complement

Complement and complement activation are involved in the development of ALD. Specifically, C1q, the recognition subunit of the first complement component, binds to apoptotic cells.

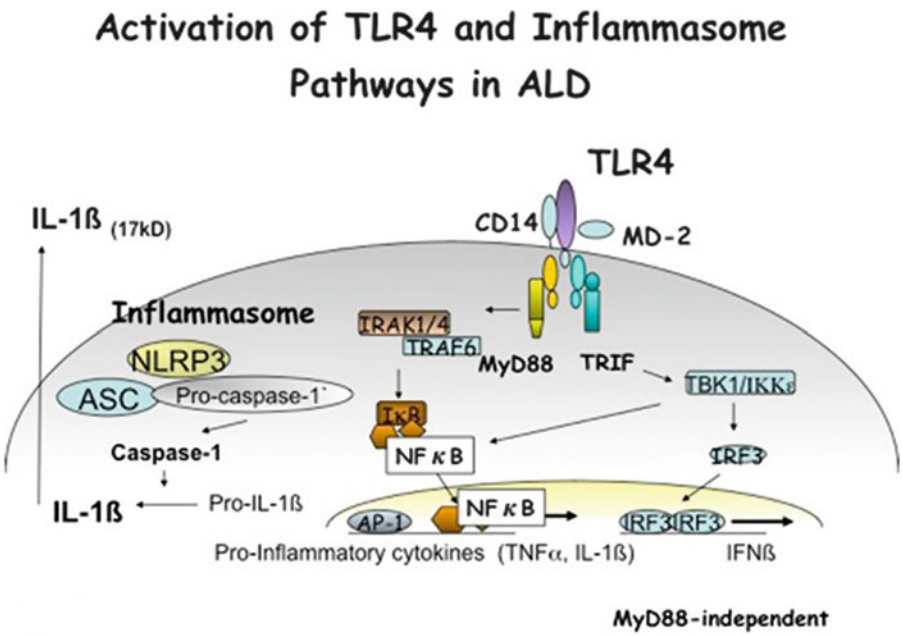

Fig. 22.3 Activation of TLR4 and inflammasomes in ALD. Pattern recognition receptors (PRRs) are activated by danger signals, resulting in the production of inflammatory cytokines

A recent study indicated that ethanol activates the classical complement pathway via C1q binding to apoptotic cells in the liver and thereby plays a role in the early stages of ALD [48, 53, 66].

Chemokines

Monocyte chemoattractant protein (MCP)-1, a CXC chemokine, contributes to recruitment of monocytes and macrophages to the liver in ALD [53, 67, 68]. Monocyte production of MCP-1 is increased in AAH [69]. MCP-1 also has direct effects on hepatocytes as it induces lipid accumulation [49]. It has been proposed that MCP-1 exerts its lipogenic effect via induction of the hypoxia-inducible factor-1 (HIF-1) in hepatocytes [70]. In a recent study, total body deficiency in MCP-1 in mice resulted in attenuation of alcohol-induced liver steatosis and inflammation [68]. It has been proposed that MCP-1 modulated PAPR-γ activity in hepatocytes as a mechanism for lipid accumulation in hepatocytes [68].

IL-8 is involved in many steps of neutrophil recruitment and activation. Increased levels of IL-8 were found in patients with alcoholic hepatitis while IL-8 was only moderately increased in patients with alcoholic cirrhosis [71].

Cytokines

The critical role of pro-inflammatory cytokines has been validated by several studies in ALD [51, 53, 72]. Pro-inflammatory cytokines not only mediate the pathogenesis of ALD but also account for many of the clinical symptoms in these patients. TNF-α has been identified as a central mediator of ALD [8, 9, 73, 74]. There is evidence for increased circulating and liver levels of TNF-α, IL-6, IL-8, and IL-1 [7, 9, 12–14]. Isolated monocytes from patients with alcoholic hepatitis produce increased levels of these pro-inflammatory cytokines [8, 9, 75]. In animal models, increased gene expression and liver and circulating protein levels of TNF-α, IL-1β, MCP-1, and IL-6 were found in several studies [49, 58, 67, 68]. In the liver, Kupffer cells have been identified as the major source of the pro-inflammatory cytokine production [23, 48, 54]. The mechanistic role of pro-inflammatory cytokines is suggested by experiments that featured cytokine knockout mice and found that deficiency either in TNF receptor 1 (TNFR1), MCP-1, or IL-1 receptor (IL-1R) ameliorated ALD [49, 68]. 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 [49]. These observations indicate that pro-inflammatory cytokine production is upregulated at multiple levels in ALD and that there is a positive amplification loop between these cytokines to perpetuate inflammation.

In addition to fueling inflammation, TNF-α, IL-1, and IL-6 have important effects on hepatocytes that contribute to the pathogenesis of ALD [58, 67]. By engaging its receptors on normal hepatocytes, TNF-α does not induce apoptosis. In injured hepatocytes that are present in the alcohol-exposed liver, TNF-α can trigger the death pathway [76]. The role of TNF-α is more complex, however, as it is also involved in liver regeneration that is a major element in compensation in liver homeostasis in the alcohol-exposed organ [58].

IL-1β is an endogenous pyrogen, an inducer of other pro-inflammatory mediators [77]. It also has direct effects on hepatocytes by inducing steatosis [49]. Furthermore, IL-1β sensitizes hepatocytes to the killing effect of TNF-α, thereby fueling a synergistic effect between pro-inflammatory cytokines on hepatocyte injury [49].

IL-6 also promotes fat accumulation in hepatocytes and, most importantly, has protective effects on the liver in steatohepatitis including ALD [59].

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 [78]. Furthermore, administration of recombinant IL-22 resulted in hepatoprotection in an acute alcohol binge drinking model, and the protective effects of IL-22 were attributed to STAT3 activation in the hepatocytes [65, 79].

Immune Cells

Neutrophil Leukocytes

In human ALD, the histopathological pattern of alcoholic hepatitis includes infiltration of neutrophil leukocytes, hepatocyte degeneration ballooning, and oncotic necrosis [31, 80]. Induction of chemokines (IL-8, cytokine-induced neutrophil chemoattractant (CINC)) and cytokines in addition to apoptosis of hepatocytes has been suggested as a mechanism for neutrophil infiltration [81].

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-induced liver intoxication [82]. They found that ALD patients showed a significant increase in both IL-17 plasma titers and frequency of IL-17$^+$ T cells and displayed a correlation between liver infiltration of neutrophils and Th17 cells. Furthermore, they found that Th17 cells produced IL-8 as well as GRO-α and that these factors were both necessary and sufficient to induce recruitment of neutrophils [82].

Kupffer Cells, Macrophages, and Monocytes

A central role has been suggested for Kupffer cells (KC) in ALD. KCs are liver resident macrophages that express surface markers of F4/80 and are enriched in livers of chronic alcoholics and alcohol-fed mice [49, 83]. There is an increase in the number of F4/80 cells that most likely represent KCs and/or newly recruited macrophages. Blood monocytes are activated in ALD and produce cytokines [75].

The tremendous plasticity in the phenotype of macrophages has recently been recognized. Depending on the tissue environment, danger signals, and cytokine milieu, blood monocytes differentiate into M1 or M2 macrophages or similar phenotypes. M1 macrophages are "classically" activated by LPS, IFN-γ, or pro-inflammatory cytokines and have high phagocytic activity while M2 macrophage differentiation is triggered by IL-4, IL-10, TGF-β, or adiponectin [84, 85]. M2 macrophages are "alternately activated" macrophages and express CD206, CD163, as well as arginase-1 [86, 87]. The role of the M1 and M2 macrophages in ALD is yet to be explored.

Most investigations focused on KCs have found that KCs isolated from ALD are in vivo "sensitized" to stimulation with LPS to produce increased amounts of TNF-α [88]. This has been linked to increased expression of NF-κB, ERK, and MAPK pathways [48, 89–91]. In vivo studies elegantly demonstrated that elimination of KC by gadolinium chloride in rats or clodronate in mice attenuated alcohol-induced liver injury [54, 92]. Recent studies using bone marrow transplantation corroborated the early findings to demonstrate the critical role of bone marrow-derived inflammatory and Kupffer cells in ALD. For example, while mice deficient in caspase-1 or IRF3, molecules that mediate IL-1β and TNF-α, respectively, are protected from ALD [19, 49], alcohol feeding after transplantation of these mice with wild-type bone marrow resulted in steatosis, liver damage, and inflammation [19].

Human studies from patients with ALD demonstrated increased production of monocyte IL-1β, TNF-α, and IL-6 [8, 9]. Furthermore, NF-κB activation was also observed in circulating monocytes from patients with ALD [8, 9, 75].

Dendritic Cells

Dysfunction of dendritic cells (DCs) including their antigen presentation capacity in inducing antigen-specific T cell activation, immunomodulatory cytokines (IL-12) production, and expression of co-stimulatory molecules is altered by acute and chronic alcohol use [50, 52, 93]. The composition of the dendritic cell population was changed in the liver in mice after alcohol administration, and DC functions were also altered in favor of an immature DC phenotype that is characterized by reduced antigen presentation capacity [50].

Adaptive Immunity

It has been shown that T cell, NK cell, and B cell functions are altered by chronic alcohol use [48, 53, 67]. In the liver, there is enrichment of T lymphocytes although their specific role to the local tissue pathology is less clear. In ALD, the formation of protein adducts was shown as a result of ROS-induced modification. Reactive acetaldehyde, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) can bind to proteins to form adducts [94]. These adducts are recognized by KCs, endothelial cells, and stellate cells in the liver via the scavenger receptor and induce cytokines [94]. In addition, protein adducts elicit antibody responses, in response to protein adducts [94, 95].

Table 22.1 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

Signaling Pathways

Pattern Recognition Receptors

Innate immune responses are triggered by danger signals from pathogens or injured self through recognition by pattern recognition receptors (PRRs) (Table 22.1). 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) [59, 60, 63, 96, 97]. Ample evidence demonstrates that activation of TLRs and NLRs is a pivotal element in the pathogenesis of ALD (Fig. 22.4). 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

Recent advances in the understanding of ALD show the contribution of the different members of these receptors. Of the 13 TLRs, TLRs 1–6 are expressed on the cell surface recognize extracellular PAMPs, while intracellularly localized TLRs (TLR3, 7, 8, 9) sense nucleic acid sequences [59, 62, 63, 98]. 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, 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 pro-inflammatory cytokine genes reviewed in [63, 99, 100]. 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

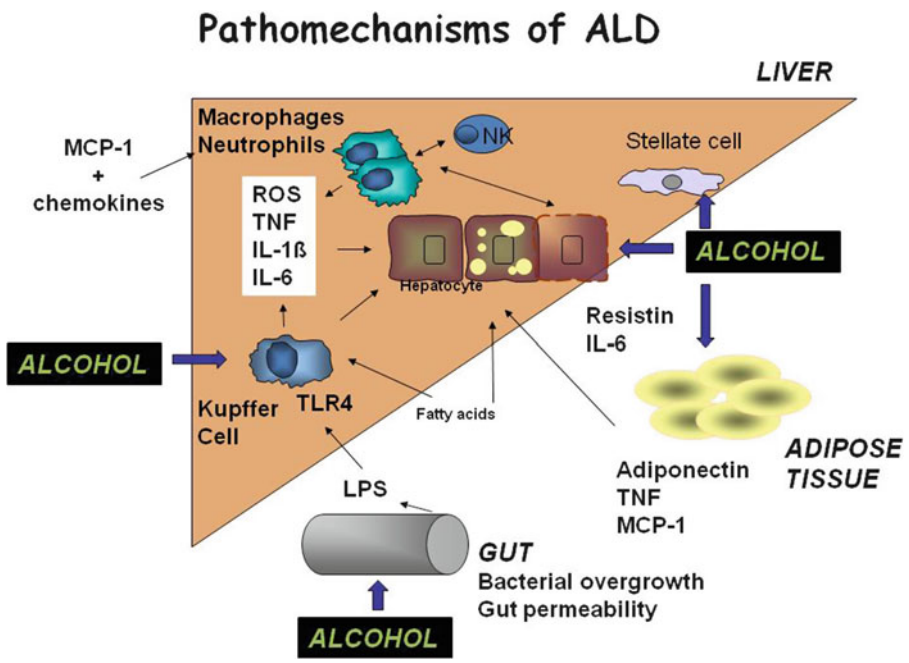

Fig. 22.4 Pathomechanisms of ALD. Both hepatic and immune-derived cells are involved in the pathogenesis of ALD. Mediators include cytokines, chemokines, and reactive oxygen species. Cell types are shown in blue, whereas extracellular mediators are shown in black

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 (Nan, Campoy, and Bird 1997, 471–481) and TLR9 recognizes CpG-rich DNA reviewed in [63, 99, 100]. All TLRs are broadly expressed in the liver in different cell populations across immune and parenchymal cells [63].

TLR4, the receptor that senses LPS, plays a central role in ALD. TLR4 recognition of LPS is facilitated by the co-receptors 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 [96]. 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) [96].

Studies in animal models demonstrated that mutation in TLR4 or deficiency (knockout) of TLR4 attenuated alcohol-induced liver steatosis, inflammation, and injury [22, 49]. The TLR4 receptor complex includes the TLR4 co-receptors CD14 and MD2 that contribute to alcohol-related liver damage [101]. 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 [98]. NF-κB activation has been shown in ALD. NF-κB has a complex role in ALD, including protecting hepatocytes from apoptosis and pro-inflammatory cytokine activation in Kupffer and immune cells [51, 53]. Nuclear translocation of the NF-κB p65/p50 dimer in immune cells correlates with pro-inflammatory cytokine induction in ALD [51]. 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. Recent studies evaluated the involvement of TLR4, MyD88, and IRF3 in a mouse model of ALD and found that TLR4 and IRF3 were critical in the development of liver steatosis, inflammation, and liver damage after chronic alcohol feeding in mice [19, 22, 102]. Bone marrow chimera experiments revealed a cell-specific role for IRF3. Specifically, the absence of IRF3 in bone marrow-derived cells resulted in protection from alcohol-induced steatosis, inflammation, and liver damage. Conversely, IRF3 deficiency in the liver parenchymal cells promoted alcohol-induced liver injury [19].

NOD-Like Receptors and the Inflammasome

Inflammasomes are multiprotein complexes that include NLR sensors, adapter molecules, and pro-caspase-1 that cleave pro-caspase-1 into active caspase-1 upon ligand engagement [97]. 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 [103]. 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 [97, 103]. 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 [104]. 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 [77]. Indeed, Il-1β levels are also increased in a mouse model of ALD while IL-1α, which is mostly cell-associated, is not elevated. Recent investigations revealed that IL-1β increase in ALD is due to inflammasome activation as caspase-1-deficient mice had significantly attenuated alcoholic liver steatosis, inflammation, and liver damage [49]. Interestingly, interruption of inflammasome activation prevented alcohol-induced increase in MCP-1 and TNF-α, suggesting amplification between these pro-inflammatory cytokines [49].

Nuclear Receptors

Most nuclear receptors that have received attention in ALD are involved in regulation of both lipid metabolism and inflammation [105]. Hypoxia has been shown to play a role in the pathogenesis of ALD [64]. Hypoxia-inducible factor-1 (HIF-1α) messenger RNA was increased in livers of chronic alcoholics and in mice after chronic alcohol administration [70]. Alcohol-induced steatosis was mediated by HIF-1α, and involvement of HIF-1α activation was found in both hepatocytes and liver immune cells [70].

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 [106–109].

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 [61, 110]. 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 [111]. SREBP contributes to lipophilic pathway in ALD [112].

MicroRNAs in ALD

MicroRNAs (miRNAs) are a class of evolutionarily conserved, single-stranded, noncoding RNAs of 19–24 nucleotides that control gene expression at the posttranscriptional levels [113]. MicroRNAs contribute to the regulation of liver parenchymal and immune cells [114]. The expression and potentially the function of many miRNAs are changed in ALD in mice [114, 115]. MicroRNAs also regulate stem cell differentiation, regeneration, and cell death [116]. 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 [117]. MiR-155 positively regulates TNF-α through enhancing its translation [114, 118]. One of the important effects of alcohol is sensitization of KCs to LPS-induced TNF-α production [8]. 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 [119]. Increased miR-155 expression was particularly prominent in Kupffer cells after chronic alcohol administration and it had a causative role in increased TNF-α production by KCs [119].

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 [120]. Epigenetic regulation of miR-34 has recently been linked to miR-34 expression and fibrosis in ALD [121].

MicroRNAs are present in the circulation and are stable in the serum and plasma, making them attractive targets in biomarker discovery [114, 122]. For example, mir-122 represents 80 % of the total liver miRNAs and is abundantly expressed in hepatocytes where it regulates fat metabolism [123]. Recent reports demonstrated that circulating miR-122 is increased in different forms of liver injury, and in a mouse model of ALD, increased circulating miRNA-122 correlated with reduced levels of miR-122 in the liver [11]. The utility of circulating miRNAs as biomarkers in AAH and ALD is an area of active research [124–126].

Treatment for Alcoholic Liver Disease

Abstinence

Cessation of alcohol intake is the first-line intervention in patients with alcoholic hepatitis [127]. This fully depends on the patient's motivation and often requires participation in detox programs and a supportive domestic environment.

Steatosis and early steatohepatitis are reversible, while cirrhosis may not regress after discontinuation of alcohol use.

Current Medical Treatment

Alcoholic hepatitis (AH), the most severe form of ALD, has high morbidity and limited treatment options [128]. While corticosteroid treatment improves short-term survival, it increases the risk of infections [129]. The standard of care is prednisolone 40 mg daily for 28 days. A recent study demonstrated that using the Lille score at day 7 of steroid treatment, patients can be stratified to those who respond to therapy where continued treatment has benefits in contrast to those who show no decrease in serum bilirubin after 7 days of prednisone treatment [130]. In the latter group steroids should be discontinued.

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 [131, 132]. A recent study investigated the combination of steroids and pentoxifylline and found no benefits over single therapy except for a small population of patients with hepatorenal syndrome as well as in animal models of ALD [133, 134].

Liver Transplantation in ALD

In the USA, patients with AAH that is linked to recent alcohol abuse are not considered candidates for liver transplantation. Most transplant centers in the USA require at least 6 months of abstinence and participation in support groups for eligibility for listing for liver transplantation. These rules obviously eliminate many patients because of the high 6-month mortality associated with AAH. In a recent multicenter study in the European Union, liver transplantation was effective as a treatment in patients with AAH [135]. While in pre-transplant all of the recipients heavily used alcohol, <10 % had relapse in alcohol use after liver transplantation for AAH [135].

Liver transplantation for alcohol-induced 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 [136].

Potential Therapeutic Targets and Considerations in Future Therapies

Advances in the understanding of the cellular and molecular mechanism of ALD in the last decades provide multiple attractive therapeutic targets in ALD. Table 22.2 lists the most actively studied potential targets in the pathogenesis of ALD that may provide the basis for new therapeutic interventions. For example, considering that AAH is a state of hepatic and systemic pro-inflammatory cascade activation with hepatocyte/liver dysfunction, approaches to interrupt these vicious cycles are highly attractive. In addition, molecular mechanisms and biomarkers that distinguish the development of AAH from stable ALD are yet to be delineated.

Table 22.2 Current therapies and emerging therapeutic targets in alcoholic liver disease

Target	Functional effect
Current therapies	
Steroid	Anti-inflammatory
Pentoxifylline	Phosphodiesterase inhibitor
Liver transplantation	Healthy liver
Zinc	Intestinal barrier
Emerging therapeutic targets	
TNFA	Inflammation, hepatocyte death
IL-1β	Inflammation, steatosis
IL-1 receptor antagonist	Inflammation, steatosis
IL-17	Inflammation, hepatocyte death
IL-22	
IL-6	Inflammation, regeneration
Chemokines	
MCP-1	Inflammatory cell recruitment steatosis
IL-8	Neutrophil recruitment
GRO-α	Neutrophil recruitment
Osteopontin	Inflammation, regeneration
Signaling molecules	
TLR4	Inflammation, fibrosis
IRF3	TLR signaling
NF-κB	Inflammation, cell survival
Caspase-1	IL-1β production
Heat shock protein 90	Steatosis, inflammation
Hypoxia-inducible factor-1	Steatosis
Heme-oxygenase1	Inflammation
SIRT1	ROS steatosis, inflammation
PPAR-α	Steatosis
Cell death	
Fas	Apoptosis
Bcl-2	Apoptosis
Microbiome	
LPS	Inflammation
Pro-/prebiotics	Inflammation of gut phase

Previous studies identified TNF-α as a central mediator of ALD and TNF-α was increased both in the serum and liver in human alcoholic hepatitis [8, 9, 58]. While TNF-α blockade showed protection in animal models, human clinical trials using anti-TNF antibodies with steroids were discontinued due to infectious complications [137–140]. These studies had several limitations including high doses of anti-TNF-α and co-administration with steroids that increased immunosuppression. Pro-inflammatory

cytokines, other than TNF-α, are also increased in AH including IL-6, IL-8, and IL-1.

Recent preclinical data demonstrated upregulation of IL-1β in the liver after chronic alcohol administration and showed amelioration of liver steatosis and inflammation after therapeutic blockade of IL-1-mediated signaling. This may provide basis for translation to clinical application by evaluation of the therapeutic utility of IL-1R blockade or anti-IL-1 antibodies in ALD. There are several reasons for this. First, IL-1 inhibition can prevent the autoregulatory amplification loop of IL-1α and IL-1β upregulation. Second, inhibition of IL-1 should attenuate TNF-α induction and break the vicious cycle of pro-inflammatory cytokine cascade activation in AH. Third, because IL-1 induces steatosis and sensitizes hepatocytes to the cytotoxic effects of TNF-α, IL-1 inhibition should attenuate hepatocyte damage in AH [141].

Inhibition of MCP-1 could be another attractive approach considering that MCP-1 is an early mediator in ALD that contributes to steatosis and inflammatory cell recruitment. Additional potential targets are listed in Table 22.2; all of these potential therapeutic targets were identified based on experimental evidence and their role in the pathomechanisms of ALD and further preclinical and potential clinical investigations.

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Nonalcoholic Fatty Liver Disease

23

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Key Points

- NAFLD is primary hepatic steatosis with inflammation and ballooning hepatocyte injury with or without fibrosis.
- Insulin resistance and chronic low-grade inflammation lead to the development of NAFLD in a genetically predisposed individual.
- Hepatic steatosis is the initiating event, whereas ER stress, oxidative stress, and mitochondrial injury propagate the liver injury.
- Chronic inflammation in NAFLD starts within the adipose tissue.
- It persists as free fatty acids and intestinal microbiome activate TLRs and inflammasome.
- Inflammation worsens insulin resistance and NAFLD.

Defining Nonalcoholic Fatty Liver Disease: Phenotypes of the Disease

Nonalcoholic fatty liver disease (NAFLD) is defined by fat deposition in the liver in the absence of secondary causes for steatosis. The disease spectrum of NAFLD varies from simple steatosis, through steatosis with inflammation with or without hepatocyte injury, to cirrhosis at the other end of the spectrum [1]. Nonalcoholic steatohepatitis (NASH) is a part of NAFLD spectrum and is characterized by the presence of hepatic fat deposition, inflammation, and most importantly hepatocyte damage in the form of characteristic ballooning injury. Current AASLD consensus guidelines require the presence of liver injury in the form of ballooning to distinguish NASH from other disorders of the NAFLD disease spectrum. On the other hand, the term nonalcoholic fatty liver (NAFL) is classically used to describe steatosis in the absence of ballooning [2–4]. The histological criterion for diagnosing NAFLD is fat infiltration in more than 5 % of the hepatocytes. The accumulation of fat usually starts in zone 3 that is the peri-sinusoidal region. Although hepatic steatosis or inflammation in itself does not define NASH, both have been associated with liver-related mortality. Steatosis has been linked to increased cardiovascular mortality [4]. Some studies have determined that inflammation that extends beyond the portal tracks has been correlated with advanced fibrosis, while others have not found this relation. Similarly, evidence suggests that pan-acinar steatosis is predictive of fibrosis [5, 6]. Age and degree of inflammation on biopsy performed at diagnosis have been correlated to progression of fibrosis in a systematic review of several clinical trials. Of the several histological systems proposed for NAFLD diagnosis, those incorporating fibrosis are predictive of long-term mortality. Fibrosis is the only histological feature that is individually related to prognosis [5–8]. On the other hand, clinical presence of obesity and type 2 diabetes has been associated with disease progression [1, 9]. Outcomes of advanced NAFLD (Child-Pugh B and C) have prognosis comparable to those with similar stage of hepatitis C-related liver disease [10, 11].

The pathology in NAFLD arises from the complex interaction of environmental factors such as sedentary lifestyle and excess energy intake in a genetically susceptible host. NAFLD is associated with metabolic syndrome and insulin resistance. This has been well established by several animal and human studies. The role of the immune system in NASH, in terms of its relationship to prognosis, has been observed from several animal studies and human data. However, the role of inflammation in NAFLD etiopathogenesis in terms of the origin, initiation and propagation of inflammation, the involved tissues, cell types, and inflammatory mediators is only beginning to be understood. In this chapter we summarize the current evidence with respect to activation of the innate immune system in NAFLD and its implications on preventing the progression of disease and therapeutic options.

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Diagnosis and Epidemiology

NAFLD is now the most common cause of liver disease in the world. The worldwide prevalence of NAFLD varies from 15 to 45 %. Ultrasound-based studies have reported the prevalence of NASH from 17 to 46 % [12]. On the other hand, histologically confirmed NASH in potential organ donors has ranged from 20 to 51 % [13]. A higher prevalence has been reported in developed countries where its prevalence corresponds to the increasing prevalence of metabolic syndrome. Although NAFLD has been historically known as a disease of the developed world, accumulating evidence supports increasing incidence in several countries of the Asia-Pacific region [12, 14]. The difference in prevalence noted by different studies depends upon the diagnostic tool used by that particular study and the population under consideration [15, 16]. Diagnosing the disease continues to remain a challenge given the limitation of liver enzymes and ultrasound to appropriately identify patients. Several new diagnostic tools have been developed including noninvasive assessment of liver fat by magnetic resonance imaging and spectroscopy and transient elastography, clinical scoring systems, and plasma CK-18 levels [17, 18]. While promising, these diagnostic options need further validation by large-scale studies and are currently reserved as research tools. The gold standard for diagnosis is still liver biopsy, which is rarely performed except in specialized centers. Given these limitations, current AASLD guidelines recommend against a routine screening of patients for NAFLD [2].

Overall mortality in NAFLD patients is two times that of the general population [19]. Morbidity and mortality from hepatic dysfunction in NAFLD vary with the histological severity of the disease [6]. While simple steatosis, the most common pathology seen in NAFLD, is not known to be related to increased disease-related mortality, it is frequently associated with metabolic syndrome and complications thereof. At the same time, steatosis puts patients at an increased risk of morbidity and mortality from other chronic liver diseases. Depending on the length of observation, studies have noted that a third to a half of all patients with simple steatosis eventually progress to NASH. Cirrhosis occurs in up to 15 % of all patients of NAFLD, and about a fifth of the patients with NASH-related cirrhosis develop hepatocellular cancer. This is significant as the third most common cause of mortality in people with NAFLD is liver related compared to 13th in the general population. In fact most cases of cryptogenic cirrhosis are now attributed to NAFLD [4, 10, 20]. This increase mortality is particularly significant given the widespread prevalence and increasing incidence of the disease as determined by population studies. This represents a pressing need for the scientific community to accurately determine the factors that determine disease progression and poor outcome and device preventive and therapeutic measures.

Pathogenesis of NAFLD Disease Initiation: Hepatic Steatosis

Hepatic fat infiltration is central to the pathogenesis of NAFLD. The factors that lead to initiation of hepatic steatosis and those that cause the disease to progress are interrelated and work in concert. The initial two-hit hypothesis proposed to explain NAFLD pathogenesis has now been largely rejected due to inability of currently available data to pinpoint precise triggers for disease initiation vs. progression. Insulin resistance and a state of low-grade chronic inflammation contribute to the pathogenesis of NAFLD, but the precise sequence of events which leads to disease progression to more severe phenotypes in not entirely understood (Fig. 23.1).

Composition of Intrahepatic Fat in NAFLD

Triglycerides (TG) represent the predominant type of fat that is deposited in the liver in NAFLD (Table 23.1). Lipidomic studies in humans have revealed that NAFLD is associated with an increase in diacylglycerol (DAG), triacylglycerol (TAG), and free cholesterol and an increase in omega-6 unsaturated fatty acids with a relative decrease in omega-3 unsaturated fatty acids [21]. In vivo evidence from animal models shows that mice genetically engineered to selectively overexpress diacylglycerol acyltransferase (DGAT) 2, the enzyme that catalyzes the final step in TG formation, had hepatic steatosis with increased amounts of TG compared to controls; however, the animals did not develop insulin resistance [22]. In another in vivo study, feeding a methionine and choline-deficient (MCD) diet to mice that are genetically prone to obesity results in the animals developing the entire spectrum of NASH but with a decrease in hepatic triglyceride content over time. More interestingly, blocking DGAT2 expression produced an expected reduction in hepatic TG content accompanied by an increase in hepatic free fatty acid (FFA) content which was associated with worsening of hepatic inflammation, lipid peroxidation, oxidative stress, hepatocyte injury, and fibrosis [22–24]. Thus, TG accumulation may in fact represent a protective mechanism against FFA-induced lipotoxicity. FFAs are the building blocks for hepatic steatosis [25].

Among FFAs, saturated long-chain fatty acids (such as palmitic and stearic acids) have been shown to be toxic, whereas monounsaturated FFAs are likely to be protective in NASH [26–28]. Cells cultured in the presence of unsaturated

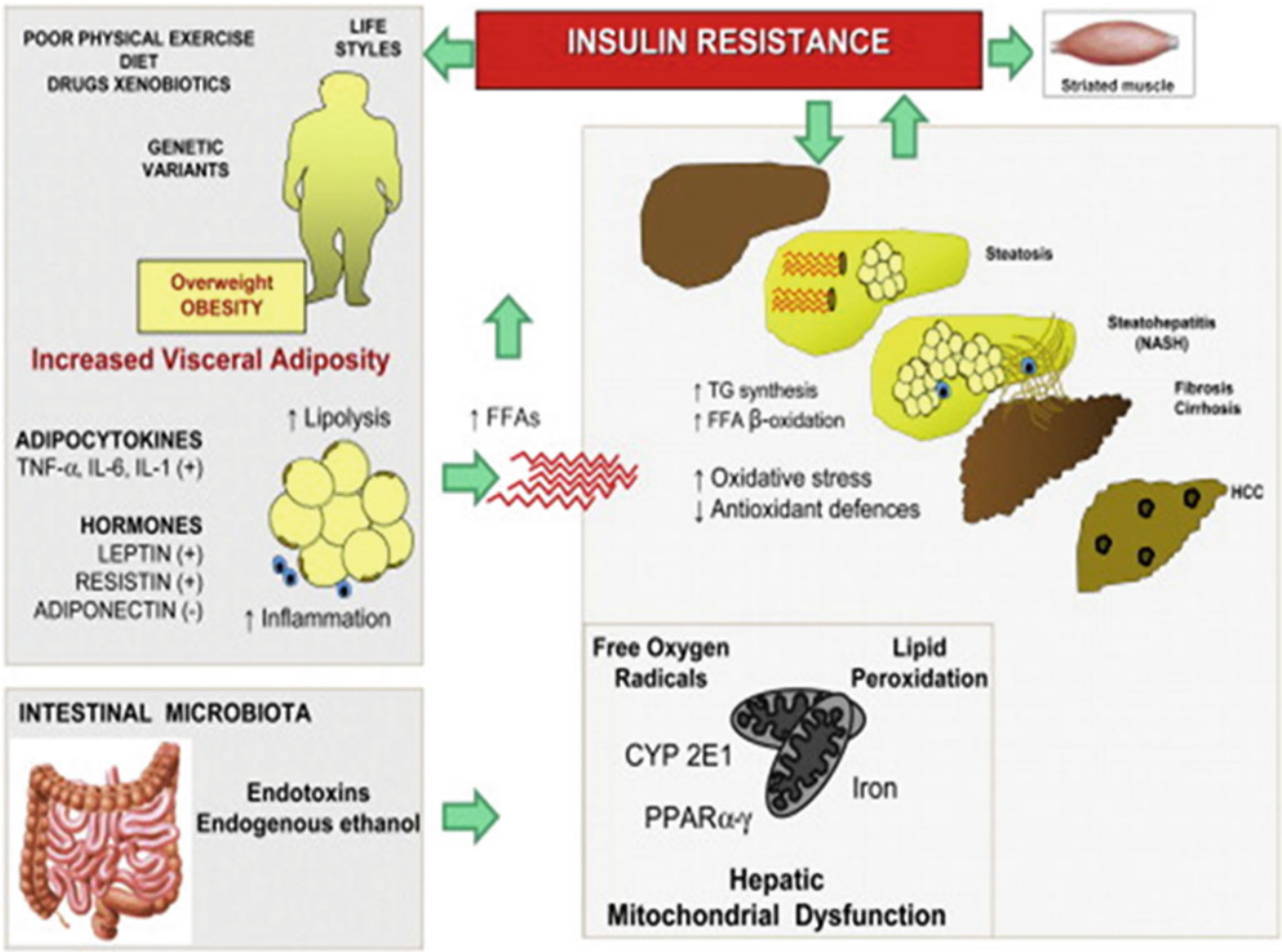

Fig. 23.1 Overview of NAFLD Pathogenesis: The complex interaction involving increased visceral adiposity, altered adipocytokines, adipose tissue inflammation, increased lipolysis and flux of FFAs to the liver, the intestinal microflora, increased hepatic-free oxygen radicals and lipid peroxidation lead to the pathology seen in NAFLD. *TG* triglycerides, *FFA* free fatty acids, *TNF* tumor necrosis factor, *PPARs* peroxisome proliferator-activated receptors, *HCC* hepatocellular carcinoma. *Symbols*: increased (↑); decreased (↓); increased/positive effect (+), decreased/inhibitory effect (−). Adapted from Krawczyk et al. 10.1016/j.bbr.2011.03.031, 2010

Table 23.1 Definition of obesity and its classification based on body mass index

BMI (kg/m^2)	Classification
• <18.5	Underweight
• 18.5–24.9	Normal weight
• 25.0–29.9	Overweight
• 30.0–34.9	Class I obesity
• 35.0–39.9	Class II obesity
• ≥40.0	Class III obesity

Obesity is defined as a BMI more than or equal to 30 kg/m^2
Note: class III obesity is also referred to as severe or morbid obesity

FFA had no change in viability but accumulated significant amounts of TG. On the other hand, saturated fatty acid (SFA) treatment resulted in an increase in apoptotic death without an increase in the amount of intracellular TG accumulation. In addition, FFAs exert hepatotoxicity via several mechanisms which includes formation of lysophosphatidylcholine, reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, c-Jun N-terminal kinase (JNK) activation, and mitochondrial and lysosomal cell death pathway and stimulates pro-inflammatory signals via direct interaction with Toll-like receptors (TLRs) and interferes with insulin signaling [26–28].

Evidence also suggests that FC is pathogenic in NAFLD. It stimulates macrophage JNK activation and depletes mitochondrial-reduced glutathione rendering hepatocytes susceptible to TNF-α or Fas-mediated apoptosis [24, 25].

Although intrahepatic FFAs are not increased in NAFLD as discussed above, serum FFAs, particularly the SFA, palmitate, are increased significantly in human studies of NAFLD [29–31]. FFAs that lead to steatosis are derived from the combined effect of diet, adipose tissue lipolysis, and de novo fatty acid synthesis. In NAFLD, 15 % of liver fat derives from dietary FFA, but de novo lipogenesis increases

from 5 % in healthy subjects to 26 % in NAFLD. However, the largest source of hepatic FFAs (60–80 %) is influx of FFA from adipose tissue as a result of adipose tissue lipolysis.

In summary, there is mounting evidence to suggest that non-TG lipid molecules, especially FFA and free cholesterol (FC), play a key role in the pathogenesis of NASH by leading to lipotoxicity. Fat infiltration in the liver does not necessarily correspond to inflammation. The quality of the fat deposits and not just the quantity is what seems to determine the pathogenesis of NAFLD.

Obesity, Metabolic Syndrome, Insulin Resistance (IR), and Their Relationship to NAFLD

Obesity is defined as a body mass index (BMI) of more than or equal to 30 (Table 23.2). Several clinical studies have demonstrated the association between obesity and NAFLD [32, 33], and as described above, an improvement in the disease is noted with diet- and/or exercise-induced weight loss [34–36]. Not unlike other diseases that fall into spectrum of metabolic syndrome, NASH correlates better with visceral obesity when compared to BMI [37–39]. See Table 23.2 for definition of metabolic syndrome. Our lab has previously published that both visceral fat and dorsocervical lipohypertrophy are associated with severity of disease in NAFLD [40].

IR is the central physiological mechanism of metabolic syndrome, including NAFLD. IR is characterized by an inability of tissues to respond to insulin despite a relative abundance of insulin [41]. The result that IR has depends upon the organ under consideration and the function of insulin in that organ. Peripheral IR results in poor glucose uptake and utilization by skeletal muscle and decreased suppression of lipolysis in adipose tissue leading to hyperglycemia and an increased FFA delivery to the liver [41, 42]. In the liver, IR results in hyperglycemia by impairment of glycogenesis and an increase in gluconeogenesis and glycogenolysis [41, 43]. In addition, the effect of insulin on several intracellular transcription factors involved in lipid homeostasis is altered. Hepatic IR leads to an increase in the activity of the liver X receptor (LXR), carbohydrate-responsive element-binding protein (ChREBP), and sterol-responsive element-binding protein 1c (SREBP-1c), thus increasing hepatic lipogenesis [44, 45]. Nuclear receptors, LXR and retinoid X receptor, work in concert to activate ChREBP and SREBP-1c, which in turn transcriptionally regulate as fatty acid synthase (FAS) and acetyl-CoA carboxylase, the key enzymes needed for de novo fatty acid synthesis in the liver [44, 45]. Another nuclear receptor, peroxisome proliferator-activated receptor-γ (PPAR-γ), leads to steatosis [46–48] but increases insulin sensitivity and suppresses inflammation by increasing serum adiponectin levels [49–51]. In addition, IR-led hyperinsulinemia induces oxidative stress, causes upregulation of connective growth factor and stimulates hepatic stellate cells (HSC) to proliferate, and secretes extracellular matrix [42, 52].

Table 23.2 Key cytokines in NAFLD

TNF-α	Pro-inflammatory, proapoptotic, promotes insulin resistance, activates neutrophils, and opposes adiponectin secretion by adipose
	Circulating TNF-α levels are significantly higher in NAFLD compared to obese controls, and its hepatic expression correlates with the severity of fibrosis. While some studies have noted that TNF levels predict disease severity in NAFLD, several studies have not found a difference in TNF levels across the disease spectrum of NAFLD
IL-6	Chronic elevation is proapoptotic, pro-fibrotic, worsens liver injury and insulin resistance. It activates STAT-3 leading to further inflammatory cytokine secretion. In human studies, serum IL-6 levels increase in patients with NAFLD, and its hepatic levels correlate with degree of steatosis, hepatocyte injury, and fibrosis
IL-4	Leptin-deficient and diet-induced obesity mice have decreased numbers of IL-4-producing NKT cells which correlates with severity of liver disease. Replacement of IL-4-producing NKT cells results in improvement in hepatic steatosis in these mice
MCP-1	Secreted by adipocytes and binds to its macrophage receptor CCR-2. In animal studies, deficiency of either MCP-1 or CCR-2 results in protection from macrophage infiltration into adipose tissue, diet-induced hepatic steatosis, and insulin resistance. Hepatic levels may correlate with NAFLD severity
CCR-2	See above
IL-1β	Activated by caspase-1 as part of inflammasome as well as NF and AP-1 and leads to neutrophil recruitment and insulin resistance via the IKK and JNK pathway
IL-18	As a part of inflammasome, same as above
MIP-1	Secreted by adipose tissue and recruits neutrophils; increased in animal models of NAFLD
Visfatin	Predominantly expressed in visceral adipose tissue and its levels decrease in serum and visceral adipose tissue in NAFLD, and its levels negatively correlate to the degree of hepatic steatosis

Role of Diet in NAFLD

Several animal models have been developed to study the role of dietary factors in NAFLD. Several types of diet have been used to generate these animal models. More commonly used steatosis-inducing diets include MCD diet, high-fat diet with varying amounts of cholesterol, and diets containing high amounts of fructose. Feeding a high-carbohydrate, HF diet with 0.2 % cholesterol to animals that are genetically prone to develop diabetes and hypo-adiponectinemia

leads to classical NASH with fibrosis [53–55], whereas chow-fed animals develop only steatosis. WT C57B6 mice also develop NASH but with diets containing higher percentage of cholesterol 1 or 2 %. In these mice the degree of liver injury is more pronounced with increasing percentage of dietary cholesterol [55–57]. Finally, an HF diet rich in trans saturated fats combined with high-fructose corn syrup equivalent also caused obesity-related steatosis with moderate necroinflammatory change; however, this failed to reproduce ballooning and fibrosis. Conversely, elimination of cholesterol from the HF diet or treatment with drugs that lower hepatic cholesterol results in decreased severity of steatohepatitis [55, 58].

Human studies evaluating dietary intake in NAFLD have shown that patients typically consume a diet with excess amount of cholesterol and saturated fat but lower in polyunsaturated fats, vitamins C and E, and fiber. This disproportionally high consumption of saturated fats by NAFLD patients has been confirmed by other reports [59]. Compared to patients with simple steatosis, subjects with NASH consume more carbohydrates but a lower amount of proteins and zinc [57]. Consumption of a fast food-based high-calorie diet is associated with increase in ALT and hepatic steatosis even in healthy subjects [60]. Several studies have noted an improvement in liver enzymes with diet- and exercise-induced weight loss in NAFLD patients [34–36].

Genetic Predisposition to NAFLD

Obesity, IR, and sedentary lifestyle are all risk factors for NAFLD that are quite widespread in the general population. In spite of this, only a small fraction of people develop steatosis and an even small percentage progress to NASH. This observation indicates that certain individuals are probably genetic predisposed to develop the disease. However, given the complexity of the disease, a Mendelian pattern of inheritance is unlikely, and both familial- and population-based studies can be helpful in understanding the inheritance pattern of NAFLD.

Familial clustering of NASH has been noted although a specific pattern of inheritance has not been identified. In a familial study, 20 % of patients were identified as having first-degree relatives with NASH [61]. In another report, hepatic steatosis was seen in 17 % of siblings and 37 % of parents of overweight children without NAFLD compared to 59 and 78 % in siblings and parents, respectively, of children with NAFLD [62].

The most important mutation identified that predisposes an individual to NAFLD is in the gene encoding patatin-like phospholipase domain-containing (PNPLA) 3 gene. This gene is regulated by insulin and increased with obesity in animals. It is also expressed predominantly in the adipose tissue and liver making it an interesting candidate gene. The single-nucleotide polymorphism (SNP) rs738409[G] of PNPLA3 encoding I148M (rs738409[G]) correlates with degree of steatosis and inflammation in NAFLD [63]. Other SNPs have been identified in PNPLA3 that predict heritability and ethnic differences in NAFLD.

Population-based studies have identified several other candidate genes in NAFLD. The SNP rs1801278 in insulin receptor substrate 1 (IRS1) that affects insulin receptor activity, predisposes to liver damage and decreases hepatic insulin signaling in patients with NAFLD [64]. Similarly, SNPs in adiponectin gene 45GT and 276GT and the SNP rs2241766 of adiponectin C1Q and collagen domain containing (ADIPOQ) are associated with NAFLD [65, 66]. Polymorphisms in apolipoprotein C3 (APOC3) and apolipoprotein E genes have been shown to increase risk for development of fatty liver disease, insulin resistance, and plasma triglyceride levels [67, 68]. Similarly, genetic polymorphisms of genes encoding Kruppel-like factor 6, microsomal triglyceride transfer protein, and manganese superoxide dismutase (MnSOD) have been associated with NAFLD. Kruppel-like factor 6 (wild type) predicts fibrotic severity of NASH while T/T genotype of MnSOD was noted to be more frequent in NASH patients compared to controls. This is plausible as MnSOD deficiency results in an accumulation of superoxide anion resulting in increased oxidative stress [69]. Several candidate genes involved in lipid metabolism, inflammation, oxidative stress, and insulin sensitivity have been identified to potentially play a role in inheritance and progression of metabolic syndrome and NAFLD and have recently been extensively reviewed [70].

Role of Oxidative Stress

Excess FFAs that accumulate as a result of the processes that are described above, in an insulin-resistant state, are further metabolized by physiologic β-oxidation in mitochondria. Mitochondria have structural and functional defects in NAFLD. Uncoupling of oxidation and phosphorylation leads to generation of ROS [31]. Peroxisomal oxidation of very long-chain fatty acids and the ER induction of cytochromes P450 [CYP] 2E1 and 4A also contribute to the ROS load in NAFLD [71–74]. These ROS are central to the pathogenesis of NAFLD. They drive cell injury by interfering with mitochondrial electron transport chain, damage mitochondrial DNA, block ATP generation, and cause peroxidation of cellular lipids leading to membrane defects. Several studies in human NASH livers have shown the presence of lipid peroxidation products [74]. Polyunsaturaed fatty acids (PUFA) are especially important in the context.

The aldehyde products generated as a result of PUFA peroxidation not only retain prooxidant properties but have a longer half-life and by diffusing to surrounding tissues stimulate stellate cell proliferation leading to fibrosis and neutrophil phagocytosis [75].

However, animal models of high-fat diet-induced obesity have failed to demonstrate a clear contribution of oxidative stress in liver injury in NAFLD. In a major clinical trial, PIVENS, treatment with antioxidant vitamin E treatment in NAFLD resulted in improved disease severity in patients without cirrhosis or diabetes mellitus. In children vitamin E improved NASH but was not associated with sustained improvement in liver enzymes [76]. Thus, oxidative stress may contribute to liver injury in NAFLD but is not the sole mechanism involved.

Endoplasmic Reticulum (ER) Stress Response

The unfolded protein response (UPR) is the physiological pathway triggered by the ER to eliminate excess or mis-/unfolded proteins within the cell. It can also be triggered by ER calcium depletion and cellular energy depletion, both of which are seen in NAFLD. Mis-/unfolded proteins, sequester glucose-regulated protein 70 kDa (GRP78) from the three UPR sensors, inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6). The three UPR sensors undergo activation by phosphorylation and dimerization (or cleavage in case of ATF-6). IRE1α and PERK in turn activate X-box-binding protein 1 (XBP1S) and ATF-4, respectively, which together with cleaved ATF-6 comprise the effector molecules for the UPR response. These molecules lead to protein folding via increased transcription of GRP78 and stimulate the endoplasmic-reticulum-associated protein degradation (ERAD) pathway by which mis-/unfolded proteins are eliminated [77, 78].

