Pooja Jain
Lishomwa C. Ndhlovu *Editors*

Advanced Concepts in Human Immunology: Prospects for Disease Control

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The Chief Editor, Professor Pooja Jain, dedicates this book to her parents (Mr. Deshraj Jain and Mrs. Kiran Jain) from India as well as family/friends in the United States who have always supported and motivated her to work hard and maintain enthusiasm and balance toward her career and personal life!

The Coeditor, Professor Lishomwa C. Ndhlovu, dedicates this book to the family and circle of friends who impart indelible enlightening experiences to his life explorations and for whom he has indescribable gratitude and hopes they enjoy being a part of the readership!

Foreword

Immunological disorders afflict a considerable number of people across the world. A majority of immunological findings have been derived from the animal studies primarily rodents that comprise a majority of available textbooks on this topic. This book attempts to compile information derived primarily from human studies while keeping important research findings irrespective of the source. This text covers a wide range of topics from host-pathogen interactions to the evolution of the host immune response against cancer, allergic and autoimmune diseases, as well as neuroinflammatory disorders. The readers are provided with the latest information with clinical data related to these topics in addition to an in-depth discussion on the immune checkpoint inhibitors in the context of cancer, infection, and neuroinflammation. A detailed account on the immunoproteomics technology in cancer and infectious diseases represents a unique aspect of this book so as clinical perspectives on the polycystic ovarian syndrome (PCOS), which is rarely covered in the scientific literature.

Preface

This book covers a range of topics related to human immunology such as host-virus interactions, innate and adaptive immunity to allergens and self-antigens, current status of immune checkpoint inhibitors in cancer and infection as well as neuroinflammation, chimeric antigen receptor (CAR) T-cell therapy, and neuroprotective immunity against diseases of the central nervous system. In addition, the power of immunoproteomics technology has been highlighted for immunotherapy, and clinical perspectives on the polycystic ovarian syndrome (PCOS) have been provided as unique aspects of this book. The first chapter of the book covers health burden, viral pathogenesis, host immune response, current treatment, and status of future trends in research on antiviral immunoprophylaxis and pharmacotherapy. This chapter also provides a detailed account on coronaviruses with the last-minute addition on COVID-19 that has shaken global health causing worldwide mortality at an unprecedented rate. When the pandemic hit, this book was at the production state; thus, we felt obliged to include all plausible information pertaining to the intended audience.

The second chapter covers applications of immunoproteomic technology in peptide-based T-cell immunotherapy against cancer, infections, and autoimmune disease, while third and fourth chapters focus on the innate and adaptive immunity against allergic and autoimmune diseases. The cellular and molecular mechanisms underlying the immunopathogenesis of allergic eye disease are highlighted. The pharmacotherapy and immunotherapy of allergic eye disease have been discussed in detail along with the emerging role of microbiota in allergy. Autoimmune disease, in which adaptive immune system causes considerable disruption of normal cells and tissue due to loss of tolerance to self-antigens as depicted by type 1 diabetes, is discussed in detail in this textbook. Immune cells and molecules that play a role in the pathogenesis of type 1 diabetes with particular focus on loss of immune tolerance to beta cells of the pancreas and the subsequent destruction of these cells are also highlighted.

The fifth chapter highlights mechanisms of dendritic cell (DC)-regulated T-cell immunity and tolerance against acute myeloid leukemia (AML), a common hematologic malignancy in adult. Immunotherapies such as immune checkpoint inhibi-

tors and DC-based vaccination, which are potentially effective for treating and preventing AML, have been discussed in this chapter. Negative immune checkpoint receptors maintain homeostasis; however, an imbalance between activation and inhibition that biases toward overexpression of negative checkpoint regulators (NCR) results in impairment of T-cell-mediated immunity. Thus, sixth chapter discusses novel immunotherapies that inhibit NCR and reverse immune perturbation in these diseases. Along the same line, seventh chapter provides an up-to-date account on the power, limitations, and future of CAR T-cell therapy as personalized medicine and immunotherapy.

The eighth chapter on the neuroprotective immunity focuses on the role of innate and adaptive immune responses in the pathobiology of neurodegenerative and neuroinflammatory disorders. In addition, the role of nanomedicine in the diagnosis and treatment of cancer with emphasis on the use of nanoparticles to aid in the delivery of anticancer therapeutic agents to target cells in cancer and the use of nanotechnology to deliver diagnostic agents to enhance visibility of target cells in cancer during imaging studies are highlighted in this chapter. The last or ninth chapter focuses on PCOS, which is an endocrine abnormality and is the most common cause of anovulatory infertility in women of reproductive age. It has been suggested to be associated with autoimmune disease such as systemic lupus erythematosus. The chapter on PCOS covers the pathophysiology, comorbidities, diagnosis, and treatment of this condition.

In view of our theme, we have made considerable efforts to compile information derived primarily from human models with particular reference to humans. It is our hope that the readers of this textbook would have a broader and deeper understanding of the aspects of immunology relevant to humans that cover topics on viral pathogenesis and host immune response to viruses, interaction between cancer and host immune response, as well as overactive adaptive immune response to innocuous substances and self-antigens.

Pooja Jain
Lishomwa C. Ndhlovu

Introduction

The human immune system is comprised of both immune surveillance and immune defense mechanisms. Both antiviral and antitumor immunities mediated mainly by natural killer (NK) cells and T cells constitute an important innate and adaptive interface in immune defense, whereas recognition of tumor and viral antigens serves as the principal drivers of immune surveillance. Current standard of care for cancers include chemotherapy, radiotherapy, and surgery whereas pharmacotherapy is the standard of care for viral infections. Immunotherapy involving modulating the immune system to induce immunity against tumor cells and viruses has recently emerged as effective therapeutic against resistant cancer to standard of care options. Immune checkpoint blockade and CAR T-cell therapy are the two current immunotherapeutic strategies that demonstrated significant clinical efficacy against a variety of cancers. Generally, immune checkpoint inhibitor is a passive immunotherapeutic strategy whereas CAR T-cell therapy is an active form of immunotherapy.

T-cell activation involves the action of cell surface co-signaling coinhibitory or costimulatory molecules. The balance between costimulatory and coinhibitory signals determines whether T cells would be activated or tolerated. Immune checkpoints are coinhibitory molecules expressed on immune cells at different stages of immune cell activation and play a role in maintaining immune homeostasis and peripheral immune tolerance. Chronic stimulation of the immune system by tumor antigens and viral antigens triggers overexpression of immune checkpoints on CD8 tumor-infiltrating lymphocytes (TIL) and virus-specific T cells, respectively. Tumor cells and viruses use immune checkpoints to enhance their progression and dissemination. Expression of immune checkpoint receptors on activated T cells in the setting of chronic viral infection and cancer correlates with T-cell exhaustion, a state of T-cell dysfunction, progression of infection, and tumor growth. However, blockade of immune checkpoint receptors with monoclonal antibodies targeting receptor-ligand interactions has been shown to restore immune function of exhausted T cells. Studies have demonstrated that blockade of CTLA-4 and PD-1 enhances antiviral immunity and antitumor immune response. It has been shown that brain metastases contain high levels of PD-1 due to high density of TIL in these tumors and this

makes them susceptible to immune checkpoint inhibitors. Unlike PD-1 inhibition, blockade of CTLA-4 is associated with depletion of regulatory T cells and loss of peripheral immune tolerance. Although both anti-PD-1- and anti-CTLA-4-based immunotherapies are FDA-approved as solid tumor cancer immunotherapies, not all forms of cancer respond to them. As such, there is heightened interest in biomedical research to develop immune checkpoint inhibitors against these other forms of cancer that are unresponsive to anti-PD-1 and anti-CTLA-4 due to factors such as varying expression of immune checkpoint levels as well as the safety and tolerability of these forms of therapy. The use of anti-CTLA-4, anti-TIM-3, and anti-PD-1 is associated with significant immune-related adverse effects, thus current research is focusing on discovering immune checkpoint inhibitors with a better side effect profile. It is of note that inhibiting TIGIT, B7-H3, and VISTA is associated with less systemic side effects. In addition, organ (GI, liver, thyroid, etc.) toxicity may also occur with use of immune checkpoint inhibitors but these side effects are generally not life-threatening.

CAR T cell, a genetically engineered method of immunotherapy, is a personalized therapeutic modality designed to target cells expressing specific antigens for elimination. In oncology, these modified T cells with laboratory-generated immune receptors have been shown to target transformed cells expressing surface-specific antigens. CAR T cell is a formidable therapeutic armament in the fight against cancer. Unlike T-cell receptor-mediated antigen identification involving major histocompatibility complex (MHC), CAR T cells recognize antigen in the absence of MHC. There are four generations of CAR T cells based on the presence of costimulatory domains. The genetically engineered receptors of CAR T cells consist of antigen identification domain that recognizes tumor antigen, hinge domain, T-cell receptor transmembrane domain, and intracellular signaling domain. Unlike the first-generation CAR T-cell therapy that had four domains, the second and third generation CAR T cells have co-stimulatory domains. The fourth generation CAR T cells have an additional element called TRUCK (T-cell redirected for universal cytokine-mediated killing) that enhances T-cell function and development of T-memory stem cells. These CAR T cells also overcome the tumor-induced immunosuppressive microenvironment, which is a major limitation associated with previous therapy. Chimeric autoantibody receptor CAAR T cell expresses autoantigens recognized by autoimmune B cells, and as such, this therapy could be beneficial for patients with autoimmune disease. The adverse effects of CAR T cells include cytokine releases syndrome, neurotoxicity, and anaphylaxis. These side effects are likely due to the release of pro-inflammatory cytokines by activated CAR T cells. High cost of treatment, resistance to CAR T-cell therapy due to antigen modulation, adverse effects of CAR T cell, type of tumor, and lymphodepletion pose a challenge to the use of CAR T cell in cancer immunotherapy. Cancer immunotherapy is undergoing rapid evolution with current and future basic and translational research being carried out to expand their clinical application in the field of oncology and beyond. In addition to the clinical use of CAR T cell in oncology medicine, it has clinical application in allergy, autoimmunity, and infection. Combination immunotherapies

are considered an attractive immunotherapy modality that allows for reduced dosing with increased efficacy, and it is hoped to have reduced adverse effect profile.

Loss of peripheral tolerance with associated immune-related adverse effects, therapeutic resistance, limited clinical therapeutic index, and cost of treatment are a few limitations to clinical application of immune checkpoint inhibitors as immunotherapies for cancer and chronic viral infection. Despite significant advance in immune checkpoint inhibitor immunotherapy, there is a need for ongoing research into ways of mitigating the above limitations. There are ongoing phase1/2 clinical trials focusing on safety, tolerability, and efficacy of immune checkpoint inhibitors and their clinical application as an immunotherapeutic strategy in cancer and chronic viral infection. Furthermore, discovery of new immune checkpoints could enhance immunotherapeutic strategies against cancer, chronic viral infection, and neurological disease.

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Contents

Human Acute and Chronic Viruses: Host-Pathogen Interactions and Therapeutics

Matilde Hoffman, DeGaulle I. Chigbu, Brenndan L. Crumley, Ravi Sharma, Sergey Pustylnikov, Thomas Crilley, Rashida Ginwala, Ronak Loonawat, Julie Joseph, Dominic Sales, Sydney Wilson, and Pooja Jain

Abstract Viruses are obligate intracellular pathogens that cause infection in susceptible host cells. Virus infections could be lytic, chronic, latent or immortalizing. Viruses causing latent infection are associated with high morbidity and mortality worldwide. The human body is protected from viral infection by physical and chemical barriers. However, when these barriers are breached, the body generates an antiviral immune response mediated by Natural killer (NK) cells, monocytes, Dendritic Cells (DCs), type I interferon (IFN), neutralizing antibodies, and T cells. DCs play an important role in generating a cell-mediated adaptive immune response to viruses, with conventional DCs playing a crucial role in the interactions between DCs and viruses. Crosstalk between NK cells and DCs facilitates DC maturation in antiviral innate immunity whereas crosstalk between DCs and T cells in antiviral adaptive immunity amplifies the function of mature DC. Viruses employ various strategies to evade the host immune system. They can block Pattern Recognition

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Receptors (PRRs) - mediated production of type I IFNs, inhibit maturation and functionality of DCs, and interfere with cell-mediated immunity. Here we will focus on important human disease-causing viruses including latest COVID19 that caused worldwide pandemic. Because of the high mortality rate associated with viral diseases, there is an urgent need to re-evaluate current antiviral agents with more research focusing on developing alternative anti-viral therapies with an enhanced therapeutic index and safety profiles. Future directions in approaching the development of vaccines should focus on specific vaccines that can induce $CD8^+$T cell responses and produce IFN-gamma to promote a Th1-biased $CD4^+$T-cell response.

Keywords Viruses · Dendritic cells · Viral infections · T cells · Treatment · Immunoprophylaxis

1 Introduction

Viruses are the smallest infectious agent that cause infectious disease with high morbidity and mortality on a global scale. Viruses are classified on the basis of the genomic type as either DNA or RNA viruses. There is further classification of the viral nucleic acid as either single-stranded or double-stranded. Viruses are able to cause infection in host cells with appropriate specific host cell receptors that facilitate viral attachment, entry, and invasion of the host cell. Viruses require host cells to replicate and survive. Destruction of the host cell by viral replication, along with immune response to the virus, results in clinical manifestations of viral infection. DNA viruses usually undergo replication in the nucleus of the host cell whereas majority of viruses with an RNA genome replicates in the cytoplasm of the host cell. Viral replication in the host cell results in disruption of normal cellular function, culminating in damage and death of the infected cell. When virus infection causes immediate cell death, this is termed lytic infection, wherein the infected cell is lysed as the virions emerge. However, many viruses are also known to cause persistent viral infection, which may take the form of a latent or persistent productive infection. In latent infection, viral nucleic acid persists inside the host cell without undergoing replication or killing the cell. Persistent productive infection, or immortalization, is associated with host cell senescence because of active viral replication. This occurs mostly with RNA viruses such as HTLV-1, during which the infected host cell becomes immortalized due to interference with normal cell cycle, this results in host cell transformation [1]. These forms of viral lifestyle are associated with the development of virus specific $CD8^+$T cells, which become stimulated during viral reactivation [2].

The human body is protected from viral infection by physical and chemical barriers. However, when these barriers are breached, different arms of the immune system come into action. The immune response involves the innate immune system mediated by Dendritic cells (DCs), Natural Killer (NK) cells, monocytes, and type I interferons (IFNs), and the adaptive immune response carried out by neutralizing antibodies and T cells. A complex interplay between different types of immune cellular and soluble factors of the innate and adaptive immune systems exists in

preventing and controlling chronic virus infections. NK cells, complement proteins and cytokines are specific components of the antiviral innate immune responses, with DCs operating as immunosurveillance and immunostimulatory cells that process and present viral antigen to T cells [3]. DCs are divided into plas-macytoid and conventional DCs. Plasmacytoid DCs respond to viral DNA and RNA to produce type I IFN that inhibit viral replication in both infected and noninfected cells [4, 5]. Conventional DCs are one of the first immune cells to encounter viruses at their port of entry into the host [6]. The three subsets of conventional dendritic cells, important in viral infection, include tissue-derived migratory DC, lymphoid-resident DC, and monocyte-derived DC [4, 7]. Tissue-derived migratory DCs include Langerhans' cells and dermal/interstitial DCs that reside and survey the skin and mucoepithelial tissue. Lymphoid-resident DCs exist as immature DCs in the lymph nodes, spleen, and thymus. Monocyte derived or inflammatory DCs are those that are generated from monocytes under inflammatory conditions [4, 7].

Dendritic cells are located in strategic parts of the body to ensure optimal performance of immunosurveillance and immunostimulatory functions with the intent of triggering both innate and adaptive immune response to the presence of viruses that breach the anatomical and chemical defense barrier of the host [7]. Siglec-1 (CD169), DC-SIGN, mannose receptor, Langerin, immune dendritic cell receptor (DCIR), heparan sulfate proteoglycan, FC gamma receptors, and syndecan-3 are surface attachment receptors on DCs that facilitate uptake of viruses by DCs [6]. Virus interaction with DCs can cause degradation of the virus within the cell to allow for antigen-MHC complex formation for presentation to T cells. This trigger the T cell mediated antiviral immune response [6, 7]. Viral antigens are either generated during intracellular replication in virus infected cells or generated from recognition of viral components from other infected cells [7]. Some viruses can bind to and replicate within DCs, with DCs acting as permissive cells that facilitate viral spread to other cells or tissues in the host, resulting in trans-infection of lymphocytes in regional lymph nodes. This type of infection is particularly seen when viral antigens are not complexed to MHC molecules [6] as another way to alert the immune system and activate T cells (Table 1).

Activation of PRRs on DCs by viral Pathogen Associated Molecular Patterns (PAMPs) is associated with maturation of DCs, processing and presenting of virus-derived peptide complexed to MHC class I molecules, upregulation of MHC molecules and costimulatory signals, production of antiviral pro-inflammatory cytokines, and induction of the adaptive immune response. Viral nucleic acid, capsomer, peplomer on viral capsid or envelope, and RNA replication intermediates are viral PAMPs that interact with PRRs [6]. The efficacy of PRR activation determines the level of viral titers and duration of infection [7–9]. PRRs have dual function of detecting viral PAMPs and alerting the immune system about the breach of the passive innate immune defense mechanism [7]. Toll-like receptors (TLR) on DCs are PRRs that undergo morphological and biochemical changes when they interact with cognate ligands [3]. TLR involved in viral recognition can induce the production of type I interferons. Intracellularly located nucleic acid sensing TLRs (TLR3, TLR7, TLR8, TLR9) within the endosome facilitate recognition of viral nucleic acid or viral genome. Activation of TLR3 results in the induction of phosphorylation of IRF3, whereas activation of TLR7 and TLR9 is associated with IRF7

Table 1 Epidemiology, virulence factors, clincal features, pathophysiology and management of chronic viruses

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
Measles	Children, unvaccinated, malnourished, and immune compromised individuals are most at risk. Measles was declared eradicated in the US in 2000 but there are still spordic outbreaks. Still epidemics in Africa, parts of South East Asia, Eastern Europe and some parts of Western Pacific Area. Between 2000-2014, measles incidence decreased by 73% worldwide, and measles deaths fell by 79%, from 546,800 to 114,900.	It is a negative sense ssRNA virus that belongs to the Paramyxoviridae family. There is one serotype. H and F proteins are necessary for attachment and fusion with DCs and lymphoid cells. V and C proteins are indispensible for in-vivo infection. Measles virus induces cell-to-cell fusion, which results in multinucleated giant cells (syncytia) formation. It is highly infectious and transmitted thorugh aerosolized droplets and contact with respiratory secretions.	There is a 10–14 day incubation. Clinical manifestations include cough, fever, Koplik's spots, and maculopapular rash. Complications include pneumonia and bacterial superinfection. Patients with severe immunosuppression develop giant-cell pneumonia without a rash. Post-infectious encephalomyelitis (PIE) and subacute sclerosing panencephalitis (SSPE) are rare sequela of measles infection.	MV-H binds DC-SIGN on DCs resulting in upregulation of CD150 expression. Infected DC travels to lymph node and cause the infection of activated T and B cells via CD150. Infected T and B cells travel to other secondary sites such as the lungs where they enter epithelial cells at basolateral side binding PVLR4/Nectin4.	Live attenuated vaccine (Schwartz or Moraten variants of Edmonston B strain) administered as two doses after 1 year of age provides protective immunity. Immune serum globulin is used for post-exposure prophylaxis.	Studies have been conducted that compare the injected versus the aerosolized MV vaccine. Research that focuses on exploring other vaccine options of measles virus is required.

Dengue	Vectors include Aedes aegypti and Aedes albopictus mosquitoes. It is endemic in tropical and subtropical areas, e.g., Puerto Rico, Mexico, and Asia. It is especially endemic in areas with standing water and poor vector control. Transmission is highest between August & November. Vertical transmission from mother to newborn is possible. 2.5 billion people are currently at risk. WHO estimated an incidence of 50–100 infections/year and a death rate of 20,000 deaths/year.	Dengue virus is a spherical virus with positive sense ssRNA genome. It belongs to the Flaviviridae family & Flavivirus genus (related to Zika). It is transmitted by vectors through the blood (most active during the day). There are four serotypes, each a (+) sense RNA virus, with DENV2 considered the most virulent. The three structural proteins include Capsid, Envelope, and PrM/pre-membrane. Dengue virus encodes seven nonstructural (NS) proteins. E protein is critical for fusion of host and virus membranes.	Clinical course of DENV includes febrile, critical, and recovery phase. Febrile phase (~1 week) is characterized by hemorrhage/hemolysis, joint & muscle pain, and most cases resolve after this phase. Critical phase is characterized by systemic vascular hyperpermeability and subsequent vascular leakage (dengue shock syndrome). It lasts about 48-72 hours, after which vascular permeability normalizes (occasional fatigue follows).	DENV targets DC, macrophages, monocytes, and endothelial cells. It binds to a target cell via heparan sulfate, DC-SIGN, and mannose receptor. Virion binding is followed by clathrin-mediated endocytosis into the host cell with E protein inducing viral and host membrane fusion to host cell. New virions mature in the trans Golgi by furin protease and a re released by exocytosis. DCs exposed to the virus travel to lymph nodes, where they activate T cells. DENV blocks IFN I production. CTLs have low level of granulation but produce pro-inflammatory cytokines. Exposure to one serotype only provides immunity to that serotype. DENV infection is associated with increased severity of infection with another serotype via antibody-dependent enhancement (ADE). DENV-IgG complexes interact with FcγRIIA receptors on DCs, which leads to generation of pro-inflammatory cytokines.	No current prophylactic vaccines or DENV-specific treatment. Treatment is based on maintaining fluid balance in patients with DHF/DSS. Preventative methods are mainly centered around vector control e.g. limiting mosquito populations/infectious potential.	Sanofi Pasteur's CYD-TDV chimeric vaccine has been shown in trials to protect against all serotypes but DENV2 (currently in phase III). Papaya leaves have been reported to be a possible treatment/protective agent but active ingredient has yet to be identified. Most research is centered around adapting current therapies for other infections e.g. minocycline has been reportrd to have a dose-dependent activity in reducing DENV RNA.

(continued)

Table 1 (continued)

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
Zika Virus	ZIKV acquired global health significance when it caused significant outbreaks in Yap Island, French Polynesia, and South America in 2007, 2014, and 2015 respectively. Aedes aegypti and Aedes albopictus are vectors of ZIKV with Aedes aegypti considered the primary vector responsible for the ZIKV outbreaks. Non-vector mode of transmission includes mother-to-fetus, sexual contact, blood transfusion and breastfeeding. WHO declared ZIKV a public health emergency of global concern due to the temporal and geographical association between ZIKV infection and microcephaly.	Zika virus is a enveloped virus with a nonsegmented positive sense, single stranded RNA genome and icosahedral capsid. It has three structural proteins (viral capsid, viral membrane protein, envelope glycoprotein) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). RNA replication is enzymatically mediated by NS3 and NS5 and processing of polyprotein is mediated by the interaction between NS3 and NS2B. Phylogenetic analysis of ZIKV strain identified the presence of African lineage and Asian lineage ZIKV strain.	The clinical manifestation of ZIKV infection include transient low-grade fever, pruritic maculopapular rash, arthralgia, nonpurulent conjunctivitis, retro-orbital pain, headache, myalgia, lymphadenopathy, fatigue, lower back pain, hematospermia, and subcutaneous bleeding. Guillain-Barre syndrome (GBS) and microcephaly are the major neurological complications associated with outbreaks of ZIKV infection. Current diagnostic test for ZIKV include IgM class capture enzyme-linked immunosorbent assay (MAC-ELISA) and Trioplex reverse transcriptase polymerase chain reaction.	Neurons, astrocytes, trophoblasts, microglia, macrophages, dendritic cells, endothelial cells, skin keratinocytes, and dermal fibroblasts are permissive cells that support ZIKV replication. Generating type I IFN is required for suppressing ZIKV replication in innate immunity. Neutralizing antibodies primarily directed at E protein play a major role in providing humoral protection against ZIKV infection. Cell-mediated immunity against ZIKV involves ZIKV-specific CD8 T cell and CD4 T cell.	There are currently no FDA approved therapeutic agent for treating ZIKV infection. The main goal of prevention is to avoid contact with Aedes mosquito as well as minimize risk of non-vector transmission. Pregnant women or women trying to conceive should avoid travel to Zika virus endemic areas and unprotected sexual contact with partners who are at risk for Zika virus infection.	Han and colleagues demonstrated that amodiaquine, an antimalarial drug could be therapeutically repurposed for ZIKV infection due to its antiviral capabilities against ZIKV. Mesci and colleagues demonstrated that Sofosbuvir (SOF), a FDA-approved RNA-dependent RNA polymerase (RdRp) inhibitor, had anti-ZIKV activity in both in vitro and in vivo models of ZIKV. Current ZIKV platform using prM/E as vaccine antigen are designed to provide correlates of immune protection based on generating neutralizing antibodies.

Adenovirus	Mode of transmission includes direct contact, aerosolized virus, fecal-oral route, and adenoviral contaminated swimming pool/water. Transmission rates are higher in day care centers and long term care facilities. Most prevalent adenovirus in civilian population is adenovirus type 3. Most prevalent adenovirus among military personnel is adenovirus type 4. In 1997, there was an outbreak of adenoviral-associated respiratory infection among military recruits in the USA. In 2011, there was an outbreak of adenovirus-related acute respiratory disease in Tayside, UK with a case mortality rate of 23%. Risk factors include recent solid-organ transplantation, hematopoietic stem cell transplantation, and immunocompromised state. Major disease burden include reactivation of a latent adenoviral infection and unavailability of suitable anti-adenoviral therapy for immunocompromised patients.	Adenovirus is a nonenveloped lytic viruses with a 36 kb linear double-stranded DNA genome that encodes more than 40 different proteins. it has an icosahedral capsid made up of 240 hexon proteins and 12 penton base proteins. Major structural proteins include hexon, penton base, and fiber protein whereas minor capsiid proteins include protein VI, protein III1, protein VIII, ad protein IX. Core proteins are terminal protein (Tp), protein MU, protein VII, protein Iva2, and protein V. Genus is Mastadenovirus and family is adenoviridae. There are seven groups (A through G) based on hemagglutination properties, serology, phylogenomics, host range, genomic composition, DNA homology, receptor usage, and tissue tropism.	Tissue and viral tropism are linked to affinity of host cellular receptor for fiber knob and penton base. Persistence of adenovirus in lymphoid tissue serves as a focal point of viral spread following viral reactivation. Pharyngoconjunctival fever is caused by HAdV type 1, 2, 3, 4, 5, and 7 and it is an ocular infection associated with swimming in insufficiently chlorinated swimming pool. Epidemic keratoconjunctivitis is caused by adenoviral types 8, 19, 37, 53 and 54. Respiratory tract disease is caused by HAdV type 1, 2, 3, 4, 5, 6, 7, 14, and 21. Gastrointestinal tract (GIT) disease is caused by adenovirus type 2, 3, 8, 31, 40 and 41. Adenoviral-induced genitourinary tract disease is caused by HAdV type 7, 11, and 21.	Viral entry is mediated by CAR/CD46-Fiber knob interaction. Viral internalization is via clathrin-mediated endocytosis via penton-integrin interaction, which is folowed by viral uncoating and release of vertex proteins as well as endosomalysis. This is followed by transportation of viral genome into the nucleus, where DNA replication and generation of progeny DNA. The last stage in replication is viral assembly and maturation. Innate immunity is mediated by NK cells, monocytes, and type 1 interferon. Adenoviral DNA interact with TLR9 on DC to signal through the MyD88 pathway to generate type I interferon. IgA-mediated mucosal immunity prevents productive adenoviral infection. Cell-mediated cytotoxicity by CTL and NK cells cause apoptosis of adenoviral-infected cells. Immune evasion strategies include inhibition of interferon response, TNF-alpha-mediated viral cytolysis, and MHC class I molecule expression on surface of virally infected cells.	No FDA-approved antiviral agents are available. Cidofovir, Ribavirin, and Brincidofovir have been shown to be beneficial for immunocompromised individuals. Maintenance of adequate or optimal levels of chlorinated swimming pools and improved personal hygiene can minimize transmission. Immunoprophylaxis via live oral vaccine are beneficial for preventing adenovirus types 4 and 7-induced respiratory infections in military recruits.	Current and future diresctions incluude the following: Ritonavir has been reported to be an inhibitor of adenoviral cysteine enzyme. RNAi-mediated viral gene silencing inhibits replication of adenovirus. RNAi-mediated suppression of CAR expression inhibits viral entry. Intravenous immunoglobulin (IVIG) against primary infection. Adoptive transfer of HAdV-specific immunity.

(continued)

Table 1 (continued)

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
HBV	HBV has tropism for herpatocytes resulting in liver infection and subsequent liver failure. Transmitted via mother-to-infant and exposure to infected bodily fluids including genital fluids and blood. Acute HBV infection is usually cleared within 6 months of onset, whereas chronic HBV infection is associated with viral persistence due to failure of the immune system to clear the virus. According to WHO estimates, 257 million people have chronic HBV infection worldwide. In 2015, there were 887,000 recorded dates secondary to HBV-induced cirrhosis and/or hepatocellular carcinoma. The economic bursden associated with chronic HBV infection is in the neighborhood of $9 billion.	HBV is a non-cytolytic virus witth a dsDNA genome. The four major proteins include Polymerase, Core, Surface antigen, and HBx. HBV polymerase possess both DNA-dependent DNA polymerase and reverse transcriptase (RNA-dependent DNA ploymerase) acitvities. Surface protein (HBsAg) a biomarker of HBV infection in sera of HBV infected individuals. Core is a structural protein found in the capsid. HBx is a regulatory protein that plays a role in viral replication. HBeAg plays a role in promoting viral persistence.	In acute HBV infection, the immune system clears the infection. Chronic HBV infection is associated with viral persistence. Acute HBV infection is less damaging to the liver. Chronic HBV infection has four phases – immune tolerance phase, immune clearance phase, inactive HBsAg carrier state, and reactive phase (HBeAg-negative chronic hepatitis B phase). Intense liver inflammtion occurs in the reactivation phase.	HBsAg interacts with TLR4 to lead to production of proinflammatory cytokines tha recruit immune cells. HBV nucleocapsid interacts with TLR2 to generate type I IFN. Interaction between pgRNA and RIG-I generates type III IFN. HBsAg inhibits type I and III IFN production. HBx degrades MAVS, TRIF and IRF3. NK cells are effective via their cytolytic action on HBV infected hepatocytes. Antibody response is generated against HBsAg surface antigens. Cd4 and CD8 T cells provide adaptive cellular immune response.	Chronic HBV infection is resistant to therapy. Interferon-based therapy (e.g. conventional interferon therapy and PEGylated interferon-α therapy) boosts the immune system to clear HBeAg and HBsAg. Nuceoside analogs (e.g. lamivudine, adefovir, entecavir, tenofovir, and telbivudine) inhibits viral replication. HBV vaccine based on HBsAg provides protective immunity. Post-Exposure Prophylaxis uses HBV immunoglobulin and it is given to individuals exposed to HBV infected bodily fluids. Prevention strategies include safe sex practices, screening blood for HBV, and vaccination.	Increasing the efficacy of existing drugs such as PEG-interferon and nucelotide analogs. Generation of Tenofovir alafenamide that targets NTCP. Cyclosporin A inhibiting interaction between NTCP and HBV surface protein. Direct acting siRNA and HBsAg inhibitors are being developed.

HCV	HCV has tropism for the liver resulting in hepatitis and progression in a few HCV chronically infected individuals leads to the development of hepatocellular carcinoma. HCV is contracted via exposure to infected blood and sexual intercourse. HCV becomes chronic when the virus persists due to failure of clearance by the immune system. 71 milliomn of individuals on the earth have chronic HCV infection. Approximately 399,000 peolpe with chronic HCV die from liver failure and hepatocellular carcinoma. About $300 million is spent on liver transplant on patients with HCV-induced liver failure.	HCV belongs to the Flaviviridae family. It is an enveloped virus with a ssRNA genome. The large polyprotein is cleaved into structural (core, envelope E1, and envelope E2) and nostructural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Genetic makeup of the host has an impact on virulence of the virus.	Approximately 30% of patients with acute HCV have symptoms such as weakness, anorexia and jaundice. Persistence of HCV RNA in the blood for more than 6 months is characteristic of chronic HCV infection. Disease progression is slower with low ALT levels whereas very high ALT levels is associated with rapid disease progression. HCV coinfection with HBV or HIV is associated with increased risk of developing hepatocellular carcinoma.	Innate immunity is mediated by type I IFN. type III IFN and NK cells. CTL induce the apoptosis of HCV-infected hepatocytes through the action of perforin and granzyme. CTL and Th1 cells through the action of IFNγ inducing cytokine-mediated antiviral action, which inhibits viral replication. Neutralizing antibodies against E1 and E2 are formed but have a limited role in immunity to HCV.	Treatment directed at patients with chronic HCV infection. Direct acting antivirals (e.g., elbasvir/grazoprevir and sofosbuvir/velpatasir) target NS5B polymerase, NS3/4 protease, and NS5A protein; these proteins are essential for HCV replication cycle. No FDA-approved vaccines. Prevention strategies include needle awareness strategies, safe sex practices, and screening blood for HCV.	Bovine viral diarrhea virus is being investigated for its abilioty to HCV replication cycle. Polyphenols have been shown to inhibit HCV RNA replication. Caffeine has been reported to target HCV replication cycle without causing non-toxic adverse effects. Clinical trials are ongoing with focus on Samatasvir and Odulasavir, which have been reported to target polymerase and protease.

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Table 1 (continued)

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
HPIV	HPIV is the second most common cause of acute respiratory infections in infants and children. HPIV is transmitted through contact or inhalation of respiratory droplets. It causes infections ranging from croup and bronchiolitis to pneumonia. Disease burden is most significant with HPIV3, as it causes severe respiratory tract infection in infants, elderly and immunocompromised individuals. Infants, the elderly and immunocompromised patients are at an increased risk due to immature or decreased function in the immune system.	It is an enveloped non-segmented negative sense ssRNA virus that replicates in the cytoplasm. Binding of HN with sialic acid on cellular membrane proteins of host cells initiates the infectious process of HPIV. F protein mediates fusion between the viral envelope and cell plasma membrane of the host cell. Release of the viral genome bound by nucleocapsid proteins (nucleoprotein, phosphoprotein and RNA-dependent RNA polymerase) allows RNA-dependent RNA polymerase to direct RNA genome replication. V and C proteins are important for survival of the virus through suppression of IFN induction and cell signaling in HPIV infected cells.	HPIV has an incubation period of 2–6 days and lasts 1–2 days after symptoms begin. HPIV Types 1-3 cause of severe lower respiratory tract infection in children and infants. HPIV Type 1 & 2 cause croup and HPIV Type 3 cause bronchiolitis or pneumonia in children. Laryngotracheobronchitis (croup) is the more severe manifestation of HPIV.	HPIV activates pattern recognition receptors leading to creation of an antiviral microenvironment. Mucosal IgA mediated protection is short lived but the longevity of the mucosal IgA response is enhanced with subsequent HPIV infections. Serum and mucosal neutralizing antibodies that target HN and F glycoprotein provide long-term protection against HPIV. CD8 and CD4 T cells provide cellular immunity that can clear the virus but T cell-mediated viral clearance following reinfection wanes over a few months.	There are no specific antiviral medications used. No live or attenuated vaccines have been effective HPIV can be inactivated by dryness and acid, and treatment is supportive.	National Institute of Allergy and Infectious Disease (NIAID) and MedImmune are working on a vaccine against HPIV infection. Majority of the HPIV3 vaccine variants have been shown to be safe and immunogenic. Phase I clinical trial of Bovine PIV3 (BPIV3) and HPIV3 vaccine revealed modest seroconversion rate for HPIV3. Recombinant Bovine/HPIV3 was well-tolerated in young children with a seroconversion of 100% in children under the age of 2 years.

Mumps	Mumps can cause infections ranging from mild parotitis to more severe systemic infections such as meningitis and encephalitis. MuV usually causes self-limited infection with fatalities only related to the 1% of infections that lead to encephalitis. It is of significant health burden particularly in regions with low MMR vaccination rates.	MuV are spherical, enveloped, negative sense, ss RNA viruses with only one serotype. They are human pathogens transmitted through aerosols or contact with infected respiratory secretions. MuV expresses the HN protein that allows it to interact with target host cells. MuV spreads through viremia and can affect multiple organ systems. MuV causes a lytic infection of epithelial cells of the URT. Cell-mediated immunity is necessary for controlling infection. Protein V is crucial in immune evasion.	MuV also causes systemic infection. It has an incubation period of 2–4 weeks, but one third of infections are usually asymptomatic. Mumps is associated with parotitis, orchitis, meningitis, encephalitis, encephalitis, transient sensorineural hearing loss, pancreatitis, pericarditis and myocarditis.	Dendritic cells, alveolar macrophages, T cells, and B cells are main players in the immune response to mumps virus infection. DC and alveolar macrophages initiate the immune response to measles and mumps. MuV has specific proteins that aid in establishing or spreading the infection. Small-hydrophobic (SH) proteins of MuV suppress TNF-α production. V protein blocks the production of type I IFN and IL-6.	Live MMR vaccine provides lifelong immunity.	Research in post-exposure prophylaxis of mumps. Innate immunity is mediated by type I interferon and NK cells while CTL provide cell-mediated immunity against the virus. NS1 and NS2 block generation of type I interferon. RSV impairs myeloid-derived DC presentation function. RSV blocks signaling via TLR and suppresses cell-mediated cytotoxicity. Ribavirin is beneficial for high risk patients.

(continued)

Table 1 (continued)

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
RSV	RSV is the major cause of upper and lower respiratory tract infection in young children and elderly individuals. RSV is highly contagious and prevalent in the winter months. It is of significant public health concern because RSV causes fatal respiratory tract infections in infants and immunocompromised individuals. RSV poses a significant health burden due to inability of the infection to confer long-term protective immunity.	RSV is an enveloped negative sense ssRNA that contains 10 genes that encode 9 nonstructural proteins and 2 structural proteins. RSV invades the respiratory epithelium and forms syncytia in infected mucoepithelial tissue of the respiratory tract. RSV G proteins bind to a target cell. NS1 and NS2 interact with the immune system. Fusion (F) and matrix (M) proteins are highly conserves between RSV serotypes. Surface glycoprotein (G) protein is not fully conserved between RSV serotypes.	RSV is the most common cause of acute respiratory tract infections in infants and young children. Typical patient presents with a low-grade fever, tachypnea, tachycardia, and expiratory wheezes over the lungs. Bronchiolitis is usually self-limited, but it can be more dangerous in infants.	Innate immunity is mediated by type I interferon and NK cells while CTL provide cell-mediated immunity against the virus. Neutralizing antibodies against major surface glycoproteins restricts reinfection of RSV. RSV can inhibit Th1 polarization. RSV promotes creation of an immunosuppressive microenvironment that favors viral persistence. RSV G protein induces Th2 polarization and eosinophilia in infected lungs. NS1 and NS2 proteins decreases DC maturation. NS1 suppresses proliferation and activation of CD8+ T cells. RSV Fusion protein inhibits activation of T cells. NS1 and NS2 block generation of type I interferon.	Ribavirin for the treatment of high-risk patients such as pre-term infants, or those with poor respiratory tract development. Palivizumab and Respiratory Syncytial Virus Immune Globulin Intravenous (RSV-IGIV) are FDA approved for treating RSV infection in infants and young children with chronic cardiopulmonary disease. There is no vaccine available for RSV.	There is ongoing research to develop vaccines for women in the third trimester of pregnancy. Subunit RSV-A vaccines containing purified Fusion, matrix, and G proteins have been demonstrated to be safe and immunogenic in the over 65-age group. Developing a RSV vaccine that minimizes Th2 mediated immunopathology is a challenge.

HTLV	HTLV-1 is the most clinically harmful subtype and it is endemic in Japan, Brazil, Iran, and parts of Sub-Saharan Africa. Probability of contracting HTLV-1 through blood transfusion and organ transplantation renders HTLV-1 a significant health burden. HTLV-1's main routes of transmission include unprotected sex, infected blood, and vertically between mother and baby.	HLTV is an enveloped positive sense ssRNA virus. HTLV-1 infects CD4 T cells. HTLV-1 genome carries several structural genes (gag, pol, & env). Genome contains genes such as Tax and HBZ (HTLV Zipper Factor). The trans-activating Tax protein initiates immune response against HTLV-1-infected cells. HBZ triggers is associated with chronic infection. Both Tax and HBZ have been a major focus of HTLV research. HTLV-1 is transmitted through an immunological synapse between DC and T cell.	ATL is a multi-organ system disease. It is characterized by atypically shaped lymphocytes. Patients present with erythema and plaques. Immunosuppression is common in the less aggressive subtypes. HLTV-1 infected patients are susceptible to opportunistic infections. HAM/TSP exerts a neurotoxic effect that leads to spinal cord inflammation. Patient's serum and CSF showing antibodies against HTLV-1 is diagnostic for HAM/TSP. GLUT-1 is utilized by HTLV virions to infect cells.	T cell is one of the key immune cells implicated in HTLV. Infected T cells express the Tax protein that suppresses their proliferation. HBZ protein enhances progression from HLTV-1 infection to ATL or HAM/TSP. DCs also play an important role in HLTV pathogenesis. DC-mediated transmission of HTLV-1 from infected DC to T cells. HTLV infected T cells and DCs are dysfunctional.	There is no FDA-approved therapy for HTLV-1 infection. Chemotherapy to counteract symptoms of leukemia and lymphoma. Combination therapy of AZT and interferon-alpha is effective in some patients with ATL. Education focused on safe sex practices and needle-sharing are preventative strategies.	Research is focused on what differentiates asymptomatic carriers from patients who progress to ATL and HAM/TSP. Further studies on the benefits of cyclosporine is required. Another area of research could focus on novel testing protocols for HTLV.

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Table 1 (continued)

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
HSV	In 2012, the global burden of HSV-1 was 3.7 billion people with age less than 50 years. Maximum prevalence in Africa, South-East Asia and western pacific countries. Globally 417 million people in 15–49 year age group are infected HSV-2, with incidence rate of 19 million per year. An estimated 25–50% of HIV infections Are attributable to HSV-2 in high prevalence regions. Initially, HSV-1 was primary thought to be associated with oral infections, while HSV-2 with genital insult, but HSV-1 has now been shown to provoke first episode of genital herpes and neonatal herpes in developed world. HSV is acquired mainly through direct exposure of mucous membranes or abraded skin to the lesions or mucosal secretions of actively infected individuals.	HSV-1 and HSV-2 are members of the neurotrophic alpha-herpesviridae subfamily. HSV is an enveloped virus with a linear dsDNA genome. HSV gD mediate adhesion of the virus to the host cell surface. Fusion is facilitated by gD, gB, gH and gH glycoproteins. Following fusion, tegument proteins and nucleocapsid enter the host cell cytosol. HSV genes α, β and γ regulate viral genome translation and transcription of viral transcription factors. Vhs protein degrades host mRNA in infected cells.	Immunocompetent children and adolescents are susceptible to acquiring primary HSV infection. Primary HSV clinical features include painful and ulcerative vesicles in the skin and mucous membranes. HSV infects neurons and mucoepithelial cells. HSV is a neurotrophic virus that travels via retrograde transport to the trigeminal ganglia to establish latent infection. Immunocompromised individuals are prone to developing HSV associated complications because of HSV reactivation and recurrent HSV disease.	TLR2, TLR3, TLR7 and TLR9 recognize HSV PAMP to initiate the antiviral innate immune response. NK cells and type I IFNs mediate the antiviral innate immune response to HSV. CD8 T cells are the major players in cell-mediated immunity to HSV. VHS suppresses type I interferon and reduce production of pro-inflammatory cytokines. DC are prone to HSV infection because they express nectin-1, nectin-2 and HVEM. HSV downregulates costimulatory molecules and induce apoptosis of DC. HSV inhibits Th1 polarization.	FDA-approved nucleoside analogues for treating HSV infection include acyclovir, valacyclovir, penciclovir, and famciclovir. There is no viable therapeutic or preventive HSV vaccine. Development of resistance to nucleoside analogues in immunocompromised individuals is a health burden.	There is current research into developing an effective preventive and therapeutic HSV Vaccine. HSV vaccine success in animal models has not been translatable in human studies. GEN-003, a subunit vaccine of gD2 and ICP4, has been reported to decrease virus shedding by 55% in a phase IIb clinical trial.

HPV	HPV infection is of significant health burden because of the carcinogenic nature of HPV 16 and HPV 18. HPV is the most common sexually transmitted infection worldwide particularly in young individuals. HPV 16 and HPV 18 cause anal cancer, penile cancer, and cancers of the vagina, vulva, and cervix. Estimated financial burden of health in the U.S. is $8 billion.	HPV is a non-enveloped doubled-stranded DNA virus. The alpha subtype of HPV affects mucosal epithelium while the beta subtype of HPV infects skin. The dsDNA genome encodes 8 genes: E1, E2, E4, E5, E6, E7, and E8, L1, and L2. Viral proteins E6 and E7 proteins are necessary for cell immortalization via their interactions with p53 and pRB respectively. The icosahedral capsid of HPV consists of L1 major and L2 minor capsid proteins. Interaction between L1 and heparan sulfate proteoglycans (HSPGs) initiates the viral attachment. Internalization of HPV is followed by disassemble of viral capsid and L2 complexing with viral DNA to form L2-DNA complex. Viral DNA replication occurs in the nucleus.	The clinical presentation of HPV depends HPV subtype, the area of skin affected, and the immune status of the individual. The majority of HPV infections are asymptomatic. Persistent HPV infection manifests as warts. Anogenital warts are caused by HPV 6 and 11. Common warts are caused by HPV types 1, 4, and 7. Plantar warts are associated with HPV types 1 and 4. Condyloma acuminatum are caused by HPV 6 and 11. Giant condyloma acuminatum of buschke and löwenstein is caused by HPV 6, 11 and 16.	TLR-mediated interaction with HPV PAMP initiates innate immune responses to HPV. TLR3, TLR7 and TLR9 recognize HPV PAMP to initiate the antiviral innate immune response via production of type I interferon. NK cell-mediated immune response controls HPV infection in innate immunity. HPV downregulates antiviral innate immunity mediated by type I interferon. It decreases production of pro-inflammatory cytokine by keratinocytes. It downregulates MHC class I expression. HPV generates an IL-10 immunosuppressive environment.	There is no FDA-approved cure for HPV infection. Pharmacotherapy is focused on amelioration of symptoms Education on safe-sex practices are crucial in preventing HPV infection. L1 VLP vaccines include Cervarix that targets HPV16/18 and Gardasil that targets HPV6/11/16/18. A CDC study showed a 56% decrease in vaccine-related HPV strains for U.S. girls age 14-19. HPV vaccination is associated with a decrease in anogenital HPV-related disease.	Current vaccine research is looking at L2-based vaccines. Current research into therapeutic vaccines aim to generate cytotoxic T cell responses against the HPV early viral gene. Radio-immunotherapy targeted at E6 and E7 proteins is promising treatment.

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Table 1 (continued)

Virus	Epidemiology and disease burden	Characteristics and virulence factors	Clinical manifestations	Pathophysiology and immunology of disease	Treatment and prevention	Current clinical trials and research studies
HIV	Injecting drug users (IDUs), men who have sex with men (MSM), and people practicing high-risk sexual behavior are most at risk. HIV is a global epidemic: over 1 million people die annually and over 3 million people were infected in the last 5 years. Pandemic is stabilized in almost all regions of the world (number of newly infected people is not growing). Between 2010 and 2015 HIV treatment coverage increased from slightly over 20% to almost 50% and number of AIDS-related deaths decreased from about 1.5–1.1 million.	Retroviridae family, two positive sense single-stranded RNA, at least 7 serotypes. Env, trimer of heterodimers of gp120 and gp41 glycoproteins, is necessary for attachment and fusion with the target cells. Induce CD4 T cell death and severe immunodeficiency associated with opportunistic infections. Mode of transmission includes through infected blood, sexual contacts, and mother-to-fetus.	Four periods of disease: incubation (1-2 weeks), acute period (2-4 weeks), chronic infection (1-20 years), and HIV-induced AIDS (deadly if not treated). Complications: opportunistic infections (oral candidiasis, tuberculosis, herpes zoster, and bacterial pneumonia). Increased risk of disorders in cardiovascular, digestive, excretory, musculoskeletal and central nervous systems. HIV-related local and systemic inflammatory reactions. HIV-associated neurocognitive disorders.	HIV binds receptors, coreceptors and adhesive molecules on the target cells. In sexual transmission, mucosal DCs can transfer HIV to the lymph nodes, but mucosal T cells are also infected. In systemic infection, main places of HIV replication are the follicular T cells in the lymph nodes. In patients on ART the main viral reservoirs are the memory T cells.	HIV prevention tools include HIV counselling, testing and treatment in the high-risk groups, education in the general population. Number of vaccines have been tested, but only one has shown efficiency of about 30%; the protective effect was temporary.	The HIV vaccine RV144 has shown 31.2% efficacy and provided only temporary defense. Developing HIV vaccines that generate CD8 Treg cell response to prevent CD4 T cell activation and viral replication. There are therapeutic approaches on bNAbs being studied for passive immunotherapy and immunotherapy based on checkpoint blockers. Developing adoptive therapy with CAR T cells for development of antigen-specific cell-mediated response and use of single-chain CARs based on bNAbs.

Coronavirus	Coronavirus (CoV) was first identified in the 1960's and there are seven human CoV (HCoV) of medical importance. HCoV are responsible for up to 30% of common cold, which are self-limiting and harmless infections. The main mode of human-to-human transmission is through aerosolized droplets containing viral particles from coughing and sneezing as well as contact with surfaces contaminated with HCoV. The SARS epidemic of 2002–2003, which infected 8098 people across 29 countries and left 774 people dead. As of January 2020, a global total of 2519 laboratory-confirmed cases of MERS and 866 MERS-associated fatalities across 27 countries have been reported.	Coronavirus belongs to the subfamily *Orthocorona virinae*, family *Coronaviridae,* order *Nidovirales.* The genera include alphacoronavirus, betacoronavirus, deltacoronavirus, & gammacoronavirus. Coronavirus has a helically symmetrical nucleocapsid and an envelope with club-shaped spike glycoprotein. It has a non-segmented positive-sense, single-stranded RNA genome of 30 kb. Spike, Envelope, Membrane, and Nucleocapsid protein are structural proteins, which play a role in virion assembly and infection. HCoV can survive on surfaces for days and remain viable in aerosols for hours. HCoV lose their infectivity when exposed to UV radiation and high temperature.	The clinical manifestations of Human CoV include mild upper respiratory tract infection, fever, nonproductive dry cough, conjunctivitis, and croup in 80% of individuals. Severe forms of acute respiratory illness with dyspnea occurs in less than 20% of infected individuals. HCoV-OC43, HCoV-NL63, HCoV-229E, and HCoV-HKU1 are HCoV that cause mild, self-limiting upper respiratory tract infection in immunocompetent individuals; however, it causes lower respiratory tract infection in infants, elderly, and immunocompromised individuals. SARS-CoV, SARS-CoV2, and MERS-CoV are betaCoV that cause severe forms of respiratory tract infection and extra-respiratory manifestations.	The host cellular receptors for HCoV-OC43 & HCoV-HKU1 is 9-O-Acetylated sialic acid. ACE2 is the host receptor for HCoV-NL63 & SARS-CoV. APN is the host receptor for HCoV-229E. DPP4 is the host cell receptor for MERS-CoV. S1 subunit of the Spike glycoprotein binds to host cell receptors to promote viral attachment. S2 protein mediates viral fusion with host cell membrane. The first two-thirds of the CoV genome consists of two overlapping open reading frames ORF1a and ORF1b that undergo translation to yield polyprotein 1a and polyprotein1b. RTC drives replication of viral genomic RNA and synthesis of subgenomic mRNA. The final third of the viral genomic RNA at the 3′ consists of ORFs that encode structural and accessory proteins. IFN-α/β & NK cells provide innate immunity. $CD4^{+}$T cells and $CD8^{+}$T cells provide cellular immunity. Spike glycoprotein induces the generation of SARS-CoV specific neutralizing antibodies.	There is no FDA-approved antiviral vaccine for human CoV. Treatment of human CoV is mainly supportive. Recently, the FDA authorized the compassionate use of remdesivir for treating individuals with severe forms of SARS-CoV2 infection. Patients with severe respiratory illness benefit from the use of oxygen therapy and mechanical ventilation. Preventive strategies to contain the infection via quarantine and practice of good hygiene should be encouraged.	There is ongoing research aimed at developing antivirals that target specific enzymes of human CoV such as viral protease, polymerase, and entry protein. Plasma and antibodies obtained from convalescent patients are beneficial. Adoptive transfer of SARS-CoV-specific Th1 cells and CTL cells could clear the virus and increase survival rates. Developing vaccines and antiviral therapeutics that target viral entry and replication.

phosphoryla-tion. Phosphorylation of IRF3 and IRF7 are associated with the transcription of type I IFN genes and subsequent translation of type I IFN proteins [9]. Interaction between viral dsRNA and TLR3 on myeloid DCs (mDC) are associated with IL-12p70 production that induce naïve $CD4^+$ T cell to polarize into IFN-γ-producing $CD4^+$T cells, thereby promoting Th1 polarization. The IFN-γ released by Th1 cells enhances DC functionality [3].

A stable DC-T cell interaction following viral infection is required for shaping and driving an efficient activation, proliferation and differentiation of T cells into viral specific T cells, as well as optimizing expansion of memory T cell pool [4]. DC-T cell crosstalk requires the formation of an immunological synapse to facilitate the process of reciprocal activation of T cell and DC, particularly the T cell dependent activation of DC. IL-12 produced by DCs during viral infection induces a Th1 response, wherein IFN−γ produced by Th1 cells activate and amplify the function of the mature DCs [10]. $CD4^+$T cells are essential for optimal priming of CD8 T cells to generate virus specific cytotoxic T lymphocytes and maintenance of memory $CD8^+$T cells. It also provides help to B cells to generate virus specific neutralizing antibodies, long-lived plasma cells, and memory B cells. Neutralizing antibodies bind to viral envelope or capsid antigens to prevent virus from attaching to and/or the entering host cell during reinfection and during release of virions from lyseda host cell, with the intent of blocking cell-to-cell spread of virus. Thus, antiviral humoral immunity can prevent reinfection, but it cannot eradicate an established viral infection. The early activation of tissue resident Th1-polarizing memory $CD4^+$T cell enhances antiviral innate immune response to induce innate inflammatory response upon reinfection with virus, while simultaneously promoting viral specific adaptive immunity through interaction with DCs. This form of DC activation correlates with reduced duration of viral infection due to reduced viral titers [8]. Immune system evasion by viruses adds an additional layer to the fight against viral infections. Viruses employ various strategies to evade recognition and elimination by the host's immune system. Viruses can escape host innate defense mechanisms by interfering with PRR-mediated production of antiviral pro-inflammatory cytokines and type I IFN [11]. Some viruses interfere with maturation and functionality of DCs [6]. Some viruses produce immunosuppressive molecules that can antagonize the function of pro-inflammatory cytokines as well as cell-mediated immunity. Impairment of lymphocyte function by preventing $CD8^+$T cell mediated killing (e.g. HSV), infecting and killing $CD4^+$T cells (e.g. HIV), and suppression of NK cells and B cells (e.g. measles) are other viral-induced immune evasion strategies [5]. Viral evasion and latency occur as mechanisms for facilitating colonization and survival of the virus within the host [7].

The review herein will discuss important viruses that cause acute and chronic viral infections associated with significant health burdens such as Cornoviruses, Hepatitis viruses, HIV, HPIV, HPV, measles, mumps, adenovirus, RSV, human coronavirus, Dengue virus, HSV and HTLV-1. Epidemiology and disease burden, virulence factors, and clinical presentation will be presented in detail. The interaction of the virus with the immune system and pathophysiology of disease caused by the above viruses will be the focus of each section, with emphasis on the interaction between these viruses and dendritic cells. Finally, current and ongoing research on antiviral therapies will be reviewed.

2 Adenovirus and Respiratory Viruses

Adenovirus is part of the *adenoviridae* family. Human Parainfluenza Virus (HPIV), Respiratory Syncitial Virus (RSV), Measles Virus (MV), Human Coronavirus (HCoV), and Mumps virus (MuV) are respiratory viruses. They all cause common infections in children: adenovirus causes respiratory illness ranging from the common cold to bronchitis [12], RSV, HPIV, and HCoV are the most common cause of respiratory tract infections (RTIs) in infants and children. MeV is one of the causes of pediatric infectious rashes and respiratory symptoms, and MuV is a common cause of paroti-tis and orchitis [13, 14]. These viruses can also cause more severe disease in infants and immunocompromised individuals, such as those receiving chemotherapy, solid-organ and hematopoietic stem cell transplantation recipients, and AIDS patients.

2.1 Adenovirus

Adenovirus is a member of the Adenoviridae family that causes lytic, latent, and persistent infection of the mucosal tissues of the respiratory tract, gastrointestinal tract, genitourinary tract, and the eyes of humans. It is a non-enveloped DNA virus that causes a broad range of diseases, such as epidemic keratoconjunctivitis, adenoviral-induced genitourinary tract infection, adenovirus-associated respiratory illness, and gastrointestinal tract infection [15, 16]. Chronic adenoviral infection is attributed to the presence of adenovirus DNA within the cell without continuous production of infectious virions and/or persistence of adenoviruses in lymphoid tissue [12, 17]. NK cells, macrophages, and type I interferon provide antiviral innate immunity against adenoviruses [18]. NK cells and CTLs are responsible for the cell-mediated cytotoxic destruction of adenovirus infected cells with CTL providing the majority of the cell-mediated cytotoxicity against adenovirus infected cells [19–22]. Inhibition of interferon response, TNF-α-mediated viral cytolysis, apoptotic pathways, and expression of MHC class I molecules on the surface of virally infected cells are immune evasive mechanisms used by adenovirus [23]. There are no FDA-approved antiviral pharmaceutical agents for treating adenoviral infection [12, 16]. Because of the high mortality and morbidity rate of adenovirus infection in young children and immunocompromised individuals, there is an urgent need to focus current research on developing anti-adenoviral therapy with enhanced therapeutic index and safety profile [24, 25].

2.1.1 Epidemiology and Disease Burden

Human adenovrus (HAdV) is a ubiquitous virus that infects the mucosa of the respiratory tract, gastrointestinal tract, genitourinary tract, and eye [15, 16]. Adenovirus causes lytic, latent and persistent infection in humans; however, some strains with oncogenic properties cause immortalization of animal cells [12]. The mode of

transmission include direct contact, aerosolized virus, fecal-oral route and adenoviral contaminated swimming pool/water [18]. Sporadic outbreaks of adenoviral infections occur in crowded communities, wherein children play a prominent role in transmitting the virus to others [26]. Epidemiological studies in the USA have revealed that most respiratory tract infections in infants and young children were caused by adenovirus type 1–5. However, studies in military personnel revealed that outbreaks of adenovirus-associated respiratory infection were caused by adenovirus type 3, 4, 7, and 21 [16]. The most prevalent adenovirus in the civilian population is adenovirus type 3, while type 4 is more common among military recruits [27]. The age groups most at risk for fatal adenoviral infections are infants; however, other individuals with weakened immune systems can be at risk as well [18]. HAdV is an emerging opportunistic pathogen in immunocompromised individuals due to the enhanced potential for dissemination and increased risk for fatal outcomes [16].

2.1.2 Characteristics, Morphology and Virulence Factors

Adenoviruses are non-enveloped lytic viruses with a DNA genome and icosahedral symmetric capsid [16, 28]. There are more than 100 types of adenoviruses, classified into seven groups (A through G) based on the hemagglutination properties, serology, phylogenomics, host range, genomic composition, DNA homology, receptor usage, tissue tropism, and adenovirus associated RNA [18, 25, 28–31, 34, 35]. Adenovirus genus Mastadenovirus and family adenoviridae infects humans [29]. HAdV is 70–90 nanometers in diameter with a 36-kb linear double-stranded DNA genome that encodes more than 40 different proteins [34, 35]. HAdV consist of an icosahedral capsid made up of 252 capsomeres comprised of 240 hexon proteins and 12 penton base proteins [35]. The major structural proteins of adenovirus include hexons, penton bases, and fiber proteins [29]. Penton base protein plays a role in hemagglutination and its RGD (arginine-glycine-aspartate) loop serves as a site for binding to integrin $\alpha\nu\beta3$ and $\alpha\nu\beta5$ to facilitate internalization of adenovirus into host cells [18, 29, 34, 36]. Fiber protein plays a major role in interaction between viruses and primary host cellular receptors [16]. Minor capsid proteins include protein VI, protein III, protein VIII, ad protein IX, whereas terminal protein (Tp), protein MU, protein VII, protein Iva2, and protein V are core proteins of HAdV [29].

2.1.3 Clinical Manifestations

Adenoviruses cause a broad range of diseases in humans and the port of entry of the virus determines the primary site of the infection [16]. The majority of adenoviral infections are subclinical, with some being mildly symptomatic and self-limiting in immunocompetent individuals; however, fatal infections do occur in infants and immuno-compromised individuals [18]. The affinity of the host cellular receptor to the virus' fiber knob, in addition to the interaction between the penton base protein

and the integrin on the host cell surface, determine viral tropism [16]. Furthermore, fiber knob, penton base protein, and cellular receptors are important determinants of tissue tropism for adenoviruses, with binding affinity for host cellular receptors contributing to tissue tropism [12, 15, 28, 31]. Tissue tropism of adenovirus is also group dependent, with HAdV D species causing ocular disease whereas HAdV A and F species causing gastrointestinal tract infections [16]. HAdV type 1-5 and 7 cause pharyngoconjunctival fever, an ocular disease characterized by follicular conjunctivitis, low-grade fever, and pharyngitis. This ocular infection is usually associated with swimming in insufficiently chlorinated swimming pools and lakes [12, 16]. Epidemic keratoconjunctivitis (EKC) is a highly contagious ocular infection that is characterized by follicular conjunctivitis, pseudomembrane and pre-auricular lymphadenopathy (Fig. 1). It is caused by adenoviral types 8, 37, 53, 54, and 64; however, it is usually self-limiting with corneal sub-epithelial opacities formed later in the course of the disease and lasting for many months [16, 18]. Most epidemic outbreaks of EKC are associated with spread from contaminated instruments and solutions in eye clinics [18, 35]. Respiratory tract disease is commonly caused by HAdV types 1 – 7, 14, and 21 and it mostly affects infants and young children [12, 16]. HAdV-E4 and HAdV-E7 are associated with adenoviral-induced respiratory illness in military recruitment camps and dormitories [18, 37]. Gastrointestinal tract (GIT) disease is associated with persistent shedding of adenovirus in stool. Adenoviral-induced genitourinary tract disease is characterized by hematuria and dysuria that may persist for many days. Hemorrhagic cystitis syndrome is a self-limiting adenoviral infection that affects children and also includes hematuria and dysuria [18]. Additionally, adenovirus could be associated with meningitis, meningoencephalitis, myocarditis, arthritis, and pancreatitis [16].

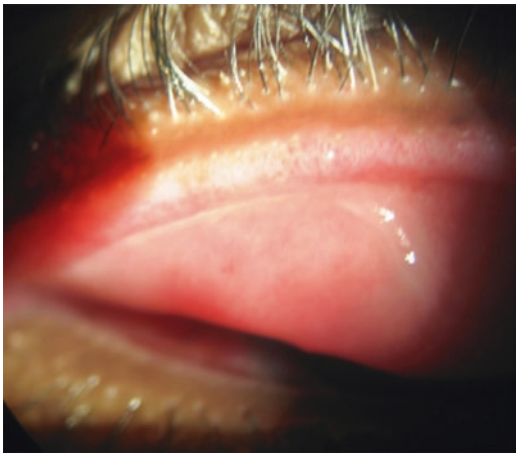

Fig. 1 Pseudomembrane in adenoviral keratoconjunctivitis. Pseudomembrane is a hallmark feature of epidemic keratoconjunctivitis and it is due to exudation of serum, fibrin and leukocytes from dilated conjunctival capillaries, which are deposited on the inflamed surface of the palpebral conjunctiva (white arrow)

2.1.4 Interactions with the Immune System and Pathophysiology of Disease

Many cells that are susceptible to adenoviral infection express primary cellular receptors such as coxsackie and adenovirus receptor (CAR), CD46, Sialic acid, desmoglein-2, Heparin sulfate proteoglycans (HSPG), CD80, CD86, and others [38, 39]. Activated macrophages and epithelial cells secrete CXCL8 that binds to CXCR1/2 receptors that triggers relocation of CAR and αvβ3 integrin from the basolateral surface to the apical surface to facilitate adenovirus attachment from the apical plasma membrane [30, 38]. Many epithelial cells express αvβ3 and αvβ5, and these integrins interact with penton base proteins to promote internalization of adenoviruses 29, 32, 36, 38]. Penton base-integrin interaction activates intracellular signaling molecules including phosphoinositide-3-OH kinase (PI3K) and p130CAS, which in turn, activate the Rho family of small GTPases, inducing polymerization of actin filaments, a process required for internalization of adenovirus into clathrin-coated vesicles [29, 34, 40, 41]. Following internalization of the adenoviral nucleocapsid into the endosome, the acidification of the endosome enables the process of uncoating of the virus, and subsequent transportation of the viral genome into the nucleus [34]. Viral DNA genome and protein VI are transported into the nucleus where adenoviral early region 1A (E1A) is transcribed. The EIA protein generated trans-activates expression of early gene E1B, E2, E3, and E4 involved in the early stage of the replication cycle [39, 42–45]. E1A protein blocks type I inducible gene expression as well as blocks induction of human leukocyte antigen (HLA) class II genes by IFN-γ [46]. E1B protein encoded by E1B gene facilitates the completion of adenovirus replication by preventing apoptosis of the infected cell via inactivation of p53 [47]. The early gene-proteins function to disrupt the immune system response and enhance viral replication, increasing polymerase transcription, blocking MHC class I function, lowering NK cell surface receptors, and shutting off host cell protein synthesis [39, 43, 46, 48]. Adenain (23K cysteine protease) encoded by L3 gene, is a proteolytic enzyme that is required for the cleavage and release of protein IIIa, pVI, protein VII, protein VIII, protein X (Mu), and terminal protein (TP) [34, 39]. It is involved in disassembly of the viral capsid, release of viral DNA, as well as maturation of adenovirus [43, 49, 50]. Viral protease cleaves pTP into TP in the final stages of DNA replication, which results in generation of progeny DNA that is packaged into virions [48].

The immune response to adenoviral infection involves the innate immune system mediated by NK cells, monocytes, and type 1 IFNs [18]. Additionally, epithelial cells, macrophages, plasmacytoid DCs and conventional DCs participate in the innate immune response to adenovirus [51]. Antimicrobial peptides such as defensins, defensin-like chemokines, and cathelicidin participate in antiviral innate immune response as well [23]. Components of the adenoviral capsid act as virus-associated molecular patterns (VAMP) that interact with pattern recognition receptors (PRRs) to initiate an innate immune response [52]. DC-SIGN is involved in the capture and uptake of adenovirus by DCs, and such interaction favors the development of a Th2-mediated immune response. This leads to establishment of a chronic infectious state due to impaired clearance of adenovirus [53]. HAdV-infected DCs can induce cellular immune response by stimulating $CD8^{+}$T cells [19]. Antibodies

and T cells mediate the humoral and cellular adaptive immune response respectively. TNF-α and IFN-γ released by macrophages and NK cells, enhance the ability of DCs to induce the adaptive immune response [18]. Neutralizing antibody directed against fiber protein plays a role in resolution of lytic adenovirus infection with the potential to protect the individual from re-infection with the actual HAdV type that generated the neutralizing antibodies [12]. IgA-mediated mucosal immunity to adenovirus prevents productive adenoviral infection by preventing interaction between adenovirus and host cellular receptors. Furthermore, antibody-mediated cytolysis of infected cells involves complement-mediated cell lysis and antibody dependent cell-mediated cytotoxicity. CTLs and NK cells play a primary role in cell-mediated cytotoxicity via the release of perforins and granzymes that cause apoptosis of adenoviral-infected cells. IFN-γ and TNF-α released by Th1 cells and CTLs mediate non-cytolytic clearance of adenoviral infection without lysing infected cells. Killing of adenoviral-infected cells before the replicative cycle is complete, significantly reduces the production of infective progeny virions [20–22].

Cellular immunity limits growth of adenovirus, however, adenovirus is also able to evade the immune response resulting in persistence in the host. Adenovirus species such as HAdV-D evade immune-mediated cytolysis by down-regulating expression of ligands (CD112 and CD155) that activate NK cell receptor DNAM-1 (DNAX accessory molecule/CD226). Adenovirus also evades killing by NK cells by up-regulating the expression of HLA-E. Engagement of HLA-E is associated with inhibition of subset of NK cells expressing NKG2A. Furthermore, down-regulation of HLA-A/B/C expression is a strategy used by the virus to modulate MHC class I expression in adenoviral-infected epithelial cells [54].

2.1.5 Treatment and Prevention

Adenovirus represents a significant health burden, especially in the immunocompromised population, due to increased rates of dissemination with multi-organ involvement, reactivation of latent infection, and unavailability of suitable anti-adenoviral therapy [18]. There are no FDA-approved antiviral agents for treating adenoviral infections [16]. Cidofovir and ribavirin have been used as antiviral therapy in immune-compromised individuals [12]. Cidofovir is an acyclic nucleoside phosphonate derivative of cytosine that incorporates into the adenoviral DNA, inhibits polymerase, and stopping transcription [35, 42, 45]. Cidofovir has been shown to have anti-HAdV activity against all types, particularly in recipients of bone marrow transplant and/or solid organ transplant. It is most efficacious when administered intravenously. It produces significant side effects such as nephrotoxicity, myelosuppression, and uveitis [25, 35]. Brincidofovir is a lipid-linked derivative of cidofovir that has enhanced oral bioavailability with an improved safety profile [35, 45]. Ribavirin is a broad-spectrum nucleoside analog of guanosine that has antiviral activity against HAdV group C species [45]. Maintenance of adequate or optimal levels of chlorinated swimming pools and improved personal hygiene can minimize transmission [55]. Immune-prophylaxis via live oral vaccine are beneficial for preventing adenovirus types 4 and 7-induced respiratory infections in military recruits [12].

2.1.6 Future Direction: Clinical Trials and Current Research

Human adenovirus infection in immunocompromised individuals is associated with a high risk of developing disseminated and fatal disease; therefore, there is an urgent need to evaluate current antiviral therapies for efficacy in treating patients with adenoviral infections. Research should also focus on developing alternative anti-adenoviral therapies with enhanced therapeutic index and safety profiles [24, 25]. RNAi-mediated viral gene silencing is a potential method of inhibiting replication of adenovirus [44]. The inhibitory effect of siRNA-mediated adenoviral gene silencing on DNA replication was also shown via blockade of the gene that encodes DNA polymerase [49]. The artificial microRNA directed against adenoviral E1A, DNA polymerase, and preterminal protein mRNA could inhibit replication of adenovirus in vitro [24]. Furthermore, shRNA delivered by non-viral or viral vector systems have been shown to undergo intracellular processing into mature functional active siRNA that possess activity against adenoviral E1A and DNA polymerase. siRNA and shRNA can induce RNAi-mediated suppression of CAR expression, which results in the inability of adenoviral species that use CAR as a primary cellular receptor to cause productive infection [35]. Thus, siRNA could be good candidates for RNAi-mediated inhibition of adenoviral multiplication.

Cysteine protease adenain lacks human homologues and could therefore be a great target for therapy with an inhibitor of adenoviral cysteine enzyme such as ritonavir [25, 50]. Adenoviral infection can be inhibited using a soluble virus receptor trap, such as soluble CAR consisting of extracellular domain D1 and D2 of CAR fused to Fc portion of human IgG1 to generate soluble CAR-Fc. The D1 domain binds to the fiber knob of adenoviruses and the Fc portion of sCAR-Fc binds to macrophages to facilitate virus clearance [35]. Suppression of adenoviral replication was enhanced by co-administration of anti-adenoviral siRNA, virus receptor trap sCAR-Fc and Cidofovir [42]. Furthermore, donor-derived adenoviral specific T cells infused in children with post-Stem Cell Transplantation systemic adenoviral infection has been demonstrated to be an effective means of inducing adoptive transfer of HAdV-specific immunity, reducing viral replication and increasing protection from HAdV-related complications [56]. While intravenous immunoglobulin (IVIG) containing neutralizing antibodies against common adenoviral serotypes could be effective against primary adenoviral infection, it may not provide antiviral protection against reactivating viruses [28].

2.2 Human Parainfluenza Virus

HPIV is an enveloped non-segmented negative sense ssRNA virus that causes lower and upper respiratory tract infection that can be more severe in infants, elderly, and immunocompromised individuals. HPIV causes causing significant health burden [13, 57–59]. Young children are susceptible to HPIV-associated acute respiratory illness due to immunological immaturity, underdeveloped immune system, and

HPIV-specific maternal antibodies suppressing vaccine-induced antibody response [57]. Sialic acids are host cell receptors that facilitate HPIV infection through interaction with HPIV viral attachment proteins (hemagglutinin/neuraminidase) or glycoprotein (G protein) [13, 58, 59]. NK cells and type I interferons provide antiviral innate immunity; however, C and V viral proteins block induction of type I IFNs that are essential for activating NK cells and providing an antiviral microenvironment [57, 60]. C-protein expressed by HPIV type 1 and 3 suppresses the induction of IFN [57]. Similar immunosuppressive effects occur with V protein expressed by HPIV type 2 and 4 [57]. There are no FDA-approved antiviral prophylactic and therapeutic agents for HPIV; however, infected patients respond to supportive therapy with nebulizer steam therapy [57].

2.2.1 Epidemiology and Disease Burden

After RSV, HPIV is the second most common cause of acute respiratory infections in infants and children. It is prevalent during the fall months and it is the cause of approximately 23,000 hospitalizations per year [57]. There are four major strains of the virus causing infections ranging from croup and bronchiolitis to pneumonia. Disease burden is most significant with HPIV3 than with HPIV1 and HPIV2 [61]. No vaccine is yet available for this virus, but it would be beneficial to reduce the quantity of office or hospital visits caused by this infection [57, 62].

2.2.2 Characteristics, Morphology, and Virulence Factors

HPIV, like the other paramyxoviruses, is an enveloped non-segmented negative sense ssRNA virus. HPIV replicates in the cytoplasm and is transmitted by respiratory droplets or person-to-person contact. The genome of all paramyxoviruses encodes for six basic genes, with some variation amongst the members of this family. The basic mRNA is 3'-N-P-M-F-HN-L transcribed in sequence into separate RNAs [13, 58, 59]. The nucleocapsid is formed by the nucleocapsid protein (N). The large protein (L) and phosphoprotein (P) attach to the nucleocapsid and form the RNA-dependent RNA polymerase protein. The P protein can go through mRNA editing to produce C and V proteins that are important for virus interactions with the host immune system. The matrix protein (M) is essential for assembly and budding-off of the virus during infection. The Fusion (F) protein and Hemagglutinin/Neuroaminidase (H/HN) or G proteins are embedded in the viral membrane. H/HN/G are the attachment proteins. They bind to sialic acids on the surface of the target cell and allow for internalization of the virus [13, 58, 59]. Binding of HN with sialic acid on cellular membrane proteins of host cells initiates the infectious process of HPIV, followed by F protein mediated fusion between the viral envelope and cell plasma membrane of the host cell. This fusion is followed by release of the viral genome bound by nucleocapsid proteins (nucleoprotein, phosphoprotein and RNA-dependent RNA polymerase). The RNA-dependent RNA polymerase directs the

generation of mRNA transcripts from viral genes and RNA genome replication. There is assembly of nucleocapsids that are packaged into virions with matrix proteins coating the inner surface of the viral envelope and surface F and HN projecting from the envelope [61]. The neuroaminidase portion of HN cleaves sialic acids on the target cell surface preventing re-infection of the same cell [58, 59]. HPIV 2 and 4 don't express C proteins, while HPIV1 and 3 don't express V proteins. V and C proteins are important for survival of the virus through suppression of IFN induction and cell signaling in HPIV infected cells [57]. All of the members of the *Paramyxoviridae* family cause cell-to-cell fusion, leading to the formation of multinucleated giant cells. This allows for viruses to easily transfer from cell to cell and evade the immune system [63].

2.2.3 Clinical Manifestations

HPIV has an incubation period of 2–6 days and lasts 1–2 days after symptoms begin. There are four major types of HPIV. Types 1–3 are the cause of severe lower respiratory tract infection in children and infants. Type 1 and 2 are the strains that can lead to croup. Type 3 is more likely to cause bronchiolitis or pneumonia in children. Less is known about type 4 HPIV but it seems to cause milder upper respiratory infections in children and adults [64–66]. HPIV is transmitted through contact or inhalation of respiratory droplets. HPIV travels through the paranasal sinuses, to the larynx and to the bronchi, which can then also lead to obstruction of the eustachian tube and cause otitis media [57]. Laryngotracheobronchitis, or more commonly known as croup, is the more severe manifestation of HPIV that is caused by inflammation, leading to sub-glottal swelling and blockage of the airways. The typical patient presents with hoarseness, cough, tachypnea, tachycardia, and suprasternal retractions [13, 57, 66]. Infants, the elderly, and immunocompromised patients are at an increased risk due to immature or decreased function in the immune system [57].

2.2.4 Interactions with the Immune System and Pathophysiology of Disease

When HPIV circulates through the host respiratory system, it activates viral receptors such as melanoma differentiation-associated gene 5 (MDA5), RIG-I, and PKR. The activation of these receptors leads to transcription of IRF3 and NF-κB, as well as activation of IFNs that create an antiviral microenvironment. The major players that interfere with this system are HPIV proteins C and V. Both HPIV1 C protein and HPIV 2 V protein block IFN production and inhibit apoptosis through interference with MDA5 and laboratory of genetics and physiology 2 (LGP2) [57, 60]. The HPIV3 C protein decreases the innate immune response by slowing down viral replication. It also binds to STAT1 and prevents IFN signaling. HPIV 4 hasn't been shown to affect IFN signaling, and this could be a reason for its milder manifestations [57]. Additionally, HPIV C protein suppresses activation of NF-κB and

IRF3 that play a role in inducing antiviral and inflammatory response to HPIV infection [57]. Secretory IgA secreted from the respiratory tract epithelium can be protective, but it is short lived. A couple or more of subsequent HPIV infections are required to enhance longevity of the mucosal IgA response [61]. Although IgA provides better correlate of immune protection, both serum and mucosal neutralizing antibodies that target HN and F glycoprotein provide long-term protection against HPIV [57]. $CD8^+$T and $CD4^+$T cells provide cellular immunity that can clear the virus [57]. Cellular immunity developed during primary HPIV infection can confer short-term protection against re-infection but this T cell-mediated viral clearance following reinfection wanes over a few months [61]. Histopathology of RSV respiratory complication, such as bronchiolitis and pneumonia are characterized by obstruction of small airways by inflammatory debris and edema as well as hyperplastic lymphoid follicles compressing the bronchioles [57]. HPIV 1-3 infect the apical superficial layer of epithelial cells of the respiratory tract, which not only contain the infection but also prevents antigen presentation, which could be one of the reasons for recurrent infections [57, 67].

2.2.5 Treatment and Prevention

HPIV can be inactivated by dryness and acid. RT-PCR of respiratory secretions is the best way to detect and quantify viral load [13]. Treatment of croup is supportive, which include steam nebulizer treatments, administration of racemic epinephrine, and monitoring for airway patency. Rarely, a patient may need to be intubated if inflammation is so severe as to compromise the airways. There are no specific antiviral medications used. No live or attenuated vaccines have been effective [57].

2.2.6 Future Direction: Clinical Trials and Current Research

There are ongoing clinical trials sponsored by the National Institute of Allergy and Infectious Disease (NIAID) and MedImmune to develop a vaccine against HPIV infection. The majority of the HPIV3 vaccine variants have been shown to be safe and immunogenic, however, only HPIV3cp45 is in phase II clinical trials. Phase I clinical trial of Bovine PIV3 (BPIV3) and HPIV3 vaccine revealed modest seroconversion rate for HPIV3. Recombinant Bovine/HPIV3, a bivalent vaccine containing RSV F protein and HPIV antigen, was well-tolerated in young children with a seroconversion of 100% in children under the age of 2 years [61]. Evaluation of live attenuated HPIV vaccine (rHPIV-1/84/del 170/942A) in adults and children revealed that the vaccine was effective in adults but ineffective in seronegative children because it was insufficiently immunogenic. Therefore, further research is necessary to develop a live-attenuated HPIV vaccine that is highly immunogenic and not over-attenuated [68]. Because HPIV3 is the cause of the most serious form of respiratory tract infection in young children, HPIV3 vaccine would be ideal for infants who are at increased risk of HPIV infection [61].

2.3 Measles and Mumps Virus

Measles (MV) and mumps (MuV) are enveloped negative sense ssRNA viruses transmitted through aerosolized viral particles inhalation or contact with infected respiratory secretions [69–72]. Because infection caused by measles and mumps virus is associated with high mortality and morbidity, it is of significant health burden particularly in regions with low MMR vaccination rates [73]. Immunoprophylaxis with MMR vaccines have significantly decreased the global incidence [74]. DCs and alveolar macrophages initiate the immune response to measles and mumps similar to other viruses that target the respiratory mucoepithelial cells. DC-SIGN on DCs mediate spread of infection, whereas CD46 expressed on all nucleated cells mediate viral interaction with epithelial cells [69, 75]. Clinical manifestations of measles-associated infection include respiratory tract infection, giant cell pneumonitis, encephalitis, conjunctivitis, and subacute sclerosing panencephalitis (SSPE). Mumps is associated with parotitis, orchitis, meningitis, encephalitis, transient sensorineural hearing loss, pancreatitis, pericarditis, and myocarditis [76]. Blockade of type I interferon production is associated with MV-induced suppression of antiviral innate immunity[69]. Protein C and V mediate immune evasion via blocking T cell and antibody effector function [63]. V protein blocks effector function of plasmacytoid DC generation [77, 78]. FDA-approved MMR vaccine provides long-lasting immunity against measles and mumps [79].

2.3.1 Epidemiology and Disease Burden

Measles and Mumps infections are most commonly seen in children and immunocompromised individuals; they are highly infectious and can cause serious long-term complications. With the advent of the Measles-Mumps-Rubella (MMR) vaccine as part of standard childhood vaccinations, the morbidity and mortality from these diseases have decreased worldwide. Measles, also called rubeola, is a worldwide, highly infectious viral disease that causes rash, respiratory symptoms, and long-term neurological disease. Despite vaccination programs, measles virus still caused 164,000 deaths in 2008 [74], and according to one of the latest WHO reports, between 2013 and 2014 there were 280,795 cases and 40 cases per million population [80]. Measles is still endemic in many developing countries with poor health and sanitation systems, and where strict vaccine regiments aren't well established [81, 82]. The mortality associated with measles can be attributed to the immune suppression caused by MV, predisposing patients to fatal secondary respiratory or gastrointestinal infections [73]. During the period between 2000 and 2014, there was a decline in measles incidence by 73% worldwide, and estimated deaths due to measles decreased by 79%, from 546,800 to 114,900 [80]. The U.S. declared measles elimination in 2000, even though there have been outbreaks since then, mostly due to decreased herd immunity following refusal of vaccinations by parents [83]. According to epidemiologic data from the WHO, there are 23 genotypes of MeV: A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, E, F, G1, G2, G3, H1, and H2. In areas

where measles is still endemic, such as Africa, there are only one or two different genotypes being transmitted (for example, West Africa only has infections with the B3 genotype). In countries where measles has been eliminated, there are many different genotypes seen during outbreaks due to exposure of the virus from outside of the country itself, such as in North America and Western Europe [84].

Mumps can cause infections ranging from mild parotitis to more severe systemic infections such as meningitis and encephalitis. With institution of MuV vaccination programs, infections have declined to <0.01 cases/100000 population in 2001 [76]. There have been some recent outbreaks of MuV, even among vaccinated populations, such as the one in 2009–2010 in NY/NJ. Recent outbreaks have identified genotype G as the culprit [85]. Currently, the most common genotypes worldwide are G, H, C, F, K, and D [86]. MuV usually causes self-limited infection with fatalities only related to the 1% of infections that lead to encephalitis [76].

2.3.2 Characteristics, Morphology, and Virulence Factors

MV and MuV are spherical, enveloped, negative sense, ssRNA viruses with only one serotype. They are human pathogens transmitted through aerosols or contact with infected respiratory secretions. In the temperate zones, outbreaks of both diseases usually occur in winter and early spring, when individuals are more likely to congregate in close quarters (e.g. inside schools in the winter) [69–72]. Like other paramyxoviruses, their genome encodes NP, P, L, H, F, M, C, V proteins that allow it to attach and enter its target cells, replicate its genome, translate it, re-assemble, and infect other cells. The H and F proteins are key in the pathogenesis of measles for entry into target cells and spread to other host cells. The H glycoprotein is immunogenic, causing an immune response reaction, and conferring life-long immunity [70]. MuV, like HPIV, expresses the HN protein that allows it to interact with target host cells.

MV also causes a transient suppression of the immune system, affecting T cell signaling and B cell production of antibodies, and predisposing affected individuals to superinfections with other pathogens. Proteins V and C have been implicated in this immunosuppression. MV can cause a persistent infection. It can remain dormant in cells such as those of the CNS, increasing the risk for post-measles infection sequelae like encephalitis [63]. MuV spreads through viremia and can affect multiple organ systems. Unlike other viruses in the *paramyxoviridae* family, MuV causes a lytic infection of epithelial cells of the URT. Cell-mediated immunity is necessary for controlling infection. Protein V is crucial in immune system evasion [76, 85].

2.3.3 Clinical Manifestations

Measles is a systemic infection. MV typically has an incubation period of 10–14 days. The first signs of the disease are fever and respiratory distress, or the 3 Cs: cough, coryza, and conjunctivitis. One of the cardinal features of MV infection

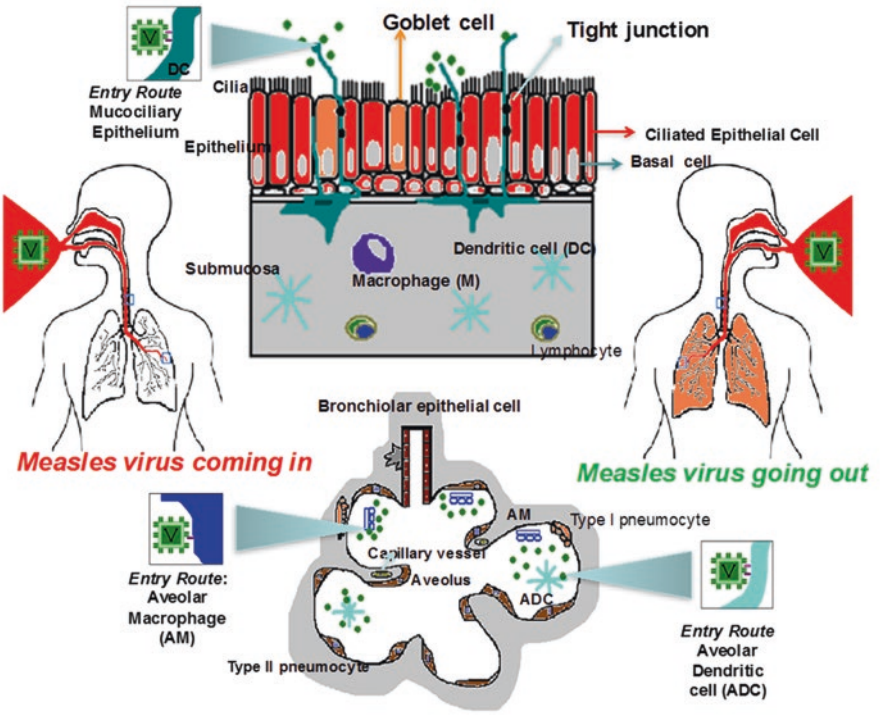

Fig. 2 Mechanism of entry and establishment of infection of Measles Virus. MV – Measles virus; DC – Dendritic cells; AM – Alveolar macrophage; ADC – Alveolar Dendritic cells). Measles virus infects respiratory tract epithelium by attaching to CD46 and nectin 4 expressed on epithelial cells. This is followed by local replication in the epithelium and subsequent spread via lymphatics to secondary lymphoid tissue and non-lymphoid tissue. Measles virus also interacts with SLAM receptors on lymphocytes in the respiratory tract to facilitate entry and subsequent replication in the lymphocytes. The virus spreads via infected lymphocytes and viremia to other parts of the host

is the appearance of Koplik spots in the buccal mucosa, which are blue-grey spots on the inside of the mouth; they usually appear 1–2 days before the onset of the other common feature of measles, the maculopapular rash. This rash extends from the head and neck, including the face, and moves downward over the whole body, eventually coalescing; it usually appears 14 days post infection, and can last up to a week [69, 70, 74, 87]. The presence of the rash signifies a good immune response to the virus replicating in the skin. Someone infected with measles is infectious even before the onset of the rash, making it difficult to contain the spread of the virus. Patients also remain infectious a few days after the advent of the rash. A patient is most infectious when the respiratory symptoms are most severe due to its mode of transmission through respiratory secretions [63] (Fig. 2).

Giant cell pneumonitis can be a more severe presentation of measles if patients have a dysfunctional cell-mediated immune response, are immunosuppressed, or malnourished. Another possible but rare outcome is encephalitis, which usually

presents 2 weeks after the initial infection and can include headache, seizures, and fever. This is usually due to an autoimmune response against myelinated neurons. Subacute sclerosing panecephalitis (SSPE) is an even more rare sequela caused by a persistent infection with a measles virus variant. This presents with seizures but also with progressive behavioral, motor and cognitive dysfunction, causing death 5–15 years post-viral infection. These serious complications have diminished since the introduction of the MMR vaccine [69, 74, 81].

MuV also causes systemic infection but one third of infections are usually asymptomatic. It is rarely seen in countries that promote the live attenuated vaccine but without vaccine, 90% people will be infected with MuV by 15 years of age [13, 76]. Symptomatic MuV initially presents with a mild fever, headache, and malaise. Once infection in the respiratory tract has been established, MuV can infect the parotid gland by travelling through the Stensen duct or by viremia. Parotitis is usually bilateral and accompanied by high fever. It appears 2–3 weeks after exposure to the virus and lasts a few days. MuV causes a self-limiting illness but if viremia occurs, it can lead to orchitis, meningitis, or encephalitis, as well as possible spread of infection to the ovaries and thyroid [76]. Orchitis is the most common manifestation outside of the respiratory tract: it is usually unilateral and affects 10–20% of post-pubertal men who have been infected with Mumps. Mumps orchitis can be one of the causes of hypofertility, but rarely causes sterility. MuV is neurotropic, with 10% of infections leading to meningitis and <1% encephalitis. It has been postulated that MuV spreads to the brain parenchyma either through viremia or CSF from the choroid plexus. Cases of pancreatitis, pericarditis, and myocarditis due to MuV infection have also been reported. Unilateral sensorineural hearing loss has also been observed in about 4% of MuV infections, but this is transient, and children usually recover well [76].

2.3.4 Interactions with the Immune System and Pathophysiology of Disease

The main players in the course of measles virus infection are dendritic cells (DCs), alveolar macrophages, T cells, B cells, and mature DCs. The main receptors involved in the spread of infection are DC-SIGN; CD150/signaling-lymphocyte-activation molecule (SLAM); normally expressed on activated T and B cells, macrophages, and mature DCs; CD46, a complement regulatory molecule that is expressed on all nucleated cells, and nectin-4/ poliovirus receptor-related 4 (PVRLA4), which is part of the E-Cadherin family and is expressed within the adherens junctions of epithelial cells [69, 73]. Wild-type measles virus infects cells mainly using SLAM, but vaccine strains also bind to CD46 [70]. MV uses the H and F glycoproteins to enter alveolar macrophages and DCs. MV-H binds to DC-SIGN, which causes these cells to increase expression of CD150/SLAM through activation of acid sphingomyelinase [69, 75]. MV-H binds to SLAM, causing a conformational change in F, which allows membrane fusion to occur. Infected DCs and alveolar macrophages travel to draining lymph nodes where they encounter and infect activated T and B cells,

allowing the virus to replicate, and spread. Infected T and B cells exit the lymph node and travel to secondary lymphoid sites and then disseminate to other tissues, causing some of the clinical symptoms already discussed [69]. In the respiratory tract, virions coating T cells, B cells, and DCs enter the basolateral surface of the epithelium via the nectin-4/PVRLA4 receptor at junctions, disrupting the barrier and allowing for widespread infection to occur [69, 73, 88, 89].

Interactions of host proteins such as the heat-shock protein 72 (HSP72), casein kinase II, and Peroxiredoxin1 (Prdx1) with MV proteins have been shown to cause an increase in viral replication [69]. HSP72 interacts with N protein, causing increased levels of MV RNA expression [90]; Prdx1 interacts with the N and P proteins, altering the MV-N-RdRp complex, and consequently enhancing mRNA synthesis and regulation [91]. MV is also associated with lymphopenia; lymphocyte numbers go back to normal about a week after infection resolves, but the immune suppression extends for several weeks more [92]. MV interaction with host immune cells causes a decrease in production of IFN-α and IFN-β, which are normally necessary to mount a good innate immune response against viruses, and this could be one of the causes for the immunosuppression [69]. When the virus enters the host cell, its RNA is sensed by RNA helicases like RIG-I, MDA5, and LGP2. MV P protein binds to MDA5 and opposes the synthesis of IFNs, especially IFN-α. When LGP2 interacts with the V protein, RIG-I is inhibited. In addition, MV P protein also decreases the signals through toll-like receptor 4 (TLR4) [69]. These interactions down-regulate the host's sensors for viral particles, diminishing the immune response. Usually, virus RNA activates transcription factors such as interferon regulatory factors 3 and 7 (IRF3 and IRF7), and nuclear factor of the kappa light chain enhancer of B cells (NF-κB), which causes the secretion of IFN-β and other inflammatory cytokines. In plasmacytoid DCs, V protein interacts with IRF3 and IRF7 and inhibits their action. P, V, and C proteins also individually bind NF-κB subunits to inhibit signaling through the JAK/STAT pathway [74, 93].

Additional mechanisms contributing to the immunosuppression include the persistence of an immature profile of DCs and a switch from a Th1 to Th2 immune response. Immature DCs have decreased expression of co-stimulatory molecules for T-cell activation and a reduced level of CCR7 expression, preventing them from homing to secondary lymphoid sites and stimulating T cells [77, 78]. The Th2 immune response decreases IFN-γ secretion as well as the immunological synapse proteins such as plexins and neuropilins that aid in T cell activation, while increasing secretion of IL-10, downregulating the immune response as a whole [77, 94, 95].

MuV usually causes mild parotitis, but there are strains that cause infections of the meninges or brain parenchyma. MuV, like other members of its family, has specific proteins that aid in establishing or spreading the infection. The small-hydrophobic (SH) proteins have been shown to suppress TNF-α production [76]. Also, V protein interrupts the IFN signalling pathway causing the degradation of STAT1, and blocking the production of IL-6 by degradation of STAT3 [85]. No specific set of proteins have been found that transforms an MuV strain to a neurovirulent strain; viral loads in the CSF have been correlated with severity of infection [96].

2.3.5 Treatment and Prevention

Knowing the immune status of patients is critical in managing a measles infection. It is beneficial for patients with immune-deficiencies to receive immunoglobulins after measles exposure [87]. Immunocompromised patients should not be administered an attenuated live-virus measles vaccine; exceptions exist for HIV patients who have a high enough CD4+ T cell count [87]. There are no efficacious treatments for Measles, but IFN-α, ribavirin and other antiviral therapies have been used. Administration of vitamin A has been proven to reduce morbidity and mortality in those affected by MV. Usually a physician will give daily doses for two consecutive days based on age and nutritional status. If a patient is already vitamin A deficient, a longer course of therapy will be necessary [97]. Because of passive immunity acquired from maternal antibodies, infants are protected from measles virus for a few months after birth. Because MV has only one serotype, and the immunogenic H glycoprotein remains constant, the vaccine created years ago is still effective and protection is maintained [81, 87]. The measles vaccine is a live-attenuated viral vaccine; there were three strains that were developed -the Edmonston vaccine licensed in 1963, the Schwarz vaccine in 1965, and the final Moraten strain in 1968. Each time, the vaccine has been progressively more attenuated, causing less side effects [70]. MuV can be recovered from saliva, urine, pharynx, secretions from the Stensen duct, and CSF. Serologic tests or ELISA can be done to look for a 4-fold increase in antibody titer when suspecting an acute infection. A live attenuated vaccine, together with Measles and Rubella, was introduced in 1967, and is part of the recommended childhood immunizations [13, 76].

2.3.6 Future Directions: Clinical Trials and Current Research

Studies have been conducted that compare the injected versus the aerosolized MV vaccine, but the latter has been found to be inferior to the original. Many developing countries where measles is still endemic would benefit from a vaccine that is easier to administer, easier to store, and less expensive, therefore more studies are needed to explore other vaccine options [79].

Another ongoing area of research is the use of measles virus for oncolytic therapy. The receptor tropism of MV makes it a great candidate to target many cancer cells due to over expression of CD46 in cancer cells (this receptor is used by the Edmonston-B vaccine measles strain), and especially lung adenocarcinoma and squamous cell carcinomas, as well as ovarian and breast cancers due to their high expression of Nectin-4. The syncytia formation induced by MV is an asset in the spread of MV engineered to target tumor cells to allow for infection of many cancer cells efficiently. In addition, MV strictly replicates in the cytoplasm and does not interact with host genome eliminating potential safety issues with recombination and transformation [98]. “Virotherapy” [99] has been proven to be safe in humans; however, trials are still ongoing to prove its true efficacy. Improving specificity to tumor cells while evading preformed immune mechanisms against the virus is a

challenge [99]. For example, in the case of measles virus, the H and F proteins can be slightly modified into glycoproteins of a virus of the same genus to avoid neutralizing antibodies present from previous vaccination or infection. To improve efficacy, but maintain safety, miRNAs can be used to augment viral replication in tumor-specific cells already expressing miRNAs, increasing the oncolytic effect of the virus [99]. Lastly, using viruses to carry chemo-, radio-, and immune-therapy genes could improve overall efficacy of these therapies. There are ongoing Phase I clinical trials using NIS-expressing measles virus (human sodium-iodide symporter) for ovarian cancer, myeloma, mesothelioma, and head and neck cancers. This kind of engineered therapy not only uses radioactive isotope targeting for cancer cells, but also allows for in-vivo tracking of viral replication in tumor cells using single-photon emission computed tomography (SPECT) and positron emission tomography (PET) .

2.4 Respiratory Syncytial Virus

RSV is an enveloped negative sense ssRNA that contains 10 genes that encode 9 nonstructural proteins found in infected cells and virus. It also encodes 2 structural proteins that are also found in infected cells [100]. RSV forms syncytia in infected mucoepithelial tissue of the respiratory tract. Because RSV causes fatal respiratory tract infections in infants, patients with cardiopulmonary abnormalities, elderly individuals, and immunocompromised individuals, it is of significant public health concern [13, 65]. Innate immunity is mediated by type I interferon and NK cells while CTL provide cell-mediated immunity against the virus. NS1 and NS2 are important viral proteins that block type I interferon via blockade of interferon regulatory factor 3. RSV impairs both plasmacytoid DC effector function of producing type I interferon microenvironment and myeloid-derived DC presentation function. RSV also blocks signaling via TLR and RIG-1 with consequential reduction of proinflammatory antiviral cytokines. Furthermore, it suppresses cell-mediated cytotoxicity via blockade of $CD8^{+}$T cell and Th1 cell effector function [14]. Ribavirin is a guanosine analogue that is therapeutically beneficial for high risk patients, particularly preterm infants and patients with cardiopulmonary disease. Premature infants also benefit from the use of antibody prophylaxis [13, 65].

2.4.1 Epidemiology and Disease Burden

Respiratory Syncytial Virus (RSV) is the major cause of upper and lower respiratory tract infection in young children and elderly individuals [101]. It is associated with pulmonary complications such as bronchiolitis, pneumonia-related illness, and exacerbations of underlying pulmonary diseases [101, 102]. RSV is highly

contagious and prevalent in the winter months. In 2005, RSV caused 34 million cases of Lower RTIs (LRTI) in children less than 5 years of age [14, 65]. RSV causes RTIs, with no serious long-term sequelae or intensive treatments. However, it still causes a considerable portion of children hospitalizations and health burden. Complications from RSV are only seen in developing countries [65]. Infants with poorly developed airway system or those born pre-term are at higher risk of RSV infection; also, a low maternal antibody titer has been correlated to higher RSV infection risk. Exposure to second-hand smoke, pollution, and daycare exposure are additional environmental factors that increase the risk of infection with RSV. There is no vaccine available and recurrence is high throughout life, especially in the elderly [65]. Some studies suggest a correlation between bronchiolitis in infants and asthma later in life, but usually RSV does not cause long-term consequences [103]. RSV poses a significant health burden because it is linked to the development and exacerbation of airway hyper-responsiveness in infected children [102]. Additionally, RSV is of significant health burden because reinfection with RSV is common due to inability of the infection to confer long-term protective immunity [101].

2.4.2 Characteristics, Morphology, and Virulence Factors

G proteins expressed by RSV bind to target cells but don't possess hemagglutinin action [58, 59]. RSV also expresses unique proteins that allow it to interact with the immune system: these are the non-structural proteins (NS1 and NS2) [104]. The fusion (F) and matrix (M) proteins are highly conserved between RSV serotypes; however, surface glycoprotein (G) is not fully conserved between RSV serotypes [101]. SH proteins have been studied in RSV and mumps in relation to their interactions with the immune system as well.

2.4.3 Clinical Manifestations

RSV is the most common cause of acute respiratory tract infections in infants and young children. Everyone below the age of 2 will be infected at least once, and it often recurs, especially in the elderly. It may be fatal in premature infants, persons with underlying lung disease, and immunocompromised people [13, 65]. RSV is transmitted through contact or respiratory droplets; it invades the respiratory epithelium directly, causing immunologically mediated cell injury. This can lead to necrosis of the bronchi and bronchioles, and eventually to plugs of mucus, fibrin, and necrotic material in the smaller airways. Infants are innately at an increased risk from RSV infection due to the small size of their airways. This can lead to respiratory impairment [65, 104]. The typical patient usually presents with a low-grade fever, tachypnea, tachycardia, and expiratory wheezes over the lungs. Bronchiolitis is usually self-limited, but it can be dangerous in infants.

2.4.4 Interactions with the Immune System and Pathophysiology of Disease

Humoral immune response restricts reinfection of RSV via the action of neutralizing antibodies against major surface glycoproteins. The immunopathology of RSV associated with pulmonary inflammation is attributed to Th2-derived cytokines that induce eosinophilia in the lungs of RSV infected individuals [100]. RSV can inhibit the production of IL-12 and IFN-γ by DCs with consequential suppression of Th1 polarization and creation of an immunosuppressive microenvironment that favors viral persistence [6]. NS1 and NS2 proteins both decrease the maturation of DCs, and therefore their ability to further stimulate the immune system. NS1 suppresses proliferation and activation of CD103+ $CD8^+$T cells and Th17 cells, which are important in anti-viral cytotoxicity and memory, while also promoting activation of the Th2 pathway, with increased production of IL-4 and other interleukins that are not helpful in fighting viral infections [105]. Other studies have shown that RSV infection impairs the ability of pDCs to produce IFN-α, IL-6, TNF-α, CCL3, CCL4, and CCL5 in response to TLR9 interaction. RSV also impair mDC presentation to $CD4^+$T cells and production of other cytokines [14]. RSV expresses proteins that allow it to evade the immune system and establish infection. G protein contributes to immune evasion and subsequent continued viral replication and/or viral persistence in the host by modifying the magnitude of chemokine and cytokine generated in response to RSV infection. Additionally, it modifies immune cells expressing CX3CR1 receptors and induction of Substance P, which contributes to dysregulation of the immune response to RSV [102]. G protein cannot elicit a CTL response because it lacks a MHC class I restricted epitope, and as such, immune response to G protein is associated with Th2 polarization and eosinophilia [100]. NS1 and NS2 proteins block IFN regulatory factor 3 activation, inhibiting type I IFN induced signaling, leading to a block of IFN-α and IFN-β production from the target cells. NS1 and NS2 have also been found to activate phosphoinositide 3-kinase pathway, inhibiting apoptosis of the infected cells. In addition, RSV inhibits TLR signaling and RIG-I signaling, leading to decreased production of other immune modulators such as NF-κB and other cytokines [65, 105]. Antigenic variation could play a minor role in immune evasion. Inhibition of T cell activation by fusion protein is a major contribution to immune evasion by RSV. Additionally, the secretion of soluble G protein that shifts response from Th1-mediated immunity to a Th2-mediated immunopathologic response is another immune evasion strategy used by RSV [100].

2.4.5 Treatment and Prevention

Treatment of healthy infants/children is usually supportive, consisting of administration of oxygen, intravenous fluids, and possibly steam nebulizer treatments. Ribavirin has been used for the treatment of high-risk patients such as pre-term infants, or those with poor respiratory tract development; it is administered through a nebulizer [65, 106]. Palivizumab is a humanized immunoglobulin G monoclonal

antibody that targets the fusion protein of RSV. Prior to FDA-approval of palivizumab, RespiGram (RSV-immune globulin) was used as passive immunoprophylaxis in high risk children under the age of 2 year (100). Palivizumab and Respiratory Syncytial Virus Immune Globulin Intravenous (RSV-IGIV) are FDA approved passive immunoprophylactic agents for RSV infection in infants and young children with chronic cardiopulmonary disease [107]. If hospitalized, isolation of the infected child is crucial to containing the infection. Infection-control methods include hand washing as well as wearing protective gowns, gloves, and mask [13, 65]. When a vaccine was introduced in the 1960s, it actually caused more severe infections when patient was re-exposed to the virus, and was therefore not approved as a preventative measure [65].

2.4.6 Future Directions: Clinical Trials and Current Research

One of the challenges for RSV vaccine development is to create a vaccine that maximizes immune protection and minimizes Th2 mediated immunopathology. Another major hurdle for RSV vaccine development for the pediatric population is the infants immature immune system and presence of maternal antibodies that could suppress vaccine-induced immunity [100]. The original FI-RSV vaccine was associated with vaccine-enhanced RSV disease (ERD) and it was believed that it induced the deposition of immune complexes and complement components in small airways of the affected infants. It was also associated with the development of Th2-mediated immunopathology with eosinophilia in the lungs of these infants who received the vaccination [108]. Subunit RSV-A vaccines containing purified Fusion, matrix, and G proteins have been demonstrated to be safe and immunogenic in the over 65-age group [101]. It has been reported that RSV vaccines using DNA plasmids that express fusion proteins or glycoproteins had low immunogenicity with limited immune protection [100].

Because of the lack of vaccine-ERD in individuals with memory immune response to natural infection with RSV and potential adverse effects of developing vaccine-ERD, only RSV vaccines that induce optimal antibody response and Th1 cell-mediated immune response should be developed particularly for antigenic-naïve infants. However, vaccine-induced ERD is not of great concern for patients with pre-existing RSV immunity [108]. RSV disease in infants is of high public health concern, and as such, there are benefits of providing child-bearing women RSV vaccination with the intent of generating RSV-specific maternal antibodies that provide immune protection and reduce maternal-infant transmission of RSV and acquiring RSV infection in the first months of life. High levels of RSV-specific maternal antibody titer correlates with reduced incidence of RSV in infants. There is ongoing research to develop vaccines for women in the third trimester of pregnancy such as RSV F nanoparticle vaccine which is currently in the final phase of the clinical trial [109]. It is of note that RSV infection does not confer long-term protective immunity, therefore, there is ongoing research to determine effect of RNAi on host immune response to RSV reinfection with the same or different strain

[102]. Future directions in approaching the development of RSV vaccines should focus on vaccines that can induce $CD8^+$T-cell responses in antigen-naïve infants to clear RSV and produce IFN-γ to promote a Th1-biased cell-mediated immune response [108]. Thus, a deep understanding of the immune evasion strategy used by RSV and RSV-associated immunopathology are required in developing a safe and effective RSV vaccine [100].

2.5 Human Coronavirus

Coronavirus (CoV) was first identified in the 1960's and there are seven human CoV (HCoV) of medical importance. In the winter of 2020, the WHO declared the disease caused by SARS-CoV2 a public health emergency of international concern. On March 11, 2020, The WHO declared the disease caused by SARS-CoV2 a pandemic. The transmission of CoV involves animal-to-human as well as human-to-human transmission [110]. Coronavirus is a positive-sense, single-stranded RNA virus with club-shaped spikes emanating from the viral envelope [110]. Coronaviruses are subdivided into four genera based on phylogenetic clustering. There genera are alphacoronavirus (alphaCoV), betacoronavirus (betaCoV), deltacoronavirus (deltaCoV), and gammacoronavirus (gammaCoV) [111, 112]. Host cellular receptors such as aminopeptidase N (APN), angiotensin converting enzyme 2 receptor (ACE2), dipeptidyl-peptidase 4 (DPP4), and 9-O-acetylated sialic acid via their interaction with spike protein of CoV play a role in pathogenesis and tissue tropism [111]. Many alphaCoV bind to aminopeptidase N (APN) on host cell receptors in order to gain entry into permissive human cells. SARS-CoV and HCoV-NL63 bind to ACE2 receptors to enter into host cells. MERS-CoV utilize dipeptidyl-peptidase 4 (DPP4) for host cell receptor binding [113].

The earlier human CoV caused up to 30% of mild self-limiting respiratory tract infection on an annual basis [113]. HCoV-229E, HCoV-NL63, HCoV HKU1, and HCoV-OC43 cause mild respiratory tract infection, which can progress to lower respiratory tract infection in elderly and immunocompromised individuals. Severe acute respiratory syndrome (SARS) is usually associated with SARS-CoV and MERS-CoV infection [114]. It is important to note that a mild self-limiting CoV infection will run its course. RT-PCT is a diagnostic test of choice for identifying human CoV [113, 115]. NK cells and type I interferon (IFN) provide antiviral innate immunity whereas antibodies such as IgG provide humoral immunity. However, humoral immune response mediated by IgM, IgA, and IgG is short lived. $CD4^+$ T cells and $CD8^+$ T cells provide cellular immunity [116]. Nonstructural and accessory proteins of HCoV interfere with antiviral innate immunity [111].

There are no FDA-approved antivirals for human CoV. The main treatment is generally supportive [114]. Because of the high mortality of SARS-CoV2 in high-risk individuals based on advancing age and pre-existing conditions, there is an urgent need to develop antiviral therapeutics and vaccines.

2.5.1 Epidemiology and Disease Burden

Coronaviruses were not considered highly pathogenic until the outbreak of two strains, SARS-CoV and MERS-CoV, which had zoonotic origins. SARS-CoV and MERS-CoV were responsible for the outbreaks in 2002 and 2012 respectively. The newly emerged SARS-CoV-2 triggered the 2019 pandemic causing CoVID-19 [117]. HCoV are responsible for up to 30% of common cold, which are self-limiting and harmless infections [111, 118]. The transmission of CoV involves animal-to-human as well as human-to-human transmission. The main mode of human-to-human transmission is through aerosolized droplets containing viral particles from coughing and sneezing as well as contact with surfaces contaminated with human CoV. The reproduction number (RO – R naught) of human CoV is more than 2.0, in which each infected individual can transmit the infection to more than 2 individuals [110, 119, 120]. The SARS epidemic of 2002–2003, which infected 8098 people across 29 countries and left 774 people dead, was caused by the severe acute respiratory syndrome-associated coronavirus, SARS-COV [121]. The Middle East Respiratory Syndrome coronavirus (MERS-CoV) is a virus that causes severe respiratory disease and has proven to be highly lethal with a fatality rate of nearly 40% [122]. MERS-CoV was first isolated and discovered in April 2012 from a 60-year-old man who was initially admitted to a Jordanian hospital for acute pneumonia, but subsequently died 11 days later from respiratory and renal failure [123]. Followed by MERS diagnoses in three hospital patients in the United Kingdom, cluster outbreaks of MERS-CoV in hospitals across Saudi Arabia were retrospectively diagnosed and raised a global concern as the number of MERS cases began to spread to countries outside of Saudi Arabia. As of January 2020, a global total of 2519 laboratory-confirmed cases of MERS and 866 MERS-associated fatalities across 27 countries have been reported [124, 125]. MERS-CoV transmission can occur between dromedary camels, camels-to-humans, and human-to-human, although human-to-human transmission is thought to be rare as it requires close contact and exposure to significant amounts of viral shedding [126]. This observation is substantiated by the majority of MERS-CoV infections occurring within hospitals where transmission between patients accounted for 62–79% of infection routes and transmission between family members accounted for 13–21% of infections [125].

2.5.2 Classification, Morphology and Virulence Factor

Coronavirus belongs to the subfamily *Orthocoronavirinae*, family *Coronaviridae*, order *Nidovirales*. Coronavirus are categorized into four genera based on phylogenetic clustering. The genera include alphacoronavirus, betacoronavirus, deltacoronavirus, and gammacoronavirus. Human coronaviruses are either alphaCoV or betaCoV [111, 112]. Coronavirus has a helically symmetrical nucleocapsid and an envelope with club-shaped spike glycoprotein emanating from the viral envelope [111, 127–129]. It contains a non-segmented positive-sense, single-stranded RNA

genome of 30 kb with a 5′ cap and 3′ poly (A) tail. The first two-thirds of the genome, constituting the replicase gene consisting of open reading frame 1a and ORF1b, is responsible for coding nonstructural proteins (NSP) [113]. Nonstructural proteins of HCoV interfere with antiviral innate immunity [111] as well as play a role in RNA synthesis and processing [130]. It is important to note that CoV undergoes recombination using both homologous and nonhomologous recombination [131]. The final third of the genome encodes structural and accessory proteins [113]. Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N) protein are structural proteins, which play a role in virion assembly and infection [114]. The Spike protein of HCoV is involved in receptor binding via the action of receptor binding domain (RBD) in S1 subunit of Spike protein binding to host receptor and viral fusion process via the S2 subunit of Spike protein [132]. Membrane and Envelope protein play a role in the formation of the viral envelope. E protein promotes release of the virus [113, 133]. Nucleocapsid protein is involved in viral pathogenesis and it inhibits production of type I interferon (IFN) [134, 135]. The accessory proteins play a role in viral pathogenesis [113]. There is more genome sequence alignment of homology in the NSP coding region than in the structural protein coding region, which translates into the NSP region being more conserved compared to the structural protein. This may explain the adaptability of the virus to a new host, this adaptability could be attributed to the diverse nature of the structure protein region [114]. MERS-CoV is a zoonotic virus, and serological studies determined the source of the virus was dromedary camels. This animal is the most likely reservoir for MERS-CoV [126]. Phylogenetic studies of 182 full-length genomes from humans and dromedary camels revealed greater than 99% sequence identity between these two species, suggesting low rates of genetic mutation and the potential for MERS-CoV to infect many mammalian species [122]. HCoV can survive on surfaces for days and remain viable in aerosols for hours [136]. HCoV lose their infectivity when exposed to ultraviolet radiation and high temperature, whereas lipid solvents inactivate the virus [110].

2.5.3 Clinical Manifestation

AlphaCoV and betaCoV usually infect mammals whereas gammaCoV and deltaCoV have been known to infect birds and fish. HCoV-229E and HCoV-NL63 are alphaCoV whereas SARS-CoV, MERS-CoV, and HCoV-HKU1 are betaCoV [114]. The clinical manifestations of Human CoV include mild upper respiratory tract infection, fever, nonproductive dry cough, conjunctivitis, and croup in 80% of individuals and severe forms of respiratory illness with dyspnea in less than 20% of infected individuals. Severe forms of HCoV infection is characterized by blood oxygen saturation of $\leq$93% and/or lung infiltrates. The critical form of the disease is characterized by respiratory failure, septic shock, and/or multiple organ dysfunction [137]. HCoV-OC43, HCoV-NL63, HCoV-229E, and HCoV-HKU1 are Human CoV that cause mild, self-limiting upper respiratory tract infection in immunocompetent individuals as well as lower respiratory tract infection in infants, elderly

individuals, and immunocompromised individuals. SARS-CoV, SARS-CoV2, and MERS-CoV are betaCoV that cause severe forms of respiratory tract infection and extra-respiratory manifestations with varying rates of mortality [110, 114]. Individuals infected with SARS-CoV initially present with fever, a non-productive cough, sore throat, and myalgia, with dyspnea becoming a prominent feature within 7–14 days of the illness. The second phase of the illness results in dyspnea and hypoxia with continued fever often accompanied by diarrhea. In some cases, they develop acute respiratory distress syndrome often requiring mechanical respiration by the third week. Death from SARS-CoV may occur anywhere from 4–108 days post infection depending on patient age, immune status, and underlying conditions [138]. It is important to note that patients infected with MERS-CoV can be asymptomatic or experience acute illness such as coughing, diarrhea, and vomiting with most hospitalized patients commonly present with pneumonia, acute respiratory disease syndrome (ARDS), and multiorgan failure [126]. Individuals that are more susceptible to the development of severe forms of the infection associated with SARS-CoV, SARS-CoV2, and MERS-CoV have comorbidities that include obesity, diabetes, and lung disease with risk factors associated with male sex and older age. Due to these complications, 50–89% of MERS patients require intensive care wherein 75% of these patients present with at least one comorbidity. Additionally, patients with comorbidities account for 86% of MERS-associated fatalities [122] where the overall fatality rate is approximately 36% [125]. Reinfection occurs in the presence of antibodies to the virus [139].

2.5.4 Interaction with the Immune System and Pathophysiology

The host cellular receptors for HCoV-OC43 and HCoV-HKU1 is 9-O-Acetylated sialic acid. ACE2 receptor is the host cellular receptor for HCoV-NL63 and SARS-CoV. Human aminopeptidase N (CD13) is a host receptor for HCoV-229E, whereas DPP4 is the host cell receptor for MERS-CoV [111]. Host cell furin-like protease cleave the spike protein into two subunits noted as S1 and S2 subunits [113, 127–129]. Either cathepsin or TMPRRS2 mediate the acid-dependent proteolytic cleavage of S protein that facilitate entry of the virus into the host cytosol. Cleavage of the S protein yields a receptor binding domain (RBD) and fusion domain of the S protein [113, 140]. The interaction between the S1 subunit of the spike glycoprotein with the host cell receptor promotes attachment of the virus, which in turn, triggers a conformational change in the Spike protein. This is followed by a second cleavage at S2 protein that exposes the fusion peptide [113, 140]. The fusion peptide inserts into the host cell membrane, which facilitates the mixing of membrane lipids of the virus envelope and host cell membrane to mediate fusion between viral membrane and host cell membrane [111, 113]. This conformational change also triggers the formation of a fusion pore that promotes entry of the virus into the host cell [141]. The fusion allows for the virus to utilize receptor-mediated endocytosis to gain access into the cytoplasm of the host cell via an endosomal pathway [111]. Fusion between viral membrane and cell membrane as well as endosomal pathway of entry is made possible by the acidic pH of the cellular microenvironment and

pH-dependent endosomal cysteine protease cathepsin [111, 142]. The non-endosomal pathway of entry into the host cell is used by some CoVs. Transmembrane protease serine 2 (TMPRSS2) and TTPRSS11D are host cell proteases that activate and cleave Spike protein into S1 and S2 subunits to facilitate the non-endosomal pathway of viral entry at the host cell membrane during HCoV-229E and SARS-CoV infection [111, 142]. Additionally, proteases such as trypsin and thermolysin facilitates the adsorption of SARS-CoV onto the cell surface to mediate the non-endosomal direct entry from the site of viral attachment. It was demonstrated that protease-mediated entry of SARS-CoV from the cell surface was more efficient than the endosomal pathway of virus entry [143]. Interferon inducible transmembrane protein (IFTM) is a host restriction factor that blocks entry of enveloped viruses by inhibiting the fusion between the viral envelope and the plasma membrane of the host cell. These host cell restriction factors are induced by type I and II interferon [111, 141]. IFITM3 accumulation at the site of fusion of viral membrane and host membrane prevents the formation of a fusion pore, thereby trapping the virus in the hemifusion stage [141, 144]. The fusion is followed by the release of viral genome into the host cytoplasm [113].

Following entry into the host cell, the uncoating process in the cytoplasm involves removing the viral capsid to release the viral genomic RNA into the cytoplasm [111, 145]. The positive-sense viral genomic RNA is used directly as a messenger RNA template for translation of polyprotein 1a/1b (pp1a and pp1b) [111]. CoV have six open-reading frames (ORF), of which, the first two-thirds of the CoV genome consists of two overlapping open reading frames ORF1a and ORF1b that undergo translation to yield polyprotein1a and polyprotein1b [111]. ORF1b undergoes ribosomal frameshift to yield polyprotein1b [130]. The subsequent cleavage of these polypeptides by viral encoded protease yield 16 non-structural proteins that assemble to form the replication-transcription complex (RTC) [146]. The RTC then localizes to modified intracellular membranes derived from the rough endoplasmic reticulum [147]. The RTC drives replication of viral genomic RNA and synthesis of subgenomic mRNA. RTC consists of RNA-dependent RNA polymerase and other nonstructural proteins, which are involved in the synthesis of viral RNA [146]. Papain-like protease, serine type protease, and main protease are proteases that cleave the replicase polyproteins. Many non-structural proteins make up the replicase-transcriptase complex (RTC). NSP1 promotes the degradation of cellular mRNA [148]. It also inhibits IFN signaling [114]. NSP3 is a large multi-domain transmembrane protein that promotes cytokine expression and blocks host innate immune response [113, 114, 149]. NSP5 encodes main protease (Mpro), which plays a role in cleaving viral polyprotein [113, 150]. NSP7, NSP8, and NSP12 assemble to form the NSP7/NSP8/NSP12 tripartite complex known as the RNA-dependent RNA polymerase [151]. NSP9 is an RNA binding protein [152], whereas NSP12 is the catalytic subunit of RNA-dependent RNA polymerase [113, 153, 154]. NSP13 is a multifunctional protein that possess RNA helicase and 5′ triphosphatase activity [113, 151, 155]. NSP14 is a bifunctional protein that has proofreading and 5'-RNA capping activity [156]. The association between NSP7/NSP8/NSP12 polymerase complex and NSP14 is required during replication of the viral

genomic RNA. Exoribonuclease subunit of NSP14 mediates proofreading activity, which is required during replication to maintain the genomic RNA integrity from damage or mutation [157]. N7-guanine methyltransferase is required for viral RNA capping and methylation [156, 158]. NSP15 evades the innate sensor of dsRNA [114]. NSP10/16 complex plays a role in the viral RNA methylation [159]. The final third of the viral genomic RNA at the 3′ consists of ORFs that encode structural and accessory proteins. Furthermore, RTC is required for transcribing the full length of positive-strand genomic RNA into a full length negative-strand RNA template, which are required for generating new genomic RNA and negative-strand subgenomic RNAs. It is important to note that discontinuous transcription yields negative strand subgenomic RNA. These subgenomic RNAs are transcribed into subgenomic mRNAs, which are translated into structural and accessory proteins [111, 114, 145, 146, 160, 161]. Additionally, negative sense subgenomic serves as a template for synthesizing positive sense subgenomic RNA [162]. The four main structural proteins, which include Spike, Envelope, Membrane, and Nucleocapsid proteins, protein along with accessory proteins are translated from subgenomic RNA [111, 163, 164]. S protein is a viral attachment protein that facilitates attachment of the virus to a host cell receptor [114]. M protein has three transmembrane domains. It gives the virus its shape and binds to the nucleocapsid. M protein plays a role in the assembly of the virion [113, 165] and promoting curvature of the viral membrane [114]. The E protein is required for viral pathogenesis and plays a role in the assembly and release of the virus [113, 114, 133]. Nucleocapsid protein is a multifunctional protein that enhances the efficiency of viral transcription and assembly. Nucleocapsid protein also plays a role in viral envelope formation and budding process. It interacts with other structural proteins and host membrane derived from the site of budding to facilitate assembly of the virus [166]. Nucleocapsid protein binds to non-structural protein 3 (NSP3) and M protein. It is present in the viral nucleocapsid [113]. The assembly process of the virion involves the encapsulation of the viral genomic RNA in nucleocapsid protein and the subsequent interaction of Nucleocapsid protein with Spike, Envelope, and Membrane proteins. This assembly process occurs in the endoplasmic reticulum-Golgi-intermediate compartment [147]. Following virion assembly, it is transported in cell surface vesicles and leaves the infected host cell via budding of the virus through the membrane. Interaction between M protein and E protein induces the budding and egress of the virion in vesicles. The vesicles containing the virion are released from the cell surface by exocytosis [111, 113] (Fig. 3).

Host-HCoV interaction involves strategies to mount an effective defense against the virus and modalities utilized by the virus to evade the host immune response. The innate immune system uses pattern recognition receptors to detect pathogen-associated molecular patterns expressed by the HCoV. Toll-like receptors and retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) are pattern recognition receptors that engage HCoV PAMPs. Viral PAMPs such as dsRNA are recognized by TLR3 whereas ssRNA is recognized by both TLR7 and TLR8. Interaction between viral PAMPS and TLR induce the recruitment of MyD88 and TRIF, which in turn, stimulate the MAPK and NFκB pathway to facilitate the production of IFN

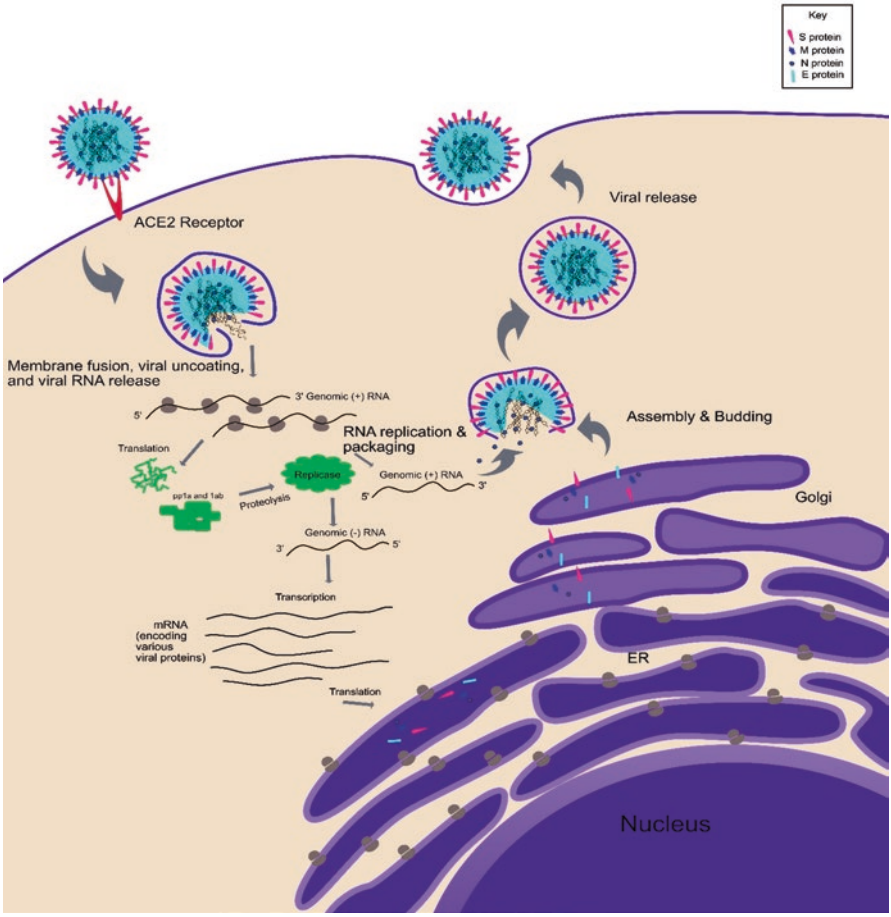

Fig. 3 SARS-CoV Lifecycle. The SARS-CoV virus enters host cells through the endosomal pathway mediated by the angiotensin concentering enzyme 2 (ACE2) receptor. The S proteins bind to the receptor and following membrane fusion into the host cell, the viral RNA is released into the cytoplasm. The positive sense (+) RNA is then translated to produce pp1a and p1ab polyproteins that are then proteolytically cleaved to yield an RNA replicase-transcriptase complex. This complex then drives the production of (−) RNA to be used as a template for (+) genome and for the generation of subgenomic mRNA encoding all the structural proteins necessary for the virus to assemble through discontinuous transcription. Viral nucleocapsids and N protein are assembled in the cytoplasm, followed by assembly, and budding into the lumen of the ER-Golgi intermediate compartment. The virion is then released by exocytosis

and pro-inflammatory cytokines [111, 167]. RIG-I and melanoma differentiation associated factor 5 (MDA5) are members of RLR, which interact with viral RNA to induce conformational changes, resulting in the recruitment and the activation of mitochondrial antiviral signaling adaptor (MAVS). The activated MAVS phosphorylates interferon regulatory factor 1 (IRF1) and IRF3 to induce the expression of type I interferons as well as phosphorylate NFκB to generate pro-inflammatory

cytokines [111, 168]. IFN-α/β induce the maturation of conventional dendritic cells and NK cells as well as induce an antiviral microenvironment at the site of HCoV infection [111, 169].

Nonstructural protein (NSP) 1 of both MERS-CoV and SARS-CoV facilitate the cleavage of host messenger RNA [111, 170, 171]. NSP3 is a large viral multifunctional protein that suppresses the antiviral activity of IFN. This has been demonstrated in HCoV-229E and SARS-CoV, via inhibition of antiviral interferon activity mediated by ADP-ribose 1′ monophosphatase activity [172]. These are viral escape mechanisms that allow the virus to downregulate the expression of antiviral innate immune factors [171]. Unlike HCoV-HKU1, the Membrane protein of SARS-CoV mediates the suppression of antiviral innate immune response via type I IFN antagonism [173]. Membrane protein suppression of type I IFN is not conserved among all strains of HCoV [111]. Additionally, the nucleocapsid protein of SARS-CoV plays a role in suppressing innate immunity by blocking IRF3 function (IFR3 activates the expression of interferon genes) [134] and block the expression of IFN-β [174].

$CD4^+$T cells and $CD8^+$T cells provide cellular immunity that plays a role in inhibiting viral replication and eliminating virus infected cells [116]. $CD8^+$T cells play a pivotal role in viral clearance and cytotoxicity [132]. IFN-γ released by both Th1 cells and NK cells inhibit viral replication, whereas granzyme B released by both $CD8^+$ T cells and NK cells destroy HCoV-infected epithelial cells [116]. The Spike glycoprotein is a major antigen for both humoral and cellular immune response against HCoV [175], since it induces the generation of SARS-CoV specific neutralizing antibodies within 2 weeks of the onset of infection. The high titer of antiviral neutralizing antibodies is retained for up to 6 months [132, 176]. Virus shedding from the respiratory tract peaks around day 10, and the presence of neutralizing antibodies is associated with a decline in viral load [138]. The SARS-CoV specific antibody response does not last for a long time, and as such, the humoral immune response mediated by IgM, IgA, and IgG is short lived. $CD4^+$ T cells and $CD8^+$ T cells recognize immunogenic epitopes present in Spike and Nucleocapsid protein of SARS-CoV. In the acute phase of SARS-CoV infection, there is a reduced number of virus-specific T cells, likely due to impaired function of dendritic cells with its associated reduced priming of T cells [116].

Resolution of the acute phase of cell-mediated immune response is followed by generation and maintenance of a pool of virus-specific memory T cells. Virus-specific memory $CD4^+$ T cells and $CD8^+$ T cells are present in SARS-CoV patients who recover from acute infections [116]. Yang et al. [177] demonstrated the presence of long-lived effector/central memory T cells in individuals who recovered from SARS-CoV infection. The study found that most memory $CD4^+$ T cells consist of SARS-CoV Spike protein specific central memory $CD4^+$ T cells that secrete IFN-γ. The study also showed that a majority of memory $CD8^+$ T cells were SARS-CoV Spike protein specific effector memory $CD8^+$ T cells. This shows that $CD4^+$ T cells and $CD8^+$ T cells mediate cellular immunity against the Spike protein of SARS-CoV [177]. Cellular immunity mediated by T cells lasts for a long time [116].