However, in case of excess protein synthesis, the adaptive UPR response fails resulting in the accumulation of mis-/unfolded proteins within the ER. This precipitates ER stress by which the ER sets off signals that lead to cell senescence and death by apoptosis, but the process may increase inflammation. IRE1α can activate the extrinsic apoptosis pathway via JNK and caspase-12 activation. ATF6 and ATF4 can induce C/EBP-homologous protein (CHOP) expression which inhibits B-cell lymphoma 2 and induces proapoptotic Bim, thus leading apoptosis via the intrinsic apoptotic pathway. Although in obesity, markers of ER stress are increased in liver along with other tissues [79, 80], in NAFLD the current evidence that ER stress plays a major role in the pathogenesis of NAFLD is inconsistent [81–83] (Fig. 23.2).

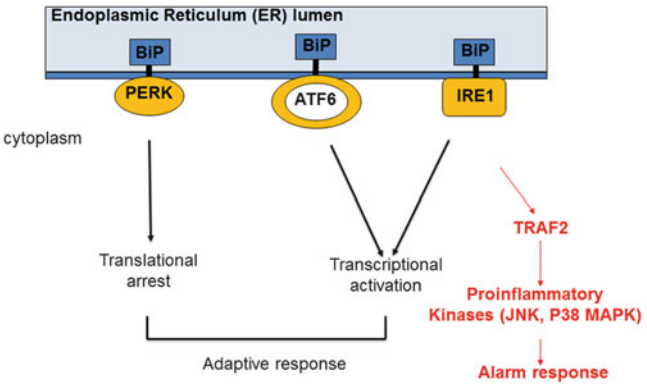

Fig. 23.2 The unfolded protein response (UPR): The three UPR sensors, inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6). Once activated the UPR sensors, lead to arrest of further protein synthesis and folding response. If the ER response fails, it leads to ER stress via TRAF2 activation

Mitochondrial Injury

Mitochondria in NAFLD have structural and functional defects including defects in its DNA, ATP depletion, and uncoupling of oxidative phosphorylation by overexpression of uncoupling proteins such as UCP-2 [84, 85]. Mitochondria respond to injury or energy depletion by mitophagy, a form organelle restricted autophagy. This process avoids excessive inflammation. However, ROS accumulation within hepatocytes can induce mitochondrial membrane permeability termed mitochondrial permeability transition (MPT). The MPT pore leads to mitochondrial death by intrinsic apoptotic pathway, but at the same time the MPT pore propagates further ROS formation and necrosis [86]. Loss of mitochondrial membrane integrity leads to a loss of the transmembrane potential required for sustaining electron transport chain. The failure to link oxidation to phosphorylation results in ROS generation. ROS have many biological effects described earlier in the paper including activation of NF-κB and inflammasome leading to inflammation and insulin resistance. MPT pore induces necrosis, and necrosis by itself can drive further inflammation.

Role of Innate Immunity in the Pathogenesis of NAFLD

The innate immune system is the first line of defense against foreign substances entering a host organism. It is essentially composed of epithelial barriers, certain proteins, and phagocytic cells that are capable of delivering a rapid defense against potential threat to the organism. Unlike the adaptive

pathway that is initially slow to recognize but remembers a potential pathogen, innate immune responses are nonspecific and rapid. Innate immune responses are initiated when the body recognizes molecular patterns on the invading substance as foreign. Many of these molecules are recognized by TLR proteins, which are highly conserved across from plants to vertebrates and expressed by several cells mediating innate immunity. Exposure to these triggers then leads to activation of phagocytic and antigen-presenting cells including macrophages, natural killer (NK) T cells, and dendritic cells. The phagocytic cells once activated release a slew of chemicals including enzymes, antimicrobial peptides, and ROS that leads to kill the invading microorganisms and/or metabolism of the foreign material. In vertebrates, microbial surface molecules also activate complement system. Ultimately, these mediators of innate immunity signal an inflammatory response and trigger activation of adaptive immunity. Several components of innate immune system are activated in NAFLD as described below.

Activators of Innate Immunity in NAFLD

Adipokines and Cytokines

Over the past decade we have learned that adipose tissue is not just a depot for storage of fat but rather a dynamic organ that secretes several cytokines, termed adipokines. Accumulation of visceral adiposity leads to worsening of metabolic syndrome leading to a low-grade chronic inflammatory state. Increased deposits of visceral fat by imaging studies have been correlated with adverse NAFLD outcomes [38–40]. Both visceral and subcutaneous adipose tissues have a variable propensity to induce insulin resistance; visceral fat is inherently more inflammatory than subcutaneous fat. This is attributed in part to a difference in the maturity of adipocytes at the two sites [87]. In the physiological state the predominant adipokine secreted by adipose tissue is adiponectin, which functions to sensitize the peripheral tissues to insulin. On the other hand, in obesity the levels of adiponectin decline, whereas there is an increase in several inflammatory cytokines such as leptin, resistin, interleukin (IL)-6, IL-1B, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1. The net result of adipose dysfunction is propagation of systemic inflammation and peripheral insulin resistance via NF-κB and JNK activation [88]. Below we summarize the role of some of the key adipokines in NAFLD.

Adiponectin

Leptin-deficient obese mice, genetically engineered to produce high levels of adiponectin, have a greater overall amount of adipose tissue but interestingly a lower number of macrophages in adipose tissue and lower levels of systemic IL-6 and TNF-α [89]. Overexpression of adiponectin in obese mice results in a greater proportion of alternatively activated M2 macrophages in their adipose tissue. These studies suggest that adiponectin promotes a decrease in macrophage infiltration of adipose tissue and favors their M2 differentiation [90]. In human studies, adiponectin levels correlate inversely to degree of steatosis, necroinflammation and fibrosis in NAFLD, BMI, percentage of body fat, fasting insulin concentration, and plasma triglyceride levels. Similarly deficiency has been noted of the hepatic receptor for adiponectin, AdipoR2. AdipoR2 expression is lower NASH liver compared to controls and correlates inversely with the severity of steatosis and fibrosis in NASH [91, 92]. Thus the evidence strongly suggests that a relative deficiency of adiponectin contributes to progressive inflammation and overall disease in NAFLD making it an attractive therapeutic option.

Leptin

Leptin is anorexigenic and promotes expenditure of energy. However in obesity and NAFLD serum, leptin levels are increased. Experimental evidence suggests that leptin may promote immune cell activation and phagocytosis and can stimulate hepatic fibrosis by stellate cell activation. Treatment with leptin in human studies is associated with improvement in steatosis and hepatocyte injury suggesting that elevated leptin levels in NAFLD may in fact be representative of a state of resistance to the hormone, not unlike an insulin-resistant state [88, 92–94]. Additional cytokines are summarized in Table 23.3.

Intestinal Microbiome

The total number of bacteria in our gut is nine times that of the number of cells in our body and 15,000–35,000 species of bacteria reside in human gut [95, 96]. It is intuitive hence that these bacteria have a significant influence on our health and disease states and are collectively referred to as the intestinal microbiome.

The intestinal microbiome affects the nutritional state of the host [95, 96]. Chow-fed conventionally reared mice have a 40 % higher body fat than gnotobiotic mice in spite of consumption of fewer calories. Transplanting bacteria from obese mice to lean mice, without a change in diet, resulted in the latter rapidly gaining weight. In addition to influencing the nutritional state of the host, the gut microbiome presents a large amount of endotoxin load to the liver via the portal circulation [97, 98]. In NAFLD the size of the microbiome is increased, and its composition is distinct from controls. Also NAFLD is associated with increased intestinal permeability from defects in tight junctions. The net result is endotoxinemia which via activating TLR signaling in the liver contributes to the development of NAFLD [99, 100].

Table 23.3 Lipids implicated in the pathology of NAFLD

Triglycerides	Largest type of fat that is deposited in the liver in NAFLD. Represents an adaptive or protective change. Does not cause tissue injury or inflammation/fibrosis. May play a role in promoting insulin resistance
Diacylglycerol	Leads to insulin resistance via protein kinase C activation
Free fatty acid	Long-chain, saturated FFA, i.e., palmitic acid leads to in vitro ROS generation, pro-inflammatory (activates JNK), and causes lipoapoptosis in hepatocytes. Promote TLR activation in Kupffer cells
	In animal models, FFA leads to blockade of TG synthesis and worsening of steatohepatitis
	Diet worsens insulin resistance and liver pathology
Lysophosphatidylcholine	Apoptosis of hepatocytes
Ceramide	Increased in NAFLD in lipidomic studies
Polyunsaturated fatty acids	Protective in NAFLD, especially omega-3 unsaturated fatty acids. Anti-inflammatory, inhibit hepatic stellate cells and Kupffer cell activation
Free cholesterol	Pro-inflammatory (activates JNK), promotes ROS formation, depletes mitochondrial GSH rendering hepatocytes susceptible to TNF-α or Fas-mediated apoptosis

Recently a lot of attention has been brought to an additional mechanism by which the microbiome may propensate NAFLD. Endogenous ethanol and acetaldehyde are produced by gut microflora and have been observed in obese subjects, patients with intestinal blind loops, and in those with small intestinal bacterial overgrowth [101, 102]. These can enter the liver by the portal system and initiate hepatic steatosis by several well-studied mechanisms of liver injury [103].

Probiotics have been used in animal and human studies of NAFLD with reports of improvement in overall disease [104, 105], but further studies are warranted before they can be adapted in clinical practice.

Cellular Elements of Innate Immunity Involved in NAFLD Pathogenesis

Role of Adipose Tissue Macrophages: Adipose Tissue-Liver Signaling

Adipose tissue is inherently pro-inflammatory in obesity. However the questions that still remain unanswered are whether adipose tissue inflammation leads to NASH, and if so how? An interesting animal study has helped shed light on this question. In mice fed a high-fat cholesterol-rich diet for 26 weeks, inflammatory signals were detected from adipose tissue between 6 and 16 weeks before their appearance in the liver at 16–26 weeks, indicating that macrophages in adipose tissue are activated in the adipose tissue before a similar process occurs in the liver [106]. However other studies have confirmed that adipose tissue inflammation, once started continues throughout the pathological spectrum of NASH and once hepatic inflammation is established despite deletion of Kupffer cells, inflammation in NASH fails to resolve. It is interesting that deletion of Kupffer cells before onset of hepatic inflammation prevents the onset of NAFLD despite high-fat diet-induced obesity, systemic inflammation, and insulin [107]. These data indicate that inflammation may originate in adipose tissue, but once established it is further driven by both the adipose tissue and hepatic macrophages [108].

Altered balance between pro- and anti-inflammatory adipokines leads to activation of resident macrophages in the adipose tissue and additional recruitment of macrophages from the circulation. For the latter process, monocyte chemoattractant protein-1 (MCP-1) and TNF-α are particularly important. MCP-1 binds to C-C chemokine receptor-2 (CCR2) receptors on macrophages and triggers their activation [109]. Adipose tissue histology in obesity shows clustering of macrophages in the pathognomic "crown-like" clusters surrounding necrotic adipose tissue cells and these are believed to propagate the cycle of inflammation. Tissue macrophages have been functionally classified into M1/M2 macrophages homologous to Th1/Th2 phenotype to T cells. While resident macrophages in healthy adipose tissue mostly express the M2 phenotype, in obesity and diabetes mellitus, they are typically pro-inflammatory or of the M1 phenotype [110, 111].

Role of Kupffer Cells

Similar to adipose macrophages, Kupffer cells have also been proposed to play a significant role in pathogenesis of NAFLD [112, 113]. In MCD diet-induced NAFLD in mice, liposome-encapsulated dichloromethylene bisphosphonate (clodronate) eliminates macrophages and prevents development of steatohepatitis [112]. In metabolic syndrome, an increased number of monocytes have been identified in circulation [114]. Also, the overall number of macrophages has been shown to increase in the liver in NAFLD patients and this correlates with the severity of disease [115]. Interestingly while simple steatosis has a more diffuse distribution of Kupffer cells, in NASH the increased numbers of Kupffer cells are mostly present in the perivenular region [115]. However it is unclear whether the increased macrophages in the liver in NAFLD are derived from blood monocytes or represent an expansion of resident hepatic Kupffer cells as currently reliable markers to distinguish between the two do

not exist. Interestingly, although the number of Kupffer cells is increased in NAFLD, imaging studies utilizing superparamagnetic iron oxide (SPIO)-magnetic resonance imaging which relies on uptake of labeled iron for detection of macrophages demonstrated decreased uptake suggesting impaired phagocytic function of Kupffer cells in NAFLD [116].

Subcellular Pathways of the Innate Immune Pathway in NAFLD

TLR Signaling and Its Role in NAFLD

TLRs are a group of extra- and intracellular receptors that are capable of recognizing nonprotein microbial sequences and damaged or altered host molecules. Of the 13 types of TLRs known to exist in mammals, so far 8 have been identified in human liver and are expressed by several cells within the liver including hepatocytes, Kupffer cells, and HSC [117, 118]. The ligand sequences that bind to and activate TLRs are called pathogen-associated molecular patterns (PAMPs) or disease-associated molecular patterns (DAMPs) depending upon whether they are nonself or originate within the host organism. TLRs recognize PAMPs from a wide variety of pathogens including protein and nonprotein molecules of bacterial, viral, and fungal origins [119, 120]. Most important of these is lipopolysaccharide, a component of the cell wall of Gram-negative bacteria which results in activation of TLR4 [119]. Downstream targets of TLR4 activation depend on the adaptor molecules recruited in the activation process [121–123]. TLR4 activation leads to activation of nuclear factor (NF)-κB and AP-1 by engaging myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein or MyD88 adaptor-like (TIRAP/Mal). TLR4 also signals via TIR domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) leading to activation interferon regulatory factor 3 (IRF3) and thus transcription of interferon-β [117, 118]. Binding of these ligands to TLRs triggers a signaling cascade that results in activation of transcription factors involved in inflammatory pathways such as NF-κB, AP-1, and interferon-responsive factors (IRF). SFAs have been shown to activate TLR4 signaling in macrophages through both Myd88-dependent and TRIF-dependent pathways. By contrast, polyunsaturated fatty acids inhibit these pathways [124, 125]. TLR4-mediated cellular events escalate liver injury in several forms of hepatic steatosis [117]. LPS levels are elevated in several animal models of NAFLD including the high-fat (HF) diet, fructose-rich diet, MCD diet, and choline-deficient amino acid-defined (CDAA) diet, and treating with antibiotics or TLR4 mutation protects the animals from hepatic steatosis [112, 126].

TLR9 may also play a significant role in NAFLD. It recognizes DNA containing an unmethylated-CpG motif on DNA that is characteristic of bacterial DNA. A recent murine study reported that bacterial DNA is detectable in the blood in NASH, even without cirrhosis, and that bacterial DNA binding to TLR9 contributes to the development of steatohepatitis. WT mice on a CDAA-defined diet developed severe steatohepatitis with insulin resistance. In contrast, TLR9-deficient mice had less steatohepatitis even though bacterial DNA was present in the blood [127, 128] (Fig. 23.3).

Probiotics can improve NAFLD in animals and humans and one proposed mechanism is via suppressing TLR activation [104, 105]. While SFAs promote TLR signaling, polyunsaturated fatty acids improve steatohepatitis by inhibiting TLR signaling [129] (Fig. 23.3).

Role on Inflammasome in NAFLD

The nucleotide-binding domain, leucine-rich repeat-containing (NLRP3) inflammasome, also known as cryopyrin or NALP-3, is a multimeric structure and is expressed by myeloid cells that regulates inflammation [130]. Once the complex which requires pro-caspase and adaptor protein recruitment is assembled in the cytosol, caspase-1 is released. Caspase-1 then promotes the cleavage of pro-inflammatory cytokines, namely, pro-IL-1β, pro-IL-18, and IL33, to their respective active forms.

The inflammasome is activated by several stimuli including PAMPs and DAMPs. SFAs, such as palmitate, are well-recognized DAMPs, which, via mitochondrial ROS formation, activate NLRP3 inflammasome to release IL-1β and IL-18. In addition, palmitate-conditioned hepatocytes activate the inflammasome in liver lymphocytes and macrophages to augment release of IL-1β and TNF-α [130–132]. In vivo studies reveal that inflammasome is activated in mice with MCD diet-induced fatty liver, but not in HF diet-induced simple steatosis [132]. A recent study shed more light on this interesting topic as the authors showed that mice lacking inflammasomes NLRP6 and NLRP3 and IL-18 develop progressive NAFLD and metabolic syndrome. Moreover, cohousing inflammasome-deficient mice with wild-type mice led to worsening of hepatic steatosis and obesity [133].

Innate Immunity and Insulin Resistance

We have previously explained that NAFLD is a disorder characterized by insulin resistance [30, 31, 134]. The insulin receptor is a transmembrane tetrameric complex, which upon binding to insulin signals autophosphorylation of tyrosine residues and sets off a signaling cascade including phosphorylation of the Janus-activated kinases (JAK) which leads to phosphorylation and activation of insulin receptor substrates (IRS)-1 and IRS-2 that mediate various intracellular functions

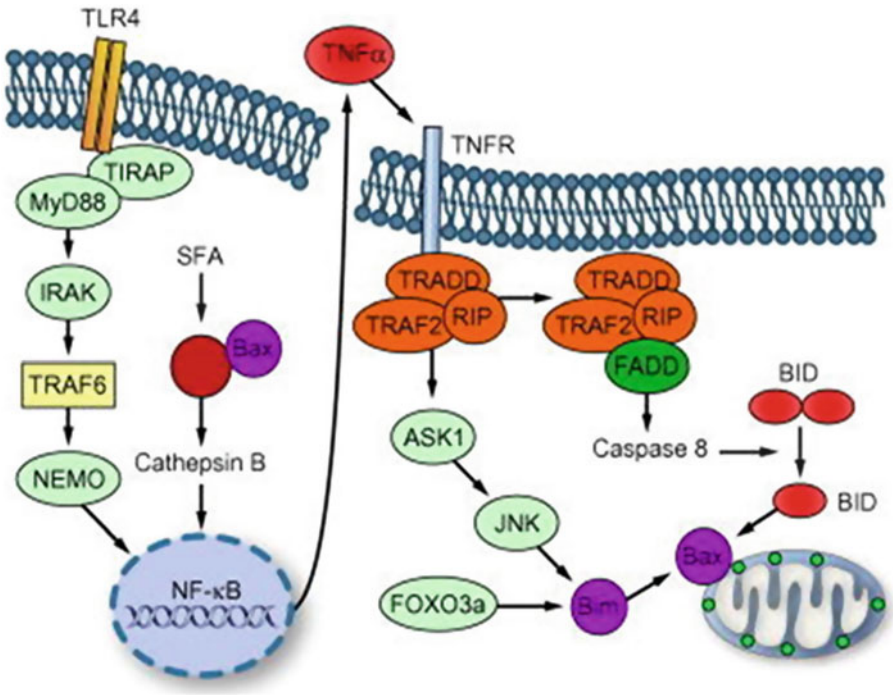

Fig. 23.3 TLR4 activation recruits several downstream adaptor molecules ultimately leading to NFκB activation and TNFα production. TNFα binds to its transmembrane receptors and causes downstream activation of proapoptotic pathways. *SFA* saturated fatty acid, *Bim* Bcl-2 protein family member, *ASK1* apoptosis signal-regulating kinase, *1Bax* B-cell lymphoma 2-associated X protein, *TIRAP* Toll/IL-1 receptor domain containing adaptor protein, *MyD88* myeloid differentiation factor 88, *IRAK* interleukin 1 receptor-associated kinase, *TRAF2/6* TNF receptor-associated factor 2/6, *NEMO* NFκB essential modulator, *TRADD* TNF receptor-associated death domain protein, *RIP* receptor interacting protein, *FADD* Fas-associated protein with death domain, *BID* proapoptotic BCL-2 interacting domain, *FoxO3a* forkhead box-containing protein, class O member 3a, *TNFα* tumor necrosis factor α, *NFκB* nuclear factor κ B. Adapted from Fuchs and Sanyal, J Hepatolology, 2011

of insulin. Serine–threonine kinases via phosphorylation and activation of IRS-1 and IRS-2 can lead to direct activation of the pathway and interfere with normal insulin signaling, thus leading to insulin resistance [135–137]. Fatty acids can activate IRS-1 and IRS-2 causing insulin resistance. Several other factors that exist in NAFLD lead to activation of these kinases including hyperinsulinemia pro-inflammatory cytokines, oxidative stress, and TLR activation. The three serine kinases that have been linked to insulin resistance are JNK, inhibitor of nuclear factor κB (NF-κB) kinase (IKK), and certain isoforms of protein kinase C (PKC) [138–140]. Among these, JNK and IKK are known to stimulate inflammatory pathways through their activation of activator protein-1 (AP-1) and NF-κB, respectively. JNK and IKK promote the expression of lipogenic genes, cytokines, and cell-adhesion molecules and mediate SFA-induced apoptosis of hepatocytes [137, 141].

Another group of molecules in this context is the suppressors of cytokine signaling (SOCS). By competing for insulin-binding sites, SOCS can directly lead to IRS-1 and IRS-2 activation and thus IR [142–144]. Hence, signaling molecules of the innate immune system mediate propagation of insulin resistance in NAFLD (Fig. 23.4).

Innate Immune Mechanisms Promote Hepatic Fibrosis in NAFLD

LPS is elevated in the systemic and portal circulation in patients with cirrhosis [145]. Reduction of gut microflora by nonabsorbable broad-spectrum antibiotics results in a decrease in serum LPS levels and inhibits experimental liver fibrosis. TLR signaling has been implicated in stimulating HSC and inducing hepatic fibrosis in several models of chronic liver injury [146]. TLR4 signaling promotes activation of quiescent HSC via an MyD88-dependent pathway leading to increased chemokine production and leads to KC chemotaxis. Mice mutant in TLR co-receptors had lesser degree of hepatic fibrosis despite a similar level of plasma LPS [147]. Another proposed mechanism for hepatic fibrosis via TLR signaling is via the adaptor molecule MAP3K tumor progression locus-2 (Tpl2). TLR4 and TLR9 activation leads to downstream activation of Tpl2 that ultimately leads to ERK signaling and increased expression of fibrogenic genes in HSC in vitro. Tpl2 knockout mice on an MCD diet have a significant reduction in fibrosis compared with wild-type controls [148].

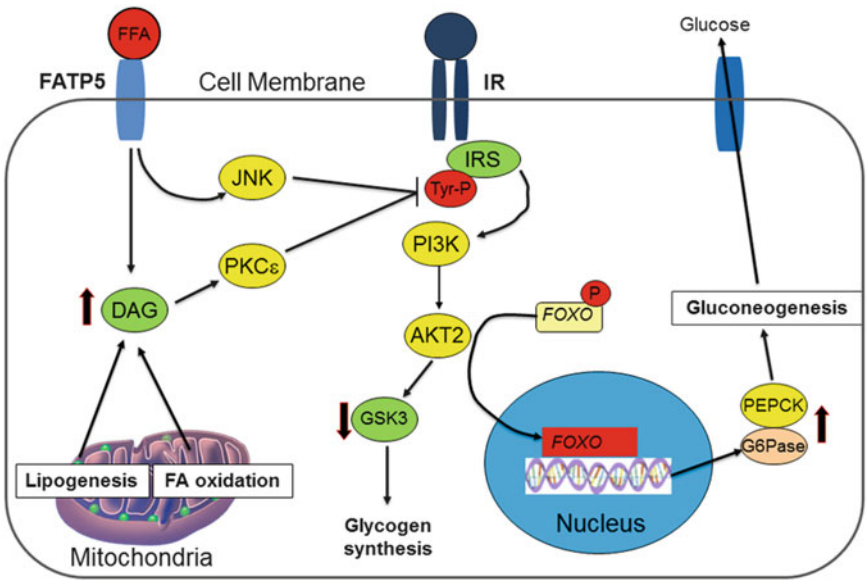

Fig. 23.4 Mechanism for lipid induced insulin resistance. Free fatty acids (FFA) and Diacylglycerol (DAG) increase from diet, lipogenesis, and β-oxidation of fatty acids. Both can lead to activation of insulin receptor substrates (IRS-1 and -2) via protein kinase-ε (PK-ε) and Janus kinase (JKN)-mediated pathways. The net result is worsening of insulin resistance due to decreased glycogen synthesis, increased FOXO-1 phosphorylation and nuclear translocation resulting in increased gluconeogenesis

Conclusion

Our insight into the pathophysiology of NAFLD has expanded tremendously over the past decade. We now understand that hepatic pathology in NAFLD evolves in a genetically susceptible individual exposed to an environment of nutrient excess and sedentary lifestyle. NAFLD is not just a liver exclusive disease, rather a hepatic manifestation of a systemic disease state characterized by insulin resistance and chronic low-grade inflammation. Innate immune responses, once initiated, undergo further amplification via interrelated pathways of the innate and adaptive immune systems. IKK and JNK activated by several intracellular pathways described above or via TLR signaling converge to stimulate hepatocytes, Kupffer cells, and possibly several other resident liver cells to produce cytokines and chemokines which can then further compound the process of inflammation, insulin resistance, and hepatocellular cell damage.

While our knowledge continues to increase on the topic, several questions still remain unanswered. We have yet to generate practical tools for making the diagnosis of NAFLD easier and have just started developing effective therapies that may help arrest the disease progression and repair damage. And although we do know that in NAFLD, there exists a dysregulation of immune system, we have still not determined which comes first, immune activation or insulin resistance, and whether this originates in the adipose tissue or gut microbiome. Nevertheless, ways to regulate the immune imbalance that occurs in NAFLD will hold the key to ultimately treating one of the root causes of the disease. The rapidly increasing worldwide prevalence of NAFLD only makes these questions all the more intriguing and the challenge more formidable at the same time.

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Paediatric Liver Disease

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Giorgina Mieli-Vergani, Rodrigo Liberal, and Diego Vergani

Key Points

- There are two main types of autoimmune liver disease (AILD) in childhood: autoimmune hepatitis (AIH) and AIH/sclerosing cholangitis overlap syndrome (autoimmune sclerosing cholangitis, ASC).
- AIH is divided into type 1, positive for anti-nuclear (ANA) and/or anti-smooth muscle (SMA) antibodies, and type 2, positive for anti-liver kidney microsomal antibody type 1 (LKM1).
- Most patients with ASC are positive for ANA and/or SMA.
- Anti-neutrophil antibodies are positive in a similar proportion of children with ASC and AIH type 1, but are usually negative in AIH type 2.
- In at least 20 % of patients with ASC, the diagnosis can be achieved only if a cholangiography is performed, because the histological picture is identical to that of AIH.
- Immunofluorescence titres of ≥1:20 of ANA and SMA and of ≥1:10 of anti-LKM-1 antibodies are significant in paediatrics, because autoantibodies are rare in healthy children.
- Presence of antibody to soluble liver antigen (SLA) is associated to worse disease severity in all types of AILD.
- Both AIH and ASC respond to treatment with prednisolone ± azathioprine, but bile duct damage in ASC may progress despite treatment.
- Autoantibody immunofluorescence titre and immunoglobulin G (IgG) levels are good markers of disease activity and can be used to monitor response to treatment.
- Twenty percent of children with AIH type 1 or ASC, but none with AIH type 2, can eventually stop treatment with no relapse.

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Introduction

In paediatrics, there are two liver disorders in which liver damage most likely stems from an autoimmune attack: "classical" autoimmune hepatitis (AIH) and the AIH/sclerosing cholangitis overlap syndrome (also known as autoimmune sclerosing cholangitis, ASC). Autoimmunity has also been implicated in the pathogenesis of de novo AIH (d-AIH) arising after liver transplantation.

The presentation of childhood autoimmune liver disease (AILD) is non-specific and can mimic most other liver disorders. Since prompt treatment is life saving, it is important to suspect AILD and perform appropriate investigations in all children who present with cryptogenic liver disorders.

According to data collected at the King's College Hospital Paediatric Hepatology tertiary referral centre, there is an increase in the yearly incidence of juvenile AILD, which can only be partially explained by referral bias. Thus, in the 1990s, the yearly incidence of children over 4 months of age referred with AILD accounted for 2.3 % of about 400 new cases, whereas in the new millennium the yearly incidence has increased to 12 %.

Autoimmune Hepatitis

AIH is a progressive inflammatory liver disorder characterised by female preponderance, hypergammaglobulinaemia, seropositivity for circulating autoantibodies and a histological picture of interface hepatitis (Fig. 24.1), in the absence of a known aetiology [1–3]. Compared to adult and

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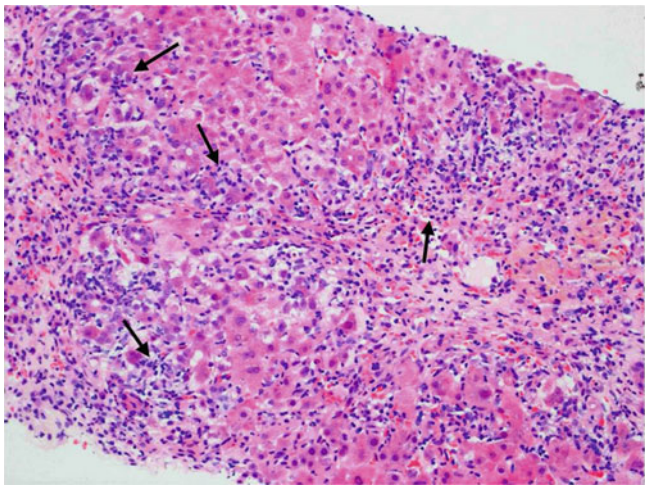

Fig. 24.1 Histology of autoimmune hepatitis. The portal and periportal inflammatory infiltrate characteristic of autoimmune hepatitis is composed of lymphocytes, monocytes/macrophages and plasma cells (*arrows* showing plasma cell infiltration), and is known as interface hepatitis. Haematoxylin & eosin staining (picture kindly provided by Dr Alberto Quaglia, Institute of Liver Studies, King's College Hospital)

Table 24.1 Clinical presentation of childhood autoimmune liver disease [6, 12]

Parameter	AIH-1	AIH-2	ASC
Median age in years	11	7	12
Mode of presentation (%)			
Acute hepatitis	47	40	37
Acute liver failure	3	25	0
Insidious onset	38	25	37
Complication of chronic liver disease	12	10	26
Associated immune diseases (%)	22	20	48
Inflammatory bowel disease (%)	20	12	44
Abnormal cholangiogram (%)	0	0	100
ANA/SMA (%)	100	25	96
Anti-LKM-1 (%)	0	100	4
pANNA (%)	45	11	74
Anti-SLA (%)	58	58	41
Interface hepatitis (%)	92	94	60
Biliary features (%)	28	6	35

AIH autoimmune hepatitis, *ANA* anti-nuclear antibodies, *ASC* autoimmune sclerosing cholangitis, *SMA* anti-smooth muscle antibodies, *anti-LKM-1* anti-liver kidney microsomal type 1 antibody, *pANNA* peripheral anti-nuclear neutrophil antibodies, *SLA* soluble liver antigen, *IgG* immunoglobulin G

elderly patients, children and adolescents more frequently present acutely and follow a more aggressive course. AIH usually responds to immunosuppressive treatment, which should be instituted as soon as the diagnosis is made. If left untreated, AIH usually progresses to liver failure requiring transplantation [3].

Two types of AIH are recognised: type 1 AIH (AIH-1), positive for anti-nuclear (ANA) and/or smooth muscle antibodies (SMA), and type 2 AIH (AIH-2), defined by the positivity for liver kidney microsomal type 1 (anti-LKM-1) and/or anti-liver cytosol type 1 autoantibodies (anti-LC-1) [4, 5]. While AIH-1 affects children and adults equally frequently, AIH-2 is predominantly a paediatric condition. In fact, anti-LKM-1-positive disease is rare, though not absent, in adults.

The epidemiology of childhood AIH is unknown, but AIH-1, accounting for some 60 % of cases, often presents around puberty, whereas AIH-2 tends to affect younger children, even infants [6, 7]. Anti-LKM-1-positive patients more commonly present with fulminant hepatic failure compared to ANA/SMA-positive AIH patients, and they tend to have higher serum bilirubin and transaminase levels. Excluding children with fulminant presentation, a severely impaired hepatic synthetic function, as assessed by the presence of both prolonged prothrombin time and hypoalbuminaemia, is more common in ANA/SMA-positive patients compared to those anti-LKM-1-positive (Table 24.1). The vast majority (80 %) of patients have elevated levels of IgG, but normal IgG levels cannot exclude the diagnosis of AIH. Partial IgA deficiency is more common in AIH-2 than in AIH-1 [6]. The severity of interface hepatitis at diagnosis is similar in both types, but cirrhosis on initial biopsy is more frequent in AIH-1, which is suggestive of a more chronic disease course [6].

In both AIH-1 and AIH-2, a more severe disease course and a higher tendency to relapse are associated with the possession of autoantibodies to soluble liver antigen (SLA), which are present at diagnosis in approximately 50 % of patients with both types of diseases [8].

AIH is three times more likely to occur in females than in males. A family history of autoimmune diseases is observable in 40 % of cases [6]. In addition, at least 20 % of AIH patients have concomitant autoimmune conditions or will develop them during follow-up [6]. These include thyroiditis, ulcerative colitis, insulin-dependent diabetes, vitiligo, nephritic syndrome, hypoparathyroidism and Addison disease, the latter two being observed in particular in children with AIH-2 or in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED) [9].

The heterogeneous and fluctuating course of AIH leads to highly variable modes of presentation and clinical manifestations [10]. In the paediatric setting, the presentation of the disease usually follows one of the three patterns:

1. In 40 % of patients, the presentation is indistinguishable from that of an acute viral hepatitis, being associated with non-specific symptoms of malaise, nausea/vomiting, anorexia and abdominal pain, followed by jaundice, dark urine and pale stools. Some children, particularly those who are anti-LKM-1-positive, develop acute liver failure

with grade II to IV hepatic encephalopathy within 2–8 weeks from the onset of symptoms.
2. In 25–40 % of patients, the onset is insidious, with an illness characterised by progressive fatigue, relapsing jaundice, headache, anorexia, amenorrhea and weight loss, lasting for several months and even years before diagnosis.
3. In 10 % of patients, there is no history of jaundice, and the diagnosis follows a presentation with complications of portal hypertension, such as upper gastrointestinal bleeding or hypersplenism [11].

The mode of presentation of AIH in childhood is therefore variable, and the disease should be suspected and excluded in all children presenting with symptoms and signs of liver disease not ascribable to more common pathologies. The course of the disease can be fluctuating, with flares and spontaneous remissions occasionally resulting in delayed referral and diagnosis. The majority of children, however, on physical examination have clinical signs of an underlying chronic liver disease, including cutaneous stigmata (spider nevi, palmar erythema, leuconychia, striae), firm liver and splenomegaly. At ultrasound, the liver parenchyma of these patients is often nodular and heterogeneous [11].

Autoimmune Sclerosing Cholangitis

In children and young adults, sclerosing cholangitis is often associated with florid autoimmune features, including elevated autoantibody titre, especially ANA and SMA, hypergammaglobulinaemia and interface hepatitis on liver biopsy (Table 24.1) [3].

Since these features are shared with AIH and are often independent of elevated alkaline phosphatase (AP) or gamma-glutamyl transpeptidase (GGT) levels at disease onset, the diagnosis of sclerosing cholangitis relies on cholangiographic studies. In a prospective study of 16-year duration, all children with the serological—positive autoantibodies and high IgG levels—and histological—interface hepatitis—features of AILD were examined by cholangiogram at the time of presentation [12]. In this study, ASC was as common as AIH; approximately half of the cases had bile duct changes characteristic of sclerosing cholangitis (Figs. 24.2 and 24.3), though these were generally less advanced than those observed in adult primary sclerosing cholangitis (PSC), and they were therefore diagnosed with ASC. Importantly, a quarter of children with ASC had no histological features pointing to a bile duct involvement (Fig. 24.4), despite abnormal cholangiograms, therefore the diagnosis of ASC was heavily reliant upon the cholangiographic studies. Virtually all ASC patients were seropositive for ANA and/or SMA. In contrast to AIH, which is a predominantly a disease of females, ASC affects boys and girls equally frequently [12].

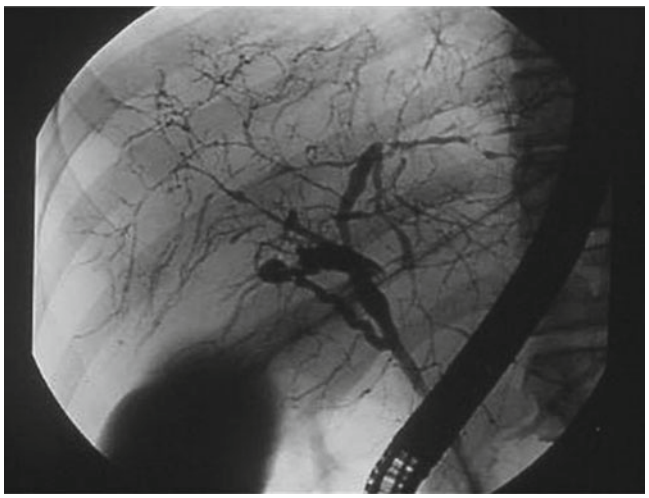

Fig. 24.2 Endoscopic retrograde cholangiopancreatography (ERCP) in autoimmune sclerosing cholangitis. ERCP showing widespread bile duct strictures and dilatations in a child with autoimmune sclerosing cholangitis

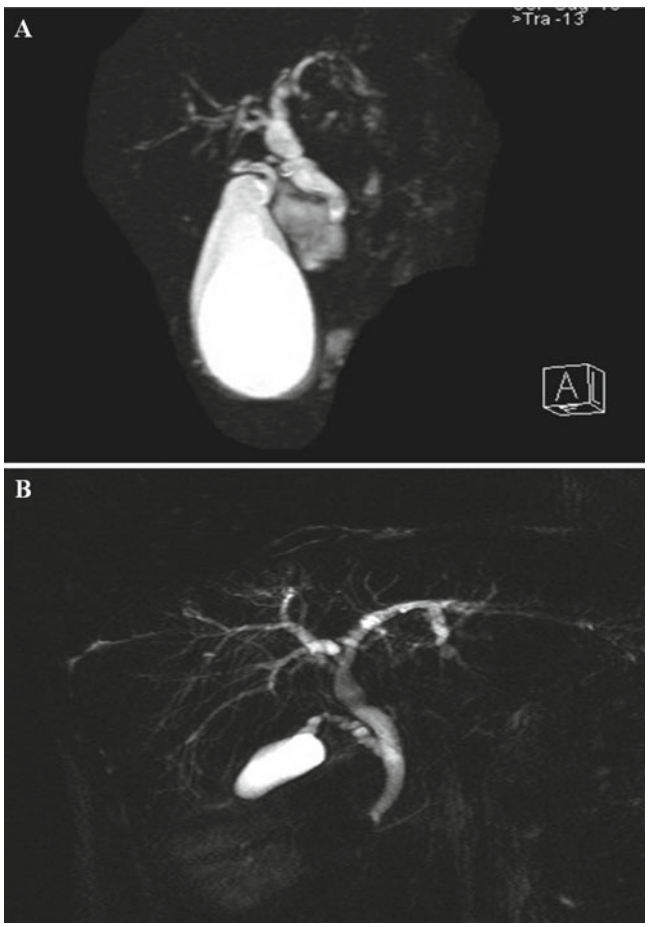

Fig. 24.3 Magnetic resonance cholangiopancreatography (MRCP) in autoimmune sclerosing cholangitis. MRCP showing only gross (**a**), and both gross and subtle biliary changes (**b**) in children with autoimmune sclerosing cholangitis

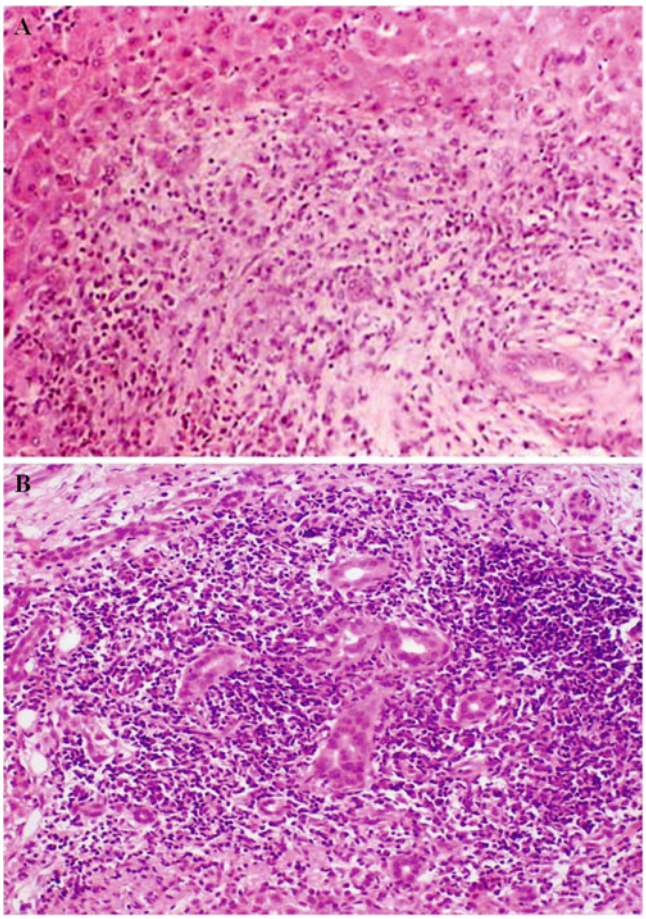

Fig. 24.4 Histology of autoimmune sclerosing cholangitis. Interface hepatitis without obvious biliary changes in a patient with autoimmune sclerosing cholangitis (*upper panel*). Interface hepatitis with abundant plasma cell infiltration and clear bile duct damage in another child with autoimmune sclerosing cholangitis (*bottom panel*)

The 16-year follow-up study showed that the mode of presentation of ASC was similar to that of AIH-1, although association with IBD was far more typical of ASC (45 %) than AIH-1 (20 %). At the time of presentation, liver function tests could not discriminate between AIH and ASC, although the AP/AST ratio was significantly higher in ASC (Table 24.2). Notably, atypical perinuclear anti-neutrophil cytoplasmic antibody (atypical pANCA, also termed pANNA) positivity was found in 74 % of children with ASC but only 45 % of those with AIH-1 and 11 % of those with AIH-2 [12]. Clinical, laboratory and histological features of AIH-1, AIH-2 and ASC are compared in Table 24.1.

Interestingly, in the same study, ASC was far more common than sclerosing cholangitis without autoimmune features; autoantibody-negative sclerosing cholangitis was observed in only nine children referred over the 16-year period [12].

Evolution from AIH to ASC has been documented, suggesting that AIH and ASC are part of the same nosological spectrum [12]. Whether the same can be said of childhood

Table 24.2 Biochemical presentation of childhood autoimmune liver disease [6, 12]

	AIH	ASC
Bilirubin (nv < 20 mmol/L)	35 (4–306)	20 (4–179)
Albumin (nv > 35 g/L)	35 (25–47)	39 (27–54)
AST (nv < 50 IU/L)	333 (24–4830)	102 (18–1215)
INR (nv < 1.2)	1.2 (0.96–2.5)	1.1 (0.9–1.6)
GGT (nv < 50 IU/L)	76 (29–383)	129 (13–948)
AP (nv < 350 IU/L)	356 (131–878)	303 (104–1710)
AP/AST ratio	1.14 (0.05–14.75)	3.96 (0.20–14.20)

AIH autoimmune hepatitis, *ASC* autoimmune sclerosing cholangitis, *AST* aspartate aminotransferase, *INR* international normalised ratio, *GGT* gamma-glutamyl transpeptidase, *AP* alkaline phosphatase, *nv* normal values

ASC and adult PSC is not known, since prospective studies in large cohorts of patients investigating the presence of bile duct damage at disease onset in adults with features of AILD are lacking. In one retrospective study, however, a high proportion of adults initially diagnosed with AIH-1 were found to have sclerosing cholangitis on magnetic resonance cholangiography during follow-up [13].

Genetic Predisposition

Akin to adult AIH-1, possession of the human leukocyte antigen (HLA) DRB1*03 is associated with AIH-1 in paediatric patients in Northern Europe [6, 14, 15]. In contrast to adult patients, possession of DRB1*04 does not predispose to AIH in childhood and can even exert a protective role [6]. AIH-2 is associated with possession of DRB1*07 [16]. In South America, possession of the HLA DRB1*1301 allele, which predisposes to paediatric AIH-1, is also associated with persistent infection with the endemic hepatitis A virus [17, 18].

Paediatric patients with AIH, whether anti-LKM-1- or ANA/SMA-positive, have isolated partial deficiency of the HLA class III complement component C4, which is genetically determined [19].

AIH-2 can be associated with the APECED syndrome, an autosomal recessive monogenic disorder in which liver disease is reportedly present in some 20 % of cases [9].

In the United Kingdom, susceptibility to ASC is conferred by the possession of the HLA DRB1*1301 allele [20].

Pathogenesis

The aetiology of AIH is unknown, although both genetic and environmental factors are involved in its expression [21]. Etiological hypotheses and possible mechanisms leading to the liver autoimmune attack are described in Chap. 19.

Diagnosis

The diagnosis of AIH is based on the summation of a series of positive and negative scores [22, 23]. Liver biopsy is necessary to establish diagnosis and to assess the extent of liver damage. The typical histological picture includes dense mononuclear and plasma cell infiltration of the portal areas, which expands into the liver lobule; destruction of the hepatocytes at the periphery of the lobule with erosion of the limiting plate (interface hepatitis) (Fig. 24.1); connective tissue collapse resulting from hepatocyte death and expanding from the portal area into the lobule (bridging collapse); and hepatic regeneration with "rosette" formation [11]. In addition to this typical histological pattern, other positive criteria include elevated serum transaminase and IgG levels and the presence of ANA, SMA, anti-LKM-1, anti-LC-1 and/or anti-SLA. The diagnosis of AIH has been aided by criteria developed by the International Autoimmune Hepatitis Group (IAIHG), who also acknowledge negative indices such as infection with hepatitis B or C virus or evidence of Wilson disease and alcohol consumption [22, 23]. The IAIHG has provided a useful scoring system for the diagnosis of AIH for research purposes. A simplified scoring system, recently designed for ease of use in clinical practice, is based on autoantibody seropositivity, elevated IgG, interface hepatitis on histology, and the exclusion of viral hepatitis [24]. Neither scoring system is, however, ideally suited to the juvenile form of the disease, where diagnostically relevant autoantibodies often have titres lower than the cut-off value considered positive in adults. Moreover, neither system distinguishes between AIH and ASC, which can only be differentiated if a cholangiogram is performed at the time of presentation. Criteria for the diagnosis of childhood AILD are depicted in Table 24.3.