HCoV infects and induces the apoptosis of epithelial cells, macrophages, monocytes, lymphocytes, and dendritic cells [111, 178, 179]. APN is a type II transmembrane peptidase expressed on the cell surface of epithelial cells of intestines and

respiratory tract. HCoV-229e infects APN positive expressing cells such as epithelial cells, granulocytes, fibroblasts, endothelial cells, and cerebral pericytes at the blood-brain barrier [180, 181]. HCoV-229E can infect and destroy monocyte-derived dendritic cells; however, the migration of infected dendritic cells into the draining lymph nodes could play a role in the spread of the virus [111, 182]. As such, the destruction of infected dendritic cells could serve as a host immune strategy to prevent viral spread [182]. TNF-α and IL-1 induce the maturation of dendritic cells, a key regulator of the immune response against viral infection [183]. Mesel-Lemoine et al. demonstrated that HCoV-229E infects and destroys monocyte-derived dendritic cells but had no cytopathic effect on monocytes [182]. Thus, monocyte-derived dendritic cells support the efficient replication of HCoV-229E, but the exposure of these cells to IFN-β prior to infection with HCoV-229E prevented permissiveness of these cells to HCoV-229E. These researchers demonstrated that monocyte-derived dendritic cells and CD34-positive stem cell-derived dendritic cells are susceptible to infection and destruction by HC0V-229E. Thus, HC0V-229E infected conventional dendritic cells are susceptible to caspase-3-activation induced apoptosis [182]. HCoV-OC43 and HCoV-HKU1 infect 9-O-Acetylated sialic acid expressing epithelial cells of the trachea and intestines as well as mucoepithelial tissue of the lungs [184]. SARS-CoV infects ACE2 positive expressing cells such as alveoli epithelial cells, bronchial epithelial cells, bronchial serous gland epithelial cells, monocytes/macrophages, gastric parietal epithelial cells, small intestine epithelial cells, myocardial cells, distal convoluted renal tubules, adrenal cortical cells, pancreatic islet cells, sweat gland epithelial cells, and acidophilic cells of the pituitary [178]. The acute injury to the lungs observed in individuals with SARS-CoV infection is attributed to infiltration of macrophages and desquamated epithelial cells along with destruction of epithelial cells and pneumocytes of the lung in the setting of upregulation of expression of pro-inflammatory cytokines in patients with SARS-CoV infection [111, 178]. Ziegler et al. [185] demonstrated that epithelial cells of the lung are permissive to infection and replication of SARS-CoV; however, SARS-CoV infected monocyte-derived dendritic cells failed to upregulate the expression of MHC class II molecules and/or CD86. Thus, the suboptimal or non-efficient infection and subsequent replication of monocyte-derived dendritic cells and macrophages was observed. This could explain the suboptimal antiviral immune response to SARS-CoV. Additionally, the little/no expression of IFN-β, a major antiviral innate cytokine, by SARS-CoV infected macrophages may explain the poor immune response to SARS-CoV [185, 186]. ACE2 receptor is not expressed on dendritic cells; however, Spiegal et al. [183] demonstrated low level replication of SARS-CoV in both immature and mature dendritic cells. The uptake of SARS-CoV by dendritic cells is mediated by CD209 (DC-SIGN) [187] and CD209L (L-SIGN) [188]. These C-type lectin receptors likely serve as alternative receptors for entry of SARS-CoV into dendritic cells [183].

SARS-CoV caused an epidemic in 2002–2003, and it had an inefficient transmissibility. This could explain the reason for the control of the infection with quarantine. SARS-CoV infects epithelial cells, macrophages, and dendritic cells, and this

results in production of pro-inflammatory cytokines by these infected cells. It is important to note that these pro-inflammatory cytokines contribute to the immunopathological mechanism of the disease. Additionally, suboptimal virus-specific T cell response has been shown to contribute to immunopathological changes in individuals with SARS-CoV infection [113, 189].

There is an increase in expression of cytokines such as IL-1β, TNF-α, TGF-β1, IL-6, and MCP-1 in patients with SARS-CoV infection [178]. TNF-α and TGF-β1 can induce the apoptosis of epithelial cells, pneumocytes, and lymphocytes. TGF-β1 induces Fas-mediated apoptosis of infected epithelial cells (alveoli epithelial cells), lymphocytes, and platelets with lymphopenia and thrombocytopenia resulting from destruction of lymphocytes and platelets respectively [111, 190, 191]. It is important to note that TGF-β1 induces Fas-mediated apoptosis of infected epithelial cells of the lung via caspase-3-activation [191]. TNF-α induces fibrosis of pulmonary tissue since it activates pulmonary fibroblasts to undergo proliferation to produce and dump excessive amounts of collagen and extracellular matrix in the affected lungs [111].

2.5.5 Treatment and Prevention

There is no FDA-approved antiviral vaccine for human CoV. Treatment of human CoV is mainly supportive [113]. Recently, the FDA authorized the compassionate use of remdesivir for treating individuals with severe forms of SARS-CoV2 infection. This drug has been shown to inhibit RNA-dependent RNA polymerase (RdRp) [153, 192, 193]. Additionally, in patients with severe respiratory illness, the use of oxygen therapy is of great benefit. Individuals with septic shock respond to hemodynamic support, whereas mechanical ventilation is the treatment of choice for patients with respiratory failure recalcitrant to oxygen therapy. Protective mechanical ventilation with rapid sequence intubation is indicated for individuals with severe forms of SARS-CoV induced respiratory failure. High flow nasal oxygen is beneficial for individuals with mild-to-moderate SARS-CoV induced respiratory distress [110]. In the absence of antiviral therapy or vaccine, preventive strategies to contain the infection via quarantine and practice of good hygiene should be encouraged [110, 113]. The following recommendations have been issued by the WHO: avoid close contact with individuals with acute respiratory infections; wash your hands following contact with infected people or a contaminated environment infected individuals with symptoms of acute respiratory infection should cover cough and/or sneeze with disposable tissue and wash their hands; maintain strict hygiene measures for prevention and control of infection in hospital department; maintain social distancing to cut down the spread of the infection; high risk individuals should avoid public gatherings; and healthcare workers caring for infected individuals should wear personal protective equipment (PPE) to prevent viral transmission [110, 114].

2.5.6 Future Direction: Clinical Trials and Current Research

Our understanding of coronaviruses and its pathogenesis are constantly evolving. Trying new strategies for treatment based on this evolving knowledge will provide opportunities to target viral replication or immunopathology. Though treatments are being developed, prevention of viral transmission is key to reducing the burden of the virus. There is ongoing research aimed at developing antivirals that target specific enzymes of human CoV such as viral protease, polymerase, and entry protein [114]. Furthermore, plasma and antibodies obtained from convalescent patients have been shown to be therapeutically beneficial [194]. Because IL-6 is a pro-inflammatory cytokine implicated in the pathology of cytokine release syndrome (CRS), the therapeutic benefit of humanized IgG1 monoclonal antibody directed against IL-6 receptor (tocilizumab) has been investigated [110, 195]. Furthermore, human monoclonal antibodies that bind to SARS-CoV 2 RBD have been shown to block interaction between human ACE2 receptor and SARS-CoV RBD. As such, it should be explored as a possible prophylactic and therapeutic agent against SARS-CoV [196]. It has been suggested that IFNβ1 is a safe and effective antiviral therapeutic agent for treating the early stages of COVID-19 [197]. Additionally, combinations of IFNβ1b and liponavir/ritonavir have been suggested for therapeutic use in individuals with MERS-CoV [198]. Adoptive transfer of SARS-CoV-specific Th1 cells and CTL cells could clear the virus and increase survival rates. This therapeutic option could be beneficial for patients with life-threatening SARS-CoV infections [116, 199], particularly in elderly individuals with reduced levels of virus-specific T cell mediated immunity [116].

Vaccine options against HCoV include recombinant attenuated virus, live virus vector, or individual viral protein expressed from DNA plasmids [113, 115]. Spike protein-based vaccines include full-length Spike protein-based vaccines that induce effective neutralizing antibodies and T cell immunity in animal models [175, 200]. Yiu Wing Kan et al. demonstrated that recombinant trimeric Spike protein induced protective immunity and production of neutralizing antibody [201]. SARS-CoV Spike protein plays a role in viral attachment and entry, thus it is an important target for the development of vaccines or antiviral therapeutics [132]. Furthermore, SARS-CoV neutralizing antibodies could be utilized for prevention and therapeutic applications. CoV is mainly a mucosal infection, and as such, it is difficult to develop an effective vaccine against the virus because natural infection of mucosal tissue does not prevent reinfection. Another challenge to developing a vaccine is the tendency of these viruses to undergo recombination to yield new viral strains. In the absence of approved effective therapeutic or vaccine, rapid diagnostic testing and quarantine remains the most effective control measure [113].

Governments and communities taking early action to prevent emerging viruses from turning into pandemics, increasing measures limiting transmission in hospitals as well as having the necessary equipment to combat the virus and protecting vulnerable populations are all key reducing the human and economic burden a virus" with "populations are all key in reducing the human and economic burden a virus generates. There is progress in developing vaccines and therapeutics, but further research and rigorous testing is necessary to fight novel viruses.

3 Flaviviruses

Dengue virus (DENV), Yellow Fever virus, West Nile virus (WNV), St Louis Encephalitis virus (SLE), and Zika Virus (ZIKV) are arboviruses that belong to the flaviviridae family [202]. ZIKV associated infection was declared a public health emergency by the WHO [203]. Ticks and mosquitos are vectors for these viruses and the infections are cause significant morbidity and mortality across the world due to some of the devastating manifestations of these viral infections such as hemorrhagic fever and encephalitis. If not caught early, these diseases progress very rapidly and can have long-term sequelae or even death, such as congenital birth defects if a woman is infected with ZIKV during pregnancy. Immunoprophylaxis exists for Yellow Fever Virus but the other members of the flaviviridae family require control of environmental factors that promote their transmission [202]. Here we discuss DENV, as a prototype to explain some of the immunology and pathophysiology behind the diseases caused by flaviviruses. One of the important players are Nonstructural proteins (NS) encoded by the different flavivirus genomes; they interact with different members of the immune system to down regulate it or evade it, leading to spread of infection [203]. Dengue virus is an enveloped positive sense single-stranded RNA virus that rely on *Aedes aegyptus* and *Aedes albopictus* mosquitoes as vectors for facilitating arboviral infection characterized by dengue hemorrhage fever and dengue shock syndrome in susceptible individuals in endemic areas [202]. Because of the predominance of Aedes mosquitoes south of the US border, the CDC declared DENV infection a serious US public health concern [2]. Monocytes and dendritic cells are primary targets of DENV wherein interaction between DC-SIGN and DENV attachment protein results in clathrin-mediated endocytosis of the virion [204]. DENV-associated immune evasion strategies include inhibition of IFN-γ production. [205]. There is no FDA-approved antiviral agents for DENV. However, vector control is the main preventive strategy utilized in reducing DENV transmission [206]. Treatment is mostly supportive including restoring normal vascular permeability and maintaining hemodynamic stability. Repurposing or adaptation of current antimicrobial therapies for DENV such as minocycline, a commonly used antibacterial, has been found to have antiviral capability against DENV infection [207]. Current research into immunoprophylaxis focuses on developing vaccines that are immunoprotective against all serotypes of DENV [208, 209]. ZIKV is an enveloped virus with a linear positive sense, single-stranded RNA genome and icosahedral viral capsid. It acquired global health significance when it caused significant outbreaks in Yap Island, French Polynesia, and South America [210]. DC-SIGN expressed on macrophages, Langerhans' cells, and dendritic cells is a primary target for ZIKV. TIM (T-cell immunoglobulin and mucin domain) and TAM (Tyro3, AXL, and Mer) family of receptors are also attachment molecules for ZIKV [211]. AXL and Tyro3 facilitate ZIKV infection of astrocytes, epidermal keratinocytes, skin fibroblasts, endothelial cells, trophoblast cells, fibroblasts, amniotic epithelial cells, trophoblast progenitor cells, macrophage, and microglia cells [212, 213]. The clinical manifestation of ZIKV infection include transient low-grade fever, pruritic

maculopapular rash, arthralgia, nonpurulent conjunctivitis, myalgia, lymphadenopathy, hematospermia, and subcutaneous bleeding [210, 214, 215]. Guillain-Barre syndrome (GBS) and microcephaly are neurological complications associated with outbreaks of ZIKV infection [210]. There are currently no FDA-approved therapeutic agents for treating ZIKV infection [214, 215]. The preventive measures include minimizing risk of both vector and non-vector transmission [214]. Current research into ZIKV vaccines is focused on developing nonreplicative vaccine strategies using prM/E as vaccine antigen [216].

3.1 Dengue Virus

3.1.1 Epidemiology and Disease Burden

Dengue virus (DENV) is an arthropod-transmitted spherical virus endemic in over 100 countries that originated in monkeys and was only found in Africa and Southeast Asia until the middle of the twentieth century [217, 218]. DENV's vectors are the *Aedes aegypti* and *Aedes albopictus* mosquitoes, and both species acquire the virus by biting infected humans and transmiting it to uninfected humans [219]. The virus has four serotypes, with *DENV-2* causing the most severe manifestations of the disease: dengue hemorrhagic fever (DHF) and shock syndrome (DSS) [202]. Roughly 2.5 billion people live in areas considered at-risk for DENV, with the WHO estimating an incidence of 50–100 million infections and a death rate of 20,000 per year [218]. *A. aegypti* is the primary vector in Puerto Rico, the U.S. Virgin Islands, Samoa, and Guam, where DENV is endemic, so the virus could be transmitted when an infected individual travel to the continental U.S. [218]. The WHO tracks outbreaks of the disease based on reported surveillance data with an DENV outbreak in Uruguay on March 10, 2016 [220]. The disease causes high burden because hospitalization is necessary even if only mild symptoms are present since these could be warning signs for the development of DHF or DSS [221]. There is currently no DENV vaccine available for clinical use, though several are in clinical trials, with Sanofi Pasteur's tetravalent vaccine viewed as the most promising [202].

3.1.2 Characteristics, Morphology, and Disease Factors

DENV is a single-stranded positive-sense RNA spherical virus classified as a flavivirus (family Flaviviridae, genus *Flavivirus*). It is also classified under the family of arboviruses due to the nature of its vectors. ZIKV is part of the same family, and interactions between the two are possible [222]. Dengue is transmitted between hosts through blood via the bite of an infected female *A. aegypus* or *A. albopictus*. They are very active during the day, and *A. aegyptus* is often found in domesticated environments due to household storage of water [223]. Each of the four DENV serotypes can be distinguished from one another based on cell surface proteins

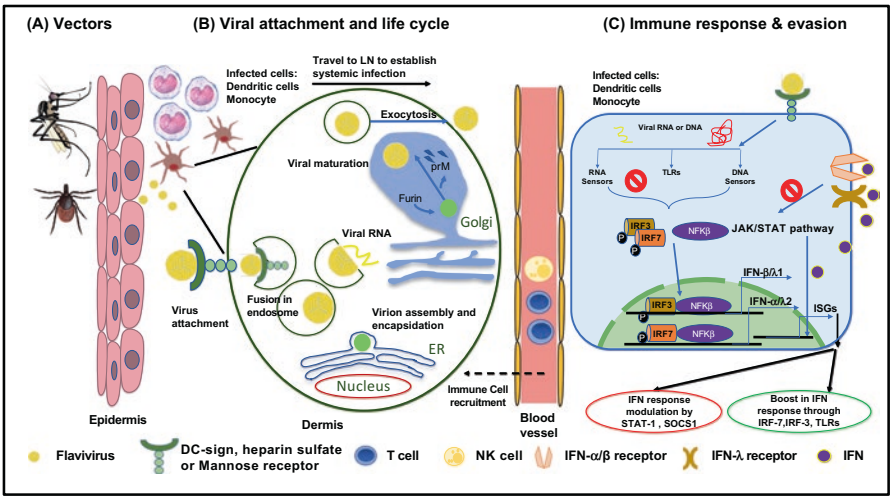

Fig. 4 Flavivirus; entry, life cycle, and immune system interaction: (**A**) Flavivirus infections are generally transmitted through the bite of an infected mosquito or tick. (**B**) Flaviviruses usually infect host monocytes and dendritic cells. Viral replication and assembly of the immature virion occurs in the endoplasmic reticulum (ER) followed by generation of mature virions in the trans-Golgi network which is then released by exocytosis. (**C**) Following viral infection, release of type I IFNs leads to expression of interferon stimulated genes (ISGs) by the JAK/STAT pathway. Release of pro-inflammatory cytokines leads to recruitment of immune cells like T cells and NK cells to site of infection. Red 'No entry' signs reflect stages and pathways of IFN and ISG production that are inhibited by flaviviruses to evade anti-viral immune responses

[220]. The three structural proteins encoded by DENV's genome are the Capsid (C) protein, envelope (E) protein, and PrM/M or (pre) membrane protein [204]. There are also seven nonstructural NS proteins that are encoded by the genome and have various functions in DENV's life cycle and pathogenesis [224]. Of the four serotypes, DENV-2 and DENV-3 have NS5 proteins that allow them to enter the nucleus, while DENV-1 and DENV-4 copies of NS5 do not have this function due to a defect in the Nuclear Localization Signal (NLS) [224].

The primary targets for DENV are immune cells i.e. monocytes and dendritic cells, though new research suggests that endothelial cells are targeted as well [204, 220]. When dengue virions reach a target cell, they bind via attachment factors e.g. heparan sulfate, DC-SIGN (a C-type lectin), and a mannose receptor. Once a virion attaches to its potential host, it is endocytosed through clathrin-coated endocytic vesicles. These join with the host cell in the late endosome, where DENV's E protein facilitates fusion of the viral and host membranes [204]. Once the viral genome begins transcription and translation, new virions mature in the *trans*-Golgi, via the action of furin protease before being exocytosed [204] (Fig. 4).

Infection with one DENV serotype doesn't confer immunity to another serotype – the patient has protective immunity only against the serotype with which they are infected. Actually, if a patient was exposed to any of the other three serotypes,

their symptoms could be worse than after the original infection, i.e. they could develop DSS or DHF [222]. A truly effective DENV vaccine would have to provide immunity against all four serotypes.

3.1.3 Clinical Manifestations

The clinical course of DENV has been described as having three phases: the febrile, critical, and recovery phases [225]. The febrile phase is clinically characterized by fever and mild hemorrhagic symptoms; on laboratory evaluation, you would see values of RBC destruction and blood loss such as thrombocytopenia, elevated liver enzymes, and hemoconcentration. This phase lasts approximately 1 week [225]. Sometimes patients may experience a mild rash or joint and muscle aches, though these are not amongst the more severe symptoms. Most DENV infections resolve spontaneously, but a minority of them progress to the critical phase. Patients will present with systemic vascular leakage due to endothelial hyper-permeability and other associated symptoms. In response to the loss of fluids, the pulse pressure decreases, though if it decreases too much the patient's peripheral vessels may collapse, leading to DSS [225]. While a patient is in the critical phase, the healthcare team must be vigilant for signs of deterioration: vomiting, serosal effusion, hemorrhagic manifestations (e.g. mucosal bleeding), and impaired clotting activity (e.g. increased PT and PTT) are all indicators that a blood transfusion is necessary to save the patient's life [225]. While the critical phase is the most dangerous, it is also the shortest, usually lasting around 48–72 h. As the patient moves into the recovery phase, their vascular permeability returns to normal, though occasionally they may develop a rash. After this, the only symptom the patient experiences is fatigue [225].

3.1.4 Interactions with the Immune System and Pathophysiology of Disease

The major players in DENV pathogenesis are DCs. Other important immune cells involved in DENV infection are macrophages/monocytes, T cells, and different varieties of APCs [226]. Once the host has been infected by DENV, the virus inhibits the body's antiviral response through inhibition of IFN-γ production via inhibition of the STAT2 pathway, with DENV-2 being the most effective at this mechanism of immune evasion [226].

Even though no receptor has yet been found that is specific for DENV recognition, a few candidates have been studied. DC-SIGN receptors are involved in DENV pathogenesis by its interaction with DENV glycoproteins and facilitating infection of monocyte-derived DCs (moDCs); an already infected moDC cannot be infected again [226]. Another important receptor in the pathogenesis of dengue is the Fc-receptor, more specifically the Fcγ subtype. These receptors bind DENV-IgG immune complexes, and send activating or inhibiting signals to monocytes (and moDCs) via immunoreceptor Tyrosine-based activation (ITAMs) and

immunoreceptor Tyrosine-based inhibitory motifs (ITIMs) [226]. If an activating signal is sent, this makes the moDC more likely to undergo antibody-dependent enhancement, or ADE. This is notable with FcγRIIa receptors, which actually internalize DENV – though the moDC must have already matured for this increase to occur [226]. ADE is part of the reason that secondary DENV infection usually leads to more severe symptoms than primary infection [226]. In antibody-dependent enhancement of disease as seen in dengue virus infection, prior infection with one serotype of dengue virus may lead to the development of suboptimal neutralizing antibodies that do not fully neutralize other dengue virus serotypes. As such, ADE can enhance viral infection of myeloid cells via Fcγ receptor-mediated process, which leads to more severe viral disease [227]). DENV immune complexes that enter conventional DCs via FC*γR*IIA receptors rather than the DC-SIGN can lead to the generation of proinflammatory cytokines that feature heavily in DV immunopathology [6].

T cells play the largest role in the cellular immune response to DENV. In primary DENV, $CD8^+$T cells exhibit a low level of pro-inflammatory cytokines, such as IFN-γ and TNF-*α*, and a high level of CD107a, indicating the $CD8^+$T cell has already released perforin substance toward a target cell [205]. In patients with DSS and DHF, $CD8^+$T cells' function switches, producing mainly pro-inflammatory cytokines, and enhancing the immune response [205]. Memory T cells are thought to play a role in the endothelial hyperpermeability associated with severe DENV [205]; they are also important because they have higher affinity for the primary infection's serotype than the serotype(s) of a secondary infection [205].

3.1.5 Treatment and Prevention

Currently, there is no infection-specific treatment for DENV, nor is there a DENV-specific vaccine. Prevention is mainly focused on vector control, diminishing *A. aegypti* or *A. albopictus* mosquito bites that actually transmit DENV to humans. Poor living conditions, lack of preventative measures, such as emptying containers of standing water, low/no use of mosquito repellent, and lack of knowledge of DENV were found to be risk factors for Dengue contraction [206]. Taken in combination with the fact that efficacious Dengue vaccines are still in development, this suggests that the best way to fight DENV is to prevent people from contracting it in the first place, educating communities, implementing ways to control mosquito infestations, and prevent bites [228]. Prevention can also be effected through the use of sterile insect technique or SIT, where sterile male mosquitos are released into the environment and compete with wild-type males, causing the mosquito population to gradually decrease [229]. This form of vector control is a good starting point and will be supplemented as research efforts discover new ways to control spread of DENV vectors, and develop an effective vaccine against the virus.

As for treatment, clinical guidelines generally focus on keeping the patient hemodynamically stable and maintaining adequate perfusion. This can be done through blood transfusion, as well as by attempting to restore normal vascular permeability and coagulation. Current treatments only provide symptomatic relief and

control of DENV infection, but do not eliminate it completely. DENV is very effective at suppressing host antiviral responses, and finding optimal ways to target this, will be the focus of future research to find treatments.

3.1.6 Future Directions: Clinical Trials and Current Research

The main focus of DENV research is on finding effective vaccines and therapeutic agents for the virus, especially finding a tetravalent vaccine that provides immunity against all four DENV serotypes. On the basis that severe cases of DENV (DSS or DHF) present with endothelial dysfunction leading to edema and hemorrhage, much of the treatment-targeted research has focused on returning the endothelium to normal function. A double-blinded clinical trial investigated the use of statins, shown to protect the endothelium from inflammation, as a possible treatment for severe DENV; unfortunately, in the context of the clinical trial, statins do not provide a clear clinical benefit [230, 231]. It was suggested that statins might need to be introduced earlier than 72 h after diagnosis, but further research would be needed to investigate this [231].

Development of an effective vaccine is an important avenue of DENV-related research. Currently, the most promising vaccine has been a live attenuated Yellow fever (YF)-dengue chimeric vaccine (CYD-TDV) in development by Sanofi-Pasteur that incorporates genes for DENV preM and E proteins into the cDNA of a YF 17D vaccine (used to prevent yellow fever) [209]. Studies based on this trial have found that it protects against DENV 1/3/4 infection at a statistically significant level, but not against DENV-2 (which could be considered one of the more dangerous serotypes) [208, 209]. While this vaccine has progressed to phase III trials, it does not contain any of the NS proteins, which hold most of the epitopes recognized by T cells [208]. Regarding treatment and preventative research, there have been studies and case reports suggesting that a substance in papaya leaves could be an effective treatment for DENV and protective agent against *A. aegypti* mosquitos. However, it has been reported that these findings pointed out that identification of the active agent in papaya leaves, as well as a way to standardize dosage, are critically necessary before any true clinical trials can begin [232].

There has also been focus on adapting current therapies for use as anti-DENV agents. For example, minocycline, a commonly used antibacterial agent, has demonstrated antiviral effect against DENV infection [207]. Extensive *in vitro* examination of the effect of minocycline on DENV found that, in a dose-dependent manner, it reduces DENV RNA activity as well as different steps of its pathogenesis. It has been shown to down regulates ERK phosphorylation, across all four serotypes [233]. This finding is significant because ERK phosphorylation normally decreases the antiviral IFN response, and as such, inhibiting ERK phosphorylation would keep DENV from hampering the immune response. On a genetic level, minocycline was found to increase the expression of *OAS1* and *OAS3* genes, which also play a role in the IFN antiviral response.

3.2 Zika Virus

3.2.1 Epidemiology and Disease Burden

Zika virus (ZIKV) is an arthropod-borne virus that belong to the Flaviviridae family and is closely related to mosquito-borne flaviviruses of global medical importance such as DENV and West Nile virus. It was first isolated in nonhuman primates in Uganda in 1947. ZIKV acquired global health significance when it caused significant outbreaks in Yap Island, French Polynesia, and South America [210]. Serosurvey have shown residents of Nigeria, Vietnam, Uganda, Gabon, India, Pakistan, Malaysia, Philippines, Egypt, and Indonesia have varying levels of seroprevalence and antibodies against ZIKV [234, 235]. Prior to 2007, there were few cases of confirmed human infection caused by ZIKV [234]. Outbreaks of ZIKV infection in 2007, 2013, 2014, and 2015 affected thousands of residents in the Yap State (an island in the Federated State of Micronesia), French Polynesia, and South America [234, 235]. The first recorded ZIKV outbreak outside Africa and Asia occurred in Yap Island, in which infected individuals had clinical manifestations such as transient low grade fever, pruritic maculopapular rash, arthralgia, and nonpurulent conjunctivitis [210]. In 2013–2014, a major outbreak of ZIKV in French Polynesia was associated with neurological complications such as Guillain-Barre syndrome. Visitors from USA, France, Norway, and Italy who were exposed to the ZIKV whilst in the Pacific Island developed symptoms associated with ZIKV infection [210]. The ZIKV outbreak in South America in 2015 was associated with high incidence of newborn infants presenting with microcephaly. The outbreak of ZIKV with associated neurological complications prompted the WHO to declare ZIKV a disease of global health emergency. The ZIKV isolate that was responsible for the Zika infection outbreak in South America was confirmed to have a high degree of homology to ZIKV that caused the ZIKV outbreak in French Polynesia based on sequence analysis [210]. Cases of ZIKV in nonendemic areas such as Europe and North America are attributed to travel-related cases with more than 95% of ZIKV infections in the USA due to travel related ZIKV infections [214].

The two major transmission cycles of ZIKV are sylvatic cycle and urban cycle [210]. ZIKV is a mosquito borne disease that existed in Africa in a sylvatic transmission cycle involving Aedes mosquito and nonhuman primates [234]. Sylvatic cycle involves ZIKV transmission between nonhuman primates and forest dwelling mosquitoes, in which humans became the accidental host [210, 234]. The spread of ZIKV in a human-Aedes mosquito-human transmission cycle constitutes the suburban-urban transmission cycle [234]. Urban cycle involves transmission to humans in the urban setting via bite of arboreal mosquito infected following blood meal from an infected nonhuman primate. Major mode of transmission of ZIKV is via mosquito bite. Aedes aegypti and Aedes albopictus are vectors of ZIKV with Aedes aegypti considered the primary vector responsible for the ZIKV outbreaks [210]. In the USA, Aedes aegypti is endemic in the US Virgin Islands and Puerto Rico, whereas Aedes albopictus is seen in the eastern part of the USA. Both species of Aedes mosquitoes are daytime feeders on human blood and are readily found in

tropical and subtropical regions of the world [234]. Non-vector modes of transmission, in which ZIKV is transmitted from human-to-human, include an infected mother transmitting ZIKV to the fetus during pregnancy, mother to infant transmission, sexual transmission with replicative viral particles and viral RNA present in sperm, and transmission via breastfeeding particularly in the setting of high titers of Zika viral particles in breastmilk [210, 234, 235]. Although non-vector modes of transmission exist, vector-borne transmission is considered the major route of transmission [210].

The WHO declared ZIKV a public health emergency of global concern due to the temporal and geographical association between ZIKV infection and neurological complications such as Guillain-Barre syndrome (GBS) and microcephaly [215]. The increased prevalence of ZIKV and its association with larger sporadic outbreak of the disease is an additional reason for the WHO declaring it a disease of public health emergency of international concern [210]. Additionally, the presence of Aedes aegypti and Aedes albopictus in the state of Florida constitutes an increased risk for ZIKV to become endemic in the southern states of the USA. Furthermore, the high vectoral capacity of Aedes aegypti heightens the concern that it can increase the spread of ZIKV in the southern states of USA in the future due to its potential to infect many individuals during blood meal [210].

Characteristics, Morphology, and Virulence Factors

Phylogenetic analysis of ZIKV identified the presence of African lineage and Asian lineage with ZIKV outbreak in French Polynesia and South America attributed to the Asian lineage ZIKV strain [215, 236, 237]. The genetic diversity of ZIKV is attributable to the subtle differences in the envelope glycoprotein sequence of the African and Asian lineage, a difference that may affect virulence of ZIKV (121). ZIKV is an enveloped virus with a linear positive sense, single-stranded RNA genome and icosahedral viral capsid. It has three N-terminal structural proteins (viral capsid, viral membrane protein, envelope glycoprotein) and seven C-terminal nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [210]. The icosahedral capsid is surrounded by enveloped (E) glycoprotein and membrane/precursor membrane (prM) protein [215]. Structural proteins are responsible for development of infectivity of the virion [210]. RNA replication is mediated by NS3 and NS5 and processing of polyprotein is mediated by the interaction between NS3 and NS2B [215]. NS3 consist of an N-terminal serine protease domain and C-terminal RNA helicase domain [210, 238, 239]. NS5 consists of an N-terminal methyltransferase domain and C-terminal RNA-dependent RNA polymerase domain [210, 239, 240]. NS5 also inhibits interferon response [212, 215]. NS4A also aids in polyprotein processing and immune evasion [212].

Viral cellular tropism is determined by the ability of ZIKV to bind to host cell surface receptors [213]. Dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) or CD209 expressed on macrophages and dendritic cells is a primary target for ZIKV. TIM (T-cell immunoglobulin and mucin

domain) and TAM (Tyro3, AXL, and Mer) family of receptors are also attachment molecules for ZIKV [211, 241]. Tyro3, AXL, and TIM-1 are ZIKV cell surface receptors expressed on human placenta cells, endothelial cells, trophoblast cells, fibroblasts, amniotic epithelial cells, trophoblast progenitor cells, and macrophages [212, 241]. Additionally, AXL and Tyro3 facilitates ZIKV infection of astrocytes, epidermal keratinocytes, skin fibroblasts, and microglia cells [213].

ZIKV envelope glycoprotein bind to host cell surface receptors on permissive cells to initiate attachment and subsequent internalization into an endosome through clathrin-mediated endocytosis. Once inside the endosome, acidic pH induced changes to viral envelope glycoprotein enables fusion between viral membrane and endosomal membrane, and subsequent release of a viral RNA genome into the host cell cytoplasm [210, 214, 236]. Once inside the cytoplasm, viral disassembly occurs to release the RNA genome for replication, which occurs in association with replicative complexes [203, 210, 214, 236]. Because the RNA genome is a positive sense nucleic acid, the positive strand RNA acts as a mRNA that is directly translated on human ribosomes into a large polyprotein that undergoes co-translation and post-translation by cellular and viral proteases into structural and nonstructural proteins [203, 214, 242, 243]. The negative strand RNA is used for generating additional mRNA and replicating the genome [214]. Budding of viral genome RNA-C protein complex into the endoplasmic reticulum initiates assembly of immature virion. Enveloping of the virion with viral precursor membrane protein (prM) and envelope protein occurs in the endoplasmic reticulum. The immature virion undergoes post-translational modification in the trans Golgi network with production of membrane (M) protein from prM. The mature virion is released from the infected cell via exocytosis [210].

3.2.2 Clinical Manifestations

The clinical aspect of ZIKV infection encompasses acute febrile illness, neurological complications and Zika associated fetal outcomes [234]. The clinical manifestation of ZIKV infection usually develops after an incubation period of 4–10 days with up to 25% of infected individuals presenting with transient low-grade fever, pruritic maculopapular rash, arthralgia, nonpurulent conjunctivitis, retro-orbital pain, headache, myalgia, vomiting, lymphadenopathy, fatigue, lower back pain, swelling of the extremities, hematospermia (blood in the ejaculate), hearing difficulties and subcutaneous bleeding [210, 214, 215]. Viremic phase may be associated with increase in erythrocyte sedimentation rate, raised C-reactive protein levels, and thrombocytopenia in some patients [214]. ZIKV can replicate in immune privilege sites and cause persistent infection of the central nervous system (CNS) [244]. Neurological correlates of ZIKV infection include Guillain-Barre syndrome (GBS), encephalitis, microcephaly, acute myelitis, and meningoencephalitis [234]. GBS and microcephaly are the major neurological complications associated with outbreaks of ZIKV infection [210]. GBS, an autoimmune characterized by immune-mediated attack of peripheral nervous system, has been documented with other arboviral infections; however, the association of GBS with ZIKV was observed

during the ZIKV infection outbreak in French Polynesia due to the presence of neutralizing anti-ZIKV antibodies in majority of patients with GBS [210].

Temporal and geographical associations of increase in incidence of microcephaly and ZIKV infection in 2015, identification of ZIKV nucleic acid in brain of deceased infants with microcephaly, and demonstration of inhibition of growth of human neural progenitor cells are evidences to support the role of ZIKV infection in microcephaly [210, 215, 234, 245]. The greatest risk of developing microcephaly is when a pregnant woman has an acute Zika viral infection in the first trimester [234] and microcephaly is worse when infection is acquired in early stages of pregnancy [214]. ZIKV infection of microglia cells is associated with an inflammatory response with high levels of IL-1β. TNF-α, and IL-6, which in turn, plays a role in the neuropathogenesis of ZIKV infection characterized by impaired proliferation and differentiation of neural precursor cells (NPC) [246]. Up to 50% of infants with presumed or confirmed congenital ZIKV-associated microcephaly present with iris coloboma, lens subluxation, cataract, glaucoma, intraocular calcifications, microphthalmia, esotropia, focal retinal pigment epithelial mottling, retinochoriodal atrophy, and hypoplastic optic nerve head [234, 247, 248].

Diagnostic tests for ZIKV include Trioplex reverse transcriptase polymerase chain reaction (RT-PCR) and IgM class capture enzyme-linked immunosorbent assay (MAC-ELISA) [215]. RT-PCR can detect viral nucleic acid in urine, saliva, semen, CSF, amniotic fluid, and plasma [214]. Enzyme-linked immunosorbent assay or immunofluorescence assay are useful for identifying IgM or IgG antibodies in serum [214]. Inhibition ELISA method (IEM) is used in detecting and determining total ZIKV-specific antibody titer in serum. Zika NS1 blockade-of-binding (BOB) ELISA measures IgG antibody titers and it is useful for diagnostic and seroprevalence studies [249].

3.2.3 Interactions with the Immune System and Pathophysiology

Neural stem cells, oligodendrocyte precursor cells, cranial neural crest cells, neurons, astrocytes, trophoblasts, microglia, macrophages, dendritic cells, endothelial cells, skin keratinocytes, and dermal fibroblasts are ZIKV permissive cells that support ZIKV replication and propagation of ZIKV to other cells and tissues [211]. ZIKV has been shown to be capable of infecting and inducing the apoptosis of human neural progenitor cells, which are crucial for driving the development of the fetal brain cortex [210]. Epidermal keratinocytes and skin fibroblasts are highly permissive cells that support ZIKV replication, and subsequent ZIKV-induced apoptosis of infected cells and viral dissemination [213].

Aedes mosquitoes become infected with ZIKV following blood meal from an infected individual [214]. The infected Aedes mosquito transmits ZIKV to uninfected humans during blood meal, which infects fibroblasts, keratinocytes, Langerhans cells, and macrophages. It is noteworthy that Tyro3, AXL and TIM-1 are cell surface receptors for ZIKV on fibroblasts and keratinocytes whereas DC-SIGN is an entry receptor for ZIKV on Langerhans' cells [214, 250]. Pattern

recognition receptors for ZIKV include TLR3, RIG-1, and MDA5 found on fibroblasts and keratinocytes, which when engaged by ZIKV PAMP results in the activated keratinocyte and fibroblast generating type I IFN that induces an antiviral microenvironment and activates NK cells [214]. Generating an antiviral microenvironment including IFN production is required for suppressing ZIKV replication and this constitutes the major anti-ZIKV innate immune response [215]. ZIKV infected Langerhans' cells traffic to the regional lymph node to activate naïve T cells to become ZIKV-specific T cells. ZIKV spreads from infected cells that support viral replication to other tissues and organs via bloodstream and lymphatics [214]. In ZIKV infection, NS5 induces the degradation of STAT2 in a proteasome-dependent manner, which results in suppression of type I interferon production [251]. Furthermore, ZIKV evades host immune response via the blockade of type I interferon production mediated by NS1 and NS4B [252]. Thus, NS1, NS4A and NS5 proteins mediate the inhibition of the antiviral immune response by blocking the production of type I interferon [251].

In non-human primate models, detectable levels of viral RNA are observed 6–7 days post-infection with a decline in plasma viral RNA levels coinciding with the presence of rising titer of ZIKV neutralizing antibodies [215, 253]. Neutralizing antibodies primarily directed at determinants in domain 3 or the fusogenic loop of domain 2 in E protein play a major role in providing humoral protection against ZIKV infection [227]. Neutralizing antibodies are protective against ZIKV; however, the concern is antibodies against flavivirus such as DENV are highly cross-reactive [215]. Cross-reactive neutralizing antibodies against ZIKV and DENV bind to conserved epitopes on both viruses [216, 254]. The cross-reactivity between ZIKV and DENV is based on the similarity of their envelope glycoprotein sequences, which can trigger the development of antibody-dependent enhancement (ADE) of clinical disease. ADE is mediated through sub-neutralizing or non-neutralizing antibodies that enhance viral entry and infectivity by FcγR-mediated process when Fc regions of these antibodies engage FcγR expressed on myeloid cells [215, 241, 255, 256]. There is a theoretical concern that neutralizing antibodies induced during primary DENV infection may cross-react with subsequent infection by ZIKV leading to increased viral load in circulation, and associated damage to blood vessel endothelial lining and promotion of inflammation within the microenvironment [216]. This could be associated with a significant risk for individuals with ZIKV infection presenting with severe clinical manifestations [244]. Serological cross-reactivity is of great concern in diagnosing ZIKV infection. This serological cross-reactivity between ZIKV and other flaviviruses constitutes a considerable hindrance to seroprevalence studies and diagnosis of ZIKV in geographical areas with co-circulating members of the flavivirus genus [215, 227]. It is of note that prior exposure to DENV may interfere with serological diagnosis of ZIKV producing a false positive result for ZIKV infection [214, 234]. In cases wherein ZIKV yields a positive MAC-ELISA result for DENV, plaque reduction neutralization test (PRNT) is beneficial in differentiating between antibodies against ZIKV and DENV [234]. Cell-mediated immunity against ZIKV involves ZIKV-specific $CD8^{+}$T cells and $CD4^{+}$T cells (121). Th1 cells produce IFN-γ, TNF-α and IL-2. Th1 cells mediate type 1 immune

response against ZIKV. They also play an important role in helping to induce proliferation and differentiation of $CD8^{+}$T cells as well as driving IgG production. $CD8^{+}$T cells are essential for immune protection and clearance of ZIKV from the CNS [244]. T cell response to NS1 and envelope glycoprotein has been demonstrated in ZIKV infected human [216, 257].

3.2.4 Treatment and Prevention

There are currently no FDA approved therapeutic agents for treating ZIKV infection [214, 215]. Pharmacotherapy is nonspecific and geared towards alleviating symptoms associated with ZIKV infection [214]. Ribavirin has demonstrated broad spectrum antiviral activity against several RNA viruses but it has not shown antiviral activity against ZIKV [258]. The main goal of prevention is to minimize risk of both vector and non-vector transmission. Such preventive measures aimed at avoiding vector transmission include draining mosquito breeding grounds, use of insecticide and Picaridin-containing insect repellent. Travelers to endemic areas should use insect repellent while outdoors, wear long sleeve shirt and pants, and use or wear permethrin-treated clothes [214]. During a ZIKV outbreak, discontinuation of all blood donations in endemic areas is recommended and it is necessary to seek travel history for blood donors from non-endemic areas [214]. Male partners returning from ZIKV endemic regions should practice safe sex to prevent sexual transmission of ZIKV [214]. Pregnant women or women trying to conceive should avoid travel to ZIKV endemic areas as well as avoid unprotected sexual contact with partners who are at risk for ZIKV infection [234]. To minimize the risk of sexual transmission of ZIKV, men and women with history of ZIKV exposure should avoid unprotected sex for at least six months and eight weeks respectively [259]. Furthermore, pregnant women or women trying to be pregnant should adopt strict personal protective methods to limit or avoid contact with mosquitoes if resident in ZIKV endemic areas [214].

3.2.5 Current Clinical Trials and Research Studies

Han and colleagues [258] demonstrated that an FDA-approved antimalarial drugs such as Amodiaquine dihydrochloride dihydrate (AQ), chloroquine phosphate (CQ), and mycophenolic acid (MPA) were capable of inhibiting ZIKV RNA replication in Vero cell cultures infected with ZIKV. They demonstrated that amodiaquine, an antimalarial drug could be therapeutically repurposed for ZIKV infection due to its antiviral capabilities against ZIKV. Although AQ is a desirable agent due to its safety profile in pregnant women, clinical trials are required to evaluate its efficacy and safe dose range in treating ZIKV infection [258]. Mesci and colleagues [260] demonstrated that Sofosbuvir (SOF), an FDA-approved RNA-dependent RNA polymerase (RdRp) inhibitor for treating chronic hepatitis C virus (HCV), had anti-ZIKV activity in both in vitro and in vivo models of ZIKV. SOF is considered a FDA pregnancy category B antiviral agent. It is of note that HCV is a member of

the Hepacivirus genus of the Flaviviridae family. Because both HVC NS5B and ZIKV NS5 sites for SOF were structurally similar and conserved, SOF could inhibit the function of NS5 via targeting the C-terminal RNA-dependent RNA polymerase domain on NS5. ZIKV has a considerable health impact on pregnant women with the heightened risk of delivering infants with microcephaly, and as such, further studies are necessary to evaluate the therapeutic benefit of SOF in preventing congenital ZIKV microcephaly [260].

Antibody-based therapies against ZIKV in the form of monoclonal antibodies that target the envelope glycoprotein to block viral attachment to host cellular surface receptors and prevent fusion of viral membrane proteins with host cellular membrane are potential viable target-based therapeutics. However, antibody-dependent enhancement of infection with associated increase in viral entry and infectivity may occur due to the development of suboptimal neutralizing antibodies that bind to the Fcγ receptors on myeloid cells such as monocytes, dendritic cells, and macrophages. This is a major safety concern and challenge to developing antibody-targeted therapy [261]. Because T cells play a major role in anti-ZIKV adaptive immune response, ZIKV vaccine platforms should trigger both humoral and cell-mediated immune response against ZIKV [262]. Current research into ZIKV vaccines are focused on developing nonreplicative vaccine strategies due to their enhanced safety profile in pregnant women and women of childbearing age [216, 244]. Current ZIKV platforms using prM/E as a vaccine antigen are designed to provide correlates of immune protection based on generating neutralizing antibodies against nonstructural proteins [216]. Incorporating immunodominant epitopes of T cells into the nonreplicative vaccine strategy could boost T cell response to nonstructural proteins. Additionally, utilizing pre-immune cross-reactive T cells to other flaviviruses could yield a strategy of formulating a combination vaccine that contains closely related flaviviruses that could increase efficacy of the prospective vaccine [241, 244].

There are in excess of 40 vaccines against ZIKV under evaluation for their safety, immunogenicity and tolerability in phase 1 clinical trials ([215, 241, 262]. ZIKV vaccine platforms under development include DNA-Based Vaccines, Vector-Based Vaccines, mRNA-Based Vaccines, Live Attenuated Virus Vaccines, Purified Inactivated Virus Vaccines, and In Silico Approaches to Vaccine Design [262]. The limited diversity among ZIKV strains is likely to be associated with reduced severity of disease in secondary ZIKV infection, and as such, neutralizing antibodies against one strain is probably going to be protective against another ZIKV strain [244]. Challenges for handling the global threat of ZIKV infection include identifying appropriate correlates of immune protection that are necessary for developing a ZIKV vaccine with high efficacy of protecting against congenital ZIKV syndrome as well as devising a means of overcoming the cross-reactive immune response among flaviviruses [215, 216]. Because pregnant women, the primary target for developing anti-ZIKV pharmacotherapy are generally excluded from clinical trials, investigational drugs should constitute very minimal risk to pregnant women and fetus [215]. Research directed towards developing point-of-care diagnostics as well as vaccine and antivirals safe for use in pregnant women and/or nursing mother should be of the utmost priority [214].

4 Hepatitis viruses

4.1 Hepatitis B Virus

4.1.1 Epidemiology and Disease Burden

The liver is the metabolism hub of the body, which is responsible for majorly all anabolic and catabolic activities for survival. Any kind of stress or damage to the liver will significantly lower the functional efficacy of the affected organism. A couple of ways in which liver could be affected is, by inflammation and hepatocarcinogenesis [263]. Along the lines of co-evolution, viruses have adopted ways to take advantage of the liver, benefit themselves while simultaneously transforming the host organ. There are various hepatic viruses which work on similar lines such as, Woodchuck hepatitis virus, Duck hepatitis B virus, Hepatitis A-E, Ground Squirrel hepatitis virus etc. [264]. Out of these viruses, Hepatitis B (HBV) and Hepatitis C (HCV) stand out to be the most dangerous and potent species to infect humans and primates. In early 1960s, Baruch Blumberg discovered the 'Australian Antigen' which was technically the surface antigen from Hepatitis B virus, which eventually lead to an entire field of unchartered viral research [265]. Hepatitis B virus is tropic to the liver and hence cause inflammation of the liver, which can lead to liver failure, liver cirrhosis, and hepatocellular carcinoma.

Hepatitis B viruses do not differentiate between continental boundaries and thus can be found in almost all places of human habitation. For Hepatitis B Virus, western Pacific and African region make up a larger bias for prevalence by contributing 6.2% and 6.1% of the total infections respectively [266]. Number of infected people with HBV is relatively higher than most of the viruses and that is one of the reasons why these two viruses are considered to be clinically very important. According to the WHO, the number of chronically infected patients with HBV is 257 million and for that of HCV is 71 million worldwide [266, 267]. The terminology of acute and chronic infection is a major criterion to classify the type and severity of the infection. Acute infection means the body is able to clear the virus within 6 months of incidence, whereas in chronic infection, the immune system is unable to nullify the threat and the virus is persistent [268]. Some of these infections are co-infection with different viruses such as HIV, which furthers the complication [269, 270]. The number of deaths attributed to chronic infection with HBV is astonishing which is corroborated by reports of 887,000 deaths due to HBV related cirrhosis and/or hepatocellular carcinoma in 2015 alone [271]. As the numbers suggest, these viruses are already an overloading economic burden for the healthcare system and hence actual expenditure statistics are monumental. For HBV, the economic burden for lifetime carriers crosses the $9 Billion with $360 million spent on chronic infections per year [272]. All these reasons make up a powerful stimulus to study and eradicate these viruses.

4.1.2 Characteristics, Morphology, and Virulence:

HBV is a spherical enveloped virus within Hepadnaviridae family [273]. Measuring 42nm in diameter, it is one of the smallest DNA virus known, with a compact genome size of 3.2Kb. Due to its compactness, the genome of HBV has an overlapping genome which is partially double-stranded, relaxed circular in nature [274]. It consists of 4 open reading frames which translate to 4 major proteins namely: Polymerase, Core, Surface antigen, and HBx [275]. Interesting fact about Polymerase is that it also acts as a reverse transcriptase, which is unusual, as HBV is a DNA virus. Polymerase is also the largest transcript generated by HBV [276, 277]. Another protein known as the Surface antigen which is also known as (HBsAg) was previously thought as Australian antigen during the discovery of HBV by Baruch Blumberg, it is now used as identification marker of HBV infection in the patient sera [278]. Core is a structural protein which makes up the viral capsid. It also acts as a unique marker for assays like Western blot and Native gel analysis. Lastly, HBx is the smallest regulator protein translated by HBV and has been shown to be involved in replication of the virus both ex vivo and in vivo [275, 279–282]. HBV is a non-cytopathic virus, which does not kill the hepatocyte, as opposed to some lytic viruses which are cytopathic in nature [283]. There are a number of reasons for viral persistence- first being the innate immune evasion like a stealth virus, where HBV does not expose its genetic material to the host cytoplasm [284]. This mechanism used by HBV is very successful in long term persistence in the host cell. Another reason could also be the expression of HBeAg, a protein produced by core ORF and its vertical transfer from mother to offspring. As new borns are the major victim population suffering from chronic version of HBV, HBeAg is recently hailed as one of the most promising reasons for chronic infection and viral persistence [285].

4.1.3 Clinical Manifestation

Children born to HBV infected mother have the highest chance of getting chronic HBV infection due to the high rate of vertical transmission [286]. Other means of getting HBV infection is through the exchange of bodily fluids such as blood. Blood banks have a mandatory rule for testing for HBV before donating the blood to somebody in need. HBV infections are divided into two main sections: Acute infection and Chronic Infection. Acute infections are considered to be the less damaging as compared to Chronic infections because adults are able to clear the infection with the help of their immune system whereas in chronic infections, the virus always persist in the body, evading the immune response [287]. Chronic HBV can be divided into 4 categories: Immune tolerance phase, Immune clearance phase, Inactive HBsAg carrier stage, and HBeAg-negative [288]. HBsAg is a clear mark for HBV infection and its sustenance for more than 6 months after initial infection is a sign of chronic infection. During the immune tolerance phase, there is little to no liver inflammation for decades, normal aminotransferases levels (ALT), but it is

associated with high titers of HBV DNA [289]. Another phase of chronic inflammation criterion is immune clearance phase. Progression of fibrosis and liver inflammation is observed in this phase with high HBV DNA and HBeAg expression. Increased ALT levels seen in this phase are often associated with CTLs (Cytotoxic T-lymphocytes) mediated response. Seroconversion to anti-HBe is a significant response to control the viral detection in serum. Inactive HBsAg carrier phase is majorly characterized by loss of HBeAg expression and seroconversion to anti-HBs. ALT levels are found to be normal and low levels of the liver disease appear with this phase. Finally, there is a reactivation phase which is called HBeAg negative phase, which is marked by increased inflammation and HBeAg negative. A significant increase in the HBV DNA levels is detected along with high tissue necrosis [290]. Long-term chronic HBV infection also leads to HCC, but the exact mechanism of its progression is not known.

4.1.4 Interactions with the Immune System and Pathophysiology

HBV is known as Stealth virus because of its ability to be invisible to the innate immune response while its invasion of the hepatocyte [291]. Recently, it was implicated that human sodium taurocholate co-transporting peptide [hNTCP] was the HBV receptor through which it infects the human hepatocyte [292]. Clathrin-mediated endocytosis is a potential candidate to explain the entry of HBV [293]. From initial entry to nuclear localization, HBV does not expose its genetic material to the intracellular immune response, as the nucleocapsid is still intact. Nucleocapsid disintegrates at the nucleus pore, releasing relaxed circular HBV DNA with the polymerase [294]. Once inside the nucleus, it forms cccDNA with help from host factors such as histones [295]. Transcription of this cccDNA results in the formation of pgRNA, which when translated produces core and polymerase for packaging. This pgRNA is also used by Reverse transcriptase to make HBV DNA for completion of the HBV replication cycle. The action of Reverse transcriptase takes place inside the nucleocapsid. Some of the HBV DNA is recycled back into the nucleus and a majority of it is packaged out via the ER pathway. Even though there is so little to no exposure of HBV genetic material in the cytosol, there is some activation of innate immune response. Some studies show that 5' region of the pgRNA sensed by RIG-1 or MDA5 leads to the production of IFN0-λ [296]. Over the course of evolution, HBV has acquired some tactics to interfere with the host's immune responses. Some of these innate immune evasion tactics include the blocking of TLR3 and RIG-1 induced Interferon response factors, inhibition of STING pathway, and blocking nuclear translocation of STAT1 [297]. All these hindrances are towards innate responses which are important during the initial stages of infection. Talking about other responses, there are few cells which are capable of fighting off HBV infection. NK cells, T cells, and antibodies are the major players to counteract HBV infection with the latter two forming the adaptive immune arm. NK cells are

cytolytic or cytotoxic in nature and they also produce IFN-γ to promote inflammation. HBV infection reduces the production of IFN-γ [297]. Antibodies are produced towards HBsAg- surface antigens providing protective immunity. Both $CD4^+$T cells and $CD8^+$T cells are very important in clearing of HBV, but particularly $CD8^+$T-cells are the ones known for their cytolytic activity against HBV infected cells. Absence of $CD8^+$T cells is known to prolong HBV infection. Interaction and upregulation of the PD-1/PD-ligand leads to $CD8^+$T cell exhaustion, which might be the reason why during chronic infection, $CD8^+$T response is dysfunctional (Reviewed in Ref [297]).

4.1.5 Treatment and Prevention

Treatments for HBV aims to clear the viral titers and/or neutralizing the surface antigens. Once the chronic phase of HBV kicks in, complete clearance of HBV is almost impossible because of its presence inside the nuclei of the host cell, but it could be contained to a minimum level [298]. Treatments are considered necessary during liver tissue damages phases, which occur during chronic infection. There are 7 treatment options available for chronic HBV infection: 2 interferon-based therapies and 5 Nucleoside analogs (NUC). Immuno-based therapies offer a boost up to the host's immune system and the nucleoside analogs interrupt viral replication. The two interferon-based therapies include conventional interferon therapy and PEGylated interferon-alpha therapy. These therapies enhance the power of the immune system to clear HBeAg and HBsAg as these will be encountered by the immune cells, which are activated through interferon signaling pathways [299]. Side effects of these therapies are greater than that of NUCs. There are 5 Analogs namely lamivudine, adefovir, entecavir, tenofovir, and telbivudine. Analogs act at the replication level to block the formation of the new virions and hence are a very efficient way to control the viral load. Combination therapies have a risk of developing resistance to one of the drugs used. Adefovir is often used in place of lamivudine where there is resistance to the latter [299].

There are preventive measures in place for HBV. The vaccination program has been very successful in preventing HBV transmission, which leads to a gradual decrease in HBV load on the population [300]. The HBV vaccine is based on the HBsAg, which will generate anti-HBs in the recipient and hence provides protective immunity to an individual. Since 1992 WHO have recommended all countries to incorporate the HBV vaccine program into their routine immunization format [301]. There is also Post-exposure prophylaxis present for HBV, which uses HBV immunoglobulin (HBIG). This could be used after the individual is exposed to the contaminated blood/ bodily fluids and it can also be used to prevent HBV vertical transfer from mother to child [302]. Apart from these preventions strategies, routine blood tests, screening of blood products, or use of condoms could greatly reduce the risk of HBV exposure [303].

4.1.6 Future Directions: Clinical trials and Current Research

Although advances in Hepatitis research have taken place, definitive answers are yet to follow. Developing and improving therapies like PEG-Interferon and nucleoide or nucleoside analogs are still the central focus of HBV research. Using these technologies, it is currently impossible to remove complete cccDNA from a patient's liver cells [304]. So the markers which are used for antiviral treatments are HBsAg, ALT levels, negative serum HBV DNA, and negative HBeAg respectively. There is also a need to use alternatives or different NUCs as using NUCs lead to the development of resistance and NUC related syndromes. Adefovir related Fanconi syndrome is associated with renal failure and hence while designing NUCs, these side effects should be taken into consideration [305]. Some of the future treatment options include generation of Tenofovir alafenamide, treatment of HBV ccc DNA and targeting NTCP. Tenofovir alafenamide demonstrated better antiviral activity and reduced the exposure of Tenofovir that leads to lower renal failures [305, 306]. Clearly, ccc DNA, was reported by APOBEC 3A and 3B related agents which will need further validation [304]. APOBEC has been known to target the viral genome as it mutates the bases during synthesis of new viral genetic material. NTCP, the recently found receptor for HBV entry, is also the latest target for HBV treatment. Some early reports suggest that cyclosporine A inhibits the interaction between NTCP and HBV surface proteins, and this could be an important breakthrough in controlling HBV infection in the future [307]. Also from the $CD8^{+}$T point of view, there has been an emphasis on anti PD-1 therapies which would block the exhaustion of the CTL activity and help in clearance of the virus. There are around 30 different drugs which are in the clinical trials which differ from NUC and Interferons [308]. Recent success of the Hepatitis C viral containment, and there is excitement in the HBV field for new directions. All the drugs which are in the clinical phase drug pipeline are either Direct acting that target virus or Indirect acting that target human host. For Direct acting siRNA, entry inhibitors, capsid inhibitors, and HBsAg inhibitors are the main drugs being developed [308]. And from Indirect acting drugs point of view, increasing the host's immunity is the answer to contain the viral infection (Reviewed in Ref [308]).

4.2 Hepatitis C Virus

4.2.1 Epidemiology and Disease Burden

Like the Hepatitis B virus, the Hepatitis C virus is tropic to the liver and hence causes inflammation of the liver, which can lead to liver failure, liver cirrhosis, and hepatocellular carcinoma. The Hepatitis C virus does not differentiate between continental boundaries and thus can be found in almost all places of human habitation. For Hepatitis C, Eastern Mediterranean and the European region take the major share of 2.3% and 1.5% respectively [267]. According to the WHO, the number of chronically infected patients with HCV is 71 million worldwide [266, 267]. The

number of deaths due to liver inflammation attributed to chronic infection with HCV is about 399,000 people each year [271]. As the numbers suggest, these viruses are already an overloading economic burden for the healthcare system and hence actual expenditure statistics are monumental. Unlike HBV, the numbers are not far less for HCV, around $300 million is spent on liver transplant every year and the lifetime infected patient healthcare cost reaching about $9 billion [272].

4.2.2 Characteristics, Morphology, and Virulence:

Discovered in 1989 at Chiron, HCV when compared to HBV, is a larger virus consisting of 9.6kb of single-stranded RNA genome under a lipid bilayer envelope. It also forms larger 45nm–65nm virion particles than HBV [309]. It is a member of Flaviviridae family, which hosts members like Dengue virus and Zika virus [310]. IRES-containing uncapped 5' UTR region translates a positive-stranded RNA into one large polyprotein which eventually gives rise to 10 different proteins [311]. The proteins are divided into 2 main categories: Structural and Non-structural proteins and one separate entity: viroporin p7. Structural proteins are core, envelope (E1 & E2), and Non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, & NS5B) [309, 312]. NS3, NS4A, NS4B, NS5A, and NS5B forms the replicase machinery, and NS2 and p7 are essential for viral assembly and release [311]. HCV virulence is well known and it is aptly called the 'hard to kill virus' [313]. Virulence of HCV may be attributed to the types of genotypes it has and the different role every genotype plays in accordance with the pathogenesis. For example, the Type 1 genotype is more aggressive and more directly linked to HCC and cirrhosis. Type 3 is associated with steatosis and fibrosis [314]. Also, the genetic makeup of the host is a major factor in virulence of HCV. The HAVCR1 gene shows variable susceptibility towards different genotypes of HCV. In HIV co-infected patients, IL28B CC was shown to be associated with chronic hepatitis C infection in patients infected with HCV genotype 3 than HCV genotype 1 or 4 [315]. Recently one more factor leading to HCV's virulence has come to light, involving stimulation of Drp1 by HCV, which leads to uneven fragmentation of Mitochondria [313].

4.2.3 Clinical Manifestation

Similar to the Hepatitis B virus' clinical manifestations, the Hepatitis C virus shows two types of disease progression: Acute and Chronic Infections. Majority of HCV acute patients remain asymptomatic. Around 20–30% of acutely infected patients develop clinical symptoms [316]. These symptoms may include weakness, anorexia, and jaundice. Levels of ALT rise 10 times the normal limit which is necrotically damaging to the liver tissue [317]. In self-containing acute infections, the levels of ALT and the HCV RNA both go down with time. Detection of HCV acute infection is dependent upon anti-HCV produced by the body within 1–3 months after onset [318]. Late or undetectable levels of anti-HCV antibodies could lead to major liver damage. Majority of HCV infected individuals develop HCV chronic infection with

HCV RNA. Presence of HCV RNA in the blood for more than 6 months after the onset of HCV infection is called chronic HCV infection. It depends on factors such as demography, ethnicity, and gender of the affected individual. Factors like age, HIV infection, alcohol consumption, prior exposure to jaundice etc. are linked to developing HCV infection (Reviewed in Ref [318]). Levels of ALT also plays a role in the rate of disease progression. If ALT is at normal levels, then the disease progression is slower than what is associated with the upregulated levels of ALT. Hepatocellular Carcinoma is also associated with chronic HCV infection under various conditions. HCV coinfection with HBV leads to a higher chance of developing HCC [319]. Co-infection with HIV and host genetic factors are associated with HCV associated HCC development.

4.2.4 Interactions with the Immune system and Pathophysiology

The HCV being the lipid centric virus has two envelope glycoproteins E1 and E2. These two proteins are involved in the entry mechanism of the HCV. These two glycoproteins interact with CD81 and various other surface proteins such as claudin [263, 280, 290], occludin, and epidermal growth factor receptor to enter the host cell [320]. Clathrin-mediated endocytosis is how the virus enters the target cell and releases the nucleocapsid into the cytoplasm. Due to the release of the nucleocapsid in the cytoplasm, the genomic material of the HCV is exposed to the host's immune machinery, which counteracts the HCV in several ways. Positive-strand RNA bearing Internal Ribosome Binding Site (IRES) is used for translation of HCV proteins. The HCV translates a large polyprotein, which is broken down into individual proteins later during ER related processing. Breaking down of polyprotein requires help from two cellular peptidases: Signalase and Signal peptide peptidase and two viral peptidases: NS2 and NS3/4A [321]. NS5B and helicase domain of NS3 are regulators of HCV replication. They assist with unwinding and stabilizing the HCV RNA in the replication complex [322, 323]. NS4B plays a role in the formation of compartments for HCV replication by producing 'membranous web' structures [309]. Certain host factors also assist in HCV replication such as; microRNA-122 which binds to IRES to increase the efficiency of translation whereas Cyclophilin A interacts with NS5A and NS5B to increase HCV replication [309]. HCV also uses Fatty acid pathways and VLDL production for assembly and release [324].

Compared to HBV, HCV initiates a better innate response due to the exposure of its genetic material in the cytoplasm. Major players in HCV induced immune responses are IFNs I and III, ISGs, NK cells, T cells, and antibody type response. During HCV infection the levels of IFNs and ISGs are upregulated in the cell. Generally, they have an inflammatory response towards the threat, but in the case of HCV, components like ubiquitin-specific peptidase 18 (USP18) and ISG15 negatively regulate the downstream signaling pathways of interferon signaling and help to prolong the persistence of HCV in the cell [325]. USP18, downregulates the production of IFNα through interaction with IFNAR signaling [326]. ISG15 is abundant in the cell during HCV infection and it also stabilizes USP18 which relates

to poor IFNα processing [327]. Moving on from internal response towards a more cellular response, NK cells are paramount in HCV infection. During prolonged HCV infection there is a decrease in production of IFN-γ and increase in cytolytic enzymes. This also results in host tissue damages due to cytotoxic effects of the NK cells. Upregulation of KIR receptors, which are found on NK cells and are markers for lysis of target cells, is seen during HCV infection indicating the importance of NK cells [328]. Due to the hypervariable regions in E1 and E2 glycoproteins and high mutation rates, T cell and B cell responses are short and not efficient enough. E1 and E2 glycoproteins are the major targets of neutralizing antibodies. However, these antibodies are short-lived, and are not persistent during chronic stages of the infection. Due to direct cell to cell transmission of HCV, it often escapes the antibodies and is difficult to neutralize [312, 329]. Neutralizing antibodies are thought to have a lesser role in controlling HCV infection, as they are detected in chronic stages rather than after acute infections [330]. As far as T cells go in accordance with HCV, $CD8^+$T cells are the frontrunners in combating the viral threat. They are active during the acute phase and relatively slow during the chronic phase of HCV infection [331, 332]. A vigorous IFN-γ response is seen by $CD8^+$T cells, which helps in providing an antiviral response at the site. Mutation in HCV also leads to its escape from T cells detection, but the response is still important during an earlier stage of infection. $CD4^+$T cells are diminished cell-mediated immune response during chronic infection due to reduced IL-2 production [312, 331]. The $CD8^+$T cell-mediated immune response is enhanced via the assistance of $CD4^+$T cells during the acute stage of infection.

4.2.5 Treatment and Prevention

Since spontaneous clearance of HCV is seen in acute infection, there is no treatment authorized for acute infections. Patients with raised levels of liver enzymes are able to clear the infection more rapidly than those with lower levels. If the infection is not cleared, IFN monotherapy is used for rescue therapy. Sustained Virological response (SVR) is over 90% in these conditions [333]. This means that patients are aviremic for over 24 weeks after the treatment. Use of DAA (Direct Acting Antivirals) in acute infection is not the ideal treatment option, but it has 100% SVR (Sustained Virological Response) detected 12 weeks after the end of therapy [309]. DAAs are effective drugs for chronic hepatitis treatment DAA target three essential proteins in the HCV replication cycle: NS5B polymerase, NS3/4A protease, and NS5A protein [309]. All patients with detectable levels of viral RNA in their serum should be treated with DAA therapy. Combinations of more than one DAA work significantly better than taking an individual DAA. The nature of the regimen also depends on the genotype and the geographical area. Examples of approved DAAs in North America and Europe are elbasvir/grazoprevir and sofosbuvir/velpatasvir. The combination of IFN and DAA are some of the preferred methods of treatment. Vaccines are currently not available for Hepatitis C virus. Post-exposure prophylaxis preventive measures are difficult to undertake

for the Hepatitis C virus, and as such, the focus is on how to prevent the spread of Hepatitis C virus in the first place. Screening for blood before transfusion, needle awareness strategies and Sex education are the primary preventive measures which need to be addressed. Identifying the people infected with HCV and treating them is the best option to prevent it further from spreading [309].

4.2.6 Future Directions: Clinical Trials and Current Research

Introduction of DAAs (Direct Acting Antivirals) has revolutionized the way we think about treating HCV infection, it is now known as a 'curable' disease. Even though DAAs are the best option for treatment of HCV infection, resistance and potential side effects are the reasons for which there is a continuity into HCV research. There are various new strategies for HCV treatment options that are being evaluated. Researchers are using similar viruses to solve for HCV related complications. Using bovine viral diarrhea virus (BVDV), which is similar to HCV in many aspects, researchers have studied different targets for HCV replication cycle, genetic makeup, and general biology [334]. Another interesting area for HCV research is the generation of antivirals from marine invertebrates. Isolation of extracts from the Bacillus genus showed antiviral potential during the viral adsorption phase [335]. Also extracts from plants are being used for potential antivirals as their side effects are minimal for human usage. Majority of the plant extracts tested so for antivirals, target RNA levels. For example; Polyphenols from Chinese mangroves exhibited inhibition of HCV's RNA replication [336]. Caffeine has been shown to improve the liver functionality of chronically infected patients with HCV, it targets the replication phase of HCV with non-toxic concentrations [337]. So all these new and exciting areas which are being explored for HCV treatment shape up the current and future endeavors for this particular field. Due to the effectiveness of the current regimen for HCV treatment, not many drugs are undergoing clinical trials, but there a few of them which are aimed at overcoming all the potential pitfalls of the current treatment options. In 2016, Harvoni and Viekira Pak were the highest prescribed HCV medications with a 95% success rate for eliminating HCV (Reviewed in Ref [308]). Drugs like Samatasvir, Odalasvir, MK-3682 etc, are top of the line for their respective clinical trials (Reviewed in Ref [308]). These drugs are still targeting the viral components such as polymerase and proteases, but their success rates during clinical phases has strengthened their overall reliability.

5 Retroviruses

Retroviruses are enveloped positive sense ssRNA viruses that belong to the retroviridae family. HIV and HTLV-1 are retroviruses that will be the focus of the discussion on retroviruses because they pose a significant health burden [338]. The two viruses not only have similar structures and mechanisms of infection, but they are

also more likely to be transmitted when someone engages in risky behaviors, such as unprotected sex and sharing of contaminated needles, as well as breastfeeding by infected mothers. HIV primarily infects macrophages, CD4 $^{+}$T cells, and neurons [338]. The difficulty with completely eradicating HIV from the host is due to HIV's ability to integrate its genome into T cells, macrophages, and DCs, making it a global health problem [339, 340]. HTLV-1 infects CD4 T cells. The probability or contracting HTLV-1 through blood transfusion of organ transplantation renders HTLV-1 a significant health burden. As such, it is imperative to have adequate blood and/or organ screening processes in place to prevent the transmission of HTLV-1 [341]. For the last several years, our group has studied host-pathogen interactions between HTLV-1 and the immune system with a focus on viral transactivator protein Tax and dendritic cells [342–351]. These studies have identified key aspects of viral pathogenesis with respect to both ATLL and HAM/TSP paving ways for novel immunotherapeutic strategies currently underway in our research team.

5.1 Human Immunodeficiency Virus

HIV is a member of the retroviridae family that is responsible for a global epidemic with an annual mortality of more than one million [352]. HIV glycoprotein interacts with CD4 (on CD4 $^{+}$T cells, monocytes, macrophages, and DCs) and CCR5 (on T cells, macrophages, and DCs) to mediate attachment and internalization of the virus into permissive host cells [338]. Furthermore, HIV can infect granulocytes (neutrophils, eosinophils, and basophils) as well as macrophages, microglial, and astrocytes in the central nervous system especially the brain [353, 354]. In the absence of an effective vaccine, clinical management is focused on prevention, education, and anti-retroviral therapy. Research on HIV therapy is focused on developing immunotherapy and immunomodulators. Challenges to developing an effective HIV vaccine constitutes a global health burden because of HIV's ability to integrate and cause a latent infection, as well as its ability to evade neutralizing antibody-mediated humoral immunity and cell-mediated cytotoxicity. An enormous barrier to the control of HIV spread is the lack of effective HIV vaccines and unaffordability of breakthrough on anti-HIV therapy in resource limited countries. In the absence of early diagnosis and treatment, HIV can lead to secondary or opportunistic infections and malignancy [355–357].

5.1.1 Epidemiology and Disease Burden

HIV is a global epidemic, killing over 1 million people annually and causing 3.4 million new infections in the last 5 years [352]. Untreated HIV infection causes acquired immunodeficiency syndrome (AIDS), and the increased susceptibility to opportunistic infections usually leads to AIDS-related death. The only effective way to manage HIV infection focuses on preventative measures to decrease transmission

and lifelong antiretroviral therapy (ART) for those infected with HIV [358, 359]. HIV infection is associated with high-risk groups including injection drug users (IDUs), men who have sex with men (MSM), and people practicing high-risk sexual behavior [360]. UNAIDS, the main international organization dedicated to the control of HIV/AIDS epidemic, set goals that include having fewer than 50,000 newly infected people and fewer than 500,000 AIDS-related deaths by 2020, and elimination of AIDS epidemic by 2030 [361]. HIV treatment coverage is increasing steadily from slightly over 20% in 2010 to almost 50% in 2015, also mirrored by decreasing numbers of AIDS-related deaths: about 1.5 million in 2010 and 1.1 million in 2015. The number of new HIV infections in most regions, besides Eastern Europe and Central Asia, has stabilized. HIV mutates during active infection, making the adaptive immune response ineffective and complicating vaccine development. In addition, the virus acquires drug resistance during therapy and it varies in different regions, making it difficult to find sustainable, worldwide treatments. There are two types of HIV genomes: HIV-1 (causing over 90% infections), which includes groups M, N, O and P, and HIV-2, which includes groups A and B [362].

5.1.2 Characteristics, Morphology, and Virulence Factors

HIV belongs to the *retroviridae* family, genus *Lentivirus*. The virus is enveloped, with positive sense single stranded RNA genome in a spherical capsid 119−207 nm in diameter [363, 364]. HIV bears several Env spikes on its surface that allow for cell infection [363]. HIV genome encodes 16 proteins. They include structural proteins: Matrix, Capsid, Nucleocapsid, and p6; enzymes: Protease (PR), Reverse Transcriptase (RT), and Integrase (IN); regulatory proteins: Tat and Rev; accessory proteins: Vif, Vpr, Nef, and Vpu/Vpx (in HIV-1/HIV-2); and envelope glycoproteins gp120 and gp41 [365]. Three heterodimers gp120/gp41 form a trimer: the envelope glycoprotein Env that mediate cell entry. Its immunogenicity is low due to massive glycan shield preventing access of antibodies to over 97% of Env surface [338].

Initial HIV infection induces strong activation of innate and adaptive immune response, but mutations of the virus gradually lead to immune system evasion and elimination of CD4 $^{+}$T cells [360]. Complete elimination of HIV in infected individuals using ART is not possible due to latent residency of the viral particles and/or integrated HIV genomes in memory T cells and possibly some populations of macrophages and dendritic cells [339, 340]. HIV is transmitted through blood, sexual secretions, and vertically from mother to baby. When transmitted through infected blood, ie. needles, macrophages, dendritic cells, and CD4 $^{+}$T cells take up the virions and spread the infection. When the virus in semen, cervicovagina, and rectal mucus comes in contact with the mucosa of non-infected individuals, it can infect the periluminal T cells, DCs, and monocytes/macrophages of the mucosal epithelium [366]. During vertical transmission, the fetus may swallow infected maternal fluids or become infected via breastfeeding [367].

5.1.3 Clinical Manifestations of HIV/AIDS

HIV has an incubation period of 1–2 weeks, which marks the primary acute infection, and manifests itself with flu-like symptoms for 2–4 weeks that can go unrecognized as HIV infection. The next stage, the chronic/latent infection, significantly varies from 1 to 20 years [368]. The last stage of infection is AIDS, marked by the development of opportunistic infections [369]. The potential for infected individuals to transmit HIV is dependent on the viral load [366]; asymptomatic individuals and those who do not have anti-HIV antibodies in their plasma could still be infected and have the potential to infect others [370]. Babies born from HIV-infected mothers have the highest chance of being infected if the mothers were not on ART [360]. The few individuals who are resistant to HIV/AIDS are called long-term non-progressors, defined as an absence in CD4 cell count, and HIV elite controllers, meaning no viremia [371]. Decreasing CD4 $^{+}$T cell count is an important diagnostic marker.

AIDS-associated opportunistic infections include oral candidiasis, tuberculosis, herpes zoster, and fungal pneumonia in both the ART-naïve people and people who receive ART. ART reduces the risk of 15 most prevalent opportunistic infections by 57–91%, with the greatest reduction shown for oral candidiasis, toxoplasmosis, and *Pneumocystis* pneumonia [372]. In developed countries, HIV-infected people on ART have only slightly reduced life expectancy compared to the general population [373, 374]. However, HIV-infected individuals on ART still have increased risk of cardiovascular, digestive, excretory, musculoskeletal, and central nervous system diseases. One of the consequences of HIV infection is HIV-associated dementia (HAD) that develops in 20–30% of HIV-infected people not on ART, and also HIV-associated neurocognitive disorders (HAND) affecting 18-50% of patients with chronic HIV on ART [375].

5.1.4 Interactions with the Immune System and Pathophysiology

HIV targets CD4 $^{+}$T lymphocytes, macrophages, and DCs [376]. T cell infection is initiated when the cell surface receptor CD4 and co-receptors CCR4 or CXCR5 interact with viral glycoprotein gp120. After this initial interaction, the Env complex undergoes a conformational change and viral fusion peptide, a part of gp41, interacts with the cellular membrane; eventually the viral and cellular membranes fuse and the capsid contents are transported inside the T cell [377]. Gut CD4 $^{+}$T cells with high expression of CCR5 [378] and T-follicular helper cells [379] are T cell populations highly vulnerable to infection. Other types of T cells serve as long-term viral reservoirs, such as subsets of memory T cells [379, 380].

Following host cell entrance, HIV proteins interact with numerous host cell proteins [381] and re-program the cell functions [382]. HIV virion core changes conformation to reverse transcription complex (RTC) [364]. RTC allows for transcription and recombination to occur using the two HIV RNA molecules as templates to build the chimeric double-stranded DNA [383]. RTC then binds nuclear membrane

proteins, undergoes uncoating, and allows the pre-integration complex (PIC) to reach the nucleus via the nuclear pore complex (NPC) [384]. The viral genome is then integrated into the host genome into the active chromatin segments [364, 384]. Host cell proteins aid in transcription of the HIV genes, splicing, translation, and virion budding [364]. Activity of HIV replication in the $^{+}$T cells is dependent on the activation status: resting T cells prevent HIV replication at different levels, while activated T cells allow multiple rounds of HIV replication [385].

Macrophages are the second most important cell type in HIV infection. Macrophages bind and take up virions, and while they rarely allow for HIV replication, they do influence the immune responses [386], promoting inflammation, disrupting the blood-brain barrier [387], and serving as both long-term reservoirs of infection (245) and means for trans-infection of T cells [388]. CD4 [389], CCR5 [390] and CXCR4 [391], mannose receptor (MR) [388], purinergic receptors [392], syndecan, cysteine-rich scavenger receptor, gp340, elastase, glycolipid GalCer, heparan sulfate, disulfide isomerase protein [386], and DC-SIGN [393] mediate binding and capture of HIV in macrophages. Another route of macrophage infection is capturing HIV-infected T cells [394]. DCs are another key cell type in HIV infection. Myeloid DCs are infected non-productively, and they can provide trans-mucosal transmission of HIV and mediate trans-infection of T cells [395]. HIV infects mucosal DC via CCR5 and CXCR4 on DC, and this promotes spread and transmission of HIV to trans-infect CD4 $^{+}$T cell in the draining lymph nodes [3]. Immature forms of myeloid DCs express high levels of CCR5 but express high levels of CXCR4 in their mature state. The immature form promotes HIV transmission because they are susceptible to HIV infection [396]. However, conventional DC with low expression of CXCR4 and CCR5 do not support efficient replication of HIV [3]. HIV endocytosis by DCs result in partial degradation of the virus with some virus retaining the infectious capability. This could explain why these APCs promote infection of CD4 $^{+}$T cell, as these captured nondegraded HIV molecules can be transferred to CD4 $^{+}$T cell in a process called trans-infection through infectious synapses [396]. Macrophages and DCs mediate trans-infection through receptors including Siglec-1 (CD169), DC-SIGN, MR, Langerin, immune dendritic cell receptor (DCIR), heparan sulfate proteoglycan, syndecan-3, and galactosylceramide [397]. The infectious synapse allows transfer of HIV from conventional DC to CD4 $^{+}$T cells via a gp120 and CD4 dependent interaction. HIV interaction with DC-SIGN on DCs may skew T cells response toward generating Th2 cells that favor development of inflammation and fibrosis during viral infection [6]. In spite of known primary role of Siglec-1 in myeloid-to-T cell trans-infection, its absence does not influence HIV acquisition and disease outcomes in Siglec-1 null individuals, demonstrating that other transmission mechanisms are sufficient (T cell-to-T cell) for development of HIV infection [398].

HIV evades intracellular recognition and secures replication, assembly and viral release by multiple mechanisms. For example, HIV genetic material is surrounded by proteins that are inaccessible for the host cell receptors before it is translocated inside the nucleus [399]. HIV protein Nef promotes T cell activation, HIV replication and evasion of the infected cells from cytolytic immune response [385]. Viral

proteins Nef and Vpu induce decline in CD4 and BST2 expression on the cell surface that reduce efficiency of antibody-dependent cell-mediated cytotoxicity [400]. Vpu protein also inhibits Theterin function, which normally prevents the release of virions, activates antiviral signaling and cytokine release [401]. HIV capsid interacts with CPSF6 (Cleavage and Polyadenylation Specificity Factor subunit 6) and cyclophilins of monocyte-derived macrophages to block type 1 interferon production and allow viral replication in these cells [402]. HIV-infected T cells die by caspase-1-mediated pyroptosis, an inflammatory form of programmed cell death with release of cytoplasmic contents and cytokines. This mechanism of T cell elimination is supposed to be a cause of chronic inflammation in HIV [403]. HIV infection also causes dysregulation of B cells and antibody production [404, 405], and impairs regulatory and suppressive functions of HIV-infected T regulatory cells [406]. HIV evades recognition and inhibits the ability of pDCs to generate IFN-α by the use of gp120 to suppress the induction of CpG-induced activation of pDCs [3]. HIV may reduce IL-12 and IFN-γ production by DCs and NK cells respectively, consequently resulting in reduced Th1 polarization [11]. HIV non-progressors and controllers possess diverse mechanisms of immune defense. CD4 $^{+}$T cells and macrophages from HIV controllers are less susceptible to infection [407]. DCs of HIV controllers are less susceptible to HIV infection but more potent in viral uptake compared to the DCs of healthy individuals [408]; DCs of elite controllers are also able to recognize HIV and stimulate antigen-specific reactions in the T cells [409]. CD8 $^{+}$T cells from HIV controllers contain highly functional HIV-specific pool [410] effective at elimination of infected CD4 T cells [411]. Moreover, CD8 regulatory T cells of elite controllers that express KIR3DL1 gene can repress HIV replication in Bw4-80Ile-expressing CD4 $^{+}$T cells by preventing helper T-cell activation [412]. Another immune response in elite controllers is the production of poly-functional antibodies with increased effector activities and production of IgG1/3, but not of IgG2/4 subclass [413].

5.1.5 Treatment and Prevention

HIV treatment should be started immediately after diagnosis, and effectiveness of ART should be continuously monitored. There is a wide diversity of ART algorithms based on age, maternal status, disease stage, and viral drug resistance status. Most patients will be on a combination of three different anti-retroviral drugs to increase effectiveness and reduce resistance. People living with HIV not on ART are recommended to follow preventative measures to decrease transmission and to seek immediate care for opportunistic infections [355]. In the absence of an effective vaccine, HIV prevention is based on education, testing, and treatment. Harm reduction programs targeting high-risk groups have proven efficacious: providing IDUs with clean syringes to prevent blood-to-blood transmission, promotion of condom use among sex workers, HIV counseling, and testing and treatment in all high-risk groups [356, 357].

5.1.6 Future Directions: Clinical Trials and Current Research

Vaccine development encompasses a number of different approaches: vaccines based on a wide range of immunogenic types from peptides to nanoparticles [414], DNA vaccines [415], dendritic cell vaccines [416], and therapeutic vaccines [417]. An important obstacle to the development of an HIV vaccine is the need to create an HIV-specific T cell pool to support HIV-specific antibody production, with this pool still being the most vulnerable to infection and promotion of HIV replication [418]. The HIV vaccine RV144 has shown 31.2% efficacy and provided only temporary defense [419]. For a long time, the development of HIV vaccines was mainly focused on the generation of broadly neutralizing antibodies (bNAbs) and cytotoxic T lymphocytes (CTLs) [420]. Induction of neutralizing antibodies is considered important since they usually correlate with the protective effect of vaccines and provide sterilizing immunity [414]. Induction of pathogen-specific CTLs is considered important based on research on efficacy correlates of SIV vaccines [420], and after analysis of the mechanisms of RV144 efficacy that included cytotoxic effects of non-neutralizing antibodies [420, 421]. Another novel aim in the development of HIV vaccines is generation of CD8 regulatory T cell response to prevent $CD4^+$T cell activation and viral replication [412].

Development of anti-HIV drugs is always ongoing. Besides the great number of small molecules being tested in preclinical and clinical trials [353], there are therapeutic approaches on bNAbs being studied for passive immunotherapy, including those that demonstrate efficacy in human subjects [422–424], and immunotherapy based on checkpoint blockers [417]. More complex HIV therapies are also proposed, such as adoptive therapy with chimeric antigen receptor (CAR) T cells for development of antigen-specific cell-mediated responses [425, 426], use of single-chain CARs based on bNAbs as neutralizing antibodies [427], gene therapy using CRISPR/Cas9-mediated removal of HIV DNA from latently infected $CD4^+$T cells [428], or genetic inactivation of HIV co-receptors production in CD4 T cells to prevent infection [429, 430]. Another future direction is the development of therapies aimed at modulating the host response to HIV [431]: cell and gene therapy have reached the stages of clinical trials [430, 432, 433]. Despite some recent breakthroughs, many of the new developments are highly complex and the costs of some of these treatments are not affordable in resource-limited countries.

5.2 Human T-Cell Leukemia Virus Type 1 (HTLV-1)

HTLV-1 is endemic in Japan, Brazil, Iran and parts of Sub-Saharan Africa [434]. It is a unique retrovirus that is transmitted through an immunological synapse wherein infected T cells form synapse with an uninfected cell to facilitate transfer of genetic material. Other forms of viral transmission include infection of dendritic cell, co-infection with HIV, and through virological synapse [435]. Education focused on safe sex practices and needle exchange programs are few preventative strategies to

limit the spread of HTLV-1. Treatment is geared towards using chemotherapy to counteract symptoms of leukemia and lymphoma. A combination therapy of AZT and interferon-alpha is effective in some patients with ATL. There is no FDA-approved therapy for HTLV-1 infections [436].

5.2.1 Epidemiology and Disease Burden

HTLV was discovered in the 1980s by a team of researchers led by Robert Gallo. It is the cause of varying burden of disease in many countries [437]. HTLV-1 is the most clinically harmful subtype and it is endemic in Japan, Brazil, Iran, and parts of Sub-Saharan Africa with an estimated prevalence of 20 million cases as of 2017 [434]. While there are four recognized subtypes of HTLV, HTLV-3 and HTLV-4 have only been recently discovered, and have a very low global prevalence [438]. Between HTLV-1 & HTLV-2, HTLV-1 has received more research attention, and carries a larger burden of disease [438]. While this may seem perplexing given that HTLV-2 is more prevalent than HTLV-1 in some areas (like the US), HTLV-1 is the subtype that can progress to more deleterious conditions [439].

Some areas in Japan and South America have about 5% prevalence of HTLV-1, while European and North American countries barely have any reported cases, and of those, most are associated with travel to and from endemic areas [440]. HTLV-1's main routes of transmission are through secretions during unprotected sex, through infected blood, such as the sharing of contaminated needles, through the donation and transfusion of contaminated blood, and vertically between mother and baby [434]. As such, HTLV infection has significant social implications. This is especially important for HTLV-1, as it is the subtype that can progress to clinical disease. Higher male-specific virulence in areas like Japan could be due to an increased prevalence of breastfeeding and a persistence of women as viral reservoir for transmission [441]. This phenomenon has the potential to influence a perceived risk of contracting the virus, partially due to its similarities with HIV.

5.2.2 Characteristics, Morphology, and Disease Factors

HLTV is a member of the family *Retroviridae*, the subfamily *Orthoretrovirinae*, and the genus *Deltaretrovirus*, placing it in the same genus as the bovine leukemia virus and simian T-lymphocytic virus (STLV), the latter of which is considered to have evolved into HTLV after interspecies transmission [442]. The HTLV-1 genome carries several structural genes (i.e. *gag, pol, & env*), but it also contains an accessory pX region at its 3' end [443]. This portion of the genome contains genes such as *Tax* and *HBZ* (HTLV Zipper Factor), which encode proteins that are key to HTLV's interaction with the immune system. The *trans*-activating Tax protein is responsible for initiating an immune CTL response against HTLV-1-infected cells, while HBZ triggers immunological senescence, inducing chronic infection that leads to pathology of clinical disease [241, 443]. Both Tax and HBZ have been a major focus of HTLV research because fully characterizing their function and interaction with the

immune system may lead to effective treatment or prevention of HTLV and related diseases.

HTLV-1 is primarily transmitted through an immunological synapse, directing structural Gag and Env proteins to create a bridge with the uninfected cell, after which the infected T-cell sends its genetic material across the bridge. There are other ways the virus could be transmitted, such as by membrane extensions or by infection via DCs and, alongside HIV, through the typical virological synapse as well [241, 435]. Vertical transmission is the most common mechanism of the spread of infection in endemic areas [435, 444]. While this type of transfer can occur through breastfeeding and through the placenta, in the case of HTLV-1, a clear majority occurs through breastfeeding, with risk of transmission increasing the longer a mother breastfeeds [444]. Once breast milk is ingested, infected cells can penetrate intestinal mucosa or oral epithelium, and infect various cells in the child's immune system; penetration of mucosal epithelium is crucial for this type of transmission [435]. If the virus is spread through contaminated blood products, it doesn't need to cross mucosal barriers because it is already in the host's blood and can immediately initiate virological synapses with uninfected cells. This increased ease of infection drives home the importance of preventing blood-borne transmission of HTLV [435, 445].

Genetic factors play a role in HTLV's clinical course as well, though their role is more prominent in determining risk of progression to symptomatic disease. Certain HLA alleles can be risk factors or protective factors for ATL and HAM/TSP. Data suggests that *HLA-A*02* and *HLA-Cw*08* can be protective against HAM/TSP, while *HLA-DRB1*0101* and *HLA-B*5401* increase the risk of progressing to HAM/TSP [446]. However, *HLA-Cw*08* (along with *HLA-DR1*) can actually increase risk of HAM/TSP in other areas (Southern Japan) suggesting that environment al factors can influence the effect of the HLA haplotype on HTLV-related risks [446].

5.2.3 Clinical Manifestations

HTLV-1 is the only subtype that has been found to be associated with disease, though this may mean that an association between the three other subtypes and clinical disease hasn't been discovered yet [438]. Roughly 5% of patients do develop either ATL or HAM/TSP, both of which can have severe clinical consequences, including severe immunosuppression, the development of autoimmune diseases, and death [442]. ATL, characterized by atypically shaped lymphocytes or flower cells, which express CD3 but not CD20 [447], have four recognized clinical subtypes: acute, chronic, smoldering, and lymphoma [448, 449]. ATL is diagnosed through serological and cytological testing, the latter being sputum analysis for malignant T cells [447, 449]. ATL is a multi-organ system disease. The skin is one of the most commonly affected organs, with patients presenting with erythema and plaques. Immunosuppression tends to occur more often in the less aggressive (chronic and smoldering) subtypes [448]. HLTV-1 alters $CD4^+$T cell behavior leading to increased inflammation and disinhibition of the immune system, increasing

susceptibility for opportunistic infections [442, 448]. Immune system dysregulation can alter risk and clinical course of autoimmune diseases. For example, patients with Sjögren's syndrome have higher levels of anti-HTLV-1 antibodies, and if co-infected with HTLV-1, they have a higher amount of infiltrates and worse outcomes than if they had Sjögren's syndrome alone, implying that HTLV-1 has the potential to imbalance the entire immune system [442].

While ATL presents with diffuse effects, HAM/TSP is system-focused, mainly affecting the nervous system. Through the production of TNF-α, IFN-γ, and other cytokines, HAM/TSP exerts a neurotoxic effect that leads to spinal cord inflammation, especially in the thoracic region [450]. This inflammation leads to demyelination and axon dysfunction, which damage nerves innervating the thoracic spinal cord resulting in, among other symptoms, bladder dysfunction and possible paralysis [450]. HAM/TSP is diagnosed through the evaluation of the patient's serum and CSF showing antibodies against HTLV-1 [451, 452]. A neuroradiological study conducted in early 2017 produced data suggesting that HTLV-1 and HAM/TSP are associated with alterations in brain glucose metabolism, which can be detected via neuroimaging [451]. The glucose transporter GLUT-1, found in the blood-brain barrier among other places, is utilized by HTLV virions to infect cells, thereby competing with glucose for the transporter.

5.2.4 Interactions with the Immune System and Pathophysiology of Disease

One of the key immune cells implicated in HTLV is the T cell. When infected, T cells initially express the Tax protein, which suppresses their proliferation and enables their recognition by $CD8^+$T cells [453, 454]. It has been shown that infected cells can spread Tax through exosomes, a potential route for dissemination of the infection [454]. Tax is therefore necessary for an HTLV-1 infection to be successfully eradicated. It is thought that the immune systems of asymptomatic HTLV-1 carriers are more responsive to Tax, and that changing this responsiveness can induce progression to ATL or HAM/TSP [453, 455]. Studies show that patients who have progressed from latent HTLV to ATL or HAM/TSP initially had high expression of Tax and corresponding stimulation of the HTLV-specific immune response, but eventually something happens in their immune system circuitry, preventing every infected cell from being destroyed. This leads to viral persistence without future immunologic triggering, and proliferation of infected cells that can lead to ATL and HAM/TSP [341].

There are two prevailing schools of thought for this mechanism. On one side, it is hypothesized that T cell exhaustion plays a role in this transformation. Normally, the immune system shuts off once the invading pathogen is eradicated, however, exhaustion forces the response to shut off prematurely, allowing infected cells to persist in the host [456, 457]. PD-1 binds PD-L1 and induces exhaustion by suppressing proliferation of the infected cell and preventing it from interacting with other immune cells. Some studies have looked for ways to reverse exhaustion or

prevent it altogether, possibly through blockade of PD-1, in order to keep the immune response active [453]. On the other side, HBZ protein is considered to be a main player in the progression from HLTV-1 infection to ATL or HAM/TSP. HBZ is expressed more in ATL patients than in carriers, contrary to Tax [341, 443, 458, 459]. HBZ acts to stimulate infected T cells and induce their proliferation, allowing them to outlast the immune response, eventually leading to ATL. It is also possible that the 'true' mechanism of ATL and/or HAM/TSP pathogenesis involves both exhaustion and the action of HBZ.

DCs also play an important role in HLTV pathogenesis. They are among the most potent APCs in the human body, and are crucial for the propagation of the anti-HTLV immune response [455]. DCs can become infected with HTLV-1, but they express different receptors necessary for their immunological function [455]. DC-mediated transmission of HTLV-1 from infected DC to T cells involve heparin sulfate proteoglycan and neuropilin-1, and consequential transformation of HTLV-1-infected T cells [6]. Patients whose HTLV-1 progresses to ATL and HAM/TSP were found to have significantly decreased amounts of pDCs compared to both carriers and uninfected controls [455]. Infected T cells may be unable to attack HTLV, and infected DCs may be unable to start the attack. DCs are also crucial for the immunogenic action of Tax. In a 2014 study examining the effect of DC presence and absence on Tax showed that Tax requires DCs to initiate the immune response against HTLV, and if the immune response does not start it is more likely that patients will progress to clinical disease [460].

5.2.5 Treatment and Prevention

Because HTLV infection is usually asymptomatic, treatment is only started if it progresses to ATL or HAM/TSP. ATL is mainly treated with chemotherapy to counteract the symptoms of leukemia and lymphoma, though patients have varying responses to this treatment [461]. Due to the generally dismal results of chemotherapy, some clinicians advocate to wait for less severe subtypes of ATL to become more aggressive, as smoldering forms are likely to remain asymptomatic. Chronic ATL produces milder symptoms than do lymphoma-type or acute ATL [436, 448]. A 2012 study found that a combination of antiviral and interferon therapy may prove more effective than chemotherapy, especially because the latter therapy can lead to ATL relapse and further complications [436]. Unlike ATL, there is no currently recognized treatment for HAM/TSP. Because HAM/TSP leads to neurological impairment of varying degrees including paralysis, discovering a viable treatment is important for all HTLV-1-infected patients, even those who remain asymptomatic.

Presently, preventing transmission is the only way to decrease HTLV infection. Prevention follows the same guidelines as those of any blood-borne pathogen, as well as education on safe sex practices and needle-sharing, providing access to barrier contraception, and increasing awareness of the consequences of HLTV-1 infection [462]. Acquiring the virus from blood transfusion or solid organ

transplant is possible, and as such, screening for HTLV in donated blood and other organs should be a priority [463]. Vertical transmission is one of the more significant routes of HTLV transmission. In some endemic countries (e.g. Japan) public health officials urge people with poor access to testing to avoid breastfeeding their infants. If the healthcare infrastructure is sufficiently developed, a mother's cessation of breastfeeding would not significantly affect her children's health; however, this is not the case in areas where people have less access to healthcare resources [444]. In such areas, researchers have acknowledged that complete avoidance of breastfeeding can harm more than help their children's health, and encourage breastfeeding only during the first few months of the infant's life [444]. There is currently no vaccine approved by the FDA or any other regulatory body for HTLV, ATL or HAM/TSP. A large amount of HTLV-related research is targeted towards vaccine development. Some studies have used Tax to model an HTLV vaccine, though a search of the literature suggests this has not yet led to its use as a clinical vaccine [453, 454, 460].

5.2.6 Future Directions: Clinical Trials and Current Research

The brunt of HTLV-related research is focused more on ATL and HAM/TSP than the virus itself, owing to these two diseases comprising most of HTLV's disease burden. There is some research oriented towards HTLV, but it is mainly focused to further determine what differentiates asymptomatic carriers from patients who progress to ATL and HAM/TSP. Some research has served to redirect the focus of molecular HTLV investigations from the Tax protein to HBZ. The Tax protein is important for the initial development of HTLV infection and can be used as a marker that infection has progressed to ATL or HAM/TSP [453]. As an oncogenic protein, Tax is certainly critical for the immune response to HTLV infection. However, because both ATL and HAM/TSP rely on a dampened or even silenced immune response to HTLV for their development, researchers have shifted their focus to HBZ due to its role in allowing infected cells to persist through an immune response. Some research has suggested this occurs via HBZ decreasing the activity of T-cell inhibitory receptors such as PD-1 and TIGIT that normally blunt the immune response, either by decreasing their expression or decreasing their function [459, 464]. This blunted inhibition leads to stimulation of infected T cell proliferation, with excessive proliferation and suppression of other cell types being part of the clinical definition of ATL; HBZ stimulates proliferation by initiating a cascade that results in the phosphorylation of molecules like CD3ζ and ZAP-70 that induce T cell proliferation [459]. Furthermore, Tax has been found to not be expressed by a majority of T cells in ATL cases, suggesting its oncogenicity and immunogenicity are turned off once an infected carrier progresses to ATL [453].

A smaller amount of current research is devoted to HAM/TSP. A cross-sectional analysis of Brazilian HAM/TSP patients stratified by age generated data suggesting that younger HAM/TSP patients are more likely to be clinically depressed [465], pointing to the importance of the psychological effects of the viral infection in

addition to its physiological effects. Finally, while HAM/TSP treatment is not universally effective in all patient populations, a 2015 case report highlighted the use of cyclosporine to treat early myelopathy cases [466]. Future studies will be needed to show whether this treatment can be expanded to other stages of the disease, and to research ways to build on it and develop even more effective treatments.

Another area of research could focus on novel testing protocols for HTLV and its associated diseases. In the case of HIV, for example, oratory-based testing is being supplanted in favor of rapid testing that can be done right in the clinic, or even in the patient's home [467]. These tests must meet the WHO requirements to be approved for clinical use. If researchers develop a similar kind of test for HTLV then patients who live in endemic areas with limited access to clinicians would be more likely to get tested and get the treatment they need.

6 Sexually Transmitted Infections

Sexually transmitted infections (STIs) affect individuals of all ages, race, backgrounds, and cultures. They can have some serious long-term sequelae, such as infertility, pregnancy complications, cancer, and super-infections, causing significant health burden on our society. HIV as an STIs has been discussed above, but two other major viruses that can be transmitted through sexual intercourse and contact are Herpes Simplex Virus (HSV) and Human Papilloma Virus (HPV). They both interact with Langerhans' cells and DCs of the skin, and cause infection of mucosal surfaces. Both HSV and HPV often go undetected and untreated because the infected individual may be asymptomatic with no visible outbreaks. HSV remains latent in the neurons, while HPV can be cleared by the body but may also cause cancer.

6.1 Herpes Simplex Virus

HSV is an enveloped dsDNA virus that causes a wide spectrum of diseases ranging from orofacial herpes and keratitis, to genital ulcerations, meningitis, and encephalitis. HSV genital infection is considered a STI of global health significance because HSV-associated genital mucoepithelial inflammation increases the risk of HIV transmission [468]. HSV glycoprotein D (gD) is essential for fusion to host cell receptors whereas VP16, a tegument protein promotes immune evasion and viral gene transcription [469]. VHS destabilizes host proteins. It also promotes immune evasion by suppressing type I interferon and reducing production of pro-inflammatory cytokines. VHS impairs expression of MHC I and MHC II, which helps the virus to evade antiviral cell-mediated immunity [470]. There are FDA-approved nucleoside analogues for treating herpes simplex infection; however, there is no viable therapeutic or preventive HSV vaccine. Another health burden of HSV is the

development of resistance to currently FDA-approved nucleoside analogues and/or inability to adhere to recommended therapeutic protocol putting immunocompromised individuals at a great risk of developing devastating complications [471]. There is current research into developing an effective preventive and therapeutic HSV Vaccine.

6.1.1 Epidemiology and Disease Burden

Herpes virus (Greek word meaning to creep or to crawl) and its diseases can be traced back to ancient Greece. Although the nature of the lesions was already established for many centuries, it wasn't until the late nineteenth century that scientists established the 'virus' mode of transmission: person to person contact. Half a billion people all over the world are affected by genital herpes infection [352]. HSV-1 primary route of transmission is through contact with oral mucosa, which leads to orolabial herpes and cold sores. HSV-2 is mostly sexually transmitted by skin to skin contact and causes genital herpes [352]. HSV-1 and HSV-2 cause a wide spectrum of diseases ranging from recurrent, painful oral and genital ulcers, to meningitis, encephalitis, neonatal infection, and keratitis [472]. These two subtypes share more than 80% amino acid sequence similarity in proteins and can cause oral or genital infections [473]. Initially, HSV-1 was primarily thought to be associated with oral infections, while HSV-2 was linked to genital infection, but HSV-1 has now been shown to cause the first episode of genital herpes and neonatal herpes in the developed world [474, 475]. In 2012, the global burden of HSV-1 was 3.7 billion people with age less than 50 years, which makes about 66% of total world population in this age group, with maximum prevalence in Africa, South East Asia, and western pacific countries [352]. Additionally, about 140 million in the 15–49 years age group are infected with genital HSV-1 infection. Globally 417 million people in 15–49 year age group are HSV-2 sero-positive, with incidence rate of 19 million per year [476].

HSV-2 infection increases the chances of HIV acquisition by 3 times [477] through genital mucosal inflammation associated with HSV-2 [478, 479]. Furthermore, genital ulcerative disease also increases the risk of HIV transmission [468]. An estimated 25–50% of HIV infections are attributable to HSV-2 in high prevalence regions [480, 481]. HSV-2 infection has an estimated $540 million total lifetime cost, which ranks 3rd after HIV and HPV among major sexually transmitted infections in the US [482]. The expenses on hospitalization in neonatal herpes [483] and contribution of HSV-1 and HSV-2 infections in HIV acquisition add further to this burden.

6.1.2 Characteristics, Morphology, and Virulence Factors

HSV-1 and HSV-2 are members of the neurotrophic alpha-herpesviridae subfamily, which is a hierarchical progeny of herpesviridae family of viruses. Besides HSV, Varicella Zoster Virus, Cytomegalovirus, and Epstein-Barr Virus are few well

known members of this family. Approximately, human herpesviruses measure about 200 nm in diameter and contain a linear, 150kb dsDNA genetic material. The genetic material is enclosed by a protein capsid, which is covered by a tegument and a glycoprotein rich envelope. In HSV, genes α (immediate early), β (early), and γ (late) regulate viral genome translation, transcription of viral transcription factors, and dynamics of viral particles via infected cells [484]. The glycoprotein D (gD) on the viral envelope is central to the process of adhesion of the virus to the host cell surface. Fusion is facilitated by gB, gH and gL glycoproteins [485]. After the viruses fuse with the host cell membrane, tegument proteins and the nucleocapsid are able to enter the host cell cytosol. Tegument proteins (such as VP16) hijack the host cell replication and translation machinery, to facilitate immune evasion and viral gene transcription [469]. Virion host shutoff protein (Vhs), a major viral endonuclease, is a member of the tegument protein family and degrades host mRNA in infected cells while protecting the viral mRNA at the same time [486]. The same protein blocks type I interferon system, dendritic cell functions, and inflammatory immune response in the affected individuals [487, 488]. This allows effective establishment of an infection in the infected individual. Despite considerable DNA sequence similarity between HSV-1 and HSV-2, the difference in envelope proteins endows them with distinct antigenicity [484]. HSV is acquired mainly through direct exposure of mucous membranes or abraded skin to the lesions or mucosal secretions of actively infected individuals. A major virulence factor of HSV is protein γ134.5, which has been found to play a significant role in inhibition of dendritic cell maturation [489]. This protein inhibits p65/RelA phosphorylation, NFkB activation, and nuclear translocation, which results in inhibition of dendritic cell maturation in HSV infection [489]. Collectively all these HSV proteins and factors help the virus to successfully establish an infection, especially in immunocompromised individuals.

6.1.3 Clinical Manifestations of Herpes

Immunocompetent children and adolescents are the most susceptible to acquiring primary HSV infection. The clinical features of primary HSV infection include painful and ulcerative vesicles in the skin and mucous membranes of the affected regions. These visible manifestations may follow a prodromal phase, which includes loss of appetite, malaise, muscle pain, and fever. The primary orolabial infection occurs on non-keratinized mucosal surfaces such as the labial and buccal mucosa, sparing the keratinized surfaces (gingival, hard palate or tongue dorsum) [490]. HSV is a neurotrophic virus, which infects epithelial cells at skin or mucosal surfaces, and travels via retrograde transport to the nerve ganglion, where it finally establishes persistent latent infection. When the virus moves from neurons to mucosa, asymptomatic shedding of viral particles follows. Although immunocompetent individuals rarely manifest severe complications from HSV-1 or HSV-2 infection, it can cause significant morbidity and mortality if the disease progresses to Bell palsy, meningitis, or encephalitis [470]. Immunocompromised individuals are the main victims of more complicated manifestations of HSV disease in terms of duration, reactivation, recurrence, and dissemination of disease.

6.1.4 HSV Interactions with the Immune System and Pathophysiology of Disease

TLR2 and TLR9 are the main players of the innate immune response to HSV by recognizing viral glycoprotein and DNA, respectively [491]. This interaction leads to the production of type I IFNs, which control initial infection via apoptosis of infected cells, recruitment of immune cells, and inhibition of viral protein expression. Few recent studies have shown increased susceptibility to herpes encephalitis in genetically TLR3 deficient individuals [492, 493]. This TRL3 deficiency in CNS cells results in poor type I interferon response, which may explain the increased susceptibility of the CNS to HSV infection [494]. The loss of pDCs and NK cells, which are heavyweights in immune response to HSV, also results in increased susceptibility and worse disease development [491, 495]. In addition to production of IFN-α leading to recruitment of NK cells and T cells, pDC play a role in stimulation of neighboring CD8 $^{+}$T cells even in absence of infection; thereby contributing to adaptive immunity [496]. Infected Langerhans' cells transmit HSV-1 and HSV-2 to human skin DCs expressing CD141 and DC-SIGN, which present antigens to various T-cell subsets [497]. Still, nothing concrete is known about the involvement and roles of (a) various DC subsets in CD4 $^{+}$T and CD8 $^{+}$T cell priming; (b) regional versus migratory DCs in T cell priming; and (c) DC subset critically important in context of HSV antigen.

The virus employs a variety of strategies to evade the host immune response in order to establish successful primary infection. The damage to various components of the immune system such as complement proteins, NK cells, major histocompatibility complex I or II molecules, or antibodies are few of the most commonly employed strategies [498]. The virion's attachment to host cells involves the interaction of HSV-1 glycoproteins B and C with heparan-sulfate proteoglycans in the host [499]. Interestingly, the ability of glycoprotein B to bind immunoglobulin like type 2 receptor alpha endows HSV-1 with infectivity towards cells lacking heparin sulfate glycans [500].

In HSV infection of skin or mucosa, dermal/interstitial DCs play a key role in presenting the viral antigen for priming CD8 $^{+}$T cells in regional lymph node [7]. Monocyte-derived DCs can be infected by HSV because they express HSV receptors nectin-1, nectin-2, and HVEM. Additionally, C-type lectin DC-SIGN on immature DCs can enhance infection of DCs by HSV. Immature DC infected with HSV do not progress to maturity and lose their antigen presenting capability because HSV downregulates expression of MHC I and costimulatory molecules like CD40, CD80, and CD86. HSV infected DCs do not respond to stimulus that induce or promote maturation such as TNF-α. Because HSV infected DCs have decreased capability to generate IL-12, Th1 immune response may not be generated, which likely favors viral spread [396]. HSV-1 and HSV-2 induce apoptosis of DCs, and apoptotic HSV-infected DCs along with HSV antigen are engulfed by uninfected bystander cells for subsequent cross-presentation to CD8 $^{+}$T cells in the lymphoid tissue. This counteracts the immune evasive strategy of HSV-induced downregulation of costimulatory signal and apoptosis of DC [11, 501]. Infected cell protein 47

(ICP47) is a protein in HSV that mediates the downregulation of MHC class I [7]. Although HSV downregulates MHC class I expression on epidermal keratinocytes, IFN-γ secreted by CD4 $^{+}$T cells upregulate suppressed MHC class I expression on HSV infected cells enabling recognition by CD8 $^{+}$T cells [396].

6.1.5 Treatment and Prevention

Treatment options are based on careful and thorough evaluation of clinical symptoms, immunocompetency status, site of infection, and primary or recurrent state of infection. Nucleoside analogues form the most potent category of drugs of choice against HSV. They range from the first drug against HSV-1, acyclovir, or its pro-drug valacyclovir to penciclovir or its pro-drug famciclovir [471]. The pro-drug formulations increase bioavailability of drug molecules in the body. Another treatment choice using acyclovir in combination with hydrocortisone has shown prevention of full-blown HSV outbreak, as it resulted in pre-outbreak symptoms only [502]. The major preventive measures for HSV include public education regarding the infectious nature and autoinoculation potential of the disease as well as promotion of barrier strategies for genital herpes infections such as condoms [484]. There is an urgent need for vaccine development because the current treatments mostly function to decreases the time to healing of lesions. In fact, treatment has minimal effect on the outbreak if viral replication has already started; this period can be as low as 8 hours after an encounter [503]. Immunocompromised individuals are even more burdened by this, because development of resistance to nucleoside analogues in these patients can lead to increased severity of disease ranging from the presence of life threatening atypical lesions and greater dissemination of virus [471].

6.1.6 Future Directions: Clinical Trials and Current Research

Major effort is needed to find both preventive and therapeutic vaccine candidates for HSV-2. Owing to homology in the two types, vaccines against HSV-2 may work against HSV-1. A number of reports have shown promise towards vaccine development in animal models using strategies ranging from replication of defective mutants and live attenuated strains, to neutralization antibodies and subunit vaccines [72, 504–506]. Unfortunately, none of these strategies have worked out at the translational stage. The most widely utilized candidates for HSV-2 vaccine are glycoprotein subunit vaccines. Surface glycoprotein D is responsible for most antibody neutralizing activity, making it a good target. The largest clinical trial of HSV subunit vaccine, Herpevac, didn't show efficacy against HSV-2, although it prevented genital HSV-1 disease with an efficacy of 58% [507]. GEN-003, a subunit vaccine of gD2 and ICP4, along with a matrix to stimulate T cell immunity, has been shown to decrease viral shedding by 55% in a phase IIb clinical trial [508]. These encouraging outcomes may lead to a successful vaccine development in future.

6.2 *Human Papilloma Virus*

HPV is a member of the *Papillomaviridae* family, a distinct nonenveloped virus family with dsDNA genomes. It is a causative agent of sexually transmitted disease in a majority of sexually active individuals via contact with lesions [509]. Low risk HPV types are associated with genital warts while high risk HPV types correlate with genital cancer [510]. Because of the carcinogenic nature of some subtypes of HPV, the virus is considered a significant health burden. HPV 16 and HPV 18 are the two most prevalent carcinogenic subtypes of HPV that are responsible for 71% of cervical cancers [510, 511]. HPV-associated immune evasion strategy includes downregulating antiviral innate immunity mediated by type I interferon, decreasing production of pro-inflammatory cytokines by keratinocytes, and increasing the expression of immunosuppressive cytokines with consequential downregulation of MHC class I expression [512]. There is no FDA-approved cure for HPV infection; however, current treatment options include tissue destruction by thermal or chemical methods. Immunoprophylaxis of HPV include quadrivalent HPV vaccine, trivalent HPV vaccine, etc [513].

6.2.1 Epidemiology and Disease Burden

HPV is the most common sexually transmitted infection worldwide [514]. The majority of sexually active individuals will likely acquire it at some point during their lives. Cervical HPV has an 11.7% prevalence worldwide, with some variation depending on geographical region; higher rates are observed at younger ages, with a general downward trend as age increases [509].

HPV carries a high health burden since some subtypes of HPV have been shown to be carcinogenic [515]. HPV has been shown to be a leading cause of cervical, head and neck squamous cell cancers (HNSCC) and other ano-genital cancers [511]. Persistent infection with HPV is a risk factor across all types of genital cancers. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 have been classified as high-risk, linked to high-grade dysplasia and particular cancer subtypes; HPV 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81 are classified as low risk associated with warts and mild dysplasia [510]. These low-risk HPV types are eventually cleared by the immune system. However, in immunocompromised patients, these infections can lead to papillomatosis and cancer [516]. HPV 16 represents the most prevalent carcinogenic type, followed by HPV 18 [510]. Together, these two subtypes represent 73% of malignancy of the anus, 38% of penile cancers, 58% of vagina cancers, 36% of the vulva cancers, and 71% of cervical cancers [510, 511]. Estimates of HNSCC attributable to HPV is 25.6% of cases [511]. Worldwide this accounted for 530,000 cases of cervical cancer and 21,400 of oropharyngeal cancer in 2008 directly attributed to an HPV infection [511]. Estimated financial burden in the U.S. is $8 billion: $6.5 billion attributable to cervical cancer screenings and follow ups, $1 billion for actual cancer treatment, and $288 million for treatment of genital warts and recurrent respiratory papillomatosis (RRP) [517].

6.2.2 Characteristics, Morphology, and Virulence Factors

Papillomaviridae is a distinct virus family of non-enveloped doubled-stranded DNA viruses [517]. The human subtypes of this virus family are limited to five genera: alpha, beta, gamma, mu, and nu [517]. The alpha subtype affects the mucosal epithelium while the beta genus infects the skin [518]. HPV is about 55 nm in diameter and contains an 8kb BP doubled stranded DNA circular genome which encodes 8 genes: E1, E2, E4, E5, E6, E7, E8, L1, and L2 [518, 519]. The icosahedral capsid of HPV consists of L1 major and L2 minor capsid proteins. L1 major capsid protein consists of 360 molecules organized into 762 pentamers whereas L2 minor capsid consists of between 12 and 72 molecules [520]. It is hypothesized that initial HPV binding involves interaction between viral protein L1 and heparan sulfate proteoglycans (HSPGs) on either the epithelial cell surface or basement membrane [518, 520]. This binding induces a conformational change mediated by cyclophilin B (CyPB), an endoplasmic protein that when secreted, is associated with HPSGs.

Because binding of HPV16 to HSPGs/CyPB does not mediate endocytosis, a second, yet to be identified receptor must be involved for infectious internalization [520]. Several candidates have been identified, including α_6 integrin and growth factor receptor (GFR) [520]. Once internalized, the viral capsids disassemble in the late endosome or lysosomes in a pH dependent manner. L2, which complexes with viral DNA, interacts with cellular machinery proteins such as sorting nexin 17, essential for lysosomal escape. L2 then targets the viral DNA to the perinuclear region of the cell through a pathway that utilizes Dynein-mediated transport and endocytic retromer components, leading the L2-DNA complex to the trans-Golgi network. Viral entry into the nucleus requires mitosis, which causes the barrier between nucleoplasm and cytosol to be removed and the trans-Golgi network to become dispersed [521]. Once in the nucleus, viral DNA replicates together with basal cell chromosomes. The early promoter is located in the upstream regulatory region adjacent to the E6 open reading frame (ORF) and is active early in infection. It directs expression of the E1 and E2 that leads to establishment of viral genomes as stable episomes with about 50–100 copies per cell, and tightly regulates E6 and E7 expression. E1 viral protein has DNA helicase and ATPase activities that catalyze the unwinding of DNA and recruits cellular replication machinery to viral origins. E2 is a DNA-binding protein that helps to load E1 onto the origins and ropes chromosomes and viral DNA during segregation. As HPV-infected cells divide and differentiate, the late HPV promoter found in the middle of the E7 ORF is activated causing expression of late gene products such as E4, E5, L1, and L2, as well as increased levels of E1 and E2 leading to genome amplification, virion assembly, and virion release. Viral proteins E6 and E7 are necessary for the differentiation-dependent life cycle of HPV and cell immortalization via their interactions with p53 and pRB respectively. How the overall differentiation-dependent life cycle of HPV is regulated is still not fully understood, and as such, it is under investigation, however, some studies show ATM DNA damage responses (DDRs) play an important role [522].

6.2.3 Clinical Manifestations of HPV

The clinical presentation of HPV depends on several factors such as the HPV type, the area of skin affected, and the immune status of the individual. The majority of HPV infections are asymptomatic and clear within 1 year [523]. If infection persists, it usually manifests as warts. Anogenital warts are most commonly caused by HPV 6 and 11. The warts are most often benign though can cause pain, discomfort, and itching; they are highly infectious, with 65% of infected patients passing it onto their sexual partners [524]. Common warts are typically caused by HPV types 1, 4, and 7, and are found more commonly in children and in immunosuppressed patients. These manifest as small, dome-shaped papules with a verrucous and kerotic surface. Plantar warts, associated mostly with HPV types 1 and 4, are found primarily on the soles of the feet and more commonly seen in children. Condyloma acuminatum presents as exophytic papillomatous lesions infecting the anogenital region and are often caused by HPV 6 and 11. Giant condyloma acuminatum of buschke and löwenstein is a large exophytic tumor that has a cauliflower-like appearance and is caused by HPV 6, 11, and 16. It can also present as undifferentiated intraepithelial neoplasia (grade I, II, or III) which can be distinguished into warty or basaloid subtypes. Epidermodysplasia verruciformis presents due to a rare, autosomal recessively inherited susceptibility to β-HPV subtypes. Respiratory papillomatosis, which is caused primarily by HPV 6 and 11, is a rare and life-threatening condition that can develop from failure to clear the virus. Finally, infection can manifest as intraepithelial neoplasia that can progress to malignancy. Most malignant cases are linked to HPV 16 and 18 [525].

6.2.4 Interactions with the Immune System and Pathophysiology of Disease

There is evidence that HPV infects epithelial cells and keratinocytes. The virus replication cycle takes a minimum of 3 weeks from infection to viral release, which equals the period of time for a differentiated keratinocyte to undergo complete differentiation and ultimately desquamate. Activation of TLRs on keratinocytes leads to signaling pathways mediated by Mal/Myd88 or TRIM/TRIF that initiate both the innate and adaptive immune responses. Genital tract keratinocytes have TLRs located either on its cell surface, 1-6, or in the endosome, 3 and 9. Activation of TLR3 by doubled stranded DNA such as HPV results in up regulation of TLR7, which then triggers release of type I interferons, α and β, leading to a predominantly Th1 cell-mediated immune response. Activation of NLR, another PRR on keratinocytes, leads to pro-inflammatory signaling pathways and pro-caspase 1, which ultimately cleaves pro-IL-1β and pro-IL-18. Keratinocytes can also be induced to make other important cytokines including IL1, IL6, IL10, and TNF-α. The secretion of these pro-inflammatory cytokines are essential for the activation of resident immune cells like Langerhans cells and macrophages, and for recruitment of effector T cells, which ultimately starts the adaptive immunity [526]. NK cell-mediated immunity is

important in controlling HPV, as loss of NK cell antiviral function is associated with loss of control of human HPV infection [527].

HPV employs various techniques to down regulate the immune response. The viral protein E7 inhibits IFN-α signaling pathway by binding to P48/IRF-9, preventing its translocation to the nucleus and formation of the ISGF-3 transcription complex. Additionally, E7 binds to IRF-1, inhibiting its activation of the IFN-β promoter for recruitment of histone deacetylases, thus preventing transcription. Experimentally, this has been shown with a reduction in TAP1, IFN-β, and MCP-1 genes, which are all IRF-1 target genes. Viral protein E6 binds to TYK2, which prevents it from binding to the cytoplasmic domain of the IFN receptor, preventing downstream phosphorylation of TYK2, STAT1, and STAT2 impairing the JAK-STAT pathway blocking IFN-α-mediated signaling [526]. Additionally, soon after infection, HPV up regulates the cellular deubiquitinase ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), which impairs PRR-induced NFκB activation by upstream interference with TRAF3, TRAF6 and NEMO [512]. Research showed that UCHL1 removes activation K63-linked ubiquitin molecules from TRAF3, suppressing the type 1 IFN pathway, and when bound to TRAF6, UCHL1 mediates enhanced degradation of NEMO, which suppresses the NF-κB pathway [528]. Additionally, in an in vitro model, it was shown that HPV up regulated the epidermal growth factor receptor (EGFR), which induced overexpression of interferon-related developmental regulator 1 (IFRD1). Ultimately, this impairs the acetylation of NFκB/RelA K310 in the keratinocytes, leading to a decreased pro-inflammatory cytokine production and immune cell attraction in response to stimuli of either the innate or adaptive immune pathways [512]. HPV viral proteins E2, E6, and E7 have been shown to increase IL-10 and other cytokines such as TGF-β production through trans-activation of different cell types, causing a decrease in MHC1 expression and limiting the effects of the immune response. Other research shows that high-grade cervical lesions correlate with a high viral load that produces an IL-10 immunosuppressive environment, with increased Treg cells [529]. Despite these defense mechanisms, 80–90% of genital infections resolve, typically with a cell-mediated immune response directed at E2 and E6 [526].

Persistent infection is often associated with disease development; it is the strongest risk factor for high grade cervical cancer as it is associated with cervical intraepithelial neoplasia (CIN) of grade 2 and 3, viewed as essential for the progression to cancer [523]. The switch from premalignant to malignant infection occurs by incorporating the viral genome into the cell's chromosomal DNA at fragile sites commonly targeted for deletion; E6 and E7 have been consistently shown to be expressed in cervical carcinomas. E6 and E7, acting as oncogenes, interfere with the normal functions of pRB and p53 at cell-cycle checkpoints, evading normal apoptosis pathways; they also act as potent mitotic mutators, increasing the likelihood of acquiring additional mutations necessary for cancer development [530]. Both of these factors are necessary in tumor progression in the human population.

6.2.5 Treatment and Prevention

Currently, two commercially available L1 VLP vaccines are Cervarix, a bivalent vaccine targeting HPV16/18, and Gardasil, a quadrivalent vaccine against HPV6/11/16/18. These vaccines are delivered intra-muscularly at three separate times [531]. Since 2006, 133 countries have licensed the vaccine and over 40 have introduced some kind of vaccine program with high efficacy. In Germany, incidence of anogenital warts was reduced by 47% for girls age 16 and 35% for girls age 18 by 2008 [532]. A CDC study showed a 56% decrease in vaccine-related HPV strains for U.S. girls ages 14–19 [533]. Countries with vaccination programs have also shown a decrease in anogenital HPV-related diseases in women and in men due to both the vaccination and herd immunity [534]. Additional research showed that vaccines stimulate effects of IL-15, DCs, and natural killer cells, leading to production of pro-inflammatory cytokines and generation of cytotoxic activity against HPV-positive tumor cells, which includes increases in antigen specific T-cell responses [535].

In October 2016, after FDA approval, the Advisory Committee on Immunization Practices (ACIP) began recommending a two-dose vaccination schedule beginning at age 9 through 14 for both boys and girls. If vaccination is started after 15 years of age up until 26, the three-dose schedule should be followed [536]. Current vaccine research is looking at L2-based vaccines since L2 peptides are conserved across HPV subtypes, and could lead to the further studies for a 9-valent vaccine [537, 538]. Despite vaccine efficacy as a prophylactic measure, treatment still remains quite limited and focused on amelioration of symptoms. There is no cure for HPV infection; in most cases, patients with HPV infection are asymptomatic and those that do present with symptoms will see those spontaneously resolve over time. Promotion of barrier protection during sexual intercourse and education on safe-sex practices are still crucial in preventing HPV infection. With the advent of the PAP smear, cytological findings can be used to determine if any high-risk HPV subtype is present and if the HPV has already begun to cause dysplasia, allowing for early detection of cancer. Current treatment options for patients with benign lesions include tissue destruction by thermal, chemical, or electrical methods, and more invasive surgical approaches for larger and more extensive lesions [513].

6.2.6 Future Directions: Clinical Trials and Current Research

Current research into therapeutic vaccines aim to generate cytotoxic T cell responses against the HPV early viral gene products E1, E2, E5, E6, and E7, since L1 and L2 are not seen in basal epithelial cells that have persistent infection. A few early trials focused on a vaccine for both prophylactic and therapeutic use via fusion of L2 and E7 or L2 and E6/E7; this showed some promise in early clinical trials, but therapeutic efficacy has not yet been proven. Early attempts to incorporate early viral proteins into L1 VLPs showed some promise by inducing a cellular response, but the response failed to reduce intraepithelial neoplasia [539]. Currently, after chemotherapy and radiation treatments fail, the 5-year overall survival rate of cervical

cancer is at 3.2–13%. Radio-immunotherapy targeted at E6 and E7 proteins have been shown as a promising treatment candidates in head, neck, and cervical cancers [540].

7 Conclusion

Viruses are obligate intracellular pathogens that come in different shapes, sizes and characteristics that give them unique abilities to invade and infect a host. The human body has physical and chemical barriers that attempt to block a virus from entering, but when these barriers are breached, the virus triggers our immune system to fight back. The innate immune response is led by NK cells, DCs, cytokines, and Interferons. The presence of PRRs on DCs serves an important immunosurveillance function, detecting potentially foreign particles and alerting the immune system of a breach in the immune defense mechanism. Together with viral PAMPs, this allows for initiation of the antiviral adaptive immune response [7]. During a viral infection response, DCs form immunological synapses with NK cells and T cells. DCs release cytokines that activate innate immune cells such as NK cells during the early phase of viral infection, followed by antigen presentation to T cells in order to drive adaptive immunity [11]. NK cells are activated by IL-12 and IFN-α produced by myeloid DCs and pDCs respectively [11]. The Dendritic Cell-Natural Killer cell crosstalk involves the activation of NK cells by cytokines (IL-12, IL-15, IL-18, and type I interferons) secreted by DCs at the site of viral infection [7]. DC-NK cell cross talk yields reciprocal activation, wherein IL-12/IFN-α activate NK cells and IFN-γ and TNF-α produced by NK cells facilitate the maturation of DCs [11]. Type I interferons generated during the innate immune response to a virus creates an antiviral microenvironment that inhibits viral infection via inhibition of viral replication in both infected and noninfected cells [5]. Cell mediated antiviral immunity is mediated predominantly by effector $CD8^+$T cells. CTL-mediated antiviral function includes killing of infected cell via the perforin/granzyme pathway, leading to secretion of antiviral pro-inflammatory cytokine IFN-γ, and other mechanisms [5].

The interaction between virus and DC leads to different outcomes with viruses triggering T cell mediated antiviral immune response via various mechanisms of antigen presentation [6, 7]. This interaction promotes activation and amplification of T cell immune response to viral infection, such as virally infected tissue-derived migratory DCs presenting endogenously derived viral antigen complexed to MHC class I and II molecule to $CD8^+$T cells and $CD4^+$T cells respectively [4, 7]. Furthermore, endogenous viral antigens detected in lymph nodes could be derived from free virus draining from the peripheral tissue via afferent lymphatics. On the other hand, tissue-derived migratory DCs may take up antigens from virally infected epithelial cells that have undergone apoptosis, and cross-present these exogenously derived viral antigens complexed to MHC I to $CD8^+$T cells in the regional lymph node [4]. When tissue-derived migratory DCs carrying processed viral antigen undergo apoptosis, the apoptotic tissue-derived migratory DC with components of the viral antigen could be taken up by an uninfected bystander DCs for onward

trafficking to the regional lymph node for further presentation to naïve T cells [7]. However, under certain conditions, lymphoid-resident DCs may either take up exogenously generated viral antigen from infected tissue-derived migratory DCs or engulf fragments of apoptotic infected tissue-derived migratory DCs loaded with exogenously generated viral peptide and cross present these viral peptides via MHC class I molecules to $CD8^+$T cells [4, 7]. Thus, dendritic cells play a central role in priming naïve T cells to generate virus-specific T cells via expression of costimulatory molecules, presentation of endogenously and exogenously-derived viral antigens complexed to MHC class I molecules to $CD8^+$T cells, presentation of MHC class II restricted viral peptides to $CD4^+$T cells, and polarizing naïve $CD4^+$T cells to generate Th1 cells [4, 6].

It was mentioned earlier that surface attachment receptors such as DC-SIGN, mannose receptor, Langerin, and immune dendritic cell receptor (DCIR) facilitates the uptake of viruses. Binding of virus (e.g. HSV, DV, Ebola virus, HCV) to DC-SIGN on DC results in the development of an infectious synapse that facilitate the transfer of virus from DCs to lymphocytes and secretion of DC-derived antiviral pro-inflammatory cytokines [6]. Langerhans cells do not express DC-SIGN but express langerin that mediates internalization and degradation of HIV in Langerhans cells. DEC-205 (CD205) is a pan-DC specific surface marker that is significantly upregulated on mature DC and function to target antigens to MHC I and II molecules with intent of priming naïve T cells. DCIR is a C-type lectin surface receptor that is involved in virus uptake by DC [6].

Stability of the DC-T cell interaction following viral infection shapes and drives activation and differentiation of T cells into viral specific T cells as well as optimizes the expansion of memory T cell pool [4]. $CD4^+$T cells provide primary and secondary antiviral protective immunity through various mechanisms. Effector $CD4^+$T cells are important in antiviral adaptive and innate immunity because they promote the generation of cytotoxic $CD8^+$T cell response and maintain the memory $CD8^+$T cell pool as well as enhances innate antiviral function [8]. Memory $CD4^+$T and $CD8^+$T cells (tissue-resident memory T cells) residing at the site of infection provide first line defense against viral re-infection by facilitating rapid recruitment and activation of innate immune cells capable of controlling initial viral titers, while simultaneously interacting with DCs to promote the generation of viral specific adaptive immunity. As such, DCs could be activated through both PRR-mediated activation and recognition of viral antigen by memory $CD4^+$T cells such as tissue-resident Th1-polarizing memory $CD4^+$T cells [8]. Activation of DCs by virus specific tissue resident memory CD4 T cells is associated with heightened intense early inflammatory response and optimal upregulation of MHCII, CD40, and CD86 [8]. When DC laden with viral antigens complexed to MHC class I molecules interact with $CD8^+$T cells in the lymph nodes, DCs and $CD4^+$T cells mediate amplification of memory $CD8^+$T cell recall. As such, DCs are capable of presenting endogenous and exogenous viral antigen peptide to T cells due to the presence of MHC and costimulatory molecules required to supply the necessary signals to ensure T cell activation, proliferation, and differentiation into effector T cells [4].

When viruses invade and colonize a host, they employ immune evasive mechanisms that enhance their survival within the host. Viruses can evade innate immunity

by interfering with PRR-mediated activation of dendritic cells and production of antiviral pro-inflammatory cytokines [11]. Some viruses inhibit the function of antiviral pro-inflammatory cytokines as well as antagonize cell-mediated immunity. Viruses may evade CTL-mediated killing by interfering with antigen processing, transportation, and presenting, which culminates in the non-presentation of viral peptide complexed to MHC I molecule on the surface of infected cells [5]. Although infected cells with absent or altered MHC I molecules are susceptible to killing by NK cells, HIV and HSV are capable of evading NK cell mediated killing [527]. Some viruses such as HSV and HIV may cause latent and cell-to-cell infection as a strategy of evading humoral immune recognition [5]. In latency, there is little/no viral replication and effector $CD8^+$T cells are maintained to inhibit viral reactivation [2]. In viruses with persistent replicative lifestyles, the constant stimulation of viral antigens correlate with impaired development of memory $CD8^+$T cells, exhaustion of virus-specific $CD8^+$T cell due to chronic $CD8^+$T cell stimulation with reduced antiviral pro-inflammatory cytokine production, and $CD8^+$T cell-mediated tissue damage [2]. The failure of the CTL-mediated cytotoxic immune response allows for the persistence of the virus in the host [5]. It is of note that DC and virus interaction determines T helper (Th) polarization and viral pathogenesis; however, some viruses favor viral persistence by polarizing $CD4^+$T helper response toward Th2-mediated immune response and generation of cytokines that suppress Th1-mediated immune response [6]. Moreover, transient IL-10 production during virus infection under conditions of high levels of viral antigen and costimulatory signals can promote or contribute to viral persistence by preventing excessive activation of DCs and T cells, which might limit efficacy of the antiviral immune response as well as prevent inflammation and immunopathology [3, 8].