A key diagnostic criterion encompassed by all scoring systems is the detection of autoantibodies (ANA, SMA, anti-LKM-1, anti-LC-1, anti-SLA). Autoantibody detection not only assists in the diagnosis but also allows differentiation of AIH types. ANA and SMA, which characterise AIH-1, and anti-LKM-1, which with anti-LC-1 defines AIH-2, are practically mutually exclusive; in those rare instances when they are present simultaneously, the clinical course is similar to that of AIH-2 [5]. It is important to note that positivity for ANA and/or SMA is not sufficient for the diagnosis of AIH because seropositivity can be found, usually at low titre, in other liver disorders such as viral hepatitis [25, 26], Wilson disease [27] and non-alcoholic steatohepatitis [28].

Routine testing of autoantibodies relevant to AIH should be performed by indirect immunofluorescence on freshly prepared rodent substrate, including kidney, liver and stomach tissue, which allows the detection of ANA, SMA, anti-LKM-1, anti-LC-1 and anti-mitochondrial antibody (AMA) [5], the serological hallmark of primary biliary cirrhosis, a disease typically affecting adults. Recognition and interpretation of the immunofluorescence patterns is not always straightforward because this is largely operator dependent. Moreover, the relative rarity of AIH occasionally leads to errors in autoantibody reporting, particularly for those less frequently encountered such as anti-LKM-1, whose pattern is often confused with AMA. Problems in laboratory reporting and clinical interpretation of results partly depend on insufficient standardisation of tests but also on a degree of unfamiliarity of some clinicians with the disease spectrum of AIH. In regard to standardisation, guidelines have been drawn by the IAIHG serology committee [5]. Importantly, titres of autoantibodies pertaining to diagnostic significance differ in the adult and paediatric settings. Because on rodent substrate healthy adults may show reactivity at the conventional starting serum dilution of 1/10, the arbitrary dilution of 1/40 has been considered clinically significant by the IAIHG. In contrast, healthy children are rarely autoantibody seropositive; therefore, titres of 1/20 for ANA and SMA and 1/10 for anti-LKM-1 are clinically relevant. It is critical for diagnostic laboratories to validate commercially available tissue sections, because some are fixed to extend shelf life and are consequently unsuitable for the recognition of diagnostic autoantibodies at low titre. It is advisable for the laboratory to report any level of positivity ≥1/10 in children and ≥1/40 in adults and for the attending physician to interpret the result within the clinical context [29].

Less commonly tested autoantibodies that are of diagnostic importance include anti-LC-1, anti-neutrophil cytoplasm antibody (ANCA) and SLA. Anti-LC-1, an additional marker of AIH-2, frequently occurs in association with anti-LKM-1, although it can be present on its own. There are three types

Table 24.3 Criteria for the diagnosis of autoimmune liver disease in childhood

Elevated transaminases
Positivity for circulating autoantibodies: • ANA and/or SMA (titre ≥ 1:20) = AIH-1 or ASC • Anti-LKM-1 (titre ≥ 1:10) = AIH-2 • Anti-LC-1 = AIH-2
Elevated immunoglobulin G (in 80 % of cases)
Liver biopsy: • Interface hepatitis • Multilobular collapse
Exclusion of viral hepatitis
Exclusion of Wilson disease
Exclusion of non-alcoholic steatohepatitis
Cholangiogram: • Normal = AIH • Abnormal = ASC

AIH autoimmune hepatitis, *ASC* autoimmune sclerosing cholangitis, *ANA* anti-nuclear antibodies, *SMA* anti-smooth muscle antibodies, *anti-LKM-1* anti-liver kidney microsomal type 1 antibody, *anti-LC-1* anti-liver cytosol type antibody

of anti-neutrophil cytoplasmic autoantibodies: cytoplasmic (cANCA), perinuclear (pANCA) and atypical perinuclear, the target of which is a peripheral nuclear and not cytoplasmic perinuclear antigen (hence the suggested name of peripheral antinuclear neutrophil antibody [pANNA]). The type occurring in AIH-1 is pANNA, which is also found in inflammatory bowel disease and sclerosing cholangitis; it is virtually absent in AIH-2. Anti-SLA, which was originally described as the hallmark of a third type of AIH [30], is also found in 50 % of patients with type 1 and type 2 AIH, where it defines a more severe disease course [8]. Anti-SLA is not detectable by immunofluorescence, but the definition of its molecular target as UGA transfer RNA (tRNA) suppressor-associated antigenic protein has enabled the establishment of molecularly based diagnostic assays [31].

Only a small proportion of AIH patients do not have detectable autoantibody seropositivity. In adults, the rare seronegative patients respond to immunosuppression like their seropositive counterparts [32]. The prevalence and clinical characteristics of seronegative AIH remain to be defined in children [29].

Treatment

Remission is defined as clinical recovery, return to normal of transaminase and IgG levels, negative or very low autoantibody titre by immunofluorescence (≤1:20 for ANA and SMA; ≤1:10 for anti-LKM-1) and histological resolution of inflammation [33]. Clinical/biochemical remission does not necessarily reflect histological resolution. In fact, the histological response lags behind the biochemical response [34]. A lowering of the intensity of portal inflammation is observed in up to 95 % of AIH cases after, on average, 4 years of treatment and this is accompanied by improved fibrosis scores [34]. Relapse during treatment—defined by increased serum aminotransferase levels after remission has been achieved—is common, occurring in about 40 % of patients and requiring a temporary increase in the steroid dose [6]. An important contributor to this high rate of relapse is non-adherence, particularly in adolescents [35]. In more aggressive cases, the risk of relapse is higher if steroids are administered on an alternate-day schedule, which is often instituted in the belief that negative effects on the child's growth are reduced. In fact, small daily doses are more effective in maintaining disease control while minimising the need for high-dose steroid pulses during relapse (which consequently have more severe side effects) and do not affect final height [36].

Both AIH and ASC respond well to immunosuppression and treatment should be initiated promptly to avoid progression of disease. The goal of treatment is to improve symptoms, induce remission, reduce or eliminate liver inflammation and prolong survival [33]. The rapidity and degree of the response depends on disease severity at presentation. In AIH, though cirrhosis is found in between 44 and 80 % of children at the time at diagnosis, progression to end-stage liver disease requiring liver transplantation is rare, most children remaining clinically stable, with a good quality of life on long-term treatment [6, 37]. The prognosis is worse in ASC, where bile duct disease progresses despite treatment in some 50 % of cases [12].

With the exception of a fulminant presentation with encephalopathy, where liver transplant is usually required, AIH and ASC respond satisfactorily to immunosuppressive treatment whatever the degree of liver impairment, with a reported remission rate exceeding 80 % [33].

Autoimmune Hepatitis

Standard Treatment

The conventional treatment of childhood AIH consists of prednisolone (or prednisone) at 2 mg/kg/day (maximum 60 mg/day), which is gradually decreased over a period of 4–8 weeks, in parallel to the declining transaminase levels, towards a maintenance dose of 2.5–5 mg/day, depending on age and weight [33, 38]. Within the initial 2 months of treatment, an 80 % decrease in serum aminotransferase levels is commonly achieved, but complete normalisation can take several months [6]. During the first 6–8 weeks of treatment, liver function tests should be performed often to allow weekly dose adjustments while avoiding severe steroid side effects [33]. At the King's College Hospital Paediatric Liver Centre, azathioprine is added as a steroid-sparing agent when the transaminase level stops decreasing on prednisolone alone or, rarely, in the presence of early, serious steroid side effects (e.g., psychosis). Azathioprine is used at a starting dose of 0.5 mg/kg/day, which in the absence of signs of toxicity is gradually increased up to a maximum of 2.0–2.5 mg/kg/day until biochemical control of the disease is achieved. Centres differ in terms of the time at which azathioprine is utilised; in some it is added in all cases at a dose of 0.5–2 mg/kg/day after a few weeks of steroid treatment, and in others a combination of steroids and azathioprine is used from the outset. However, caution is recommended, particularly in severely jaundiced patients, given the hepatotoxic properties of azathioprine. Regardless of the initial choice of treatment protocol, 85 % of patients eventually require the addition of azathioprine [33].

Measurement of thiopurine methyltransferase (TPMP) activity level has often been advocated to predict azathioprine metabolism and toxicity before the initiation of azathioprine therapy [39], although only patients with near-zero

erythrocyte concentrations of TPMP activity are at risk of myelosuppression during azathioprine treatment [39, 40]. Thus, determination of the enzyme activity is warranted only when there is pre- or intra-treatment cytopenia or the need for particularly high doses of azathioprine [40]. Measurement of the azathioprine metabolites 6-thioguanine and 6-methylmercaptopurine has helped to identify drug toxicity and non-adherence to treatment and to achieve therapeutic levels of 6-thioguanine in inflammatory bowel disease. However, an ideal therapeutic level for AIH has not been determined [41].

Alternative Treatment

Some alternative treatment regimes have been proposed. Firstly, remission has been induced in treatment-naïve children using cyclosporine A alone, before the addition of prednisone and azathioprine after 6 months. Cyclosporine was discontinued from this treatment regimen 1 month later [42]. However, whether this protocol has any advantage over standard treatment has not yet been evaluated in controlled studies. Secondly, budesonide has been used in a large European study in combination with azathioprine [43]. Budesonide is an attractive alternative to prednisolone, as it has a hepatic first-pass clearance of >90 % of oral dose and fewer side effects, although it cannot be used in cirrhotic patients, who represent a large proportion of AIH cases. However, the results within the paediatric cohort of this study are disappointing, with similarly low remission rates in the budesonide/azathioprine and prednisone/azathioprine arms (16 % and 15 % after 6 months of treatment and 50 % and 42 % after 12 months of treatment, respectively) [44]. The poor response rate to prednisolone/azathioprine in this study compared to that observed with standard treatment [33] is likely to depend on the low fixed initial dose of prednisone used. Despite this, budesonide could be a valid alternative in selected noncirrhotic patients at risk of adverse steroid side effects.

Treatment of Refractory Cases

Mycophenolate mofetil (MMF) is a purine antagonist that selectively inhibits proliferation of activated lymphocytes but is not dependent on TPMP activity [45]. It improves various symptoms of AIH, but many patients may also experience drug intolerance—headache, diarrhoea, nausea, dizziness, hair loss and neutropenia [33]. MMF has been successfully used in those children (up to 10 %) resistant to standard immunosuppression or intolerant to azathioprine in association with predniso(lo)ne [46]. In patients for whom standard immunosuppression fails to induce stable remission, or who are intolerant to azathioprine, MMF, together with prednisolone, is currently the treatment of choice [38].

Calcineurin inhibitors, cyclosporine and tacrolimus, have been used as a rescue treatment for difficult-to-treat cases of AIH. As large studies in this subgroup of patients are lacking, they should be used with caution [33].

Autoimmune Sclerosing Cholangitis

ASC responds to the same immunosuppressive treatment used for AIH when treatment is initiated early. Abnormal liver function tests generally resolve within a few months of treatment, although medium- to long-term prognosis is worse than that of AIH because bile duct disease continues to progress despite treatment in approximately 50 % of patients [12]. Ursodeoxycholic acid (UDCA) is usually added to the conventional AIH treatment regimen in ASC, but whether this actually helps arrest the progression of bile duct disease remains to be established. In adults with PSC high-dose UDCA was reported to be more beneficial than standard doses [47], but a randomised double-blind controlled study from the Mayo Clinic revealed negative long-term implications of high-dose UDCA [48]. It is prudent, therefore, to use a dose of 15 mg/kg/day. ASC is commonly associated with inflammatory bowel disease which should be investigated even in the absence of symptoms and appropriately treated, as progression of bile duct disease is associated to persistent intestinal inflammatory damage. Flare-ups of liver disease often follow exacerbations of intestinal manifestations [12].

Duration of Treatment and Prognosis

The optimal duration of immunosuppressive treatment for AIH is unknown, although treatment withdrawal is successful only in the presence of histological resolution of inflammation. Hence, cessation of treatment can be considered when there is little or no inflammatory evidence on biopsy after 1–2 years of normal liver function tests, normal IgG levels and negative or low titre autoantibodies. In our centre, treatment withdrawal is never considered within 3 years of diagnosis or during/immediately before puberty, when relapses are more common. In AIH-1, but not AIH-2, some 20 % of juvenile patients can successfully and permanently stop treatment [6]. Long-term treatment, therefore, is required for the majority of patients, and patients should be counselled accordingly. In the paediatric setting, it is of particular importance to monitor the response to treatment of both AIH and ASC patients by assessing IgG levels and

autoantibody titres, the fluctuation of which correlates with disease activity [49]. In patients with high IgG levels especially, an observable decrease is a reliable, objective and inexpensive measure of disease control.

For those children with AIH who respond to immunosuppressive treatment, prognosis is generally good, with the majority surviving long term with an excellent quality of life on low-dose medication. However, progression to end-stage liver disease requiring liver transplantation, despite treatment, has been reported 8–14 years after diagnosis in 8.5 % of children with AIH [6]. The medium- to long-term prognosis of children with ASC is worse than that of children with AIH because of the continuation of bile duct damage in approximately 50 % of cases [12, 50].

Liver Transplantation

Approximately 10 % of children with AIH and 20 % of those with ASC require liver transplantation. Liver transplantation is indicated in patients who present with fulminant hepatic failure (with encephalopathy) and in those who develop end-stage liver disease despite treatment. After transplantation, recurrent AIH has been described in about 20 % of cases [51] and recurrent ASC in about 70 % [50]. Diagnosis of recurrence is based on biochemical abnormalities, seropositivity for autoantibodies, interface hepatitis on histology, steroid dependence and, for ASC, the presence of cholangiopathy. Recurrence of ASC after transplant is more common in the presence of active inflammatory bowel disease. Recurrence may even appear years after transplantation; therefore, steroid-based immunosuppression should be maintained at a higher dose than that used for patients transplanted for non-AILDs [51].

An algorithm summarising the management of juvenile AILD is shown in Fig. 24.5.

De Novo Autoimmune Hepatitis After Liver Transplantation

The first description of post-transplant d-AIH was in 1998. In contrast to the recurrence of the disease in patients transplanted for AIH, this condition affects patients transplanted for disorders other than AIH [51]. According to the first report describing d-AIH, seven children (4 % of 180 LT recipients) developed a form of graft dysfunction with features identical to those of classical AIH over a 5-year period, namely hypergammaglobulinaemia, positivity for circulating autoantibodies (one ANA, two ANA and SMA, one gastric parietal cell antibody and three atypical anti-LKM-1) and histological features of chronic hepatitis with portal and periportal inflammation [52].

Importantly, the index case only responded to the classical treatment of AIH, rather than the short course of high-dose steroids used to treat classical rejection. Moreover, none of the children were transplanted for AILD, they were all hepatitis C virus negative, and they all had serum concentrations of cyclosporine A or tacrolimus within the therapeutic anti-rejection levels at the time of diagnosis of d-AIH [52]. Since that report, several other groups have reported the occurrence of d-AIH after LT. Its prevalence in children ranges from 2.35 to 6.2 %; the indications for LT so far reported include extra-hepatic biliary atresia, Alagille syndrome, acute liver failure, alpha-1 antitrypsin deficiency, primary familial intra-hepatic cholestasis, PSC and Budd-Chiari syndrome [53–58].

Several reports have investigated whether the development of d-AIH is associated with the possession of specific major histocompatibility complex (MHC) antigens either by the recipient or by the donor. In the original report, five of the seven children received livers from donors who were HLA DR3- or DR4-positive [52]. In adults, Heneghan et al. found HLA DR3 or DR4 in either donors or recipients in all cases [59], and Salcedo et al. noted an over-representation of DR3 in recipients [60]. In an attempt to identify possible risk factors leading to d-AIH, a study by Miyagawa-Hayashino et al. showed that in 69 % of patients at least one episode of acute cellular rejection had been identified before the development of d-AIH [57]; however, other series reported that d-AIH was preceded by acute cellular rejection in only 20–50 % of patients [53, 56, 59]. Venick et al. in a matched case–control study found that previous episodes of ACR and steroid dependence constituted risk factors for the development of paediatric d-AIH [58].

Awareness that treatment with prednisolone alone or in combination with azathioprine or MMF is successful in d-AIH has led to excellent graft and patient survival [60]. It is of interest that these patients do not respond satisfactorily to short courses of high-dose steroids for cellular rejection, making it essential to reach an early diagnosis to avoid graft loss. It is therefore important to stress that the protocol for AIH should be applied in patients with d-AIH. Children should be given a starting dose of 1–2 mg/kg predniso(lo)ne, without exceeding a daily dose of 60 mg, in combination with azathioprine (1–2 mg/kg); the steroids should then tapered over 4–8 weeks, to reach a maintenance dose of 5–10 mg/day. In the absence of response, azathioprine should be replaced by MMF [51]. The importance of maintenance therapy with steroids was shown in a study comparing treatment with and without steroids; while all steroid-untreated patients developed cirrhosis and either died or required re-transplantation, none of the steroid-treated patients had progressive disease [60].

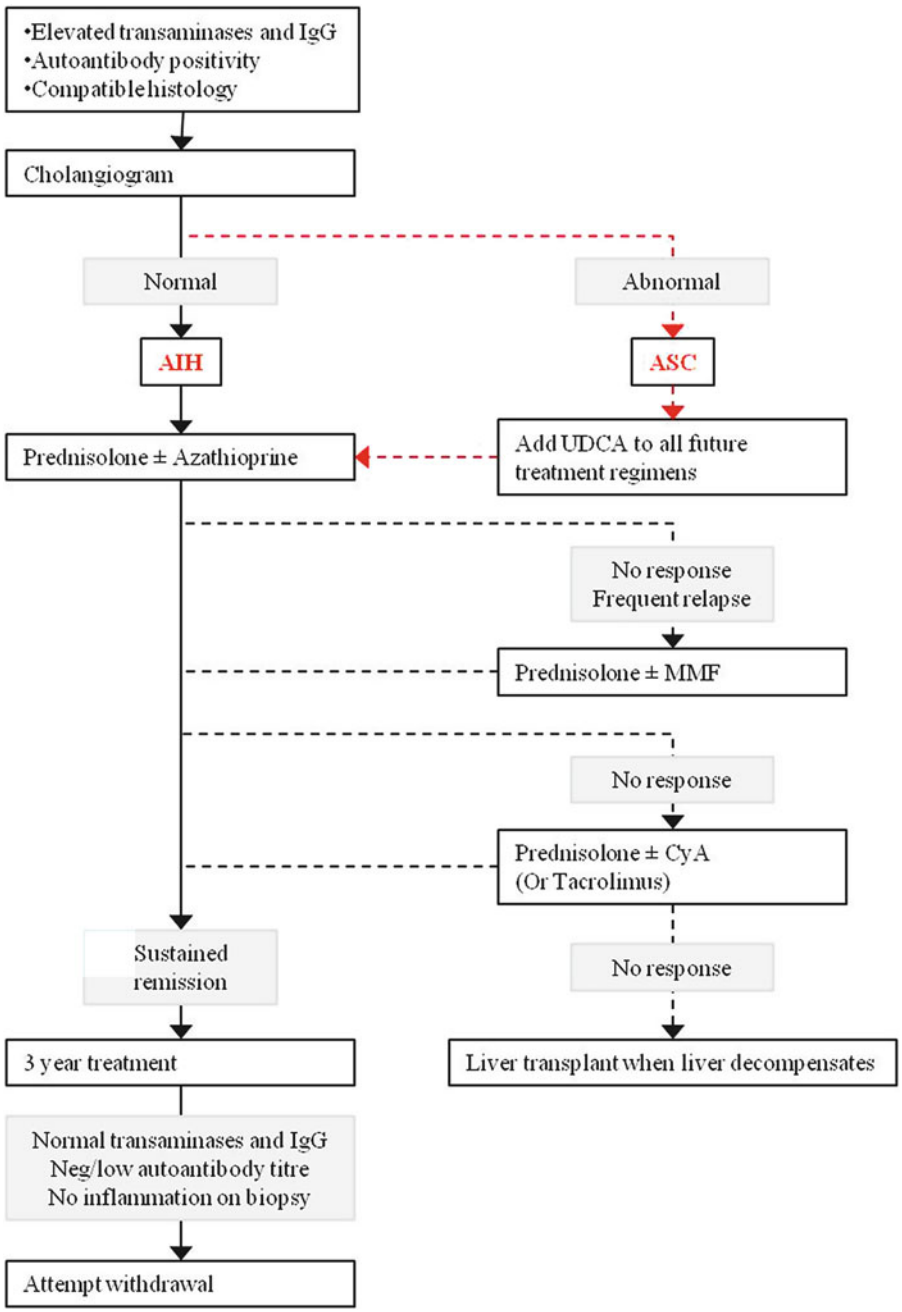

Fig. 24.5 Algorithm for treatment decision in children with autoimmune liver disease. *IgG* immunoglobulin G, *AIH* autoimmune hepatitis, *ASC* autoimmune sclerosing cholangitis, *UDCA* ursodeoxycholic acid, *MMF* mycophenolate mofetil, *CyA* cyclosporine A, *neg* negative. Adapted from Mieli-Vergani G, Vergani D. Best Pract Res Clin Gastroenterol. 2011; 25: 783-795

Concluding Remarks

Over the past few decades, the frequency of children being diagnosed with AILD has increased. Whether this is due to a real increase in incidence or merely to better awareness of the disease remains to be clarified. It is important to consider AILD in the differential diagnosis of any increase in liver enzyme levels. With prompt immunosuppressive treatment, the prognosis for patients with AIH is very good, with symptom-free long-term survival in the majority of cases. The prognosis of ASC patients is worse, with a higher proportion of patients requiring transplantation in the medium term and a greater risk of disease recurrence after transplantation. The immunosuppressive regimens currently available for AILD are non-specific and are marred by unpleasant side effects. A deeper understanding of the pathogenic mechanisms leading to AIH and ASC will hopefully lead to a targeted, more efficient and less toxic therapeutic approach.

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Mechanisms of Acute Liver Failure

25

Christian Trautwein and Alexander Koch

Key Points

- Acute liver failure (ALF) is characterized by the sudden onset of liver failure in a patient without evidence of chronic liver disease.
- Mainly 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 renal failure are important clinical complications limiting the survival of the patients.
- Ammonia levels can be used for risk stratification in patients with ALF and subsequent hepatic encephalopathy.
- Intravenous administration of *N*-acetylcysteine improves transplant-free survival in patients with early stage non-acetaminophen-related ALF.
- Mild hypothermia might improve the outcome of patients with ALF by reduction of intracranial pressure and improvement of disturbed autoregulation in cerebral blood flow.
- Cytokines are involved in the pathogenesis of ALF as well as in controlling the balance between survival and proliferation of hepatocytes.
- The mode of liver cell death which 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.
- Cytokeratin (CK)-18 and the CK-18 modified MELD appear to be novel promising tools for ALF patients to predict the prognosis in the clinical routine.

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Introduction

Acute liver failure (ALF) is characterized by the sudden onset of liver failure in a patient without evidence of chronic liver disease. This definition is important, as it differentiates patients with ALF from patients who suffer from liver failure owing to end-stage chronic liver disease [1].

Clinically, patients present with severe liver failure (icterus and coagulation failure, as reflected by an international normalized ration (INR) $\geq$1.5) and hepatic encephalopathy. The time between the first symptoms and the manifestation of hepatic encephalopathy has been shown to affect prognosis of these patients. Therefore several groups have included in their definition the time frame between the onset of symptoms and start of encephalopathy. The definition of the US ALF study group uses the term ALF as an umbrella and differentiates between three subgroups: hyperacute, acute, and subacute. The time between 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 [2–4] (Fig. 25.1). More generally, ALF can be defined as a severe liver injury, clinically characterized by coagulopathy and hepatic encephalopathy within 26 weeks of symptom onset in previously healthy subjects [5]. Owing to loss of hepatic function, ALF results in hepatic encephalopathy, coagulopathy, and multiorgan failure within a short period of time.

However, the mortality rate is high and ALF accounts for 6–8 % of liver transplantations in the United States and in Europe [4]. Data of the US ALF study group are depicted in Fig. 25.2; spontaneous survival occurs in approximately

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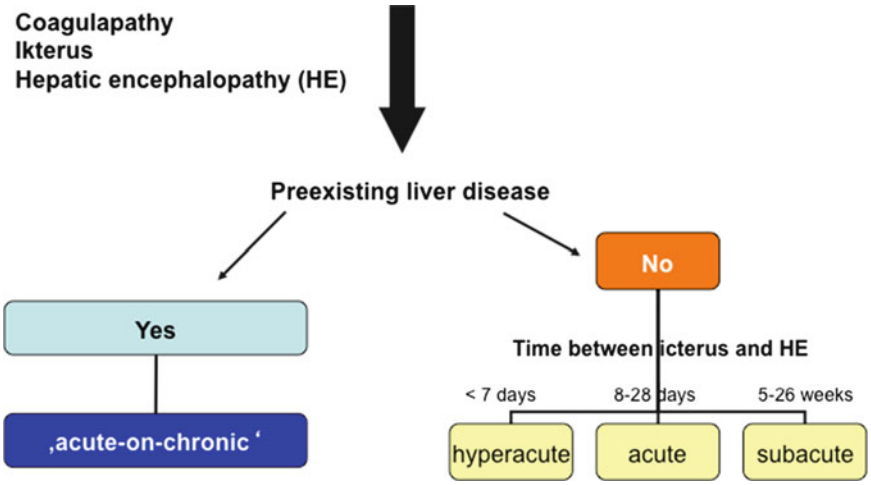

Fig. 25.1 Definition of ALF. ALF is defined as a severe liver injury, clinically characterized by coagulopathy and hepatic encephalopathy within 26 weeks of symptom onset in previously healthy subjects

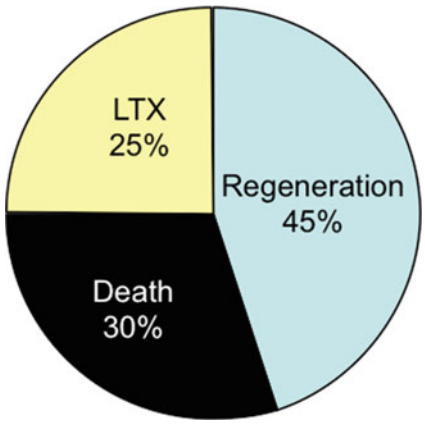

Fig. 25.2 Natural history of ALF. Liver regeneration with spontaneous survival occurs in approximately 45 %, liver transplantation in 25 %, and death without transplantation in 30 % of adults with ALF (Data from the United States); LTX, liver transplantation [4]

45 %, liver transplantation in 25 %, and death without transplantation in 30 % of adults with ALF [4].

Mechanisms of Disease

Different causes may result in ALF. In principal four different classes can be differentiated: (a) infectious (mostly viral), (b) drugs/toxins/chemicals, (c) cardiovascular, and (d) metabolic [6] (Table 25.1).

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 important in determining prognosis: (1) the metabolic consequences resulting from the loss of liver cell mass, (2) the release of mediators and toxic metabolites from liver tissue,

Table 25.1 Causes of acute liver failure

Infectious (viral)
Hepatitis A
Hepatitis B
Hepatitis C
Hepatitis D
Hepatitis E
Hepatitis non-A/non-B
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/toxins/chemicals
Acetaminophen
Amanita phalloides
Halothane
Isoniazid
Sodium valproate
Tetracycline
Nonsteroidal anti-inflammatory drugs (NSAIDs)
Pirprofen
Ketoconazole
Cardiovascular
Budd–Chiari syndrome
Hypotension (circulatory shock)
Heart failure (e.g., right ventricular)
Hyperthermia
Malignant tumors
Veno-occlusive disease
Portal vein thrombosis
Sepsis

(continued)

Table 25.1 (continued)

Metabolic
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 25.2 Specific therapeutic options in ALF

Cause of ALF	Treatment	Dosage
Acetaminophen	*N*-acetylcysteine	600 mg/kg/day total dose
Amanita poisoning	Silibinin	20–50 mg/kg/day
Acute hepatitis B	Lamivudine	100–300 mg/day
	Entecavir	0.5–1 mg/day
	Tenofovir	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	3×10 mg/kg/day

Source: Modified from ref. [10]

and (3) the capacity of the remaining vital hepatocytes to restore liver mass [3, 7].

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 outcome. Etiology of ALF and coma grade on admission are two prominent factors influencing prognosis. ALF due to acetaminophen toxicity, hepatitis A, ischemia, and pregnancy are associated with at least 60 % short-term transplant-free survival, whereas drug-induced liver injury, hepatitis B, autoimmune hepatitis, and indeterminate causes are associated with a spontaneous recovery rate of only 30 % [8]. Patients presenting with early grades of hepatic encephalopathy in ALF (independent of etiology) have usually a more favorable outcome than those with established stupor or coma [9]. Liver transplantation, intensive care medicine, and specific therapeutic options [10] (Table 25.2) can improve prognosis.

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, 6, 7]. In the developing world, infections with hepatitis A, B, and E viruses are accounting for most cases of ALF. For Europe, most recent data from the ELTR database reveal that liver transplantation for ALF due to hepatitis A virus (HAV) and hepatitis B virus (HBV) decreased significantly in the last 5 years (from 1 % to 0.5 % and from 17.9 % to 13.2 %, respectively) [11].

Hepatitis A Virus

Due to effective use of vaccination, infections with the HAV have declined over the last decade and they accounted for 3 % of ALF cases in the United States [12]. The proportion of patients with ALF is higher in older than in younger patients. This is of relevance, as over the last decades in Western countries, HAV infection has occurred more frequently in older patients, and thus the risk of ALF increases in this population [13, 14]. Moreover, patients with underlying chronic liver disease, especially chronic hepatitis C, have an increased risk of ALF [15].

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.

Hepatitis B Virus

The risk of ALF of all patients who are hospitalized because of an acute HBV infection is around 1 % [16]. Fulminant HBV is the most predominant viral cause of ALF in Western countries [8, 17] and accounts for 7–10 % of ALF in Europe and for 7 % in the United States [4, 11]. Due to the implementation of routine vaccination, the incidence of fulminant HBV has been decreased. In fulminant HBV infection, antiviral therapy with lamivudine, entecavir, or tenofovir has been proven efficient and safe, with significant reduction of HBsAg concentrations [18, 19] (Table 25.2). Reactivation of HBV or infection with highly replicative HBV harboring precore and core-promoter gene mutations become a more important cause of ALF [20, 21]. Virus reactivation is associated with a much higher risk of ALF than novel acute HBV infection, and antiviral prophylaxis should be administered to HBsAg-positive patients who are about to receive immunosuppressive therapy [22].

In general, the virus itself is not cytopathic, but the immune response directed against the virus is essential [23]. Frequently at the time of hospitalization, the viral load is already decreasing while transaminases are still rising. This may reflect the possibility that different factors contribute to the elimination of the virus. 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, T cells infiltrate the liver and destroy hepatocytes [24]. Therefore activation of HBV-specific T cells is essential to determine the degree of hepatic injury during ALF.

In the case of HBV/hepatitis D virus (HDV) coinfection, the risk of ALF is increased [25]. 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 [3]. In Japan in particular cases of HCV-related ALF have been documented [26]. As there are only a few reports in the literature, the pathogenesis of HCV-related ALF is incompletely understood. However, there is evidence that elimination of HCV-specific T cells is associated with chronic HCV infection [27]. This indicates the HCV-specific immune response is involved during acute infection and thus is most likely also the determining factor during ALF.

Hepatitis E Virus

ALF owing to hepatitis E virus (HEV) infection is seldom seen in Western countries. However, hepatitis E has a predilection for older men in whom it causes substantial morbidity and mortality. Based on a poor prognosis in combination with preexisting liver disease, patients with unexplained hepatitis should be tested for HEV [1]. Epidemic outbreaks are known in developing countries including patients with ALF. In India and Pakistan, China, and southeast Asia, HEV infection is the most predominant cause of ALF [1]. Pregnant women, especially in the third trimester, have been regarded to be at high risk for ALF (up to 20 %) [28].

However, recent data indicate that pregnancy does not affect outcome of ALF resulting from HBE infection [29]. 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 (Table 25.2), human herpes virus type 6, cytomegalovirus, varicella virus, Epstein–Barr virus, and parvovirus B19. A few cases of ALF related to an infection with the toga-, paramyxo-, 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 lead to ALF (Table 25.1), 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 of the cases, acetaminophen was taken in a suicide attempt. Patients who consume alcohol chronically may be more susceptible for acetaminophen toxicity as cytochrome P450 has been induced in their liver [30]. Measurement of serum acetaminophen-protein adducts can reliably identify acetaminophen toxicity in cases of ALF in which no clinical or historic data are given that would reveal the cause [31]. At present, these analyses are only available in specialized laboratories.

The pathogenesis of acetaminophen injury is related to the formation of toxic metabolites through the cytochrome P450 enzymes, especially cytochrome P450 2E1 [32, 33]. These toxic metabolites are normally conjugated and inactivated through glutathione. However, when glutathione stores are depleted, these toxic metabolites accumulate and result in hepatocyte injury. Necrosis has been shown to be the more prominent form of cell death in acetaminophen toxicity [34]; however, in vitro data and animal data suggest that also apoptosis contributes to acetaminophen-induced ALF [35–37].

N-acetylcysteine (NAC), the standard antidote for acetaminophen overdose, exerts its therapeutic effects by restoration of depleted hepatic glutathione stores [38] (Table 25.2). 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 [39].

Mushroom (*Amanita*) Poisoning

Mushroom poisoning, mainly through the species *Amanita phalloides*, frequently leads to ALF, especially in the fall. The clinical spectrum of *Amanita* poisoning varies from acute gastroenteritis to development of ALF. Amanatoxin and phalloidin are the two distinct toxins produced by mushrooms. Whereas phalloidin is not absorbed in the gastrointestinal tract, the dose-dependent systemic and hepatotoxic effect of amanatoxin is mediated through inhibition of mRNA synthesis [6, 40–42]. Although there a no controlled trials proving its efficiency, silibinin is used in Europe because of cytoprotective effects against amanatoxin and has been reported to be more effective than penicillin G in *Amanita* poisoning (silibinin is not available as a licensed drug in the United States) [43, 44] (Table 25.2). Despite advances in intensive care

therapy, the morality rate in patients who develop ALF after *Amanita* ingestion is high [43].

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 development of sensitization to both autoantigens (including CYP2D6) and halothane-altered liver cell determinants [45]. 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 for ischemic events are severe hypotension or heart failure. 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 found in females [46]. Depending on the progression of the disease, Budd–Chiari syndrome may result in ALF when sudden closening of at least two main liver veins occurs. Typically, acute Budd–Chiari syndrome presents with ascites, abdominal pain, jaundice, and hepatomegaly [47]. Budd–Chiari syndrome is frequently associated with primary myeloproliferative disorders, a factor V Leiden mutation, anticardiolipin antibodies, and protein C and S deficiencies that increase the risk of thrombotic complications [48]. In general, the course of disease in Budd–Chiari syndrome leads to liver transplantation. Transjugular portosystemic stent shunt (TIPSS) or percutaneous transjugular direct porto-caval shunt, in patients with inaccessible hepatic veins, seems to be a therapeutic option to decrease the portal pressure gradient, improve synthetic functions, reduce transaminase levels, and control ascites [49, 50] (Table 25.2).

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 the HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome may develop. Patients with HELLP syndrome typically presents with LDH, ALT, and bilirubin elevation. Immediate termination of pregnancy and delivery usually reverses hepatopathy, but patients are at increased risk for complications in future pregnancies [51] (Table 25.2).

Wilson's Disease

Wilson's disease is an autosomal recessive genetic disorder of copper metabolism and a rare cause of ALF. The Wilson gene is a copper-transporting P-type ATPase involved in copper transport across cell membranes, with over 200 known mutations, although its precise location and function is not known [52, 53]. In general, patients with ALF owing to Wilson's disease present with only moderately elevated aminotransferases, low alkaline, but very high bilirubin. Hemolytic anemia induced by copper ions leaking from necrotic hepatocytes into the circulation and causing lysis of erythrocytes is another typical clinical feature of Wilson's disease [54]. The patients frequently already have liver cirrhosis and are therefore not in accordance with the "real" definition of ALF. However, many of the patients were healthy before onset of the disease and therefore are treated like patients with ALF [55].

There is evidence that elevated copper levels are directly toxic for the cell and involve CD95-mediated apoptosis [56]. The current hypothesis postulates that excess copper generates free radicals that deplete cellular stores of glutathione and oxidize lipids, enzymes, and cytoskeletal proteins.

Mechanisms of Organ Failure

As a consequence of ALF, multiorgan failure (MOV) develops rapidly (Fig. 25.3). Different factors contribute to MOV. 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 cardio-vascular and kidney failure; pulmonary and metabolic complications also develop.

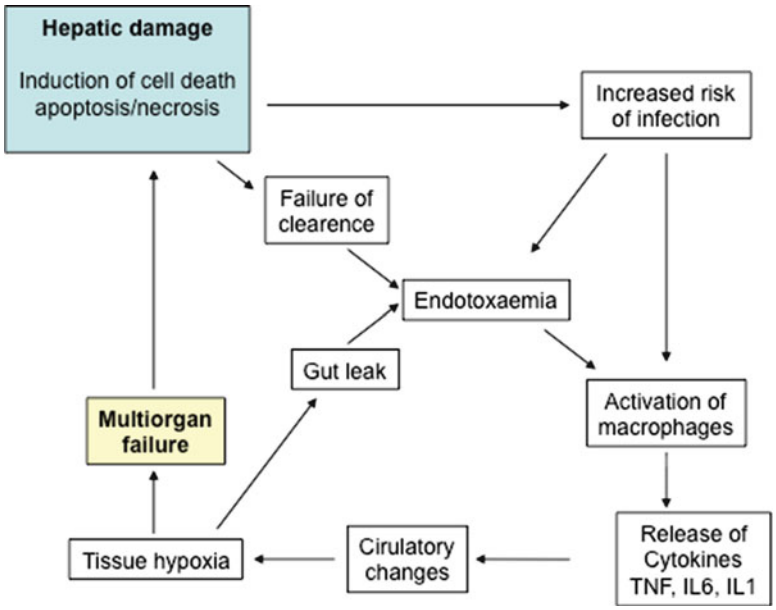

Fig. 25.3 Mechanisms that contribute to multiorgan failure during acute liver failure

Table 25.3 Stages of acute hepatic encephalopathy

Stage	Mental state
I, Prodrome	Mild confusion, slurred speech, slowness of mentation, disordered sleep rhythm, euphoria/depression
II, Impending coma	Accentuation of stage I, drowsy but speaking, inappropriate, behavior, incontinence
III, Stupor	Sleeps most of the time but rousable, incoherent or no speech, marked confusion
IV, Coma	Patient may (stage IVA) or may not (stage IVB) respond to painful stimuli

Source: Modified from ref. [74]

Encephalopathy and Cerebral Edema

Hepatic encephalopathy is essential for the diagnosis of ALF and is subdivided into four different grades, I–IV (Table 25.3). In 75–80 % of the patients in stage IV, cerebral edema develops independent of the cause of ALF.

The precise pathophysiological mechanisms that lead to hepatic encephalopathy are incompletely understood. However, laboratory studies indicate that the cause is an ammonia-induced deficit in neurotransmitter synthesis rather than a primary deficit in cerebral energy metabolism [57]. Most likely the astrocytes and the pre- and postsynaptic neurons contribute to the clinical picture of hepatic encephalopathy (Fig. 25.4). Astrocytic swelling during ALF determines the degree of cerebral edema and thus the degree of cerebral dysfunction [58].

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 rise 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 showed significantly higher serum ammonia levels than patients 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 [59].

Ammonia has direct effects on cerebral function by direct and indirect mechanisms (Table 25.4). There is clear evidence that arterial ammonia concentrations directly correlate with cerebral edema and thus herniation [60]. 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 [61, 62].

Higher ammonia concentrations have a direct effect on the glutamate neurotransmitter system. Glutamate is the major excitatory neurotransmitter in the mammalian brain (Fig. 25.4). After 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 uptake of glutamate in astrocytes via GLT-1, it is transformed into glutamine. Ammonia downregulates GLT-1

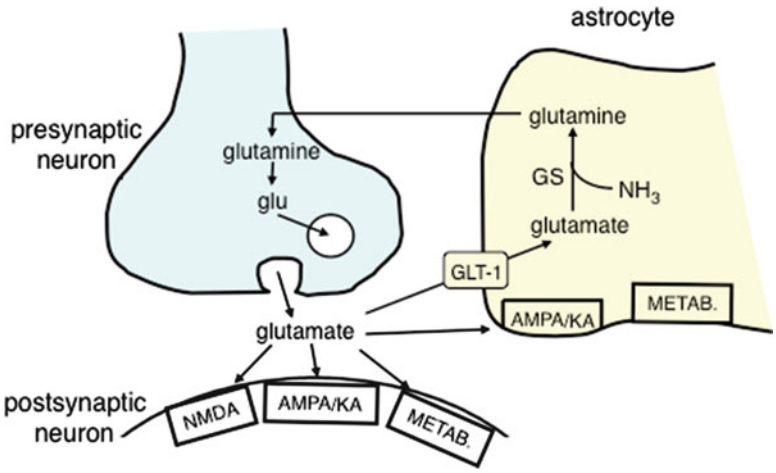

Fig. 25.4 The role of glutamate/glutamine in the brain. Shown are the localizations of the glutamate transporter (GLT-1) and glutamate receptor subtypes (NMDA, AMPA/KA, METAB) on astrocytes and neurons involved in glutamatergic neurotransmission are shown. *Glu* glutamate (Modified from ref. [62])

Table 25.4 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 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

Source: Data from ref. [62]

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 for hepatic encephalopathy during ALF [61, 62].

Other neurotransmitters that participate in hepatic encephalopathy are GABA, serotonin, and the opioid system. Systemic circulation of proinflammatory 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 [63].

A few uncontrolled studies [64–66] 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, 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 [67]; in contrast, hypothermia might also lead to impaired liver regeneration. Further research and controlled clinical studies are required to clarify the significance of hypothermia in ALF.

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).

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 is a major problem 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 [68]. Studies from the King's Collage Hospital group clearly indicated that monitoring by daily cultures (sputum, urine, blood) identifies bacteria in up to 90 % and fungal infections in around 30 % of the patients [69, 70]. Frequently the classical signs (fever, leukocytosis, biochemical

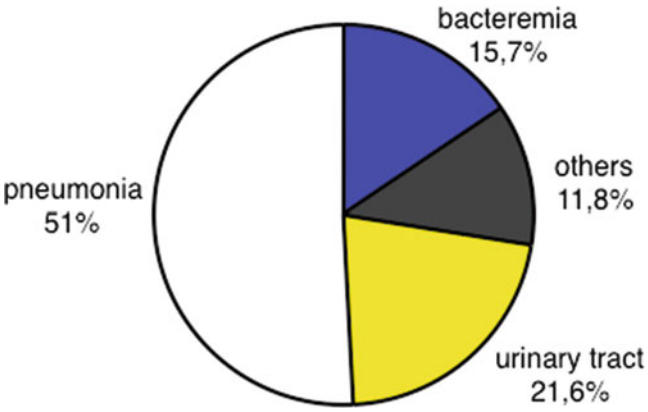

Fig. 25.5 Sites of infections during acute liver failure (From ref. [69])

parameters like 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. 25.5). If antibiotic or antifungal treatment is necessary in these patients, the potential of further liver injury caused by antibiotic drugs should be considered.

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, 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, activity of polymorphonuclear leukocytes and complement-mediated opsonization is reduced. Therefore phagocytosis and killing of polymorphonuclear cells is inhibited in patients with ALF. Through the portal circulation, bacterial toxins are regularly brought to the liver tissue that is 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 the 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 [6, 69].

Pulmonary Complications

Pulmonary complications are frequent [71]. Different mechanisms contribute to this observation. Up to 50 % of the patients have infections, especially after intubation and subsequent mechanical ventilation (Fig. 25.3) [72]. The possible consequent capillary leakage can result in an ARDS-like syndrome that is further augmented by the often required infusion of albumin, fresh frozen plasma (FFP), and coagulation factors.

Besides these local mechanisms, systemic causes, as a result of liver failure, also lead to intrapulmonary vasodilatation and pulmonary, which further increase the risk of hypoxic complications [73].

Renal Failure

Renal failure with oliguria and anuria is found in 40–50 % of patients with ALF [44, 45]. In acetaminophen and *Amanita* poisoning, a direct toxic effect additionally contributes to kidney failure. Therefore, in these patients the rate of kidney failure is increased up to 70 %.

The association of liver failure and kidney failure is functional and known as hepatorenal syndrome. The syndrome is characterized by a contraction of the vessels with a distinctively reduced renal perfusion. At this stage the kidney impairment is completely reversible. In the further course of the disease, at a more advanced stage, hepatorenal syndrome may progress to tubulus necrosis, which is not reversible [74].

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 [75].

As SIRS has been recently 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 [76].

Metabolic Complications

The liver is essential for several metabolic functions. Two particular problems are frequent 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 consequently reduced disintegration of insulin 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 between hypoglycemia and hepatic encephalopathy as possible causes for disturbed mental status at certain stages.

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 is found in up to 30 % of patients with acetaminophen-dependent ALF. In patients with a different etiology, acidosis is evident in only 5 %, in which lactate acidosis is present because of tissue hypoxia owing to a disturbed microcirculation and the inability of the injured liver tissue to metabolize lactate.

Coagulation Disorders

Because of the central role of the liver in coagulation and thrombolysis, severe coagulation disorders are a major problem in ALF. As a result of reduced coagulation factors and a deficit of inhibitors of fibrinolysis, the hemostasis situation in ALF is complex [77, 78].

Factors I, II, V, VII, IX, and X are synthesized in the liver. Therefore prothrombin time is a useful parameter—besides the measurement of single factors—to assess the lack of production of coagulation factors. An additional factor that may contribute to the decrease in blood coagulation factors is disseminated intravasal coagulation (DIC), which may be associated with sepsis during ALF.

Antithrombin-III (AT-III) is also synthesized in the liver and is thus reduced. The decrease in AT-III concentration further contributes to coagulation problems.

The number of blood platelets is frequently decreased, and additionally the function and morphology of blood platelets are impaired. Together, these changes result in adhesion abnormalities, leading to decreased aggregation and increased adhesion. Without clinical signs of bleeding, the application of FFP, single coagulation factors, or platelets is not indicated.

Pathophysiological Aspects of Acute Liver Failure

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: (a) direct cellular damage and activation of cell signalling cascade pathways, resulting in disturbance of intracellular homeostasis, and (b) innate and adaptive immune responses leading to immune-mediated liver injury.

Similar to sepsis, patients with ALF commonly display immune paralysis with characteristic features of systemic inflammation and cellular immune depression contributing to severe extrahepatic complications, such as multiple organ failure [68, 79]. In this context cytokines exert crucial pathophysiological functions in ALF, comprising hepatocellular death, extrahepatic complications, and hepatocellular regeneration.

Dysregulation of the Cytokine Network in Acute Liver Failure

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 molecules 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. We focus here 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, which results in the activation of Janus kinases and in turn in 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 (Fig. 25.4) [80]. 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 [81, 82].

One of the simplest models to study the loss of liver tissue is 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, 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 [83], as it 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 [84, 85]. The model of how IL-6 and TNF may work in concert during liver regeneration after partial hepatectomy is shown in Fig. 25.5.

In humans suffering from ALF, IL-6 serum levels are highly elevated, and in the liver infiltrating cells express tremendous (tenfold higher compared with controls) amounts

of IL-6 [81, 82, 86]. In animal models of ALF, IL-6 serum levels are also greatly increased [87], and treatment with a hyper-IL-6 designer molecule reduces liver cell damage in several animal models [88, 89]. Therefore, not only during liver regeneration after partial hepatectomy, but also during ALF it is obvious that IL-6 plays a protective role for hepatocytes; cDNA arrays further demonstrate that IL-6 activates antiapoptotic pathways, e.g., Bcl-xl in hepatocytes [90, 91]. Our group generated a hepatocyte-specific knockout mouse for gp130.

Most IL-6 data in animal models show that gp130-dependent pathways in hepatocytes activate protective mechanisms [81, 82], 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 for 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 (Fig. 25.6). In case of TNF-R1, first the molecule TNF-R-associated death domain (TRADD) and then additional molecules bind that activate the caspase cascade either via Fas-associated death domain (FADD) or via TNF-associated factor-/receptor-interacting protein (TRAF/RIP) jun kinase (JNK) and nuclear factor-kB (NF-kB) [92].

Recently, it has become evident that TNF—besides inducing apoptosis—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 prognosis in ALF patients [86]. 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 proinflammatory genes, e.g., chemokines, nitric oxide, and adhesion molecules like intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin [93–95]. These changes in the liver are essential to trigger the extravasation of neutrophils into the liver parenchyma, which results in cytotoxic liver cell damage. During this scenario a stepwise cascade has been described consisting of three events: (1) sequestration of neutrophils in the liver vasculature, (2) transendothelial migration, and (3) adherence-dependent cytotoxicity against hepatocytes [96].

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

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 [97]. GaIN will directly inhibit 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 pathway does not seem to be involved in the pathogenesis of liver damage, and also non-parenchymal cells play no role. In this model, simple administration of an adenoviral construct expressing a dominant molecule blocking the FADD pathway is protective [86]. 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 [98]. 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 to trigger the early phase of ConA-induced liver injury [99, 100]. Consecutively, CD4-positive and polymorphonuclear cells are attracted to the hepatic sinus and trigger an increase of cytokines like 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, as anti-TNF-α and anti-IFN-γ antibodies protect from ConA-induced liver injury [101, 102] 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 like 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 [103].

Recently, it has been shown that hepatocyte-specific caspase-8 knockout mice are more susceptible to ConA-induced liver injury [104]. 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 Acute Liver Failure

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 initiation, execution, and regulation of apoptotic pathways. Effector caspases (e.g., caspase-2, caspase-6, caspase-7) cleave various cellular proteins (e.g., cytokeratin-18) [105] and initiator caspases (e.g., caspase-8, caspase-9, caspase-10) exhibit regulatory functions by activation of downstream effector caspases [106]. The major signalling routes for caspase activation are the extrinsic death receptor and the intrinsic mitochondrial pathway [107] (Fig. 25.6).

Death receptors are transmembrane proteins that consist of the following domains: (a) extracellular ligand-interacting domain, (b) transmembrane domain, and (c) intracellular death domain. Typically involved in ALF are death receptors CD95 (Fas), tumor necrosis factor-receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand receptors 1 and 2 (TRAIL-R), and death receptors 3 and 6. Binding of death ligands such as TRAIL, CD95L, or TNF to their specific receptors leads to the recruitment of the adapter protein FADD and caspase-8 into death-inducing signalling complex (DISC), wherein caspase-8 is activated [108]. 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 proapoptotic Bcl-2 family protein). Subsequently, together with the Bcl-2 family members Bak and Bax, the release of proapoptotic mediators from the mitochondria is initiated [109].

ALF, induced by agonistic CD95 antibody, could be abolished by silencing of CD95 or caspase-8 protected mice [110, 111]. On the other side, CD95 and caspase-8 are involved in liver regeneration by inducing differentiation of stellate cells and other non-parenchymal liver cells [112, 113]. TNF-α plays a key role in liver regeneration by activation of NF-kB, which exerts antiapoptotic functions in the liver [114].

Necrosis is mediated by opening of the mitochondrial membrane permeability transition (MPT) pore, leading to disruption of ATP formation and finally resulting in mitochondrial swelling and rupture of the outer mitochondrial membrane. Interestingly, recently it has been shown that TNF

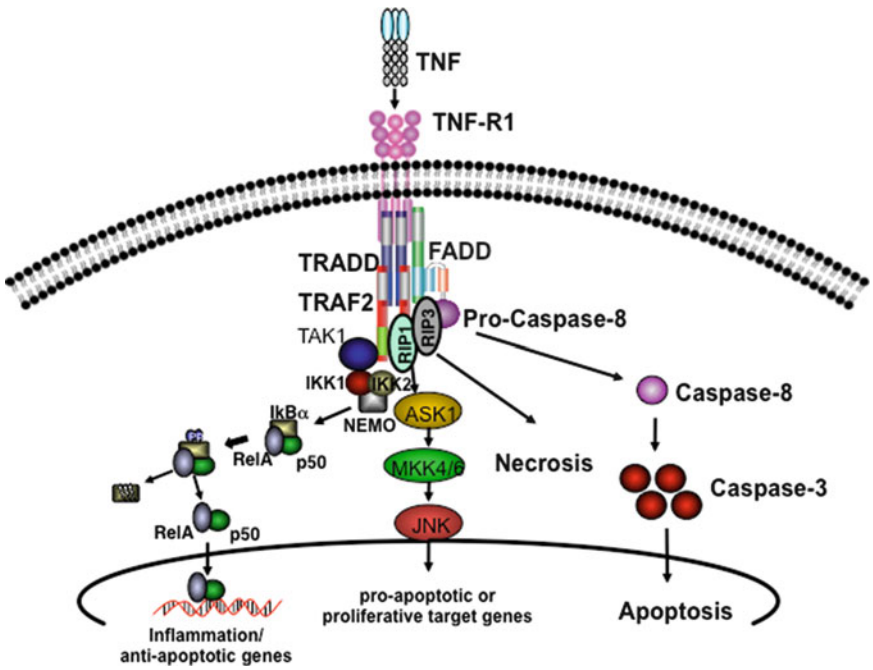

Fig. 25.6 TNF-dependent signalling pathways. The molecules and pathways that are involved in TNF/TNF-R1-dependent signalling are depicted. After TNF/TNF-R1 interaction different adaptor proteins bind to the intracellular part of TNF-R1. As a consequence at least four pathways (NF-kB, jun kinase (JNK), 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

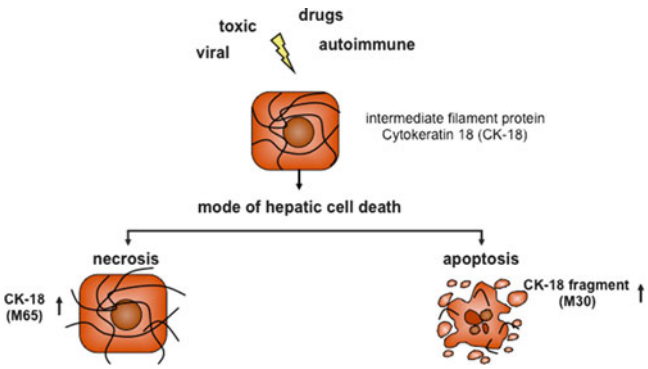

Fig. 25.7 Cytokeratin-18 is associated with the mode of hepatic cell death. In apoptotic cell death (induced by toxin, drugs, viruses, or autoimmune etiology), cytokeratin (CK)-18 is cleaved by caspases into specific fragments, which can be measured in serum by the M30 ELISA. Whereas cleaved CK-18 levels represent apoptosis, uncleaved CK-18 (M65 ELISA) reflects necrosis

can also induce controlled necrosis. Therefore now necrosis is 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 [115, 116]. Necrosis is associated with inflammation, as rupture of necrotic cells 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 release of intracellular contents.

Cytokeratin-18 as a Novel Prognostic Biomarker in ALF

CK-18 is a filament protein, which is cleaved by caspases into specific fragments, which can be measured in serum by the M30 ELISA (Fig. 25.7). CK-18 levels at the time of admission have been demonstrated to be a predictor of mortality in patients with ALF with a prognostic impact that is comparable to the model for end-stage liver disease (MELD). Additionally, a modified MELD score where uncleaved necrotic CK-18 (M65 ELISA) substituted bilirubin predicted significantly better the prognosis of ALF patients compared with the current MELD score [117].

The observation that ALF patients who died or required transplantation displayed increased serum levels of total CK-18, but reduced levels of caspase-cleaved fragments indicate that necrosis and not apoptosis is the more prominent cell death mode in these most critically ill ALF patients [118]. In line with this, patients with acetaminophen-induced liver injury, where necrosis is the predominant cell death mode, showed higher levels of total CK-18 than caspase-cleaved CK-18.

Translation of Experimental Data into Therapeutic Approaches in Humans

The current data in animal models and humans indicate that TNF plays an essential role in the pathogenesis of ALF. However, as demonstrated for the three animal models discussed—depending on the pathogenesis—the intracellular pathways that are 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 is potentially depending 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. On the other hand, 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.

Potentially, differentiation of necrosis and apoptosis might be novel tools to early identify 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 needed to better understand the molecular mechanisms determining the mode of cell death during ALF.

In mouse models the administration of cyclooxygenase (COX) inhibitors resulted in decreased oxidative stress and a reduction of hepatic necrosis [119]. Therefore, COX inhibitors could be further investigated as potential agents in the prevention of ALF.

Another promising novel target in acetaminophen-induced ALF is cyclophilin A. Cyclophilin A is an intracellular protein that is proinflammatory when released by cells. In an animal model of acetaminophen-induced liver injury, it has been demonstrated that cyclophilin A acts as a damage-associated molecular pattern (DAMP) to mediate acetaminophen toxicity and that experimental inhibition of cyclophilin A ameliorates acetaminophen-induced liver injury [120].

Concluding Remarks and Open Questions

ALF is characterized by sudden onset in patients without evidence of chronic liver disease, by which ALF is differentiated from end-stage chronic liver disease. According to the time between 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 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 United States. Other important mechanisms are viral hepatitis, 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 to assess the extent of remaining liver function.

Intensive care therapy is crucial for patients with ALF to manage multiorgan failure, and mild hypothermia to reduce cerebral edema should be considered. Further research and controlled clinical studies are needed 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 novel prognostic tool for instant identification of 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.

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Nonimmune-Mediated Drug-Induced Hepatotoxicity

26

Christian P. Strassburg

Scope of Hepatic Drug Toxicity

Although drug-mediated hepatotoxicity is of considerable importance and an important factor in the differential diagnosis of biochemical and structural liver disease, not much of its epidemiology in clinical practice is backed by high-quality data. It is assumed that the incidence of drug reactions leading to hepatic toxicity ranges from 1 in 10,000 to 1 in 100,000 of drug-exposed individuals or patients [1, 2].

During the diagnostic approach to liver abnormalities or diseases, drug-induced etiologies are far less reported or documented. It can be speculated whether this reflects a process in which the establishment of an accepted classical liver disease and the documentation of defined diagnostic serological, virological, genetic, histological, or biochemical indicators decrease vigilance to consider drug-mediated effects or co-occurrence with liver diseases. In addition, there is no accepted or practical gold standard for the diagnosis of drug-mediated liver disease that is as easily applicable in routine clinical practice in comparison to common hepatic diseases and hepatic injury [3]. This is particularly confounded by the fact that toxic drug reactions do not share a common phenotype and can mimic all known entities of hepatic injury found with other etiologies of liver disease (Table 26.1). This leaves the clinician with the difficult process of a diagnosis by exclusion [4].

One prospective population-based study from France reported a crude annual incidence of hepatic drug reactions to amount to 14 cases in 100,000 [5]. This number significantly exceeded that estimated by drug regulatory agencies and indicates that drug-mediated toxicity is likely to be substantially underestimated. Estimates suggest that up to 10 % of all cases of jaundice in general hospital settings may be related to toxicity reactions and around 1 % of all inpatients are believed to suffer from (idiosyncratic) drug reactions. Among the most implicated drug groups are antibiotics, non-steroidal anti-inflammatory drugs (NSAID), and anticonvulsants [1, 2, 5] (Table 26.2). In the Western Hemisphere the combination of amoxicillin with clavulanic acid is the single most frequent inducer of severe hepatotoxicity.

In addition to prescribed drugs, herbal preparations most likely represent an important yet difficult to quantify factor in hepatic drug toxicity. This can proceed as a toxic reaction of herbal compounds by themselves or as a result of interactions resulting from induction or inhibition of biotransformation and affecting coadministered drugs specifically those with a narrow therapeutic/toxic corridor [6–8]. Toxicity associated with herbal preparations appears to be rising and to account for about 10 % of toxic drug reactions. In Asian countries, where herbal remedies are much more common, the incidence of hepatotoxicity related to their use is much more prevalent.

Fulminant hepatotoxic drug reactions are also the most frequent cause of acute liver failure in Europe and the USA accounting for around 50 % of all fulminant hepatic failures [9, 10].

Who Is at Risk for Drug Toxicity?

There is a large body of literature analyzing potential risk factors for hepatic drug toxicity. These include the usually implicated factors such as age, gender, nicotine consumption, alcohol use, preexisting liver disease, genetic factors, and coadministered drugs in complex drug treatment regimens [1, 2, 11]. However, there is no common denominator indicating a predisposition to drug toxicity. Drug toxicity occurs in a situation defined by three principal factors: (a) the drug itself, its class, drug–drug interactions, dose, and duration of treatment; (b) environmental effectors including use of alcohol, nicotine and coffee, chemical exposure, and dietary effects; and (c) host factors including individual

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Table 26.1 Examples of the variable presentation of drug-associated hepatotoxicity

Presentation	Characteristics	Drugs
Acute hepatic necrosis	Necrosis of liver tissue	Isoniazid and many others
Acute liver failure	Extensive necrosis, encephalopathy, elevations of INR	Isoniazid, acetaminophen, antibiotics, and many others
Autoimmune hepatitis-like	Autoantibodies, plasmacellular infiltrates, interface hepatitis, necrosis	Minocycline, α-methyldopa, nitrofurantoin
Cholestasis	Pruritus, hyperbilirubinemia	Estrogens, anabolic steroids
Cholestatic hepatitis	Elevated AP, bilirubin	Amoxicillin + clavulanic acid
Acute fatty liver	Microvesicular steatosis (mitochondrial injury and dysfunction)	Valproic acid and others
Cirrhosis	Collagen deposits	Methotrexate
Vanishing bile duct syndrome	Reduced number of bile ducts, cholestasis	β-Lactam antibiotics
Isolated hyperbilirubinemia	No histological alterations, unconjugated hyperbilirubinemia, Gilbert syndrome present	Atazanavir, indinavir

Table 26.2 Hepatotoxicity in Europe (Spain) and the USA and the drug groups implicated

Origin	Spain	USA
Year of study	1994–2008	2004–2007
Type of study	45 sites	Prospective, 5 sites
Numbers	603	300
Mean age/range (years)	54 (13–88)	48 ± 18
Men (%)	51	40
In hospital (%)	54	54
Type of injury		
Hepatocellular (%)	55	56
Mixed (%)	21	20
Cholestatic (%)	25	24
Deaths or transplants (%)	5.4	10.1
Chronic injury (%)	16.9	13.6
Most common agents (%)	Antibiotics (39)	Antimicrobials (45.5)
	CNS agents (15)	CNS agents (15)
	Painkillers (11)	Herbs (9)
		Immunomod. (5.5)
		Painkillers (5)

CNS central nervous system, *Immunomod*. immunomodulators

Table 26.3 Factors influencing the disposition and severity of drug-associated hepatotoxicity

Drug compound	Drug recipient	Environmental factors
Chemical class	Age	Diet
Dose	Gender	Alcohol consumption
Duration	Obesity	Coffee consumption
Pharmacokinetics	Antioxidant defense	Smoking
Coadministered drugs	Genetic variability (drug-metabolizing enzymes, transporters, immune system, mitochondrial genes, etc.)	Use of herbal preparations
Herbal preparations	Viral hepatitis	Microbiome
Metabolism of drug	Genetic liver diseases	Pollution
	Infections	Circadian rhythms
	Nutritional status (GSH depletion)	
	Kidney function	

genetic drug disposition, individual metabolism and biotransformation, coexisting diseases, age, gender, and obesity [12] (Table 26.3).

These factors influence each other and can lead to differential susceptibility characteristics. In the case of isoniazid-related hepatotoxicity, advanced age is a significant risk factor [13]. In contrast, aspirin-induced Reye's syndrome and valproic acid-induced hepatotoxicity are more prevalent in children [14]. Differences in race suggest that genetic effectors exist regarding a higher probability of anticonvulsant hypersensitivity in African black individuals and a higher risk of flucloxacillin hepatotoxicity in Caucasian white individuals. An increased risk of hepatotoxicity in females is controversial and not backed by more extensive studies. It can be speculated whether this effect is partly explained by the use of a broader spectrum of drugs in females; however, this has not been analyzed in prospective population studies.

Individual examples have been provided for genetically defined risk factors. The *HLAB5701* allele, which is present in about 4 % of Caucasian Whites in Europe, has been linked to flucloxacillin-associated hepatotoxicity [15]. *HLAB5701* is well known because of its association with susceptibility reactions to abacavir. Valproic acid toxicity has been associated with variants in the mitochondrial DNA polymerase gamma (*POLG1*) gene. The *POLG1* Q1236H amino acid substitution was more often found in patients experiencing valproic acid-induced liver injury mediated by mitochondrial injury [16]. Cholestatic injury mediated by estrogen exposure has been associated with variants of the *ABCB11* gene (BSEP, bile salt export pump) a biliary ABC transport pump. Polymorphisms in the mitochondrial manganese superoxide dismutase 2 (*SOD2*) gene [17] encoding

a protein responsible for mitochondrial superoxide scavenging have been linked to hepatotoxicity of antituberculosis drugs in Asia. Variants leading to a higher activity of the SOD2 protein most likely lead to increased hydrogen peroxide generation as a breakdown product of superoxide [18, 19]. Glutathione peroxidase I (*GPX1*) gene variants have also been linked to drug-induced liver injury in Spanish patients. These genetic data show that mechanisms altering oxidative stress and radical oxygen species (ROS) formation are linked to the susceptibility of drug-induced hepatotoxicity. A null variant of glutathione transferase present in the cytosol (*GST* MI T1) has been linked to liver toxicity with NSAID und antibacterial drugs [20]. GST is a major enzyme for the prevention of excessive oxidative stress and cell injury.

Genetic predisposition can also affect the major players of biotransformation, namely, the cytochrome P450 (CYP) enzymes and the conjugating enzymes (e.g., UDP-glucuronosyltransferase (UGT)). *CYP2E1* genetic variants have been linked to antituberculosis drug reactions. CYP2E1 has been related to the production of the hepatotoxic acetaminophen metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). Deletion of *CYP2E1* and *CYP1A2* in mice has been shown to prevent acetaminophen toxicity reactions indicating that genetic alterations of these *CYP* are likely to affect the disposition of patients to toxicity reactions [21]. Other CYP involved in the metabolism of most commonly used drugs are CYP3A4, CYP3A5, CYP2C8/C9, and CYP2D6, which have also been associated with drug-induced hepatotoxicity although this appears to be a rare occurrence. NSAID hepatotoxicity (diclofenac) has also been associated with the *UGT2B7*2* variant affecting the activity of a conjugating enzyme [22]. Commonly, UGT lead to less toxic metabolites, which are easily eliminated via bile and urine [23, 24]. UGT are characterized by over 200 genetic variants [23]. However, instable 1-*O*-acyl glucuronides can react with biological nucleophiles, but a role of reactive acyl glucuronides and hepatotoxicity is not yet well established.

An important consideration in the risk for hepatotoxicity is the demographic development of obesity in the Western Hemisphere. This not only makes the diagnosis more difficult because a considerable proportion of the population will exhibit elevated liver enzymes because of obesity-related metabolic abnormalities, but it also impacts the potential of drugs leading to reactive intermediates to initiate toxic reactions. Steatosis is associated with a higher probability and rate of lipid peroxidation leading to hepatic injury, inflammation, and fibrogenesis. In Germany, 67 % of men and 53 % of women are overweight (BMI >25), and 23 % of men and 24 % of women are obese (BMI >30). In the USA 72 % of men and 64 % of women are overweight (BMI >25), and about one-third of the population is obese (BMI >30). Apart from the chemical class effect, genetic disposition, and co-medication, the presence of a metabolic syndrome with insulin resistance, hepatic steatosis, and the downstream mechanisms of lipid-induced injury is likely to play a very prominent role for the risk of hepatotoxicity. An analysis of the prospective US cohort of the DILIN network identified diabetes as a risk factor for serious disease progression. In addition to lifestyle-dependent risks, the presence of other forms of acute or chronic liver disease is likely to influence the cause and severity of hepatotoxicity and requires careful exclusion and diagnostic identification (Table 26.4).

Table 26.4 Baseline clinical workup in suspected hepatotoxicity for ruling out prevalent hepatic disease

BMI, careful history, lipids, HbA1c	Metabolic syndrome, hepatitis
Viral serology	Viral hepatitis
Anti-HAV IgM	
Anti-HBc IgM	
Anti-HCV, HCV-RNA	
IgM anti-HEV, HEV-RNA	
CMV IgM	
EBV IgM	
Autoantibodies (ANA, ANCA, AMA, SMA, SLA, anti-LKM-1), IgG, histology	Autoimmune hepatitis Primary biliary cirrhosis
Ceruloplasmin, urinary copper (patients <40 years)	Wilson's disease
Alfa-1 antitrypsin (PIZZ)	Alfa-1 antitrypsin deficiency
Transferrin saturation	Hemochromatosis
UGT1A1*28 variant	Gilbert syndrome
Hypotension, shock, heart failure, vascular disease, older patients	Ischemic hepatitis
Imaging techniques: radiographic/endoscopic (abdominal US, CAT, cholangioresonance, ERCP)	Biliary obstruction

BMI body mass index, *PIZZ* alfa-1-antitrypsin genotype

Presentation of Drug-Induced Hepatotoxicity

The most frequent clinical presentation of hepatotoxicity is similar to acute viral hepatitis. Elevations of alanine aminotransferase (ALT), alkaline phosphates (AP), and conjugated serum bilirubin are encountered. This ranges from mild elevations to the presentation as fulminant hepatic failure. The non-biochemical features are unspecific and overlap with those of any acute liver condition. They include anorexia, nausea, abdominal pain, fever, jaundice, dark urine, and asthenia. In general the most often encountered symptom is fatigue. In hypersensitivity reactions, rash, eosinophilia, and edema can be present indicating an immune-mediated component [3, 25].

The diagnosis is further confounded by the promiscuity of clinicopathological presentations (Table 26.1). Hepatotoxicity induced by drugs is capable of presenting similar to any known acute or chronic liver disease. This necessitates a careful diagnostic approach to exclude causes of nondrug-induced liver diseases (Table 26.4). Isoniazid toxicity can present like acute viral hepatitis-like liver injury or acute liver failure, methotrexate toxicity can present with liver cirrhosis, valproate toxicity includes acute fatty liver with lactic acidosis, and nitrofurantoin and methyldopa toxicity can present similar to autoimmune hepatitis [25]. A vanishing bile duct syndrome can be encountered in β-lactam antibiotic therapy and a sinusoidal obstruction syndrome as a result of pyrrolizidine alkaloid and cyclophosphamide toxicity. Treatment with protease inhibitors such as atazanavir can result in unconjugated uncomplicated hyperbilirubinemia without evidence of structural liver damage in susceptible individuals carrying variants of (bilirubin) conjugating UGT1A [26].

In clinical practice the suspicion of drug-induced hepatotoxicity is raised after drug exposure and an elevation of ALT, conjugated serum bilirubin, or combined with AP to 2×the upper limit of normal (ULN).

In view of the increasing prevalence of elevated aminotransferase activities as a result of the rising incidence of nonalcoholic steatohepatitis, a threshold of 2×ULN is likely to be too low to adequately assess hepatotoxicity. Therefore, recent suggestions include elevating the ALT threshold to 5×ULN although this is controversially discussed. Along this line an increase of ALT to 5×ULN or an ALT/AP ratio (ALT×normal/AP×normal) of ≥5 indicates a hepatocellular toxicity type. A cholestatic toxicity profile is represented by an AP>2×ULN or an ALT/AP ratio of ≤2, and a mixed profile by an ALT and AP>2×ULN or an ALT/AP ratio of 2–5.

In those patients with already elevated aminotransferase activities prior to the initiation of drug therapy, the above said requires an adjustment according to the baseline values of ALT and AP. The biochemical profile can change during the course of the hepatotoxic reaction and develop from hepatocellular to cholestatic or the other way around.

The classification of the biochemical profile serves prognostic purposes. In general a cholestatic profile carries a higher risk. A cholestatic presentation of hepatotoxicity is more frequent in males above 60 years, while a hepatocellular presentation is more frequent in females under the age of 60.

In many if not most cases of drug-associated hepatotoxicity, biochemical abnormalities resolve completely with no evidence of sustained or permanent hepatic damage. In 6 % biochemical data show evidence of chronicity. An initial pattern of cholestatic injury or mixed cholestatic/hepatocellular injury indicates a significantly higher rate of chronicity. The hepatocellular presentation in turn is associated with a higher rate of chronic hepatitis and cirrhosis development.

Classification of Drug-Induced Hepatotoxicity

A general classification distinguishes (a) intrinsic (or direct) toxicity from (b) idiosyncratic toxicity, which is further subclassified into an allergic and a nonallergic group. One difference between (a) and (b) is the assumption that immune-mediated mechanisms are less involved in intrinsic hepatotoxicity. Intrinsic hepatotoxicity is characterized by dose dependency and a predictable response above a threshold drug dose and is more easily reproducible in animal models. In contrast, idiosyncratic drug reactions are not easily predictable, occur without an obvious dose dependency and often with considerable latency, and are not easily reproducible in animal models. In the allergic subgroup of idiosyncratic hepatotoxicity, typical symptoms of the adaptive immune system are observed including fever, rash, autoantibody formation, and eosinophilia. While this classification has merits regarding a mechanistical approach to hepatotoxicity, many examples exist, where a distinction between nonimmune-mediated hepatotoxicity and immune-mediated toxicity is not clear and the features actually overlap. Although the title of this chapter suggests that nonimmune-mediated hepatotoxicity can be defined and characterized as a distinct and definable entity, clinical data and observations suggest that such a clear mechanistic division is artificial and not likely in most instances. When metabolic and biotransformation-associated events leading to oxidative stress and ROS are studied as the effectors of nonimmune-mediated hepatic injury, it is important to realize that the antioxidative balance, adduct formation, and haptenization are potent inducers of immune-mediated processes, which are capable of playing a key role in the overall presentation and consequences of hepatotoxicity (Fig. 26.1).

In this context it is interesting to acknowledge that in general, drug-induced hepatotoxic reactions are indeed dose dependent. A review of drugs that were discontinued or subject to black box label warnings showed that drugs administered in doses above 50 mg per day were associated with hepatotoxicity [27]. A correlation exists between daily dose of oral drugs and the incidence of liver failure, mortality, and liver transplantation. Therefore, the risk of drug-induced hepatotoxicity appears to be low in compounds administered below a daily oral dose of 50 mg. Because the majority of these reactions can be classified as idiosyncratic drug reactions or at least exhibit an idiosyncratic component attributable to immune-mediated processes, these epidemiological data argue for a dose dependency in any type of drug reaction. Idiosyncratic drug reaction below a daily dose of 10 mg is extremely rare.

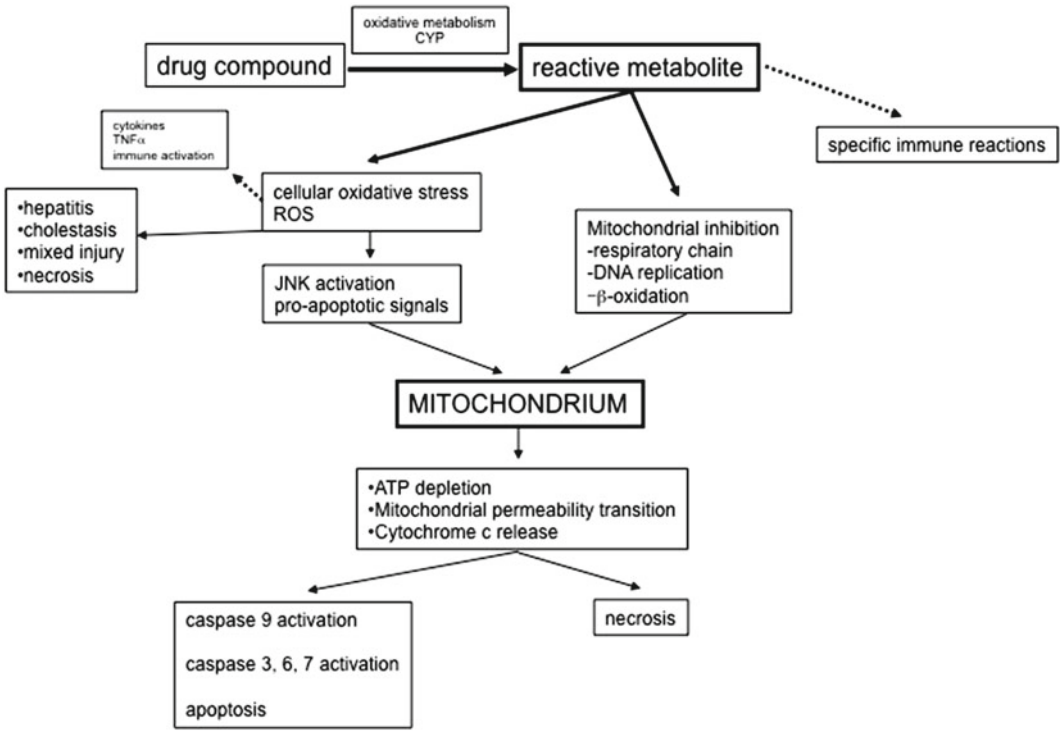

Fig. 26.1 Simplified diagram showing the process of hepatotoxicity by activated drug metabolites leading to oxidative stress, reactive oxygen species (ROS), mitochondrial damage and apoptosis. This flow diagram focuses on nonimmune mechanisms. However, oxidative stress and ROS are powerful inducers of inflammation and specific reactions of the adaptive immunity, and from a mechanistic point of view nonimmune mediated and immune mediated mechanisms cannot be strictly separated

Oxidative Stress and Mitochondrial Toxicity as Initial Mechanisms of Nonimmune-Mediated Drug Toxicity

The most likely mechanism inducing hepatotoxic drug reactions is the formation of reactive metabolites, ROS and adduct formation with subcellular and molecular cellular structures (Fig. 26.1). Therapeutic drugs are xenobiotics, which in almost all instances undergo some degree of host metabolism to enable their subsequent elimination from the host organism. Therefore, drug toxicity is intrinsically linked to this process and its alterations, specifically to the ability of the host organism to regulate a process of generating more reactive intermediate metabolites followed by the production of excretable or removable less toxic breakdown products. The most important enzyme system for this process is the CYP superfamily of proteins and their counterpart in biotransformation the conjugating enzymes (phase II metabolism), which include UGT, GST, and acyltransferases. The balance of phase I (oxidative metabolism) and phase II (conjugation, detoxification, elimination) defines the outcome of metabolic processes affecting the potential for hepatotoxicity [28, 29]. A third process involves hepatobiliary transport (also designated phase III by some authors), which indicates the dependency on an effective usually energy-dependent transport of metabolites from the hepatocyte into the biliary system. This process is performed by transporter proteins such as the multidrug resistance protein (*ABCB4*), the multidrug resistance-associated protein (*ABCC2*), the canalicular bile salt export pump (*ABCB11*), and the multidrug resistance-1 P glycoprotein (*ABCB1*). For *ABCB4*, *ABCB11* genetically defined cholestasis syndromes (benign recurrent intrahepatic cholestasis, BRIC; progressive familial intrahepatic cholestasis, PFIC) exist indicating that genetic variation is capable of producing cholestatic phenotypes, which are also likely to impact the disposition to drugs depending on their requirement of elimination via these pathways.

A distinctive feature observed after the treatment with specific drugs including tetracycline, amiodarone, nucleoside analogues, or valproic acid is microvesicular steatosis of the liver [16, 30]. This feature is characterized by the microvesicular accumulation of fat in the hepatocyte in addition to a paucity of mitochondria. Further progress of toxicity can subsequently develop to macrovesicular steatosis. This feature of microvesicular fat accumulation is associated with and typical of mitochondrial toxicity. The cellular generation of oxidative metabolites, glutathione (GSH) depletion, and adduct formation with lipids, enzymes, nucleic acids, and

subcellular macromolecules can lead to cellular accumulation of reactive substrates. As a result of or independent of this, mitochondria can be indirectly or directly injured (Fig. 26.1). Processes capable of uncoupling or inhibiting the mitochondrial respiratory chain lead to ATP depletion and increase ROS. It is assumed that, e.g., valproic acid is capable of inhibiting mitochondrial β-oxidation of fatty acids leading to a decrease of mitochondrial function. The inhibition of β-oxidation and steatosis, damage to mitochondrial DNA, and alterations of mitochondrial DNA replication are features of mitochondrial injury. Data associating hepatotoxicity with variations of *SOD2* and *POLG1* relevant to mitochondrial defense further support mitochondrial toxicity as a central process of reactive metabolite and ROS-mediated hepatotoxicity. In the course of mitochondrial injury and the interference with mitochondrial DNA replication, mitochondrial permeability transition (MPT) by opening of the inner membrane MPT pore is an important process of injury. MPT leads to the release of cytochrome c and the subsequent activation of apoptosis via caspase 9 and downstream caspases (caspase 3, 6, 7). This is believed to be a result of a critical downregulation of the electron transport chain and an increase in cytosolic ROS in addition to a critical elevation of c-Jun N-terminal kinase (JNK) signaling. However, MPT can also proceed by immune-mediated processes involving tumor necrosis factor α/Fas ligand (TNFα/FasL) by the activation of caspase 8, Bid, and ceramides. This illustrates that pure non-immune processes are not likely to proceed by themselves but rather act synergistically and in combination towards the induction of apparent hepatotoxicity. Hepatotoxicity with isoniazid is an example in which intrinsic toxicity with a mild phenotype and idiosyncratic toxicity with a fulminant phenotype can be evident by exposure to the same drug.

The hypothesis of reactive intermediates and adduct formation as a central mechanism is supported by the fact that 62 % of drugs (13 of 22) withdrawn from the market because of severe hepatotoxicity can be shown to lead to reactive intermediate metabolites [31]. It is difficult to screen for this feature during the preclinical development of drugs, and it is interesting that in a majority of drugs associated with the development of hepatotoxicity, drug-protein adduct formation cannot be detected [32].

Examples of Intrinsic Drug-Mediated Hepatotoxicity

The classical intrinsic hepatotoxin in the Western Hemisphere is acetaminophen [33]. NSAIDs are the most widely prescribed and used drugs and are available as over-the-counter drugs in most countries [34]. About 30 million individuals consume NSAID, and it is estimated that 25 % of the population in the USA have experienced NSAID-related side effects, which includes gastrointestinal complications, and not only hepatotoxic side effects. Although hepatotoxicity is relatively rare, NSAID-associated hepatotoxicity is estimated to be responsible for around 10 % of all cases of drug-induced hepatotoxicity. Nearly all cases of hepatotoxicity by NSAID are caused by 8 compounds: diclofenac, ibuprofen, sulindac, aspirin, naproxen, piroxicam, nimesulide, and of course acetaminophen (Fig. 26.2).

NSAID belong to different chemical groups and can be classified (in the order of frequency) as acetic acid derivatives, propionic acid derivatives, salicylates, enolic acid derivatives, and sulfoanilides (Fig. 26.2). The diversity of chemical structures suggests that differing metabolic processes are required and this opens the possibility of differing presentations of hepatotoxicity (Table 26.5). The risk to develop acute liver failure when NSAID-associated hepatotoxicity is present is highest with ibuprofen (9.4 %), followed by aspirin (8.2 %), naproxen (8.1 %), nimesulide (6.6 %), diclofenac (5.1 %), piroxicam (4.1 %), and sulindac (2.5 %) [33]. The overall risk to proceed to acute liver failure from NSAID-induced hepatotoxicity is estimated at 6 %. The risk is difficult to assess precisely because co-medication with other potentially hepatotoxic drugs, dietary supplements affecting biotransformation, and the coexistence of chronic or acute liver diseases including the effects of obesity are likely to influence the incidence significantly.

When chemical classes are considered, about 50 % of all instances of hepatotoxicity occur with acetic acid derivatives (carboxylic acids). Of note, the glucuronidation of carboxylic acid groups leads to the formation of acyl glucuronides which are electrophilic and can lead to reactive aldehyde intermediates. The glucuronidation of the carboxylic group of most NSAID drugs leading to acyl glucuronide formation is the principal elimination pathway in humans. The formation of non-acyl glucuronides conversely leads to less active or inactive glucuronides which undergo elimination in bile and urine [23].

Different NSAID lead to different patterns of hepatotoxicity. Hepatocellular damage is encountered with diclofenac, ibuprofen, indomethacin, ketoprofen, nimesulide, piroxicam, and sulindac. A cholestatic toxicity profile is observed with celecoxib, ibuprofen, naproxen, piroxicam, and sulindac, and a mixed type can be seen with ibuprofen, piroxicam, rofecoxib, and sulindac. This list illustrates that the profile alone does not allow for a clear distinction of toxicity profile and corresponding responsible drug, and it shows a wide overlap of potential toxicity phenotypes. In addition, the mechanism of toxicity varies with the individual drug and also within a single drug type. Aspirin can lead to acute hepatitis in a dose-dependent manner but also to Reye's syndrome with higher doses. Diclofenac can lead to an acute or

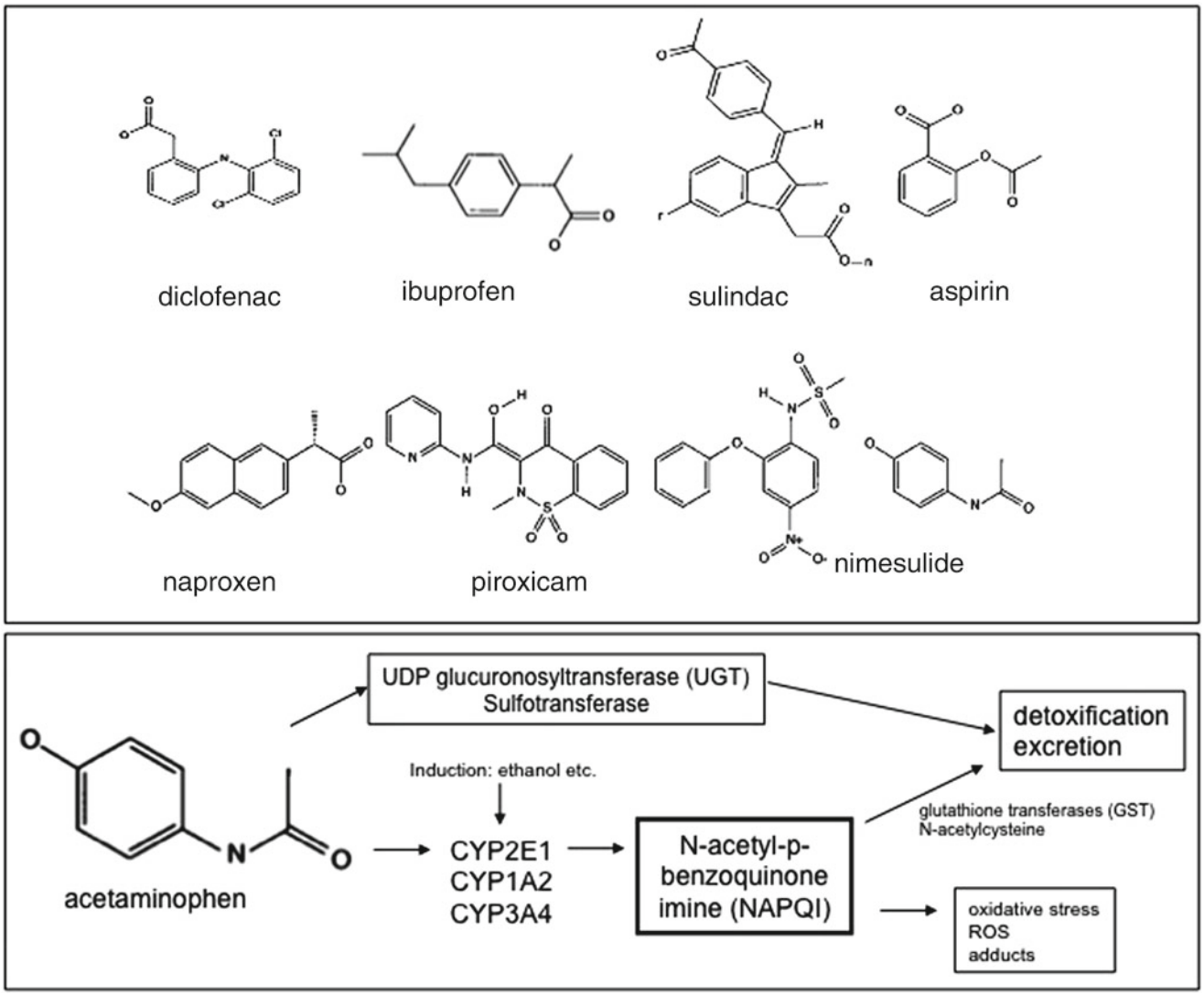

Fig. 26.2 Structural formulas of the seven most common nonsteroidal anti-inflammatory drugs leading to hepatotoxicity. In addition acetaminophen is shown including the metabolic mechanisms leading to hepatotoxicity

Table 26.5 Toxicity with nonsteroidal anti-inflammatory drugs

Compound	Injury presentation	Mechanism
Aspirin	Acute/chronic hepatitis	Dose dependent
	Reye's syndrome	
Ibuprofen	Acute hepatitis, vanishing bile ducts	Metabolism
Diclofenac	Acute/chronic hepatitis	Metabolism
	Mixed hepatitis/cholestatic	Immune mediated
Naproxen	Mixed hepatocellular/ cholestatic	Metabolism
Coxibs	Mixed hepatocellular/ cholestatic	Unclear, metabolism
Sulindac	Acute hepatitis/mixed hepatocellular/cholestatic	Immune mediated

chronic hepatitis believed to be related to metabolic activation but can also lead to an immune-mediated cholestatic injury type. Hepatotoxicity of ibuprofen, naproxen, oxycams, and nimesulide is predominantly dependent on metabolic processes, whereas hepatotoxicity of sulindac represents a hypersensitivity reaction.

The number of therapeutic drugs potentially leading to intrinsic hepatotoxicity is legion. A few selected examples will be discussed in more detail below.

Acetaminophen toxicity. Acetaminophen toxicity follows a classical intrinsic mode of dose-dependent hepatotoxicity. A small proportion of acetaminophen is metabolized in the liver by CYP1A2 and CYP2E1 to form variable amounts of NAPQI.

CYP2E1 is ethanol inducible and therefore coadministration of ethanol increases the rate of NAPQI formation and is therefore a potent regulator of acetaminophen-mediated hepatotoxicity. The major pathway of acetaminophen elimination is performed by phase II biotransformation (Fig. 26.2). This is catalyzed by glucuronidation (UGT) and sulfation utilizing phase I-generated metabolites and leading to inactive metabolites eliminated in the bile and urine. NAPQI is subsequently conjugated to glutathione and physiologically detoxified to mercapturic acid. When this pathway is overwhelmed by excessive dosing or an excessive activation of phase I metabolism, NAPQI accumulation leads to the intracellular depletion of glutathione stores. NAPQI is highly reactive and can covalently bind to subcellular structures and proteins. It also dysregulates mitochondrial function, leading to the above delineated mechanisms of mitochondrial toxicity and apoptosis induction via ROS (Fig. 26.1). The treatment options for fulminant hepatotoxicity or acute liver failure therefore only offer a competitive provision of sulfhydryl groups, specifically the administration of *N*-acetylcysteine to saturate the effects of NAPQI and to prevent the depletion of glutathione, thus decreasing the likelihood of downstream effects of mitochondrial damage and apoptosis.

The description of the mechanism of metabolic activation and hepatotoxicity in acetaminophen exposure suggests that this process is nonimmune mediated. However, two aspects deserve consideration. First, the downstream effects of mitochondrial damage and ROS accumulation in the hepatocyte are very likely to activate adaptive immune-mediated reactions. Second, a mouse model lacking CD44 encoding a cell adhesion molecule expressed on lymphocytes and involved in cell–matrix interactions was reported to show a considerably decreased susceptibility to acetaminophen toxicity [35]. In humans, genetic variants of the CD44 gene were also associated with decreased serum ALT activities following the intake of acetaminophen [35]. These data suggest that although the mechanism of acetaminophen toxicity is primarily dependent upon the regulation of phase I and phase II biotransformation, it is unlikely that immune mechanisms do not partake in this process and possibly contribute to define the susceptibility of individuals at risk for hepatotoxicity or acute liver failure.

Aspirin. Hepatotoxicity associated with aspirin appears to be dose dependent. The usual presentation is hepatocellular and only rarely cholestatic. Aminotransferase activities appear to correlate with salicylate levels. Animal studies have suggested that salicylic acid impairs mitochondrial function leading to ATP depletion, which can result in hepatic injury by lipid peroxidation. In addition, free fatty acid levels can rise leading to massive microvesicular hepatic steatosis (Reye's syndrome). Although these data point to a nonimmune-mediated mechanism, the exclusion of a significant effect of the immune system is not possible. The majority of patients taking aspirin suffer from immune-mediated diseases such as rheumatoid arthritis. It is difficult to assess whether aspirin toxicity is also a function of the underlying immune-mediated disease and requires an immune component for its development. Aspirin-related hepatotoxicity is rare.

Diclofenac. Worldwide, diclofenac is one of the most commonly used NSAID but only retrospective studies exist linking it to hepatotoxicity. Following the hydroxylation of diclofenac by CYP on the aromatic ring, this leads to the formation of para-hydroxydiclofenac isomers. These can undergo peroxidase- or again CYP-mediated oxidation to form quinone imines. Electrophilic quinone imines then require GSH for further metabolism leading to GSH trapping or depletion. Clinical data suggest that both an idiosyncratic injury with a long latency and a higher rate of jaundice as well as a hepatitic presentation are possible. Overall, diclofenac does not lead to a high rate of adverse reactions. In one study 16 liver-related hospitalizations per 100,000 patient-years were reported [36].