The majority of the viruses discussed in this review do not have an FDA-approved therapeutic agent and/or vaccine. Because of the high mortality and morbidity rate associated with viral diseases, there is an urgent need to re-evaluate current antiviral agents, both therapeutic and prophylactic. Further, there are no FDA-approved antiviral agents for treating adenoviral infections, cidofovir and ribavirin have, however, demonstrated anti-HAdV activity in high risk individuals [12, 16, 45]. Immune-prophylaxis via live oral vaccine have been demonstrated to be beneficial for preventing adenovirus types 4 and 7-induced respiratory infections in military recruits [12]. Although there are no efficacious antiviral agents for treating measles infection, IFN-α and ribavirin have demonstrated anti-measles activity [97]. MMR is a well-established effective live-attenuated vaccine recommended in children for the prevention of measles, mumps, and rubella infection [13, 76]. There is ongoing research into exploring ways to enhance the efficacy as well as facilitate storage and administration of current vaccines. Also, there are no FDA-approved vaccines or antiviral agents for HPIV infection [57]. Ribavirin is used as an antiviral therapeutic agent in treating patients with an RSV infection, Palivizumab and RSV-IGIV are FDA-approved passive immunoprophylactic agents for high risk groups [65, 106]. Because there are no vaccines for the pediatric respiratory pathogens RSV and HPIV [32] that cause serious respiratory tract infection in this population, a combined RSV/HPIV3 vaccine would be most appropriate for young children. It has been reported that NIAID/MedImmune programs are currently evaluating pediatric

HPIV vaccines with the intent of having one of the investigational vaccine progress to phase III clinical trials [61]. Currently, prevention of Dengue fever is focused on minimizing the exposure to vectors that facilitate transmission of Dengue virus. This may take the form of controlling mosquito infestation with the intent of preventing mosquito bites [206]. Research in Dengue virus is geared towards developing an effective vaccine that would generate appropriate correlates of immunity against all serotypes of Dengue virus [230, 231]. Studies of live-attenuated Yellow fever-Dengue chimeric vaccine has demonstrated efficacy against serotype 1, 3 and 4 of Dengue virus but not against the DENV-2- serotype [208, 209]. Because pregnant women are generally excluded from clinical trials, current research into ZIKV vaccine is focused on developing nonreplicative vaccine strategies due to their enhanced safety profile in pregnant women [216, 244].

Owing to the absence of an effective therapeutic and/or preventive HIV vaccine, management of HIV is focused on education and FDA-approved ART [356, 357]. HIV vaccine that generates broad neutralizing antibodies to prevent cell-free viruses from infecting other permissive cells in the host, and CTL to induce apoptosis of HIV infected cells would be the preferred vaccine [420]. HIV therapeutic approach may involve the use of passive immunotherapy that utilizes broad neutralizing antibodies to prevent HIV from infecting host cells [422–424]. It has been reported that combination of antiviral and IFN therapy may have anti-HTLV-1 activity [436]. HTLV-1 is a disease of significant health burden because it causes neurological impairment and bone marrow malignancies. There are no vaccines for HTLV-1, and therefore, therapy is focused on education and preventive measures to limit or restrict transmission [453, 454, 460].

Acyclovir, valacyclovir, and panciclovir are FDA-approved nucleoside analogues that provide antiviral activity against HSV. A significant burden of HSV is the potential of resistance to anti-HSV therapy, particularly in immunosuppressed individuals, and as such, efforts should be stepped up in developing an effective preventive HSV vaccine [471]. There is ongoing research geared toward developing an effective preventive and therapeutic HSV-2 vaccine. However, the focus is on education and modification of social behavior in high-risk groups that could limit HPV transmission, as well as routine screening protocols. Although there is an effective quadrivalent HPV vaccine that targets HPV 6/11/16/18, but ongoing research is focused to develop L2-based HPV vaccines [537, 538].

RNAi-mediated viral gene silencing has been shown to inhibit viral replication of adenovirus, RSV, HIV, and HSV; however, viruses such as HIV can mutate the target sequence. A mismatch of the specific target for RNAi can induce drug resistance [44, 33]. The advantage of using siRNAs is that they can target conserved regions of viral genome and sites of viral replication. In addition, efficacy of RNAi is independent of immune status of the recipient, and as such, its effective in individuals with an immunosuppressed or underdeveloped immune system [102]. Many viruses cause persistent viral infections, which may take the form of a latent, chronic infection or transformation of host cells. Conventional dendritic cells play a crucial role in interactions between DC and viruses. Crosstalk between DCs and immune cells that mediate cell-mediated cytotoxicity is crucial for generating IFN−γ that enhance maturation and functionality of DCs. Most viruses do not have

FDA-approved antiviral therapeutic and prophylactic agents. Hence, future directions should target on developing vaccines that can induce $CD8^{+}$T cell responses and produce IFN-γ to promote and secure a Th1-biased cell-mediated immune response. Because of the high mortality and morbidity rate associated with chronic viral diseases, there is an urgent need to re-assess the therapeutic index of current available antiviral agents with research focusing on developing novel anti-viral agents with enhanced efficacy and safety profile.

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Applications of Cutting-Edge Immunoproteomics Technology in Human Immunotherapy

Joseph Comber and Ramila Philip

Abstract Harnessing the ability of the immune system to mount robust and effective responses in the face of pathogenic challenge or cancer development is rapidly developing into frontline treatment for these diseases. This field, called immunotherapy, relies on the activation of antibody mediated B cell and/or cellular mediated T cell responses that directly target diseased cells and tissues. One of the most challenging aspects of developing effective immunotherapeutics, however, is first identifying the target antigens that the immune system should recognize and 'attack'. Among the many methods available today immunoproteomics is ideally suited to identify relevant target antigens. Immunoproteomics combines cutting edge proteomic methodologies to identify physiologically relevant target antigens expressed and/or produced by the diseased cells with standard immunological techniques to validate these targets. In this topic, we explore how immunoproteomics can shape the development of effective immunotherapeutics. We focus primarily on immunotherapies harnessing the cell mediated arm of the adaptive immune system and review promising clinical data on T cell-based immunotherapies in cancer, infectious diseases, and autoimmune disorders.

Keywords Major histocompatibility complex · T cell epitope · Cytotoxic T cells · Active immunotherapy · Epitope prediction · Vaccine development

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1 Introduction

The immune system has a monumental task. In the simplest terms, it must protect the host from cancers and infectious disease while carefully regulating responses so as not to inflict any long-term damage of host tissues. These immune responses are not perfect: cancers do develop even in the face of an initial immune response; infectious diseases do overwhelm the immune system and claim lives; and autoimmune diseases are a cause of significant pathology in those afflicted. Despite these imperfections, a tremendous amount of data indicates harnessing the beneficial responses of the immune system provides innovative possibilities to treat patients suffering from cancers, chronic infections, and autoimmune diseases. This field, known as immunotherapy, has rapidly developed over the past 2 decades and produced a number of effective treatments mainly in the cancer arena. Immunotherapies are based on either antibody mediated (humoral immunity) or cell mediated immunity by activating T cell immune response.

Cell mediated immunity, driven by $CD4^+$ and/or $CD8^+$ T lymphocytes, plays a critical role in in defending the host against cancers and infectious diseases. These responses begin in secondary lymphoid organs (i.e. lymph nodes or spleen) when dendritic cells and/or macrophages present fragments of protein antigens, termed peptide epitopes, to the T cells. These peptide epitopes are generated via a number of antigenic processing pathways. Antigens endocytosed from the extracellular environment are broken down in the endosomal/lysosomal system and loaded onto major histocompatibility class (MHC)-II molecules for presentation to $CD4^+$ T cells. In contrast, antigens biosynthesized inside of the presenting cell are broken down by the proteasome, peptide fragments shipped into the endoplasmic reticulum, trimmed further, and loaded onto MHC-I molecules for presentation to $CD8^+$ T cells. Importantly, there is a great deal of overlap between these pathways; endocytosed antigenic fragments can be processed by the proteasome and load onto MHC-I molecules while biosynthesized antigens can be processed in the endosomal/lysosomal system and loaded onto MHC-II molecules. This "cross-talk" undoubtedly broadens the number of targets important for an efficient cell mediated response. In normal, healthy cells, self-proteins go through these pathways and an array of peptides is displayed on the MHC molecules. These peptides are recognized as 'self' and therefore do not provoke a T cell response. However, changes in this MHC signature of cells alert T cells to changes in the host that may be associated with infection, malignant transformation, or other abnormal cellular processes, resulting in a cascade of events that induce a cell mediated immune response. In this case, when the right "match" is found, the properly matched T cell clone is activated, expands, and migrates to the tumor or site of infection to mediate effector functions.

Currently, one of the major challenges in the development of immunotherapies is the lack of clearly defined peptide epitopes capable of being recognized by T cells. The identification of such antigens in cancers, infectious diseases, and autoimmunity could provide the basis for a therapeutic vaccine, or for the stimulation of more effective T lymphocytes for adoptive immunotherapies (Fig. 1). Among the many

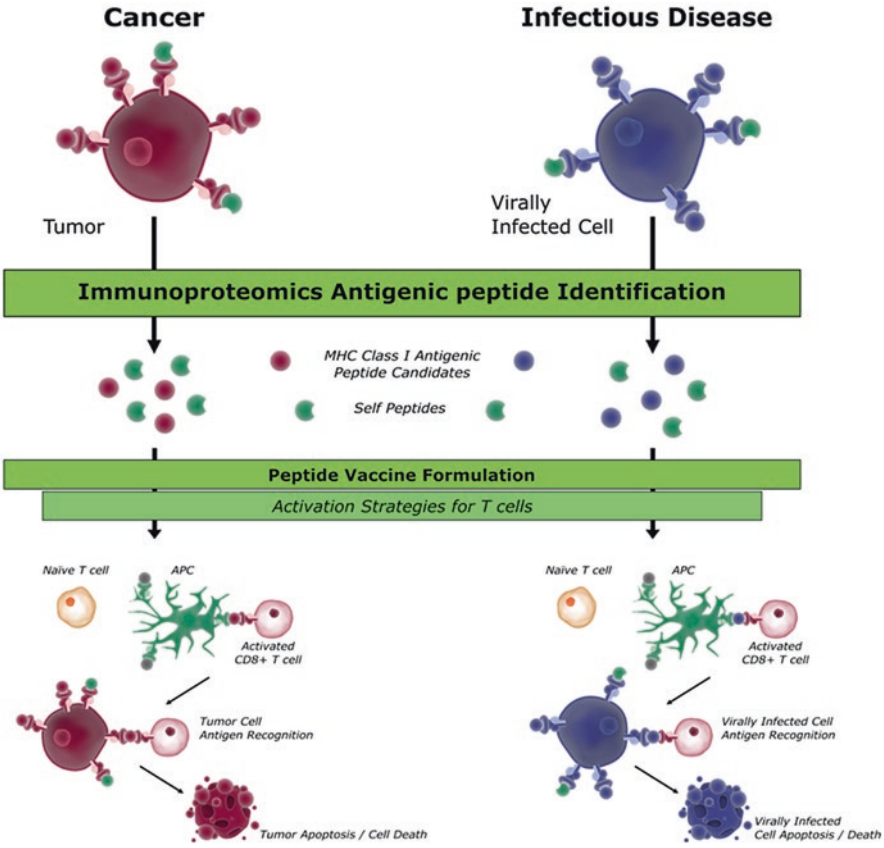

Fig. 1 Immunoproteomic approach for identifying antigens for T cell vaccines

methods available today, immunoproteomics, which is the combination of immunology and the tools of proteomics in particular mass spectrometry, is ideally suited to study these immune responses at a molecular level and identify physiologically relevant peptide epitopes. This topic explores the use of immunoproteomics as a tool for immunotherapy in cancers, infectious diseases, and autoimmune disorders. We focus primarily on immunotherapies harnessing the cell mediated arm of the adaptive immune system and review promising clinical data on T cell-based immunotherapies.

2 Immunoproteomics as a Tool to Identify T Cell Activating Epitopes

Identification of new antigens is limited by certain aspects of the currently available technologies. For example, differential genomic and proteomic approaches identify over- and under-expressed proteins but are unable to identify very low abundant

proteins that are often processed and presented by the MHC molecules as the true recognition targets for T cells. Indeed, the level of protein expression does not always correlate with MHC processing and presentation [1]. Therefore, the most appropriate method for identifying truly relevant antigenic peptides is to identify those naturally presented by the MHC molecules by direct immunoproteomics analysis.

2.1 Genetic Approaches

One of the first methods used to identify specific peptides was a genetic approach in which antigen presenting cells were transfected with cDNA from tumor cells resulting in the expression and subsequent processing and presentation of peptide epitopes. A number of epitopes were identified in melanoma using this methodology: an HLA-A1 restricted epitope from MAGE-1 [2], an HLA-A2 restricted epitope from tyrosinase [3], and an HLA-A2 restricted epitope from MART-1 [4]. However, this methodology has some major limitations including differences in the ability of transfected antigen presenting cells to post-translationally modify proteins, thereby impacting epitope discovery [5]. Perhaps more importantly, transcription and translation of cDNA in different cell types may not generate physiologically relevant epitopes. Antigen presenting cells (APCs), whether professional APCs, infected cells, or malignant cancer cells, have different levels of proteolytic activity [6, 7]. Therefore, epitopes generated in the APC transfected with the cDNA may not be the same as those generated in the infected or malignant cell itself. Although the genetic approach identified a number of cancer peptide epitopes, it was not highly successful in doing so in malignancies other than melanoma.

2.2 Overlapping Peptide Libraries

In this method, proteins (or the entire proteome) of a pathogen or tumor cells are synthesized in 9–20 amino acid stretches and overlapped to an extent that ensures every possible epitope can be presented to cognate T cells [8, 9]. The peptides are assembled into libraries, tested "matrix style" [10], and the libraries that induce T cell responses are teased apart until a number of single peptides that stimulate T cells have been positively identified. Improvements in technology have allowed for this method to be coupled with software to optimize the peptide pools [11]. In this way, the overlapping peptide method allows for the discovery of both MHC class I and class II epitopes in the context of multiple MHC alleles. However, a major disadvantage of this method is it may not identify epitopes that are naturally processed and presented during infection *in vivo*. This is largely due to the processing and presentation necessary to generate epitopes, and the peptides may not reach the appropriate intracellular compartment necessary for processing. Thus, epitopes

identified by this method may not accurately reflect the clinically relevant epitopes for immunotherapeutic formulations.

2.3 Motif Prediction Algorithms

Epitope predicting algorithms are a commonly used method for identifying T cell epitopes, screening the protein sequences for peptide segments predicted to bind to one or more HLA alleles [12, 13]. These prediction algorithms can maximize cross-HLA coverage [12] an important consideration since vaccines formulated with epitopes restricted by HLA "supertypes" might provide the broadest possible coverage for the population [14]. A number of algorithms exist for this purpose including SYFPEITHI [15], RANKPep [16, 17], and the newly developed MetaMHCpan [18]. Computerized predictors have value in identifying epitopes; however, they typically sort potential high binders based on predicted binding scores for the HLA molecule, and usually only the top scoring, or dominant peptides are chosen for further studies. The dominant peptides are then validated by screening circulating CTLs from cancer patients or virus infected individuals to ensure these peptides will activate the T cells. However, there are some significant disadvantages to peptide prediction algorithms. First, selecting only the dominant, or "top scoring" peptides will undoubtedly miss T cell activating epitopes, including those that are subdominant but still clinically relevant as we and others have previously described [19–23]. Secondly, the peptides identified by motif prediction may not be processed and presented at all *in vivo*. As is the case for genetic approaches, because different APC subsets have different processing capabilities, it is likely the epitopes generated *in vivo* may differ substantially from the dominant epitopes predicted from a linear protein sequence by these algorithms. To this end, when Zhong et al. compared motif prediction with mass spectrometry analysis in the identification of naturally processed and presented epitopes derived from influenza virus in a murine model, only 6 of the 16 epitopes that stimulated T cell response were high MHC binders [24]. Reliance only on peptide prediction algorithms is likely to miss a large majority of clinically relevant T cell epitopes.

2.4 Immunoproteomic Method

Within the past decade, direct identification of HLA associated epitopes has emerged as an alternative to the motif and overlapping peptide library methods, a technique termed immunoproteomics. This analysis is based on direct isolation of HLA-peptide complexes from infected or cancer cells and elution of the bound peptides from the HLA molecules. The eluted peptides are then subjected to high-performance liquid chromatography fractionation [25, 26] combined with mass spectrometry [27–29]. The identified peptides can be validated in a number of ways including *in*

vivo using animal models and *in vitro* with cells isolated from actively infected or seropositive individuals [19–21, 23, 30]. There are a number of significant advantages in using this approach to identify T cell activating peptides. First and most importantly, this method allows for the identification of epitopes that are naturally processed and presented during an infection or malignant transformation. As such, these epitopes represent the most physiologically relevant targets and have the potential to be clinically relevant for including in vaccine formulations. Secondly, this method allows for the identification of epitopes that can bind to multiple HLA molecules and with varying affinities (i.e. dominant vs. subdominant) without increasing experimental difficulty. In all, identifying peptides bound to different HLA alleles and/or multiple HLA alleles will be crucial for any vaccine development.

3 Immunoproteomics, Immunotherapy, and Cancer

Transformation of normal cells to malignant cells involves various pathways including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction induced by gene mutations and endogenous and exogenous factors [31]. These transformation pathways usually dysregulate proteins associated with the transformation processes and thereby alter the peptide repertoire associated with MHC molecules on the surface of the cells potentially marking them for detection and destruction by the immune system [32–35]. Thus, the interaction between cancer and the immune system plays a pivotal role in cancer development. However, immune system destruction of cancer cells is not as straight-forward as it seems. Cancer patients are immunosuppressed due to several factors including low frequency of anti-tumor reactive T cells, presence of regulatory T cells and various tumor induced soluble factors [36–38]. Based on these observations, various immunological methods that eliminate antitumor immunosuppression and/or increase antitumor immunity have been successfully developed for the treatment of various cancers.

3.1 Passive Cancer Immunotherapy

Immunotherapy based on the adoptive transfer of tumor-specific lymphocytes dates back several decades [39, 40]. Clinical studies using adoptive transfer of activated T cells, such as lymphokine activated killer (LAK), cytokine-induced killer (CIK) and tumor infiltrating lymphocytes (TIL), are the passive immunotherapy strategies that have been shown to be effective against cancer [41]. The development of adoptive cell therapy started with the generation of interleukin-2 (IL-2) activated LAK cells for cancer treatment [42]. LAK cells have been used to treat tumors such as colon cancer, pancreatic cancer, adrenal gland cancer, esophageal cancer, renal cancer, and

sarcomas in a nonspecific manner as a passive immunotherapy [43]. Although early clinical evaluation of LAK therapy in melanoma showed promising results, clinical efficacy of LAK cell immunotherapy in other cancers appeared to be relatively low and therefore, LAK cell therapy is not currently used in cancer patients. Similar to LAK cell therapy, CIK adoptive cell therapies have been tested in the clinic and showed no sustainable clinical response. The clinical ineffectiveness of these non-specific therapies may be due to the lack of antigen specificities of these T cells. In order to overcome this problem, various antigen specific adoptive cell therapies have been pursued. When antigen pulsed dendritic cells (DC) were used to activate CIK cells, there were significantly increased anti-tumor activities and an increased tumor progression free survival in patients with non-small cell lung cancer [44, 45]. Combination of DC-CIK cell therapy with high-dose chemotherapy also demonstrated progression-free and overall survival in patients with metastatic breast cancer [46]. A number of similar studies are ongoing to confirm the effectiveness of DC-CIK cell therapy. Similarly, tumor infiltrating lymphocytes (TIL) present in many cancers have been shown to play a critical role in tumor development and regression [47–49]. TILs isolated from patients and expanded *in vitro* with IL-2 have been used for clinical application by adoptive immunotherapy in various cancers and induced significant tumor regression, suggesting that adoptive cell therapy with antitumor TIL was an effective method for cancer treatment. However, it is not feasible to obtain TILs from all cancers. Therefore, genetic methods to modify T cells to increase antitumor activities for adoptive cell therapy of cancer patients have recently been developed. Two types of genetically engineered T cells currently being evaluated in clinical studies are (1) gene modified T cell receptors (TCRs) specific to tumor antigens and (2) chimeric antigen receptors (CARs) modified T cells. TCR modified T cells have shown significant anti-tumor activity in various cancers [50–52]. T cells engineered with a CD19- specific CAR induced long term eradication of B cell acute lymphoblastic leukemia (B-ALL) and primary human pre- B-cell acute lymphoblastic leukemia [53, 54]. Recent promising clinical effectiveness of adoptive cell therapy using genetically engineered T cells with antitumor activity seems to be effective in cancer treatment. CAR T cells recognize MHC-non-restricted antigens on the surfaces of target cells, whereas TCR modified T cells recognize antigens that have been processed and presented as peptide complexes with MHC molecules, thus varying clinical efficacy and limitations.

3.2 Active Cancer Immunotherapy and the Importance of Immunoproteomics

As opposed to passive immunotherapy using adoptive transfer of activated or gene modified T cells, active immunotherapy or therapeutic cancer vaccines are strategies aimed to activate a patient's own immune system to generate tumor specific T cells. These active immunotherapies require the knowledge of cancer specific antigens presented by the tumor cells and a vaccine delivery system capable of activating T cells *in*

vivo. Identification of appropriate tumor antigens has been the focus of cancer immunotherapy for many decades. Tumor development and maintenance of malignant phenotypes is driven by a wide range of abnormal cellular events including genetic mutations resulting in changes of protein coding sequences, deletions, insertions, and the abnormal expression of critical genes involved in cancer transformation pathways [55]. Antigens encoded by these dysregulated proteins in a transformed cell are likely to be unique to tumors. Effective therapeutic cancer vaccines must take advantage of these genetic changes by selecting proteins involved in these cancer pathways in order to induce tumor specific T cell responses [56]. Peptides presented by MHC class I molecules reflect the changes that occur in the transforming cell from the normal state, described as "nature's gene chip" by Shastri et al. [1], which could serve as targets for cancer immunotherapy. Therefore, surveying peptides presented by the MHC-I molecules on the diseased cell surface will reveal novel T cell targets for potential immune intervention as tumors have a distinct surface presentation of peptides compared to their normal counterparts [57]. Analysis of the peptide repertoire associated with the MHC class I molecules of cancer cells therefore provides a source for new tumor antigens for development of cancer immunotherapy (reviewed in [58]). Although normal tissues may express the antigen-coding genes, due to the differences in the regulation of expression and proteasomal processing, normal tissues in general do not present these antigenic epitopes in association with MHC-I molecules [57]. Due to the lack of presentation of the epitopes in the context of MHC molecules in normal cells, the CTLs do not recognize normal tissues and therefore are tumor specific and limit the risk of autoimmunity [59]. The large number of peptide/MHC-1 (pMHC-I) complexes expressed at the cell surface combined with multiple pathways to generate epitopes provides a great resource for identifying physiologically and clinically relevant tumor specific or tumor associated antigens. Undoubtedly, an examination of the peptides complexed with MHC-I molecules will reveal novel and immunogenic epitopes capable of inducing effective $CD8^+$ T cell responses. However, despite a growing body of literature indicating that $CD8^+$ T cells are naturally activated during an anti-tumor response [60–62], these anti-tumor T cell responses often fail to eradicate tumors, in part due to suppression in the local tumor environment [63, 64] and/or T cell induced exhaustion from continual antigen stimulation [65, 66]. However, combination therapies incorporating cancer vaccines with various drugs and checkpoint inhibitors to reverse the exhaustion phenotypes of $CD8^+$ T cells are attractive and feasible methods to generate robust anti-tumor responses [65, 67, 68].

3.3 Immunoproteomic Applications in Clinical Cancer Immunology

Cancer vaccines based on MHC class I associated peptides identified by immunoproteomics method are being tested in the clinic with promising results [69] (Table 1). To date, PROVENGE, Sipuleucel-T is the first FDA-approved therapeutic cancer vaccine for patients with metastatic prostate cancer [70]. Most of the peptide-

Table 1 Current and future areas of development for peptide vaccines

Indications	Conditions for which vaccines are in progress *or* may benefit from vaccine development
Cancer	Melanoma, Breast, Ovarian, Lung, Colon, Renal cell, Kidney, Pancreas, Gastric, Glioblastoma, Bladder, hematological malignancies,
Infectious diseases	Malaria, Falciparum Malaria, Anti-Plasmodium vivax, Influenza, HIV, HCV, HBV, CMV, Pneumococcal, genital Herpes—Herpes Simplex Type II, Tuberculosis,
Autoimmunity	Insulin dependent diabetes mellitus, T1D Diabetes Mellitus, Type One, Cat allergy, Allergy, Diabetes, Diabetes Mellitus, Type One, Cat allergy, Ragweed allergy, Grass allergy, Asthma, House dust mites – Rhinoconjunctivitis,

based vaccines tested in the late stage clinical studies include peptides identified by motif prediction methodology with fewer exceptions mainly in melanoma, renal and colon carcinoma. The majority of the peptide vaccine clinical studies were performed pre-realization of existence of regulatory T cells and checkpoint inhibitors that modulated peptide vaccine responses *in vivo*. However, a number of peptide-based vaccines with and without immune modulator combinations have shown success in various cancers.

The majority of the pioneering work was done in melanoma as many well described MHC class I restricted epitopes were identified and tested in the clinic. A clinical study with MAGE-1 peptide vaccine was the first to be tested with limited success [71]. Still, this study was important as it reinforced the idea that $CD8^+$ T cells could be induced to generate an anti-tumor response. Recent studies with peptides identified by immunoproteomic methods utilized a multi-epitope approach in order to induce a broader range of T cell specificities and potentially overcome the problem of antigen loss variants that arise during cancer progression [58, 72, 73]. In these early vaccine studies various adjuvants and cytokines were combined with multiple peptides for vaccination with some clinical efficacy [74]. Data from 115 patients with stage IV melanoma demonstrated functional responses to the peptides (as judged by IFNγ secretion) and were correlated with clinical responses including overall survival and complete and partial remission [74]. The inclusion of cytokines as adjuvants had mixed responses for peptide based vaccines in melanoma [75] with a possibility of accumulation of regulatory T cells (T_{REGs}) [76]. Dendritic cells are considered one of the most important antigen-presenting cells in initiating an immune response and as such have received much attention in designing peptide-based vaccines for cancers. Melanoma peptides, tyrosinase and gp100 pulsed dendritic cell vaccines also induced variable and limited clinical responses in metastatic melanoma patients [77, 78]. Despite these earlier variables promising clinical responses in melanoma, researchers are searching for the most tumor specific peptides and ways to improve the immune responses in patients.

In contrast to melanoma vaccines, peptide vaccines for colorectal cancer have typically relied on a single peptide injected with adjuvant, usually Montanide ISA-51. A single survivin peptide without an adjuvant showed no clinical response in patients with colon cancer [79], although a minor increase in survivin tetramer

positive $CD8^+$ T cells was observed in a few patients. However, survivin peptide with adjuvant and IFNα showed some response including stable disease and a decrease in the CEA (a marker for colon cancer) levels in patients with unresectable colon cancer [80]. Other motif predicted peptide-based vaccines have been tested clinically but these do not induce $CD8^+$ T cell responses. Notably, vaccination of patients with an extended p53 peptide induced sustained $CD4^+$ T cell responses [81] that were enhanced (i.e. higher levels of IFNγ) when administered with IFNα [82]. Early stage clinical trials were conducted using peptide antigen pulsed dendritic cells (DC). DCs were pulsed with peptides derived from CEA, Her2-neu, MAGE-2, and MAGE-3 induced $CD8^+$ T cell responses [83] with no significant clinical benefits. When DCs pulsed with the CEA peptide CAP-1 compared to DCs electroporated with CEA mRNA, $CD8^+$ T cell responses were detectable only in the electroporated group [84]. This latter study reinforces the need to identify naturally processed epitopes presented on tumor cells as it is not clear that the electroporated cells generated the CAP-1 epitope efficiently. On the contrary, 13 rationally selected colon cancer associated peptides (IMA910) identified by immunoproteomics method showed significantly longer overall survival in comparison to a matched-pair analysis of patients from the recently published phase 3 MRC COIN trials [85, 86].

Similar to colon cancer, survivin peptide based vaccine with or without adjuvant was tested in breast cancer with positive T cell responses but no clinical responses [87]. In contrast, prolonged disease free survival was observed in trials with Her2-neu antigenic peptide (E75 or GP2) immunization [88, 89]. A multi-epitope breast cancer vaccine comprised of 12 epitopes, identified by immunoproteomic methods, tested in patients with resected breast cancer generated broader $CD8^+$ T cell responses and objective prolonged diseases free survival [90]. Dendritic cells pulsed with MHC class I and II peptides derived from Her2-neu protein were also tested in breast cancer. Patients with confirmed DCIS treated with peptides pulsed DCs generated detectable $CD4^+$ and $CD8^+$ T cell responses to the vaccine, and a decrease in Her2-neu expression was detected in these patients [91] although a decrease in antigen expression is not necessarily indicative of complete elimination of the cancer. In a second study, majority of patients mounted functional CD4 and CD8 T cell responses against the tumor [92].

Similar to melanoma, renal cell carcinoma (RCC) is one of most common type of cancer [93] that are highly immunogenic. A number of immune based therapies have been tested in RCC including T cell epitope-based vaccines with promising results. Vaccine comprised of peptides derived from VEGFR1 protein generated specific CD8+ T cell responses and several partial regression and stable disease in RCC patients [94]. Antigenic peptides, identified by immunoproteomics approach, were incorporated in a multi-peptide vaccine and tested with and without cyclophosphamide treatment in patients with RCC [69]. $CD8^+$ T cell responses to multiple antigens were associated with control of the disease. Further, inclusion of cyclophosphamide three days before IMA901 injection prolonged survival and reduced the number of regulatory T cells [69]. This latter point is critical: while T_{REGs} are well represented in the tumor microenvironment, peptide-based vaccines may need

a T_{REG} depleting step prior to injection or other modulation of the anti-inflammatory environment by concomitant cytokine treatment. However, not all cytokines are ideal in this application. In trials of DC based vaccines combined with IL-2 administration, T_{REGs} were induced to significantly higher levels than before treatment, albeit transiently [95, 96].

Peptide based vaccines have also been evaluated in patients with stage III-IV non-small cell lung cancer with measurable clinical responses including stable disease and increase in overall survival [97]. Both antigen specific $CD8^+$ T cell responses and clinical responses measured by improvement in overall survival were observed in hepatocellular carcinoma patients treated with peptide derived from glypican-3 based vaccine [98]. A multi-epitope based vaccine demonstrated $CD8^+$ T cell responses and delay in progression of disease in ovarian and breast [90] and prostate cancer [99]. Finally, a multi-epitope vaccination approach was used in a Phase I trial of patients with biliary tract cancer and resulted in a detectable clinical response in 6 of the 9 patients [100]. Peptide vaccines with multiple cancer specificity have undergone clinical studies with promising immunological and clinical results. For example, HER-2/neu immunodominant peptide (lung, breast, or ovarian cancer) [101–103], Mucin-1 (MUC-1, Stimuvax), peptide (breast or colon cancer) [104, 105], Carcinoembryonic antigen (colorectal, gastric, breast, pancreatic and non-small-cell lung cancers) [106, 107], Prostate-specific membrane antigen (prostate cancer) [108–110], HPV-16 E7 peptide (cervical cancer) [111], Ras oncoprotein peptide (colorectal and pancreatic carcinomas) [112–114], and Melanoma antigens (Melanoma) [38, 115–118]. Another vaccine known as GV-1001 is under development, which is an injectable formulation of a promiscuous MHC class II peptide derived from the telomerase reverse transcriptase catalytic subunit (hTERT). GV-1001 is currently undergoing phase II clinical trials for liver cancer and NSCLC (non-small-cell lung cancer) as well as a phase III trial for pancreatic cancer [119].

3.4 Peptide Cancer Vaccines: Current Status and Trends

Tremendous amount of clinical data is currently available attesting to the efficiency of peptide-based cancer vaccines. Combination therapy is emerging as an important strategy to achieve synergistic effects in fighting cancer as a single method alone may not be efficient enough to yield positive results. Combining immunotherapy with conventional therapies such as radiation and chemotherapy or combining an anticancer peptide with a nonpeptidic cytotoxic drug is an example of this emerging field. The peptide vaccines are relatively less expensive, easy to manufacture and manipulate, are of defined structure, and being synthetic in nature do not have a problem of batch-to-batch variation. The major disadvantage of the peptide vaccines is their weak immunogenicity. Several strategies such as epitope enhancement, use of multiple T-cell epitopes, adjuvants, incorporation of costimulatory molecules, and ex vivo loading into professional antigen presenting cells are being explored to enhance the immunogenicity and efficacy of the peptide vaccines. Since the clinical

immunogenicity of the individual peptides is different, it is very hard to conclude which of these strategies was more efficient than the other. Recently, the role of immune checkpoint molecules, such as CTLA-4 and PD-1, programmed cell death-1, on antitumor immunity was clarified, and promising results have been reported in the clinical trials using combination therapies with peptide vaccines and immune checkpoint blockades [120]. Further randomized phase III trials would be essential to prove the clinical benefits of these vaccine therapies, including immune checkpoint blockade combination therapies.

4 Immunoproteomics, Immunotherapy, and Infectious Diseases

Pathogenic organisms are ubiquitous in nature and present a constant challenge for an individual's immune system. Before the development of vaccines polio virus, smallpox, measles, and whooping cough were constant threats. As vaccines were developed and distributed, morbidity and mortality caused by these organisms precipitously dropped; smallpox was completely eradicated, and polio virus may be eradicated 1 day in the near future.

Traditional vaccines offering protection against infectious organisms are prophylactic, designed to stimulate an immune response to a weakened or inactivated version of a pathogen or against macromolecular components of a pathogen (i.e. proteins, carbohydrates, etc.). The goal of prophylactic vaccination is to stimulate the innate and adaptive immune systems *before* exposure to the wild-type or circulating pathogen. This exposure should, ideally, generate memory B and T lymphocytes that can respond rapidly and robustly to a secondary challenge. Although both B and T memory responses are integral for protection against reinfection with an organism, the large majority of prophylactic vaccines in use today are designed to induce a strong B cell mediated response characterized by the secretion of antigen specific neutralizing antibodies. Immunogenicity of a vaccine is often determined by directly measuring the robustness of the B cell response [121]. Although B cell mediated responses are critical for protection, vaccines that predominantly stimulate antibody responses have their shortcomings. First, many strains of pathogens circulate in nature and it is not guaranteed that antibodies induced by one vaccine will protect against all strains. Indeed, a new influenza vaccine formulation is required almost every year due to antigenic drift or shift within circulating viruses [122]. Secondly, antibodies directed against one strain or serotype of a virus might actually enhance infectivity of a second strain/serotype. This antibody dependent enhancement is seen in patients infected with different strains of dengue virus and may lead to Dengue hemorrhagic fever and Dengue shock syndrome (DSS) [123–125].

Despite the drive to develop B cell stimulating vaccines, a large body of literature indicates that T cell responses are equally as important at controlling and eliminating infections. For example, data indicate that the robustness of the $CD4^+$ and $CD8^+$

T cell responses to Hepatitis B virus [126] and Hepatitis C virus [127] is a key determinant of whether these viruses are cleared or establish chronic infection. T cells activated in patients that resolve acute Hepatitis B virus infections recognize a broader range of epitopes and are better able to secrete key effector molecules like IFNγ [126]. Similarly, patients who mount a broad T cell response during a primary influenza virus infection are more likely to have cross-reactive T cells that can be activated during a second influenza infection despite substantial differences in the infecting strain [128]. Together the data make two important points: first, vaccines should be designed to stimulate a broad immune response by activating B cell mediated responses for antibody production as well as both $CD4^+$ and $CD8^+$ T cells for direct targeting of infected cells. Secondly, because multiple, antigenically distinct strains of pathogens circulate in nature these vaccines should stimulate B and T cell responses targeted to antigenic sequences conserved between the many circulating strains. This later point requires a new approach in vaccine development, and such an approach must be flexible enough to be easily and quickly modified if a new strain of virus (or a newly identified virus) emerges.

4.1 Cell Mediated Immunity in Infectious Diseases

B cell mediated responses are necessary during an adaptive response as the first line of response, however antibodies largely recognize antigens that exist extracellularly. Although these molecules can neutralize and eliminate infectious organisms, they cannot directly target infected cells which are often the 'factories' producing new copies of the pathogen. To destroy these factories, the cell mediated immune response consisting of $CD4^+$ and $CD8^+$ T cells is critical. $CD4^+$ and $CD8^+$ T cells are activated after their T cell receptor (TCR) recognizes a peptide epitope derived from a pathogen in complex with major histocompatibility complex (MHC) molecules. $CD4^+$ T cells recognize peptide epitopes ranging from 12–24 amino acids in conjunction with MHC class II molecules, while $CD8^+$ T cells recognize peptides ranging from 8–11 amino acids in conjunction with MHC class I molecules [129]. The generation of peptides for loading onto the appropriate MHC molecule requires degradation of proteins derived from the pathogen by proteases in the endosome or by the major cytosolic protease, the proteasome. Subsequent presentation of these peptides to T cells has the ability to activate a broad response with T cell clones targeting a number of stimulatory pathogen-specific peptide epitopes.

4.2 Influenza Virus Infection

The early experiments implicating T cell responses as critical contributors in controlling influenza virus infections were largely done in mice lacking B cell immunity [130–133]. Graham and Braciale showed adoptive transfer of influenza specific

$CD8^+$ T cells into B cell deficient mice infected with a lethal dose of influenza virus led to their full recovery while transfer of $CD4^+$ T cells lead to only modest recovery [132]. In a similar study, Epstein demonstrated mice unable to mount antibody responses to influenza virus, both $CD4^+$ and $CD8^+$ T cells played a critical role in controlling viral replication with $CD8^+$ T cell responses likely more critical than $CD4^+$ responses [133]. Although the role of $CD8^+$ T cells in controlling influenza virus in murine models is well established, the data for the importance of influenza specific $CD8^+$ T cell responses in human infection is not as abundant. Yet, a number of studies have revealed important roles for these cells during human influenza infection. Sridhar et al. followed individuals during the 2009 H1N1 pandemic in the United Kingdom and demonstrated those with pre-existing T cells directed against conserved, internal proteins of influenza virus (PB1, NP, M1) were better protected against infection. Although these T cell subsets did not protect against complete infection, the subsets limited the severity of infection as infected individuals who did not have symptoms or had minimal symptoms had higher frequencies of influenza specific IFNγ, IL-2 secreting $CD8^+$ T cells [134]. These data are in line with an earlier report from Wilkinson et al. that demonstrated less severe infection in individuals with pre-existing $CD4^+$ and $CD8^+$ T cells directed against conserved epitopes [128]. In a similar study, Wang et al. analyzed PBMCs obtained from individuals infected with a novel H7N9 influenza A virus. Recovery from this infection was associated with more robust IFNγ mediated T cell responses [135]. Interestingly, $CD8^+$ T cells were activated earlier in patients who recovered more quickly (within 18 days) while $CD4^+$ T cells were activated earlier in patients with a delayed (21–27 days) recovery. Together, these data suggest T cells are a key player in the immune response against influenza virus and vaccine formulations should elicit strong cell mediated responses directed against well conserved epitopes of influenza virus.

5 Dengue Virus Infection

Antibodies generated during dengue virus infection play an important role in neutralizing the virus and preventing future infection with the same virus serotype. However, at least four distinct serotypes of dengue virus circulate and antibodies against one serotype do not neutralize others; in fact, these antibodies have been demonstrated to enhance infection by distinct dengue virus serotypes [123–125]. With the potential for enhancing disease using a B cell mediated vaccine, newer vaccine formulations offering protection against dengue virus should stimulate robust T cell responses, ideally against antigens conserved across each of the serotypes. $CD8^+$ T cells play a major role in controlling dengue virus infections *in vivo*. DENV specific $CD8^+$ T cells have been detected after natural infection [23, 136–139], and studies have demonstrated a strong $CD8^+$ T cell response characterized by IFNγ and TNFα secretion in children who were infected but asymptomatic compared to weaker responses in symptomatic and severe infections [139]. $CD8^+$ T cells targeting each of the viral proteins are detectable after infection further suggesting

a broad T cell response is possible. Using an immunoproteomic approach in combination with an HLA-A2 humanized mouse model, our laboratory identified several novel epitopes that induce dengue virus specific, cross-reactive $CD8^+$ T cell responses [21] and of which, two capable of binding to HLA-A24 and two with the unique ability to bind to both HLA-A2 and HLA-A24. Importantly, particularly for designing vaccines for use in humans, we demonstrated these $CD8^+$ T cell subsets were detectable in dengue virus seropositive individuals and these subsets could be activated to produce IFNγ [23].

5.1 Hepatitis B Virus

For the majority of immunocompetent adults, encounter with hepatitis B virus does not lead to chronic infection. However for the remaining 5–10% of adults, neonates, and children infected, hepatitis B establishes a chronic infection that is responsible for approximately 500,000 deaths per year due to complications primarily involving the liver [140]. Individuals who fully recover from a hepatitis B virus infection display strong polyclonal and multi-specific $CD4^+$ and $CD8^+$ T cell responses [127, 141–144] targeting multiple viral proteins. Indeed, the key determinant of whether hepatitis B virus is cleared or becomes a chronic infection is based on the robustness of the immune response- individuals who resolve acute infections have greater numbers of IFNγ producing $CD4^+$ and $CD8^+$ T cells [145, 146] when compared to chronically infected patients [147]. Interestingly, chronically infected patients are also able to mount robust, broad $CD8^+$ T cells responses particularly in response to treatments like IFNα [148]. These data indicate a therapeutic vaccination stimulating a robust adaptive cell mediated immune response may be able to eradicate infected cells in these patients.

5.2 Vaccines Should Establish Protective Immunity to Infection

In order to protect an individual against infection or to stimulate an immune response in a chronically infected individual, i.e. prophylactic and therapeutic vaccines respectively must robustly stimulate the innate and adaptive immune responses. Initial stimulation of the innate immune system, driven by macromolecules derived from the pathogen, activates a relatively non-specific response designed to limit the replication of the pathogen and control its spread. Much of this is done through the secretion of pro-inflammatory cytokines and enhanced phagocytosis at the site of infection. Often, cells of the innate immune system migrate to the lymph nodes or spleen to activate the more specific and robust adaptive immune system. Within 5–7 days (reaching a peak around day 10), antigen specific B and T cells are mobilized and join the fight against the pathogen. After the pathogen is cleared, the immune response dampens and a pool of antigen specific, memory B and T cells persists and,

if the same pathogen is encountered again, they can be activated within 24–48 h. In this secondary challenge, the stronger more specific adaptive immune response is turned on earlier and prevents much of the pathogenesis that would otherwise arise during a primary infection. Any vaccine to any pathogen must induce this long-lasting immune – B cell memory to neutralize infection through antibodies production and T cell memory to perform a variety of tasks including killing of infected cells.

5.3 T Cell Induction Via Vaccines: An Alluring Alternative to Conventional Vaccines

The clearance of many viral infections is dependent upon the robust activation of $CD4^+$ and $CD8^+$ T cell responses, and T cell vaccines have great potential for use to prevent infection or to stimulate responses in chronically infected individuals. An added advantage to T cell-based vaccines is the ability to design these vaccines to induce responses against highly conserved regions of a pathogen stimulating an immune response that potentially protects against multiple strains that may be in circulation. To this end, an ideal vaccine formulation would incorporate multiple conserved targets to stimulate both $CD4^+$ and $CD8^+$ T cells as well as an adjuvant to induce robust innate immune responses. Additionally, vaccine formulations should be flexible with a stream-lined synthesis process in order to respond to emerging infections and to newly identified and potentially more protective T cell targets.

To meet the requirements of the ability to induce cross protection and be readily and quickly modified, peptide-based T cell vaccines are ideal. These vaccines can be formulated to include a variety of $CD4^+$ and $CD8^+$ T cell targets and adjuvants which stimulate a strong innate immune response to enhance processing and presentation of the associated targets to T cells. A number of promising delivery systems are currently in various stages of development including gold nanoparticles, polymeric nanoparticles, liposomes, and virus like particles (VLPs). Other favorable factors for each of these delivery systems are the customization capabilities with regard to size, shape, and antigenic targets/adjuvants. With these delivery systems and the development of more robust immunotherapies, the overall goal of driving T cell mediated immunity for prophylactic vaccine is within reach.

5.4 Peptide Based Vaccines in the Clinical Setting

Peptide based vaccines for infectious diseases are still in their infancy, but these vaccines have been tested in more depth in various cancers (as previously discussed in this chapter) and the results from both settings are promising (Table 1). In general, peptide vaccines are safe and easy to produce and depending on the backbone of the vaccine (i.e. nanoparticle vs. liposome) relatively stable [149–151]. A number of peptide vaccines for various diseases are currently in clinical trials.

A peptide-based vaccine against Hepatitis C virus (HCV) was recently tested in a phase II clinical study. This therapeutic vaccine, IC41, includes five highly conserved HLA-A2 restricted $CD8^+$ T cell epitopes and three $CD4^+$ T cell helper epitopes and is capable of inducing epitope specific IFNγ $CD8^+$ T cells in healthy non-HCV infected patients [152]. In chronically infected individuals, the vaccine stimulated an increase in epitope specific $CD8^+$ T cells in 25% of patients; however, this did not lead to increases in IFNγ production [153]. More recent studies indicate this vaccine is also able to reduce levels of HCV RNA in infected individuals after vaccination, but interestingly this reduction in RNA levels was not correlated with differences in immune responses [154].

Human Papilloma Virus is a sexually transmitted virus causing over 99% of all cervical cancers. Although most individual clear HPV infections roughly 10% are chronically infected. Developing a therapeutic vaccine in hopes to clear the virus from the body and prevent cancer is attractive. One therapeutic vaccine is composed of the E6 and E7 proteins, which are required for transformation, and emulsified with Montanide as an adjuvant. This vaccine induced epitope specific, IFNγ secreting $CD4^+$ and $CD8^+$ T cells that persisted for at least one year after vaccination [155]. A phase I clinical trial of this vaccine formulation in cervical cancer patients demonstrated it was safe, relatively non-toxic, and induced a broad T cell response [156]. Follow up studies with this vaccine (now termed HPV16-SLP) in other cancers offer similar hope. In a study of vulvar neoplasia, this vaccine induced strong T cell responses characterized by IFNγ and IL-5 secretion and resulted in a complete regression in half (10/20) of the patients [157]. Although not all patients experienced regression, in part due to the initial size of the lesion, the heightened T cell response initiated after vaccination suggests therapeutic vaccines based on peptides have promising potential.

Peptide vaccines against HIV are also under development and being tested in clinical trials. Biono Pharama developed a peptide-based vaccine (called Vacc-4x) which is composed of peptide derived from Gag p24, a major core protein of the virus. In clinical trials, this vaccine was immunogenic and decreased viral titers in infected individuals [158, 159] without a detectable impact on the generation of escape mutants [160]. Importantly, Vacc-4x induced an efficient memory response detectable for years after initial vaccination [161]. Other peptide based vaccines have not been as immunogenic [162], perhaps due to adjuvant used or delivery mechanisms. Interestingly, Vacc-4x is being tested with other adjuvants and via other delivery mechanisms; this vaccine candidate is also immunogenic when administered intranasally although the clinical significance has yet to be determined [163]. Overall, the data again indicate that generation of T cell responses are possible and that a peptide-based vaccine could be useful in treatment of HIV infections.

The majority of studies on peptide vaccines for infectious diseases have bene confined to therapeutic vaccines and to studies of pathogens that lead to cancer. A major reason for this is prophylactic vaccines to many pathogens are already licensed, approved and relatively efficacious. However, with the recent emergence of novel strains of influenza virus [164, 165] combined with the length of time it takes to make a strain specific influenza vaccine [166] makes this virus an ideal

target for a cross-reactive peptide based vaccine. To this end, Huber et al. designed a tandem epitope-based vaccine study in mice and ferrets. One vaccine was designed to stimulate B cell responses while the other was one designed to activate T cells, and both vaccine formulations were designed to target conserved regions of the genome. Huber et al. demonstrated these vaccines induced influenza specific antibody and influenza specific T cell responses, reduced viral titers in the lungs of animals, and may improve recovery time [166]. It is clear from the data that peptide-based T cell vaccines have the potential to prevent and treat viral infections, particularly in cases where antibody-based vaccines do not offer protection against all serotypes of a virus. Vaccines that offer cross-subtype efficacy could significantly prevent the spread of an emerging or re-emerging strain.

6 Immunoproteomics, Immunotherapy, and Autoimmunity

The immune system is continually tasked with clearing invading microorganisms and eliminating transformed or malignant cells from the host. The vast majority of the responses required to clear these challenges are pro-inflammatory and are accompanied by the secretion of cytokines and chemokines making the host an uninviting habitat for the pathogen or altered cells. However, these pro-inflammatory responses can be damaging to the host, and in some cases, the pathology seen during an infection is due to the immune response itself rather than the pathogen [167]. As such, these responses must be tightly regulated to avoid overt damage and pathology to host tissues. This regulation is accomplished in a variety of ways including the presence of regulatory cells that function during innate and adaptive responses. These cells release anti-inflammatory cytokines and/or directly modify the activity of responding cells in order to dampen down the immune responses and to prevent long term, systemic inflammation in the host. As pathogens are a constant threat to the immune system there must be a balance between the pro-inflammatory, pathogen clearing responses and the anti-inflammatory regulatory responses. Tipping the scales in either direction can lead to serious immunopathology and the inability to clear an invading organism. In certain disease states like cancer for example the balance is tipped in favor of regulation. Multiple regulatory mechanisms are actively preventing a robust pro-inflammatory response and therefore, an affective immune response against the malignant cells [168, 169]. However, when the balance is tipped in the other direction towards a more inflammatory environment the immune system may begin a robust attack against otherwise normal and healthy tissues, a process called autoimmunity. Undoubtedly both the innate and adaptive arms of the immune system are critical drivers of autoimmune responses [170, 171]. However, for the purpose of this section we will focus on the adaptive immune system, in particular T lymphocyte responses.

Adaptive immune cells begin their development in the bone marrow. Hematopoietic stem cells give rise to a common lymphoid progenitor that, after receiving a number of signals, begins a process of differentiation to form B and T

lymphocytes. B cell development occurs directly in the bone marrow; in contrast, T cell progenitors leave the bone marrow and migrate to the thymus to complete development. For both B and T lymphocytes, the end goal of the developmental process is the same: produce functional cells capable of recognizing and responding to foreign antigens but *not* to self-antigens. Although there are some key differences in the education process between the lymphocyte subsets, we will focus on development of T lymphocytes to illustrate these processes. T lymphocytes display a receptor, called the T cell receptor (TCR), on the surface that recognizes antigens. These antigens are breakdown products produced by an antigen presenting cell and loaded onto MHC molecules to form peptide/MHC complexes (pMHC). In the thymus, developing T cells are first 'educated' to recognize self-pMHC complexes. The affinity of this interaction determines the fate of the developing T cell: too high of an affinity and the T cell undergoes *negative* selection and is most often deleted from the repertoire; too low of an affinity and the developing T cell dies from lack of interaction. However, T cells that recognize p/MHC complexes at an appropriate affinity are *positively* selected and migrate from the thymus to lymphoid organs where these cells will respond to foreign antigens [172]. This developmental process establishes *central tolerance*. However, during development T lymphocytes do not see *all* potential self-antigens. Therefore, some cells reactive against self-pMHC complexes do escape the thymus and migrate to secondary lymphoid organs. In the periphery, a number of *peripheral tolerance* mechanisms exist to prevent fully developed T cells from reacting against these self-pMHC complexes. Peripheral tolerance mechanisms include regulatory T cells dampening or inhibiting adaptive responses, regulatory APC subsets inducing anergy in lymphocytes, and/or the deletion of T cell subsets that continually recognize self-pMHC complexes [173].

Despite central and peripheral tolerance mechanisms preventing T and B lymphocytes from responding against self-antigens, tolerance can be broken resulting in autoimmune diseases and immunopathology. The events leading to breaks in tolerance and subsequent autoimmune responses are not well understood although a number of factors are likely to play a role. First, there is clearly a genetic predisposition to autoimmunity. Expression of certain MHC (HLA) alleles is correlated with a higher likelihood of developing autoimmune disease; for example, expression of the MHC-I allele HLA-B27 is correlated with ankylosing spondylitis while the MHC-II alleles –DR3 and -DR4 are correlated with Hashimotos' thyroiditis [174]. More recently, non-HLA associated genes such as FoxP3, and PTPN22 have also been linked to autoimmune diseases [174–177]. Genetic composition alone however may not be sufficient to drive autoimmune responses, and evidence indicates environmental factors play a role as well, in particular infections. Multiple hypothesis exist as to how infections drive autoimmune responses, but the general idea is that inflammation directed against an invading pathogen leads to responses against self, either through molecular mimicry [178–180], epitope spreading, and/or bystander activation [167, 181]. In all cases, it appears inflammation serves as trigger to overcoming tolerance mechanisms. And because T cell responses are a critical driver of autoimmune diseases, immunoproteomic methodologies have the power to reveal previously unidentified epitopes that may indicate a pathogenic

driver (or enhancer) of the disease process and/or epitopes that can be used in the preparation of a tolerizing vaccine (Fig. 2).

In order to fully understand autoimmune diseases and to develop the proper immunotherapies to alleviate symptoms and, potentially, cure these disorders it is essential to identify the antigenic targets of autoimmune responses. To date, a number of T cell epitopes associated with autoimmune responses have been identified. Below, we summarize a select few of the autoimmune disorders and discovery of T cell epitopes contributing to pathogenesis.

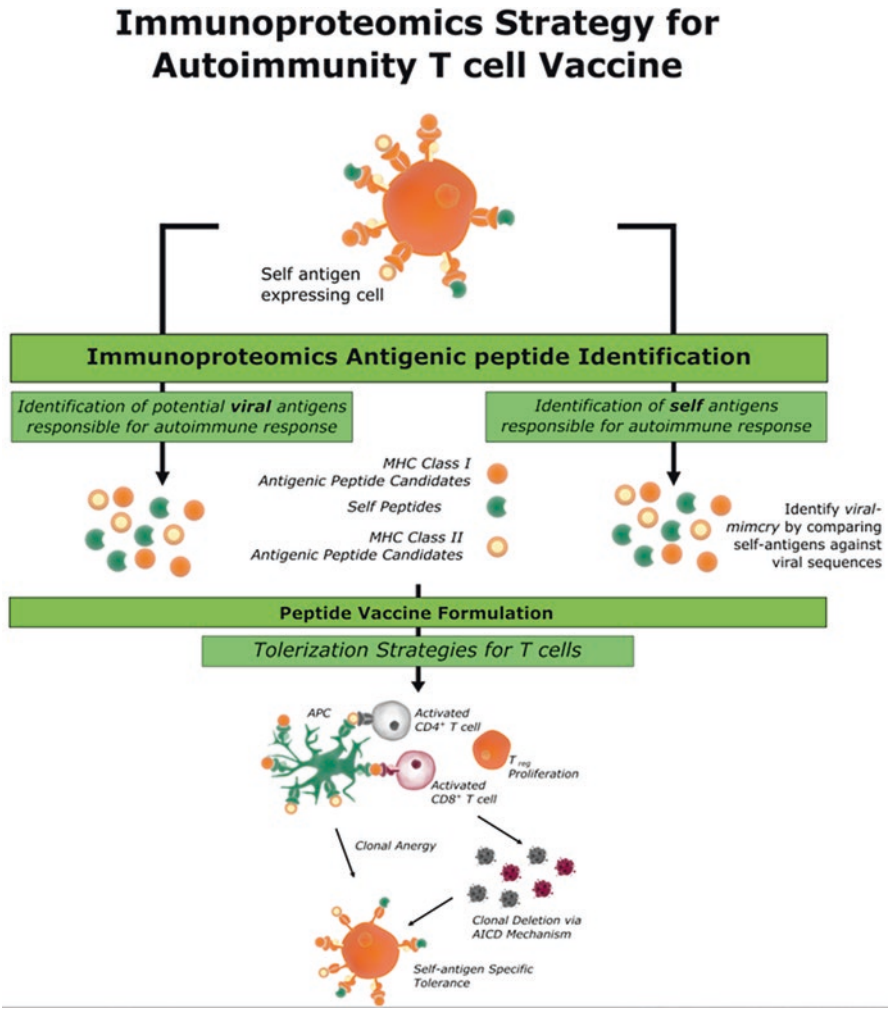

Fig. 2 Identification of novel epitopes presented during autoimmune disease progression

6.1 Type I Diabetes (T1D)

Type I diabetes is an autoimmune disorder characterized by the sequential accumulation of antibodies directed against self-antigens expressed in the pancreas [182] and a robust $CD4^+$ and $CD8^+$ T cell response that contributes to the destruction of pancreatic β cells [183]. Destruction of β cells results in a significant decrease in secretion of insulin and an inability to regulate blood glucose levels. A number of B and T cell epitopes thought to contribute to disease progression have been identified and these drive an inflammatory immune response which might be a trigger for autoimmune responses [183]. Less is understood about the infectious component of T1D; many pathogens have been linked to T1D but the broad nature of these pathogens and the absence of a leading candidate suggests infection may not be a large contributor to T1D [167, 181]. Although the evidence for infections as a trigger for the development of T1D do not point to a specific "culprit", a genetic predisposition is well described: individuals expressing the HLA-DR4 class II molecule are at a greater risk for developing T1D [184] although more recent evidence suggests that the genetic association with T1D is much more complex and involves both HLA and non-HLA related genes [185].

The genetic association with HLA-DR4 class II molecules suggests that $CD4^+$ T cell responses are a major driver of the autoimmune pathology seen in T1D. Using a humanized mouse model restricted to the HLA-DR4 background, Congia et al. identified a number of $CD4^+$ T cell epitopes derived from preproinsulin, proinsulin, and insulin including one epitope (derived from preproinsulin/proinsulin) naturally presented on HLA-DR4 expressing cells [186]. Similarly, Peakman et al. used immunoproteomic techniques to identify six naturally processed and presented MHC class II restricted and immunogenic epitopes derived from the islet antigen IA-2. Such natural processing and presentation allowed the group to identify epitopes that were immunogenic only in a HLA-DR4 restricted setting and did not stimulate non-specific T cell activation [187]. In perhaps the most physiologically relevant study, Kent et al. demonstrated a very small subset (n=3) of T1D patients had clonally expanded $CD4^+$ T cells in pancreatic draining lymph nodes while normal, non-diabetic patients did not [188]. These clonally expanded T cells recognized the insulin derived peptide $A_{1\text{-}15}$. A number of other class II restricted, $CD4^+$ T cell activating epitopes have been verified to various degrees including those derived from proinsulin, insulin, GAD-65, and a number of heat shock proteins [189].

While $CD4^+$ T cells in the pancreas, and draining lymph nodes are relevant for the inflammatory mediators they secrete, it is the $CD8^+$ T cell subsets that mediate direct cytotoxicity. In 1999 Charles Janeway's group made a seminal discovery. Using a pancreatic cDNA library, this group identified an MHC class I epitope derived from proinsulin that was a critical driver of $CD8^+$ T cell responses in T1D in a murine setting. Interestingly, the sequence of this epitope ($B_{15\text{-}23}$) is identical to the human counterpart and overlaps with a previously identified CD4 epitope ($B_{9\text{-}23}$) that contributes to T1D [190]. A few years later Hassainya et al. used a reverse immunology technique to identify ten potential $CD8^+$ T cell epitopes based on pro-

teasomal cleavage patterns and binding scores to HLA-A2 molecules [191]. Of the ten epitopes identified, seven were immunogenic in HLA-A2 transgenic mice suggesting that the T cell response during an autoimmune disease is broad. Using a similar immunoproteomic approach as others, Skowera et al. identified two naturally processed HLA-A2 restricted epitopes localized to the signal peptide region of preproinsulin. Indeed these epitopes appear clinically important as up to 50% of HLA-A2 expression T1D patients have circulating $CD8^+$ T cells directed against these epitopes [192]. Overall, the data suggests there is a broad T cell response directed against a number of protein antigens expressed in the pancreas.

6.2 Celiac Disease

Celiac disease is characterized by T cell responses in the gut directed against antigens contained in grains (wheat, barley etc.). Patients with celiac disease suffer from a number of symptoms including abdominal pain, diarrhea, fatigue, and weight loss. These and other symptoms likely result from the remodeling of the architecture of the small intestine; the continual pro-inflammatory responses lead to flattened villi and an inability to absorb nutrients properly. There is a strong correlation between genetics and the development of celiac disease as more than 95% of patients affected by celiac disease express the class II HLA allele HLA-DQ2 or HLA-DQ8 [193, 194], although it is important to note the expression of these alleles on their own has not been shown to be sufficient to drive celiac disease. Additionally, it is likely that other non-HLA genetic factors play a role in celiac disease development [195].

Due to the strong association with HLA-II alleles, the large majority of epitope identification in celiac disease has focused on $CD4^+$ T cell epitopes. A number of HLA-II restricted epitopes have been identified [196] but the most recent data suggest that a smaller number of epitopes dominate the response. An initial study by Shan et al. identified a long, 33-mer peptide derived from α-gliadin that stimulated T cells isolated from celiac disease patients. Interestingly, this peptide is found in all foods reported to negatively affect celiac suffers [197] suggesting that this peptide (or a derivative) is one of the immunodominant epitopes that drives celiac T cell responses. In an effort to identify immunodominant epitopes in celiac disease patients, Tye-Din et al. evaluated T cell responses in PBMCs obtained from celiac disease patients [194]. Celiac disease or healthy individuals consumed a wheat, barley, or rye grain diet for three days. Subsequently, PBMCs were obtained and tested in a high throughput screen to identify immunodominant epitopes using an overlapping peptide library based on the sequences derived from wheat, rye, and barley. This method led to the identification of 96 peptides capable of stimulating a T cell response. Although an unexpectedly high percentage of these epitopes were cross reactive, three immunodominant epitopes were identified independent of the grain consumed (peptide sequences were similar; derived from wheat (ω-gliadin) and barley (C-hordein)) [194]. Finally, Dorum et al. utilized an interesting HLA-II

capture method to identify novel celiac disease epitopes [198]. In this method, the gliadin protein was digested, incubated with HLA-II molecules (DQ2.5 or DQ2.2), and eluted. The resulting fractions were analyzed by mass spectrometry to identify glutenin and gliadin peptides. Similar to the results obtained by Tye-Din, Dorum's group identified a small number of dominant core peptide sequences (4 out of 86 total) associating with the HLA-DQ2.2 molecule. Two of these epitopes were novel and never before described. Together these latter studies demonstrate that despite the antigenic variation present in grain, the $CD4^+$ T cell response is directed against only a small number of peptides.

Although $CD4^+$ T cells are more strongly associated with celiac disease development, $CD8^+$ T cells also play a role, specifically when inducing lesions in the gut mucosa. Gianfrani et al. (2003) demonstrated an HLA-A2 restricted epitope derived from gliadin ($A_{123–132}$) was capable of inducing T cell responses in PBMCs obtained from celiac disease patients on a gluten free diet [199]. A follow up study using an *in vitro* organ culture system derived from celiac disease patients demonstrated an increase in activated $CD8^+$ T cells ($CD8^+CD25^+$) in the lamina propria when the gliadin $A_{123–132}$ peptide was present [200]. These cells were not detected in cultures including a control peptide or in cultures of HLA-A2 negative patients with the gliadin peptide indicating that the activation of these T cells was epitope and HLA specific. It is likely $CD8^+$ T cells play major roles in remodeling the intestinal architecture through direct cytotoxicity, and there is ample opportunity to identify novel epitopes that may drive the pathology of this disease.

6.3 Multiple Sclerosis

Multiple sclerosis (MS) is a disease in which the myelin covering of nerve fibers is destroyed and replaced by scar tissue build up. The loss of myelin in the nervous system slows down the rate at which impulses are transmitted and has profound effects in cognitive and motor functions. Although full repair to the damaged myelin is unlikely, there may be therapeutic potential to intervene and slow or stop the progression of disease, especially during the early stages. Immunotherapy may be an attractive target, especially considering the recent discovery of lymphatic tissue in the dural sinuses in the CNS [201]. A number of infections are thought to contribute to MS as enhancement of disease is observed after bacterial or viral infections of the upper respiratory tract [202]. Although it is difficult to identify a single pathogen as an environmental contributor to the onset of disease, it seems plausible that the inflammatory response generated to clear the pathogen may contribute to tolerance breakdown and attack of myelin. Like other autoimmune diseases, there is a strong correlation with HLA alleles specifically the class II molecule HLA-DR15 (DR2a and DR2b) [203, 204].

The antigenic targets of T cells in MS patients are hypothesized to be derived from myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein [205]. The first evidence that a peptide segment from myelin basic protein

may be responsible for T cell stimulation was described in 1990 by Ota et al. In this study, the authors mapped two regions of MPB [84–102 and 143–168] that were able to activate T cell lines derived from patients suffering from MS [206]. Subsequent follow up studies mapped the minimal peptide sequences required for T cell stimulation to be residues 85–99 [179] and immunohistochemistry experiments using CNS sections obtained from HLA-DR15 positive patients demonstrated microglial cells and macrophages expressed this MBP peptide in combination with the HLA-DR15 molecule [207]. Most recently Ben-Nun's group used a humanized mouse model to demonstrate that the HLA-DQ6 haplotype may also be a contributing factor to the development of MS like disease, specifically targeting proteolipid protein [208] and myelin oligodendrocyte glycoprotein [209]. Interestingly, this group demonstrated that the DQ6 allele mediated activation of T cells of a Th1/Th17 phenotype marked by secretion of IFNγ, TNFα, and IL-17. However, DR2 haplotype mediated activation of a Th2 phenotype with secretion of IL-4 [209]. Together, the overwhelming evidence suggests there is a strong HLA-II association with MS and the immunopathology is driven, in part, by a proinflammatory Th1/Th17 type CD4 response.