Ibuprofen. Ibuprofen is also one of the most commonly prescribed or purchased NSAID because of its anti-inflammatory, analgesic, and antipyretic effects. It has a high safety profile and a very low incidence of liver toxicity. Ibuprofen is available as an over-the-counter drug and is therefore widely consumed. Most instances of hepatotoxicity have been reported in individuals with other liver diseases such as hepatitis C. In contrast to the structurally related drug ibufenac, which is characterized by a high potential for hepatotoxicity, ibuprofen has a short half-life and only a low number of reported cases of toxicity. The mechanism of toxicity is likely related to the formation of an acyl glucuronides. In human plasma protein, adducts have been detected that are believed to result from ibuprofen acyl glucuronides. However, it is questionable whether acyl glucuronides and not oxidative CYP-mediated metabolism is indeed responsible for ibuprofen-mediated hepatotoxicity.

Isoniazid. The antituberculosis drug isoniazid is well recognized for its hepatotoxic potential. It can lead to mild intrinsic hepatotoxicity but is also associated with severe idiosyncratic reactions. This example again emphasizes that both immune-mediated and metabolically defined mechanisms can be induced by the same drug. As a chemical structure, isoniazid is a hydrazine. Hydrazines and/or hydrazide moieties are known inactivators of CYP enzymes, monoamine oxidases, and peroxidases. This can lead to alterations in biotransformation that are further influenced by coadministered drugs, herbals preparations, and underlying diseases. Apart from this interaction potential, the bioactivation of isoniazid leads to reactive metabolites capable of inducing metabolic hepatotoxicity. Isoniazid is metabolized

to *N*-acetyl isoniazid and *N*-acetyl hydrazine. Both metabolites have been shown to covalently bind to liver protein. *N*-acetyl hydrazine appears to be the proximate liver toxin, which is further oxidized to *N*-acetyldiazine and the more reactive ROS: the acetyl radical or the acetylonium ion. This metabolism can increase ROS levels and hepatotoxicity but obviously also initiates idiosyncratic immune-mediated hepatotoxicity in predisposed individuals.

Future Perspective

From a clinical point of view, the most important aspect of nonimmune-mediated hepatotoxicity and immune-mediated hepatotoxicity by drugs alike is increased vigilance to suspect this frequent injury to the liver. This is not an easy task because it relies on considerable clinical expertise and the exclusion of other forms of hepatic injury. No gold standard exists for the diagnosis. The preclinical process of drug development also has no robust instruments to predict and ultimately prevent hepatotoxicity reliably, which is therefore likely to continue to appear in a number of newly licensed drugs in the future.

The disposition for hepatotoxicity depends upon the drug and its metabolism; host factors including comorbidities, comedications, liver diseases, and genetic variation; as well as environmental factors. This interdependency makes it difficult to predict and to ascertain the contribution of drugs to hepatic injury in an individual clinical scenario. A high rate of acute liver failures and urgent liver transplantations on the other hand illustrates the clinical importance of this problem. In future the prediction of risk in order to implement personalized approaches would represent a significant advance. In the case of Gilbert syndrome that leads to intermittent unconjugated hyperbilirubinemia and can be tested by UGT1A*28 determinations, an example with a direct link to hyperbilirubinemia under drug treatment with protease inhibitors has been characterized [26, 37, 38]. Gilbert syndrome is actually not only a variant of the bilirubin conjugating *UGT1A1* but comprises a complex haplotype of variants altering the function and transcriptional regulation of the human *UGT1A* gene family possibly also impacting biotransformation of many drugs undergoing conjugation [39].

Based on the outlined mechanistic implications, therapeutic intervention to prevent hepatotoxicity would also be an interesting approach. Apart from the administration of *N*-acetylcysteine for the competitive prevention of GSH depletion (or the application of corticosteroids in immune-mediated mechanisms), modification of metabolic control would be a plausible and potentially successful strategy. Given the central role of reactive metabolites and ROS for cellular injury and mitochondrial dysfunction, the therapeutic modification of antioxidative mechanisms is a promising candidate strategy for the prevention and therapy of nonimmune-mediated drug-associated hepatotoxicity. The transcription factor *nuclear factor erythroid 2-related factor 2* (Nrf2) appears to represent a key regulator in oxidative stress that is activated by ROS [40, 41]. Nrf2 is a member of the Cap'n'Collar family of bZIP proteins and recognizes the antioxidant response element (ARE) in the promoter of its target genes [42]. Under normal basal conditions, Nrf2 is bound to its inhibitor, the cytoskeleton-associated protein Keap1 which represses Nrf2 by facilitating its proteasomal degradation. Upon stimulation by antioxidants such as *tert-butylhydroquinone* (*t*BHQ), Nrf2 is released from Keap1 and translocates into the nucleus, followed by heterodimerization with other transcription factors, such as Jun and small Maf. Induction of oxidative stress-related genes that protect against damage by electrophiles and ROS is a key element in the maintenance of cellular redox homeostasis and in reducing oxidative damage [43]. These genes encode various antioxidant and detoxifying enzymes and are regulated through the *cis* acting ARE in their 5′-flanking promoter regions. Nrf2 is the central transcription factor, which regulates both constitutive and inducible ARE-related gene expressions [44]. Nrf2 knockout mice have a deficiency in this protective genetic program and have a higher susceptibility to oxidative damage [45, 46]. Nrf2 knockout mice also have a higher susceptibility to liver toxicity. A recent study demonstrated that xenobiotic activation of drug metabolism by the aryl hydrocarbon receptor and Nrf2 signaling are coordinately regulated and activate conjugating enzymes (UGT), which are a large family of proteins with cytoprotective and antioxidative capabilities [47].

An interesting epidemiological observation has been reported regarding the use of coffee and liver injury. Study data suggest that coffee consumption is associated with a decreased risk for a number of diseases as well as toxicity. In 1986, Arnesen et al. observed lower gamma-glutamyltransferase activities in coffee drinkers in the Tromso Heart study [48]. This has been replicated in subsequent studies [49] including an analysis of the Third National Health and Nutrition Examination Survey (NHANESIII) in 2005 [50] that showed an inverse correlation of coffee intake and ALT activities. Coffee consumption has been associated with reduced risks for liver cirrhosis [51, 52] and disease progression in chronic hepatitis C [53]. A recent study showed in cell culture experiments and in a transgenic mouse model [39] that coffee leads to the Nrf2-mediated activation of UGT transcription and therefore leads to a potential increase of indirect antioxidative effects [54]. This is in agreement with data linking coffee consumption to the reduction of hepatic injury evidenced by lower aminotransferase activity. Coffee may therefore act as a protective regulator by inducing Nrf2-mediated gene transcription including detoxification by glucuronidation. Future research is aimed at identifying druggable inducers of this pathway to prevent or treat hepatotoxicity [55].

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Immune-Mediated Drug-Induced Liver Injury

27

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Key Points

- The traditional classification of drug-induced liver injury (DILI) into immunologic or metabolic idiosyncracy is too simplistic; the development of idiosyncratic DILI is a multistep process involving both metabolic and immunologic factors.
- Hypersensitivity or immunoallergic reactions are usually characterised by fever, rash, eosinophilia and a rapid recurrence on re-challenge; occurrence of eosinophilia in DILI implies in most cases a favourable prognosis.
- Drug-induced autoimmune hepatitis (DIAIH) is a syndrome with clinical, biochemical and histological features indistinguishable from idiopathic AIH; relapse rate after discontinuation of immunosuppressive therapy is much lower in DIAIH than in idiopathic AIH.
- 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.
- 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.

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Introduction

Drug-induced liver injury (DILI) has previously been classified into immunologic or metabolic idiosyncracy. Metabolic idiosyncrasy implies that a subject developing adverse reaction metabolises the drug in a different way than the most individuals or lacks adequate protective mechanisms to neutralise reactive metabolites formed. An immunologic idiosyncrasy implies that the susceptible individual has an immune system that would more readily recognise 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 re-challenge 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 metabolising enzymes and the immune system within the liver which may act both as a lymphoid organ and as a target for toxicity creates a setting suitable for 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 cases of DRESS (drug rash, eosinophilia and systemic symptoms) syndrome, a severe form of idiosyncratic reaction involving multiple organ systems [1, 2]. This syndrome has been associated with drugs such as phenobarbital, carbamazepine, 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 20 years ago have shown 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,

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several candidate gene and genome-wide association studies involving well-characterised 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 aetiology [5, 6]. These classical hypersensitivity reactions are usually characterised by fever, rash, eosinophilia and a rapid recurrence on re-challenge [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 favourable, 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 favourable 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 favourable prognosis [10–12]. Children with features of hypersensitivity presented earlier (25 vs. 35 days; $P=0.24$), but, had less severe disease (MELD, 16 vs. 29; $P=0.01$) and no mortality (0/16 vs. 12/23; $P<0.001$), compared to 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 favourable 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 immunologic idiosyncrasy based on their associated clinical features; this is not just simplistic and incomplete, but more importantly fails to reflect the key role immune system plays in the pathogenesis (Fig. 27.1) even when the liver injury does not 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-metabolising enzymes (phase I and II) and transporters involved in the excretion (phase III) and elimination of drug metabolites are 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.

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 anaesthetic, halothane, is the best example of a drug causing what has been considered an immunoallergic DILI. Halothane is metabolised by

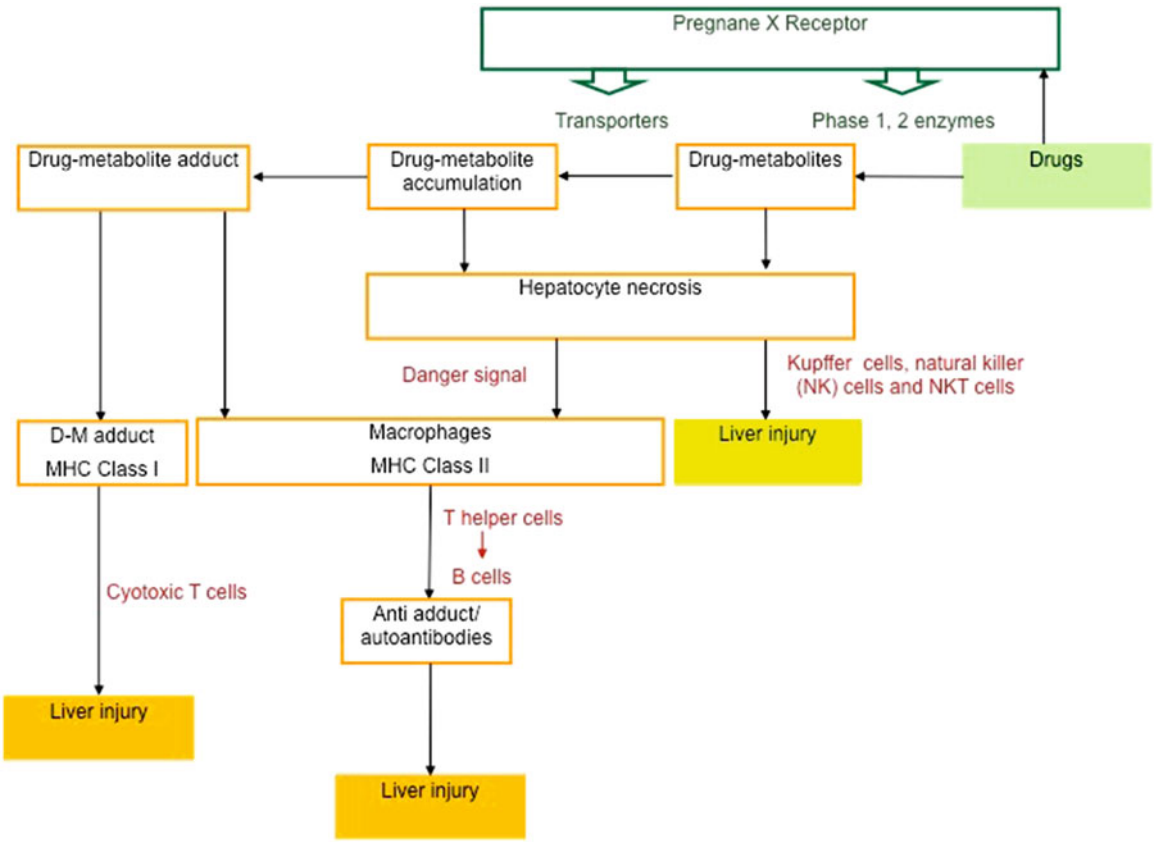

Fig. 27.1 Unifying hypothesis of pathogenesis of drug-induced liver injury highlighting immune mechanisms involved

cytochrome (CYP) 450 2E1 to form a chemically reactive acyl halide. Acyl halide targets lysine residues of proteins; antibodies that recognise auto-antigens 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.

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 signalling and cytokines, which are necessary for sustained T cell activation, proliferation and expansion [22].

Several candidate gene and genome-wide association studies (summarised in Table 27.1) have demonstrated that the human MHC plays a major role in increasing or decreasing susceptibility to DILI. A seminal genome-wide association study 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]. Flucloxacillin binds covalently to selective lysine residues on albumin and the level of protein binding determines the strength of the T cell proliferative response [24]. Consistent with the role of adaptive immune system, flucloxacillin-specific peripheral blood mononuclear cell (PBMC) responses can be detected in those who

Table 27.1 HLA genotypes increase and decrease susceptibility to drug-induced liver injury

Drug	Genotype	Hazard ratio	Cases (n = largest cohort)
Class I			
Clometacin [92]	*B*08*	–	7/30 (genotyped)
Co-amoxiclav [93, 94]	*A*0201*	2.2	201
	*B*1801*	2–8	201
Flucloxacillin [23]	*B*5701*	80.6	64
Ticlopidine [95]	*A*3303*	13	22
Tiopronine (mercaptopropionylglycine) [96]	*A*33*	–	14
Class II			
Anti TB drugs (isoniazid, rifampicin, pyrazinamide) [97]	*HLA-DQB1*0201*	1.9	56
	*HLA-DQA1*0102*[a]	0.2	56
Co-amoxiclav [26]	*DRB1*1501 DQB1*0602*	2.3–10	201
	*DRB1*07*[a]	0.18	61
Diclofenac [17]	*DRB1*13*[a]	–	24
Flucloxacillin [26]	*DRB1*0701-DQB1*0303*	7	64
	*DRB1*15*[a]		
Lapatinib [98]	*DRB1*0701-DQA1*0201*	2.6–9	37
Lumiracoxib [91]	*DRB1*1501-DQB1*0602-DRB5*0101-DQA1*0102*	5	41
Ximelagatran [25]	*DRB1*07-DQA1*02*	4.4	74

[a]Association reduces the risk of DILI

had suffered DILI; in a recent investigation, flucloxacillin-responsive CD4+ and CD8+ T cell clones were isolated and characterised from patients with hepatotoxicity. Flucloxacillin also activated naive CD8+ T cells from *HLA-B*5701*-positive volunteers [24]. These lines of evidence provide new insights into the role of adaptive immune system in the pathogenesis of DILI.

In the case of ximelagatran, the drug as well as its intermediate metabolite melagatran-ethyl can directly bind to HLA-DRB1*0701 molecule and activate an immune response [25]. In other instances, HLA variants associated with toxicity are thought to increase 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 immunologic destruction of hepatocytes. A number of studies have confirmed association of co-amoxiclav DILI with the *DRB1*1501–DQB1*0602* haplotype; recently a novel protective association of *DRB1*07* family with co-amoxiclav DILI has been demonstrated [26]. In contrast, with regards to 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 [26].

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 [17]. In contrast, in nitrofurantoin-induced DILI, CD8+ cytotoxic T cells may play a pivotal role in the pathogenesis [27].

Danger Signals

According to the ‘danger hypothesis’ [28], the primary function of immune system does not rely upon the distinction of non-self 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 [29]. 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 (Fig. 27.1) 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 [30]; 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 as well as 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' [31] and hence influence the immune equilibrium [32]. 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 [33]. 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, IL-10; tumour necrosis factor-α (TNF α); interferon-γ; and transforming growth factor-β; inflammatory molecules such as prostaglandin E2, human serum complement and activated protein C; oxidants such as buthionine sulfoximine and H_2O_2 and hyperthermia to mimic 'danger signals' [34]. 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 [34]. It is plausible that concomitant infection may contribute to susceptibility to DILI, and therefore, as a group, antimicrobials including co-amoxiclav, flucloxacillin and antituberculous 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 [35] and C [36], as well as those coinfected with human immunodeficiency virus [37].

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 Kupffer 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 TNF-α, interleukin-1β (IL-1β), IL6 and IL8 which in turn correlated with a higher number of neutrophils recruited into the liver [38]. 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 [39].

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 fourfold 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 an SNP, in the vicinity of signal transducer and activator of transcription 4 (*STAT4*); this association was replicated in an independent cohort [40]. 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 [40].

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 [41]. 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 [42, 43]. 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 [44], at least in the beginning of their disease course and, drug aetiology 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

Table 27.2 Histological features favouring AIH versus DILI

Histological features	Favouring AIH	Favouring DILI
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

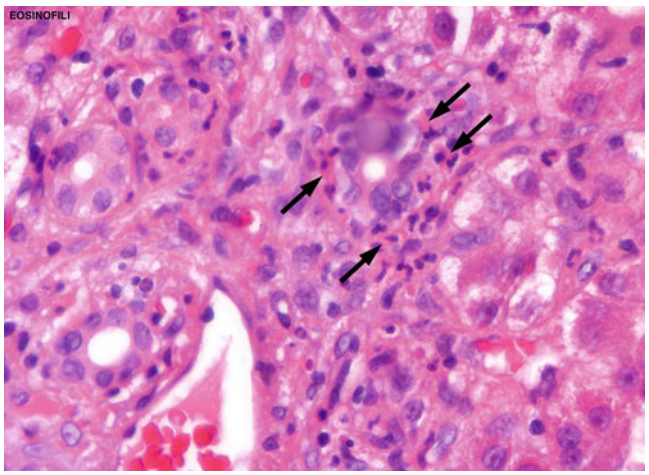

Fig. 27.2 A liver biopsy from a patient with disulfiram induced liver injury showing prominent eosinophilia, eosinophils depicted by *arrows*

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 pathologists undertook a blinded systematic evaluation of liver biopsies from a clinically well-characterised DILI and AIH cases [45]. 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 [45]. The occurrence of prominent intra-acinar lymphocytes and canalicular cholestasis favoured the diagnosis of DILI, whereas more severe portal inflammation, portal plasma cells, intra-acinar eosinophils and rosette formation favoured the diagnosis of AIH [45]. A considerable histological 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, histological 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, 46]. Prominent eosinophil infiltration which has been considered to be one of the histological 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 were in fact higher among AIH than both HS and CS type of DILI cases [45]. 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 favoured AIH over HC type of DILI [45]. It seems that different inflammatory cells may be enhanced in DILI versus AIH as prominent portal neutrophil infiltrate was favouring CS type of DILI [45]. Table 27.2 demonstrates histological features favouring AIH versus DILI. In Fig. 27.2 histology in a patient with disulfiram-induced liver injury with marked eosinophilia is shown in a liver biopsy.

Drug-Induced Autoimmune Hepatitis

Many drugs have been reported to have induced the syndrome of drug-induced autoimmune hepatitis (DIAIH) [47, 48]. Most of these drugs have appeared in case reports or small case series [47, 48]. The most common drugs previously found to provoke DIAIH were dihydralazine [49] and tienilic acid [50], both that have been removed from the market. Later on accumulating reports have been published on the occurrence of DIAIH by nitrofurantoin [47] and minocycline [51]. Figure 27.3 shows minocycline-induced AIH with portal inflammation and interphase hepatitis. More recently increasing number of reports have been on statins [52–56] and antitumour necrosis α agents [57–59] inducing DIAH. Currently, it is unclear what proportion of patients with DILI develops DIAIH. Conversely, it is not clear what proportion of patients who fulfil the criteria for AIH have DIAIH. In large series on DILI [10, 60, 61], 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 %) were considered to be induced by drugs [62]. Two drugs, nitrofurantoin ($n=11$) and minocycline ($n=11$), were the main causes in this series [62]. 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 [63]. In the best documented drugs leading to AIH-like picture, the vast majority of patients consist of females [62]. The majority of patients with idiopathic AIH not induced by drugs are females, but the female preponderance is more pronounced in DIAIH

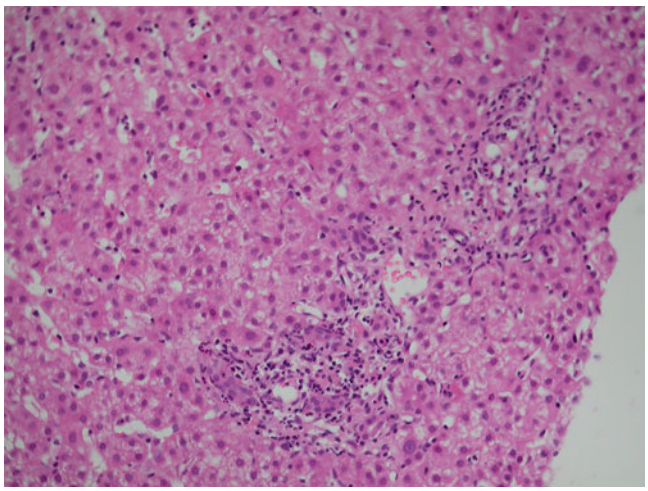

Fig. 27.3 Minocycline induced auto-immune hepatitis with portal inflammation and interphase hepatitis

[62], which is consistent with female propensity of autoimmune diseases.

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 [62], is rarely associated with the development of cirrhosis and very rarely shows relapse after steroid discontinuation, when this has been tried [62]. Two recent studies have not been able to identify any inflammatory features discriminating DIAIH and AIH [45, 62]. 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 [62]. The findings of another study are consistent with this, as advanced fibrosis was observed only in AIH but not in DIAIH cases [45]. Consistent with these, none of the patients with AIH induced by antitumour necrosis α agents had histologically proven cirrhosis at presentation [58]. A small series of nitrofurantoin-induced AIH, precirrhosis or cirrhosis was present in one case [64]. Thus, in general fibrosis and cirrhosis are less frequently observed in DIAIH cases than in idiopathic AIH [45, 62, 64].

Risk Factors for Drug-Induced Autoimmune Hepatitis

In a long-term follow-up of patients with DILI with concomitant jaundice leading to hospitalisation, AIH developed in 5/23 (22 %) patients after the initial event over a mean period of 6 years [65]. 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, ANA was detected after DILI in 6 patients and 5/6 (83 %) were females [66]. All 5 patients who developed AIH after the initial DILI were females in a long-term follow-up study, which is in line with these results [65]. In the Spanish DILI registry 9/742 (1.2 %), patients had evidence of two DILI episodes caused by different drugs [55]. An interesting finding in that series was that four out of nine cases (44 %) developed DIAIH in the second episode during follow-up [55]. This clearly exceeds the chance of association of this liver injury phenotype in the Spanish DILI registry's general patient cohort as 6 out of 9 cases in the series were AIH-like [55]. 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 pre-existing or subclinical AIH [54]. Interestingly, Sugimoto et al. reported 7 cases which were diagnosed as DILI, but features of AIH became apparent later despite discontinuation of the drug, suggesting a different pattern of aetiology [67]. Interestingly, ANA titers and immunoglobulin (Ig) G levels increased during the course [67].

The Role of Specific Drugs

Nitrofurantoin

AIH induced by nitrofurantoin was reported from the United States in a small series of 5 patients from the 1970s and six patients from the Netherlands from the 1980s [47, 68]. 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 have been published on nitrofurantoin-induced AIH before and after these series [64]. Nitrofurantoin has also been associated with other types of DILI such as acute liver failure and also liver cirrhosis [61, 64]. 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 [62]. 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 [62], which is similar to a report from the Netherlands showing no cases of nitrofurantoin-induced cirrhosis in 52 cases [47]. The changes observed on imaging showing 'cirrhotic' changes [62] might be explained by post-necrotic 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 [69, 70]. 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 [62]. In general, DIAH induced by minocycline seems to have a favourable prognosis [51, 69–71], 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 [72, 73]. In a patient with acute liver failure, requiring liver transplantation, anti-smooth muscle antibody, anti-double-stranded DNA antibody, antimitochondrial antibody and antinuclear antibody were positive, indicating an autoimmune process rather than a necrotic and/or inflammatory process in the liver [74]. 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 been demonstrated in different types of acute liver failure [74].

Statins

Although rare statin-induced hepatotoxicity has been well documented [56, 75], many case reports [52–56, 75–80] and some cases series [54, 56] 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 [52, 56]. Most of patients with DIAIH due to statins were reported to have favourable prognosis. Cross-reactivity, with development of DILI after exposure of another statin, has been reported [54, 56], but it has also been observed that another type of statin could be tolerated, and hence, the 'class effect' is not universal [56], as with other types of DILI induced by statins [75]. 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 [78, 80].

Antitumour Necrosis Factor α Agents

More than 20 cases of DIAH related to the use of these agents have been reported [57–59, 81, 82]. This has been in patients with all indications for these drugs such as psoriasis, ankylosing spondylitis, inflammatory bowel disease and rheumatoid arthritis. Taken together TNF α agents are probably the most common cause of DIAH among drugs in use nowadays. Reviews of these cases have been published [59, 81–83]. 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, aminotransferases are in most cases >10 times the ULN; they 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 [62]. After the resolution of liver injury, patients have been successfully switched to another TNF α agent without recurrence of liver injury [81–83].

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 (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, 84–87]. 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 [62]. In the largest series, the new simplified score of AIH was used to establish the diagnosis of DIAIH [58]. 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; it was higher in DIAH than in AIH [62]. Similarly, histological features were very similar in these two groups, and no single histological finding could distinguish between them [62]. 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 [45]. Marked fibrosis (Ishak score >4) was however only seen in patients with classical AIH and not in DIAIH cases [45].

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 [62]. Out of 4/9 (30 %) with DIAIH developing after a second exposure of drugs leading to DILI in the Spanish registry [55], liver tests normalised in two patients without requiring immunosuppression, and smooth muscle antibody became negative after drug discontinuation [55]. 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 DIAH in patients where this has been tried and/or reported [55, 62]. In the largest series of patients with DIAH, discontinuation was tried in 14 DIAIH cases (median follow-up 36 months), with no relapse, whereas 65 % of the AIH patients relapsed [62]. 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 DIAH 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 DIAH were associated with a relapse when immunosuppression was withdrawn [54, 71, 88]. 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 this 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.

Conclusions

Our understanding of relationship of drug metabolism in the development of primary immune response has improved substantially. 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 costimulatory 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 [89]. Understandably, potential application of these associations in pre-empting DILI has been considered [90]. 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 [91]. 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 500–1,000 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 pre-emption and primary prevention should remain the goal of translational research.

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The Immunopathogenesis of Cirrhosis

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Key Points

- There has been continued clarification of the cellular source of extracellular matrix (ECM) in hepatic fibrosis, major advances in understanding signaling and transcriptional events, and exciting insights into the biology of fibrosis progression and resolution.
- Both fibrosis and cirrhosis are the consequences of a sustained wound-healing response to chronic liver injury, and they are determined by the nature and severity of the underlying liver disease as well as the extent of hepatic fibrosis.
- Even cirrhosis may regress, and the inflammatory and immunologic determinants of reversibility are becoming identified.
- The hepatic lymphocyte populations are very diverse and are dominated by cells that are rare in other parts of the body including natural killer (NK), natural killer cells with a T cell receptor (NKT), T cells with the standard αβ T cell receptor (TCR-αβ), T cells with the γδ receptor (TCR-γδ), and B cells.
- The sinusoidal structure, low flow rates, and resident Kupffer cell population all contribute to retention of activated T cells in the liver.
- The identification of pattern recognition receptors including Toll-like receptors (TLRs) has been a crucial advance, whose impact on fibrosis progression and resolution is being clarified.
- The activated hepatic stellate cell (HSC) is the primary source of fibrosis in liver disease; however, related mesenchymal cell types from a variety of sources may also make measurable contributions.
- Degradation of interstitial, or scar, matrix is required for fibrosis regression, and Kupffer cells, or liver macrophages, may regulate this response.
- Stellate cells can amplify the inflammatory response by inducing infiltration of mono- and polymorphonuclear leukocytes.

Introduction

Fibrosis is a type of wound healing which occurs in most organs after repetitive acute or sustained chronic injury. With continued injury, fibrosis may progress to a complex set of changes which encompass matrix deposition, immunomodulation, and distortion of liver vasculature and gross architecture. Tremendous progress in understanding the pathophysiology of this wound-healing response has led to realistic expectations for treating fibrosis in patients with chronic liver disease owing to either viral hepatitis or metabolic or autoimmune diseases, among others. There have been continued clarification of the cellular source of ECM in hepatic fibrosis, major advances in understanding signaling and transcriptional events, and exciting insights into the biology of fibrosis progression and resolution [1]. The clarification of interactions between the immune system and fibrogenic response has been among the most exciting developments in fibrosis. In the liver, these advances include evidence of direct interactions between immune cell subsets and fibrogenic cells in liver, the emergence of natural killer (NK) cells as determinants of hepatic stellate apoptosis and thus fibrosis resolution, the establishment of hepatocellular apoptosis as an inflammatory and fibrogenic stimulus, and the growing recognition that HSCs contribute to the innate

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immune response and development of hepatocellular carcinoma (HCC). These and other observations underscore the prospect for eventually manipulating these interactions therapeutically. Whereas fibrosis accompanies progressive liver injury and may vary from mild to extensive, cirrhosis is the end stage of fibrosis of the hepatic parenchyma, resulting in nodule formation that can lead to altered hepatic function and blood flow which can be seen as a form of vascular remodeling.

Both fibrosis and cirrhosis are the consequences of a sustained wound-healing response to chronic liver injury, with variable clinical manifestations that are determined by the nature and severity of the underlying liver disease as well as the extent of hepatic fibrosis. Recent studies suggest that cirrhosis is a slowly progressive disease whose risk of complications accrues over time, with an annual mortality rate of 4 % in patients infected with chronic hepatitis C virus (HCV) [2]. Among patients with cirrhosis, approximately 70 % of deaths are directly attributable to liver disease, the largest fraction of which is due to HCC [3]. The overall burden of liver disease in the United States—the vast majority of which is caused by chronic disease with fibrosis—continues to expand, and it has a growing economic and social impact [4]. Remarkably, recent studies suggest that not only is fibrosis reversible, but in selected patients even cirrhosis may regress, although the determinants of reversibility and its likelihood in patients with chronic liver disease are not completely understood [5]. Moreover, the relative contribution of immune interactions to reversibility is unknown. Still, the continued clarification of how the immune system regulates both fibrosis progression and regression, combined with basic science advances in understanding of both acquired and innate immunity, augurs well for significant progress in exploiting this knowledge to the benefit of patients. This chapter will review the immune cellular components and general pathophysiology of hepatic fibrosis and then emphasize our growing knowledge of the immune and molecular mediators of fibrosis, which establish the basis for how these advances might lead to immunomodulation of liver fibrosis.

Immune Cellular Components in the Liver

The healthy liver contains a large number of immune cells and is one of the richest sources for innate immune cells, including Kupffer cells, NK cells, NKT cells, and γδ T cells [6]. Kupffer cells, liver-resident macrophages, account for 80–90 % of the total population of fixed tissue macrophages in the body and are responsible for elimination of insoluble waste by phagocytosis through a variety of receptors. During liver injury, Kupffer cells are activated and produce a wide variety of inflammatory mediators to regulate liver injury, inflammation, fibrosis, and repair. In addition, a large number of infiltrating macrophages accumulate in the liver during acute or chronic liver injury and exert many critical functions in the pathogenesis of liver disease.

The human peripheral blood contains less than 5 % NK cells, whereas human liver lymphocytes are composed of about 50 % NK cells [7]. Compared to peripheral NK cells, liver NK cells express higher levels of TRAIL and have higher levels of basal cytotoxicity against tumor cells and activated HSCs, thereby playing key roles in host defense against liver tumor and liver fibrosis [7]. During acute or chronic HCV infection, peripheral NK cells are activated, whereas the data on activation of intrahepatic NK cells have been controversial. Multiple studies showed that intrahepatic NK cells had higher levels of TRAIL, NKp46, and CD122 expression and cytotoxicity than peripheral blood NK cells of HCV patients; there levels were further elevated after IFN-γ therapy [8, 9]. However, a recent study revealed that intrahepatic NK cells exhibit reduced cytotoxicity and TRAIL expression in HCV patients when compared to the levels in patients undergoing surgery for an uncomplicated gallstone [10]. These findings suggest that the intrahepatic NK cells, which have higher basal levels of cytotoxicity than peripheral blood NK cells, are likely suppressed during chronic HCV infection, but their activities are still higher than peripheral blood NK cells.

The mouse liver lymphocyte pool contains about 30–40 % NKT cells, whereas the percentage of NKT cells in human liver lymphocytes is much lower and most studies reported less than 5 % [11]. NKT cells are a heterogeneous group of T lymphocytes that recognize lipid antigens presented by the nonclassical MHC class I-like molecule CD1. The functions of NKT cells in the pathogenesis of liver diseases have been controversial [11], for the following reasons. First, several types of NKT cells exist, including type I and type II NKT cells, which usually play different sometime opposite roles in regulating liver injury, inflammation, fibrosis, and tumorigenesis. Second, after activation, NKT cells lose their markers or become apoptotic, which makes it difficult to detect them. Third, although the exogenous lipid antigen α-GalCer, which is a strong NKT activator, has been extensively characterized, the endogenous ligands and cytokines that activate NKT cells remain largely unknown. Fourth, after activation, NKT cells become tolerant and nonresponsive to subsequent stimulation. Finally, activated NKT cells can produce a wide variety of mediators, such as pro- and anti-inflammatory cytokines and pro- and antifibrotic cytokines, which may contribute to the diverse functions of NKT cells in the liver.

TCR-γδ T cells represent a minority of T cells in lymphoid organs and peripheral blood, but a high percentage of γδ T cells is found in the liver lymphocytes, which account for 3–5 % of total liver lymphocytes and 15–25 % of total liver T cells in normal mouse liver [6]. This makes the liver one of the richest sources of γδ T cells in the body. The percentage of

γδ T cells in the liver is significantly increased in the liver of tumor-bearing mice and in the livers of patients with viral hepatitis infection, but not in patients with nonviral hepatitis. The functions of γδ T cells in the liver are not clear, but they may play a prominent role in innate defenses against viral and bacterial infection and against tumor formation. Liver lymphocytes also contain a small percentage of dendritic cells (DCs), but they are poor naïve T cell stimulators. Instead, intrahepatic DCs are more susceptible to TLR stimulation and play an important role in hepatic innate immunity [12].

Collectively, the liver contains a large number of immune cells, which interact reciprocally with liver parenchymal and nonparenchymal cells, playing many important roles in regulating the progression of liver diseases.

Pattern Recognition Receptors: General Features

The mechanisms by which complex organisms detect the presence of infectious agents have been one of the most intriguing in immunology, and the identification of the germline-encoded molecules including TLRs has been a crucial advance. These receptors are members of an expanding group of molecules known as pattern recognition receptors [13, 14]. TLRs recognize relatively invariant structures called pathogen-associated molecular patterns (PAMPs) that are shared by many pathogens but not usually expressed by the host. Examples of PAMPs include lipopolysaccharide (LPS), lipoteichoic acid (LTA), and unmethylated CPG DNA of bacteria lipoarabinomannan (LAM) of mycobacteria. These PAMPs are recognized by specific TLRs and result in a cascade of signaling molecules with upregulation of effector molecules [15]. One group of effector molecules consists of reactive oxygen intermediates and antimicrobial peptides. A second group consists of costimulatory molecules that are upregulated and increase the efficiency of activation of the adaptive immune response. A third group includes cytokines, chemokines, and adhesion molecules. As can be surmised, this activation of TLRs has far-reaching consequences on immune activation and provides a rapid response to pathogens. The TLRs are, however, only a subgroup of pattern recognition receptors, with a non-TLR group termed the caterpillar protein family. This includes the two molecules NOD1 and NOD2 as well as a group of 14 NALP proteins [16]. There has been great interest in NOD2 based on its association with susceptibility to Crohn's disease, and mutations of members of the NALP family have been shown to be responsible for rare, mostly autosomal recessive, periodic fever syndromes [17]. The role of NALPs in the immune response in the liver has been greatly increased with demonstration for their requirement in acute liver injury, alcoholic liver disease, and NASH [18–20].

Much of the conceptual drive in the discovery of TLRs centered on the inability to explain how the adaptive immune system distinguishes self from nonself, and the relatively long time that it takes to become activated. Subsequent to the identification of pathogen-derived molecules as ligands for TLRs, it has become clear that many self-molecules can also activate TLRs. A clear example of this is the fact that nuclear DNA can activate TLRs in a manner that is indistinguishable from bacterial DNA. These self-molecules with an ability to activate immune receptors are termed damage-associated molecular patterns (DAMPs) and have been shown to be important in many types of acute liver injury and also fibrosis. In general, inflammation induced by DAMPs is considered to be sterile inflammation, but due to the unique position of the liver, in the course of injury, TLR activation is likely occurring from a combination of PAMPs and DAMPs.

Cellular Pathophysiology of Hepatic Fibrosis and the Role of Hepatic Stellate Cells

The identification of the cellular sources of ECM in hepatic fibrosis has laid the groundwork for defining mechanisms of fibrosis and potential therapies. The HSC (previously called the lipocyte, Ito, fat-storing, or perisinusoidal cell) is the primary source in normal and injured liver. In addition, related mesenchymal cell types from a variety of sources may also contribute measurably to total matrix accumulation, including classical portal fibroblasts (especially in biliary fibrosis) and bone marrow cells [21–24]. HSCs are resident perisinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells [25]. They are the primary site for storing retinoids within the body. Stellate cells can be recognized by their vitamin A autofluorescence, perisinusoidal orientation, and variable expression of a number of the cytoskeletal proteins including desmin, glial acidic fibrillary protein, vimentin, and nestin, among others [26]. In strict terms, "stellate cells" may represent a heterogeneous population of mesenchymal cells with respect to cytoskeletal phenotype, vitamin A content, and localization, but collectively they are the key fibrogenic cell type in the liver. Moreover, a remarkable plasticity of the stellate cell phenotype has been documented in vivo and in culture, precluding a strict definition based only on cytoskeletal phenotype [27, 28]. Stellate cells with fibrogenic potential are not confined to the liver and have been identified in the pancreas, for example, where they contribute to desmoplasia in chronic pancreatitis and carcinoma [29, 30]. Studies in situ in both animals and humans with progressive injury have defined a gradient of changes within stellate cells that collectively are termed *activation* (Fig. 28.1). Stellate cell activation refers to the transition from a quiescent vitamin A-rich cell to a highly fibrogenic cell characterized morphologically by enlargement of rough

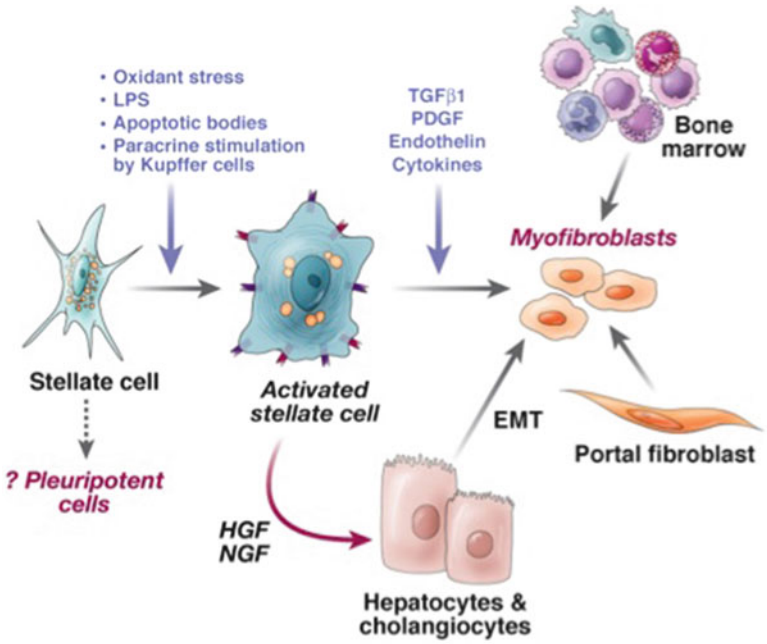

Fig. 28.1 Contributions of stellate and other fibrogenic cell types to fibrosis. A wide range of stimuli can induce activation of quiescent stellate cells. After activation stellate cells are responsive to a range of cytokines, and develop an effector phenotype known as myofibroblasts which are capable of proliferation, contractility, fibrogenesis, matrix degradation and chemotaxis. Additional cells may also contribute to the myofibroblast population, including bone marrow (gives rise to circulating fibrocytes), portal fibroblasts, and epithelial mesenchymal transition (EMT) from hepatocytes and cholangiocytes

endoplasmic reticulum, diminution of vitamin A droplets, ruffled nuclear membrane, appearance of contractile filaments, and proliferation. As noted above, proliferation of stellate cells occurs in regions of greatest injury, which is typically preceded by an influx of inflammatory cells and is associated with subsequent ECM accumulation. Conceptually, activation occurs in two phases, *initiation* and *perpetuation*, followed by *resolution* when liver injury has subsided. Initiation refers to the earliest events that render cells responsive to cytokines, and perpetuation connotes those responses to cytokines that collectively enhance scar formation (*see* below). Resolution refers to the fate of activated stellate cells when the primary insult is withdrawn or attenuated [31]. Below are some of the phenotypic and functional changes associated with stellate cell activation.

Proliferation

Platelet-derived growth factor (PDGF) is a key stellate cell mitogen [32], whose signaling pathways have been well characterized in this cell type [33]. In addition to proliferation, PDGF stimulates Na+/H+ exchange, providing a potential site for therapeutic intervention by blocking ion transport [34]. Other compounds with mitogenic activity toward stellate cells include vascular endothelial cell growth factor [35], thrombin [36, 37], epidermal growth factor (EGF), TGF-β, keratinocyte growth factor [38], and basic fibroblast growth factor (bFGF) [39]. Signaling pathways for these and other mitogens have been greatly clarified in stellate cells [40].

Chemotaxis

Stellate cells can migrate toward cytokine chemoattractants [40, 41] mediated by a number of transmembrane receptors [40, 42, 43]. One challenge of identifying such varied functions of a single cell type is to explain how they are interrelated. For example, it is not plausible that a cell is undergoing proliferation, chemotaxis, and laying down matrix. With this question in mind, adenosine was simultaneously was identified to block chemotaxis and to upregulate collagen production. As adenosine levels are high at sites of injury, this was interpreted as adenosine providing a stop signal to stellate cells and initiating the next functional stage which is laying down of collagen.

Contractility

Contractility of stellate cells may be a major determinant of early and late increases in portal resistance during liver fibrosis. Activated stellate cells impede portal blood flow both by constricting individual sinusoids and by contracting the cirrhotic liver, since the collagenous bands typical of end-stage cirrhosis contain large numbers of activated stellate cells. The major contractile stimulus toward stellate cells is endothelin-1, whose receptors are expressed on both quiescent and activated stellate cells but whose subunit composition may vary [44]. Increased endothelin levels result from increased endothelin-converting enzyme (ECE) activity due to stabilization of the ECE mRNA [45]. Another key contractile mediator in activated stellate cells is angiotensin II,

which is synthesized by activated stellate cells in an NADPH-dependent pathway [46–48]. Locally produced vasodilator substances may oppose the constrictive effects of endothelin-1 [49, 50]. Nitric oxide, which is also produced by stellate cells, is a well-characterized endogenous antagonist to endothelin. HSC contraction employs the well-characterized machinery of Rho ROCK activation, and this can also be inhibited by adenosine.

Matrix Production

Increased matrix production is the most direct way that stellate cell activation generates hepatic fibrosis. TGF-β1 is the most potent fibrogenic factor identified to date; it stimulates the production of matrix components including collagen, cellular fibronectin, and proteoglycans [51]. Signals downstream of TGF-β converge upon a family of bifunctional molecules known as Smads, which refine or enhance TGF-β's effects downstream of its receptors [52–54]. Smads 2 and 3 elicit distinct signaling responses that favor stellate cell activation and fibrogenesis [40], whereas Smad 7 is inhibitory via activity of Id protein [55], making it an attractive molecule to utilize in antifibrotic therapies [56]. The response of Smads in stellate cells differs between acute and chronic injury to further favor matrix production [55, 57, 58]. It is important to emphasize that although most analyses of TGF-β in hepatic fibrosis have focused on its potent fibrogenic activity, it is also a highly immunoregulatory molecule [59]. However, the potential importance of TGF-β's immunomodulatory activity—via effects mediated through T cell subsets or fibrogenic cells—in mediating hepatic fibrosis has been largely overlooked.

Matrix Degradation

Quantitative and qualitative changes in matrix protease activity play an important role in ECM remodeling accompanying fibrosing liver injury. An enlarging family of matrix metalloproteinases (MMPs) (also known as matrixins) has been identified, which are calcium-dependent enzymes that specifically degrade collagens and noncollagenous substrates. In the liver, "pathological" matrix degradation refers to the early disruption of the normal subendothelial matrix, which occurs through the actions of at least four enzymes: *matrix metalloproteinase-2* (*MMP-2*) (also called gelatinase A or 72-kDA type IV collagenase); *matrix metalloproteinase-9* (*MMP-9*) (gelatinase B or 92-kDa type IV collagenase), which degrade type IV collagen; *membrane-type metalloproteinase-1 or metalloproteinase-2*, which activate latent MMP-2; and *stromelysin-1*, which degrades proteoglycans and glycoproteins and also activates latent collagenases.

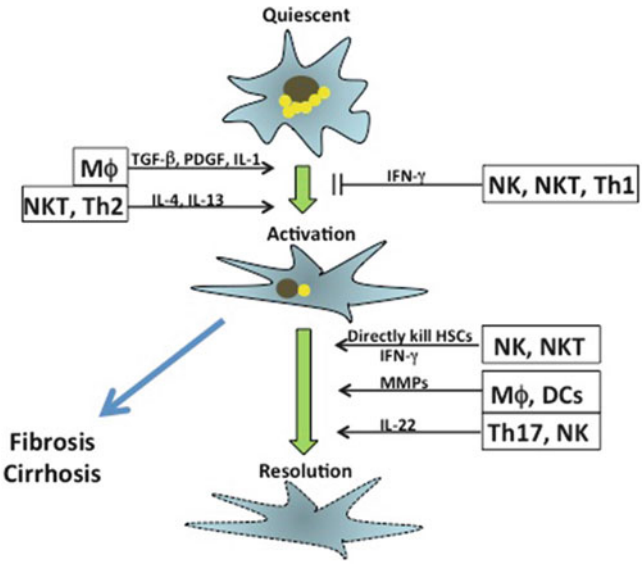

Fig. 28.2 Immune regulation of liver fibrosis. During liver injury, Mϕ are activated and produce a variety of cytokines, promoting HSC activation. NKT and Th2 cells also induce HSC activation by producing IL-4 and IL-13. In contrast, NK, NKT, and Th1 cells produce IFN-γ, thereby inhibiting HSC activation. In addition, NK, NKT, Mϕ, DCs, and Th17 cells play important roles in promoting liver fibrosis resolution by directly killing HSCs, producing MMPs that degrade matrix proteins, or producing cytokines (such as IFN-γ or IL-22) that induce HSC apoptosis or senescence

Stellate cells are a key source of MMP-2, MMP-13 in rodents, and stromelysin [60–62]. Failure to degrade the increased interstitial, or scar, matrix is a major determinant of progressive fibrosis, and Kupffer cells, or liver macrophages, have emerged as key determinants of this response. An elegant genetic model in mice has demonstrated that macrophage depletion during fibrosis progression attenuates fibrosis, whereas depletion during fibrosis regression augments fibrosis [63]. It is unknown whether these divergent responses reflect different subpopulations of macrophages or different functions of the same macrophage population (Fig. 28.2) [64]. Regardless, the findings reemphasize the potentially important role of macrophages—a key component of the hepatic immune system—in regulating fibrogenesis and point to the need for further studies of this cell type. Progressive fibrosis is associated with marked increases in tissue inhibitor of metalloproteinases TIMP-1 [65, 66] and TIMP-2 [67], leading to a net decrease in protease activity and therefore more unopposed matrix accumulation. Stellate cells are the major source of these inhibitors [33]. Sustained TIMP-1 expression is emerging as a key reason for progressive fibrosis, and its diminution is an important prerequisite to allow for reversal of fibrosis. It is unclear whether the activity of macrophages in fibrosis regression is related to interactions with or modulation of TIMP-1.

Retinoid Loss

As stellate cells activate, they lose their characteristic perinuclear retinoid (vitamin A) droplets and acquire a more fibroblastic appearance. In culture, retinoid is stored as retinyl esters, whereas stellate cells activate the retinoid released outside the cell as retinol, suggesting that there is intracellular hydrolysis of esters prior to export. However, it is unknown whether retinoid loss is required for stellate cells to activate and which retinoids might accelerate or prevent activation in vivo.

Senescence

The proposal that cells such as HSC have substantial proliferative capacity results in the obvious question of if there are limits to cell replication and what these might be. This is not simply an abstract question as it is relevant to the issue of how fibrogenesis is terminated. One obvious limit might be the phenomenon of senescence which has been described as an in vitro phenomenon, but its in vivo relevance outside of tumors was unclear. Senescence cells are in fact present in fibrotic but not healthy livers, and these are predominantly HSC [68]. Of greater interest is the fact that inhibition of senescence programs results in greater HSC proliferation and fibrosis. The importance of HSC senescence for fibrosis is also supported by the fact that senescent HSCs downregulate many of the functions associated with fibrogenesis and are themselves targets of immune surveillance and removal by NK cells. This paradigm of HSC senescence as a mechanism for limiting fibrosis is complicated by the fact that individuals with rare mutations in genes required for telomerase function have increased, not decreased, fibrosis, which is thought to be due to senescence in hepatocytes [69, 70].

Reversion

HSCs undergo activation into myofibroblasts which subsequently can undergo cell death or senescence. The question then remains: Can HSC undergo full activation into myofibroblasts and then revert into a quiescent phenotype? By lineage tracking studies, it has now been demonstrated that after resolution of fibrosis, approximately half of the activated myofibroblasts still survive and revert into a phenotype in which fibrogenic genes are downregulated and the cells have some of the characteristics of quiescent. They are however distinct from un-activated HSC as with subsequent stimuli they more rapidly undergo full activation and recover a fibrogenic phenotype [71].

Roles of HSCs in Liver Inflammation and Innate Immunity

In a healthy liver, HSCs are quiescent and store retinol, but in response to liver injury, they are activated and converted into highly proliferative, contractile myofibroblast-like cells. Activated HSCs produce many chemokines (e.g., CCL2, CCL5, CXCL2, CXCL8, CXCL10, CX3CL1), adhesion molecules (e.g., ICAM-1, VCAM-1, NCAM-1), and proinflammatory cytokines (e.g., IL-6, osteopontin). These inflammatory mediators not only promote leukocyte chemotaxis and adherence but may also induce leukocyte activation [33, 72–74]. In addition, HSCs express pattern recognition receptors such as TLR2, TLR9, and TLR4, and respond to bacterial products, which subsequently enhance the expression of chemokines and adhesion molecules on HSCs and promote liver inflammation [75]. HSCs also express TLR3 and retinoic acid-inducible gene-I (RIG-I), activation of which promotes HSCs to produce antiviral cytokines and subsequently inhibit HCV replicon replication [76], suggesting that activation of HSCs may act as important antiviral immunity to control viral hepatitis.

Coculture of NK cells and activated HSCs results in NK cell activation as demonstrated by the enhanced NK cell production of IFN-γ and NK cell degranulation [77, 78]. During activation, HSCs lose retinol and produce retinoic acid, which induces expression of retinoic acid-inducible gene 1 (Rae-1) in HSCs [79]. Rae-1, which is an important NK cell-activating ligand, promotes NK cell activation by binding NKG2D on NK cells.

In contrast to promoting inflammation and innate immunity, activated HSCs inhibit rather than promote T cell responses. HSCs have been shown to prevent graft rejection in a transplantation model by inhibiting T cell responses [80]. The immunosuppressive effects of HSCs on T cell responses are mediated by expressing the co-inhibitory molecule B7-H4 [81, 82], promoting generation of myeloid-derived suppressor cells (MDSC) [83], and inducing regulatory T cells [84, 85]. Although the immunosuppressive effects of HSCs on T cell responses have been well documented by several reports, Winau et al. provided evidence that HSCs function as APCs and present antigenic peptides to CD8(+) and CD4(+) T cells and mediate cross priming of CD8(+) T cells [85]. In this study, HSCs were isolated from the mouse liver by collagenase-pronase digestion, followed by density gradient centrifugation [85]. This study has been later questioned by the impurity of HSCs and contamination of $CD11c^+$ DCs because the flow cytometric profile in Fig. 28.1 from the original paper contained a trace of $CD11c^+$ cells [85]. By using highly purified HSCs, a recent study demonstrated that HSCs do not display an APC phenotype or

function, do not express detectable levels of MHC II or costimulatory molecules, and fail to prime naïve T cells in vitro [84].

Immunomodulation of Liver Fibrosis

The Antifibrotic Effect of NK Cells

The liver lymphocyte population contains a high percentage of natural killer (NK) cells, which play a critical role in host defense against viral infection and tumor transformation. Accumulating evidence suggests that NK cells also play a key role in controlling liver fibrogenesis by killing of activated HSCs and producing IFN-γ that induces HSC apoptosis and cell cycle arrest. Interestingly, NK cells only kill selectively early or senescence-activated HSCs but do not kill quiescent or fully activated (myofibroblasts) HSCs. This is because early or senescence-activated HSCs express upregulated NK cell-activating ligands, whereas quiescent or fully activated HSCs do not, and these activating ligands can induce NK cell activation and subsequently kill these activated HSCs [21, 68, 79, 86]. Downregulation of several NK cell inhibitory ligands in activated HSCs may also contribute to the increased susceptibility of these cells to NK cell killing [87]. The cytotoxicity of NK cells is mediated by releasing several mediators, including TRAIL, perforin, granzyme B, and Fas L. Among these mediators, TRAIL may play a major role in NK cell-mediated killing of activated HSCs [78, 86]. This is because liver NK cells express higher levels of TRAIL compared to peripheral NK cells and TRIAL receptor expression is upregulated in activated HSCs [88]. Additionally, other cytotoxicity mediators may also contribute to the NK cell killing of activated HSCs, including Fas L, granzyme B, and perforin [78, 87, 89].

NK cells likely play an important role in controlling liver fibrogenesis in human liver diseases especially viral hepatitis, for following reasons. First, human liver lymphocytes contain about 50 % NK cells, and these cells have higher cytotoxicity activities compared with peripheral NK cells. Second, peripheral NK cells are activated in the early stages of viral hepatitis although it is still debated whether liver NK cell activities are enhanced or attenuated in viral hepatitis. Third, several recent studies have shown that human NK cells can also kill activated primary human HSCs and NK cell functions correlate negatively with the degree of liver fibrosis in HCV patients [9, 21, 78, 90–92]. Fourth, the antifibrotic role of NK cells in HCV patients is augmented after IFN-α therapy [78], which likely contributes to the antifibrotic functions of IFN-α. Finally, chronic alcohol consumption and the late stages of liver fibrosis are associated with the suppression of liver NK cell functions, which contributes to the pathogenesis of alcoholic liver disease and cirrhosis [77, 93].

The Diverse Roles of NKT Cells in Liver Fibrogenesis

NKT cells are a heterogeneous group of T lymphocytes that recognize lipid antigens presented by the nonclassical MHC class I-like molecule CD1 [94]. The CD1d-dependent NKT cells include two types of cells: type I and type II NKT cells. Type I NKT cells, also known as classical or invariant NKT (iNKT) cells because they express an invariant T cell receptor-α (TCR-α) chain, comprise 95 % of hepatic NKT cells. Type II NKT cells express diverse TCRs and make up less than 5 % of liver NKT cells. NKT cells can affect target cells by releasing cytotoxic mediators (e.g., Fas ligand, perforin, TRAIL) or releasing an array of cytokines (e.g., IFN-γ, IL-4, IL-13, TNF-α, IL-17).

Liver lymphocytes are enriched in NKT cells, which seem to play complex roles in controlling liver fibrogenesis (see review [95] and references therein). NKT cells can inhibit liver fibrosis by killing of activated HSCs and producing IFN-γ [96] or enhance liver fibrogenesis by producing profibrotic cytokines (e.g., IL-4, IL-13, hedgehog ligands, and osteopontin) [97–99]. Because they have complex features, such as producing a wide variety of cytokines and cytotoxic mediators, and becoming tolerant after activation [100], NKT cells likely play multifaceted and even contrasting roles in regulating liver fibrogenesis in patients with chronic liver diseases with different phases and different etiologies. Further clinical studies are needed to clarify such complex functions of NKT cells.

The Roles of T Cells in Liver Fibrogenesis

The antifibrotic functions of Th1 cells and pro-fibrotic roles of Th2 cells have been well documented and discussed in the last version of this book and therefore are not discussed here [101, 102]. Here we focus on the roles of Th17 and T regulatory cells in liver fibrogenesis.

T helper 17 (Th17) cells are a recently identified subset of T helper cells that play significant roles in host defense against extracellular bacteria as well as in the pathogenesis of autoimmune disease [103, 104]. The functions of Th17 cells are mediated via the production of an array of cytokines, including IL-17 and IL-22. Both cytokines are elevated in several types of human chronic liver disease [105–107], but increasing evidence suggests that they play opposing roles in controlling liver fibrogenesis [108]. IL-17 is a family of six cytokines, including IL-17A, IL-17B,

IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F. These cytokines activate cells by binding to a heteromeric IL-17 receptor (IL-17R), which consists of subunits IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. For example, IL-17A and IL-17F bind to a heterodimeric receptor consisting of one IL-17RA and one IL-17RC subunit, which are expressed by most cell types in the liver. Therefore, IL-17 may affect liver fibrogenesis by targeting several types of liver cells. Indeed, it has been shown that IL-17 directly promotes HSC activation by increasing expression of collagen type I and activates immune cells such as Kupffer cells to produce pro-fibrotic cytokines (e.g., IL-6, IL-1, TNF-α, and TGF-β), thereby exacerbating liver fibrogenesis [106]. IL-22, a member of a group of IL-10 superfamily cytokines and its biological activity, is mediated by binding to a receptor complex composed of IL-10R2 and IL-22R1. Although IL-10R2 is ubiquitously expressed, the expression of IL-22R1 is restricted to epithelial cells (e.g., hepatocytes) and HSCs [109]. Immune cells do not express IL-22R1; therefore, IL-22 has little effects on immune cells. Conversely, IL-22 plays an important role in tissue repair, such as protection against hepatocyte damage and promotion of liver regeneration, thereby attenuating liver fibrosis [107, 109, 110]. In addition, IL-22 can directly induce activated HSC senescence and subsequently inhibit liver fibrogenesis [109]. Collectively, Th17 cells either promote liver fibrosis by producing IL-17 or ameliorate liver fibrosis by producing IL-22. The outcome of Th17 cells on liver fibrosis may be determined by the balance between IL-17 and IL-22 production. Under many conditions, Th17 cells preferentially produce IL-17, thereby exacerbating liver fibrogenesis [111, 112].

Regulator T cells (Treg) ($CD4^+CD25^+Foxp3^+$) are a subpopulation of T cells that suppress T cell proliferation and subsequently abrogate autoimmune disease. Liver Treg cells are elevated during viral hepatitis, which play an important role in preventing excessive immunopathological damage but may also help the virus to establish viral persistence [100]. The immunosuppressive functions of Treg cells are mediated in part via the production of IL-10 and TGF-β. Because TGF-β is a key cytokine to promote HSC activation and liver fibrosis, one may speculate that Treg cells may promote liver fibrosis via the production of TGF-β. However, a recent study showed that HCV-specific T cell-derived TGF-β inversely correlated with liver inflammation and fibrosis [113]. This study suggests that Treg cells producing TGF-β may play a more beneficial anti-inflammatory role by locally protecting against surrounding tissue damage, thereby limiting liver fibrogenesis in viral hepatitis.

The Regulatory Effect of Macrophages in Liver Fibrogenesis

Chronic liver injury is associated with infiltration of a large number of macrophages, which likely play a key role in regulating initiation, progression, and regression of liver fibrosis [114, 115]. Several lines of evidence suggest that macrophages promote liver fibrogenesis during liver injury. First, selective depletion of macrophages during ongoing injury prevents liver fibrosis [63]. Second, mice deficient in the key macrophage chemokine CCL2-CCR2 axis, which are associated with reduced monocyte/macrophage infiltration, are protected from fibrogenesis after chronic liver injury [116–118]. Third, pharmacological inhibition of hepatic monocyte/macrophage infiltration by blocking CCR2 markedly inhibits liver fibrosis in mice fed with a choline-deficient amino acid-defined (CDAA) diet [117]. Mechanistically, macrophages stimulate liver fibrosis by producing many pro-fibrotic cytokines (TGF-β, IL-1, IL-6, IL-17, IL-13, IL-4) and growth factors (e.g., PDGF) that induce HSC activation and proliferation [114, 115]. Accumulating evidence suggests that macrophages may also play an important role in promoting liver fibrosis resolution, because selective deletion of macrophages during liver injury recovery delays liver fibrosis resolution [63]. Activated macrophages are the key sources of MMPs expression. MMPs are endopeptidases, which degrade ECM proteins, such as collagen, gelatins, fibronectin, and laminin, thereby playing an important role in promoting fibrosis resolution. The different effects of macrophages on liver fibrogenesis may be carried out by divergent populations. For example, the $CD11b^{hi}F4/80^{int}Ly\text{-}6C^{lo}$ macrophage subset, which is the most abundant in livers during maximal fibrosis resolution, is different from M1 and M2 macrophages and has increased expression of MMPs, growth factors, and phagocytosis-related genes, including MMP9, MMP12, insulin-like growth factor 1, and glycoprotein (transmembrane) nmb, playing an important role in accelerating liver fibrosis resolution [119], whereas $CD11b^+F4/80^+Gr1^+$ monocyte-derived macrophages directly activate HSCs in a TGF-β-dependent manner in vitro and promote liver fibrosis [118].

The Potential Roles of Dendritic Cells (DCs) and B Cells in Liver Fibrogenesis

The liver lymphocyte pool contains a small percentage of DCs, which are poor naïve T cell stimulators but have an enhanced ability to secrete cytokines in response to TLR stimulation, acting as an important part of hepatic innate immunity. The data regarding the roles of DC's in the liver fibrogenesis have been

controversial. An earlier study suggests that after liver injury, DC promotes liver inflammation and injury by secreting proinflammatory cytokines (e.g., TNF-α) and activating HSCs, NK cells, and T cells, thereby exacerbating liver fibrosis [120]. However, a recent study suggests that DCs promote liver fibrosis resolution by producing MMP-9 [120]. Collectively, DCs, similar to macrophages, may play dual roles in controlling liver fibrosis.

In normal healthy liver, B cells comprise only a very small percentage of total liver lymphocytes. An earlier study showed that B cell-deficient ($JH^{-/-}$) mice had markedly reduced liver fibrosis after chronic CCl_4 treatment, which is mediated in an antibody- and T cell-independent manner [121]. However, the molecular mechanisms by which B cells regulate liver fibrosis remain unknown. In contrast, other studies suggest that B cells have a suppressive effect on the inflammatory response in the dnTGF-βRII model of primary biliary cirrhosis [66].

In summary, immune cells play complex roles in regulating the development and progression of liver fibrosis (Fig. 28.2). Many types of immune cells can either promote or suppress liver fibrogenesis dependent on the stage and etiologies of liver fibrosis. Understanding the functions of these immune cells in liver fibrogenesis may help us identify novel therapeutic targets for the treatment of liver fibrosis.

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Graft-Versus-Host Disease

29

Ali Raza and John M. Vierling

Key Points

1. Graft-versus-host disease (GVHD) is the major complication of allogeneic hematopoietic stem cell transplantation (HSCT).
2. GVHD causes substantial mortality and morbidity, limiting the effectiveness of HSCT.
3. Development of GVHD requires (1) immunologically competent cells in the donor graft, (2) expression of major or minor tissue antigens in the host that are not present in the donor, and (3) inability of the host to eliminate the transplanted cells from the donor.
4. Donor T cells mediate GVHD in hosts matched for major histocompatibility complex loci (HLA in human beings) by immune recognition of host minor histocompatibility antigens (MiHA).
5. Donor T cell effector mechanisms are generated in four sequential steps: (1) production of damage to host tissues caused by conditioning chemotherapy and/or irradiation, resulting in danger-associated molecular patterns (DAMPs), gut permeability to the entry of pathogen-associated molecular patterns, and generation of inflammation and the cytokines interferon-gamma (IFNγ) and proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) that result in presentation of host antigens by host antigen-presenting cells (APCs) to donor T cells; (2) host-antigen activation of donor T cells resulting in proliferation and differentiation of donor effector T cells; (3) injury of target tissues and organs mediated by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and TNFα; and (4) continued reactivation and augmentation of effector mechanisms by peptide antigens processed from injured target organs and inflammatory cytokines.
6. Tissue-specific expression of allogeneic antigen, cytokines, chemokines, and adhesion molecules skews the immunopathology to a limited number of target organs, principally skin, intestine, and liver.
7. Prevention of GVHD is not yet possible.
8. Acute and chronic GVHD are associated with significant morbidity and mortality, despite immunosuppressive therapies.
9. GVHD caused by donor T cells within transplanted livers manifests as injury to skin, intestine, and recipient bone marrow but spares the donor liver.

Introduction

Allogeneic HSCT representatives are the only curative option for patients with numerous malignant and nonmalignant diseases of the lymphatic and hematopoietic system [1]. Development of a competent immune system from the engraftment of HSCT is mandatory for clinically successful transplant. Failure to reconstitute a competent immune system from the donor graft is associated with the risk of infection with opportunist organisms (viruses, bacteria, fungi, and protozoa) and relapse of malignant diseases due to failure of the graft to destroy surviving malignant host cells. All recipients undergo ablative chemotherapy or irritation to eliminate the recipients' preexisting lymphoid immunity and lymphoid malignancies. Lymphoid immunity following HSCT engraftment is derived from the newly engrafted donor stem cells and mature B and T lymphocytes present in the graft. Polyclonal, polyantigen-specific engraftment after HSCT reduces the risks of infection and relapse of malignancy but increases the risk of allogeneic reactions to host antigens, resulting in GVHD. Attempts to deplete HSCT grafts of T cells to reduce the risk of GVHD resulted in higher rates of infection and relapse of malignancies. Thus, optimal engraftment

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requires prophylactic immunosuppression to prevent or retard development of GVHD.

The goals of this chapter are threefold. The first is to summarize the physiology of HSCT engraftment. The second is to review current understanding of the immunopathogenic mechanisms of aGVHD and cGVHD and the therapeutic options to inhibit specific mechanisms. The third is to review the clinical aspects of acute and chronic GVHD (aGVHD and cGVHD, respectively) with an emphasis on liver pathology, resulting from HSCT or, rarely, orthotopic liver transplantation (OLT).

Physiology of HSCT Engraftment

Successful HSCT engraftment leads to the development of common lymphoid progenitors of B, T, and NK cells [2]. These donor progenitors migrate to the host thymus where T cells proliferate and differentiate under the control of both cell surface molecules and cytokines expressed by thymic stromal cells. Donor T cells are positively selected and expanded if they express T cell receptors (TCRs) with specificity for peptide antigens presented by the host's major histocompatibility complex (MHC, designated HLA in human beings) molecules expressed on thymic epithelial cells. Positively selected T cells avidly reacting with self-/host antigens undergo negative selection (apoptosis) induced by interaction of their TCRs with self-/host antigens presented by activated host APCs. The progeny derived from the donor's common lymphoid progenitors undergoing host thymic selection express the differentiation markers of mature CD3/CD4 or CD3/CD8 T cells and are detectable in peripheral blood approximately 3 months following HSCT. In the peripheral lymph nodes, these donor-derived T cells undergo further expansion and differentiation resulting from activation of their TCRs with specific antigens.

Donor grafts also contain mature antigen-specific T cells, naïve T cells, and common lymphoid progenitors. Both antigen-specific and naïve T cells can proliferate in host peripheral lymphoid tissue that retain host APCs after ablation of host lymphocytes by conditioning chemotherapy and/or irradiation. Thus, the ultimate graft contains both mature T cells derived from proliferation of infused T cells and donor T cells derived from common lymphoid progenitor cells that have undergone thymic conditioning. When engraftment of mature T cell population is greater than engraftment from common lymphoid progenitors, the repertoire of TCRs is more limited but more alloreactive. Recipients of such grafts have a greater risk of GVHD but a lower risk of relapsing malignancy.

Elimination of mature donor T cells from grafts before HSCT effectively reduces GVHD, but leaves the host vulnerable to increased risks of infections and relapse of malignancy [1]. Even if grafts contain optimal amounts of mature T and B cells and common progenitor lymphocytes to engage in host thymic selection, grafts do not have the robust TCR repertoire of a normal adult. This results in inadequate responses to environmental pathogens and defective immunoregulation of immune responses.

Donor common lymphocyte progenitors also contain B lymphocytes that differentiate in the eradicated host bone marrow [2]. B cells expressing cell surface IgM migrate to peripheral lymphoid tissue where they can engage specific antigens and become antibody secreting B cells and plasma cells with the help of cytokines secreted by antigen-specific CD T cells. Defects in the antigen-specific TCR repertoire of donor CD4 T cells may cause corresponding defects in specific antibody production after HSCT.

NK cells are the earliest identifiable circulating donor lymphoid cells after HSCT and reach normal levels within 3 months [2]. NK cells express several receptors: inhibitory receptors (killer Ig-like receptors, KIRs), lectin-like CD94/NKG2 heterodimers, and activating receptors. Following HSCT, NK cells differentially express these receptors with majority expressing CD94/NKG2 and only a minority expressing KIRs. NK cells normalize their differential expression of receptors (KIRs>CD94/NKG2) 1–3 years after HSCT.

HLA Matching: The human MHC, designated HLA, is located on the short arm of chromosome 6 and expresses class I, II, and III gene products [1, 3, 4]. 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 or mismatch, serological and molecular allelic comparisons of donor and recipient are performed to quantify the degree of matching.

Minor Histocompatibility Antigens: MiHAs are produced by the degradation of normal cellular proteins into antigenic peptides [1, 5]. Intracellular proteins are processed in proteosomes and presented in antigen-binding grooves of HLA class I molecules on the cell surface. In contrast, peptides derived from the extracellular environment are processed by lysosomes and presented by HLA class II molecules. In monozygotic twins or recipients of autologous HSCT, both the graft and host are HLA-identical. Yet, MiHAs may differ between donor and recipient [6]. This may result from expression of genes active in the recipient, but not in the donor, or as a result of mutations generating 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 produced by the active Y chromosome in males. The H-Y

MiHA is recognized by female donor cells [1, 7]. 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 (GVL) reactions after HSCT [6, 8]. MiHAs may be widely expressed among different cell types or expressed uniquely within specific tissues. Tissue-specific expression of MiHAs has been postulated as a reason for the restriction of target organ involvement in GVHD. Despite using HLA-identical grafts and optimal post-HSCT prophylactic immunosuppression, presentation of host MiHAs by host professional APCs to donor T cells results in aGVHD in ~40 % of such recipients [6, 9].

Non-HLA Genes: Genetic polymorphisms in non-HLA genes may also influence the incidence and/or severity of aGVHD [1, 10]. Examples may include polymorphisms in KIRs, cytokines, and nucleotide-binding oligomerization domain containing 2 (NOD2) genes [11]. Polymorphisms in KIRs dictate whether a receptor has an inhibitory or an activating potential [12].

Cytokine Genes: Polymorphisms in the inflammatory cytokine TNFα, expressed in recipients as well as donors, have been implicated in GVHD [1]. These include TNFd3/d3 in the recipient, TNF863 and TNF857 in donor and/or recipients, and TNFd4, TNFα-1031C, and TNFRII-196R in the donors [13]. The three subtypes of IL-10 gene promoters in recipients dictate high, intermediate, and low production of the anti-inflammatory IL-10 [14]. These differences have been associated with differences in aGVHD in sibling-donor HLA-matched allogeneic HSCT. Polymorphisms 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 polymorphisms have been associated with decreased and increased rates of aGVHD, respectfully [15]. NOD2/caspase-activating recruitment domain 15 (CARD15) gene polymorphisms in recipients and donors have been associated with intestinal GVHD and all-risk mortality after HSCT from either related or unrelated donors [11, 16]. The relative effect of non-HLA gene polymorphisms in GVHD is likely to differ with variables such as the related versus unrelated source of the donor stem cells, degree of HLA matching and source of the graft (core blood versus bone marrow versus peripheral blood stem cells), and the type and intensity of recipient conditioning.

Graft-Versus-Host Disease

In 1966, Billingham [17] defined three absolute requirements for the development of GVHD: (1) the donor graft must contain immunologically competent cells; (2) the recipient must express tissue antigens that are not present in the donor; and (3) the recipient must be incapable of mounting an effective response to destroy transplanted donor cells. These criteria indicated a risk for GVHD in a variety of clinical settings when immunosuppressed or immunoincompetent hosts received tissues or solid organs containing immunocompetent donor cells, such as blood products containing leukocytes, bone marrow, or passenger leukocytes within solid organ allografts. Sackstein recently proposed adding the requirement of chemokine-mediated trafficking of activated donor T cells to target tissues to Billingham's criteria [18].

Graft-Versus-Leukemia Response

The GVL response results from the alloimmune donor T cell attack against leukemia cells in the host [19, 20]. While initially the evidence was circumstantial, the use of donor leukocyte infusions to treat relapses of leukemia after allogeneic HSCT has provided proof for the GVL effect. The initial patients with relapsed chronic myelogenous leukemia who are treated with IFNα and donor leukocyte infusion from the original donors of the failed HSCT achieved complete remission [21]. Subsequent studies achieved remission rates of 60–80 % for patients treated with donor leukocytes for relapsing chronic myelogenous leukemia [20]. The GVL response calls attention to the need for a balance between preservation of donor alloreactions capable of preventing relapse of malignant disease, while minimizing the risk of GVHD.

Overview of Acute and Chronic Graft-Versus-Host Disease

GVHD was originally subdivided into acute or chronic based solely on the time of onset after HSCT: acute <100 days and chronic >100 days [22]. Distinction between aGVHD and cGVHD is now based on validated NIH consensus working group criteria (Table 29.1) [22, 23]. The category of aGVHD includes two subcategories: (1) classic aGVHD and (2) persistent, recurrent, or late onset aGVHD defined as manifestations of aGVHD occurring >100 days or extending beyond 100 days in the absence of distinctive manifestations of cGVHD. Similarly, cGVHD includes two subcategories: (1) classical cGVHD and (2) an overlap syndrome with coexisting clinicopathological features of both aGVHD and cGVHD. In the absence of clinical or histologic features of cGVHD, new onset, recurrent, or continuation of persistent aGVHD should be classified as aGVHD regardless of the time after HSCT. Accurate classifications are increasingly important for stratification of randomized controlled therapeutic clinical trials.

The principal target organs in both aGVHD and cGVHD are skin, intestine, and liver [1, 24–26]. However, clinical manifestations of cGVHD are more protean than those of aGVHD and can involve the lacrimal glands, oral mucosa, salivary glands, lung, female genital tract, musculoskeletal system, hematological system, and immune system [27].

Pathogenesis of Acute GVHD

aGVHD represents an exaggeration of normal inflammatory mechanisms of donor lymphocytes functioning appropriately within a host with variable degrees of HLA mismatch and expression of MiHAs [1]. Importantly, donor lymphoid engraftment occurs in the setting of host inflammation and tissue/organ injury resulting from underlying disease, prior infections, and the intensity of the pre-HSCT conditioning regimen. Cumulatively, these factors can contribute to production of DAMPs and pathogen-associated molecular patterns (PAMPs) which activate innate immunity and promote the activation and proliferation of the grafted donor immune cells [28]. Based on experimental models and human studies, the pathogenesis of aGVHD can be conceptualized in four sequential steps (Fig. 29.1): (1) activation of host APCs and processing and presentation of host peptide antigens; (2) activation of donor T cells with subsequent proliferation, differentiation, and migration into target tissues; (3) destruction of target tissues; and (4) continued reactivation of donor T cells by host antigens released from injured, inflamed tissues.

Table 29.1 NIH consensus classification of acute and chronic graft-versus-host disease

Classification	Time of onset after HSCT or DLI	Features of acute GVHD	Features of chronic GVHD
Acute GVHD			
1. Classic	≤100 d	Present	Absent
2. Persistent, recurrent, or late onset	>100 d	Present	Absent
Chronic GVHD			
1. Classic	No time limit	Absent	Present
2. Overlap syndrome	No time limit	Present	Present

GVHD graft-versus-host disease, *HSCT* hematopoietic stem cell transplantation, *DLI* donor lymphocyte infusion, *d* day. Adapted from reference [22]

Step 1: Activation of Host Antigen-Presenting Cells

Activation of host APCs is a cumulative process generated by the underlying disease, prior infections, tissue and organ injury and inflammation generating DAMPs and activation of inflammasomes, intestinal permeability to pathogen-associated molecular patterns (PAMPs), and the cytotoxicity of pre-HSCT conditioning regimen [1, 28–30]. Destruction of host tissues, including malignant cells, also results in production of proinflammatory cytokines (e.g., IL-1, IL-6, and TNFα), IFNγ, chemokines and increased expression of adhesion molecules, HLA molecules, and costimulatory molecules (e.g., CD80/86 [B71/B72] and CD40) on host APCs. Increased intestinal permeability, caused by the conditioning regimen, facilitates the entry of bacterial and fungal PAMPs

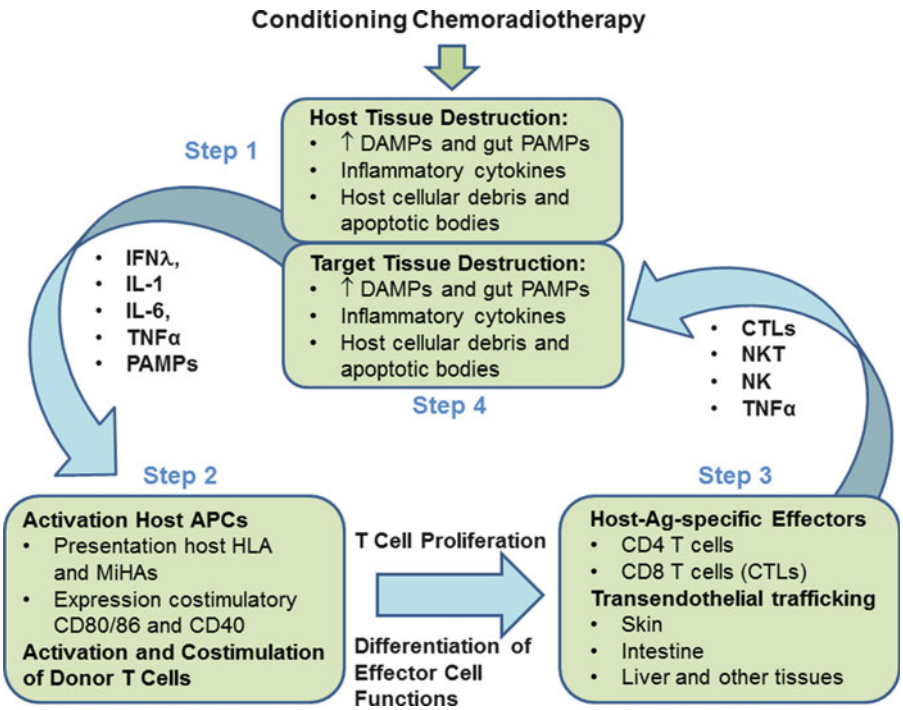

Fig. 29.1 Proposed immunopathogenesis of graft versus host disease. See text for detailed explanation. *DAMPs* danger associated molecular patterns, *PAMPs* pathogen associated molecular patterns, *IL* interleukin, *TNFα* tumor necrosis factor-alpha, *APCs* antigen presenting cells, *MiHAs* minor histocompatibility antigens, *CTLs* cytotoxic T lymphocytes

into portal venous blood flowing to the liver. PAMPs then interact with pattern recognition receptors (PPRs), such as Toll-like receptors (TLR), on cells of the immune system as well as tissues and organs inducing alteration in gene expression. In addition, the microbiome of patients prior to HSCT most often has been altered significantly by broad-spectrum antibiotic and antifungal therapies [31]. Recent evidence indicates that the composition of the microbiome contributes to the pathogenesis of GVHD [32]. This may in part be due to the fact the secondary lymphoid tissues of the gastrointestinal tract appear to be the initial site of interaction between activated host APCs and donor T cells. TLR recognition of viral DNA on activated host APCs may explain why viral infections with cytomegalovirus (CMV) are associated with an increased incidence of GVHD [33]. Two approaches have been taken to reduce the extent of host APC activation after HSCT. The first has been the reduction in the intensity of conditioning regimens [34–36], while the second has been the inclusion of non-hematopoietic stem cells, principally mesenchymal stem cells or stromal cells, that are capable of reducing donor allogeneic T cell responses [37, 38].

Step 2: Activation of Donor T Cells

Activation, proliferation, and differentiation of donor T cells in response to antigens presented by activated host APCs is referred to as the graft-versus-host reaction. Host APC expression of HLA-mismatch molecules can lead to either direct or indirect donor T cell reactions; however, with high degrees of HLA matching, a direct reaction is minimized. The direct reaction refers to donor T cell cytotoxicity mediated by populations of mature donor T cells within the graft that occur without the need for host-HLA antigen activation or proliferation. In contrast, the indirect reaction of donor T cells requires activation by the presentation of host peptide antigens derived from processed HLA-mismatch molecules in the antigen-binding groves of host (and later also donor) APCs. Similarly, processed and presented peptide MiHAs are presented by host APCs to donor T cells. Proliferation and differentiation of donor effector cell function requires costimulation of activated donor T cells through the binding of CD28 on the T cell with CD80 and CD86 (aka B7.1 and B7.2) on the APC or binding of CD150 (aka CD40-ligand) on the T cell with CD40 on the APC. In the absence of adequate costimulation, the activated T cells may exhibit antigen-specific anergy. Step 1 leads to activation of host APCs that results in increased expression of CD80/86 and CD40 costimulatory molecules, favoring polyclonal proliferation and differentiation of donor T cells into effector cells. In experimental models, donor CD4 T cells induce aGVHD to MHC class II molecules, while CD8 T cells induce aGVHD to MHC class I molecules. In HLA-matched HSCT, both CD4 and CD8 T cell subsets react to host MiHA. Thus, GVHD can occur even in HLA-identical HSCT.

Regulatory T cells (Tregs) suppress antigen-specific proliferation of activated donor T cells and can prevent GVHD in animal models [39–42]. Tregs secrete anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGFβ) and inhibit APCs by direct contact. NKT cells of both the donor and host can modulate aGVHD. Surviving host NKT cells can suppress acute GVHD in an IL-4, Th0-dependent manner. Conditioning with total lymphoid irradiation preserves host NKT cells and ameliorates GVHD [43, 44]. Donor NKT cells enhance cytotoxic GVL responses while reducing GVHD in experimental models [45].

aGVHD is associated with production of large amounts of Th1 cytokines (IFNγ, IL-2, and TNFα) [46–49]. Since IL-2 is the primary mitogenic cytokine for T cell proliferation, clinical prophylaxis and therapeutic approaches aim to reduce IL-2 production (cyclosporine and tacrolimus) [50, 51] or IL-2 signaling (sirolimus) [52]. Paradoxically, such suppression may adversely affect the generation and maintenance of beneficial CD4, CD25 Tregs as an unintended consequence [53]. IFNγ can amplify GVHD by several mechanisms [47]. These include increasing the sensitivity of APCs to PAMPs, such as LPS, accelerating intracellular signaling induced by DAMPs, increasing expression of HLA molecules, adhesion molecules, and chemokine receptors necessary for tissue trafficking of activated donor T cells. IFNγ may also amplify GVHD through direct damage to the intestine and skin [54]. In contrast, IFNγ may suppress GVHD by promoting apoptosis of activated donor T cells [55, 56]. The immunosuppressive cytokine IL-10 may also downregulate acute GVHD [14]. In contrast, TGFβ is immunosuppressive for acute GVHD but can exacerbate chronic GVHD, possibly through promotion of fibrogenesis [57].

Step 3: Cellular and Inflammatory Effector Functions

Activation of donor CD4 T cells skews toward a Th1 phenotype, providing the helper functions for the proliferation and differentiation of CD8 T cells, which function as antigen-specific CTLs. Donor NK cells also mediate cytotoxicity by recognition of stressed or injured target cells expressing reduced amounts of KIR on their membranes. Circulating inflammatory mediators include IL-1, IFNγ, TNFα, and nitric oxide. Circulating and cellular effector cells act synergistically to amplify local tissue injury and promote inflammatory target cell destruction.

CTLs and NK cells are the primary effector cells of aGVHD. CD8 CTLs mediate target cell lysis primarily through the CD95 (aka Fas)/CD95L (CD95-ligand, aka FasL) pathway in hepatic GVHD. In contrast, CD8 CTLs mediate cytolysis of enterocytes and keratinocytes through a perforin-granzyme pathway [58, 59]. Resistance of hepatocytes to perforin-granzyme-mediated cytolysis [60] may partially explain the paucity of hepatocyte cytolysis in hepatic aGVHD [61].

Table 29.2 Expression of chemokines and chemokine receptors in skin, intestine, and liver

	Skin	Intestine	Liver
Chemokines	CXCL 1, 2, 9, 10, 11	CXCL 9,10,11,16	CXCL 1,9,10,11,16
	CCL2,5,6,7,8,9,11,12,17,19,20,27	CCL2,3,5,20	CCL2,3,5,20
	XCL1	XCL1	XCL1
		CX_3CL1	
Chemokine receptors	CXCR3	CXCR3,6	CXCR2,3,6
	CCR1,2,4,5	CCR1,2,5,6	CCR1,2,5
	CCR10	CX	XCR1
	XCR1	CX_3R1	

A prerequisite for tissue and organ damage in aGVHD is transendothelial migration of differentiated effector cells from lymphoid tissues into blood and from blood into the target tissues or organs [18, 62]. Chemokine gradients produced in the target tissues by inflammatory cells and activated target tissues control the migration of activated donor T cells by activating adjacent vascular endothelial cells to display the tissue chemokines and adhesion molecules necessary to interact with chemokine receptors and adhesion molecules on circulating, activated circulating T cells. A variety of chemokines and chemokine receptors are differentially expressed in the skin, intestine, and liver (Table 29.2). Tissue and organ chemokine gradients mediate the trafficking of the cellular effector cells to target organs during experimental GVHD [62–64]. Expression of integrins, for example, α4, β7, and its ligand MadCAM-1 is involved in the homing of donor T cells to Peyer's patches during intestinal GVHD [65]. Studies of the human biliary disease, primary sclerosing cholangitis, also have indicated a potential role of MadCAM-1 in the homing of effector T cells to the peribiliary space [66].

Step 4: Feedback Loop Generated by Target Tissue Destruction

The migration of activated cytolytic effector cells into target tissues, coupled with production of TNFα, results in destruction of host cells within target organs of skin, intestine, liver, and others. Generation of apoptotic bodies, cellular debris, and chronically inflamed sites with high concentrations of cytokines and chemokines recapitulates the processes initiated by conditioning chemoradiation therapy in step 1, except that the effector mechanisms confer a more tissue-specific pathology. Thus, a positive feedback loop for continued activation of donor T cells is established, potentially resulting in the generation of effector cells reacting against a broader array of host MiHAs expressed by keratinocytes, enterocytes, and biliary epithelial cells through epitope determinant spreading.

Pathogenesis of Chronic GVHD

The pathogenesis of cGVHD is less well understood than that of aGVHD. The NIH redefinition of cGVHD as a clinicopathological entity that may occur as a continuation or recrudescence of aGVHD, as an overlap syndrome, or as a distinct de novo process requires reassessment of putative pathogenic mechanisms [22]. In contrast to aGVHD, experimental models of cGVHD are less numerous and less reflective of human cGVHD [67]. Specifically, animal models do not adequately reflect the prolonged kinetics of the immunopathology or the protean end-organ manifestations of human chronic GVHD. Some results suggest that cGVHD results from defective thymic negative selection, leading to generation of autoreactive clones that escaped peripheral tolerogenizing mechanisms [68]. This would imply that donor T cell reactions in the target tissues are against non-polymorphic peptide antigens presented by matched HLA class II and class I molecules, in addition to or instead of MiHAs. Reactivity against a common determinate of MHC class II molecules shared by donor and host in another cGVHD model also suggested autoreactivity [69]. The autoreactive cells in cGVHD were associated with evidence of thymic dysfunction, which can result from age-related involution, destruction by the conditioning regimen, or aGVHD [70].

Another possibility is that donor T cells chronically stimulated with MiHAs might evolve to mediate syndromes resembling autoimmune diseases (see Step 4 in pathogenesis) [1]. It is also possible that sustained target tissue and organ injury mediated by donor T cells leads to activation of additional T cell clones reacting against MiHA epitopes other than those that were initially immunodominant through epitope determinate spreading. It is also conceivable that chronic stimulation of the donor T cell repertoire in cGVHD might lead to disproportionate secretion TGFβ, promoting fibrosis within target organs characteristic of cGVHD. Failure to renew and maintain antigen-specific Tregs might promote donor T cell reactions against host antigens that were previously inhibited.

Replacement of host APCs within lymphoid tissues and solid organs by donor-derived dendritic cells and activated macrophages (e.g., Kupffer cells in hepatic sinusoids) could also promote processing and presentation of both host non-polymorphic and MiHA peptides [71]. Abrupt changes in the immunoregulatory milieu might facilitate loss of T cell clonal energy initially produced by inadequate costimulatory signaling during initial activation of donor T cells following HSCT.

The pathogenesis of cGVHD may also reflect a pathogenic contribution of donor alloreactive B cells as APCs and producers of antibodies specific for MiHAs [72, 73]. Anti-MiHA antibodies could mediate injury directly by binding to target cells or by promoting antibody-dependent cellular cytotoxicity of the target cell. APC processing of immune complexes of containing MiHAs could also promote generation of larger numbers of CD8 CTLs. The efficacy of rituximab in the treatment of GVHD supports a role for antibody-mediated mechanisms [72, 74].

Several other factors might play a role in the pathogenesis of cGVHD. Changes in the production of chemokines might control the timing of trafficking of donor effector cells into target tissues in cGVHD (Table 29.2). Evidence that the microbiome modulates effector functions in GVHD suggests that the microbiome can prevent or promote development of cGVHD [32]. It is interesting to speculate that alteration of the microbiome by aGVHD and its therapy may create a risk for cGVHD. Injury to the vascular endothelium in target tissues might also promote cGVHD by reducing the microvasculature of target organs and promoting ischemic fibrogenesis [75]. In addition, endothelial cells have been shown to have immunoregulatory functions in animal models of aGVHD, suggesting that loss of such functions might represent a risk factor for cGVHD [75].

Restriction of Tissue-Specific Injury to Skin, Intestine, and Liver

In both acute and chronic GVHD, the primary target tissues are cutaneous hepatocytes, intestinal epithelial cells in the villous crypts, and biliary epithelial cells lining small to medium caliber interlobular bile ducts [24, 61]. Predilection of donor effector T cells for these tissues exists even in models mismatched for MHC class I or II, where multiple tissues should potentially be targets due to ubiquitous expression of allogeneic MHC molecules.

While no unifying explanation of target restriction has been identified, three non-mutually exclusive mechanisms have been postulated. The first postulate is that the tissue specificity results from immunodominant MiHAs expressed exclusively by keratinocytes, crypt enterocytes, and the biliary epithelia lining small to medium caliber ducts. A potential human candidate MiHA is UGTIIB17 (representing a gene deletion in the UDP-glycosyltransferase 2 family), which is expressed in liver, pancreas, and enterocytes of the small bowel and colon [5]. Second, skewing of the Vα and/or Vβ chains of the TCRs of donor T cells infiltrating target tissues has been identified in humans and mice [76, 77]. A biased TCR Vβ indicates that the infiltrating donor T cells are oligoclonal, which suggests reactivity against only a small number of antigens. After induction of GVHD, patterns of tissue expression of MiHAs were qualitatively and quantitatively altered in the target organs of GVHD in mice in accord with both postulates [78]. The second postulated mechanism is that qualitative or quantitative differences in the secretion of chemokines by the target tissues result in localization of donor effector T cells and cytotoxic cytokines adjacent to the target cells. Recent evidence indicated difference in chemokine expression within the target tissues (Table 29.2) and localization of donor effector T cells bearing chemokine receptors for these ligands [5, 66, 79]. The third mechanism is that keratinocytes, intestinal epithelial cells, and biliary epithelial cells respond to IFNγ and proinflammatory cytokines IL-1, IL-6, and TNFα by secreting chemokines and polarizing Th1 cytokines, such as IL-12, to further activate effector cells. Immunohistochemical studies of biopsies and studies of immortalized biliary epithelial cells in a murine model of cGVHD support the latter mechanism [79, 80]. Further studies are required to define mechanisms restricting the target tissues and cells in GVHD.