$CD8^+$ T cells also contribute to the pathogenesis of MS [210], and similar to their CD4 counterparts activation of these cells is driven by specific MHC interactions. The class I haplotype HLA-A03 may be a contributing factor to disease especially if a patient is also HLA-DR15$^+$ [211]. Like $CD4^+$ T cells, $CD8^+$ T cells recognize fragments of myelin basic protein, proteolipid protein, and myeling oligodendrocyte glycoprotein [212]. Berthelot et al. investigated the activation of $CD8^+$ T cells in patients with MS by using a library of 188 peptides selected based on class I binding motif algorithms [213]. 69 of the 188 peptides tested activated $CD8^+$ T cells to produce IFNγ in an ELISpot assay; however, there were no activation differences between MS patients and the healthy controls. This result is in line with other observations and reaffirms the idea that the presence of autoreactive T cells is only one part of the autoimmune equation. Interestingly, the data generated by Berthelot et al. indicates binding affinity does not predict the level of T cell activation (as we have previously described [23, 30] and at least one of these 69 activating peptides (MBP200-208) was naturally processed and presented [213]. Although it is clear that $CD8^+$ T cells play a role in MS many of the specific targets have yet to be identified

6.4 Potential Vaccine Immunotherapy for Autoimmune Diseases

To date vaccine formulations that aim to induce tolerance to peptide epitopes or reduce the number of antigen specific T cells have had some success (Table 1). Using a murine model of Type I diabetes (the NOD mouse), Solvason et al. demonstrated that injection of plasmid DNA encoding the preproinsulin II gene resulted in

a reduction of insulin specific pathogenic T cells in hyperglycemic mice [214]. The effect of this DNA based vaccine was enhanced with more injections and higher doses of antigen but was not mediated by regulatory T cell function. Hyporesponsive T cell development in this model is consistent with previous studies that demonstrate T cell anergy developing under conditions of high antigen load [215, 216]. Building upon the success of this initial study, Roep et al. evaluated the $CD8^+$ T cell response in T1D patients after injection of a plasmid containing the proinsulin gene (BHT-3021) [217]. Patients enrolled in the clinical trial were given 12 weekly IM injections and their $CD8^+$ T cell responses against pancreatic and non-specific epitopes were evaluated with flow cytometry. Over the course of the 15-week study, patients receiving BHT-3021 but not the placebo plasmid, had a reduction in $CD8^+$ T cells specific for pancreas antigens. Importantly, there were no non-specific reductions of $CD8^+$ T cells and the overall safety profile was good [217]. In a similar fashion, a plasmid based DNA vaccine encoding myelin basic protein (BHT-3009) or a placebo was administered to MS patients over the course of 44 weeks [218]. Administration of BHT-3009 reduced the occurrence of new lesions appearing in the CNS (as assessed by MRI) and a reduction in autoantibodies specific for myelin antigens. Together the data clearly indicated vaccines designed to induce tolerance are feasible, but more work is to be done.

While traditional vaccine formulations induce effective protection against pathogens, there are significant limitations when designing protective or therapeutic vaccines against other immunological insults like cancer and autoimmunity. For autoimmune vaccines, the candidate vaccine of choice is currently DNA based and while effective there are still major caveats to this approach. First, the coding message must be translated, the resulting protein processed, and the epitopes generated must be loaded onto the appropriate HLA molecule. Despite efficient uptake of DNA based vaccines, there are cellular differences in antigen processing capabilities which may result in a less efficient, but still efficacious vaccine. Further, for autoimmune diseases with multiple gene targets (i.e. MS, T1D) it is not yet clear what DNA sequences are optimal to include. Because of these caveats new generation nanoparticle vaccines may be an innovative step in the right direction. As discussed in preceding sections, these vaccines can be made in various sizes and shapes, have targeting sequences added, and contain multiple epitopes targeting both the CD4 and CD8 pathological responses. Conjugation of adjuvants is also possible including the CpG derived GpG tolerizing adjuvant [219].

7 Advantages and Disadvantages of Peptide Vaccines: Where Do We Go From Here?

Peptide vaccines are gaining momentum in recent years, since they are synthetic, simple to manufacture and cost effective. A number of clinical studies ongoing and in development using peptide vaccines in various disease conditions (Table 1).

7.1 Cancer

Overall, the data discussed above indicate peptide vaccines are capable of inducing robust $CD8^+$ T cell responses that, in some cases, provide clinical benefit to patients. Peptide based vaccines have significant advantages as an immunotherapy option. First, these vaccines are flexible in their design and can accommodate many peptide epitopes in a single dose (Table 1; Fig. 1). This allows for multiple MHC class I epitopes to be included to initiate a T cell response. This is an important feature because not all individuals share the same MHC alleles; peptides that bind to single alleles (i.e. HLA-A2 or HLA-A24) and peptides that bind to multiple alleles (i.e. HLA-A2 and HLA-A24) can be included in the same formulation. Thus, a vaccine derived from naturally processed peptides can be given to individuals with a wide diversity in their MHC alleles and still be effective. Secondly, a multi-epitope vaccine may protect against tumor resistance due to antigen downregulation by inducing a broader, oligoclonal response. Although multiple epitopes from a single antigen have been identified and might overcome HLA-restriction (i.e, MAGE-n [220], survivin [87, 221], and CEA [222, 223]), it is important that the epitopes included in such a vaccine be derived from different parent proteins. This not only will increase the clonality of the T cell response but also prevent tumor cells from downregulating a single protein and escaping the T cell response induced by the vaccine. Finally, peptide-based vaccines can also incorporate MHC class II restricted epitopes to activate $CD4^+$ T cells and/or B cell epitopes to activate T helper and antibody mediated responses. Together, a complete adaptive immune response could prove to be a more effective and robust way by which to eliminate tumors. Despite these advantages, peptide-based vaccine strategies are not without their downfalls. First and foremost, in order for the vaccine to be effective the tumors must be expressing the antigens included in the vaccine formulation. Ideally, the tumors should be *presenting* the epitopes included in the vaccine, which is a major reason for using an immunoproteomic approach for the discovery and selection of antigens in vaccine development. Secondly, peptide based vaccination has been shown to induce the accumulation of immunosuppressive regulatory T cells [76, 95, 96] which would limit vaccine utility *in vivo*. Finally, in some instance peptide vaccines may not be enough to eradicate tumors from patients depending on staging of the disease. Importantly, potential solutions are being evaluated in the clinic to prevent or mitigate each of these limitations. Peptide based vaccines, despite their limited effectiveness to date, have shown promise and progress in the clinic. Identifying novel and perhaps more immunogenic peptides through an immunoproteomics approach combined with a better understanding of adjuvant and cytokine therapy should result in more clinically effective vaccine regimens (Table 1).

7.2 Infectious Diseases

It is clear vaccines of the future will require more than simply inactivating a pathogen strain. Vaccines with built-in cross-subtype efficacy could prevent significant spread of an emerging or re-emerging strain. A cross-subtype vaccine containing immunogenic consensus sequence epitopes could achieve this goal. Fortunately, technology has progressed enough to allow us to identify immunodominant and memory-inducing peptides presented by the MHC class I molecules of virus-infected cells. Armed with these peptides, vaccine formulations will now have to incorporate antigens that activate both humoral and cellular immunity with various adjuvants to drive a strong immune response with high immunogenicity. Additionally, the use of peptides offers a flexible and simple way to synthesize a vaccine. It is therefore highly likely that peptide vaccines will play a large part in overall vaccination strategies and will offer hope to universal prophylactic as well as therapeutic vaccines for protection against infection and therapy for chronic infections respectively. T cell vaccines could play a major role in viral infections such as influenza and dengue viruses where the antibody targeted vaccines have limited clinical efficacy due to significant variations in the envelope protein between various strains. Significant efforts are being directed to find conserved regions of envelope proteins of influenza strains and dengue virus serotypes to generate broad humoral immunity. With the difficulty in finding conserved antigenic regions on the virus surface some efforts are aimed at targeting conserved proteins within the virus. Antibodies cannot reach these proteins to prevent infection, and therefore, peptides derived from intracellular processed protein presented in the context of MHC class I molecules must be utilized. The concept behind this approach is to stimulate T cells to quickly kill virus infected cells before the cells can produce new virions thus limiting disease severity.

7.3 Autoimmunity

Autoimmune diseases are triggered by aberrant B and T cell responses. For a number of reasons these responses have broken tolerance and perpetuate a proinflammatory environment conducive to immune mediated destruction of otherwise normal tissue. In the case of T cells, the responses are driven by specific peptide epitopes associated with HLA molecules. By understanding the specific naturally processed and presented epitopes driving the autoimmune responses, it may be possible to dampen, skew, or completely shut off these responses. Importantly, in order to prevent wide scale immunosuppression, the epitopes specific T cells should be the target of immunotherapeutics and not the HLA alleles. The most attractive mechanism for inhibiting these responses is a vaccine that can induce tolerance and/or anergy in T cells, skew the Th phenotype from Th1/17 to Th2, or induce regulatory T cell development in patients.

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Microbiome and Cellular Players in Type 1 Diabetes: From Pathogenesis to Protection

Darshan Badal, Mahinder Paul, Neenu Jacob, and Naresh Sachdeva

Abstract Type-1 diabetes (T1D) is an autoimmune disease characterized by the loss of immune tolerance to the beta (β)-cells of the pancreas. In this disease, the islet infiltrating immune cells mainly comprising of autoreactive T cells target the β-cell associated antigens, such as preproinsulin (PPI) and in the process destroy β-cells, leading to insulin deficiency. Besides, genetically predisposing human leukocyte antigen (HLA) alleles, several environmental factors have been proposed in the initiation of T1D, as the disease develop years before the actual presentation of clinical symptoms. However, loss of tolerance to β-cells is the central event in the pathogenesis of T1D for which various cellular entities and cellular mechanisms have been implicated. This chapter provides a detailed review of involvement of these cells and mediators, right from the organogenesis of the pancreatic tissue till the destruction of the β-cells. Further, the chapter focuses on the role of various innate immune cells including, macrophages, monocytes, dendritic cells (DCs), neutrophils, natural killer (NK) cells, innate lymphoid cells (ILCs) and adaptive immune cells mainly different subsets of CD4+ and CD8+ T cells and B cells in causing β-cell damage with special focus on immune cells that infiltrate early in the pancreas during the disease process. Amongst the cellular mechanisms, factors such as endoplasmic reticulum (ER) stress and posttranslational modifications (PTM), neutrophil extracellular traps (NETosis), over-expression of major histocompatibility complex (MHC)-I, involvement of major chemokines and inflammatory cytokines have also been discussed. The latter half of the chapter discusses about various immunomodulatory cells, mainly regulatory T cells (Tregs) that are involved in the protection of β-cells and efforts to replace functional β-cells or prevent β-cell destruction. While the complete treatment of T1D is still far in sight, this chapter attempts to refresh the current knowledge on the pathogenesis of the disease from the perspective of cellular players, which might be helpful in exploring newer therapeutic approaches.

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Abbreviations

EPC	Endothelial progenitor cells
GZM	Granzyme
HSC	hematopoietic Stem cells
IDO	indoleamine 2,3-dioxygenase
IFN	Interferon-
IL-	Interleukin-
iNKT	Invariant NK T (iNKT) cells
mDC	Myeloid dendritic cell
MSC	Mesenchymal stem cell
MΦ	Macrophage
NK	Natural killer cell
NKT	Natural killer T cell
NO	Nitric oxide
pDC	Plasmacytoid dendritic cell
PFN	Perforin
PMN	Polymorphonuclear leukocytes (neutrophils)
PP	Perinatal period
Teff	Effector T cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell
W	Weeks

1 Introduction

Type-1 diabetes (T1D) or autoimmune diabetes is one of the most common autoimmune diseases affecting more than 11,10,100 children and adoloscents worldwide (IDF 2019). The disease is characterized by the loss of immune tolerance to beta (β) cells associated antigens [1]. Because of an aberrant immunological response, the β-cells are attacked and destroyed by islet infiltrating immune cells mainly comprising of autoreactive T cells. Continuous β-cell destruction leads to insulin deficiency that results in impaired blood glucose metabolism and persistent hyperglycemia. Over time, the T1D patients become prone to micro- and macro-vascular complications like nephropathy, retinopathy, neuropathy, and cardiovascular diseases [2]. The primary risk factor for β-cell autoimmunity involves genetic factors i.e. individuals with either

human leukocyte antigen (HLA)-DR3-DQ2 or HLA-DR4-DQ8 haplotypes or both HLA class II alleles are at higher risk. Among the HLA class I alleles, HLA-A∗02 and HLA-B∗39 alleles further increase the risk in individuals possessing HLA class II DR3/4-DQ8 haplotype [3, 4]. However, development of clinical T1D typically requires a trigger from the environment as well, for which multiple factors have been implicated.

Till date, insulin replacement by exogenous insulin and oral anti-hyperglycemic drug remains the mainstay of T1D management. Although this approach is useful in preventing minor and early-onset complications, serious late-onset complications do pose a major challenge as they affect a large number of patients. Moreover, exogenous insulin therapy is never able to mimic physiological insulin responses leading to chaotic glucose profiles and life-threatening hypoglycemic episodes. Based upon the pathophysiology of diabetes, it appears that preserving insulin-secreting cells and stimulating their regeneration are the essential approaches for treating diabetes [2]. Since, the current management regimens are neither able to selectively eliminate diabetogenic immune cells nor able to protect the newly formed β-cells for the long term, therefore, there is a need to develop effective treatment against major autoimmune mechanisms involved in T1D [5]. This target can be achieved by abolishing the selective pathogenic reactivity of immune cells to β-cell auto antigens as well as preserving their full capacity to generate a normal immune response against foreign antigens. In addition to stopping the β-cell destruction process such a strategy would be able to restore immune balance in a safe and long-lasting fashion [6].

2 Role of Genetic Predisposition

T1D is a polygenic disorder with more than 40 different loci accounting for disease susceptibility. The HLA region located on chromosome 6 accounts for one-half of the genetic susceptibility [7]. HLA class II locus accounts for strongest association with T1D with DRB1∗04:01-DQB1∗03:02 and DRB1∗03:01-DQB1∗02:01 alleles conferring the greatest susceptibility. Their presence marks 55% chance for developing T1D [8]. On the other hand, some alleles such as, DRB1∗15:01 and DQA1∗01:02-DQB1∗06:02 are associated with disease resistance [9]. HLA class I locus also influences risk for T1D, mostly attributed to HLA-A and HLA-B genes. The susceptible alleles include HLA-B∗39, HLA-A∗02 and HLA-A∗24 while the protective HLA alleles are A∗11:01, A∗32:01, A∗66:01, B∗07:02, B∗44:03, B∗35:02, C∗16:01 and C∗04:01 [10]. The study conducted by Type 1 Diabetes Genetics Consortium (T1DGC), showed that HLA-B∗57:01 is significantly protective for T1D [11]. Similarly, a study conducted on African population found haplotype HLA DRB1∗03:02-DQA1∗04:01-DQB1∗04:02, has protection for T1D [12]. Various HLA alleles associated with susceptibility to T1D are listed in Table 1.

The other susceptibility loci include polymorphism in variable number tandem repeat (VNTR) in the promoter region of insulin gene [25]. A gain of function mutation in the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene, which encodes for lymphoid protein tyrosine phosphatase (LYP) suppresses T-cell

Table 1 HLA susceptibility genes associated with risk of type-1 diabetes

S No:	HLA gene	Reference
1.	HLA DRB1∗04:01	[13]
2.	HLA B∗08:01	[14]
3.	HLA DRB1∗03 and DRB1∗04	[15]
4.	HLA DQA1∗05:01 and DQB1∗03:02	[16]
5.	HLA DQA1:03:01 and DQB1∗02:01	[17]
6.	HLA DPB1∗03:01 and DPB1∗02:02	[18–20]
7.	HLA A∗24	[21]
8.	HLA B∗39:06	[11, 22]
9.	HLA DRB1∗07:01-DQA1∗03:01-DQB1∗02:02	[17, 23, 24]
10.	HLA DRB1∗03-DQB1∗02:01, DQB1∗02/ DQA1∗03:01,DQB1∗03:02	[24]

receptor (TCR) signaling during thymic development, thereby allowing autoreactive T cells to escape negative selection [26]. A single nucleotide polymorphism (SNP) of the PTPN22 caused a A629T substitution in the biobreeding diabetes-prone (BBDP) rat. This resulted in 50% decrease in C-terminal Src kinase binding affinity which contributed to T cell hyper-responsiveness [27]. A study carried out in the cohort of Caucasian subjects showed increased frequency of PTPN22 C1858T polymorphism in diabetic patients [28]. A49G polymorphism has also been detected in the cytotoxic T lymphocyte associated protein (CTLA)-4 which causes a change in the primary amino acid sequence of CTLA-4 thereby reducing its surface expression on T cells [29]. Studies show that SNP CT60A/G in the CTLA-4 gene marks as a susceptibility factor for T1D [30]. A meta-analysis study involving 2238 participants from Chinese population showed a significant relationship between CTLA4 + 49A/G gene polymorphism and T1D [31]. Another gene, interferon-induced helicase 1 (IFIH1) codes for an IFN induced helicase that recognizes dsRNA from picornavirus, thus serving as a sensor for viral infection. Coxsackievirus, which is proposed to be a causative agent for T1D pathogenesis, belongs to *Picornaviridae* family. Polymorphisms in the IFIH1 gene have shown its enhanced gene expression in peripheral blood mononuclear cells in patients with T1D [32]. Studies also confirm the association of the polymorphism in IFIH1 locus with susceptibility to T1D [33] Fig. 1.

Symbol	Description
	Antibodies
	Interferon-α
	Cytokines
	Chemokines
	Granzyme
	Perforin

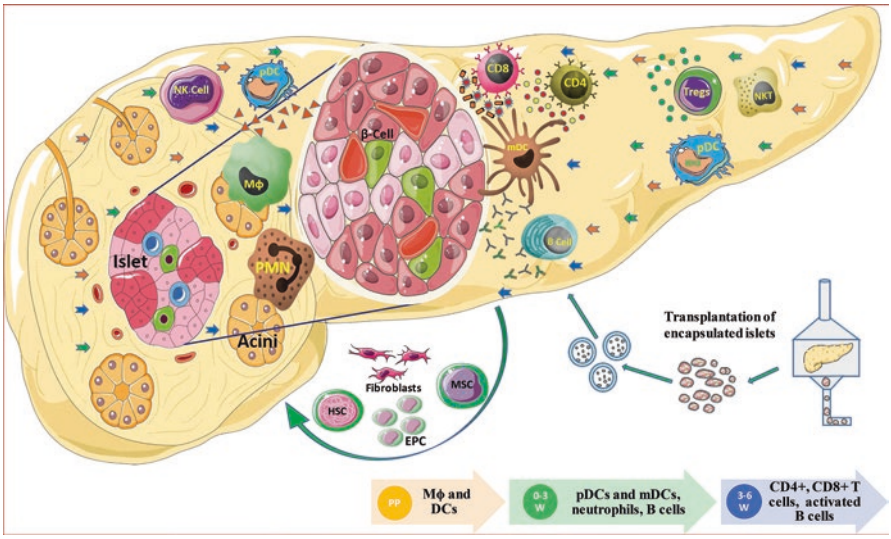

Fig. 1 Initiation of type 1 diabetes (T1D) is marked by the infiltration of innate and adaptive immune cells in pancreatic islets. Infiltrating antigen presenting cells including macrophages and myeloid dendritic cells (mDCs) capture and process β-cell antigens released following initial damage caused by inflammation, apoptosis, ER stress, viral infections or other environmental stimuli. Beta-cell destruction is primarily initiated by CD4+ T cells that recognize β-cell associated-antigens and produce IL-2 and interferon-γ (IFNγ) to activate CD8+ T cells. Cytotoxic CD8+ T cells mainly mediate the destruction of β-cells by releasing perforins and granzymes. Natural killer (NK) cells contribute to β-cell killing via release of IFNγ, granzymes and perforin. Activated macrophages can also cause β-cell death through secretion of tumour necrosis factor (TNF), IL-1β and nitric oxide. B cells present in and around the islets can present β-cell antigens to diabetogenic T cells and secrete auto-antibodies. pDCs infiltrate islets at early stages of T1D and are shown to produce IFN-α and augment Th1 responses. Neutrophils are also among the earliest islet infiltrating cells that are thought to play a role in pathogenesis through NETosis. Cells limiting β-cell damage include Tregs that inhibit effector T cells and inflammatory mDCs via various mechanisms. Indoleamine 2,3-dioxygenase (IDO) producing tolerogenic pDCs check the proliferation of effector T cells by limiting the amount of IL-2 in the milieu and by expanding Tregs. Invariant NK T (iNKT) cells can promote recruitment of tolerogenic DCs and pDCs. In the β-cell replacement cellular therapies, besides whole pancreas transplantation, islet transplantation is a safe and promising approach. Attempts are underway to encapsulate isolated islets with semi-permeable membranes or co-infuse them with accessory cells, such as endothelial progenitor cells (EPCs) or fibroblasts. Hematopoietic stem cells (HSCs) have been tried in β-cell regeneration and, MSCs due to their immunosuppressive nature are also being tried preserve the β-cell mass

3 Contribution of Environmental Factors

T1D develops years before the actual presentation of clinical symptoms [34, 35]. George S. Eisenberth in 1986, proposed a model, which suggests a steady progression in β-cell killing by autoreactive T cells that results in 80–90% of β-cell death [36]. Some of the extensive studies such as, The Environmental Determinants of Diabetes in the Young (TEDDY) [37], The Diabetes Auto Immunity Study in the

Young (DAISY) [38] and TrialNet [39], have been commenced to identify the prospective environmental triggers and biomarkers for T1D.

Multiple environmental triggers can result in autoimmunity. Viral infection has long been considered as a predisposing factor leading to T1D due to the discordance in monozygotic twins [40]. Many papers suggest enteroviruses (EV) especially coxsackievirus B (CVB) as the prime viral candidate for the precipitation of T1D. Serum antibodies against coxsackieviruses have been found in recent onset patients with T1D versus healthy controls [41]. CVB4 strain isolated from the pancreas of a deceased diabetic child, after passaging through murine cells, was found to induce diabetes after inoculation in mice [42]. After examination of pancreatic autopsy sample in patients with T1D, CVB3 RNA was detected in the islets but not in the exocrine tissue [43]. Recently this was validated by evidence of CVB5 particles exclusively in the endocrine cells but not in the exocrine cells of T1D primary human pancreatic cells [44]. A possible explanation for this difference is the higher basal and induced expression of signal transducer and activator of transcription (STAT)-1 regulated genes in alpha cells thus being able to clear viral infection more efficiently than β-cells [45]. There are mainly three pathways by which EVs have been proposed to kill β-cells, direct cytolysis of infected β-cells, local virus-induced inflammation, and molecular mimicry. A direct cytolytic effect of EVswas supported by the finding that EV can infect human β-cells *in vitro* [46, 47] and has been discovered in the islets at onset of T1D [43, 48]. Infection of β-cells, or other cells in close association to the islets, induces an inflammatory milieu [49, 50] that can be directly toxic to the islets [51, 52] or attract immune cells to the site of infection [53, 54]. The molecular mimicry that results due to the sequence homology between the EV protein 2C and the islet autoantigen glutamic acid decarboxylase (GAD)65 also results in β-cell killing [55]. The Diabetes Virus Detection study (DiViD) is the first study to examine the presence of virus in pancreatic tissue of T1D. The study was conducted on six type 1 diabetic patients, the findings of which revealed the presence of EV in pancreatic islets at the time of diagnosis [56]. Rotavirus infection has also been associated with progression of diabetes in children. Studies have shown that infection of non-obese diabetic (NOD) mice with rotavirus accelerated diabetes onset, which was evidenced by infection in the regional lymph nodes [57]. Apart from rotavirus, cytomegalovirus [58], parvovirus [59] and encephalomyocarditis virus [60] have also been found to be contributing factors for T1D.

Other environmental factors suspected to be involved in T1D is early exposure to cow's milk. The albumin in the milk cross reacts to islet cell autoantigen (ICA)-1 (p69), which is a β-cell surface protein [61]. Recent studies using hydrolyzed casein diet showed promising results in lowering T1D. Administering NOD mice with anti-diabetogenic casein hydrolyzed diet reduced the incidence of T1D. This result was corroborated with reduced levels of reactive oxygen and nitrogen species in the epithelial cells and distal intestine [62]. A study was conducted in Finland on infants with first-degree relatives with T1D. They received either hydrolyzed or conventional formula during first 4–6 months of their life. It was observed that the infants receiving hydrolyzed formula developed less autoantibodies than their counterparts [63]. However, this effect on islet autoimmunity was not confirmed in a larger phase

3 Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study [64]. The DAISY study showed that increased intake of cow's milk in children with low/moderate HLA-DR genotype increases the risk of developing islet autoimmunity and further progression to T1D [65]. Another protein gluten, which is a storage protein present in several grains such as wheat, rye and barley, has also been implicated in T1D development. Gluten peptides are incompletely digested and reach the intestinal mucosa, where they are partly resistant to enzymatic degradation resulting in continuous exposure of the protein to the intestinal immune system [66]. Some of the gluten peptides, of which gliadin is most extensively studied are known to be immunogenic in nature. Increased reactivity of peripheral blood T cells to wheat gluten has been seen in T1D especially in celiac disease and reports have shown production of proinflammatory cytokines resulting from T cell activation [67, 68]. The use of animal models such as NOD mice has been able to provide a better understanding on the effect of dietary gluten on T1D progression. The occurrence of diabetes was reduced in offspring of NOD mice, which was supplemented with gluten-free diet during pregnancy [69]. Studies have also shown that gluten-free diet increased the percentages of $CD11c^{+}$ dendritic cells (DCs) in NOD mice spleen, thus providing a new insight into the stimulatory effect of gluten-free diet on innate immune cells [70]. A pilot study carried out to assess the beneficial effects of gluten-free diet on newly diagnosed children with T1D, showed better outcomes on haemoglobin A1c (HbA1c) and insulin dose-adjusted A1c (IDAA1c) levels [71]. Studies also showed that gluten-free diet resulted in reduction in HbA1c level from 7.8% to 5.8–6.0% without insulin therapy in a subject with T1D. Even after 16 months of diagnosis the fasting blood glucose was maintained at 4.1 mmol/l [72].

Vitamin D plays a crucial role in immune modulation and thus could impact the early onset and disease progression of T1D. A nationwide Diabetes Incidence Study in Sweden (DISS) diagnosed low levels of plasma vitamin D concentration in T1D subjects, suggesting its role in disease development [73]. Supplementation of 1, 25-dihydroxyvitamin D3 [1, 25(OH) 2D3] (an active form of vitamin D) in NOD mice promoted the generation of tolerogenic mature DCs that suppressed the activation of auto reactive T cells [74]. An *in vitro* treatment of T cells from T1D subjects as well as healthy subjects with TX527, a less calcemic analog of bioactive vitamin D, promoted the induction of $CD4^{+}CD25^{high}CD127^{low}$ Tregs [75, 76]. A Cross sectional study on Caucasian children and adolescents with T1D demonstrated a high prevalence of low levels of 25-hydroxyvitamin D [77]. Low concentrations of vitamin D during pregnancy time have also been implicated in the development of T1D in their offspring [78]. A genome wide association study discovered the expression of vitamin D binding protein (VDBP) on the alpha cells of pancreatic islets. The VDBP antibodies were detected in T1D subjects which suggest that they acquired auto-antigenicity during diabetic progression and hence could be a potential T1D biomarker [79]. Although many studies have shown reduced vitamin D levels in T1D subjects, there are few studies showing contradictory results as well. A study on Finnish and Estonian children participating in the DIABIMMUNE and Type 1 Diabetes Prediction and Prevention (DIPP) studies showed no correlation of plasma 25-hydroxyvitamin D [25(OH)D] concentrations with subjects positive or negative

for β-cell autoantibodies [80]. T1D prediction and prevention study carried out in Finland showed no variation in the circulating 25(OH)D concentrations between cases and control groups [81].

At present the incidence of T1D is increasing in developed countries highlighting the influence of infections in disease protection. Infections may help in disease protection by skewing the response towards Th2, ameliorating the Th1 response [82]. Improved sanitation and infection control has hampered the immunoregulatory mechanism of our body. Strachan et al. proposed the hygiene hypothesis in 1989 that explained the rise of allergic conditions [83]. Recently an extension of this hypothesis suggested, greater access to antibiotics and vaccination and improved hygiene increased the susceptibility to autoimmune disease [84]. Studies in NOD mice show an inverse relationship between microbial exposure and incidence of diabetes [85]. NOD mice infected with live attenuated *Salmonella typhimurium* showed reduced incidence of T1D [86]. Helminth infection has shown to modulate inflammatory responses in NOD mice. Infection of *Heligmosomoides polygyrus* (helminth parasite) to NOD mice at 5 weeks of age reduced the incidence of T1D. There was marked reduction in pancreatic insulitis and the expression of IL-4, IL-10 and IL-13 as well as the frequencies of CD4+ Tregs were elevated in mesenteric lymph nodes (MLN) and pancreatic lymph nodes (PLN) in helminth infected mice [87]. Helminth infection has also been shown to prevent diabetes in NOD mice by inducing non Tregs that produce IL-10 independent of STAT 6 signaling [88]. Recently a combinatorial therapy with helminth antigen and proinsulin prevented the onset of diabetes in NOD mice. This protective effect was associated with increased frequency of Tregs within the PLNs [89].

3.1 Obesity

Obesity is a disease which is caused by excess accumulation of body fat leading to predisposition to various cardiovascular and inflammatory diseases in an individual. Several factors influence the incidence of obesity, which includes a lack of physical activity, age pattern and various socioeconomic factors [90].

3.2 Obesity and T1D

The epidemic of obesity is increasing throughout the world and is now also prevalent among young adults with T1D. Until recently, the role of obesity in the development of T1D has not been a focus of active research but the field is picking up the pace recently. A study by Liu et al. (2010) observed that youth with T1D are more prone to be obese than their peers without T1D [91]. A time trend, of which was provided by 18 years' follow-up study, which observed 47% increase in the prevalence of overweight whereas seven-fold increase in the prevalence of obesity [92].

The risk for development of T1D is increased by obesity and may occur at an earlier age among obese individuals with a predisposition as shown by a recent mendelian randomization study that found association between 23 SNPs and childhood onset T1D [93]. Higher bodyweight, obesity and insulin resistance increases the risk of T1D development even though no longitudinal studies have simultaneously assessed their association during preclinical diabetes [94]. There could be a crucial link formed by inflammatory cytokine and adipokines between obesity and T1D. Obese patients have been shown to have high levels of IL-17, IL-23 and leptin, similarly the higher production of IL-17 is observed during the early stages of T1D [95, 96]. Several studies have shown that adipokines like leptin and resistin could play a role in the development of T1D as resistin, decreases beta cell viability and has increased levels in T1D patients [97, 98]. Similarly, in murine models leptin has shown to destruct beta cells through its proinflammatory effects [99]. Pancreatic adipocytes derived proinflammatory cytokines have a direct cytotoxic effects on pancreatic islets, additionally they also aid infiltration of Th1 and Th17 cells thereby inducing persistent inflammation in islets by increase chemokine ligand (CCL) 5 expression [100]. Obesity increases the risk for comorbidities like metabolic syndrome, along with macro- and microvascular diseases among individuals with T1D, collectively speaking, prevention of obesity may slow down the development of T1D and might also prevents the late complications in T1D [101].

3.3 Gut Microbiota

The gut microbiota is a complex community of microbes belonging to at least nine divisions of Bacteria and one division of Archaea, which may vary for each individual but mostly dominated by four phyla of bacteria like *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria*, whereas archaea domain is dominated by *Methanobrevibacter smithii*, a methanogen that consumes hydrogen [102–104]. Most of them reside in large intestine which is home to estimated 10^{11} bacteria per gram of intestinal matter and plays an important role in various physiological functions such as helping in digestion and metabolism, absorption of nutrients, synthesis of several vitamins and inhibiting the growth of pathogenic microorganisms.

3.4 Gut Microbiota and Obesity

Studies in recent years especially those on germ free animals and transplant of microbiota have shed light on the influence of gut microbiota on human health and diseases and more importantly on metabolic disorders like obesity [102]. Many factors are known to affect composition of gut microbiome which can be linked to obesity like diet, genetic variations, use of antibiotics [105–107]. The initial evidence of link between obesity and gut microbiota was provided by Wostmann et al.

(1983) by their experiments on germ free animals, demonstrating that these mice require 30% more calories for sustaining their body mass than their conventional counterparts [108]. Several studies have shown increased bacteria of *Firmicutes* phyla over *Bacreroidetes* phyla, this is believed to have an association with enhanced low-grade inflammation and increased absorption of energy from food [109, 110]. The gut microbiota also plays an important role in the metabolism via the production of short chain fatty acids (SCFA) like acetate, propionate and butyrate. Several studies have shown the beneficial effects of SFCA on insulin resistance and glucose tolerance and obesity induced by diet etc. [111–113].

3.5 Gut Microbiota and T1D

The human gut microbiome has density which is highest in nature and it outnumbers his own cell number by100:1 [114]. The perfect storm for the development of T1D has been hypothesised which includes the trio factors such as an aberrant intestinal microbiota, a "leaky" intestinal mucosal barrier, and an altered intestinal immune responsiveness [115–117]. Recently gut microbial dysbiosis has been proposed as the main factor contributing to the pathogenesis of T1D. The DIPP study carried out in Finland provided a first line of evidence showing gut microbial alterations in T1D subjects with lower abundance of *Firmicutes* and increased abundance of phylum *Bacteroidetes* [118]. T1D subjects with proper glycemic control and good physical fitness displayed gut microbial profile comparable to that of matched subjects without diabetes. *Faecalibacterium* sp., *Roseburia* sp. and *Bacteroides* were the most abundant microbial species in the study cohort [119]. Studies were carried out to assess the gut microbiota in Infants from Finland and Estonia who are at risk for developing T1D. Significant alterations in the gut microbiota with shifts in both microbial phylogenetic and metabolic pathways were observed. Also increased intestinal inflammation characterized by high levels of human β-defensin 2 (hBD2) (an antimicrobial product induced by colonic epithelial during inflammation) have been observed in the study cohorts [120]. A case control study carried out in Caucasian children with T1D showed a significant difference in *Firmicutes* to *Bacteroidetes* ratio as well as difference in the number of *Bifidobacterium, Lactobacillus* and *Clostridium.* These differences correlated with glycemic level in the group with diabetes [121]. A study conducted on the comparison of fecal microbiota of Mexican children with T1D with that of controls, reported high levels of *Prevotella* in controls while *Bacteroides* dominated T1D subjects. These results were attributed to the dietary intake, where *Bacteroides* were associated with high protein and fat diet while *Prevotella* is associated with carbohydrate rich diet. The role of *Bacteroides* in thinning of the mucin layer in intestinal epithelial cells (IEC) thereby causing increased gut permeability and inflammation also supports its role in T1D development. Studies have shown a low abundance of lactate producing as well as butyrate producing species in children with β-cell specific autoimmunity. These include *Bifidobacterium adolescentis*, *Roseburia faecis* (a member of

Clostridium cluster XIVa), and *Fecalibacterium prausnitzii* (a member of Clostridium cluster IV) [122]. Diet rich in plant polysaccharide and low in fat as well as animal proteins has been found to favor the development of tolerogenic commensal bacteria. This has been proved in a comparative study between African and European children. The African children's diet comprised mainly of fiber and plant while the European children were fed on a high fat western diet. The fecal microbiota of African children consisted mainly of *Actinobacteria, Prevotella* and *Xylanibacter*, and more SCFA while the European children's microbiota comprised of *Proteobacteria* [123].

The role of gut microbiota in T1D diabetes progression has been reported in animal models as well. The absence of Myeloid differentiation primary response gene 88 (Myd88), an essential signal transducer in toll like receptor (TLR) signaling in NOD mice protected it from diabetes development [124]. But the protection against diabetes was abrogated in Myd88$^{-/-}$ mice, when it was transferred to germ free environment, however under specific pathogen free conditions (SPF) NOD Myd88$^{-/-}$ mice were protected from T1D [125]. The oral administration of broad spectrum antibiotics such as streptomycin, colistin and ampicillin) or vancomycin alone from the time of conception until adulthood resulted in increased diabetes incidence in male NOD mice [126]. Also NOD mice receiving either continuous low-dose antibiotic or pulsed therapeutic antibiotics (PAT) early in life had higher incidence of T1D as well as gut microbial alterations [127].These data indicates that antibiotic treatment as well as germ free environment disrupts the commensal microbial population that plays a major role in disease protection. Lower abundances of *Lactobacillus* and *Bifidobacterium* have been observed in BBDP as compared with healthy diabetes-resistant BioBreeding (BB) rats [128].

The gastrointestinal tract is lined by intestinal epithelial cells that act as a protective barrier against harmful antigens as well as helps in nutrient absorption. In BioBreeding rats an increased intestinal permeability was observed at an early age. This was correlated with decreased expression of tight junction protein claudin [129]. An alteration in intestinal barrier function was observed in non-celiac T1D which was associated with mucosal ultra-structural alterations [130]. Dietary microbial toxins have been shown to promote T1D by damaging beta cells thereby releasing autoantigens. Injection of bafilomycin A1 extracted from Streptomyces into mice resulted in impaired glucose tolerance and, reduced islet size and relative beta cell mass [131]. A study carried out by Bosi et al. (2006) showed significant increase in intestinal permeability in subjects with T1D compared to healthy individuals [132].

In recent years there has been a drastic change in the dietary habits of individuals due to increased consumption of processed food which are rich in carbohydrates and fats. Hence the recommended intake of dietary fibers which is 30 g daily has been reduced to one half [133]. The fluids in the gastrointestinal tract cannot digest the dietary fibers; hence they are broken down by gut microbiota into metabolites such as SCFA. A study comparing intestinal microbial composition of T1D subjects positive for at least two autoantibodies revealed low abundance of bifidobacteria and butyrate-producing species [134]. The fecal transfer from male to female NOD

mice conferred diabetes protection in female with an associated increase in butyrate producing bacteria [135, 136]. These SCFA exerts anti-inflammatory effects by producing immunosuppressive cytokines and Immunoglobulin A [137]). The SCFA especially butyrate stimulated the colonic mucus secretion in rats [138], in addition butyrate accelerated the assembly of tight junction proteins as well as increased the AMP-activated protein kinase (AMPK) activity in Caco-2 cell monolayer model [139]. In addition, SCFA can maintain immune homeostasis by modulating inflammatory responses. Butyrate and propionate suppressed the expression of lipopolysaccharide (LPS)-induced cytokines such as IL-16 and IL-12p40 [140]. Another *in vitro* study demonstrated that butyrate stimulated the DCs to express immunosuppressive enzymes such as indoleamine 2,3-dioxygenase 1 (IDO1) and aldehyde dehydrogenase 1A2 (Aldh1A2), which enabled the conversion of naïve T cells into FoxP3+ Tregs and eventually suppressed its conversion into IFNγ+ T cells [141]. Consumption of dietary fiber enhanced SCFA production in the small intestines, which induced the expression of the vitamin A-converting enzyme Aldehyde dehydrogenase 1 (RALDH1) on CD103+ tDCs in MLN. This in turn, promoted the differentiation of FoxP3+ Tregs from naive T cells [142]. Intraperitoneal administration of butyrate to NOD mice increased the pancreatic cathelicidin-related antimicrobial peptide (CRAMP) production by the beta cells. CRAMP exerts immunoregulatory effects on pancreatic macrophages and cDCs thereby maintaining immune homeostasis in pancreas via induction of Tregs. The induction of CRAMP by SCFA was mediated through G protein-coupled receptors (GPR) 43 and GPR41 expressed on beta cells [143]. Feeding NOD mice with acetate and butyrate releasing diet provided complete protection against T1D. Interestingly these two diets had their respective mode of action such as markedly decreasing autoreactive T cells in the lymph nodes as well as boosting the number and function of Tregs [144].

It is a universally accepted that providing new born with human milk protect them from infections. Human milk has the unique composition of proteins, fats, carbohydrates, vitamins and minerals as well as essential fatty acids, enzymes, hormones and many other biologically active compounds that provide health benefits [145]. Early life introduction of human milk oligosaccharides provides an interesting strategy for T1D prevention. Two population based cohort study from Norway and Denmark supports the contention that prolonging the breast feeding for more than 12 months reduced the risk for T1D [146]. There are only a few reports available on the effect of Human milk oligosaccharide (HMOS) on T1D. In breast fed infants these complex oligosaccharides can influence the composition of intestinal microbiota with abundance of *Bifidobacterium* [147]. It has been shown that *Bifidobacterium infantis* and B*ifidobacterium bifidum* grow well on HMOS as it is their only carbohydrate source [148, 149]. The HMOS grown bifidobacteria can maintain gut integrity by reducing occluding relocalization and inducing the expression of cell membrane glycoprotein. They also cause higher expression of anti-inflammatory cytokines such as IL-10 in Caco-2 cells [150]. A recent study showed the immune-modulatory potential of non-digestible short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) on human monocyte derived dendritic cells (MoDC). These scGOS/lcFOS mimicked the HMOS and promoted MoDC to release IL-10 *in vitro* [151].

It is said that the PLNs as well as the MLNs drain the pancreatic tissue. There is an immunologic connection between the gut associated lymphoid tissue (GALT) and the pancreatic islets since orally administered antigens are able to activate T cell responses in the PLNs [152]. Also the T cells activated in the gastrointestinal tract migrate to islets that express mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) [153]. In NOD mice infection with *Citrobacterium rodentum* which disrupts intestinal epithelial barrier has been found to accelerate the development of diabetes and the administration of this antigen via gastric route was found in the PLN and MLN of infected NOD mice [154]. These data suggest that enteral antigens and immune responses arising in GALT may be able to target islet beta cells for destruction.

Whether Th17 cells plays a role in pathogenesis or provides protection from T1D remains a controversial issue. A study conducted by Martin et al. (2009) on NOD mice show increased expression IL-17A or IL-7F in islets that correlated with development of insulitis [155]. Further the deficiency of IL-17 in NOD mice reduced the severity of insulitis and delayed the onset of diabetes [156]. The gut microbial modulations profoundly influence the balance between Th17 cells and Tregs that may influence intestinal immunity. A study by Ivanov et al. (2008) found that specific commensal microbiota such as Cytophaga-Flavobacter-Bacteroidetes (CFB) bacteria was required for the Th17 differentiation in Lamina Propria (LP) and the absence of these bacteria was accompanied by increased $Foxp3^{+}$ Tregs in the LP [157]. Later colonization of segmented filamentous bacteria (SFB) in the small intestine of LP in mice has been found to be potent inducers of Th17 cells [158]. Although many studies are in favor of the pathogenic role of Th17 cells in T1D, some studies also show the protective effect Th17 cells in T1D when gut microbiota is manipulated. Feeding of BBDP rats with *Lactobacillus johnsonii* strain N6.2 (LjN6.2) from Bio-Breeding diabetes-resistant rat conferred diabetes resistance to BBDP. This was correlated with TH17 cell bias within the MLNs [159, 160]. The SFB colonization in NOD female mice showed only 20% incidence in diabetes development, while those without SFB colonization had 80% incidence by 30 weeks of age. The Th17 cells in SFB positive mice correlated with SFB levels in feaces. Indeed these Th17 cells are assumed to be Foxp3+/RORũFE;t + IL-17-producing T regulatory cells that migrate to the site of inflammation and protect NOD mice from diabetes [161].

4 Development of Pancreas and Beta-Cells

Since the pathogenesis of T1D involves destruction and regeneration of the islets, it is important to have some knowledge about various cells and cellular factors involved in the ontogeny of the pancreas. The pancreatic development starts when the embryonic foregut gives rise to surrounding mesenchymal tissue by endodermal budding [162]. The intricate interactions between mesenchyme-epithelium tissues give rise to branching of pancreatic ducts and differentiation, whereas morphogenesis results in the growth of the acini and pancreatic islets. Other organ systems, particularly the circulatory and nervous systems strongly influence pancreas development [163]; signals like vascular endothelial growth factor (VEGF) are provided by blood vessels, resulting in the induction of pancreas organogenesis [164].

4.1 Beta-Cell Development in Mouse

Mouse pancreas development has been studied in much more detail and can be operationally divided in three major time periods: first, is a primary transition of embryonic day (E) (E9.5 to E12.5), second is a secondary transition (E12.5 to birth), and third and the final one is postnatal period from birth to weaning, which in mouse also coincides with adolescence onset. During the first phase, the development of pancreas initiates with endoderm thickening, followed by proliferation of the pancreatic progenitors cells at E9.5, and the evagination of dorsal and ventral pancreatic bud around E9.75 [165–167]. The pancreatic endocrine progenitor cells expressing neurogenin 3 (*Ngn3*) differentiate into β-cells [168]. Additionally, expression of several transcription factors [167] (Table 2) are required for the formation of a functional glucose-sensing and insulin-secreting β-cells [169–171]. After initial differentiation, maximum fetal β-cells remain functionally immature till late gestation period [172–174]. Beta-cells can be considered mature when they are capable of sensing physiological signals like glucose and secrete appropriate levels of insulin to match them. After birth, the β-cells of new-born mice rapidly mature to confront the new host energy sources and requirements [28]. A recent study by Sasson et al. (2016), suggested that pericytes plays an important role in the islet niche, and directly influence the maturity and functionality of β-cells. When the pericytes were depleted from the islets it resulted in the reduction of insulin content and expression. The pericyte devoid islets had impaired glucose-stimulated insulin secretion, along with a reduced expression of β-cell function and reduced levels of the MafA and Pdx1 transcription factors [175].

4.2 Role of Immune Cells during Pancreas and Beta-Cell Development

Immune cells are present in the pancreatic islets during the neonatal periods in both mice and humans, but their role during the development of pancreas and β-cells was not given much focus. There is hardly any literature on whether there is a link between the early presence of immune cells during β-cell development and pathogenesis of T1D.

4.2.1 NOD Mice Neonates

The presence of macrophages is a well-recognized component of adult pancreas in rodents, although their presence in the neonatal and fetal pancreases are not well understood. Large number of several types of macrophages especially the mature BM8+ scavenger macrophages were found to be localizing around periphery of blood vessels, ducts, nerves and islets, and also scattered in the septa and exocrine tissue in

Table 2 Factors involved in beta-cell development and maturation

Associated gene-expression changes	
Factors increased	References
Ldha	[176–179]
Npy	[179–183]
Mmp-2, Spd	[184]
Ck-19	[179, 184]
Factors decreased	References
Ins2	[185, 186]
Glut2	[186, 187]
Gck, Glp1r, Pcsk 1/3	[186]
Oxidative metabolism genes (Pyruvate carboxylase, mitochondrial shuttles, etc.)	[187]
Transcriptional regulators	
NeuroD1	[179]
MafA	[186, 188–190]
MafB	[190, 191]
Islet1	[192]
Ngn3	[193, 194]
Nkx2.2	[195, 196]
Pdx1	[197–200]
Vhl	[201, 202]
Other factors	
αvβ3 and αvβ5 integrin	[203]

pancreas of NOD control and NOD/*SCID* mice [204–207]. At the time of birth, BM8+ and ER-MP23+ macrophages, and CD11c + DCs were more abundant in the pancreas of NOD/*SCID* and NOD than C57BL/6, DBA/2 and BALB/c controls, which is suggestive of ongoing abnormal events in islet milieu [206]. Few weeks after birth, the number of macrophage progressively decline in all mouse strains till weaning and rebound subsequently only in NOD and NOD/*SCID* strains with diabetic background [206]. DC precursors like ER-MP581, Ly6C^{hi} and Ly6C^{low} were present in fetal pancreases of prediabetic NOD and control mice. Ly6C^{hi} and Ly6C^{low} DC precursors were capable of developing into CD11c + MHCII+ CD86+ DCs capable of processing DQ-OVA antigen. Additionally, ER-MP581 cells in the embryonic and pre-diabetic NOD pancreas had a higher proliferation capacity than controls [208].

Additionally, during the tissue remodeling in pancreas, apoptosis of β-cells peaks around 2 weeks of age and is significantly increased in NOD neonates as compared with controls. Although apoptosis is considered a non-immune response generating process, but certain studies have indicated that apoptotic cells can preferentially activate DCs capable of activating autoreactive T cells by presenting auto-antigens on their surface blebs and have also been shown to induce autoantibodies formation. In NOD and transgenic NOD mice, the immune cell infiltration into pancreatic islets appears around 15 days of age and coincides with neonatal β-cell apoptosis with

accelerated onset of autoimmune diabetes [209]. The NOD mice younger than 15 days of age do not develop diabetes even after the transfer of functional T-cells from adult BDC 2.5 TCR transgenic mice to 10-day-old NOD recipients, the possible reason may be the lack of autoantigens or absence of antigen presenting cells (APC) [210].

4.2.2 Human Neonates

There are very few reports on the infiltration of immune cells in humans especially during neonatal and fetal period. Infiltration of lymphocytes was observed parallel to the two successive waves of β-cell apoptosis/islet degeneration during the pancreatic development as reported in an early study of human pancreas [204, 206]. Another study by Jasen et al. (1993) showed the presence of large focal lymphocyte infiltrates, containing primarily T cells in capsule and connective tissue of septa of fetal and neonatal human pancreas. Abundant endothelial venule-like structures, macrophages and DCs were also observed in periphery of fetal islets [211]. Presence of lymphocytes and expression of MHC class II antigens were also confirmed in pancreas of human fetuses [212]. Collectively, these reports suggest that presence of lymphocytes, macrophages and DCs during developmental periods is an essential part of the pancreatic milieu, which requires special attention in understanding T1D pathogenesis. These cells have also been shown to play a role during the development of limb, nervous system, retina, kidney, gut and thymus in rodents, during various stages of organogenesis, such as angiogenesis/vasculogenesis, neurogenesis/perinatal nerve degeneration and epithelial branching. Macrophages, in particular, are well-recognized for their role during tissue remodeling, phagocytosis during embryogenesis and their interaction with apoptotic cells during developmental periods and are also known to secrete numerous factors, including, growth factors, cytokines, and extracellular matrix proteins [213] (Table 3).

In fact, the mesenchymal compartment of every organ throughout embryogenesis is populated by macrophages, where they support tissue regeneration and organogenesis by regulating remodeling of the extracellular microenvironment. Mussar et al. (2014), shed some light on their specific role in islet development by describing that M2 macrophages regulate cell cycle progression and migration of pancreatic progenitors cells by modulating adhesion receptor, neural cell adhesion molecule (NCAM) and transcription factor, paired box protein (PAX6) in the epithelium [214]. Further, the role of macrophages was also observed in β-cell proliferation following injury, where their depletion blocked connective tissue growth factor (CTGF) mediated β-cell proliferation [215].

5 Loss of Self-Tolerance

Immune tolerance is a state of unresponsiveness to antigens that can elicit an immune response. There are mainly two types of immune tolerance, central and peripheral tolerance. Central tolerance is generated at sites of lymphocyte development, such as thymus and bone marrow for T and B cells respectively. This helps to

Table 3 Growth and differentiation factors produced by macrophages involved in islet development

Factors	Synthesized by macrophages
Mesenchyme and extracellular matrix	
Activin A	+
β-Cellulin	–
Fibronectin	+
Follistatin	–
Laminin	?
Matrix metalloproteases (MMPs)	+
Cytokines and growth factors	
Epidermal growth factor (EGF)	+
Fibroblast growth factor (FGF)	+
Hepatocyte growth factor (HGF)	+
Insulin growth factors	+
Interferon-γ (IFN-γ)	+
Interleukin-6 (IL-6)	+
Keratinocyte growth factor (KGF)	–
Nerve growth factor (NGF)	+
Transforming growth factor-α or -β (TGF-α or -β)	+
Tumor necrosis factor-α or -β (TNF-α or -β)	+
Vascular endothelial growth factor (VEGF)	+

distinguish self and non-self-antigens, whereas peripheral tolerance is generated at sites of antigen recognition and processing mainly in the lymph nodes. This helps prevent over reactivity to environmental triggers such as gut microbes and allergens. Failure of central and peripheral tolerance can lead to development and expansion of effector T cells, which eventually lead to progression of autoimmunity. T1D ensues as a result of breakdown of this tolerance, which leads to commencement and progressive destruction of insulin producing β-cells. Self-reactive T cells are eliminated in the thymus by negative selection process. The thymic medulla express the transcription factor, autoimmune regulator (AIRE), which controls the transcription of broad array of organ-specific genes, including preproinsulin, thereby creating an immunological umbra in the thymus [216, 217], thereby eliminating autoreactive T cells. Yet many autoreactive T cells escape this immune regulation in the thymus. This partial clearance of autoreactive T cells in the thymus could be attributed to lower HLA binding affinity of self-peptide epitopes [218], low avidity of the TCR recognizing self-epitopes presented on the HLA molecules, and variances in post-transcriptional [219, 220] and post-translational expression regulation in peripheral tissue versus thymus [221]. The autoreactive CD8+ T cell tolerance is achieved by immunological ignorance, if the avidity of self-peptide presentation in the draining lymph node is low or by anergy or death mediated by high expression of Bim, a pro-apoptotic protein [222]. The breakdown of tolerance depends on the phenotypic and functional characteristics of DC that is whether DCs promote tolerance or present antigens in an immunological manner. Also, the avidity of interac-

tion between autoreactive TCRs and their respective cognate antigens presented by DCs must reach a certain threshold to trigger activation of autoreactive CD8 + T cells in PLNs [223]. Peripheral tolerance is also maintained by recognition of self-antigens on APCs other than DCs. Stromal cells present tissue-specific antigens in lymph nodes in association with AIRE [224, 225]. Mutations in genes encoding AIRE and PTPN22 have been involved in T1D [226, 227]. A gain-of function mutation in PTPN22 gene results in lower T-cell activation and IL-2 production [26] resulting in compromised immunoregulation by Tregs.

There is ambiguity regarding the factors involved in loss of β-cell tolerance, but it is evident that β-cells are themselves responsible for their demise rather than being an innocent victim of autoimmune attack [228]. Viral infection or ER stress provokes an immune response in β-cells leading to activation of immune system. Infiltration of leukocytes (insulitis) towards islets is preceded by hyper-expression of MHC I, IFN-α, and CXCL10, that attracts immune cells expressing CXCR3 towards the islets [229–231]. The NOD mice develop autoimmunity with overt hyperglycemia (where 70% of the β-cell have been destroyed) by around 3–5 months of age much later than the actual development of insulitis, which begins at 3 weeks of age. This delayed disease onset and occurrence of β-cell destruction has been evidenced from a study where, adoptive transfer of pathogenic polyclonal CD4+ and CD8+ T cells from the spleen of diabetic NOD mice to syngeneic immune deficient recipients resulted in diabetes incidence in these mice [232–234]. It is still unclear whether a single antigen or a repertoire of antigens is responsible for autoimmunity. Also it is unknown which candidate antigen is responsible for pathogenic auto-reactivity or bystander islet autoimmunity [235, 236]. There is still an enigma on why loss of tolerance to certain antigens expressed in islets and other tissues lead to tissue specific pathogenesis. Nonetheless, breakdown of this tolerance leads to activation and recruitment of T lymphocytes, which have an important involvement in the disease process.

5.1 Endoplasmic Reticulum (ER) Stress and Post-Translational Modifications (PTM)

During the initiation and progression of insulitis, immune cells move towards the pancreatic islets after sensing inflammation, although the factors causing this initial inflammation and infiltration are not well defined. Beta-cells are predisposed towards ER stress due to their secretory nature and rapid turnover of insulin molecules. Inflammation causes ER stress in β-cells which they try to resolve by activating unfolded protein response (UPR) pathways, but if ER stress remains prolonged and unresolved, the UPR switches from a pro-adaptive to pro-apoptotic outcome leading to the death of β-cells [237]. Several studies have suggested link between disruption of ER homeostasis and β-cell dysfunction and diabetes, as misfolded insulin was shown to induce diabetes in both mouse mod-

els and humans [238, 239]. Also, mutations in genes critical for ER function results in β-cell failure and diabetes onset both in experimental models and humans [240–242].

ER stress and dysfunction also leads to abnormal protein folding and post-translational modifications (PTM), affecting protein function and may give rise to "neo-antigens" with increased immunogenicity [243]. Coxsackie viral infection is also linked to ER stress and PTM via disruption of ER membrane and release of Ca2+ from the ER into the cytosol [244, 245]. The risk of developing T1D increases considerably with increase in number of target auto-antigens, which can happen via PTM. PTM includes phosphorylation, citrullination, acetylation, carbamylation, amidation, and oxidation [246]. Once the β-cell ER stress increases, it leads to the release of β-cell related neo-antigens which are processed and then presented by APCs to T cells in draining lymph nodes leading to the increased infiltration of auto-reactive T cells. Beta-cells under ER stress may secrete cytokines and chemokine's that attracts immune cells to islets [247]. With increase in immune infiltration into the islets the ER stress also increases progressively [248]. Increased ER stress could lead to rise in cytosolic Ca2+ that enhances the activity of tissue transglutaminase 2 (Tgase2) and Peptidylarginine deiminases (PAD) enzymes. PTM by the Ca2+ dependent enzymes Tgase2 (deamidation) or PAD (deimidation) increases the immunogenicity of several β-cell proteins [246] (Table 4). Recent study by Marre et al. (2016) demonstrated that ER stress increases immunogenicity in the human β-cells. Induction of ER stress by thapsigargin in human islets and insulinomas increases the recognition of deamidated GAD65 by 135–360 fold by human T cells and increased activation of the PTM enzyme Tgase2 was found to accompany this increase in immunogenicity [249].

Table 4 Post-translational modifications (PTM) in beta-cell associated antigens occurring during endoplasmic reticulum (ER) stress

Autoantigen	PTM	References
Phogrin	Deamidation	[250]
Proinsulin	Oxidation	[219]
CHGA (WE14)	Crosslinking/ Isospeptide bond	[251, 252]
Preproinsulin	Deamidation	[250]
ICA69	Deamidation	[250]
ZnT8	Deamidation	[250]
IA-2	Deamidation	[250]
IGRP	Deamidation	[250]
GAD65	Citrullination	[253]
	Deamidation	[250, 253]
GRP78	Citrullination	[254]

CHGA, Chromogranin A; GRP78, Glucose regulated protein 78; GAD65, glutamic acid decarboxylase 65; IA-2, insulinoma antigen-2; ICA69, islet cell autoantigens; IGRP, islet-specific glucose-6-phosphatase catalytic subunit related protein; ZnT8, zinc transporter-8

5.2 *Role of Chemokines, Cytokines and Cell Signaling Pathways*

In T1D, disease onset is preceded by leukocyte infiltration to the pancreatic islets suggesting the role of chemokines expressed in the pancreatic islets in disease pathogenesis. Pancreas produce numerous chemokines such as CXCL10, CCL5, CXCL9 and CCL2 [255, 256] implicating the recruitment of pathogenic [257] or Treg [258] cells into the pancreatic islets. Studies also indicate that chemokine receptor (CCR)7 and its ligands are important for T cell recruitment to pancreatic islets. During insulitis, β-cells secrete chemokines such as CXCL10 and CXCL9, which act as driving forces for the accumulation of cytotoxic T cells expressing CXCR3 [256]. Genes encoding chemokines, mainly CXCL10 and also CXCL9 and CXCL11 are the response genes in pancreatic β-cells that are elevated in inflammatory conditions. The circulatory levels of these chemokines are also elevated in NOD mice [259]. Islets obtained from 4 weeks old NOD/*SCID* mice showed the basal expression of several chemokine ligands. CXCL10 was predominantly expressed followed by CCL22, CCL21, CCL3, CCL17 and CCL2 [260]. Gene expression analyses detected the presence of mRNA for CCR7 as well as its ligands CCL19 and CCL21 in inflamed islets but not in uninflamed islets of NOD mice, suggesting their role in disease pathogenesis [261]. In a population-based registry of children diagnosed with T1D from 1997 to 2005, the levels of five inflammatory chemokines (CCL2, CCL3, CCL4, CCL5 and CXCL8) were analyzed from the serum samples. The levels of CCL2, CCL3, CCL4 and CXCL8 varied based on seasonal variations with higher levels during summer period. The study also showed an inverse relationship of CCL4 chemokine with age [262]. Expression of CCL2 by β-cells, recruits monocytes and macrophages thereby causing insulitis and islet cell destruction [263]. CCL2 has also been shown to attract the tolerogenic CD11c + CD11b + DC (DCs) to pancreatic islets, thereby reducing diabetes incidence in NOD mice [264]. Pancreatic islets release CXCR1/2 ligands such as CXCL1 and CXCL8 in response to inflammation [265] and the circulatory levels of these ligands are elevated in humans and mouse models of T1D reflecting an anti-islet autoimmune activity [266]. Neutrophils are the primary leukocytes expressing CXCR2 and the depletion of neutrophils in combination with CXCR1/2 inhibitors efficiently prevented diabetes in NOD mice [267].

During early islet inflammation, proinflammatory cytokines are released by a small number of early infiltrating immune cells, including, IL-1β, TNF-α, and IFN-γ. IL-1β and/or TNF-α plus IFN-γ induce β-cell apoptosis via the activation of β-cell gene networks under the control of the transcription factors nuclear factor-κB (NF-κB) and STAT(STAT-1), attracting the DCs and other immune cells to pancreatic islets [268]. NF-κB activation leads to production of nitric oxide and chemokines and depletion of ER calcium [269]. The execution of β-cell death then occurs through activation of mitogen-activated protein (MAP) kinases, via triggering of ER stress and by the release of mitochondrial death signals [268, 270]. Upon further activation, more mediators like Fas/FasL, perforin/granzyme, and pro-inflammatory cytokines come into play to produce their deleterious effects on β-cells secreted by islet invading immune cells [271].

5.3 Infiltration of Immune Cells during Early Stages of T1D

Early infiltration of immune cells in the pancreatic islets always precedes inflammation and onset of autoimmunity in both NOD mice and humans. The islets are normally encapsulated by a layer of peri-islet basement membrane and an interstitial matrix and this layer must be breached by the infiltrating immune cells to cause any β-cell damage [272]. At the same time, the islets are highly vascular in nature, providing abundant cell adhesion molecules for T cell interactions [272]. Pancreatic infiltration predominated by monocytes and B-lymphocytes indicates an early expression of autoimmune phenomena in NOD mice [273]. Infiltrating mononuclear cells consists of CD4 + T cells, CD8+ T cells, B cells, and macrophages, out of which CD8 + T cells being predominant followed by macrophages both in NOD mice and humans [274, 275]. Novel techniques like two photon and intravital microscopy gave much more clear and detailed insight of the islet infiltrates and their phenotype. T cell trafficking studies by Coppieters et al. (2010, 2012) gave us a much better insight of some of the happenings during onset of experimental T1D. According to these studies CD8+ T cells enters pancreatic islets by extravasation through post capillary venules in a random-walk fashion and they move freely in and out of the islets with no time-lag at the islet–exocrine interface [276–278].

The islets seem to be exposed to both antigen-specific and non-antigen-specific T cells, with both cell trafficking to and from the pancreas similarly [279]. One recent study by Lindsay et al. (2015) suggested that these cells halted and mostly interacted with APCs during early stages of disease [280]. These studies also suggest that some other signals in addition to chemokines and cytokines may be involved in the recruitment of T cells to the islets as many of the T cells found at islets of both mouse models and humans are non-islet antigen-specific [278]. A recent study of population dynamics of islet-infiltrating cells by Magnuson et al. (2016) found out that insulitic lesion is open to constant cell influx and turnover, predominated by B and T cells along with CD11b + c + myeloid cells. They have also shown that Tregs exist in peripheral lymph nodes but their migration towards the pancreas is slow and sluggish, which might be the reason for their decreasing proportion in islets as T1D progresses [281]. Innate immune cells, like plasmacytoid DC (pDCs) have also been implicated in initial progression of islet inflammation, especially in NOD mice, as early as 2 weeks of age [282].

6 Cellular Players and Pathological Mechanisms Involved in Beta-Cell Destruction

6.1 Innate Immune Cells

The innate immune system is the first line of defense that provides prompt response following infection or injury. The primary mediators of innate response are circulating factors and cells of non-lymphoid lineage like DCs, monocytes/macrophages, neutrophils and other rare lymphocytes. It recognizes threats by using cell surface,

intra-cellular and secreted, pattern recognition receptors (PPRs), like TLRs, nucleotide binding oligomerization domain (NOD)like receptors and RIG-I like receptors [283].

6.1.1 Dendritic Cells (DCs)

DCs are APCs with functions extending to both innate and adaptive immunity. They play a crucial role during infections and in maintaining immune tolerance to self-tissues and commensal microorganisms [284]. DCs can be divided into two main subtypes: myeloid DCs (mDCs) and pDCs.

6.1.2 Myeloid DCs (mDCs)

mDCs are CD11c + and can be further divided into two major types according to their migratory and tissues localization properties namely, migratory mDCs and lymphoid tissue-resident mDCs. Migratory mDCs are immature and sample antigens in peripheral tissues and subsequently migrate to local lymph nodes via the afferent lymphatics and develop into mature or semi-mature mDCs [285, 286]. Semi-mature mDCs are thought to induce tolerance whereas mature mDCs primarily induce immunity and have a high expression of co-stimulatory molecules and MHC II [287]. DCs found in lymphoid organs like lymph nodes are called lymphoid tissue-resident mDCs and they play a major role in priming CD4+ and CD8+ T cells.

The role of DCs in T1D is well studied; their peri-islet accumulation can be seen in NOD mice as early as 4 weeks of age and was concomitant with the influx of lymphocytes. Earlier studies found yield, function and phenotype of DCs from subjects at risk of developing T1D to be impaired. Lower yield of DCs from adherent peripheral blood mononuclear cells along with reduced expression of CD1a and co-stimulatory molecules like CD80 and CD86 was observed in T1D relatives compared to controls. Additionally, abridged stimulation potential of DCs for autologous CD4+ T cells from relatives of T1D subjects and some recently diagnosed subjects was observed [288]. Saxena et al. (2007) have shown that, the ablation of CD11b + CD11c + DCs leads to the loss of T cell activation, insulitis, and diabetes mediated by CD4+ T cells, and the same was restored when the cells were added back [289]. Decreased numbers of mDCs and pDCs with, a reduced CCR2 expression in recent-onset T1D were also observed. This abnormality of DCs in T1D may have an effect on the initiation and intensity of auto-immune responses, due to the important role that CCR2 plays in DC chemotaxis and differentiation of Th1 subsets [290]. A recent study described that DCs can also guide islet autoimmunity via processing and presentation of restricted autoantigens in a unique and a highly immuno-dominant form by the high-risk HLA-DR [291]. It has also been demonstrated that human BDCA1+ DCs from pancreas-draining lymph nodes and blood effectively engulf β-cells and induce interferon (IFN)-α/β responses and have suppressed Th2 cytokines [292].