Clinical Features of Acute GVHD

Based on clinical and biochemical features, patients with acute GVHD can be staged and graded [1]. Table 29.3 summarizes the clinical and biochemical features used to stage involvement of the target organs of skin, intestine, and liver, and Table 29.4 shows the grading of severity. Onset of aGVHD between 14 and 35 days after HSCT is common with current conventional high-intensity conditioning regimens. Clinically, aGVHD most commonly presents as a rash, while intestinal or hepatic manifestations are rarely the first or only signs [25]. Rarely, a hyperacute form of GVHD occurs in patients with significant HLA histoincompatibility or those receiving grafts containing larger numbers of T cells or those without adequate GVHD prophylaxis [81]. Hyperacute GVHD manifests as fever, diffuse erythroderma, and desquamation and often edema. Onset is generally 1 week after HSCT, and it is often fatal. Hyperacute GVHD must be distinguished from "engraftment syndrome," which also presents in the first 1–2 weeks after HSCT as fever, rash, and fluid retention [82]. It is believed to be mediated by high levels of circulating cytokines during donor engraftment and responds promptly to steroids.

Table 29.3 Clinical manifestations and staging of acute graft-versus-host disease

Organ	Clinical manifestations	Staging
Skin	Erythematous, maculopapular rash involving palms and soles, may become confluent	Stage 1: <25 % rash
	Severe disease, bullae	Stage 2: 25–50 % rash
		Stage 3: generalized erythroderma
		Stage 4: bullae
Liver	Painless jaundice with conjugated hyperbilirubinemia and increased alkaline phosphatase	Stage 1: bili 2–3 mg/dL
		Stage 2: bili 3.1–6 mg/mL
		Stage 3: bili 6.1–15 mg/dL
		Stage 4: bili >15 mg/dL
Gastrointestinal tract	Upper: nausea, vomiting, anorexia	Stage 1: diarrhea >500 mL/d
	Lower: diarrhea abdominal cramps, distension, ileus, bleeding	Stage 2: diarrhea >1,000 mL/d
		Stage 3: diarrhea >1,500 mL/d
		Stage 4: ileus, bleeding

Table 29.4 Grading severity of acute graft-versus-host disease

Overall grade	Skin	Liver	Gut
I	1–2	0	0
II	1–3	1 and/or	1
III	2–3	2–4 and/or	2–3
IV	2–4	2–4 and/or	2–4

Cutaneous Manifestations

The skin is the most commonly affected organ in acute GVHD [83]. It is most frequent after mild ablative conditioning. It presents often as a pruritic maculopapular rash, often involving the palms, soles of the feet, and ears. Severe cases can progress to a full-body erythroderma associated with bullae and desquamation. The rash may spread but most often spares the scalp. Patients report sensations of burning, tightness of the skin, or pruritus. Severe rashes may form bullae and ulcerate. Cutaneous biopsy is mandatory to confirm the diagnosis and eliminate alternatives such as drug-induced hypersensitivity or viral infections. aGVHD is associated with apoptosis at the base of dermal crypts, dyskeratosis, exocytosis of lymphocytes, lymphocytic infiltrates adjacent to dyskeratotic epidermal keratinocytes, and lymphocytic infiltration of the dermal vasculature.

Gastrointestinal Manifestations

aGVHD can involve any portion of the upper or lower gastrointestinal tract [84, 85]. Clinically, gastrointestinal aGVHD manifests as anorexia, nausea, vomiting, diarrhea (with or without blood), abdominal pain, or ileus. Vomiting without nausea is a feature of aGVHD. In contrast, gastroparesis is not associated with GVHD. Diarrhea is secretory and/or exfoliative, and blooding from mucosal ulcerations may occur. Such ulcerations increase the risk of potentially fatal systemic bacterial or fungal infections. The differential diagnosis of diarrhea includes CMV or herpes virus infections, parasitic infections, and *Clostridium difficile* toxin-induced pseudomembranous colitis.

Mucosal biopsies are required to validate the diagnosis [85]. Characteristic histologic features of gastrointestinal aGVHD include apoptotic bodies in the base of intestinal crypts, crypt abscesses, loss of crypts, and the flattening of the villi.

Hepatic Manifestations

aGVHD of the liver manifests as hyperbilirubinemia and increased serum alkaline phosphatase and aminotransferase levels [1]. In severe cases, coagulopathy and hepatic failure with ascites and hepatic encephalopathy can occur. Acute hepatic GVHD must be distinguished from hepatic complications common in patients after HSCT. Certain conditioning regimens or chemotherapies for leukemia cause sinusoidal obstructive syndrome (SOS, formerly known as veno-occlusive disease), which is clinically characterized by early onset of ascites and right upper quadrant pain due to the obstruction of hepatic arterial and portal venous flow within the sinusoids [86]. Other differential diagnostic considerations are drug-induced liver injury, viral infections (especially non-hepatotropic viruses such as CMV or herpes viruses), and cholestasis associated with the systemic inflammatory response syndrome with sepsis or cholestasis caused by total parenteral nutrition.

Liver biopsy is required to establish or refute aGVHD as a cause of abnormal liver biochemistries. The characteristic histologic features of hepatic GVHD are lymphocytic cholangitis involving the proximal small to medium caliber bile ducts, lymphocytic infiltration of the portal tracts, and endothelialitis of the portal and/or terminal hepatic veins.

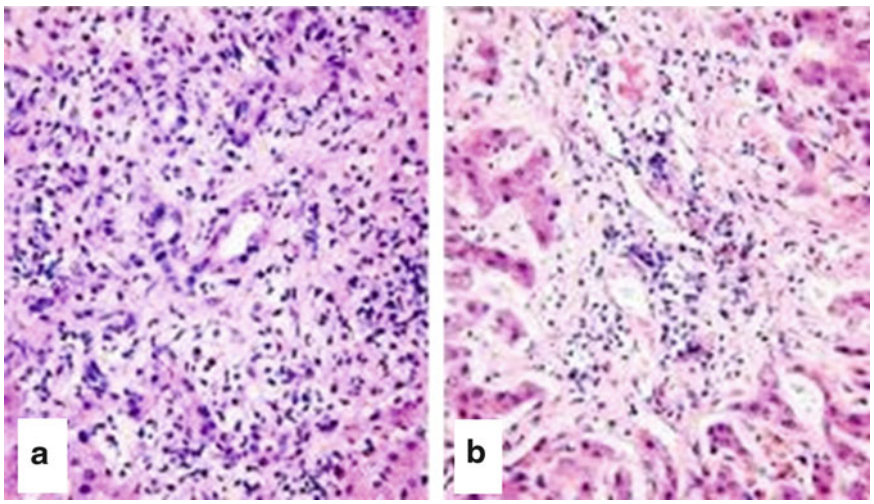

Fig. 29.2 Histopathology of hepatic graft versus host disease. (**a**) *Late onset acute GVHD*, *day123*. The portal tract is expanded with a mixed infiltrate of lymphocytes and scattered eosinophils. The interlobular bile duct exhibits lymphocytic infiltration of the biliary epithelium, segmental loss of nuclei, cytoplasmic vacuolization and nuclear dyspolarity. Ductular proliferation at the margin of the portal tract also shows features of GVHD (original magnification ×250). (**b**) Refractory untreated GVHD day 350. The portal tract contains a lymphoplasmacytic infiltrate, loss of the interlobular bile duct (ductopenia) and fibrosis (original magnification ×250). Photomicrographs adapted from reference [24] and used by permission

The lymphocytic infiltration of the interlobular bile ducts results in apoptotic segmental loss of cholangiocytes, which can culminate in the destruction of the interlobular duct (Fig. 29.2) [24, 61]. Since many patients with abnormal levels of bilirubin, alkaline phosphatase, and aminotransferases do not undergo liver biopsy, the exact incidence of hepatic aGVHD remains unclear.

Clinical Features of Chronic GVHD

cGVHD is a major cause of late mortality after HSCT that is not attributable to relapse of malignant disease [87]. Table 29.1 summarizes differences between aGVHD and cGVHD based on the NIH consensus statement [22]. The risk of cGVHD is substantially increased in patients with any prior manifestation of GVHD [26]. The incidence of cGVHD ranges from 30 to 60 % with bone marrow-derived HSCT and may be higher with transplantation of peripheral blood stem cells. The NIH consensus criteria for diagnosis and grading of severity were recently validated but had limited value in predicting clinical outcomes [23].

The clinical manifestations of cGVHD are more protean than those of aGVHD [88, 89]. Significant dysfunctions of the immune system increase 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 [90]. Thus, clinicians must diligently assess HSCT recipients on a serial basis and consult specialists whenever suspicious of a finding compatible with cGVHD [88, 89].

Cutaneous Manifestations

Skin manifestations of cGVHD differ from those observed in patients with aGVHD [26, 89]. Lesions resembling diffuse lichen planus (papulosquamous dermatitis, plaque formation, desquamation, varied pigmentation, and vitiligo) occur in up to 80 %. Alopecia and onychodysplasia may occur as a result of the destruction of dermal appendages. Severe cutaneous changes mimic scleroderma with induration and tightening of the skin, joint contractures, cutaneous atrophy, and chronic ulcerations. Each of these manifestations has counterparts in human autoimmune diseases, implicating dysregulation of the donor-derived immune system in the pathogenesis.

Gastrointestinal Manifestations

Gastrointestinal symptoms in patients with cGVHD may mimic a variety of intestinal diseases [26, 89]. In addition to manifestations observed in aGVHD, such as nausea, vomiting, and diarrhea, patients may present with signs and symptoms 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 [91].

Hepatic Manifestations

Hepatic disease associated with cGVHD classically presents as cholestatic liver test abnormalities with elevated serum alkaline phosphatase, gamma-glutamyltransferase, and variable elevations of total and direct bilirubin and aminotransferases [26]. Comparison of histopathological findings has shown that the features are similar in aGVHD and cGVHD [61]. However, the chronicity and severity of lymphocytic cholangitis results in progressive senescent changes in the biliary epithelia and destruction of the small to medium caliber proximal interlobular bile ducts (Fig. 29.2) [22, 92, 93]. Progressive destruction of interlobular bile ducts results in obstruction of bile flow and hepatocellular cholestasis. Periportal fibrosis may accompany ductopenia, but progression to biliary cirrhosis is rare.

Ocular Manifestations

Destruction of the lacrimal gland results in keratoconjunctivitis sicca with symptoms of ocular dryness, photophobia, and burning pain [93]. The conjunctivae are rarely involved in severe chronic GVHD, but such involvement has a poor prognosis. As with cutaneous manifestations, keratoconjunctivitis has its counterpart in the human autoimmune disease Sjogren's syndrome.

Oral Manifestations

Destruction of the epithelia of the salivary glands leads to xerostomia [94]. The oral mucosa may appear erythematous or exhibit white plaques leading to a misdiagnosis of candida or herpes infections. Lichenoid plaques occur with advanced disease. Food sensitivity is common with advancing oral mucosal lesions.

Pulmonary Manifestations

cGVHD is associated with bronchiolitis obliterans, which has a poor prognosis [95]. It typically presents with cough and/or dyspnea. Pulmonary function tests show obstructive airway disease and reduced DLCO. Computed tomography of the chest may demonstrate hyperinflation with ground-glass appearance but also can appear normal. Severe sclerotic cutaneous disease of the chest wall may also produce dyspnea in the absence of pulmonary disease. Chronic infections of the sinuses or lower respiratory tract may also produce symptoms.

Female Genital Tract Manifestations

cGVHD affects the vulva and vagina in 25–49 % of long-term survivors of HSCT [89, 96]. Vulvar involvement presents a median of 7–10 months after HSCT, but vaginal involvement can present concurrently or independently up to 8 years later. Genital pathology is more common after peripheral blood stem cell transplantation than with bone marrow stem cell transplantation. Sclerotic skin changes are common and stenosis of the vagina can lead to hematocolpos. Genital GVHD may be the initial manifestation of cGVHD in up to 27 % of women.

Musculoskeletal Manifestations

Musculoskeletal manifestations often occur in conjunction with skin changes in cGVHD [97]. Fasciitis may restrict the range of motion of joints. Muscle cramping is common, but myositis or elevated creatine kinase is rare. Chronic use of steroids after HSCT may result in avascular necrosis, osteopenia, or osteoporosis.

Hematopoietic Manifestations

cGVHD is commonly associated with chronic cytopenias [1]. Stromal damage of the bone marrow may decrease production, but autoimmune neutropenia, anemia, and/or thrombocytopenia have also been observed. Thrombocytopenia in association with cGVHD is a poor prognostic sign [98]. Eosinophilia is associated with development of CGVHD in children [99].

Immunologic Manifestations

cGVHD is intrinsically immunosuppressive and occurs in the setting of chronic immunosuppressive therapies [26, 89]. 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 hypogammaglobulinemia also occur.

Prevention of GVHD

Multiple strategies for ex vivo T cell depletion of donor allografts before infusion to reduce the incidence of severe GVHD were abandoned because of high rates of allograft failure, relapse of malignant disease, infections, and post-transplant lymphoproliferative disorders produced by

Epstein-Barr virus infection of B cells [10]. In vivo reduction of donor and host T cells using polyclonal antithymocyte globulin (ATG) or antilymphocyte globulin (ALG) resulted in reduction in the incidence and severity of GVHD [100–102], but survival was compromised by infections. However, one long-term follow-up study showed that thymoglobulin prevented cGVHD and chronic pulmonary disease [102]. Alemtuzumab, a monoclonal antibody against CD52 expressed on T and B lymphocytes, monocytes, and dendritic cells, reduced the rates of both acute and chronic GVHD, but these benefits were negated by high rates of both infectious complications and relapse of malignant disease [103].

Currently, the primary prophylactic strategy is pharmacological inhibition of the cytoplasmic enzyme calcineurin using either cyclosporine or tacrolimus [104]. Calcineurin inhibition reduces production of the T cell mitogenic cytokine IL-2. Clinically effective dosing is associated with adverse events of hypomagnesemia, hyperkalemia, hypertension, nephrotoxicity, dyslipidemia, and diabetes mellitus. More serious side effects include thrombotic microangiopathy and neurotoxicity. Both cyclosporine and tacrolimus have often been administered in combination with the antiproliferative agent methotrexate [104]. To avoid the neutropenia and mucositis toxicities of methotrexate, recent regimens have substituted mycophenolate mofetil [105]. Neither the incidence nor the severity of aGVHD differed between those receiving methotrexate and mycophenolate, but patients treated with mycophenolate had accelerated hematopoietic engraftment and reduced mucositis [105].

Non-myeloablative conditioning has been used to suppress the host immune system so that donor T cell engraftment can occur before ablation of the lymphohematopoietic compartment of the host [106]. Such regimens generate less tissue damage and lower levels of inflammatory cytokines, which may decrease the initial step in the pathogenesis of GVHD. This conditioning also delayed onset of aGVHD until after 100d and reduced the incidence of severe GVHD compared to full-intensity conditioning regimens in historic controls.

Sirolimus is a potent inhibitor of the mammalian target of kanamycin, which inhibits the T cell mitogen IL-2 signaling mediated by IL-2R (CD25) expressed on activated T cells. Sirolimus decreases IL-2-mediated proliferation of activated T cells and also reduces the transcription of gene products necessary for growth and differentiation of effector T cells. Recent studies have shown that sirolimus is efficacious in the prevention of aGVHD, especially in the setting of non-myeloablative or reduced-intensity conditioning [52].

The importance of chemokines in the immunopathogenesis of GVHD in humans was illustrated by the results of a recent clinical trial testing the in vitro effect of CCR5 antagonist maraviroc on lymphocyte function and chemotaxis. The positive results led to a clinical therapeutic trial of 38 high-risk patients in a single group phase I and II study of reduced-intensity allogeneic HSCT that combined maraviroc [107] with standard GVHD prophylaxis using tacrolimus and methotrexate [107]. In 35 treated patients, the cumulative incidence of aGVHD grades II–IV was low: 14.7 ± 6.2 % on day 100 and 23.6 ± 7.4 % on day 180. No involvement of intestine or liver was observed before day 100 and remained infrequent before day 180. The cumulative incidence of aGVHD grades III–IV on day 180 was also low: 5.9 ± 4.1 %. 1 year mortality (not due to relapse) was 11.7 ± 5.6 %. This study clearly demonstrated that inhibition of lymphocyte trafficking mediated by CCR5 (Table 29.2) is efficacious in preventing intestinal and hepatic aGVHD.

Treatment of Acute GVHD

aGVHD usually occurs during ongoing prophylactic treatment with cyclosporine or tacrolimus with or without methotrexate [105]. Corticosteroids should be added to an optimized regimen of calcineurin inhibitor therapy to exploit their anti-inflammatory and antilymphocyte function properties. Mild cutaneous involvement (grade I) can be treated with topical steroids alone, but high-dose systemic steroids are required for higher grades or any involvement of the intestine or liver. Unfortunately, steroids achieve complete remission in <50 % of patients.

Sirolimus has also been effective for the treatment of steroid-refractory aGVHD and cGVHD alone or in combination with tacrolimus or cyclosporine [52]. In the initial phase II study, 50 % of patients achieved sustained, complete resolution of aGVHD with sirolimus in the absence of steroids. One year overall survival was 56 % (95 % confidence interval 38–74 %).

Extracorporeal photopheresis (ECP) is commonly used to qualitatively and quantitatively suppress the functions of circulating leukocytes [108, 109]. Apheresis is used to collect circulating leukocytes that are then incubated with the DNA-intercalating agent, 8-methoxypsoralen. Following exposure to ultraviolet light, the leukocytes are reinfused into the patient. This process induces cellular apoptosis and has strong anti-inflammatory and immunosuppressive effects, including evidence of the prevention of solid organ allograft rejection. ECP also increases Tregs after HSCT [110]. A phase II clinical trial showed that ECP resolved GVHD in the majority of patients who are steroid-dependent or steroid-refractory, achieving a 50 % long-term survival [111].

Strategies to inhibit the activity of the ubiquitous proinflammatory cytokine TNFα have also been tested using the anti-TNF-α monoclonal antibody infliximab or the TNFα receptor etanercept [48, 112, 113]. Inhibition of TNFα would be expected to impact diverse events, including reduction of

Table 29.5 Investigational therapies for acute or chronic graft-versus-host disease

Mechanism	Agent
Reduce numbers of B and myeloid dendritic cells but preserve T cells for antitumor response	Milatuzumab (humanized anti-CD74 mAb)
Reduce activation of effector T cells	3,6-bromoindirubin 3′-oxime (glycogen synthase kinase inhibitor)
PPARγ inhibition	Rosiglitazone
	Bezafibrate
Inhibit costimulation of donor T cells	Lentiviral vector-mediated RNA interference
Inhibit CCL3 chemoattraction gut and liver	Evasin-1, CCL3-binding protein
CCR5 antagonist preventing chemotaxis	Maraviroc
Prevent egress of effector cells from lymph nodes	Fingolimod (FTY720)
Reduce target organ trophism	Vitamin A
Regulation of Th1 and Th17 responses	Am80 (synthetic retinoid)
Inhibition of IL-6 signaling	Anti-IL-6 receptor mAb
Prevention of aGVHD	Triterpenoid CDD0-Me
	Repifermin (keratinocyte growth factor-2)
Treatment of aGVHD	Pentostatin (potent inhibitor of adenosine deaminase)

APC activation, prevention of direct cytokine-mediated tissue damage, and failure to stimulate gene expression of the target cells for the production of chemokines attracting donor T cells. Plasma levels of TNFR I (a surrogate of TNFα) progressively rise before clinical onset of GVHD. Phase II trials have been promising in small numbers of patients. In the phase II trial of etanercept added to steroids, 70 % of patients had complete response in the skin and intestine within 1 month. Oral thalidomide, an antagonist of TNFα production, has been studied for cutaneous disease [114]. Multiple other therapies (Table 29.5) are in development, targeting a variety of pathogenetic mechanisms.

Treatment of Chronic GVHD

The incomplete understanding of the immunopathogenic mechanisms of cGVHD has hampered development of therapeutic strategies [89]. Currently, the treatment involves a variety of immunosuppressive agents; however, the response to treatment is unpredictable and variable among different affected organs in the same patient [26]. Currently, the standard of care systemic therapy is steroids with or without cyclosporine or tacrolimus. However, a randomized control trial showed no difference in the response to prednisone alone versus a combination of prednisone and cyclosporine. Chronic use of steroids results in systemic steroid-related complications and increases the risk of opportunistic infections. ECP has demonstrated significant response rates in patients with severe cGVHD, including lesions of the skin, liver, oral mucosa, eye, and lung [26].

Rituximab, a monoclonal antibody against CD20 expressed on mature B cells, has shown efficacy in the treatment of steroid-refractory cGVHD associated with sclerodermatous or other severe skin reactions, rheumatological complaints, and thrombocytopenia [115]. In a prospective study of 37 patients with steroid-refractory cGVHD, rituximab therapy resulted in 8 complete responses and 24 partial responses [116]. After 1 year of therapy, 21 patients maintained their response and were able to reduce or discontinue steroids. The effect was greatest for cutaneous, oral mucosal, and musculoskeletal manifestations of cGVHD. However, therapy was complicated by infections and relapse of malignant disease. Rituximab therapy was associated with a marked decline in CD8 T cells infiltrating the skin, indicating a role for B cells in maintaining the pathological effects of CD8 T cells [117].

Chronic GVHD of the Liver

Up to 50 % of patients with cGVHD have some degree of hepatic involvement [89]. Patients with significantly abnormal biochemical tests and/or symptoms of cholestatic liver disease (e.g., pruritus, hyperpigmentation) should be followed by a hepatologist. Evidence-based management options include high-dose methylprednisolone (2 mg/kg/d) and high-dose ursodeoxycholic acid (30 mg/kg/d) [89]. Ursodeoxycholic acid at 13 mg/kg/d was administered prospectively to 15 patients at the time of the diagnosis of hepatic GVHD [118]. After 1 year of therapy, 60 % had normal liver tests, while 40 % had reduced liver test abnormalities from baseline levels. Pruritus resolved in seven of nine patients. No adverse events were noted. Supplemental fat soluble vitamins may be required to prevent or treat coagulopathy, osteopenia, or night blindness. Complications of portal hypertension may manifest as ascites, gastroesophageal varices or portal hypertensive gastropathy, hepatic encephalopathy, and hypersplenism. First-line diuretics for edema or ascites

include spironolactone and furosemide. All patients with portal hypertension require screening for varices using endoscopy or videoesophagography. Hepatic encephalopathy should be treated with lactulose, and rifaximin can be added if lactulose is ineffective. Other investigational therapies are listed in Table 29.4.

GVHD After Orthotopic Liver Transplantation

OLT is performed between donors and recipients who are matched for ABO blood group but not for HLA class I or II antigens. Passenger leukocytes within the donor liver include T and B cells, NKT cells, and NK cells that can be stimulated by the allogeneic environment of the host. Generally, the host alloimmune response, despite being therapeutically immunosuppressed, destroys donor T cells. However, persistence of some donor cells, such as dendritic cells, often results in a clinically silent chimerism within lymphoid organs [119]. Rarely (~1 %), passenger T cells engraft in the host, causing CGVHD [120–123]. HLA matching between donor and recipient prevents the elimination of donor cells, while HLA haplotype mismatches generate alloimmune destruction of the recipient's lymphoid cells by donor T, NKT, and NK cells. Conceptually, this is analogous to F1 hybrid animal models of GVHD in which the offspring of parents with MHC mismatches recognize infused cells from one parent as self, while that parent reacts against the MHC haplotype of the other parent, resulting in GVHD. The primary risk factor is serendipitous matching of HLA class II alleles between a donor and a recipient. Incidence is inversely proportional to the number of HLA mismatches, being ≤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. Other risk factors include older recipient age of ≥60 years, African American race, mismatched sex, and CMV infection. Prevention is possible in living-related adult-to-adult OLT by identifying the degree of HLA mismatching prior to OLT.

Onset of aGVHD occurs 1–8 weeks post OLT with fever and rash involving the palms and soles [124]. In ~15 % of patients, GVHD is confined to the skin. In ~85 %, aGVHD progresses to involve the intestine and hematopoietic tissues, resulting in diarrhea, neutropenia, and thrombocytopenia. Since engrafted T cells and the liver allograft are identical for HLA and MiHA, they recognize hepatic tissue as self and do not cause hepatic GVHD. Once acute GVHD has been established, intensified corticosteroids, reduction of immunosuppression, reduction of IL-2 with calcineurin inhibitors, and strategies to neutralize TNFα have been largely unsuccessful, culminating in a mortality of 68–85 %. Infliximab treatment was reported to be successful in a single patient [125].

Future Directions

The most promising prospect is prevention of acute and chronic GVHD by refining preventive measures. The exciting report that maraviroc blockade of CCR5-mediated chemotaxis reduced the incidence and onset of visceral aGVHD highlights the importance of transendothelial migration of effector cells to their restricted target tissues [107]. This provides an important paradigm for potential exploitation in skin, intestine, and liver, where the chemokines and chemokine receptors are characterized (Table 29.2), and accumulating data indicate that the target cells, such as the biliary epithelial cells, may participate in their own destruction by secreting chemokines and Th1 and Tc1 polarizing IL-12 when stimulated by IFNγ and TNFα [80].

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Immune-Mediated Liver Disease in the Transplanted Liver

30

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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 component, including rejection, graft hepatitis and 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' which have an increased risk for poor function.
- The use of marginal donors is a risk for ischaemia–reperfusion injury (IRI), the most common reason for re-transplantation in the early post-operative period.
- Understanding the molecular mechanisms responsible for rejection and IRI will allow development of new therapeutic treatments.
- Acute cellular rejection remains the most common form of rejection although itself does not necessarily translate into poor long-term outcome and may paradoxically benefit the recipient.
- Chronic or ductopenic rejection remains a risk factor for graft loss.
- Nevertheless the liver allograft remains relatively resilient to immune-mediated injury when compared to other solid-organ allografts, in part due to its inherent tolerogenic properties and large haemopoietic organ mass.
- The reported incidence of recurrent autoimmune liver disease varies largely due a lack of codified diagnostic criteria, and liver biochemistry can remain within the normal range on a background of recurrent autoimmune liver injury.

Introduction

Liver transplantation has evolved as the treatment of choice for many patients with end-stage liver disease (Fig. 30.1). Currently, survival post-transplant is excellent with 1-, 3- and 5-year survival of 87 %, 78 % and 73 %, respectively [1]. Patients are selected for listing when the estimated survival without transplantation is less than with transplantation; and organs from deceased donors are allocated to potential recipients primarily on a needs-based system with the Model for End-Stage Liver Disease (MELD) score being the most widely used scoring system (Table 30.1) [2]. Transplant benefit is felt to outweigh procedural risk develops when the MELD score reaches 15.

While the number of patients awaiting liver transplantation has shown a steady rise over the last decade, there has been no corresponding increase in the organs available for transplantation. 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 liver allograft is susceptible to a range of complications including ischaemia–reperfusion injury (IRI), 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 towards alloantigens.

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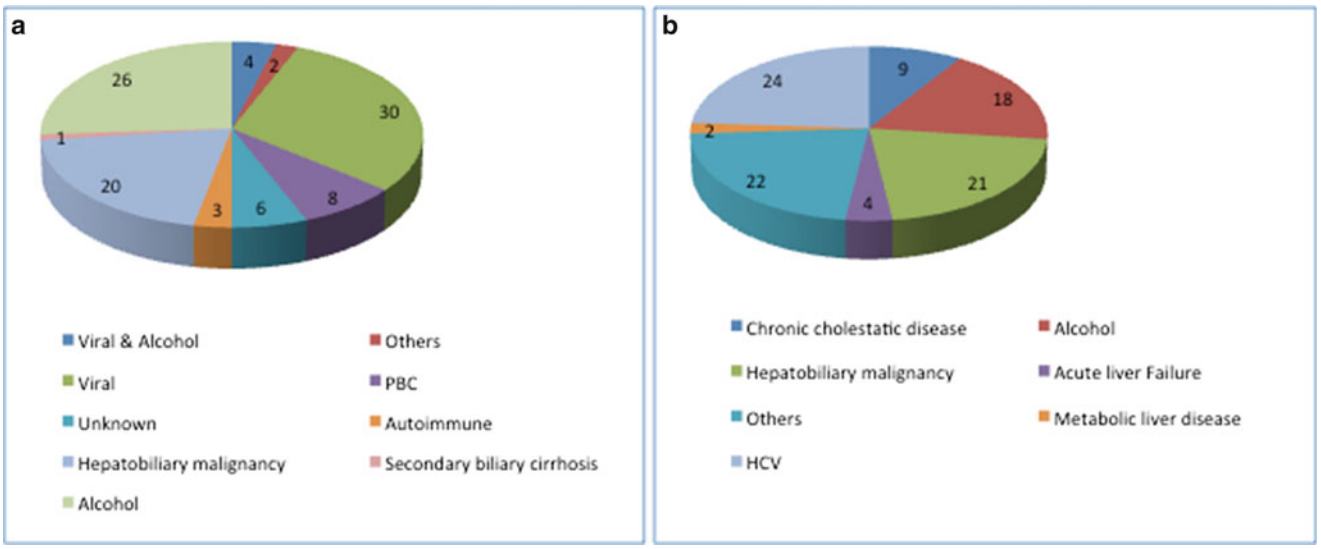

Fig. 30.1 Indications for liver transplantation (data expressed as percentage of total transplants). (**a**) Europe 1998–2010. *Source*: European Liver Transplant Registry (ELTR). (**b**) United States 2011. *Source*: UNOS OPTN/SRTR (2011) Annual Data Report

Table 30.1 Medical urgency scoring systems for prioritising receipt of a liver transplant

Score	Components/formula	Score required for listing
CTP[a]	Ascites, encephalopathy, bilirubin, albumin, prothrombin time	≥9
MELD[b]	3.78[Ln serum bilirubin (mg/dL)] + 11.2[Ln INR] + 9.57[Ln serum creatinine (mg/dL)] + 6.43	>15
MELD – Na[c]	MELD – Na [0.025 × MELD × (140 – Na)] + 140	>15
UKELD[c]	[5.395 × ln(INR)] + [1.485 × ln(creatinine)] + [3.13 × ln(bilirubin)] × [81.565 × ln(Na)] + 435	≥49

[a]The Child-Turcotte-Pugh (CTP) score integrates five empirically selected variables with a range of 5–15 points. Unfortunately the CTP score has several pitfalls in this regard, not least the nature of ascites and encephalopathy as subjective variables. Secondly, patients are not sufficiently differentiated so that waiting time impacts on prioritisation. Moreover, there is no variable reflecting renal function, a well-established prognostic marker in end-stage liver disease

[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 aetiology or complications of cirrhosis. Recent changes in United Organ Sharing Network (UNOS) policy require liver donor offers first to patients with MELD scores ≥15 within a region, before offers to local candidates with MELD <15

[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

Ischaemia–Reperfusion Injury

IRI is the main cause of both primary non-function and delayed graft function accounting for 80 % of re-transplantations during the first week. This results from a multifaceted process that combines elements of 'warm' and 'cold' injury. Warm ischaemic injury occurs when perfusion is reduced after clamping or when there is reduced liver perfusion from shock, heart failure, respiratory failure, haemorrhage, 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 grafts [3]. Although immune mechanisms are involved in IRI, the association of IRI with clinical graft rejection is conflicting.

Molecular Mechanisms of Ischaemia–Reperfusion Injury

The ischaemic injury is a localised 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 endothelium, 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 hepatic sinusoidal endothelial cell (HSEC)-associated NADPH oxidase. However, most of the oxidant stress appears to occur in the vasculature, with Kupffer cells as the main source [4]. 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 leads to an increase in cytosolic and mitochondrial calcium (Ca^{2+}). This reduces the mitochondrial transmembrane potential, and as a result, the activity of the enzyme mitochondrial ATP synthase becomes reversed in an effort to hydrolyse 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 depolarisation 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 principle cells initiating IRI in the liver allograft are Kupffer cells [5]. 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 IL-1β and TNFα which activate and induce migration of neutrophils and $CD4^+$ T-cells. These pro-inflammatory 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 IFNγ and IL-17 released by activated lymphocytes. These cytokines also activate natural killer (NK) T-cells which 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), anti-apoptotic proteins (Bcl-2, $Bcl\text{-}_{XL}$) and the NFκB pathway [6]. These modifications are followed by the release of danger-associated molecular patterns (DAMPs) which bind to Toll-like receptors (TLRs), specifically TLR4, and the receptor for advanced glycation end products (RAGE) (Fig. 30.2).

The endogenous TLR ligands are classified as:

- 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-characterised 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 h) and may depend on the direct cytotoxic effect of a soluble

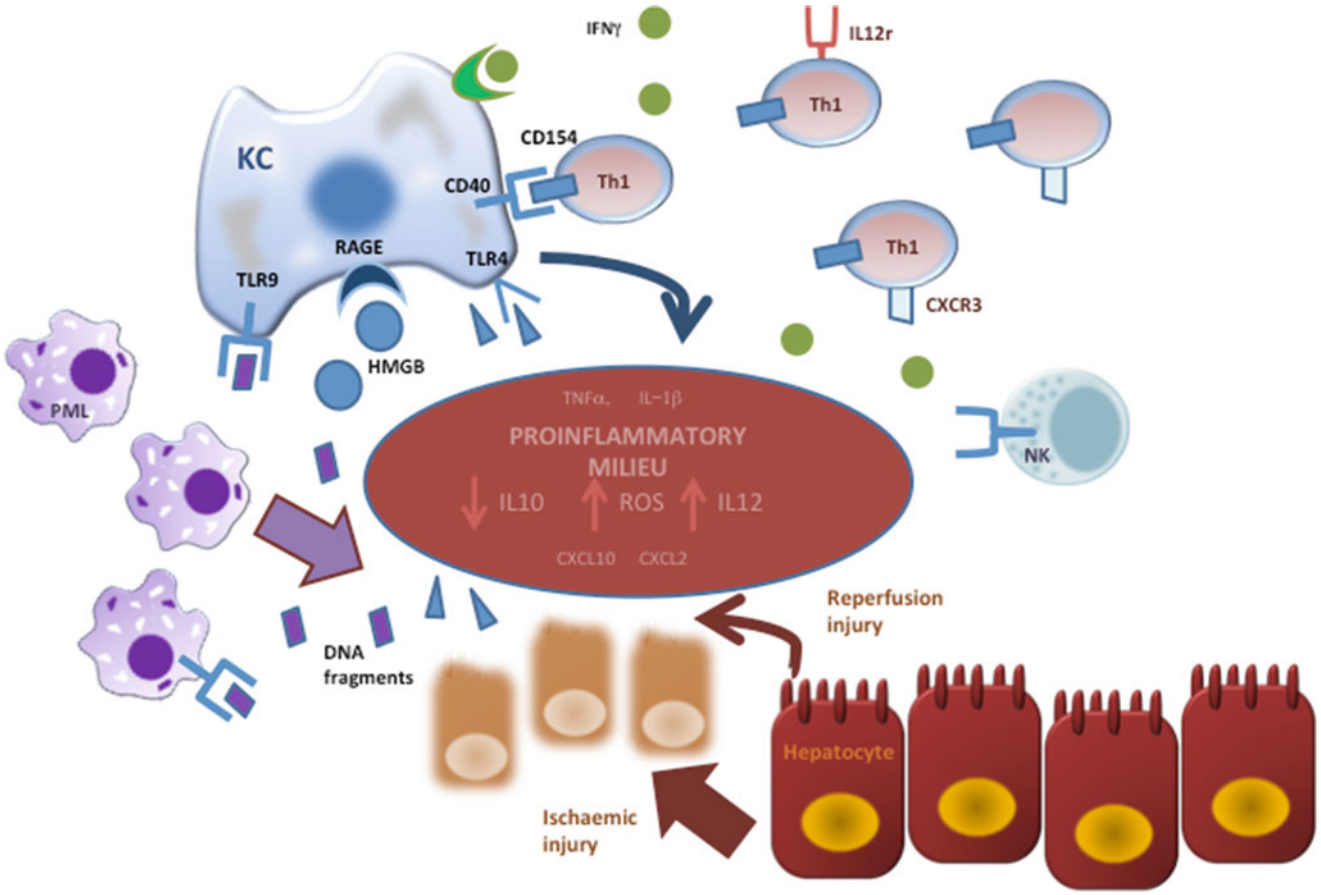

Fig. 30.2 Immune activation in hepatic ischaemia–reperfusion injury (IRI). The ischaemic insult induces necrotic cell death which in turn provides diverse 'danger'-associated molecules (e.g. HMGB1 and DNA fragments) to activate innate TLR4, RAGE and TLR9 signalling on Kupffer cells (KC) as well as dendritic 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-1b, IL-1β, CXCL10, CXCL2 and ROS recruits and activates local and circulating immune cells which promote cytotoxicity against the liver parenchyma

TNFα-enriched inflammatory milieu. In later stages (>12 h), newly recruited and activated polymorphs require MyD88 signalling 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 [7], 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]) recognise PAMPs existing within the cytosol that can also trigger local inflammatory responses and immune activation.

Potential therapeutic targets for IRI: Pro-survival 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 ischaemic 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 ischaemia [11]. 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 [8].

Clinical trials assessing NAC as a preventative agent against ischaemia during liver procurement and partial hepatectomy have reached phase IV [12]. However, despite a reduction in biochemical markers of liver injury, there remains a lack of convincing evidence that NAC administration actually improves clinical outcome. 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), an 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 ischaemia [13].

One of the most commonly investigated methods of reducing IRI has centred around ischaemic preconditioning whereby the liver is exposed to a brief period of ischaemia then reperfusion before a longer period of hepatic ischaemia [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. Ischaemic post-conditioning has also been shown to be protective against the ischaemic insult and exerts its beneficial effects through mechanisms similar to those observed in preconditioning, such as activation of the pro-survival 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 ischaemic injury (see above), 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 been disappointing [11].

Allograft Rejection

Allograft rejection—defined as an immunological reaction to a graft antigen that results in damage to the graft—involves a 'host-versus-graft reaction' whereby recipient-derived antibodies, the complement system and lymphocytes mediate immune responses to allogeneic cells leading to damage and/or the destruction of the grafted liver tissue. Despite the increasing availability of immunosuppressive agents, immune-mediated damage remains a major cause of graft and patient loss.

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 30.2).

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 1-encoded antigens. Antibodies to donor MHC-I and MHC-II can also be associated with acute and chronic graft damage which 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 anti-HLA antibodies can contribute to alterations in endothelial cell function through complement-independent mechanisms by transducing both pro-inflammatory and pro-proliferative intracellular signals. This supports a more 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. More commonly, acute allograft rejection is driven by recipient T-cells which recognise donor organ alloantigens. The accumulated injury caused by donor disease (such as hepatic steatosis), the procurement process, cold ischaemia, 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 [17]:

- *The direct pathway*: Recipient T-cells recognising 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 APCs and/or through fusion with donor APC-derived exosomes. These 'chimeric' recipient APCs stimulate recipient T-cells that recognise intact allogeneic MHC–peptide complexes.

Table 30.2 Immunes responses in allograft rejection

Immune response	Key pathological findings
AMR-*hyperacute*	Widespread haemorrhage
	Microvascular thrombosis
	Hepatocyte necrosis
	Variable infiltration of neutrophil polymorphs
AMR-*acute*	Portal tract oedema
	Ductular reaction
	Neutrophil-rich portal infiltrate
	Portal venular endothelial inflammation (neutrophil-rich)
	Severe cases: portal and periportal haemorrhage secondary to severe endothelial injury [94]
ACR-*early*	Portal tract inflammation (mixed inflammatory infiltrate)
	Centrilobular necro-inflammatory lesions involving hepatic venules and liver parenchyma (central perivenulitis) may be observed
	Bile duct inflammation varies from mild to severe
ACR-*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
CR	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

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 [18]. 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 antigen-presenting cell populations allow the liver to act as a site for lymphocyte activation [19]. The portal blood supply from the intestinal circulation leads to 'endotoxin tolerance' [20] 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 unclear although in part this depends on the site of lymphocyte activation. It has been hypothesised that activation by DCs in draining lymph nodes leads to a vigorous immune response, whereas local activation by HSEC or hepatocytes favours tolerance [21].

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 which 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 non-classical adhesion molecules which are widely expressed within the hepatic sinusoids including CD44 [22], common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) [23] and vascular adhesion protein-1 (VAP-1) which have also been demonstrated to mediate transendothelial migration [24]. 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 up-regulation of classical and non-classical 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 which 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 [25]. 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 post-transplant immune-mediated liver injuries.

Clinicopathological Features

Antibody-Mediated Rejection [26]

AMR develops in a recipient with preformed anti-donor antibodies usually against ABO blood group antigens. Severe cases present as acute fulminant hepatic failure within the first few hours (hyperacute rejection) to days after transplantation but are rarely seen beyond 2 weeks.

AMR was commonly observed in the earlier days of transplantation, being reported in 2.6 % of recipients and associated with the use of ABO-incompatible donors; however, AMR is now very rare. AMR is mediated by pre-existing antibodies specific to graft antigens, particularly ABO blood type antigens vascular endothelial cell (VEC) antigens and rarely HLA antigens. Less severe changes may be observed in the presence of other donor-reactive antibodies (e.g. anti-Kell, anti-Duffy, lymphocytotoxic) and present similarly to episodes of acute rejection. Such antibodies can recognise 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 C4d, a hydrolysis product of the complement protein C4. 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 haemorrhagic necrosis of the graft.

Pathological findings: Laboratory tests and radiological changes are not specific, but imaging is needed to exclude hepatic artery thrombosis. Histological changes are usually identified in the explanted liver and include intra-sinusoidal neutrophil and platelet aggregates and platelets lining vessels, with portal oedema, ductular reaction and a neutrophil-rich inflammatory infiltrate resembling changes seen in biliary obstruction. Portal haemorrhage occurs in more severe cases and is associated with poorer 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.

Positive C4d immunostaining is seen in up to 50 % of grafts with AMR and is significantly associated with high postoperative anti-donor A/B antibody titres and worse survival. C4d staining of portal capillaries occurs in mild/early cases with stromal staining around portal capillaries and/or biliary epithelium occurring in more extreme situations. Bilirubinostasis may develop and portal 'biliary' features suspicious of hyperacute AMR positively correlate with the extent of positive C4d staining. Portal staining can extend into periportal sinusoids, and sinusoidal C4d deposition occurs in association with areas of lobular necrosis. C4d staining in ABO-compatible transplants presenting with hyperacute rejection is more difficult to interpret given the lack of correlation of C4d staining with donor-specific antibody titre.

Treatment considerations: The outcomes of ABO-incompatible liver transplants are inferior to those of ABO-compatible donor–recipient pairings. Treatment has focussed on the prevention of antibody- and complement-mediated damage to the vascular endothelium and include attempts to reduce the donor-specific antibody titre (<1:8–16) with various combinations of preoperative high-dose intravenous steroids (methylprednisolone), depletion of donor-reactive antibodies through plasmapheresis or intravenous immunoglobulin infusion, administration of the protease inhibitor gabexate mesilate and anti-CD20 antibodies, portal infusion of prostaglandin E1 and splenectomy. Emergency re-transplantation currently remains the only viable option.

Massive haemorrhagic necrosis (MHN) is a distinct form of hyperacute liver injury and characterised by an uneventful postoperative period, only to be followed by a sudden deterioration in graft function and graft failure, haemorrhage and hepatocyte necrosis but with only mild graft inflammation and without occlusive lesions in large arteries or veins. These distinctive features differ from other recognised patterns of graft damage and comprise a unique form of graft dysfunction [27]. Histologically these livers have associated small-vessel veno-occlusive lesion disease, ductopenia and foam cell arteriopathy.

Acute Cellular Rejection

The majority of cases (~65 %) develop within the first year, with a median time of 8 days post-transplantation. ACR 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 20–40 %. In part, this is attributable to regimens using tacrolimus rather than cyclosporine [28]. Risk factors associated with ACR include:

- Indication for transplantation: Chronic HCV (69 %) infection, primary biliary cirrhosis (PBC; 63 %) and autoimmune hepatitis (AIH; 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 ACR.
- Use of anti-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 ACR.
- Specific IL10 [29] and CTLA4 [30] genetic polymorphisms are associated with a lower risk of ACR.
- Ethnicity: ACR 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 ACR than those who do not (65.9 % vs. 42.5 %).
- Longer cold ischaemia times (>15 h).
- Poor recipient performance status.

The patient with ACR may report non-specific symptoms of malaise and ill health, fever, asthenia and abdominal pain but can often be asymptomatic in the early phase. Tender (graft) hepatomegaly is described but is of little clinical utility. The bile colour may become pale.

Molecular mechanisms of ACR: In the early sensitisation stage of cellular rejection, $CD4^+$ and $CD8^+$ T-cells, via their T-cell receptors (TCR), recognise 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 (commonly known as CD80 or CD86), remains the most widely studied [31]. 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 hydrolysed 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 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 ACR 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 characterised 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 recognise alloantigens on donor tissue and kill graft cells through the release of perforin and granzymes and through Fas/FasL interactions. Antibodies can also injure the graft in ACR, 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 recognised as active participants in the acute and chronic rejection of solid tissue grafts [32, 33]. NK cells can mount a potent effector immune response without prior sensitisation 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 which 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 rejection is characterised by a predominantly cholestatic biochemical profile (elevated ALP/γGT and bilirubin), whereas late acute rejection is often more of a hepatitic picture. However, changes in liver biochemistry are non-specific and cannot reliably be used to determine the presence or severity of ACR. Peripheral blood

eosinophilia has often been reported to be associated with acute cellular rejection but is affected by the concomitant use of steroids. A fall in peripheral blood eosinophils may be an independent predictor of histological resolution of acute rejection [34]. 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. Reduced portal blood flow velocity and an increase in splenic pulsatility index are also recognised features of early acute rejection (accuracy 88 %) [35].

The histological features of acute rejection 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 ACR centres on the triad of portal inflammation, endothelialitis and non-suppurative destructive cholangitis (Snover's triad):

- Portal inflammatory response: 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 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 post-transplant and related to preservation–reperfusion injury.

Late ACR has some histological differences, notably the presence of central perivenulitis [36]. Furthermore, fibrosis can be present in late ACR but is not really a feature of early acute rejection.

- 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 ACR, portal vein and hepatic vein inflammation is 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 ACR and typically occurs without hepatic vein endothelialitis.
- Biliary infiltration: Bile duct inflammation is rarely more than mild in late ACR. Bilirubinostasis is uncommon, 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 be confirmed histologically before treatment is instigated. Moreover, treating the patient with ACR should be individually tailored and requires an expert multidisciplinary approach. For instance, in the individual transplanted for autoimmune liver disease, histological features of mild ACR may warrant more aggressive treatment than a patient with mild rejection who received a liver allograft for chronic viral hepatitis in whom the need for high-dose immunosuppression to control rejection must be balanced against the risk of increasing HCV replication and the consequent damage to the liver allograft.

In contrast to cardiac and renal transplantation, the development of early ACR is not necessarily harmful to the liver allograft (only 5 % of patients develop graft failure due to ACR [37]), and early immunological engagement may help enhance allograft tolerance [38]. Indeed 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.

Therefore current immunosuppressive protocols, although largely successful in preventing rejection, have the 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 h post-transplantation and relates to events completed by the end of the first 2 weeks. Thus, by attempting to block rejection early, there is a greater potential that induction of long-term tolerance will also be arbgrogated. 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 reaction activity index is most widely used. 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 ACR [39]. In the individual transplanted for non-viral 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. In patients transplanted for HCV infection, with near-normal liver biochemistry and mild histological features of rejection, a 'watch and wait' approach may be adopted while the possibility of recurrent HCV is excluded as high-dose steroids will enhance viral replication.

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 non-responsive episodes of ACR can be treated with further cycles of corticosteroid therapy. However, repeated rejection or non-responsive rejection 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 ACR 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 SRR 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 ACR do not affect long-term graft survival and are not associated with chronic rejection. However, late acute rejection episodes respond less well (51 % compared with 80 % for early ACR) to enhanced immunosuppression, progress to liver fibrosis more frequently and, unlike early ACR, are associated with a worse outcome and a significant risk of progression to chronic ductopenic rejection. Early ACR is not related to the development of late ACR.

Chronic (Ductopenic) Rejection

Chronic rejection (CR) is usually diagnosed in the second half of the first year but may occur at any time. The incidence of CR 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 post-transplant) 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 non-responsive ACR: Although not the end stage of ACR, both acute–late and chronic rejection may share a temporal relationship, and late ACR and CR have several overlapping histological features. While over 25 % of patients treated for late ACR develop CR, only 5–10 % of patients treated for early ACR develop CR.
- 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 CR are less well understood than for AMR and ACR although it is hypothesised that CR 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 which synergise 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 CR. A recognised feature of CR 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 which secrete mesenchymal growth factors (e.g. PDGF, TGFβ) that lead to smooth muscle proliferation in the intima of arterial walls. CR therefore reflects vascular occlusion and chronic ischaemia 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 CR are a loss of bile ducts and an obliterative arteriopathy [40]. Specifically:

- Portal inflammation: This is of a variable severity during the early stages and may encompass features of ACR, 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) which 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 on.
- Biliary inflammation: Bile duct inflammation is variable although bile duct atypia and senescence are recognised phenomena during the early stages and result in progressive duct loss. In contrast to acute rejection, ductular reactions are typically absent in CR presenting within the first year post-transplantation but may be present in cases which develop later, particularly in those with coexisting biliary fibrosis.
- Centrilobular hepatocyte damage: Ballooning and bilirubinostasis are common findings in CR. 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 recognised and include:
  - 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 recognised)

The Banff schema classifies CR into early and late stages based on the potential reversibility of rejection-related events [41]. Early CR is characterised by inflammatory and degenerative changes in bile ducts; However, in contrast to acute rejection, CR 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 CR, 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 CR 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 re-transplantation is made.

Treatment considerations: Therapeutic strategies may be effective in the ductopenic stage although the evidence supporting their use in CR is small. Nevertheless, episodes may resolve if >50 % of portal tracts have intact bile ducts, and in patients with early CR and mild/moderate cholestasis (e.g. 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 [42] and may also prevent intimal narrowing of the arteries through its action on smooth muscle. Mycophenolate has also proven efficacious in stabilising liver function in small numbers of patients [43]. End-stage CR warrants re-transplantation although there is a high risk of recurrent CR in the re-graft.

Graft Hepatitis

Unexplained inflammatory changes in late post-transplant biopsies are common with the incidence ranging from 10 to 50 % in patients undergoing liver biopsy more than 1 year post-transplant [44]. The term idiopathic 'graft hepatitis' has been adopted where biopsies demonstrate a chronic hepatitis without an otherwise obvious cause, characterised 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 ACR [45]. Another possibility is the presence of an as yet unidentified viral trigger driving the immune response. There is gathering interest in hepatitis E virus (HEV) as an under-recognised cause of chronic hepatitis in solid-organ transplant recipients. Studies from Europe demonstrate that despite its low prevalence, the presence of HEV infection can be associated with graft hepatitis and progress to advanced fibrosis or cirrhosis requiring re-transplantation [46]. Unexplained hepatitis can also be an early manifestation of recurrent autoimmune disease (see below) which may precede a definitive diagnosis by many years, and organ non-specific 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 paediatric 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 [47]. Significant fibrosis or cirrhosis has also been demonstrated in up to 27 % of adult liver recipients with graft hepatitis [48].

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 evolved a natural ability to discriminate between 'self' and 'non-self' antigens by deleting immature autoreactive bone-marrow-derived T-cells in the thymus 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}. The liver is constantly exposed to harmless food antigens via the portal vein and has consequently evolved its own, inherent tolerogenic mechanisms to prevent it being constantly inflamed by immune activation [49]. 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 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 in the transplanted liver compared to that observed with other solid-organ allografts.

Following liver transplantation, 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 which 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 co-stimulation, 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} which restrain non-deleted 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 with 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 above. The liver allograft is also an abundant source of soluble MHC class I antigens which are able to bind to alloreactive $CD8^+$ T-cells and induce activation and apoptosis in the absence of appropriate co-stimulation.

Microchimerism

The ability to function as a haemopoietic 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 non-lymphoid tissues. This phenomenon is known as 'microchimerism' and occurs to a degree with all vascularised 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 haematopoietic 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.

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 indirect pathway, persistence of donor antigen provides a constant source of alloantigen that can be presented by non-professional recipient APC in the periphery resulting in the elimination/inactivation of donor-reactive recipient T-cells. The haemopoietic 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 liver transplantation. 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 above.

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 re-exposure to antigen, thus ensuring protective immunity against pathogens upon reinfection. It is now clear that the normal pool of memory T-cells contain alloreactive T-cells even in patients that 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 naïve 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 anti-rejection 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 [50]. 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 which possesses potential for future therapeutic intervention in the induction of transplant tolerance.

Operational Tolerance Following Transplantation [51]

The immunosuppressive drugs most frequently given to patients in the early post-transplantation 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 towards the preservation of long-term graft function and minimisation 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 [52]. 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 immunosuppressive 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 SRR could severely affect graft survival.

Spontaneous long-term acceptance of transplanted livers following complete discontinuation of conventional immunosuppression has been observed in a few 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 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 non-reactivity towards 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 one-fifth of highly selected recipients. Chronic HCV-infected patients are often considered good candidates for weaning given the potentially beneficial effect on HCV immunopathogenesis. Favourable clinical markers for successful withdrawal include an increasing time since transplant (at least 2 years of stable function), low incidence of previous ACR episodes and non-autoimmune primary liver disease and few inflammatory cells on histology. More recent studies suggest that in stable recipients of liver transplants, operational tolerance might occur more frequently later (~80 % of attempted cases, >10 years after) [53]. The incidence of ACR during weaning of immunosuppression ranges from 12 to 76 %, but these episodes are usually mild and often resolve by return to baseline immunosuppression. At time of writing, Only two cases of graft loss due to chronic rejection have been reported following medication withdrawal in patients with operational tolerance. 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 and immunological characteristics identify those most likely to succeed without immunosuppression such that withdrawal would only be considered in such a suitable 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 may serve as a predictive tool for the immunosuppressive management of the post-transplant population in the near future.

- Operationally tolerant renal allograft recipients have recently been identified as having increased total B-cell numbers and naïve B-cells in the peripheral blood suggesting that these cells may be important regulators of the anti-donor immune response.
- 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 non-tolerant recipients [54]. These genes encode kappa or lambda light chains which 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 up-regulation of CD20 (a B-lymphocyte surface marker) mRNA in urine sediment cells and elevated numbers of peripheral blood naïve 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 liver transplant recipients.
- Similar studies in liver transplant recipients have identified 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 up-regulation 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. Paediatric 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.
- There also exists a marked difference in a set of genes involved in iron homeostasis, with the master regulator of iron metabolism, hepcidin, being over-expressed in operationally tolerant liver patients [55]. Levels of soluble HLA-G are also significantly higher in tolerant paediatric recipients compared to those with rejection or on stable immunosuppression therapy.
- 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 9–20-fold during an episode of rejection, and levels of the former fall rapidly after institu-

tion of methylprednisolone treatment. Moreover, these potential biomarkers may help discriminate episodes of rejection versus other causes of graft dysfunction [56].

- Recent studies have suggested that patients with low pre-transplant 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 favour their potential as new therapeutic targets, as well as in the design of new drugs directed at controlling their levels in serum [57].

Recurrent Autoimmune Disease Following Transplantation (Table 30.3)

Recurrent Primary Biliary Cirrhosis

Approximately one-third of patients with PBC may not respond to therapy with ursodeoxycholic acid (UDCA) and develop progressive cholestasis, fibrosis and cirrhosis for which liver transplantation remains the only viable option. Outcome following transplantation for PBC is excellent with reported 5-year survival between 77 and 86 % [58, 59]; however, recurrent PBC in the liver allograft has been reported to occur in up to 6–33 % of recipients [60, 61]—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 anti-mitochondrial antibodies (AMA) remain detectable following liver transplantation, even with no evidence of recurrent disease [62]. 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 post-transplant liver biochemistry and may not be of clinical significance.

Table 30.3 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 cirrhosis, *PSC* primary sclerosing cholangitis, *AIH* autoimmune hepatitis

Aetiopathogenesis and Molecular Mechanisms

Tacrolimus usage is a risk factor for early and aggressive recurrence of PBC compared with cyclosporine [63], and some have suggested that the protective effects of the latter are secondary to its putative antiviral properties [64]. 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 [63]. These findings have not been confirmed by all [65]. Other reported risk factors of recurrent PBC include family history, age and gender of donor, lack of corticosteroids in the immunosuppressive regimen and prolonged ischaemic 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 liver transplantation, whereas others have suggested a relationship between DR-locus mismatch and diseased donor transplantation [66, 67]. 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 [68]. However, whether BEC or their progenitors undergo EMT to become matrix-producing myofibroblasts during biliary fibrosis has been subject to significant ongoing controversy [69].

Treatment Considerations and Outcome

Recurrent PBC does not appear to influence long-term patient survival or graft loss [70, 71]. In patients who develop recurrence, survival remains excellent with only few reported cases of graft loss. 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 [72]. The limited available data from other centres suggests that UDCA does not seem to influence patient or graft survival [70].

Recurrent Primary Sclerosing Cholangitis

There is no currently available medical treatment for primary sclerosing cholangitis (PSC) which has consistently been proven to improve outcome [73]. The reported incidence of recurrent PSC following liver transplantation differs widely between studies, with quoted rates between 10 and 27 % [74]. 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 [75], which consists of a confirmed diagnosis of PSC prior to liver transplant and either a cholangiogram demonstrating non-anastomotic biliary strictures of the intrahepatic and/or extrahepatic biliary tree with beading and irregularity occurring >90 days post-transplantation 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 [75].

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 liver transplantation. These include ischaemia, 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 non-anastomotic strictures as seen in recurrent PSC [76]. However, other diseases can result in similar cholangiopathic appearances to recurrent PSC, which include hepatic artery thrombosis/stenosis (HAT/HAS), established ductopenia rejection, reperfusion injury, biliary sepsis, anastomotic strictures and ABO incompatibility between donor and recipient [77].

Aetiopathogenesis and Molecular Mechanisms

Risk factors for the development of recurrent PSC have been proposed to include donor–recipient gender mismatch, the presence of specific HLA haplotype (e.g. HLA-DRB1*08), episodes of SRR, the use of OKT3 for the treatment of cellular rejection, recurrent ACR 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 [78].

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 OLT [79]. 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 [80]. These events stimulate production of proinflammatory 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 favoured 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 [81].

Treatment Considerations and Outcome

There is no established medical therapy for recurrent PSC post-transplant and disease progression or development of complications may require re-transplantation. Unlike PBC, there exist many reports which indicate an increased risk of graft loss in recurrent PSC, with a median survival before re-transplantation of approximately 39 months (CI: 28–51) [82]. Furthermore, PSC patients have a higher rate of re-transplantation for graft loss and a lower survival rate compared with patients transplanted for PBC [83].

Recurrent Autoimmune Hepatitis

The majority of patients with AIH respond well to immunosuppression, usually a combination of corticosteroids and azathioprine. The reported 10-year patient survival post-transplant is approximately 75 % [84]; however, features of AIH can recur in spite of post-transplant immunosuppression

[85]. Recurrence rates vary between 12 and 50 % over 8–10 years post-transplant (median time ~2 years) depending on diagnostic criteria [74, 86]. Diagnosing recurrent AIH is challenging, and abnormal serum transaminases can be the consequence of many different insults directed towards 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 [87]. Conversely, high-titre autoantibodies and hypergammaglobulinemia may remain detectable following liver transplantation irrespective of recurrence of disease.

Aetiopathogenesis and Molecular Mechanisms

Native AIH is putatively mediated by HLA-restricted, autoantigen-reactive $CD4^+$ and $CD8^+$ T-cells [88], and data from studies looking at HLA matching in living related donor transplantations has 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 [86]. There is also evidence that recurrent AIH is dependent upon the severity of disease prior to transplantation and that chronic ductopenic and acute cellular rejections are both more commonly seen in patients with recurrent disease [89]. Recurrent AIH is significantly increased in recipients with high pre-transplant 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 [90].

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 [84]. Significant risk factors for recurrence have not been fully elucidated although patients transplanted for AIH frequently require continued steroids compared to those for other aetiologies (64 % vs. 17 %). As recurrence of AIH is an important risk factor for graft loss [72], the practice in Birmingham is often to keep all patients transplanted for AIH on low-dose corticosteroids, in addition to a calcineurin inhibitor and either azathioprine or MMF.

De Novo Autoimmune Hepatitis

Features of AIH developing in the donor liver have been reported in patients transplanted for non-immune indications [91, 92], although this is a difficult diagnosis to truly reach and its classification strains the boundary of what might be also considered a variant of rejection. Such a hypothesis stems from the association of de novo AIH with suboptimal immunosuppression, prior history of ACR, the use of immune modulating drugs (e.g. pegylated interferon) and good response to increasing doses of immunosuppression [93, 94]. 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 GSTTI genotype mismatch between donor and recipient. Hence, the immune-related damages seen in GSTT1-positive patients are directed against the graft and not the host, suggesting an alloimmune over autoimmune response [95]. Children appear more at risk than adults, but the condition is relatively uncommon. There is usually a good response to additional immunosuppression with corticosteroids, but in some cases there is progression to cirrhosis and subsequent graft failure [96].

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 cellular rejection although in contrast to other solid-organ allografts this does not influence graft or patient survival in the absence of SRR 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 CR, 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 focussed the field of research into identifying immunological and genetic signatures associated with operational tolerance in an effort to minimise the long-term complications associated with prolonged immunosuppressive therapy.

In contrast to acute rejection, our 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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The Future of Liver Immunology

31

M. Eric Gershwin, John M. Vierling, and Michael P. Manns

Take-Home Points

- The liver as a lymphoid organ is key to tolerance and successful maintenance of pregnancy but also can become a victim of autoimmune disease.
- The prevalence and incidence of immunological disorders in the liver is staggering and impacts fields as diverse as autoimmunity, bacteriology, parasitology, and virology.
- Sadly, however, with the exception of newer and aggressive therapies for hepatitis C, there is a relative paucity of both research and new therapies for patients with autoimmune liver disease.
- Diseases such as PBC and PSC are considered orphan diseases and have not generated the quality/quantity of research necessary to bring new therapeutic venues to the bedside, unlike rheumatoid arthritis.
- Newer technologies, including deep sequencing, usage and dissection of microRNA, proteomics, metabolomics, and epigenetics, should change these concepts and will hopefully, by the time of the next edition, change the profile of both diagnostic and therapeutic immunologically mediated liver diseases.

Introduction

Immunology is a relatively novel new specialty compared to the many other subdisciplines of medicine. It is difficult to begin explorations of medical history to know exactly where the driving force behind immunology began. There are clearly multiple heroes in the distant past who began with such basic concepts as allergy, anaphylaxis, and host defense. Among these was Clemens Von Pirquet. Although Von Pirquet's work has long been forgotten, other than by allergists, his tuberculin test is still in usage, although the name of the discoverer is no longer associated with it. Von Pirquet was born in 1874 in a small town near Vienna. His ancestors originally came from Belgium, and at that time, and in fact until the Battle of Waterloo in 1815, that region of Belgium was an Austrian province. Von Pirquet and his work at the Vienna School of Medicine became famous, and although he did not grasp the concept of autoimmunity, he did champion the thesis that the immune system could do harm. There is but one definitive biography of Clemens Von Pirquet and that is published by one of his last students, the famous Tufts University-New England Medical Center pediatrician, Richard Wagner [1].

Ironically, the first textbook of autoimmune disease was published by Mackay and Burnet in 1963 [2]. The editors of this textbook note with pride that Dr. Ian Mackay has once again written the foreword to this textbook. In quoting from the jacket cover, it is noted that the authors "define current usage of the many terms of immunology which are rapidly appearing in modern medical parlance. They describe immunological findings which provide the background to modern immunological theory. These theoretical concepts are then applied to the problem of how autoimmunization might occur and the nature of the diseases that result. Full descriptions are given of diseases associated with autoimmunization … The authors critically review the claims of rheumatoid arthritis, the so-called collagen diseases, multiple sclerosis, chronic liver disease, and chronic kidney disease for inclusion as autoimmune processes. The spectrum broadens as they discuss Sjogren's disease, rheumatic fever, ulcerative colitis, myasthenia gravis, pernicious anemia, and various other disease processes. As similar principles of treatment apply to most autoimmune diseases, these principles are described in a single comprehensive chapter.

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An excellent description is given of the rationale of treatment … the suppression or elimination of autoantibody producing tissues … the part played by cortisone and allied agents." This classic textbook had a total of 14 chapters and including approximately ten pages on liver disease, including what was called "active chronic and lupoid hepatitis" and a chapter on primary biliary cirrhosis (PBC).

The Textbook Herein

The WHO has shown increasing interest in the global and regional epidemiology of acute and chronic liver diseases to better understand their impact on health and productivity, as well as their role in liver-related and all-cause mortality. The fact that hepatic inflammation, due to any cause, is associated with a risk of increased all-cause mortality that is directly proportional to the level of elevation of ALT has integrated hepatobiliary diseases with the broader issue of cardiovascular disease risk and prevention. In the near future, we will likely see adoption of male- and female-specific normal ranges of ALT that reflect the increased risk of all-cause mortality in women or men with levels ≥19 or ≥30 U/L, respectively. Evidence that sustained virologic responses (cures) of hepatitis C virus (HCV) infections significantly decrease all-cause, not just liver-related, mortality should lead to investigations of the impact of therapies in liver diseases on all-cause mortality. Such analyses will allow assessment of therapeutic benefit decades earlier than would be required to assess impact on liver-related deaths and provide evidence of the cost-effectiveness of early diagnosis and treatment of chronic liver diseases. Regional and/or global disparities should allow appropriate allocations of resources to meet identified needs. Overall, progress in this area should help inculcate a concern for liver health worldwide.

In 2004–2005, one of us (J.M.V.) met with skepticism regarding a proposal to devote the AASLD Postgraduate Course to the role of inflammation and immunology in the pathogenesis of the entire spectrum of hepatobiliary diseases. The undeniable need for all hepatologists to conversant with immunology was validated by that Postgraduate Course and by the second and third editions of this book, which reflects the expanding knowledge of the seminal roles of inflammatory immunological mechanisms in the pathogenesis of all hepatobiliary diseases. We anticipate that a working knowledge of fundamentals of immunology will continue to be of value to clinicians and researchers interested in the liver. It will also be of value for understanding many non-hepatic diseases associated with inflammatory and immunological mechanisms.

Refinements in our knowledge will lead to new biomarkers to interrogate plasma, urine, saliva, and biopsy specimens for diagnostic and monitoring purposes. We are likely to develop tests to distinguish among the types of inflammation (or its absence) in the liver, refining our concepts of disease at the level of pathogenic heterogeneity. Such tests may ultimately distinguish between concurrent processes, such as recurrent viral hepatitis and acute cellular rejection after liver transplantation. Such biomarkers may also yield tests to supplant both AST and ALT. Whole genome and gene chips will likely allow prediction of the metabolism of xenobiotics and therapeutics in individual patients. Imaging should continue to evolve in precision and the use of injectable markers of metabolism or mAbs specific for malignant cells. Transcutaneous mass spectroscopy should revolutionize our understanding of hepatic physiology and pathophysiology, which is useful in diagnosis or monitoring of progression or response to therapy. Immunohistochemistry will continue to advance with identification of additional epitopes for the development of mAbs and proteonomic profiles indicative of disease activity.

Continued progress in this area should unravel the molecular and genetic controls of the inherent balance between hepatic recognition of deleterious pathogens and diseased or malignant hepatobiliary cells and maintenance of the immunosuppressive environment required to reduce the risks of food allergies, autoimmunity, and excessive systemic exposure to PAMPs. Such knowledge will likely open up novel therapeutic approaches to primary hepatobiliary diseases and primary and metastatic malignancies that are rooted in aberrant activity of normal mechanisms of surveillance. Similarly, strategies may be identified to prevent allergic, autoimmune, and other undesirable systemic consequences. In many hepatobiliary diseases it is conceivable that the trafficking of effector cells to the liver may be inhibitable by interruption of the chemokine and chemokine receptors required for immunopathology. The key role of the microbiome will likely be identified as having a regulatory function of the liver as an immune organ, as will the status of gut inflammation and permeability promoted by gluten and the gene products of bioengineered grains.

Further research is needed to understand the liver-specific aspects of sterile inflammation triggered by danger signals and those induced by PAMPs and PRRs, especially in the environment perfused by portal venous blood normally containing a variety of PAMPs in the absence of viable bacteria and subject to changes induced by gastroenteritis, changes in permeability, etc. We will likely come to appreciate the roles of commensal viruses in these hepatic events and the impact of diseases of hepatocytes and/or cholangiocytes on the homeostasis. We may also learn important information based on whether the microbiome does or does not contain parasites, as it often does globally.

The holy grail of producing long-lasting, Ag-specific tolerance by oral feeding of Ag has not been successful to date, but it remains possible that liver-mediated tolerance

may lead to the prevention of type 1 DM, MS, and other immune-mediated or autoimmune diseases.

A testable hypothesis is that all hepatocellular diseases are immune mediated, at least in the sense that they derange the normal immunological milieu of the liver as an immunological organ. Categorizing the spectrum, ranging from direct hepatocellular-specific toxicity (e.g., white phosphorous, amanita toxin) with secondary necrosis/apoptosis to hepatocyte-specific immune attack (e.g., HBV, HCV infections, autoimmune hepatitis (AIH)) at the level of pathogenesis will likely be accomplished. Such knowledge should provide evidence of an integrated or heterogeneous array of specific diseases and potentially provide new diagnostic classifications based on mechanism, rather than etiology or histopathology. Recognition of common mechanisms would be useful in targeted research into therapies to disrupt such mechanisms.

Further research will continue to solidify the concept that the biliary tract—from canaliculus to ampulla of Vater—as an organ within the liver is composed by cells of variable gene expression and has the capacity for absorption and transport from the most proximal bile ducts to the distal common bile duct. We will likely appreciate at the molecular and physiological level the importance of the bile duct unit, defined by the inseparability of the arteriole branches of equal caliber that lie near the intrahepatic bile ducts and give rise to the circumferential biliary capillary plexus and to the retrograde flow of lymph created in the space of Disse in the hepatic lobule that flows in the peribiliary space. Among the likely advances will be a formal understanding of the cholehepatic circulation and its capacity to concentrate substances in the proximal biliary-peribiliary tract, the role of paracellular pathways and tight junctional integrity in health and diseases of the bile ducts, and the impact of lymph on bile ducts. Greater understanding of Ags, expression, and degradation may clarify our understanding of the PBC vs. PSC.

A detailed understanding of the extrahepatic effects of Ag–Ab complexes derived from antigens of hepatobiliary cells or pathogens infecting hepatobiliary cells will likely occur. It should clarify similarities and differences in the pathogenesis of GN, arthritis, and cutaneous vasculitis in patients with liver diseases and non-hepatic immune-mediated diseases.

The liver is unique in its capacity to maintain a branching connective tissue conduit to contain the portal triad of portal vein, hepatic artery-arteriole, bile duct, and lymphatics with minimal fibroblast activity and to harbor in the space of Disse the hepatic stellate cells. These cells are subject to activation and proliferation as myofibroblasts, leading to production of collagens, ultimately associated with cirrhosis, the final common pathway of all chronic hepatobiliary diseases. Further understanding of hepatic fibrogenesis will likely provide therapeutic opportunities to inhibit or retard hepatobiliary fibrogenesis, while permitting the normal fibrogenesis required extrahepatically. In addition, we will likely learn the important differences between postnecrotic cirrhosis (hepatitides) and biliary cirrhosis, which may provide additional therapeutic opportunities specific for hepatocellular vs. biliary diseases.

The roles of commensal bacteria and viruses in health and disease should add to our understanding of the circumstances facilitating bacterial infections of the liver and the role derangement of the normal relationship plays in risk. Studies of the microbiome, gut inflammation, and increased permeability of the gut may provide important clues, including genetic predisposition based on these and the ability to ward off infection with innate immune responses.

Future studies will likely detect factors important in the susceptibility of the liver to parasitic infections. Progress in the prevention and development of new therapeutics for parasitic infections will likely continue.

We should be able to define the immune mechanisms—innate and adaptive—that underlie the universal ability of humans to terminate this RNA viral infection. We will also likely understand the quasispecies differences between the hepatocyte and plasma compartment. Understanding these issues should explain why adults may develop a relapse, often characterized by cholestasis, during the initial recovery period.

We will likely come to understand the mechanisms involved in the transitional selection of the HBeAg-negative mutants in previously HBeAg-positive wild-type infections, which now constitute approximately 40 % of chronic HBV infections. We will then be able to understand the nature of the silencing of innate immunity in hepatocytes and the requirements of adaptive immunity required to clear infection. We will also likely learn how a de novo infection with HBeAg-negative mutants differs from acute infection with the HBeAg-positive wild type and how the absence of HBeAg in the former influences the host immune response. Importantly, we may discover ways to eliminate HBV cccDNA from the nuclei of cells, through immune recognition. This would have an immediate impact by allowing discontinuation of therapies now used long term.

We should learn about the roles of disinhibited innate immunity and dysregulated adaptive immunity in terminating HCV infections during treatment with antiviral agents to achieve SVR in up to 100 % of treated patients. We will learn more about the mechanisms of therapeutic vaccines that can induce polyAg, polyclonal T cell responses specific for HCV-Ags in patients with poor responses prior to immunization. This may reflect Treg and/or Breg and/or other inhibitory mechanisms that could become targets of therapeutic development. We should learn how the host immune response and prior interferon-based therapies alter the quasispecies and whether or not the quasispecies differ within hepatocytes and plasma. As noted earlier, we need to overcome the present inability to identify HCV Ags in liver biopsy specimens.

Understanding of the life cycle within hepatocytes should allow identification of hepatitis D virus (HDV)-specific therapeutic options that go beyond depriving HDV of the HBV-produced HBsAg needed as the HDV envelope. We should also understand in more detail whether HDV infection, and by analogy other RNA viruses, may infect hepatocytes that do not support their full life cycle, such as was reported for HDV infection after OLT in livers devoid of HBV infection. Conceptually, this would appear not to be an infection because of the failure to shed viruses into the circulation.

Greater understanding of the emergence of HEV infections in immunocompromised and immunocompetent individuals is a high priority, as is understanding of the vectors and preventive measures. Virologically, a WHO-standardized quantitative HEV RNA PCR will be licensed to diagnose active infections and to assess the response to therapy with ribavirin.

Continued studies in humans and animal models will likely identify new opportunities to therapeutically disrupt fundamental immunological mechanisms of lymphocytic destruction of small to medium caliber bile ducts. Attention to the immune and nonimmune genetics may help identify persons at risk so that diagnosis can be made prior to the irreversible destruction of a large proportion of bile ducts. Antifibrotic therapies and therapies, such as obeticholic acid, to counteract the deleterious gene expression associated with cholestasis will likely be developed. Since PBC has such well-defined, overlapping T and B cell epitopes, it provides an opportunity for prevention were Ag-specific tolerogenic methods developed. The characterization of the initial at-risk population would be easy given the high incidence within first- and second-degree relatives with PBC. Studies of the immunopathogenesis of recurrent PBC post-OLT, especially in donor livers with high degrees of HLA mismatching, should provide important insights into the HLA restriction of the autoimmune process and whether (as proven in recurrent HCV) allogeneic T cells of the host can recognize autoAgs presented by donor allogeneic HLA class I and II molecules.

In contrast to type 2 AIH, which is mediated by reactions to overlapping epitopes in the CP2D6 molecule, we still lack identification of the Ag or more likely Ags responsible for the more prevalent type 1 AIH. This is a key priority for research, since it will afford the chance to define whether or not type 1 AIH is or is not a heterogeneous disease. It will also allow the development of more specific diagnostic tools, minimizing reliance on the nonspecific ANA and SMA autoantibodies. As already shown in type 2 AIH, the response to the autoAgs is mediated by oligoclonal T cells, as expected by the Ag-restricted repertoire of TCRs. This in turn has allowed exploration of the development of Ag-specific Tregs ex vivo to suppress the hepatic disease, while avoiding nonspecific immunosuppression and its adverse events. This may move into clinical testing soon. Once type 1 AIH Ags are identified, similar studies can be pursued. As noted above under PBC, studies of the recurrence of AIH in an allogeneic liver should lead to an understanding of mechanisms that may not require Ag presentation by HLA-identical molecules.

It is likely that the debate regarding PSC as an immune-mediated disease or an "atypical" autoimmune disease should be resolved by either defining the relevant autoAg or not. Current data, including genetic data showing more similarities between immune-mediated diseases and PSC than it has with the genetics of autoimmune diseases (including PBC), favor an immune-mediated pathogenesis involving gut-derived T cells with chemokine and adhesion receptors for aberrant ligands in the portal tracts. Further studies may provide new insights into the relevance of this to the high frequency of association with IBD, especially UC. This in turn would allow comparison of PSC with and without IBD. Additional insights will come from focused studies on the pathogenesis and mechanisms of the most common form of secondary sclerosing cholangitis, IgG4 cholangiopathy in the absence of pancreatic involvement. The issue of recurrent PSC post-OLT is unique among the "autoimmune" liver diseases in that the cumulative recurrence rate progresses for at least 10 years and affects up to 50 %. The relationship between gut, microbiome, magnitude of proinflammatory responses, and their regulation may explain this process, leading to a better understanding of the pathogenesis of PSC and therapeutic targets for prevention.

The key issue is not if clinical, biochemical, serological, pathological, and cholangiographic evidence of overlap of AIH-PBC or AIH-PSC exists, but whether they represent the concurrent presence of two independent diseases in genetically susceptible patients or a transition of pathogenic mechanisms from one disease to another. Progress in understanding the individual diseases is required to make progress in understanding the overlap syndromes.

The role of the immune system will become increasingly important in our concepts of pathogenesis and progression of alcoholic liver disease, especially in the propensity for acute alcoholic hepatitis to worsen for up to 6 weeks after cessation of alcohol. Studies of the immune mechanisms, combined with comparisons of the genetics involved in the susceptibility of only a minority of patients drinking excessive alcohol to alcoholic liver injury and the genetics of the innate and adaptive immune responses, may identify markers of risk. In addition, understanding of the pathogenic mechanisms should also provide more specific therapeutic targets than high-dose methylprednisolone for the treatment of acute alcoholic hepatitis. Such therapies are disparately needed, since the mortality rate is approximately 40 % by day 29 and in most countries these patients are excluded as OLT candidates.

The mechanisms and susceptibilities involved in the development of NASH, compared to benign steatosis, are high priorities for research, since NAFLD is increasingly prevalent worldwide and threatens to become one of the leading causes of cirrhosis, liver failure, HCC, and OLT. Only by understanding these mechanisms can rationale therapies be proposed. Research may allow prevention of NASH but not steatosis, which only partially solves the problem but would significantly reduce consequences.

The heterogeneity and complexity of extrahepatic biliary artesian will likely be clarified by study of the immune mechanisms underlying the activation and recruitment of a fibrosing inflammatory infiltrate. Breakthroughs in this disease would revolutionize the practice of pediatric hepatology and the frequency of OLT in this population. Knowledge of the impact of growth of the hepatobiliary system during normal development on the expression of disease and susceptibility to disease may provide important insights relevant to the process of hepatic regeneration during acute and chronic liver diseases of adults.

There are four key issues in ALF. The first is the need to understand and countermand the massive hepatocyte death mediated by Fas, TNFR, and perforin granzyme mechanisms during a cascade of events involving proinflammatory cytokines, IFNγ, NK, NKT, PMNs, and T cells. The second is to understand how the massive loss of hepatocytes and inflammatory milieu prevents successful regeneration despite production of high concentrations of all known growth factors required for regeneration. Third, the mechanisms and etiologies of ALF in the majority of patients, currently classified as "indeterminate," will likely be understood by developments in the area of xenobiotic injury and metabolism. Fourth, it will likely be proven that even nonimmune-mediated drug-induced liver disease (DILI) involves inflammation, cytokines, and chemokines and, at a minimum, resident innate immune cells are key to hepatocellular destruction and ALF caused by DILI. Support for this notion comes from animal studies of acetaminophen (APAP) hepatotoxicity, long believed to be mediated solely by covalent binding of APAP metabolites to ER and mitochondrial membranes, showing that lethality required NK and NKT cells.

GWAS findings of strong associations of HLA and SNPs of immunological genes in most so-called idiosyncratic DILIs have rekindled a focus of attention on immune mechanisms and biomarkers of those mechanisms. The issue of DILI induction of AIH, mimicry of AIH, or unmasking of AIH will likely be clarified, especially in patients with inadvertent reexposure to offending drugs. Studies should also help clarify why many immunological reactions are against the carrier proteins of CYPs and UGTs bound with metabolite haptens, while the AIH variants associated with DILI are almost always associated with autoantibody profiles of type 1, not type 2, AIH.

The relationship between inflammation and wound healing in the liver clearly involves activation and proliferation of hepatic stellate cells and resident fibroblasts in the connective tissue of the portal tracts. Refinement of information indicating important roles for cytokines and chemokines in the progression to cirrhosis should lead to novel therapies focused on prevention of fibrogenesis to eliminate progression to cirrhosis. In addition, the mechanisms of dissolution of hepatic fibrous tissue by collagenases appear to require a microenvironment without inflammation, as shown in chronic therapy of HBV infection and during remission of AIH. Results of the ongoing phase 2 studies of humanized mAb against lysyl oxidase-like 2 in PSC and NASH should provide definitive information about this approach.

The primary dilemma in HSCT continues to be the need for retention of alloreactive T and B cells for graft vs. tumor effects and the risk of GVHD conferred by the presence of these cells. Since the effector cells of GVHD are required for successful application of HSCT, future therapies will continue to explore ways to suppress or prevent end-organ damage without high-dose nonspecific immunosuppression with its liabilities of opportunistic infections. Promising approaches are to prevent the transendothelial migration of effector cells into the target tissues by preventing chemoattraction and recognition of chemokines by activated T cells with specific chemokine receptors. Modulation of effector cell generation by co-transplantation of mesenchymal cells from the donor may also prove beneficial. Studies will likely clarify the target tissue restriction of GVHD for the skin, gut, and liver, especially the restriction of lymphocytic cholangitis to the cholangiocytes lining the small to medium caliber bile ducts. Understanding the latter will provide useful information not only about GVHD but also hepatic allograft rejection (HVGD) and PBC.

Development of immunosuppressive therapies will continue with recent introduction of everolimus as an mTOR inhibitor and trials of belatacept. Studies designed to prevent alloreactive T cells from trafficking to portal tracts and to the cholangiocytes secreting chemoattractant and differentiating chemokines and cytokines polarizing Th1 and Tc1 responses may result in apoptosis of multiple alloreactive T cell clones through exhaustion. Such strategies, coupled with inhibition of costimulation of alloreactive T cells (belatacept), could promote a relative tolerance, requiring minimal immunosuppression. This in turn would result in lower incidence and severity of DM, HTN, and risk of CKI.

Studies of new immunosuppressive strategies, including inhibition of costimulatory signaling in T cell activation and inhibition of leukocyte trafficking to target tissues, will be conducted in solid organ transplantation and in autoimmune and immune-mediated diseases. The combination of agents acting to inhibit specific aspects of the innate and adaptive immune responses may achieve therapeutic goals, while minimizing toxicity and risk of infections due to the use of lower doses of agents in combination.

The Future: A Clinician's View

Liver immunology is the science that helps us to understand the role of the liver as part of the immune system. This is relevant not only for components of the immune system infiltrating the normal and diseased liver but also for non-parenchymal as well as parenchymal liver cells. Hepatocytes and bile duct epithelial cells both are targets of immune-mediated liver diseases. In addition, the innate as well as the adaptive immune system is significantly involved in the pathophysiology of various liver diseases including viral hepatitis, immune-mediated "DILI", autoimmune liver diseases, liver cancer, and others like steatohepatitis and even alcoholic hepatitis. Modern medicine also uses the immune system or components of it, humoral as well as cellular, to prevent, diagnose, and treat liver diseases.

Hepatitis B virus (HBV) was the first of the major hepatotropic viruses to be discovered. Originally, not the virus itself, but its surface protein synthesized and secreted in 1,000-fold excess by the patient's own liver was discovered in 1965. Subsequently the virus itself was identified, originally termed the Dane particle. This led to the first HBV vaccine produced from human plasma just over a decade later in 1980 consisting of the spherical and filamentous HBsAg particles. Subsequently, recombinant HBV vaccines became available and were widely used and demonstrated to be the first vaccine against cancer. This was shown in Taiwan in 1997 following the introduction of universal vaccination of all newborns a decade earlier. The hepatitis A virus (HAV) was discovered in human stool in 1973, but production of complete virions in tissue culture was necessary in order to obtain an active vaccine in the early 1990s. An active hepatitis E vaccine was developed around 2000, but the lack of Western government commitments to widely use this HEV vaccine prevented its final clinical development. A separate HEV vaccine development programme by a company supported by the Chinese government finally led to the availability of an active HEV vaccine, however, so far in China only. Vaccines against the two remaining major hepatotropic viruses are missing: hepatitis C and D (delta). Since hepatitis D always requires coinfection with hepatitis B, active HBV vaccination protects against de novo HDV infection as well. However, a genuine HDV vaccine would be helpful to protect the 150 million chronic HBV carriers against HDV superinfection. This would be necessary since superinfection of chronic hepatitis B patients with HDV means more rapid progression of chronic liver disease and even fulminant life-threatening acute hepatitis. In some areas of the world like the Amazonian basin, the combination of hepatitis B genotype F and hepatitis D genotype 3 often leads to fulminant hepatitis with high fatality rates. In particular in these populations, an active HDV vaccination would be very helpful. Although the estimations for chronic hepatitis D (delta) patients worldwide range between 10 and 30 million, this seems not enough motivation for the pharmaceutical industry to invest in the development of an HDV vaccine. It does seem, however, technically feasible.

The situation for HCV and its vaccine is quite different. From the first day of HCV discovery in 1988, all major efforts seemed to concentrate on the development of an active HCV vaccine. The development of a prophylactic HCV vaccine, however, proved to be more difficult than expected. Some HCV vaccine candidates were able to induce specific B and T cell responses. One promising candidate could prevent chronicity but not HCV infection itself. By now all major vaccine companies have terminated their prophylactic HCV vaccine development programmes. There are multiple reasons behind this decision: failure of several vaccine approaches and lack of unanimous government support for such a vaccine that would facilitate universal prophylactic vaccination programmes. There has been and still is a controversy about the need for such a HCV vaccine: De novo HCV infection was reduced by over 50 % due to anti-HCV antibody and later NAT screening of blood donors and blood products as well as continuous improvements in HCV therapies leading to cure of the first chronic viral infection in man in 70–80 % depending on HCV genotype. There has also been a debate in which risk population such as vaccination should be tested. No doubt the best population would be prison inmates since their de novo infection rate is high. However, this is not allowed due to ethical reasons, in particular in Germany with its history in the past. In contrast, several therapeutic vaccines for both hepatitis C and B are still being developed mainly by small- or medium-sized companies.

Recombinant interferon alfa, now in a pegylated form, is used successfully in all types of chronic viral infections: hepatitis B, D, and C.

In chronic hepatitis D interferon alfa seems to be the only effective therapy. However, this clearance of HDV RNA is achieved and maintained 24 weeks after the end of treatment in only about 25 %. Therefore novel anti-HDV drugs are urgently needed that specifically interfere with the HDV life cycle. At present prenylation and virus entry inhibitors are promising compounds. The scientific community must convince governments and as well as the industry that such HDV therapies are urgently needed.

In chronic hepatitis B several recombinant pegylated as well as non-pegylated interferons as well as oral nucleotide or nucleoside HBV polymerase inhibitors are approved. However, there is still room for innovative therapies that would allow stable HBsAg/anti-HBs seroconversion which means long-term immunological control of viral replication which is closest to cure. Innovative combination therapies should be explored that use an intelligent combination of drugs used in an individualized approach which takes

advantage of novel biomarkers to guide therapy. The combination of various biomarkers like quantitative HBV DNA, quantitative HBsAg, IP10, and others might help to guide the successful development of innovative therapies using novel interferons like interferon lambda, nucleos(t)ide HBV polymerase inhibitors, and novel drugs like therapeutic vaccines and toll-like receptor agonists, e.g., TLR 7 and TLR 9. These novel therapies should allow finite treatment paradigms associated with stable HBsAg/anti-HBs seroconversion rates in the majority of patients.

In hepatitis C therapy the future has already started, at least for HCV genotype 1 patients. Since 2011 peginterferon alfa, ribavirin, plus one of the two approved protease inhibitors are standard of care. The second-wave HCV protease inhibitors are just finishing phase 3 programmes allowing once daily dosing, shorter duration of therapy in the vast majority of patients, and less side effects than the first wave of HCV PIs. All-oral interferon-free combinations are also in phase 3 at the moment, and first all-oral regimens are to be approved in early 2014 for genotypes 2 and 3, may be even in Q4 2013. These combinations use NS 3/4A HCV protease inhibitors, nucleoside and non-nucleoside polymerase inhibitors as well as NS 5A inhibitors plus or minus ribavirin. It looks that a combination of drugs specifically intervening with different targets of the HCV life cycle is sufficient to cure this chronic viral infection since once HCV RNA clearance is achieved short term (24 weeks) after the end of treatment; this means cure. At present it seems that intervention with the immune system is not necessary to cure HCV infection. So far only the easy-to-treat HCV populations have become easier to treat with the novel direct-acting antiviral agents (DAAs) while we still wait for effective all-oral interferon-free therapies for difficult to treat special patient populations. The first all-oral interferon-free HCV therapies are expected to become approved in early 2014, however, first for HCV genotypes 2 and may be 3 only.

AIH, PBC, and primary sclerosing cholangitis (PSC) are the three major autoimmune liver diseases. Between 1987 and 2000, most of the major autoantigens targeted by disease-associated autoantibodies have been identified at a molecular level. As a consequence recombinant autoantigens became available for diagnostic testing.

These discoveries also enabled us to understand better the immunopathogenesis of autoimmune liver diseases. Animal models were developed, targeting autoantigens relevant for human disease that allowed us to better understand the mechanisms underlying the self-perpetuating disease process. However, despite all the progress made in the diagnosis and understanding of the pathogenesis of autoimmune liver diseases, progress in drug therapy for all three autoimmune liver diseases remained limited. Since decades steroids with or without azathioprine are standard of care as first-line therapy for AIH. Results are excellent as long as normal aminotransferases ALT and AST are achieved. Normalization of transaminases is associated with a normal life expectancy. The topical steroid budesonide has become an alternative to predniso(lo)ne with similar efficacy but less steroid specific side effects. In contrast, patients who fail to this stand of care find it difficult to achieve complete remission following second-line therapies. Here, mycophenolate mofetil (MMF), tacrolimus, cyclosporine, cyclophosphamide, and anti-TNF antibodies are of limited success. Maybe anti-CD3 antibodies provide an option; hopefully they are as beneficial as in several other autoimmune diseases including type 1 diabetes mellitus. Still 4 % of all liver transplants in Europe and North America are performed because of end-stage AIH; this is too much.

PBC is associated with antimitochondrial antibodies (AMA) in around 95 %. AMA and their autoantigens of the inner mitochondrial membrane were the first liver disease-associated autoantigens that were cloned in the late 1980s and thus could be defined at the molecular level. In the therapy of PBC, ursodeoxycholic acid (UDCA) is still the treatment of choice—for decades now. Obeticholic acid is at present among the very few drugs under clinical development for PBC; the drug has reached phase 3. Nor-ursodeoxycholic acid (NOR UDCA) may become another candidate. This drug is in phase 2 for PSC.

In contrast, PSC remains the “black box of hepatology”: unknown etiology, no specific autoantibodies, no benefit from immunosuppressive therapy, no effective generally accepted medical therapy at all, association with hepatic and non-hepatic malignancies for yet unknown reasons. Multiple hypotheses exist for the etiology and pathogenesis of PSC ranging from *gut leaky syndrome* to the *aberrant homing of gut-derived lymphocytes* to the biliary tree. For decades, there has been a debate as to whether PSC is an autoimmune or immune-mediated liver disease at all. However, the strongest indicators for an immune-mediated pathogenesis for PSC now come from genetic studies provided by an international consortium of PSC researchers joined in the International PSC study group (IPSCSG) chaired by the world renowned center in Oslo. DNA samples were collected from several thousand patients mainly from Northern Europe and North America. The overall results published in prestigious journals suggest that an immune-mediated pathogenesis is underlying PSC. Several genes of the MHC locus are highly associated with PSC followed by membranous bile acid receptors like TGR 5 and mediators of inflammation like MST-1. A further unsolved miracle of PSC is its over 80 % association with inflammatory bowel disease (IBD). The genetic studies also give interesting insight into the relationship between IBD and PSC. There are genes shared by both IBD and PSC while others are uniquely associated with each individual inflammatory disorder.

The liver is regarded as an immunologically privileged organ. ABO but not MHC compatible organs are used in

liver transplantation. However, lifelong immunosuppression with classical agents such as tacrolimus, cyclosporine, MMF, everolimus, and specific monoclonal antibodies is usually necessary. mTOR inhibitors have been the latest addition to the armamentarium of posttransplant immunosuppressive agents. They not only act as immunosuppressive agents but also exhibit antifibrotic and antiproliferative activity. This makes mTOR inhibitors attractive for liver transplantation in patients with liver cirrhosis and hepatocellular carcinoma. Adverse events following long-term use of immunosuppressive agents are often limiting the success of transplantation and contribute to the morbidity following transplantation. At present the pipelines of the pharmaceutical industry for new innovative immunosuppressive agents are empty. Therefore the next step must be to achieve a long-term immune tolerance towards the transplanted organ by weaning off all immunosuppressive agents. This must be the future for most if not all solid organ transplants, namely, liver transplantation. Biomarkers will become necessary that guide us in this direction. Innovative cell therapies like application of regulatory T cells (Tregs) might facilitate this long-term goal of donor organ tolerance without the long-term use of toxic drugs: A dream would come true.

Final Comments

As Yogi Berra said, “Predictions are dangerous, especially ones about the future.”

References

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