6.1.3 Plasmacytoid DCs (pDC)

The ability of pDCs to secrete copious amounts of IFN-α upon viral encounter has defined their role as front runners of virus induced adaptive immune responses [293]. pDCs once activated through TLR7 and TLR9 stimulation by CpG nucleotides containing DNA, start releasing large amounts of IFN-α [294, 295]. pDCs can also play important role as APCs and the uptake and presentation of antigen to CD4+ T cells or CD8+ T by human pDCs enhances when stimulated in the presence of antigen-specific immunoglobulins [296, 297].

The role of pDCs in autoimmune diabetes has been proposed by several studies. Increased production of IFN-α and pDCs were detected in autoimmune diabetes patients at diagnosis, along with high expression of IFN-α induced genes in prediabetic children [298–300]. One of the reasons for the infiltration of pDCs in islets during the initiation of autoimmune diabetes, could be the release of self-nucleic acids (genomic DNA, mitochondrial DNA, RNA etc.) by dying β-cells. As pDCs and monocytes can capture β-cell specific nucleic acids during normal scavenging process akin to other autoimmune diseases like systemic lupus erythematosus (SLE) and psoriasis, these cells might get activated to a pro-inflammatory phenotype [301–303]. In the islets of NOD mice accumulation of pDCs were observed as early as 2 weeks of age, where they get activated via TLR 9 by self-DNA-cathelicidin-related antimicrobial peptide (CRAMP) complexes, leading to the production of IFN-α and induction of autoimmune diabetes. Their role in the initiation of autoimmune diabetes was also confirmed by depletion treatments [282]. T1D subjects both at risk and newly diagnosed were found to have increased pDCs compared to controls. Increased IFN-α production in T1D subjects by PBMCs upon stimulation with influenza viruses was observed that correlated positively with pDC numbers. Additionally by *in vitro* studies authors also demonstrated that IFN-α produced by pDCs augments Th1 responses, as a greater proportion of IFN-γ-producing CD4+ T cells from T1D subjects were observed [304]. A potential role of TLR9 induced IFN-alpha in T1D development can be deduced, as CpG 2216 induced IFN-α production by pDCs was found to be highest in T1D relatives even though lower pDCs numbers were observed both in T1D patients and their relatives [305]. A disease-promoting role of E2–2 dependent pDCs was recently described during autoimmune diabetes in the NOD mice. After knocking out E2–2, abridged recruitment of pDCs was observed in pancreatic islets along with decreased CpG1585 induced IFN-α production that markedly reduced diabetes incidence [306].

A tolerogenic role of pDCs has also been suggested by some studies, Welzen-Coppens et al. (2013) reported the accumulation of pDCs and lymphocytes in pancreas of NOD mice 10 weeks onwards. These pDCs expressed Indoleamine-pyrrole 2,3-dioxygenase (IDO) and were found to be responsible for reduced insulitis and slow disease development [307]. In another study, ablation of DCs from NOD mice lead to accelerated insulitis, marked by the loss of pDC and localized loss of IDO, which was restored on the return of pDCs to the depleted mice [289].

6.1.4 Monocytes and Macrophages

In addition to diabetogenic T cells and B cells, several studies suggest a role for monocytes/macrophages in autoimmune mediated β-cell destruction. In a study, passively transferred diabetogenic T cells failed to induce diabetes following depletion of monocytes. Additionally, activated macrophages are also known to kill β-cells directly *in vitro* [308, 309]. Convincing evidence was provided by Martin et al. (2008) using multiple transgenic mouse models, that monocytes can induce diabetes by destroying β-cells even in the absence of functionally mature T and B cells, following their recruitment to pancreatic islets under the transgenic expression of chemokine CCL2 in β-cells [263]. Apart from their direct effect, macrophages also help in the recruitment of other cells to islets by producing chemokines CXCL1 and CXCL2, which recruit CXCR2-expressing neutrophils from the blood. This recruitment of neutrophils is important for the induction of diabetes as its blockade at early age by CXCR2 antagonist diminishes T cell responses and development of the disease [310, 311].

6.1.5 Neutrophils

Neutrophils are also part of the list of innate immune cells involved during the initial phases of T1D as their numbers are decreased in the peripheral circulation of recently diagnosed T1D subjects which may be attributed to their increased infiltration in the pancreas [312]. Additionally, neutrophil extracellular traps (apoptosis of neutrophils resulting in the release of DNA complexes or NETosis) and associated serum biomarkers like neutrophil elastase (NE) or proteinase 3 (PR3) are increased in recently diagnosed T1D subjects compared to controls [313]. Although a new study by Qin et al. (2016) contradicts the previous study and has shown that, NETosis-associated serum biomarkers, NE and PR3 are decreased in T1D subjects in association with the reduced neutrophil count [314].

6.1.6 Natural Killer (NK) Cells

NK cells are granular lymphocytes that lack B or T cell receptors and recognize their target cells via presence or absence of specific cell surface receptors like MHC molecules. They are cytotoxic in nature and destroy their target cells by exocytosis of perforin and granzyme, and are also known to secrete IFN-γ and TNF-α [315]. Some early studies suggested role of NK cells in TID by showing that NK cells are involved in destruction of islet cells in BB rat and NOD mice [315]. The mechanism of β-cell killing was further explored by Gur et al. (2010), where they identified that presence of ligand to NKp46 or NCR1 on β-cells is responsible for activation of NK cell receptor which leads to their degranulation and onset of diabetes in NOD mice [316]. Tregs are capable of regulating NK cells in islets by limiting amounts of IL-2 [317]. In humans altered frequency and phenotype of NK

cells has been observed by many studies, the first of those observing slight reduction in blood NK cells at the time of onset with very high secretion of IFN-γ [318]. NK cells from T1D children were found to be reduced in number with reduced responses to IL-2 and IL-15; finally defects in activating NK cell receptor, NKG2D were also observed [319]. A recent study by Duangchan et al. (2016), showed that NK cell subsets in long standing T1D are skewed towards more activated or less regulatory phenotype [320].

6.1.7 Natural Killer T (NKT) Cells

NKT cells are unconventional T cells that act as a link between innate and adaptive immune systems. Their best-known subset invariant-NKT (iNKT) cell expresses semi-invariant TCR, Vα14-Jα18 and Vα24-Jα18 in mice and humans respectively, and recognizes glycolipid ligands, presented by highly conserved CD1d molecule. In a recent study, they have been postulated to play regulatory role during T1D through various mechanisms [321]. Absence or abnormalities in their frequency and function relates to the acceleration of autoimmunity and diabetes, whereas their increased frequency or function prevents β-cell autoimmunity in both NOD mice and humans [322–325]. Studies on iNKT cells in NOD mice associates T1D protection with a Th2 shift in the effector T cell responses that involves IL-4 and IL-10, along with their ability to induce tolerogenic DCs that generates Tregs in PLNs [326–329]. **S**tudies in humans have shown decreased IL-4 production by iNKT cells sourced from the PLNs and peripheral blood [330]. Additionally, defective Th2 cytokine production and Th1 bias by iNKT cells was also observed by another study [331]. A recent study by Usero et al. (2016) found that iNKT cell suppression of effector T cells is defective in T1D patients. The mechanism involved was cell contact independent and IL13 was described to exert the suppressive effect [332]. Collectively these studies support the notion that exploring iNKT cell alteration in T1D could open a new path in T1D intervention.

6.1.8 Innate Lymphoid Cells (ILCs)

Innate lymphoid cells (ILCs) belong to a family of developmentally related cells that lack specific antigen receptors but can promptly mount an immune response on microbes by producing copious amounts of an array of effector cytokines. They have functions in tissue remodeling, lymphoid organogenesis, inflammation and antimicrobial immunity predominantly at mucosal barrier surfaces [333]. The family of ILCs comprises of three subsets, named as group 1, 2 or 3 ILCs, on the basis of common of surface markers, transcription factors and cytokines produced. Group 1 ILCs (ILC1s) constitutively express T-bet, secrete cytokines like IFN-γ and TNF and respond to IL-12. Group 2 ILCs (ILC2s) have high expression of GATA3, secrete IL-4, IL-5, IL-9, IL-13 and respond to IL-25, IL-33 and TSLP, Group 3 ILCs (ILC3s) expresses RORγt, secrete IL-17 and/or IL-22 and respond to IL-1β, IL-6

and IL-23 [334]. There is scant information on their role in T1D. NOD mice have an increased frequency of type 3 ILCs along with decreased frequency of type 1 ILCs in the MLN at all stages of disease and in the PLNs at 8 weeks of age [335]. A novel CD25+ ILC population in the pancreas is also been identified, but more studies are required to ascertain its role if any in T1D [336].

6.1.9 Mucosal Associated Invariant T (MAIT) Cells

Mucosal associated invariant T (MAIT) cells are innate like T cells in peripheral blood of humans and abundantly found in intestinal mucosa that display both innate and effector like functions to confer protection against microbial activity and infection. These cells express an invariant α-chain (TRAV1–2-TRAJ33/12/20 in humans and TRAV1-TRAJ33 in mice) coupled with a limited repertoire of β-chains, imparting them with the ability to recognize precursors of riboflavin of bacterial origin (vitamin-B related antigens), presented by the MHC-I related protein MR1 [337]. Recently, Rouxel et al. (2017), have suggested an important role of MAIT cells in the development of T1D. Firstly, they discovered that in recent onset T1D children, the frequency of circulating MAIT cells is significantly lower and the phenotype of these cells was also different in the recent onset T1D children, than their age matched controls [338]. In the recent onset T1D children, the MAIT cells had higher expression of activation marker, CD25 and exhaustion marker, programmed death-1 (PD-1), but lower expression of tissue homing chemokine receptor, CCR6 and adhesion molecule CD56. Additionally, upon stimulation the MAIT cells derived from these children showed lower expression of IFN-γ, but higher expression of TNF-α, IL-4 and granzyme-B, upon stimulation with PMA/ionomycin. The authors further showed that in an inflammatory milieu, as expected during islet inflammation, these cells secrete high levels of granzyme-B, in response to increased upregulation of MR1 by the pancreatic β cells, implicating their role in direct participation in β cell killing. In NOD mice as well progression to diabetes is associated with decreased production of IL-17A and IL-22 from MAIT cells in the ileum and an accumulation of IFNγ- and granzyme-B (GzB) –producing MAIT cells in the pancreatic islets. Compared to humans (6%) the frequency of MAIT cells is lower in NOD mice (0.1%) in peripheral circulation, however, such cells can be traced in pancreas or peripheral blood by using MR1 tetramers loaded with the riboflavin derivative 5-OP-RU [339, 340].

6.2 Adaptive Immune Cells

6.2.1 T Cells

T1D results from the destruction of insulin-producing pancreatic β-cells mainly by T cells recognizing the self-islet associated antigens. Best studied antigens include preproinsulin [341], GAD65 [342], insulinoma antigen-2 (IA-2) [343], ICA [344],

heat shock protein (HSP) [345], islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) [346], imogen-38 [347], zinc transporter-8 (ZnT8) [348], pancreatic duodenal homeobox factor 1 (PDX1) [349], chromogranin A (CHGA) [350] and islet amyloid polypeptide (IAPP) [351]. However, CD4+ T cells recognizing post translational modified peptides [246, 249] and hybrid insulin peptide also have been detected in NOD mice and T1D subjects [352]. Recently, Delong et al. (2016) reported that CD4+ T cells recognizing epitopes formed by covalent cross-linking of proinsulin peptides to other peptides present in β-cell secretory granules such as CHGA and IAPP can be detected in islets of T1D subjects [352].

6.2.2 CD4+ Helper T (Th) Cells and Subsets

The autoreactive CD4+ T cell is likely at the heart of this disease, as an orchestrator of the immune attack on β cells. Loss of CD4+ T cell tolerance to β-cell associated antigens is a key step involved in pathogenesis of T1D (221). CD4+ T-cells are activated upon interaction with APCs presenting β-cell autoantigens mainly in PLNs followed by a formation of specialized junction called immunological synapse at the T-cell interface [353]. Recognition of antigen by CD4+ T cells can lead to activation or anergy/death depending upon the co-stimulatory molecule involved in process. Signaling through CD28, TNF family members, CD154 (CD40L) and OX40 leads to activation of CD4+ T cells whereas CTLA4 and PD-1 inhibit T cell activation [354, 355]. Following activation, CD4 + T cells (Th1) cells secrete IL-2, which activates CD8+ T cells. At late stages of disease, autoreactive T cells become resistant to suppression by Tregs that may also have diminished regulatory capacity, ultimately leading to complete β-cell destruction [356]. It has been reported that CD4 + T cells specific for β-cell auto-antigens present more proinflammatory phenotype and secret IFN-γ and IL-17[357].

6.2.3 Th17 Cells

Several line of evidences from animal and human studies indicate that Th17 cells are involved in pathogenesis of T1D which were previously thought to be mediated by only Th1 cells [358]. Role of Th17 cells in β-cell destruction is now being explored in T1D subjects. Deficiency of IL-17 in NOD mice delayed the onset of diabetes [156]. Inhibition of Th17 cells using anti-IL-25 or anti-IL-17 decreased GAD65 autoantibody levels, increased the frequency of Tregs, significantly suppressed development of diabetes in 90% of treated animals [359, 360]. IL-23, regulator of IL-17, promotes development of diabetes in sub-diabetogenic doses of streptozotocin treatment by expansion of Th17 cells and IFN- γ production in male C57BL/6 mice [361]. Moreover, deficiency of IL-17A ameliorates streptozotocin-induced diabetes [362]. Adoptive transfer of islet associated antigen-specific Th17 cells induced diabetes in immunodeficient mice [363, 364]. Studies have reported that PLNs of T1D subjects possess increased population of Th17 cells [365].

Furthermore, increased population of IL-17 secreting T cells were observed in new onset T1D children [366]. Interestingly, circulating memory CD4+ T cells from T1D subjects showed increased IL-17 secretion and expression of IL-17, IL-22 and retinoic acid-related orphan receptor C isoform 2 (RORC2) *ex vivo,* indicating activation of IL-17 pathway *in vivo* [96]. Upon *in vitro* stimulation with β-cell autoantigens including proinsulin, insulinoma-associated protein, and GAD65 peptides, the circulating CD4+ T cells from T1D subjects have been shown to produce IL-17 [367]. These observations clearly indicate a Th17 biased response in T1D patients.

6.2.4 Th40 Cells and TCR Revision

A central paradigm of immunology holds that once T cells exit the thymus, TCR molecules do not undergo alteration. To the contrary, several laboratories have shown that peripheral T cells re-express recombination activating genes 1 (RAG1) and RAG2 proteins and subsequently alter TCR expression [368–371]. Th40 cells are subsets of Th cells defined by expression of CD40 and capable of undergoing TCR revisions [372–376], a process by which T cells can alter expression of TCR even in the periphery by inducing RAG1 and RAG2 [374–376]. Th40 cells have been shown to become highly pathogenic in autoimmune disease models [372–376]. CD40 acts as a co-stimulatory molecule on T cells, which upon engagement induces RAG1/RAG2 TCR recombination machinery via interaction with Ku proteins, DNA polymerases and helicases leading to alteration of TCR expression [374–378]. Alterations in the expression of TCR-α [73, 104] and TCR-β [370, 379, 380] in long-standing peripheral T cells occurs following the induction of RAGs [369, 374, 381]. Th40 cell numbers in spleen and peripheral lymph nodes of young NOD mice are equivalent to non-autoimmune mice, but in PLNs, Th40 cell numbers are expanded significantly as early as 3 weeks of age [375]. Pathogenicity of Th40 cells is demonstrated by their ability to transfer T1D to NOD/SCID recipients [373, 375–377]. Th40 cells are stimulated in the PLNs and are then recruited to infiltrate islets. Since Th40 cells are capable of TCR revision, the odds of increasing autoreactive T cells on site would be increased dramatically. Th40 cells are capable of producing IL-17 [377, 382, 383] and IFN- γ to drive diabetogenesis.

6.2.5 CD8+ Cytotoxic T Cells

Infiltrating CD8+ T cells recognize epitopes presented with MHC-I molecules on the surface of β-cells and destroy them. During this period there is hyperexpression of MHC-I molecules on the surface of the β-cells, allowing enhanced epitope presentation to the infiltrating CD8+ T cells [384]. Among the major epitopes recognized by the autoreactive CD8+ T cells, preproinsulin derived epitopes are the primary ones to be recognized by the CD8+ T cells, during the progression of the disease [385].

These autoreactive CD8+ T cells kill target cells mainly by releasing cytotoxic granules or interaction with TNF family-related death receptors. Cytotoxic degranulation involves release of perforin, which facilitates the entry of co-released gran-

zymes with serine protease activity into cells and thus results in rapid cell death. Fas ligand (FasL) is the best-characterized TNF family-related death receptor, binding to Fas expressed on the target cell surface and initiating a series of intracellular pathways resulting in apoptosis. It has been well established in T1D that CD8+ T cell mediated killing of β-cells predominantly use cytotoxic degranulation pathway [386, 387]. This period is also marked by a change in the phenotype of autoreactive CD8+ T cells, whereby there is a shift towards the effector phenotype and an increase in the proliferative potential [388]. Destruction of β-cells results in shedding of other islet associated antigens and presentation of these antigens leads to infiltration of pancreatic islets by diverse population of T cells (predominantly tissue specific), by a process called epitope spreading [389]. Rate of progression of β-cell destruction may vary, depending upon frequency, proliferative and pathogenic potential of CD8+ T cells [388]. Beta-cell associated antigen-specific CD8+ T cells have been characterized and shown to express memory cells markers [390]. Therefore, targeting memory T cells in T1D subjects to preserve residual β-cell mass seems plausible [391].

6.3 B Cells

B cells play an additional key role in the pathogenesis of T1D, yet their functions are less explored. B cells produce autoantibodies against insulin, GAD-65, IA-2, and ZnT8 which are commonly used as biomarkers in predicting disease onset [392], besides routine clinical diagnosis of autoimmunity in diabetes. Although they produce antibodies, these are not thought to be pathogenic, rather their islet antigen presenting capabilities appear to be critical in disease pathogenesis [393]. To explore their role in antigen presentation, a transgenic NOD mouse was generated which could not secrete immunoglobulin but present the antigen. This resulted in increased insulitis and development of diabetes in NOD mice [394]. Early therapy, with either anti-CD20 or anti-B cell activating factor (BAFF) mAb, before the onset of insulitis merely delayed disease progression in NOD mice [395, 396]. A recently identified subtype of B cells, immunosuppressive B cells, also known as B regulatory cells (Bregs) are CD1d^{high}, CD5+ and produce IL-10 [397]. Studies have shown that expansion of Bregs by tolerogenic DCs, subsequently reversed new-onset T1D in NOD mice [398].

6.4 Pathological Mechanisms Underlying Beta Cell Death in T1D

Heterogeneous population of immune cells infiltrates pancreatic islets during the progression of the disease. However, T cells comprise the major proportion of the cells causing damage to β-cells [399]. Following antigenic recognition in lymph nodes, naïve T cells expressing self-reactive TCRs become activated, proliferate and

differentiate into various subsets: central memory T cells and effector memory T cells and effector T cells. Effector T cells invade pancreatic islets and destroy β-cells. Central memory T cells persist in lymph nodes, exhibit high sensitivity to antigenic stimulation, are less dependent on co stimulation and are able to differentiate into IFN-γ producing effector cells. Effector memory T cells can home to inflamed tissue; express high levels of perforin and mount rapid effector responses [400]. Effector T cells are short lived, while long term survival of central memory and effector memory T cell subsets pose major hurdle for immunotherapeutic approaches [401]. On the other hand, CD4+ T cells also participate in activation of CD8+ T cells and B cells. Due to loss of self-tolerance to β-cell associated antigens, β-cells are targeted by immune cells by various effector mechanisms including, (1) Granzymes and perforin pathway (2) Fas-FasL pathway (3) Cytokine mediated death (4) Production of reactive oxygen species. Granzyme and perforin mediated apoptosis is the principle pathway used by CD8+ T cells to kill β cells [386]. In the presence of Ca^{2+} ions, perforin monomers inserted in membrane polymerize to form a cylindrical pore of 5–20 nm through the membrane, which assist the entry of granzymes to cytoplasm. Granzymes activate the caspase cascade resulting in apoptosis of β-cells. Pretreatment of preproinsulin specific CD8+ T cells clones with concanamycin A, which results in perforin degradation, significantly reduce the β-cell death *in vitro* [386]. Quite surprisingly, a recent report by Mollah et al. (2017), have demonstrated that Granzyme A, normally considered as a pro-apoptotic mediator of cell mediated cytotoxicity, may be associated with protection to T1D. In their finding, the authors demonstrated that Granzyme-A knock out NOD mice progressed towards diabetes much faster, implicating its role in maintenance of peripheral tolerance [402].

TNF receptor superfamily member Fas is expressed on the surface of β-cells. Islet infiltrating autoreactive T cells can also activate the caspase dependent pathways of β-cell death by binding of FasL expressed by them. Disruption of Fas-FasL signalling using targeted overexpression of a dominant negative form of Fas-associated death domain adaptor protein in pancreatic β-cells significantly delays the onset of diabetes in NOD mice, implicating a role for Fas in the early stages of autoimmune β-cell destruction [403].

Pro-inflammatory cytokines such as type II interferons including, IFNγ, IL-1 β, TNFα also induce β-cell death [404]. IFNγ is mainly secreted by Th1 subset of CD4+ T cells. Binding of IFNγ to their receptor activates the JAK STAT signaling pathway, which induces β-cell death via regulating the expression of FAS, inducible nitric oxide synthase (iNOS) and caspases. In the absence of STAT 1, major downstream transcription factor of IFNγ signaling, IFNγ mediated destruction of β-cells is disrupted in NOD mice [405]. Apart from the role of IFN- γ in pathogenesis of disease, recent study by John P et al. (2017) also reported that IFN- γ can also limits the activation of diabetogenic CD8+ T cells implicating its role in induction of tolerance [406]. Type 1 interferons, IFNα and IFNβ, also provide signals responsible for accelerating the β-cell death. Type 1 interferons regulate the effector functions and augment the cytotoxity of CD8+ T cells by rapid phosphorylation of STAT4 and induction of Granzyme B. Additionally, studies revealed that overexpression of IFN α in pancreatic β-cells of non-diabetes-prone mice regulate the onset of diabetes in

mice with severe insulitis, while expression of IFNβ in islets of NOD mice accelerated autoimmunity [407].

In another mechanism, signaling through IL-1β leads to activation of NF-κβ in rodent and human islet cells. Translocation of NF-κβ to nucleus induces the β-cell death. Prevention of NF-kβ activation by an inhibitory B (I B) "super-repressor" protects pancreatic cells against cytokine-induced apoptosis. It has been demonstrated that overexpression of NF-κβ super-repressor in rodents protect pancreatic β-cells against cytokine-induced apoptosis [404] and transgenic mice expressing an NF-κβ super-repressor are resistant against experimental diabetes induced by multiple low-doses streptozotocin [408].

TNF-α causes destruction of β-cells by activation of NF-kβ and extrinsic pathway of apoptosis. An important role for TNF-α in β-cell killing was demonstrated in TNF-R1 null mutant NOD mice, which fail to develop spontaneous diabetes [399]. Moreover, treatment of NOD mice with anti-TNF-α antibodies also prevents diabetes development implicating the role of TNF-α in β-cells destruction [409]. Reactive oxygen species e.g. nitric oxide induce β-cell death by causing DNA damage and in turn activation of p53 in a concentration dependent manner. However, reactive oxygen species seems to have a less relevant role for cytokine-induced β-cell death in humans and mice. Blocking of iNOS does not prevent cytokines induced β-cell death [410] while islets obtained from an iNOS knockout mouse are only partially protected against death induced by IL-1β and IFN- γ [411, 412].

7 Protection of Beta-Cells

Targeting immune cells that are associated with β-cell destruction remains the mainstay of most of the approaches in protecting β-cells. Initial attempts to target the immune cells were more generalized, had limited success and were associated with risks of infection. With time, as the information about the cells and factors involved in the disease process became clearer, targeted approaches have been pursued. However, till date, none of the treatment approaches has been able to achieve the goal of selective elimination of immune cells causing β-cell damage, without any compromise on the general immune responses.

7.1 Immunosuppressive Agents

It has been proven in combined outcomes of several trials that blocking T cell function in T1D leads to β-cell preservation by the use of immune-suppressive agents such as cyclosporine (CsA) and azathioprine. Although the continuous CsA treatment in patients with new-onset T1D can eliminate the need for exogenous insulin for some duration, continuous treatment and chronic CsA therapy to maintain remission has been found to be associated with toxic effects in the kidneys leading to decline in the

enthusiasm for its use in T1D patients [413]. Another promising drug, rapamycin (sirolimus) inhibits the critical mammalian target of rapamycin (mTOR) pathway which is involved in cell growth, proliferation, motility, and survival [414]. Rapamycin monotherapy has also been found to increase in serum C-peptide and a reduction in exogenous insulin requirement in patients with long-term T1D [415]. However, rapamycin in combination with IL-2 has also been shown to impair β-cell function [5, 416].

7.2 Monoclonal Antibodies (mAbs)

Among several newer immunotherapies developed in the recent past, selecting mAbs against different immune cell receptors appeared as another promising approach [5]. In an attempt to replace the use of immunosuppressive drugs globally, several agents like anti-CD3 mAb (teplizumab/otelixizumab), anti-CD20 mAb (rituximab), and CTLA-4-Ig (abatacept) directed at the co-inhibitory receptors have been evaluated in new onset T1D patients [417].

7.3 Anti-CD3 mAbs

In contrast to pharmacological immunosuppression treatment, anti-CD3 therapy transiently depletes T cells and exerts long-lasting immune regulatory effects [413]. Administration of anti-CD3 mAbs has shown substantial benefits in recently diagnosed T1D patients in the initial clinical stages. Another report revealed that this therapy particularly teplizumab and otelixizumab can help in preserving the β-cell function for more than 2 years in patients [418–420]. Otelixizumab treatment preserved insulin production for more than 3 years depending on patient age and baseline residual β-cell mass. Moreover, preservation of residual β-cell function was observed following brief teplizumab treatment as long as 5 years in a small group of patients [421]. Therefore, it seems that a short treatment course with Anti-CD3 mAbs may eliminate the need for chronic treatment by triggering lasting tolerance. However, the targeted permanent arrest of the C-peptide decline rate could not be achieved as observed in a series of immune modulation trials in new-onset T1D. Hence, it is to be evaluated whether further optimization of therapeutic antibody concentration and timing of treatment would be able to provide better outcomes or not [413]. Furthermore, the risks of T cell depletion in predisposing individuals to infectious diseases must also be evaluated.

7.4 Anti-CD20 mAb (rituximab)

Being APCs, B cells play a crucial role in the pathogenesis of T1D as these cells themselves are involved in infiltrating the pancreatic islets, presenting autoantigens to T cells and secreting autoantibodies. Therefore, anti-human (h) CD20 mAb were

used to delay or revert diabetes by depleting B cells in transgenic NOD mouse having human CD20 receptors on their B cells with positive outcomes [5, 395]. Rituximab has also been used in Phase II clinical trials. The study showed an initial improvement in T1D by promoting C-peptide levels, reducing HbA1c levels and reducing insulin dose, although this protective effect was short lived. However, continued B cell depletion and associated adverse events as well as the risk of lowering systemic immunity limit the utility of anti-hCD20 mAbs [417, 422].

7.5 CTLA-4-Ig (abatacept)

Besides the main antigen-driven signal, co-stimulatory signals are required to keep immune T cells fully activated. In humans, the susceptibility of T1D has an association with CTLA-4 locus and its immunopathogenesis is linked with T-cell autoimmunity. Therefore, modulating this co-stimulatory signal is another promising strategy in treating T1D. The target can be achieved by using abatacept, which has been observed to modulate co-stimulation and prevent full T-cell activation, as an estimated 9.6 months delay in C-peptide reduction had been achieved with continued administration of abatacept. Despite this, a continued parallel deterioration of β-cell mass as well as function was also observed, inhibiting its further use [423].

7.6 Antithymocyte Globulin (ATG)

ATG is an effective immune-depleting agent and a rabbit polyclonal gamma immunoglobulin (IgG) which is active against thymocytes of human. It is specific for various receptors presented on T cells as well as other immune cells. Short-term ATG therapy in recent onset T1D patients preserved residual C-peptide production and lowered the requirement of insulin but could not induce long-lasting remission [424].

7.7 Low Doses of Interleukin-2 (IL-2)

IL-2 also called a T cell growth factor secreted by T cells itself, can stimulate both effector T cells and Tregs in a dose dependent manner. IL-2 activates primarily STAT5 in Tregs, whereas IL-2 also induces the MAP kinases and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathways in effector T cells [425, 426]. Due to higher expression of IL-2 receptor, Tregs require less IL-2/Il-2R signaling [427]. It has also been reported that IL-2 mediated signaling is dispensable for effector T cells but not for Tregs [428]. Defects in IL-2 mediated signaling have been reported in T1D [429–431]. High dose of IL-2 is associated with many severe side effects [428, 432]. Besides side effects, high dose of IL-2 also carries risk of expansion of

effector T cells that mediate autoimmunity [428]. These key points permit the development of targeted Tregs therapy using low-dose IL-2 administration. First trial with low dose IL-2 (0.33–1 MIU/day) reported that it is well tolerated in the T1D subjects with mild side effects [433]. The minimal doses that are required for the purpose are not fully known and are being investigated in an ongoing dose-finding trial in recently diagnosed T1D children (NCT01862120).

7.8 Phytotherapeutic Approaches

As discussed earlier, prevention of the degeneration of β-cells and stimulation of endogenous islets regeneration are currently the essential approaches for the treatment of T1D. Among several antidiabetic plants investigated so far, a small fraction has been shown to pose pancreatic β-cell protection and/or regenerative properties as well (2). *Allium sativum* [434], *Azadirachta indica* [435, 436], berberine [437], *Crocus sativus* [438], *Gymnema sylvestre* [439], *Juglans regia* [440, 441], *Momordica charantia* [442] and *Nigella sativa* [443–445] have been reported to possess β-cell regenerative property [446]. Many of these agents and their extracts have also been shown to reduce insulin resistance. Hence, their consumption may help in reducing insulin dependence in diabetic patients.

8 Cell Based Treatments

As T1D is caused by functional loss in pancreatic β-cells, replacing them with functional β-cells from various sources provides a new hope for treating T1D. For this purpose, whole-pancreas transplantation, initiated in 1966 is a widely accepted therapeutic modality as evidenced by the fact that several thousand pancreatic transplants have been performed until now. Normal HbA1c levels achieved using this strategy allow long-term insulin independence over 2 years after transplant. However, pancreas transplantation is a surgical procedure that involves high risk of systemic infection that requires lifelong immunosuppression in the recipients. In order to overcome these complications, pancreatic islet cell transplantation has been introduced to replace whole organ transplantation due to new research efforts which presents as a better procedure requiring lesser invasive procedure [447]. However, the procedure requires harvesting the islet cells, preferably from the brain-dead donors and mostly requires two or three donors to achieve insulin independence. Also, to protect the transplanted islets from host's anti-donor HLA and anti-islet responses, various immune-isolation strategies, such as encapsulation in semi permeable matrices are also being explored. Further, in view of the limited availability of pancreas donors, xenografts from other sources like pig islets, have also been considered and pursued further for research.

8.1 Stem Cell-Based Therapies

Stem cells have become an important therapeutic entity due to their inherent regenerative, differentiation capacities as well as their immunomodulatory potential. While the regenerative and differentiation potential can be utilized to avail a supply of glucose-responsive insulin-producing cells for transplantation, the immunomodulatory properties of multipotent mesenchymal stromal cells and hematopoietic stem cells (HSCs) can be used to seize cell damage, preserve the remaining cell mass, promote the regeneration of endogenous cells as well as prevent graft rejection [448]. In view of these regenerative and immunomodulatory characteristics, a variety of stem cells from different sources including, embryonic, bone marrow-derived HSCs and bone marrow-derived MSCs, umbilical cord blood-derived MSCs, adipose tissue-derived MSCs (ADSCs) and pancreas-derived multipotent precursor cells as well as pancreatic cell progenitors have been tested and various studies have provided promising outcomes for the treatment of T1D as follows:

8.1.1 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that were originally identified in the bone marrow. MSCs can also be isolated from cord blood, peripheral blood, fallopian tube, fetal liver and lungs. In preclinical T1D studies [449–451], MSCs have been shown to induce and expand Tregs thereby suppressing the immune responses. MSCs can also induce immature IL-10-secreting DCs *in vitro*, thus they potentially interrupt the priming and amplification capacity of autoreactive T cells involved in tissue inflammation. These DCs can assist in the inhibition of inflammatory T cell responses to islet antigens and promoting the anti-inflammatory, regulatory responses exerted by MSCs [452]. Being non-immunogenic in nature, MSCs can also provide protection after allogeneic transplantation and hence they are more attractive for cell based therapies [453]. In spite of the source, whether bone marrow [454] or adipose tissue [455] used for their aspiration, MSCs have been proven to be well-tolerated in T1D patients. Moreover, MSCs have also been documented to improve T1D parameters such as C-peptide preservation [455].

8.1.2 Hematopoietic Stem Cells (HSCs)

In contrast to MSCs, hematopoietic stem cells (HSCs) are found in stem cell niches such as bone marrow, which are situated in the entire body or in umbilical cord blood. HSCs are comprised with the ability to initiate and promote neovascularization rather than an effective differentiation and therefore their prime use is to treat immune-related disorders [456]. Voltarelli et al. have reported increase

in β-cell function, prolonged independence from exogenous insulin in 80% of the patients after high-dose immunosuppression and autologous transplantation of hematopoietic bone marrow-derived stem cells with acceptable toxicity in newly diagnosed T1D patients [457]. Further, in another study by Couri et al. (2009) autologous nonmyeloablative HSCs transplanted in patients with newly diagnosed T1D resulted in significant increase in C-peptide levels and insulin independence in most of the patients with good glycemic control [458]. In another study by Li et al. (2012), it has been reported that autologous HSCs transplantation helps in modulating lymphocytes and preserving β-cell function in Chinese patients with new onset of T1D and diabetic ketoacidosis [459].

8.2 Regulatory T Cells (Tregs) Based Therapies

The discovery that CD4+ Tregs play indispensable role in maintaining self-tolerance [460, 461] has led to the prospect of these cells in cell based treatments to restore tolerance and treat autoimmune diseases such as T1D. These Tregs are CD4 + CD25 + Foxp3+ and suppress the proliferation of autoreactive T cells by producing cytokines, cytolysis, deprivation of cytokines and contact-induced cell modulation [462]. Two types of Tregs are engaged in maintaining the tolerance, natural Tregs (nTregs) and induced Tregs (iTregs). nTregs develop from thymic TCR high affinity T cells selection whereas iTregs are peripherally generated FoxP3+ T cells under immunogenic stimulation [463]. Both Treg subsets express CD25, FoxP3, GITR (glucocorticoid-induced TNF receptor) and CTLA-4 but nTregs exhibit a higher expression of programmed cell death-1 (PD-1), neuropillin 1(Nrp-1) and Helios compared with iTregs [464]. There are many evidences, which show that Tregs have the potential to prevent destruction of pancreatic islets, thereby protecting from T1D. Hence, strategies to increase Treg cell numbers and/or function are being explored as potential therapeutic approaches in treating T1D. In fact most of the antigenic/immunosuppressive treatment approaches to reverse diabetes in NOD mice worked via induction of Tregs or proliferation of Tregs [465–467]. Trials on therapy of T1D subjects with Tregs have indeed shown to prolong survival of pancreatic islets [468].

8.2.1 Polyclonal Versus Antigen-Specific Tregs

While considering therapy with Tregs, there are two available choices, polyclonal or antigen-specific (or epitope-specific) Tregs. Administration of polyclonal Tregs may be associated with significant off-target effects, including global immunosuppression that may compromise beneficial immune responses to infections and cancer cells. Therefore, the objective of research in recent times

has shifted to antigen-specific therapeutic approaches that can reverse the disease by selectively halting the harmful immune response without requiring lifelong immune suppression. Adoptive transfer studies suggest antigen-specificity is required by Tregs for trafficking and maintenance in inflammatory tissues such as the pancreas in T1D [389, 469]. Moreover, antigen-specific Tregs are much more potent in suppressing effector T cell responses, as demonstrated in a tumor rejection model, than polyclonal Tregs, which were only partially suppressive [470]. Another study has demonstrated that small number of *in vitro* expanded antigen-specific Tregs are sufficient to reverse T1D whereas large numbers of polyclonal Tregs are required to reverse the disease [471]. Antigen-specific Tregs have been reported to exhibit a much lower threshold for activation and may be activated by a broad range of loosely-defined analogs of their cognate antigen [472]. Besides, the site-specific mode of action, antigen-specific Tregs also have the ability to act as bystander suppressors locally in the organ under attack. It has also been shown in mice that antigen-specific Tregs treat autoimmunity without compromising antibacterial immune response [473]. However, isolation of sufficient number of antigen-specific Tregs is a major challenge, particularly when sampling is limited to peripheral blood. Moreover, success in inducing antigen-specific tolerance has been hampered by the inability to identify peptides triggering the diabetogenic versus the regulatory response. It has been established that islet-associated antigen-specific Tregs can be generated from CD4 + CD25- T cells. Alice et al. (2009) observed that GAD65 derived epitope specific Tregs suppress not only proliferation of GAD specific effector cells but also of tetanus toxoid (TT) specific effector cells when the GAD was present. Suppression was not observed when TT was present alone [474]. Therefore, these observations indicate that it might be possible to reverse autoimmune diabetes by small number of epitope-specific Tregs rather than having Tregs specific for all the diabetes associated antigens.

8.3 Dendritic Cells

Being the most specialized APCs, DCs have the ability to remove or inactivate diabetogenic T cells, convert them into Tregs or re-stimulate the preexisting Tregs [475]. Therefore, they have been chosen several times for immunomodulation in autoimmune diseases especially T1D. At present, phase 1 and phase 2 clinical trials are ongoing with the purpose to evaluate the safety and efficacy of this therapeutic strategy. Of these trials, phase 1 has been completed in one (NCT00445913), but study results have not yet been posted till date. This trial has included candidates of age ranging between 18–60 years with established diabetes. Another clinical trial (NCT02354911), which is in phase 2, is still ongoing and has included new onset T1D candidates aged between 12–35 years.

8.4 Cord Blood Derived Cells

Umbilical cord blood (UCB) is a rich source of Tregs [476, 477] besides other tolerogenic cells such as immature DC and MSCs, all of which have been shown to play key role in immune tolerance [478, 479]. UCB derived CD4 + CD25 + T cells have been shown to contain greater Foxp3 expression than their peripheral blood counterparts, suggesting the greater abundance of Tregs in UCB than peripheral blood [476]. Based upon preliminary observations, it has been found that autologous cord blood transfusion is helpful in slowing down the loss of endogenous insulin production and is a safe procedure in T1D children [480]. Further, it has also been documented that highly functional populations of Tregs are available in UCB and this increased Treg population may be available in the peripheral blood of subjects after more than 6 months of cord blood infusion as evidenced by mechanistic studies [480]. Autologous UCB transfusion in T1D pediatric patients has also been reported to be safe [481]. As the, collection and banking of UCB is becoming widespread all over the world, its utility as a source of therapeutic Tregs is expected to rise further.

8.5 Fibroblasts

Attempts to determine efficacy of stable IDO-expressing dermal fibroblasts in cellular therapy of autoimmune diabetes have been tried in NOD mice. IDO-expressing fibroblasts were found to significantly reduce islet infiltration by immune cells. Diabetes progression was reversed by inhibiting autoreactive CD8+ T cells and Th17 and through the induction of Tregs. Additionally, it was also observed that when IDO-expressing fibroblasts were cultured with islet β-cells they successfully reduced IL-1β levels and β-cell apoptosis [482].

9 Combinatorial Therapies

The accessory cells and biomaterials can provide a definite therapeutic benefit to save islets and their functional improvement. Currently, majority of the combinatorial approaches have been explored in islet transplantation, although, most of them are in experimental phases. The main goal is to recreate an islet friendly niche in a carrier or capsule to provide β-cell interactions within its native environment i.e. creating a microenvironment that includes accessory cells, proteins, as well as the local immunosuppression enclosed within a biocompatible material along with the islet cells. For the purpose, several accessary cells and therapies have been proposed and tested to achieve successful transplantation.

9.1 Cell Encapsulation

Cell encapsulation is a concept by which cells are encased within a biocompatible matrix. In this way a barrier against immune cells and cytotoxic molecules is created to prevent injury and hence avoid rejection while still allowing the active diffusion of essential molecules like oxygen, nutrients and hormones [483]. This way, other β-cell sources (e.g., xenogeneic islets and stem cell–derived β-cells) can also be used for clinical therapy [484]. In a previous report, vortex-induced silk hydrogels have been documented to provide a 3D environment for islets encapsulation *in vitro* thereby allowing the co-encapsulation of proteins found in extracellular matrix and secondary stromal cells to maintain function and viability of islet cells [485]. In a study by Borg et al. (2011) star-PEG-heparin cryogel scaffolds which are tunable in architecture, mechanical characteristics and biomolecular functionalization, and having the ability to load accessory cells, have been reported as highly promising supportive carriers for pancreatic islets in the context of transplantation in various alternate sites [484].

Although encapsulated islet transplantation has been supported in various animal model studies, the process has several limitations such as biocompatibility of encapsulation material, the damaging actions of cytokines, oxygen deficiency in implanted tissue at the transplantation sites and hindered secretion of insulin from capsules, which still remain to be solved [486]. The biggest of these problems is prevention of islet revascularization and oxygen transport to islets. This is associated with development of a hypoxic core within the islets that may result in reduced tissue function and ultimately, death. Therefore, several approaches to enhance microencapsulated islet survival and function have been proposed. For instance, incorporating a perfluorocarbon emulsion into alginate microcapsules to enhance oxygen permeability may help protect islets from hypoxia. Another approach is scattering the islets and allowing them to recluster into smaller size than the original islet. These smaller clusters are less likely to develop a necrotic core and they can function normally because of adequate oxygen supply and better cell-cell communication. Further, 10,000~20,000 IEQ/kg placed in a collagen matrix in stainless steel mesh tubes, with a polytetrafluoroethylene rod in the cassette have been successfully used in 11 T1D patients. This approach resulted in decrease in exogenous insulin requirements in more than 50% patients for up to 4 years [487]. Cadaveric human islets encapsulated in alginate microcapsules transplanted into T1D subject have also shown some beneficial effects [488]. However, fibrotic reactions still occur in alginate microcapsule leading to graft rejection.

9.2 Use of Accessory Cells

As it is known that islet transplantation is gradually becoming a popular diabetes therapeutic strategy, therefore, another emphasis of research is promoting angiogenesis and increasing blood vessels density around transplanted islets. In a recent study by Cao et al. (2016) the combination of allogeneic islet transplantation and

bone marrow mesenchymal stem cells (BM-MSCs) was pursued into NOD mice to investigate the effect of BM-MSCs in transplanted islet function and neovascularization. It was observed that BM-MSCs can migrate to transplanted islets along with promoting neovascularization. In addition, BM-MSCs enhanced immune tolerance of the allograft by improving lymphocytic chimerism of the donor [489]. The endothelium is also known to play an important role in the native islets function and revascularization process after islet transplantation. Endothelial progenitor cells (EPCs) are a population of rare circulating cells in the, cord blood, vessel walls, peripheral blood and bone marrow with the ability to adhere to endothelium at sites of hypoxia with subsequent differentiation into endothelial cells. EPCs/islet co-transplantation, have shown beneficial effects on islet transplantation in rodent models of diabetes [490, 491]. EPCs mediate their functions via direct differentiation into new vessels and pericytes, through secretion of paracrine factors (angiogenic and β-cell mitogenic) [492], via thrombospondin (Tsp)-1-mediated activation of TGF-β1, [493, 494] and through modulation of the expression of the β-cell gap junction protein connexin, a key element in coordinating β-cell function [491] resulting in enhanced insulin secretion.

The adoptive transfer of Tregs as accessory cells can be used to improve islet graft survival, as inflammatory immune response to alloantigens and recurrence of autoimmunity following islet transplantation are the major contributors to pancreatic islet transplant dysfunction. Experimental studies in murine models demonstrate that co-transfer of Tregs and islets can improve the graft survival [495]. Golab et al. (2014) have shown that, the anchoring of human *ex vivo* expanded Tregs to the surface of human pancreatic islets creates an immune barrier and decreased immunogenicity of the islets was shown *in vitro* [496] and the group is currently working on translating this work in animal models.

Alternatively, immune privilege can also be induced locally by accumulating immune-suppressive Tregs at the site of islet transplantation as done by Vågesjö et al. (2015), they co-transplanted islets with a plasmid encoding the chemokine CCL22 into the muscle of MHC-mismatched mice. Myocyte pCCL22 expression and secretion resulted in local accumulation of Tregs, which resulted in significantly fewer effector T-lymphocytes in close proximity to the islets, leading to delayed graft rejection [497]. However, data on human studies on efficacy of autologous Tregs in prevention of effector T cell mediated destruction of islets is very scarce. Several clinical trials have been completed or in process to evaluate different strategies of cell-based therapies in T1D patients some of which are summarized in Table 5.

10 Conclusions

The pathogenesis of T1D is a highly complex process involving various cellular entities and mechanisms, in addition to predisposing genetic factors and environmental triggers. While it is still unknown that how the central tolerance to β-cells is broken, the role of various immune cells infiltrating the pancreas at various stages

Table 5 Major clinical trials on cell-based therapies in type 1 diabetes

Study	Intervention	Phase	Status
NCT00873925	Transfusion of autologous umbilical cord blood plus vitamin D and omega 3 fatty acids to preserve β-cells function in children with recent onset type 1 diabetes	Phase 1	Completed (April 1, 2013)
NCT00468403	Islet transplantation in type I diabetes with LEA29Y (Belatacept) maintenance therapy (CIT-04)	Phase 2	Completed (march 9, 2016)
NCT01379729	Transplantation of encapsulated β-cells	Phase 2	Ongoing
NCT02763423	Umbilical cord mesenchymal stem cell	Phase 2	Ongoing
NCT00160732	Intraportal infusion of allogenic islet cells	Phase 1 & Phase 2	Ongoing
NCT01897688	Islet cell transplant	Phase 3	Ongoing
NCT00790257	Encapsulated human islets in a "Monolayer Cellular Device"	Phase 1	Completed (April 13, 2016)
NCT00708604	Islet after kidney transplantation (IAK)	Phase 1	Completed (July 2, 2014)
NCT02803905	Allogeneic islet cells transplanted into the Omentum	Phase 2	Ongoing
NCT00530686	Islet cell transplantation	Phase 1	Ongoing
NCT01630850	Islet transplantation in patients with "Brittle" type I diabetes		Ongoing
NCT00014911	Islet transplantation using the Edmonton protocol of steroid free immunosuppression	Phase 2	Completed (June 4, 2014)
NCT01210664	*Ex vivo* expanded human autologous polyclonal regulatory T cells	Phase 1	Ongoing
NCT00445913	Autologous dendritic cell therapy for type 1 diabetes suppression: A safety study	Phase 1	Completed (February12, 2016)
NCT02354911	Immunoregulatory dendritic cells	Phase 2	Ongoing
NCT02772679	Treg+IL-2	Phase 1	Ongoing

of disease process is getting clearer. Availability of latest technologies such as two photon and intravital microscopy, multicolor flowcytometry, single cell analysis and proteomics have thrown more light and provided more clearer and detailed insight of the islet infiltrates and their phenotype. Studies with animal models, mainly NOD mice and human subjects have provided abundant information and data about the mediators of the disease. Most of the studies have confirmed the role of T cells as principle mediators of β-cell damage, however, at the same time the role of previously unknown immune cells such as pDCs, NKT cells, ILCs is also coming into picture. The previously known CD4+ T and CD8+ T effector cells are now characterized in a better way and novel auto-antigens and modifications in antigens, such as PTM and peptide fusion have been identified. All this information has provided newer therapeutic targets and novel cellular modalities in targeting the disease. It is now becoming clear that antigen specific approaches, such as induction of PPI spe-

cific Tregs have better prospects in immunoprotection of β-cells, as compared to generalized approaches. Further, improvements in islet isolation and use of accessory cells in various clinical studies have provided momentum in strategies aimed at β-cell replacement or regeneration. Although, we are still far away from the ultimate goal i.e. complete treatment of T1D, recent developments have been quite encouraging and show better prospects for the future.

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Immune Mechanisms, Pathology, and Management of Allergic Ocular Diseases

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Abstract Allergic eye diseases are mediated primarily by immunoglobulin E (IgE) and/or Th2 cells along with cytokines, chemokines, histamine, prostaglandins, and leukotrienes that participate in the immunopathogenesis and immunopathology. Dendritic cells initiate the immune response to allergens by processing and presenting them as peptides to naïve T cells, which in turn, develops into allergen-specific Th2 cells that play a crucial role in the allergic immune process via action of Th2-derived cytokines that induce B cells to become allergen-specific IgE-producing plasma cells. This chapter provides a comprehensive review of the current understanding of the immunopathogenesis and immunopathology of allergic eye diseases including allergic conjunctivitis, giant papillary conjunctivitis, atopic keratoconjunctivitis, and vernal keratoconjunctivitis. The immunopathological process is responsible for the clinical manifestations of allergic eye diseases as well as damage and remodeling of the ocular surface. Furthermore, the role of immune cells and mediators in allergic ocular surface inflammation will be discussed in great detail with particular focus on T cells, eosinophils, mast cells, histamine, cytokines, chemokines, and eosinophil-derived mediators. Finally, clinical management of allergic eye diseases with pharmacotherapy that targets cells and mediators of allergic eye diseases as well as potential future therapeutic directions such as potential adjunctive therapeutic benefits of resident ocular microbiome that modulate the ocular mucosal immunity will be discussed. The main objective of this chapter is to highlight the immunopathology of allergic eye disease with a view to focus interest in developing therapeutic agents that target cells and mediators of allergic immune response and consequential immunopathological processes.

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1 Introduction

The eye is an important sensory organ of the human body that participates in the process of detecting visual stimuli, which subsequently undergo neural processing in the retina and brain [1]. However, the normal function of the eye could be affected by both infectious and non-infectious diseases along with the associated inflammatory state that develops in response to these threats. The ocular surface of the eye is exposed to the external environment with obvious threats from infection, trauma, and immunogenic factors of particular concern. Infectious and non-infectious diseases that affect the conjunctiva and cornea are listed in Table 1. The focus of this chapter is on allergic eye diseases with particular emphasis on the immunopathogenesis and immunopathological mechanisms. It is noteworthy that the immune system functions to distinguish between self and nonself, and as such, it tolerates self-antigens but recognize and remove non-self-antigens. However, innocuous substances can generate immunological memory following first encounter, and subsequently cause inflammation and tissue damage on re-exposure to the inciting innocuous substance. Overreaction of the adaptive immune system to innocuous environmental antigens along with associated inflammation and damage to the tissue are characteristics of the allergic immune response. Allergic eye diseases are predominantly due to type I hypersensitivity reactions triggered by allergen-specific

Table 1 Diseases of the conjunctiva and cornea

Diseases of the conjunctiva	Diseases of the cornea
Acute bacterial conjunctivitis	Fungal keratitis
Staphylococcal marginal keratitis	Bacterial keratitis
Follicular conjunctivitis	Acanthamoeba keratitis
Pharyngoconjunctival fever	Microsporidial keratoconjunctivitis
Acute hemorrhagic conjunctivitis	Herpes simplex keratitis
Epidemic keratoconjunctivitis	
Herpes simplex conjunctivitis	
Acute hemorrhagic conjunctivitis	
Trachoma	
Chemical conjunctivitis	Peripheral ulcerative keratitis
Amyloidosis conjunctivitis	Vernal keratoconjunctivitis
Toxic follicular conjunctivitis	Thygeson's superficial punctate keratitis
Cicatricial conjunctivitis	Superior limbic keratoconjunctivitis
Allergic conjunctivitis	Interstitial keratitis
Phlyctenular keratoconjunctivitis	Mooren's ulcer
Giant papillary conjunctivitis	Neurothrophic keratitis

IgE bound to Ig-like domain of the alpha chain of high affinity IgE receptor type 1 (FcεRI) on sensitized mast cells and basophils. There is also a type IV hypersensitivity reaction component in allergic eye disease, in which the chronic nature of the clinical manifestation of allergic eye disease is mediated predominantly by Th2 cells. Approximately 40% of individuals in the Western world are atopic with incidence of allergy on the increase in people who reside in developed nations [2]. Allergic eye diseases can be acute or chronic based on the underlying immunopathology and clinical manifestations of the allergen-induced immune response. The acute form includes allergic conjunctivitis (AC), which is predominantly a type I hypersensitivity response to innocuous substances such as allergens. The chronic form includes vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). Giant papillary conjunctivitis (GPC) has an immunological component to its pathogenesis; however, it is mostly due to microtrauma of the palpebral conjunctiva. Pollen is a major environmental aeroallergen associated with seasonal form of AC while dust mites and molds are indoor allergens associated with the perennial form of AC. Dendritic cells (DC), the initiator of the immune response to allergens, process and present allergen peptides to naïve T cells, which in turn, become activated to undergo proliferation and differentiation into allergen-specific Th2 cells that play a crucial role in allergic immune process via action of Th2-derived cytokines that induce allergen-specific B cells to proliferate and differentiate into allergen-specific IgE-producing plasma cells. The major effector cell involved in inducing the clinical manifestations of the allergen-induced immune response is the mast cell and its mediators such as histamine, tryptase, leukotrienes, prostaglandins, cytokines, and chemokines. Clinical manifestations of allergic eye disease include itching, tearing, eyelid edema, hyperemia, chemosis, papillary hypertrophy, corneal epithelial defects, and remodeling of the ocular surface. The management of allergic eye disease includes nonpharmacological and pharmacological therapy. Pharmacological therapy includes antihistamine, mast cell stabilizers, multimodal anti-allergic agents, and corticosteroids [3, 4].

This topic will discuss the immunology of the ocular surface with particular emphasis on conjunctival-associated lymphoid tissue (CALT), immunoregulation of the ocular surface, and factors that contribute to the immune privilege status of the cornea. Additionally, cells and mediators that play a role in the immunopathogenesis and immunopathology of allergic eye diseases will be discussed in detail with greater focus on antigen-presenting cells of the ocular surface, the part played by epithelial cells and fibroblasts in allergic eye diseases, the role of eosinophils and mast cells and their mediators, and the central role of Th2 cells and Th2-derived cytokines. Furthermore, the immune mechanisms of AC as a disease are characterized by IgE-mediated mast cell degranulation, which occurs in three successive stages (sensitization, early phase, late phase) will be discussed. Moreover, the multifactorial nature of the immune and pathological mechanisms of VKC and AKC along with the T cell-mediated immune mechanisms will be reviewed. There will also be a brief discussion of the immunological and mechanical aspects of the pathological mechanisms of GPC involving the actions of Th2 cells. Finally, in the end, approved and pipeline anti- allergic agents will be discussed.

2 Cells and Mediators of Allergic Eye Diseases

2.1 Allergens

Allergens are small antigens that are capable of diffusing across the mucosal surface to induce Th2 cell-mediated response that is characteristic of allergic eye diseases [5]. House dust mites, molds, and pollen allergens possess proteolytic enzymatic activity that promote allergenicity [6]. House dust mites are indoor allergens that possess cysteine and serine protease activity [7]. It is noteworthy that Dermatophagoides pteronyssinus 1 (Der p 1) and Der p 3 produce cysteine protease, whereas Der p 6 and Der p 9 allergens secrete serine protease [6]. Proteolytic enzymes released by allergens disrupt the barrier function of the conjunctival epithelium, this in turn, facilitates the access of allergen into the conjunctival subepithelial layer where it is taken up by antigen-presenting cell (APC), such as DCs that become activated to initiate the generation of allergen-specific Th2 cells and allergen-specific IgE [5, 7–9]. Thus, proteolytic enzymes produced by allergens play an important role in the pathogenesis of allergic eye disease, since it enhances the access of allergens to APCs in the conjunctiva epithelium and subepithelial layer, which in turn, facilitates the differentiation of naive T cells into allergen-specific T cells [6]. Allergens in contact with epithelial cells can trigger these cells to express thymic stromal lymphopoietin (TSLP), an IL-7-like epithelial cell-derived pro-allergic cytokine that activates DCs to promote the generation of Th2 cell immune response and associated allergic inflammation via TSLP-TSLPR (TSLP receptor chain) signaling pathways [10]. Additionally, mast cells, fibroblasts, and DCs can also secrete TSLP [11]. TSLP can activate eosinophils to express ICAM-1, which in turn, enhances the adhesion of eosinophils to vascular endothelium and its influx into the site of allergic inflammation [12]. Thus, TSLP has a role to play in the immunopathogenesis of allergic eye disease through the activation of DCs, eosinophils or mast cells in synergy with proinflammatory cytokines such as IL-1 and TNF-α [13, 14].

2.2 Antigen Presenting Cells

Antigen presenting cells (APCs) are cells that are capable of engulfing antigens and subsequently processing and presenting it on major histocompatibility complex (MHC) molecules as a peptide:MHC complex that is recognized by T cells [15, 16]. MHC is a cell surface molecule that plays a crucial role in displaying processed antigens in a form that is recognized by T cells [17, 18]. Nucleated cells express MHC class I molecules that present peptide antigen to $CD8^+$T cell; however, B cells, dendritic cells, and macrophages express MHC class II molecules that present peptide antigen to $CD4^+$T cell [19]. APCs bear costimulatory molecules that interact with costimulatory ligands on naïve T cells to provide co-stimulatory/survival signals during the process of T cell activation [20]. APCs are classified into professional and nonprofessional APCs based

on the level of constitutive expression of MHC class II molecules. DCs, macrophages and B cells are professional APCs that express high levels of MHC II antigen and costimulatory molecules. Nonprofessional APCs such as vascular endothelial cells, epithelial cells, and fibroblasts do not constitutively express costimulatory and MHC class II molecules, but these are upregulated when induced by pro-inflammatory mediators [21, 22]. Langerhans' cells are immature DCs located in the epithelium of the conjunctiva, limbus, and cornea. They take up antigen to become mature Langerhans' cells expressing MHC class II-positive with $CD80^+$ and $CD86^+$ that interact with naïve T cells to mediate Th2 cell polarization [23–25]. Conventional DCs are important APCs found in peripheral tissues including the cornea and conjunctiva. They are involved in the initiation and modulation of the allergic response, as well as in determining the nature of the immune response to allergens [23]. Immature DC take up antigen to undergo the maturation process to become mature DCs displaying processed peptide antigen and upregulating the expression of CCR7, CD80, and CD86. CCL21-CCR7 interaction directs chemokine-mediated migration of mature DCs to regional lymph nodes [12, 21]. Toll-like receptors (TLRs) are glycoprotein cell receptors that recognize exogenous and endogenous molecules. It is a pattern recognition receptor that triggers an innate immune response, which culminates in linking the innate and adaptive arms of the immune system [22, 26]. TLRs are expressed on non-immune cells such as epithelial cells and immune cells such as eosinophils, neutrophils, macrophages, monocytes, and DCs. TLR2, TLR4, and TLR6 are expressed on mast cells. TLR2- and TLR4-mediated mast cell degranulation is associated with the release of inflammatory mediators that may exacerbate inflammatory response in chronic allergic eye diseases such as atopic keratoconjunctivitis [26, 27]. TLRs located on DCs play an essential role as an innate immune surveillance system that initiates the innate immune response in the eye as well as induces the development of humoral and cellular immune responses [28]. Because DCs detect and present allergens to naïve T cells in regional secondary lymphoid organs with the intent of linking the innate and adaptive immune systems, they play an essential role in the proliferation and differentiation of naive T cells into effector T cells that participate in the allergic immune response of the ocular surface [19, 29].

2.3 T Cells

T cells are immune cells produced by common lymphoid progenitor cells in the bone marrow that undergo development and maturation in the thymus. These immune cells play an important role in generating an adaptive immune response to mediate antigen-specific effector immune responses and regulate activity of other immune and non-immune cells via the action of T cell-derived cytokines [18, 30]. The three crucial roles of effector T cells include killing, activation, and regulation [19, 31]. $CD8^+$T cells differentiate into cytotoxic T-lymphocytes that destroy intracellular pathogens especially viruses [25] while CD4T cells differentiate into Th1, Th2, Th17, and regulatory T cells [32]. $CD4^+$T helper cells provide signals in the form of cytokines that activate antigen-specific B cells, macrophages, and $CD8^+$T cells [25].

2.4 Cytokines

Cytokines are highly potent proteins secreted by immune and nonimmune cells that mediate cell division, inflammation, cytotoxicity, differentiation, migration, and repair. Cytokines include interleukins (ILs), colony stimulating factors (CSF), tumor necrosis factor (TNF), and interferons (IFN) [31]. IL-l (IL-lα and IL-lβ) is an important pro-inflammatory cytokine produced by immune (e.g. macrophage) and non-immune cells (e.g. epithelial cells). It plays a role in the immunopathogenesis of allergic disease [31, 33]. IL-2 facilitates the proliferation of activated T cells and B cells [25, 31, 34]. IL-4 is produced by Th2 cells and mast cells and it induces the production of IgE-secreting plasma cells [25, 31]. IL-13 is produced by Th2 cells and mast cells, and it induces the synthesis of IgE [31]. IL-4 and IL-13 induce tissue remodeling by triggering conjunctival fibroblasts to proliferate and produce collagen and vascular endothelial growth factor (VEGF), which in turn, results in papillary formation with new vessels [35]. IL-5, produced by Th2-lymphocytes and mast cells, plays a role in eosinophil activation and recruitment [36]. IL-9 is secreted by T-lymphocytes, eosinophils, and mast cells. It synergizes with IL-4 in the production of IgE and in the promotion of conjunctival tissue remodeling [31, 37, 38]. TNF-α is a multifunctional proinflammatory cytokine produced by monocytes, macrophages, DCs, mast cells, and T cells. It induces the expression of adhesion molecules on vascular endothelial cells and facilitates chemokine synthesis by immune and non-immune cells (e.g. epithelial cells) [39]. Th17 cells play a significant role in acute inflammatory response [25], since antigen-specific Th17 cells produce cytokines (IL-17A, IL-17F, IL-21, and IL-22) that trigger non-immune cells such as epithelial cells and fibroblasts to express pro-inflammatory cytokines and chemokines that induce the recruitment of immune cells such as neutrophils to the site of allergic response [29, 31]. Regulatory T cells that act to control immune responses could be either natural regulatory T cells committed to an immunoregulatory fate while still in the thymus [40] or induced variety of regulatory T cells that differentiate from naïve T cells in response to antigen [25]. Regulatory T cells via the action of immunosuppressive cytokines such as IL-10 and TGF-β are capable of downregulating the expression of B7 on DCs, which in turn, affects downstream allergen-induced activation of T cells and subsequent production of IgE [40].

2.5 Antibodies

Antibodies are immunoglobulins produced by effector B cells in response to antigenic stimulation [18, 41]. Membrane-bound immunoglobulin (mIg) or surface immunoglobulin (slg) is the antigen receptor of B cells [19]. Antibodies consist of five different types and include immunoglobulin G (IgG), IgM, IgA, IgD, and IgE. Antibodies are effector molecules that mediate humoral-mediated immune

responses, such as neutralization, opsonization, and complement activation. Th1-derived cytokines stimulate antigen-specific B cells to produce IgG-secreting plasma cells, whereas Th2-derived cytokines (e.g. IL-4 and IL-13) enhance proliferation and differentiation of antigen-specific B cells into IgE-producing plasma cells [25]. IgG is the principal antibody in serum and non-mucosal surfaces, whereas IgA is the primary antibody that participates in mucosal immune protection [42]. IgE, a major antibody that participates in type 1 hypersensitivity reactions characteristic of allergic eye diseases, binds to FCεRI found on mast cells, basophils, B cells, activated eosinophils, and follicular dendritic cells. FcεRII are present on B cells, activated T cells, monocytes, eosinophils, and follicular dendritic cells [5, 43]. Th2 cells play an important role in allergic eye diseases, since its cytokines are involved in the production of IgE, mast cell activation, and activation of eosinophils [44].

2.6 Co-stimulatory Molecules

Co-stimulatory molecules or ligands are cell surface proteins on immune cells that are involved in signal transmission. The generation of effector T cells require costimulation signals provided by interaction between costimulatory receptors and their ligands [45, 46]. CD28 is a co-stimulatory receptor on the surface of naïve T cells that binds co-stimulatory ligand B7 expressed by DCs to facilitate the activation of naïve T cells [45, 47]. It has been demonstrated that CD28/CD86 costimulatory pathway participates in production of Th2-derived cytokines that mediate eosinophil activation and production of IgE in allergic inflammation [45]. Eosinophil and Th2 cell recruitment to the site of allergic inflammation requires the action of adhesion molecules, which mediate the interaction between leukocytes and vascular endothelial cells [48].

2.7 Adhesion Molecules

Adhesion molecules are classified into three main categories: (a) the integrins, (b) the selectins, and (c) the immunoglobulin gene superfamily [48, 49]. The integrin family of adhesion molecules include lymphocyte function associated antigen-1 (LFA-1) and Very Late-activation Antigen-4 (VLA-4). LFA-1 binds to intercellular adhesion molecule -1 (ICAM-1) and ICAM-2 to form strong adhesion between leukocytes and endothelial cells on the inflamed vascular endothelium, which results in the extravasation of leukocytes [48]. The selectin family includes E-selectin, P-selectin, and L-selectin [50]. E-selectin (CD62E) is expressed on the endothelium, whereas P-selectin (CD62P) is expressed on platelets and the endothelium. E-selectin plays a role in mediating the rolling of leukocytes, such as neutrophils on the endothelium. L-selectin (CD62L) is expressed on leukocytes and it guides the

exit of leukocytes in circulation into the tissue by mediating their rolling along the vascular endothelium [49, 50]. Leukotriene B4 (LTB4), histamine, and TNF-α can activate the vascular endothelium to upregulate the expression of P-selectin and E-selectin. These selectins initiate endothelium-leukocyte interaction, that culminates in the reversible binding of leukocytes to the wall of the blood vessel [48]. ICAM-1 (CD54), ICAM-2 (CD102), vascular cell adhesion molecule-1 (VCAM-1/CD106), platelet and endothelial cell adhesion molecule-1 (PECAM-1/CD3l), and the mucosal vascular address in cell adhesion molecule 1 (MAdCAM-1) are members of the immunoglobulin gene superfamily that plays an important role in the recruitment of leukocytes such as T cells to the site of allergic inflammation [50, 51]. ICAMs on the endothelium facilitate the tight adhesion of leukocytes to the endothelium [48, 49]. TNF-α, interferon gamma (IFN-γ) and interleukin (IL)-1β can induce the expression of ICAM-1 [51], whereas TNFα can also induce the upregulation of ICAM-2 [48]. Additionally, ICAM-I is expressed on mononuclear cells, granulocytes, lymphocytes, APCs, fibroblasts, and epithelial cells. Although ICAM-1 is not expressed on normal conjunctival epithelial cells, it is upregulated on conjunctival epithelial cells following an allergic reaction, which in turn, facilitates the migration of inflammatory cells into the site of allergic inflammation [39]. Furthermore, ICAM-1 plays an important role in homing and migration of eosinophils that are involved in the inflammatory process in allergic eye disease [52]. ICAM-2 and PECAM-1 are expressed on endothelial cells and participate in adherence of leukocytes to the endothelium. PECAM-1 is also found on platelets and leukocytes [49, 50]. Vascular adhesion protein-1 (VAP-I) is an endothelial adhesion molecule. PECAM-1 and VAP-1 participate in the adhesion and transmigration of lymphocytes [50, 53]. There is an increased expression of ICAM-1, E-selectin, and VCAM-1 in allergic eye diseases [39]. It is important to note that cytokines and chemokines are capable of upregulating the expression of adhesion molecules on epithelial cells and vascular endothelium during the immunopathological process in allergic eye diseases [39] Thus, adhesion molecules are involved in mediating the three-step process involved in the allergen-induced accumulation of inflammatory cells and molecules at the site of allergic inflammation [11].

2.8 Epithelial Cells and Fibroblasts

Epithelium represents a physical barrier that protects against the intrusion of antigens through the function of tight junctions that play a vital role in the formation and maintenance of epithelial barriers [8, 54]. Epithelial cells are located at the port of entry of allergens and actively participate in allergic inflammation via the expression of cytokines, chemokines, and adhesion molecules. These expressed mediators promote the infiltration of immune cells such as eosinophils and Th2 cells to the site of allergic inflammation in the conjunctiva [39, 55, 56]. Fibroblast, a cell that produces extracellular matrix (ECM), acts as an immune modulator in allergic conditions by producing pro-inflammatory mediators in response to cytokines. Fibroblasts

in the conjunctiva and cornea in response to late phase mediators undergo increased proliferation as well as produce ECM and inflammatory mediators. In chronic allergic eye diseases, IL-4 and IL-13 can activate corneal fibroblasts to secrete eotaxin, thymus- and activation-regulated chemokine (TARC or CCL17), matrix metalloproteinase (MMP), VCAM-1, and ICAM-1. TARC is a potent chemoattractant for Th2-lymphocytes. Excessive ECM production, vascular endothelial growth factor (VEGF) secretion, and proliferation of conjunctival fibroblasts that occurs in response to IL-4 are major contributory factors to the formation of giant papillae [3, 35, 57–59]. Thus, epithelial cells and fibroblasts of the cornea and conjunctiva participate in the immunopathogenesis as well as immunopathological process that results in tissue damage and remodeling in allergic eye diseases.

2.9 Mast Cells

Mast cells, located in mucosal and epithelial tissue, are derived from mast cell progenitors that differentiate from hematopoietic stem cells under the effect of stem cell factor (SCF) [2, 60]. Additionally, IL-3 and IL-9 play a role in the growth and development of mast cells [5]. Mast cells are usually found in vascularized connective tissues in the subepithelial layer [42].There are two types of mast cells based on their location and protease content, mucosal mast cells (Tryptase-positive (MC_T) and connective tissue mast cells (tryptase and chymase-positive (MC_{TC}) with the conjunctiva containing mainly connective tissue mast cells [2, 61, 62]. Mast cells are not present in the cornea but are predominant in the conjunctiva, where they play a pivotal role in allergic eye diseases [2]. In the normal conjunctiva, the mast cells are concentrated in the conjunctival substantia propria [63–65]. In the healthy human conjunctiva, there are more than 10,000 mast cells/mm^3 located in the conjunctival stroma (substantia propria) with the number of mast cells significantly increased in chronic forms of allergic eye disease [8]. The cytoplasm of the mast cells contain up to 200 large granules with each granule containing preformed mediators such as histamine, heparin, proteases (tryptase and chymase), major basic protein, acid hydrolases, peroxidase, and phospholipases [2, 66]. Following the activation and subsequent degranulation of sensitized conjunctival mast cells, preformed mediators such as histamine and protease are released immediately followed later by the production of lipid mediators, cytokines, and chemokines. Lipid mediators include leukotrienes (LT) B4, LTC4, prostaglandin (PG) E2, PGD2, and platelet-activating factor (PAF). Cytokines released include TNF-α, IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-9, IL-13, IL-25, and SCF; whereas chemokines released following activation and degranulation of mast cells include CXCL8, CCL5, CCL11, and CCL17. Growth factors include vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) [66]. These mediators along with histamine, leukotriene, and prostaglandins released following mast cell degranulation play a major role in the immunopathological process in acute and chronic forms of ocular allergy.

2.10 Histamine

Histamine, a low molecular weight vasoactive mediator stored in mast cells and basophils, is synthesized by the decarboxylation of histidine by histidine decarboxylase [67]. Its biological actions are achieved by interacting with four G-protein coupled receptors. These receptors include histamine 1 receptor (H1R), 2, 3 and 4 [68] with H1R, H2R and H4R playing a major role in allergic eye diseases [8]. H1R and H2R are expressed on immune cells (e.g. lymphocytes), non-immune cells (e.g. epithelial cells), vascular smooth muscle cells, and endothelial cells. H1R and H2R on the conjunctiva vasculature mediate vasodilation and increased vasopermeability, whereas H1R on conjunctival sensory fibers mediate ocular itch in the setting of allergic eye diseases [3, 8, 69]. H4R are expressed mainly on immune cells (mast cells, eosinophils, T cells, and DCs) [8], and as such, histamine/H4R interaction mediates recruitment of immune cells to the site of allergic inflammation in the conjunctiva resulting in exacerbation of the allergic response [8, 70]. Histamine released following mast cell degranulation affects conjunctival blood vessels, nerve endings, epithelial cells, and fibroblasts [69]. Conjunctival epithelium and fibroblasts secrete cytokines, chemokines, and adhesion molecules in response to histamine/histamine receptor interaction [71]. Thus, histamine participates in the clinical expression of the allergic response [72, 73].

2.11 Lipid Mediators

Leukotriene (LT), a potent lipid mediator synthesized via the lipoxygenase pathway of arachidonic acid metabolism, is involved in the pathological mechanism of allergic eye disease [74]. LTC4, LTD4 and LTE4 are released by degranulated mast cells and eosinophils [5, 74]. In allergic eye disease, leukotrienes have been shown to cause increased vascular leakage and increased secretion of mucus [3, 61, 75, 76]. Prostaglandin is a potent lipid mediator synthesized via the cyclooxygenase pathway of arachidonic acid metabolism [15, 64]. Prostaglandin D2 (PGD2) is produced following activation and subsequent degranulation of mast cells and it is an important mediator of ocular allergy [5, 77]. It is expressed on human eosinophils and Th2 cells. Fujishima and colleagues [77] used flow cytometry analysis to demonstrate the expression of chemoattractant receptor-homologous molecule expressed on Th2 cells and eosinophils. They were able to demonstrate that the interaction between PGD2 and CRTH2 on eosinophils resulted in PGD2-mediated recruitment of eosinophils and secretion of cytokines by Th2 cells in allergic eye diseases [77, 78]. Platelet activating factor (PAF) is a lipid mediator that plays a role in the chemotaxis of eosinophils. It is synthesized by most inflammatory cells and is involved in the pathological mechanism of allergic eye diseases [5, 79]. Okumura and colleagues [80] used liquid chromatography-tandem mass spectrometry to demonstrate the presence of PAF in patients with AC. Thus, PAF plays an important role in allergic eye diseases [80].

2.12 Chemokines

Chemokines are low molecular weight chemotactic cytokines that mediate the attraction and activation of leukocytes such as monocytes, neutrophils, lymphocytes, and other effector cells to the site of inflammation [39, 48]. CXC, CC, XC and CX3C are chemokine ligands that act on different sets of chemokine receptors. There are more than 40 chemokine ligands in humans that promote the directional migration of immune cells [81]. CCL11, produced by stromal cells and immune cells, binds to CCR3 on human eosinophils to promote their release from bone marrow and migration to the site of allergic inflammation [12]. Interleukin (IL)-8/CXCL8 is produced by immune (e.g. monocytes, macrophages) and nonimmune (e.g. fibroblasts, epithelial cells, and endothelial cells) cells. CXCR1 and CXCR2 are receptors for CXCL8 [48]. T cells, endothelial cells, and platelets secrete CCL5 that interacts with CCR1, CCR3, and CCR5 to mediate the recruitment of T cells, basophils, and eosinophils [39, 48]. Th2 cells express CCR4, CX3CR1, and CRTH2, whereas Th1 cells express CCR5 and CXCR3. These chemokine receptors and their ligands participate in Th1-type and Th2-type adaptive immune response [12, 81]. Thus, chemokines produced by activated immune and nonimmune cells play a role in allergen-induced ocular surface inflammation as well as in the recruitment of effector immune cells, such as eosinophils that contribute to the tissue remodeling and damage in the chronic forms of allergic eye diseases [82].

2.13 Eosinophils

These contain arginine-rich basic proteins and they can also secrete enzymes, IL-4, IL-6, IL-12, IL-13, CCL2, CCL11, CCL17, CXCL8, lipid mediators, eosinophil peroxidase, major basic protein, eosinophil collagenases, and matrix metalloproteinase-9. Eosinophil major basic protein is a major cause of corneal epithelial toxicity in chronic allergic eye conditions [5, 83]. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that are expressed in physiologic and pathologic conditions [84]. They are key enzymes for breakdown of ECM associated with inflammatory reactions and wound repair [85]. Kumagai and colleagues demonstrated that active forms of MMP-2 and MMP-9 are significantly raised in patients with allergic eye disease, and as such, these active forms of MMP are responsible for the inflammatory reactions and tissue remodeling in the ocular surface of these patients [86, 87]. Eosinophils in the non-active state do not express surface markers but when activated by cytokines and chemokines, they upregulate the expression of FcεRI, major histocompatibility class II molecules, CD86, and CD40, and as such, they are capable of presenting antigens to activated $CD4^{+}$T cells [83, 88]. The recruitment of eosinophils is associated with their infiltration of the conjunctiva of patients with allergic eye diseases [39, 89, 90].

2.14 Neutrophils

Neutrophils are important innate effector immune cells that act as phagocytes. These short-lived immune cells secrete prostaglandin, leukotriene, IL-1β, TNF-α, MMP-9, neutrophil elastase, and myeloperoxidase [15, 32, 48, 91]. Neutrophils are the most abundant immune cells in innate immunity that participate in acute inflammatory reactions and it has been shown to be increased in patients with chronic forms of allergic eye diseases, such as vernal keratoconjunctivitis and atopic keratoconjunctivitis [19, 92, 93].

3 Biology of the Ocular Surface

The anatomy, physiology, and immunology of the conjunctiva, limbus and cornea will be discussed, since the conjunctiva, cornea and limbus are ocular structures affected by allergic inflammation. The epithelium of these structures forms a physical barrier that prevents foreign substances such as allergens from gaining access to the subepithelial tissues [94, 95].

3.1 Structure and Function of the Ocular Surface

The conjunctiva is a highly vascularized, immunologically active mucosal tissue; however, there are several regulatory mechanisms in place to control the immune response in order to prevent tissue damage [96]. The conjunctiva consists of an epithelium and stromal layer. The epithelium of the conjunctiva is a non-keratinized mucous membrane that houses goblet cells and intraepithelial leukocytes. The epithelial cells are held together by tight junctions. The conjunctival stroma consists of collagen, fibroblasts, vasculature, lymphocytes, macrophages, DCs, and mast cells. It is noteworthy that eosinophils are not present in the healthy conjunctiva [39, 97, 98]. The limbus is an annulus of tissue, which acts as a junctional barrier that separates the cornea from the conjunctiva. It consists of a vascular network of palisades of Vogt that contain stem cells and Langerhans' cells. It is noteworthy that the removal of damaged epithelial cells via constant shedding of the superficial epithelial cells and their replacement by stem cells contribute to the ocular surface's immune protection [97, 99–102]. As such, the limbus contributes to the immune-surveillance of the ocular surface [15, 103]. The cornea consists of the epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium. The corneal epithelium consists of Langerhans' cells, superficial cells, wing cells, and basal cells. The corneal stroma constitutes 90% of the entire corneal thickness and it consists of macrophages, collagen, fibroblasts, and immature DCs in the peripheral cornea. Corneal APCs exist in the immature immunological state and they include

Langerhans' cells and DCs. Langerhans' cells express MHC-II$^-$, CD80$^-$, and CD86$^-$ while dendritic cells express CD45$^+$, CD11b$^+$, CD11c$^+$, DC-SIGN$^-$, MHC-II$^-$, CD80$^-$, and CD86$^-$. Macrophages in the cornea express CD45$^+$, CD11b$^+$, CD11c$^-$, HLA-DR$^-$, F4/80$^+$, and DC-SIGN$^-$. Following a breach in the corneal epithelial barrier, CD45$^+$ CD11b$^+$ CD11c$^-$ macrophages located in the posterior stroma of the cornea provides an initial defense against foreign substances by producing cytokines and chemokines that participate in the innate immune response [23, 104]. Sensory nerve fibers derived from the ophthalmic division of the trigeminal nerve supply to the cornea and the vasoactive intestinal peptide (VIP) secreted by theses nerves contributes to immune regulation via increasing production of immunoregulatory cytokines and blocking the expression of pro-inflammatory cytokines [21, 96]. Corneal epithelial cells are joined together by a tight junction complex that links the cytoskeletons of adjacent epithelial cells, and as such, the tight junction contributes to the physiological barrier function of the epithelium via blockade of access of allergens to immune cells in the sub-epithelial layer [8, 54, 105, 106].

3.2 Immunology of the Ocular Surface

The epithelium of the ocular surface utilizes effector mechanisms of both arms of the immune system to provide immune surveillance and immunoregulation [107–109]. Conjunctival-associated lymphoid tissue (CALT) is a component of the Eye-associated lymphoid tissue (EALT). CALT is an ocular surface immune protection system that consists of diffuse lymphoid effector tissue and conjunctival lymphoid follicles (CLF). It maintains balance between immune tolerance and inflammation, which is tilted toward immune tolerance via the action of regulatory T cells and immunosuppressive cytokines. Diffuse lymphoid effector tissue, an efferent arm of the CALT, consists of DCs, mast cells, macrophages, IgA-secreting plasma cells, and intraepithelial and lamina propria effector T cells. Conjunctival lymphoid follicles, an afferent arm of the EALT, are interspersed within the diffuse lymphoid effector tissue. CLF consists of B cells, parafollicular T cells associated with lymph vessels and high endothelial venules (HEV), and apical follicle-associated epithelium (FAE) with M cells for antigen transport. Thus, CALT provides immunosurveillance for the ocular surface through its ability to detect antigens and generate effector immune cells in response to invasion of the ocular surface [3, 98, 108, 110]. Afferent and efferent immunoregulatory mechanisms of the ocular surface involve the action of mediators and cells of the immune system. The afferent immunoregulatory mechanisms include controlling maturation of DCs, controlling production of pro-inflammatory cytokines by epithelium and DCs via TLR-mediated activation pathways, and reducing expression of cell adhesion molecules by vascular endothelial cells. The efferent immunoregulatory mechanism is mediated by regulatory T cells and immunosuppressive or immunoregulatory cytokines [96]. Langerhans' cells, DCs and macrophages in the conjunctiva and cornea are the main APCs of the ocular surface that participate in the innate immune surveillance system. They constitute the first line of defense of the ocular surface to foreign sub-

stance when the physicochemical barrier is breached [111]. Regulatory T cells inhibit effector $CD4^+$T cell-mediated ocular surface inflammation whereas immunoregulatory cytokines (e.g. IL-10 and TGF-β) direct DCs to promote differentiation of naïve $CD4^+$T cells into regulatory T cells. Additionally, TGF-β inhibits proliferation of damaged epithelial cells [21, 96, 112]. Tear film provides a physicochemical barrier that prevents adherence of allergens to the epithelium of the conjunctiva via flushing and diluting these allergens in the tear film [108]. Complement system consists of plasma and membrane-bound protein and plays an important role in innate immune surveillance. It consists of proteins that initiate and activate the complement pathway and complement proteins that regulate complement activities. Decay activating factor (DAF, CD50), membrane cofactor protein (MCP, CD46), complement receptor 1 (CR1, CD35), and membrane inhibitor of reactive lysis (MIRL, CD59) are complement regulatory proteins that block various complement functions at different stages of the complement cascade [113]. Expression of complement regulatory proteins such as DAF and CD59 by epithelial cells of the ocular surface protect the cornea from complement-mediated inflammation and cytolysis respectively [48, 114, 115]. Furthermore, Fas ligand (FasL; CD95L) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expressed on the epithelium mediates immunoregulatory processes by preventing immune-mediated inflammation through the induction of apoptosis of infiltrating immune cells that express the receptor for FasL (Fas; CD95) and TRAIL [96, 115]. Deregulation of resident lymphocytes of CALT results in ocular surface inflammation, as pro-inflammatory cytokines can activate epithelial cells of the ocular surface to express adhesion molecules, proteases, co-stimulatory molecules, and MHC class II molecules. The expression of costimulatory molecules and MHC class II on epithelial cells facilitate their interaction with $CD4^+$ T cells. Additionally, increased levels of proteases such as MMPs, can cause a breach of the epithelial physical barrier, which allows allergens access to immune cells in the subepithelial layer [21, 98]. Thus, deregulation of the CALT results in immune-mediated ocular surface inflammation [108, 110]. The cornea is an immunologically privileged tissue, and corneal immune privilege status maintains homeostasis and prevents the cornea from immune mediated damage. Although cornea is an immune privileged tissue, it does contain immune cells, such as DCs and macrophages in the immature state [23]. Corneal immune privilege status is attributed to lack of corneal vasculature and lymphatics and absence of mature DCs in the cornea. Additionally, the lack of TLRs on the apical layer of the corneal epithelium provides an immunosilent environment for the epithelium, and contribute to corneal immune privilege status; however, TLR-mediated innate immune response can occur when the corneal epithelial barrier is breached [21, 22, 115, 116]. The lack of MHC classII molecules in the healthy cornea constitutes part of the cornea's immune privilege status. Antigen presentation to T cells by immature DCs leads to the generation of anergic T cells and subsequent induction of immune tolerance, and this protects the cornea from T cell-mediated ocular surface damage [23]. The cornea and conjunctiva are located at the port of entry for allergens, and their epithelial cells, APCs, and fibroblasts participate in immune mechanism by secreting inflammatory mediators during allergic inflammation [39].

4 Immunopathology of Allergic Eye Disease

This section will discuss the immunopathogenesis and immunopathology of the major types of allergic eye disease. Allergic conjunctivitis is an ocular surface disease characterized by IgE-mediated mast cell degranulation, and sensitization, early phase, and late phase of the allergic immune response involved in the pathological mechanism will be discussed. Additionally, the pathological mechanisms of GPC as part immunologic and part mechanical involving the actions of Th2 cells will be discussed. The immunopathogenesis and immunopathology of AKC is multifactorial involving chronic IgE-mediated mast cell degranulation and T cell-mediated immune mechanisms. The immunopathogenesis and immunopathology of VKC is multifactorial involving Th2 cells and Th2-derived cytokines, chemokines, adhesion molecules and inflammatory enzymes. Beside these, the immunopathology along with clinical correlates of allergic eye disease, therapy and clinical outcomes will be discussed.

4.1 Allergic Conjunctivitis

AC is a bilateral inflammatory process that involves the conjunctiva [117]. It is the most prevalent form of allergic eye disease that causes clinical manifestations when IgE bound to sensitized mast cells are cross-linked by allergen. AC constitutes over 90% of all forms of allergic eye disease, and it has a seasonal variant (seasonal AC) due to outdoor allergens and perennial variant (perennial AC) due to indoor allergens [18, 118]. Tree pollen and grass pollen are associated with seasonal AC whereas pet dander and dust mite are associated with perennial AC [119, 120]. Environmental allergens such as pollen, mold and dust mite secrete proteolytic enzymes that promote allergenicity of the allergen and disrupt the barrier function of the ocular surface. Itching, conjunctival hyperemia, chemosis, mucoid discharge, tearing, burning and eyelid swelling are hallmark features of AC [5–8]. In this condition, histamine, leukotrienes, cytokines, chemokines, proteases and prostaglandins produced by activated mast cells play a crucial role in conjunctival inflammation associated with AC [4, 121].

4.1.1 Immunopathogenesis and Immunopathology

The immune mechanism of AC occurs in three phases following exposure of the conjunctiva to allergens. In the sensitization phase, allergens are able to gain access to immune cells in the subepithelial layer via the action of their protease activating protease-activated receptor-2 (PAR-2) in the conjunctiva and the subsequent degradation of the tight junction between epithelial cells [122]. DCs in their immature state will engulf and process these allergens resulting in maturation of DCs with

upregulation of MHC class II molecules, CD80, and CD86. The processed allergen is displayed on the mature DC as a peptide complex to MHC class II molecule. Mature DC with peptide-MHC class II complex migrate to the regional lymph node where they interact with naïve T cells resulting in the activation of T cells and their subsequent proliferation and differentiation into Th2 cells. IL-4 and IL-5 are important cytokines released by allergen-specific Th2 cells. IL-4 induces the proliferation and differentiation of allergen-specific B cells into IgE producing plasma cells, which is preceded by T cell-dependent B cell activation involving peptide-MHC class II complex on BCR and CD40 on allergen-specific B cells interacting with TCR and CD40L on T cells respectively [3, 123]. Allergen-specific IgE binds via their Fc region to Ig-like domain of alpha chain of FcεRI located on the surface of mast cells in the conjunctiva leading to the induction of mast cell sensitization. Primed mast cells participate in type I hypersensitivity immune reaction that are characteristic of AC [2, 3, 124, 125]. Additionally, TSLPs play a role in generating IgE-producing plasma cells. Allergens in contact with epithelial cells of the conjunctiva, induce these cells to produce TSLP that interact with TSLP receptors on DCs. TSLP-activated DCs induce naïve T cells to differentiate into Th2 cells that produce IL-4 that induce allergen specific B cells to undergo proliferation and differentiation into IgE-secreting plasma cells. Thus, conjunctival epithelial cells via the action of TSLP participate in the initiation of the sensitization phase of AC. The binding of these allergen-specific IgE to FcεRI on mast cells completes the process of mast cell priming (Fig. 1) [3, 10, 11, 18, 123, 125]. The elicitation or activation phase of AC occurs when previously sensitized eyes are exposed to allergens, culminating in multivalent allergen binding and inducing crosslinking of IgE-FcεRI complex on sensitized mast cells in the conjunctiva. Crosslinking leads to activation and subsequent degranulation of primed mast cells and release of histamine, which is followed by the synthesis of lipid mediators and cytokines (Fig. 2) [3, 120, 123, 126, 127]. Additionally, chemokines and adhesion molecules are released by degranulated mast cells. When histamine bind to their receptors on the conjunctival epithelium, it results the in disruption of the barrier function of the conjunctival epithelium as well as activation of conjunctival epithelial cells with subsequent release of adhesion molecules, chemokines, and pro-inflammatory cytokines. The clinical manifestations of the early phase of the allergic reaction, such as itching, edema, hyperemia, and tearing are attributed to the action of histamine on vascular endothelium, sensory nerve fibers, immune cells, and conjunctival epithelium [120, 123, 127–131]. Additionally, tryptase is also released and it induces proliferation of fibroblasts in the conjunctiva. It is of note that histamine and tryptase are biomarkers of IgE-mediated allergic reaction in AC [3, 120]. The late phase of AC is mediated by prostaglandins, leukotrienes, and cytokines. Prostaglandin induces vasodilation and intensifies the histamine-mediated ocular pruritus, whereas leukotrienes induce vasodilation and increased vascular permeability [123]. Degranulated mast cells release cytokines such as IL-4, IL-5, IL-13, and TNF-α as well as chemokines such as CXCL8, CCL3, CCL5, CCL11, and CCL17. Cytokines play a crucial role in the activation of immune cells such as eosinophils, lymphocytes, and neutrophils. Chemokines recruit eosinophils and other immune cells to the site of

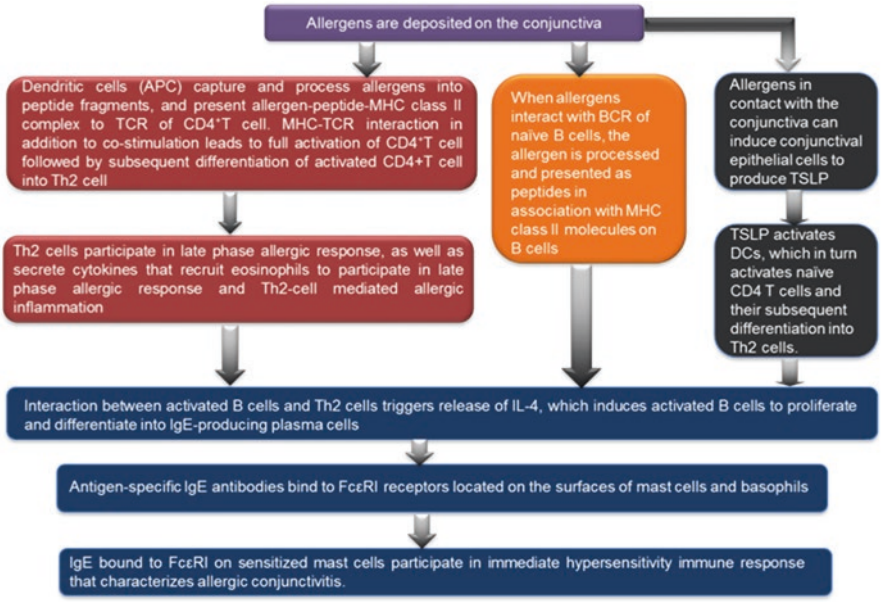

Fig. 1 Sensitization phase of allergic immune response that involves allergens interacting with DC, B cells and conjunctival epithelial cells, and subsequent interaction between DCs and $CD4^{+}$T cells that leads to the generation of Th2 cells

allergen-induced inflammation in the conjunctiva [60, 66, 121]. Vasodilation mediated by leukotrienes, prostaglandins, and histamine causes conjunctival hyperemia, whereas vascular permeability mediated by histamine and leukotrienes results in an influx of fluid from leaky conjunctival vessels into the mucosal tissue causing conjunctival edema or chemosis [118, 120, 123]. Cytokine and chemokines mediate cellular infiltration of the conjunctiva, in which inflammatory cells and mediators released by recruited inflammatory cells exacerbate the conjunctival inflammation in AC. Conjunctival fibroproliferative lesions seen in AC is attributed to the action of recruited immune cells to inflamed conjunctiva. Lipid mediators play a major role in the early stages of late phases of AC whereas cytokine and chemokines are involved in sustaining the inflammation in the late phase [3, 18, 118, 123, 132].

4.1.2 Diagnosis, Management, and Prognosis

Diagnosis of AC is mainly clinical with skin prick testing or radioallergosorbent test (RAST) used for confirmatory diagnosis and identification of the offending agent [117]. Consulting with an allergist to assist in identifying the causative agents may be beneficial. Avoidance of the offending environmental allergen by staying indoors, wearing a filter mask when outdoors, avoiding freshly cut grass constitutes the first line therapy of AC. Saline irrigation, avoidance of eye rubbing, and palliative therapy

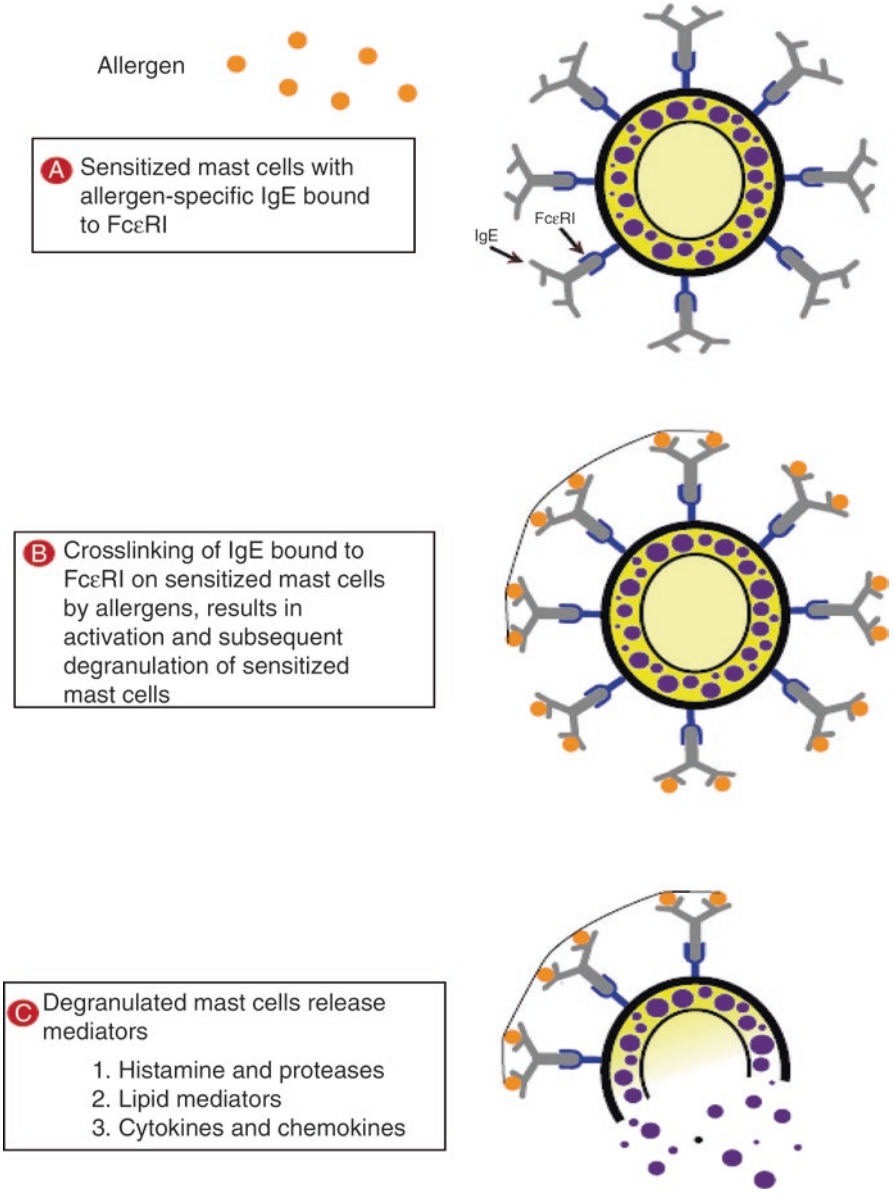

Fig. 2 Early phase allergic response occurs when crosslinking of IgE-FcεRI complex on primed mast cells leads to activation and subsequent degranulation of conjunctival mast cells and release of histamine, lipid mediators and cytokines

with cool compress are non-pharmacological management modalities that are beneficial for patients with AC. The application or administration of refrigerated preservative-free ocular lubricants in conjunction with cool compresses can induce vasoconstriction, which in turn can counteract the allergen-induced vasodilation effect associated with conjunctival hyperemia. Furthermore, preservative-free ocular lubricant can dilute and flush away environmental allergens and possibly other inflammatory mediators on the ocular surface without having any impact on the activity of histamine, tryptase, and other inflammatory mediators [117, 132]. Oral antihistamines play a role in the pharmacotherapy of AC via reduction of allergic sensitivity. Topical ophthalmic pharmaceuticals such as antihistamine, non-steroidal anti-inflammatory agents, corticosteroids, mast cell stabilizers, and antihistamine/mast cell stabilizer combination are used for treating patients with AC [124]. Majority of cases respond to antihistamine or topical antihistamine/mast cell stabilizer combination; however, an individual presenting with hyperacute expression of AC, severe AC, or recalcitrant AC would benefit from mast cell stabilizer or antihistamine/mast cell stabilizer combination and pulse topical steroidal therapy along with a tapering schedule. Anti-allergic nasal sprays such as azelastine hydrochloride nasal spray or oral antihistamine along with topical antihistamine/mast cell stabilizer combination ophthalmic agents would be beneficial for individuals who have allergic rhinoconjunctivitis. It is of note that oral antihistamines can reduce the aqueous component of the tear film; however, they usually have a long duration of therapeutic effect with delayed onset of action. As such, it is recommended that clinicians should prescribe topical antihistamine or antihistamine/mast cell stabilizer combination ophthalmic agents with rapid onset of action and more than 8 hours of therapeutic effect. The antihistamine in topical antihistamine/mast cell stabilizer combination ophthalmic agents provide an immediate therapeutic resolution of histamine-induced allergic expression, whereas the mast cell stabilizer provides long term anti-allergic prophylaxis [133–135]. AC has a favorable prognosis but tends to reoccur. However, untreated AC is associated with disruption of barrier function of the conjunctival epithelium, which results in persistent activation and degranulation of primed conjunctival mast cells [117, 136].

4.2 Giant Papillary Conjunctivitis

Giant papillary conjunctivitis (GPC) is not strictly an allergic disease, but an inflammatory condition characterized by papillary hypertrophy of the superior tarsal conjunctiva with little or no corneal involvement [45, 137]. It may be due to persistent mechanical ocular surface irritation or microtrauma from contact lens, ocular prostheses, exposed sutures after ocular surgery, or elevated corneal deposits. Additionally, hypersensitivity reaction to antigenic material derived from protein deposits on contact lens or ocular prosthesis [45, 138–141]. This section on GPC will focus on contact lens induced papillary conjunctivitis (CLPC) or contact lens induced GPC. CLPC may result from an immune-mediated hypersensitivity response to protein deposits on the contact lens surface and/or ocular surface irritation due to damaged contact lens,

poorly fitted contact lens, and/or irregular contact lens edge [137, 142]. CLPC may affect both atopic and non-atopic individuals with no gender or age predilection [138, 139]. CLPC occurs earlier in individuals wearing silicone soft hydrogel contact lenses than in individuals wearing rigid contact lenses. However, individuals wearing silicone soft hydrogel contact lenses are more susceptible to developing CLPC than individuals wearing soft hydrogels [61, 141, 143]. It is of note that CLPC is more likely to occur in patients wearing contact lenses made of ionic material compared to those wearing contact lenses made of non-ionic material [144].

4.2.1 Immunopathogenesis and Immunopathology

CLPC is a multifactorial ocular surface inflammation with a pathogenesis that is partly immunologic and partly mechanical. The immunologic aspect of the pathogenesis of CLPC occurs when proteinaceous deposits on a contact lens surface becomes antigenic, which results in the generation of antigenic-specific Th2-mediated immune response. Th2 cells provide signals to activated B cells to produce IgE-secreting plasma cells [145]. Additionally, the activation of complement leads to the generation of complement mediators of inflammation (C3a and C5a) that act on vascular endothelium to upregulate the expression of adhesion molecules and also acts on mast cells to induce their activation and degranulation, and subsequent release of pro-allergic (e.g. histamine) and pro-inflammatory (e.g. cytokines and chemokines) mediators [48, 91, 141]. Szczotka and colleagues [146] demonstrated that tear levels of DAF were significantly reduced in individuals with CLPC, and as such, the reduced levels of DAF allows for the activation of complement and subsequent generation of complement mediators of inflammation. C5a can upregulate the expression of adhesion molecules on vascular endothelium leading to vasodilation and vascular permeability. Because mast cells express receptors for C3a and C5a, these complement mediators of inflammation can induce the chemotaxis and activation of conjunctival mast cells [147]. As such, interactions between complement mediators of inflammation and their respective receptors on mast cells are likely to be involved in the immunopathology of CLPC. The mechanical aspect of the pathogenesis of CLPC involves the generation of proinflammatory cytokines in response to mechanical trauma or irritation of the conjunctiva. IL-8 released by traumatized conjunctival epithelial cells attracts neutrophils to the site of inflammation. Because both IL-1 and TNF-α released by the damaged epithelial cells can induce both epithelial and vascular endothelial cells to secrete chemokines and adhesion molecules respectively, they plays a role in recruitment of immune cells to the site of conjunctival inflammation [39, 61]. Furthermore, C5a-C5a receptor interaction on monocytes and neutrophils induces their recruitment to the site of traumatized conjunctiva [147]. Thus, interaction of complement mediators of inflammation and IgE with mast cells triggers the release of pro-inflammatory mediators. The presence of IgE and IL-8 in the tears of individuals with CLPC is suggestive of immune-mediated and mechanical-induced CLPC respectively [39, 61]. This is supportive of the multifactorial nature of the pathogenesis of CLPC. Histopathological examination of con-

junctiva in patients with CLPC reveals infiltration with lymphocytes, neutrophils, and eosinophils. Although, eosinophils are present in the conjunctiva of patients with CLPC, it has little/no role in the immunopathology of CLPC, since the levels of eotaxin and eosinophil cationic protein (ECP) are not significantly increased in these patients with GPC [45, 138, 148]. Patients with CLPC have significantly less eosinophils and eosinophil major basic protein (EMBP) than patients with VKC [138, 140]. Thus, the immunologic component of the pathogenesis of CLPC is due to increased levels of IgE, chemokines, cytokines, $CD4^+$T cells, and complement mediators of inflammation [141]. The normal tarsal conjunctiva has a satin appearance and it is a pink mucous membrane with fine vessels radiating perpendicular to the tarsal margin [141]. The symptoms of CLPC include contact lens awareness, itching, excessive contact lens movement, decreased contact lens tolerance, and blurred vision from coatings on the contact lens surface [138, 140, 144]. There is little/no obscuration of normal conjunctival vascular pattern in the mild form of CLPC. However, there is significant obscuration of normal conjunctival vascular pattern in moderate and severe forms of CLPC. Patients with CLPC present with subconjunctival scarring, fibrosis of the apices of papillae, papillary hypertrophy, and hyperemia of tarsal conjunctiva (Fig. 3) [141]. In severe giant papillary conjunctivitis, the papillae on the upper tarsal conjunctiva are large (1 mm or larger) with flattened, scarred apices that stain positively with sodium fluorescein [138, 141].

4.2.2 Diagnosis, Management, and Prognosis

The presence of papillae (0.3 mm in diameter or larger) on the superior palpebral conjunctiva induced by immune response to antigens and/or mechanical irritation from contact lenses is diagnostic of CLPC [139, 149]. The goal of management of CLPC is to remove the trigger factors via discontinuation of contact lens wear until the inflammatory reaction subsides and the patient becomes asymptom-

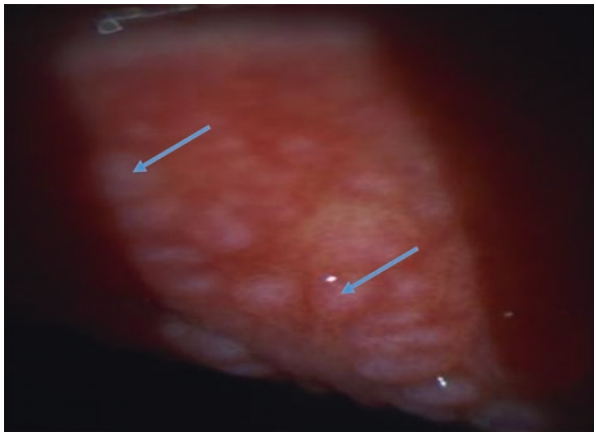

Fig. 3 Giant papillae on the superior palpebral conjunctiva in a patient with CLPC (blue arrow)

atic. When this is achieved, contact lens wear could be resumed with therapeutic intervention that does not interfere with contact lens wear. Anti-allergic and anti-inflammatory therapies would be necessary to control the ocular hypersensitivity and inflammatory cascade in CLPC [61, 138]. An important aspect of contact lens hygiene is to keep contact lens deposition to a minimum via weekly enzymatic contact lens cleaning. Maintaining a resolved state of CLPC would entail refitting the patient with new contact lens either in the same or different material and design, decreasing contact lens wear time, regularly replacing contact lenses, and instituting regular contact lens cleaning and disinfection [140, 144]. It is important to educate the patient of not resuming contact wear lens until inflammatory reaction, corneal epithelial defect and apical staining of the conjunctival papillae are completely resolved. Patients who do not respond to conventional therapy require short-term topical therapy [150]. Bartlett and colleagues [137] demonstrated the efficacy of loteprednol etabonate in treating CLPC. Kymionis and associates [151] reported success with using topical tacrolimus 0.03% ointment to treat severe GPC that was unresponsive to conventional therapy. Non-pharmacological management strategies are usually effective in CLPC; however, pharmacological therapy would become necessary when CLPC does not respond to non-pharmacological management strategies. Maintenance pharmacologic therapy may involve the use of once-daily or twice-daily dosed anti-allergic medication on a long term basis, which could be started prior to the allergy season if the patient has a history of atopy [132]. The long-term prognosis of a patient with CLPC is mostly good; however, ocular complications due to chronic inflammation or treatment side effects may ensue [151]. Although the prognosis is typically good for cases that require non-pharmacologic and pharmacologic intervention, prevention is the best strategy.

4.3 Atopic Keratoconjunctivitis

Atopic keratoconjunctivitis (AKC) is a sight-threatening, chronic inflammatory disease of the ocular surface and periocular tissue. It is characterized by chronic conjunctivitis, progressive infiltration of the cornea, and corneal vascularization and fibrosis [117, 152–154]. It is associated with atopic dermatitis and other allergic conditions. It is the most severe form of chronic ocular surface allergy with a great potential to cause ocular surface complications and damage [153, 155]. AKC is more common in men and it usually begins in late teens or early twenties with the clinical course of the disease persisting until the fourth or fifth decade of life [156, 157]. Patients with AKC have an inherited predisposition to atopy with a positive family history of allergic disorders such as asthma. The systemic disorders associated with AKC include hay fever, bronchial asthma, atopic dermatitis, food allergies, urticaria and nonhereditary angioedema [157].

4.3.1 Immunopathogenesis and Immunopathology

AKC has a multifactorial pathogenesis with T cells, cytokines, hormonal factors, genetic factors, and conjunctival hyperreactivity having an impact on the pathogenesis [45]. The immunopathology of AKC involves chronic IgE-mediated mast cell activation, T cell-mediated inflammation, T cell-derived cytokines, eosinophils, basophils, and other inflammatory cells [124, 158]. The histopathological finding in the conjunctiva of patients with AKC reveals elevated levels of T cell-derived cytokines, significant levels of toxic mediators released from degranulated eosinophils and neutrophils, increased goblet cell proliferation, presence of regulated on activation, normal T cell expressed and secreted (RANTES) and ICAM-1, and high levels of IgE in tears [45, 156, 159, 160]. Thus, the damage to the ocular surface in AKC is due to chronic expression of pro-allergic mediators, inflammatory mediators, and infiltration of effector cells [161, 162]. Patients with AKC usually present with papillary hypertrophy of the lower palpebral conjunctiva, eyelid edema, limbal gelatinous hyperplasia, chemosis, conjunctival hyperemia, stringy discharge, chronic ocular itch, meibomian gland dysfunction, and corneal epithelial defects [117, 156, 157, 163]. In moderate to severe forms of AKC, there is conjunctival subepithelial fibrosis, fornix foreshortening, and persistent corneal epithelial defects [164]. The decrease in mucin 5AC (MUC5AC) levels are usually associated with conjunctiva squamous metaplasia, tear film instability, and significant reduction in goblet cell density. Tear film instability in AKC is due to meibomian gland dysfunction that causes alteration in the composition of tear film lipids [45, 165]. Eyelid and periorbital involvement in AKC are seen as hyperpigmentation of the periorbital skin, keratinization of the eyelid margin, trichiasis, eyelid edema, and eczema of the periocular skin [154, 157, 166]. Conjunctival manifestations in AKC include papillary hypertrophy of the inferior palpebral conjunctiva, conjunctival subepithelial fibrosis, fornix foreshortening, and conjunctival chemosis and hyperemia. Limbal involvement in AKC includes perilimbal gelatinous hyperplasia and limbal hyperemia [154, 164, 166]. Corneal involvement is usually secondary to effects of inflammatory mediators on the ocular surface and tear film, and these corneal signs include persistent corneal defects, filamentary keratitis, corneal ulceration, peripheral micropannus, corneal neovascularization, and pseudogerontoxon [52, 154, 156, 157, 166]. The crystalline lens opacification in AKC is predominantly anterior subcapsular cataract that may progress into complete lenticular opacification [156, 167].

4.3.2 Diagnosis, Management and Prognosis

Diagnosis is based on clinical manifestations indicative of AKC with a skin prick test or a RAST to identify the allergen-specific IgE [117]. The goal of management in AKC is to eliminate or avoid the offending agent, control ocular surface inflammation, reduce exacerbations, and prevent ocular surface and periocular tissue damage. Because AKC is a chronic ocular surface inflammatory disease, anti-allergic and anti-inflammatory pharmacotherapy in addition to supportive therapy are nec-

essary. Supportive therapy in allergic eye disease involves avoidance of inciting agents, cool compress application, and ocular surface lubrication with preservative-free ocular lubricants [52, 157, 166, 168, 169]. Steroidal therapy is necessary and it should be used with caution due to the increased risk of infection, cataract, corneal melting, and elevated intraocular pressure [157]. There are therapeutic benefits of using systemic cyclosporine in treating AKC that is refractory to conventional therapy [152]. Nivenius and colleagues [170] demonstrated the therapeutic potential of tacrolimus as a suitable alternative to topical steroidal therapy for treating periocular eczema; however, during therapy with tacrolimus, the patient should avoid ultraviolet exposure. García and colleagues [171] demonstrated the therapeutic potential of tacrolimus 0.03% dermatologic ointment for treating AKC that is unresponsive to conventional treatment. AKC is a chronic, immune-mediated ocular surface disease that has the potential to cause ocular surface and periocular tissue damage if left untreated. Complications of AKC include subepithelial fibrosis, decreased tear production, lid margin keratinization and malposition, fornix foreshortening, symblepharon formation, corneal neovascularization and corneal ulceration [153, 162, 169]. The corneal lesion in AKC may be multifactorial with a mechanical component associated with trauma to corneal epithelium by giant papillae and inflammatory component due to inflammatory mediators released by eosinophils, T cells, basophils, and/or mast cells [172]. Thus, without prompt and appropriate management, it will progress to a potentially sight-threatening sequelae.

4.4 Vernal Keratoconjunctivitis

Vernal keratoconjunctivitis (VKC) is a multifactorial ocular surface inflammatory condition that is associated with genetic, immune, and environmental factors [173]. It is predominantly a Th2 cell-mediated chronic inflammatory disease with nonspecific hypersensitivity responses characterized by conjunctival fibroproliferative lesions such as giant papillae of the superior palpebral conjunctiva and/or gelatinous limbal papillary hyperplasia, as well as itching, limbal infiltration, conjunctival hyperemia, and corneal involvement [174–176]. VKC affects mainly children and young adults with preponderance in males [85, 177]. The three forms of VKC based on the main site of papillary reaction include limbal, mixed, and palpebral VKC [119, 178, 179]. The tarsal form of VKC is characterized by the presence of papillary hypertrophy that over time may assume a cobblestone appearance on the upper palpebral conjunctiva. This form is common in temperate regions, and vernal ulcers and plaques are usually a common complication [82, 86]. Gelatinous limbal papillary hyperplasia is a hallmark feature of the limbal form of VKC [180]. Although VKC usually resolves within 4–10 years after onset, it could progress to atopic keratoconjunctivitis in the late teens and early twenties [82, 119, 177, 181]. The perennial variant of VKC is common in warm climates while the seasonal variant is usually common in temperate region with flare ups occurring in the spring and summer [177]. However, this variation in presentation is dependent on the allergic disposition of the patient and climate [119]. Approximately 50% of patients

with VKC have a positive family history of atopy, which confirms that non-IgE-mediated mechanisms are involved in the immunopathology of VKC [76, 181, 182].

4.4.1 Immunopathogenesis and Immunopathology

VKC is predominantly a Th2 cell-mediated allergic inflammatory disease characterized by over expression of Th2-derived cytokines, chemokines, adhesion molecules, histamine, eosinophils, growth factors, enzymes, mast cells, macrophages, and dendritic cells [68, 82, 85]. Mast cells and Th2 cells in the conjunctiva of allergic eye disease release IL-4, IL-5 and IL-13 that play a pivotal role in the immunopathology of VKC [39, 183–185]. TNF-α is also released by degranulated conjunctival mast cells [184, 185]. Because there is an increased level of dendritic cells in the cornea and conjunctiva in VKC, dendritic cells play a predominant role in the immunopathogenesis of VKC [186]. Climatic, environmental, hormonal, genetic, and neural factors may influence the pathogenesis of VKC [178, 187, 188]. The involvement of neural factors in the pathogenesis of VKC is demonstrated by the overexpression of nerve growth factor (NGF) in serum and NGF receptors on the conjunctiva. Hormonal factors are characterized by the overexpression of estrogen and progesterone receptors on the conjunctiva, suggestive of a potential role of sex hormone in VKC pathogenesis [76, 187, 189]. Estrogen and androgen exert immune-enhancing and immunosuppressive effects on the humoral and cellular immune response respectively. As such, androgen could be considered a natural anti-inflammatory hormone. It has been hypothesized that recovery of VKC at puberty or spontaneous remission of VKC in late puberty could be due to the immunosuppressive and protective function of androgen. The effect of these steroid hormones may explain the difference in the course of VKC in males and females [57]. Tears of patients with VKC have significantly increased levels of hemopexin, a type II acute phase reactant glycoprotein upregulated by IL-6. Hemopexin has serine protease and pro-inflammatory activity, and as such, it may have a role in tissue remodeling in VKC [190]. Hemopexin possess antioxidant properties, and increased levels of hemopexin correlate with pathological changes in the cornea, conjunctiva, and limbus [191]. Abelson and associates [192] demonstrated high levels of histamine in tears and attributed this to a defect in histaminase. This increased tear level of histamine and its effects on the ocular surface could be exacerbated by effects of chronic eye rubbing [82]. Allergic inflammatory mediators such as histamine can trigger the epithelium of the ocular surface to express ICAM-1, cytokines, and chemokines [193]. Chemokines expressed by activated epithelial cells participate in the recruitment of immune cells such as T cells, neutrophils, and eosinophils to the ocular surface perpetuating and exacerbating inflammatory reaction at the site of allergic inflammation [184, 193]. The expression of MMPs by these activated epithelial cells can facilitate the access of inflammatory mediators into the subepithelial tissue via degradation of the ECM and cell-to-cell junctions [193]. Fibroblasts in the conjunctiva and cornea are involved in formation of giant tarsal papillae and gelatinous limbal thickening. It also exacerbates and perpetuates the allergic process via the release of cytokines, chemokines, and adhesion molecules such as VCAM-1 [194]. During allergic inflammation, corneal

fibroblasts express CCL17, eotaxin, ICAM-1, and VCAM-1, which promotes the recruitment and infiltration of eosinophils and other immune cells. Thus, corneal and conjunctival fibroblasts have a pivotal role to play in the immunopathology of VKC. CCL17 and CCL22 are chemokines that play an important role in Th2 cell-mediated inflammation in VKC, as it induces the recruitment of Th2 cells to the site of conjunctival allergic inflammation. These chemokines are produced by corneal fibroblasts activated by TNF-α and/or IL-4 during the allergic response [57].

In VKC, there are increased levels of eotaxin and IL-8, which correlate with increased infiltration of eosinophil and neutrophil in the conjunctiva [194]. Levels of activated eosinophils in tears, serum, and conjunctiva of patients with VKC is significantly higher compared to other allergic eye diseases [194, 195]. Additionally, CCL11/CCR3, CCL24/CCR3, and CCL26/CCR3 interactions are responsible for eosinophil infiltration in VKC. CCL11 (eotaxin-1), CCL24 (eotaxin-2), and CCL26 (eotaxin-3) expressed on ocular surface epithelial cells create a chemokine gradient that facilitates access of eosinophils to the conjunctiva and cornea in patients with VKC [196]. Conjunctival fibroblasts are known to prolong the survival of eosinophil that accumulate in the conjunctiva [57]. Eosinophil-derived granule proteins are epitheliotoxic to the corneal epithelium, and as such, they are responsible for breaching the corneal barrier function in VKC [174]. Eosinophil-derived granules such as eosinophil cationic protein (ECP) and eosinophil major basic protein (EMBP) disrupt the corneal epithelium while MMPs degrade the corneal basement membrane and stroma [185, 193]. The presence of ECP in the tears is an indication of increased levels of activated eosinophils [193]. MMP-2 and MMP-9 in tears of VKC patients mediates the degradation of collagen type IV and laminin in the corneal basement membrane. Activated eosinophils, epithelial cells, and fibroblasts are known to express MMP-2 and MMP-9, resulting in an increase in the level of MMPs at the ocular surface [57, 194]. Eosinophil-derived granule proteins and MMPs participate in the pathological mechanism of corneal damage by causing damage or disruption to the barrier function of the cornea, which allows them access to corneal stroma to activate fibroblast to release chemokines [185]. A balance between synthesis and degradation of ECM protein is an important aspect of the metabolism of ECM protein and proteoglycan that is necessary for maintaining the structure [57]. Increased synthesis of ECM proteins by activated fibroblasts under allergic inflammatory condition is associated with an imbalance between MMP and tissue inhibitor of metalloproteinase (TIMP) that results in increased collagen deposition and extracellular matrix hyperplasia, which causes proliferative changes that lead to the formation of limbal papillary hyperplasia and giant papillae [57, 194]. Histologically, the proliferative lesion is composed of eosinophils, mast cells, Th2 cells, fibronectin, neutrophils, goblet cells, plasma cells, and collagen type I and III [35, 57]. Giant papillae are characterized by squamous hyperplasia of the conjunctival epithelium and presence of dense fibrotic tissue [193]. ECM proteins participate in allergic inflammation by augmenting the expression of pro-inflammatory cytokines by both eosinophils and macrophages attached to them. Additionally, ECM is a reservoir for cytokine and chemokine, and as such, release of ECM proteins by corneal and conjunctival fibroblasts activated by IL-4 in the setting of allergic reaction contributes to the persistent activation of inflammatory cells during allergic

inflammation of the ocular surface [57]. As such, conjunctival tissue inflammation, corneal damage, and conjunctival tissue remodeling in VKC are associated with increased deposition of collagen type I, III, IV, and V in the conjunctiva [194]. Growth factors such as vascular endothelial growth factor (VEGF) are expressed by conjunctival epithelial cells and fibroblasts, mast cells, macrophages, and eosinophils [57, 59, 119, 197]. VEGF plays a crucial role in the immunopathology of VKC, as it induces angiogenesis and vasopermeability. The leakage of plasma from these leaky capillaries into the extravascular space leads to edema and significant modifications in the ECM of the conjunctiva [119, 198]. Th2 cell-mediated tissue remodeling is responsible for the development of giant papillae, hyperplasia of the epithelium, extensive deposition of ECM components in the conjunctiva, peripheral corneal fibrovascular proliferation, and other corneal changes [68, 119, 198]. Eosinophils, mast cells and Th2 cells accumulate in the conjunctiva, when all these cells are activated, they produce mediators that are associated with tissue damage [191].

Inflammatory reactions and clinical manifestations in VKC occur as a result of the action of histamine, arachidonic acid metabolites, as well as cytokines and chemokines released by mast cells, T cells, and eosinophils [176, 184]. Chronic inflammation in VKC is associated with tissue remodeling, conjunctival fibroproliferative lesions/papillary hypertrophy, limbal stem cell deficiency, squamous metaplasia of the ocular surface, and corneal epithelial defects [176, 199]. The clinical features of VKC include burning sensation, tearing, photophobia, blepharospasm, mucoid discharge, eyelid edema, conjunctival hyperemia and chemosis, perilimbal bulbar conjunctival hyperpigmentation, pseudogerontoxon, Horner-Tranta dots, shield ulcer, persistent corneal epithelial defects, and vernal plaques [68, 200–202]. In moderate to severe forms of VKC, hyperemia and papillary hypertrophy of the superior palpebral conjunctiva may partially obscure visualization of the deep tarsal conjunctival vessels [201, 203]. Perilimbal bulbar conjunctival or circumcorneal hyperpigmentation, seen as a fine golden brown pigmented perilimbal thickening, is due to excess pigment production by activated and proliferating melanocytes in the limbus [204]. Perilimbal bulbar conjunctival hyperpigmentation is associated with VKC, and it is an indication of limbal involvement in the immunopathology of VKC [205]. Limbal VKC is characterized by the presence of multiple gelatinous limbal infiltrates, Horner-Tranta dots, and pannus of the limbus (Fig. 4) [82]. Horner-Tranta dots found on the limbus are composed of clumps of necrotic eosinophils, epithelial cells, and neutrophils, and they usually disappear when the inflammatory reaction abates [8, 156, 206]. Conjunctival giant papillae on the superior palpebral conjunctiva and limbus are considered the hallmark features of VKC [178]. Corneal involvement is present in more than 50% of VKC patients; however, the cornea is an immunological privileged tissue that consist of epithelial cells, fibroblasts, endothelial cells, macrophages, and dendritic cells [59]. Activated corneal epithelial cells and fibroblasts participate in the immunopathology of VKC that leads to the development of corneal findings such as persistent corneal epithelial defects, epithelial macroerosion, shield corneal ulcer, corneal plaque, cornea ectasia, pseudogerontoxon, and corneal fibrosis [57, 59, 68, 119, 180, 207, 208]. Chronic inflammation of the ocular surface mediated by eosinophil-derived granule proteins and mediators released by inflammatory cells have an adverse effect on the limbal

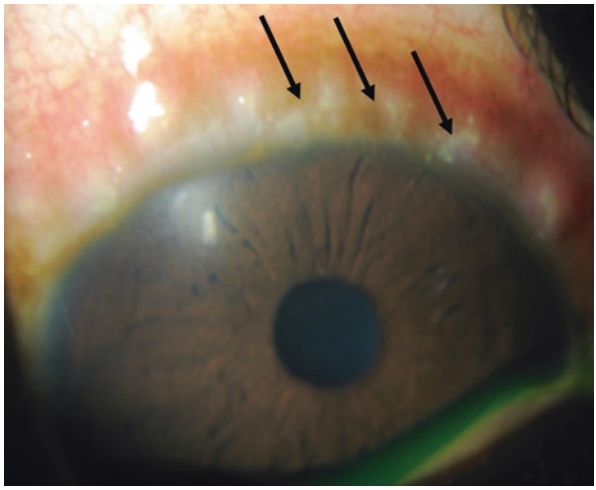

Fig. 4 Limbal VKC characterized by the presence of gelatinous limbal infiltrates with Horner-Tranta dots (black arrow)

epithelium and stroma causing direct damage to progenitor limbal stem cells, which results in the development of limbal stem cell dysfunction [8, 185, 199, 205]. Corneal or vernal shield ulcer in VKC is a vision-threatening oval shaped ulcer usually located in the superior third of the cornea [68, 76, 178, 180, 209]. Corneal shield ulcer has been reported to be more common in patients with the tarsal form of VKC [178]. The pathogenesis of vernal shield ulcer is believed to be due to: (1) chronic mechanical abrading of the corneal epithelium by giant papillae on the superior palpebral conjunctiva associated with the blink action, (2) breakdown of the barrier function of the corneal epithelium and degradation of corneal basement membrane, and (3) stroma caused by mediators produced by inflammatory cells and degranulated mast cells, and MMPs secreted by activated corneal fibroblasts and eosinophils [122, 180, 210–212]. Corneal plaque is formed when VKC-induced ulcer takes on a translucent appearance due to the deposition of inflammatory debris composed mainly of eosinophil-derived cytotoxic mediators at the base of the ulcer [180, 213, 214]. VKC is associated with corneal ectatic disorder such as keratoconus, pellucid marginal degeneration, and keratoglobus [194]. Corneal ectasia in VKC is due to thinning of the central cornea that results from apoptosis of keratocytes in the corneal stroma and/or degradation of corneal stroma due to the increase in matrix-degradative enzymatic activity [193]. Pseudogerontoxon, a gray-white lipid deposit in the peripheral cornea, is considered clinical evidence of previous allergic eye disease that results from prolonged infiltration of the limbus [68, 82, 215]. Shield ulcer, corneal plaque, corneal ectasia, and limbal stem cell deficiency are complications that involves the cornea in patients with VKC [185].

4.4.2 Diagnosis, Management, and Prognosis

The diagnosis of VKC is based on the presence of clinical signs and symptoms due to Th2 cell-mediated immunopathology. The main objective of clinical management in VKC is to suppress the allergic inflammatory process using non-pharmacological and

pharmacological treatment modalities. Avoidance of trigger factors and eye rubbing is important, since chronic eye rubbing can mechanically degranulate conjunctival mast cells leading to release of inflammatory mediators that play a role in the immunopathology of VKC [82, 195]. Although patients with VKC may benefit from supportive therapy and antihistamine/mast cell stabilizer combination, pulse topical steroidal therapy is usually necessary to control the inflammatory process. Prednisolone acetate 1.0% ophthalmic suspension is usually the drug of choice when loteprednol etabonate 0.5% is not therapeutically effective in controlling the allergic inflammation [132, 178]. The main objective of treating VKC-induced shield ulcer is to promote re-epithelialization by inhibiting the release of pro-inflammatory mediators, eliminating or minimizing damage to corneal epithelium by mechanical trauma from giant papillae during blinking, and promoting healthy corneal epithelium by removing inflammatory material [210]. Therapeutic bandage contact lens and/or prophylactic antibiotic ointment with anti-MMP activity are beneficial as adjunctive therapy in a VKC-related ulcer, since it reduces pain and promotes corneal re-epithelialization via protection of the fragile corneal epithelium during the corneal wound repair process [216]. Additionally, therapeutic bandage contact lens reduces the effect of blink action, which prevents giant papillae on the superior palpebral conjunctiva from inducing mechanical abrasion of the corneal epithelium [217]. There may be a role for amniotic membrane transplantation (AMT) in the management of VKC-associated shield ulceration, as it facilitates re-epithelialization by reinforcing adhesion of corneal epithelial cells as well as minimizing corneal scarring associated with proliferation of activated corneal fibroblasts. Additionally, AMT protects the corneal epithelium from mechanical trauma of giant papillae during blink action and prevents access of eosinophil-derived granule proteins to the cornea [210]. Majority of corneal vernal plaque requires anti-inflammatory therapy and surgical therapy to remove the plaque [180]. Disease-related complications of VKC that induce visual impairment are due to central corneal scar, shield ulcer, limbal stem cell deficiency, irregular cornea, and corneal ectasia (e.g. keratoconus). Treatment-related complications include steroid-induced cataracts and raised intraocular pressure [68, 177, 181, 210]. Long term prognosis is usually good with majority of patients with VKC having spontaneous resolution of the disease 4–10 years after onset [76].

5 Pharmacotherapy of Allergic Eye Disease: Current and Future

The management of allergic eye disease ranges from non-pharmacological to pharmacological modalities. The primary management of allergic eye disease is to remove or avoid the offending agent that triggers the allergic response in the eye. Management of allergic eye disease with pharmaceuticals becomes necessary when non-pharmacological approach fails to control the allergic immune response [117]. The following paragraphs will review most of the pharmaceutical agents used in the management of allergic eye disease (Table 2).

Table 2 Ophthalmic agents used in pharmacotherapy of allergic eye disease

Medication generic (brand)	Mechanism of action	Clinical uses	Daily Dosage
Alcaftadine 0.25% Rx (Lastacaft)	H1 receptor antagonist and mast cell stabilizer	Prevention of itching associated with AC	1 drop QD
Azelastine hydrochloride 0.05% Rx (Optivar)	H1 receptor antagonist and mast cell stabilizer	Treatment of itching associated with AC	1 drop BID
Bepotastine besilate 1.5% (Bepreve), Rx	H1 receptor antagonist and mast cell stabilizer	Treatment of itching associated with AC	1 drop BID
Epinastine hydrochloride 0.05% (Elestat), Rx	H1 receptor antagonist and mast cell stabilizer	Prevention of itching associated with AC	1 drop BID
Ketotifen fumarate 0.025% (Zaditor)	H1 receptor antagonist and mast cell stabilizer	Prevention of itching associated with AC	1 drop BID
Olopatadine hydrochloride 0.1% (Patanol), Rx	H1 receptor antagonist and mast cell stabilizer	Treatment of signs and symptoms of AC	1 drop BID
Olopatadine hydrochloride 0.2% (Pataday), Rx	H1 receptor antagonist and mast cell stabilizer	Treatment of ocular itch associated with allergic conjunctivitis	1 drop QD
Olopatadine hydrochloride 0.7% (Pazeo), Rx	H1 receptor antagonist and mast cell stabilizer	Treatment of ocular itch associated with allergic conjunctivitis	1 drop QD
Nedocromil sodium 2.0% (Alocril), Rx	Mast cell stabilizer	Treatment of itching associated with allergic conjunctivitis	1 drop BID
Ketorolac tromethamine 0.5% (Acular), Rx	COX inhibitor	Temporary relief of itching in AC	1 drop QID
Fluorometholone ophthalmic (FML 0.1%, FML Forte 0.25%), Rx	Inhibit edema, capillary dilation, leukocyte migration, and fibroblast formation associated with inflammation	Treatment of steroid-responsive inflammation of the conjunctiva, cornea, and anterior segment conditions	1 drop QID
Loteprednol etabonate ophthalmic 0.2% (Alrex), Rx	Inhibit edema, capillary dilation, leukocyte migration, and fibroblast formation associated with inflammation	Treatment of signs and symptoms of AC	1 drop QID
Loteprednol etabonate ophthalmic 0.5% (Lotemax), Rx	Inhibit edema, capillary dilation, leukocyte migration, and fibroblast formation associated with inflammation	Treatment of steroid-responsive inflammation of the conjunctiva, cornea, and anterior segment conditions	1 drop QID
Prednisolone acetate ophthalmic (Pred Forte 1%), Rx	Inhibit edema, capillary dilation, leukocyte migration, and fibroblast formation associated with inflammation	Treatment of steroid-responsive inflammation of the conjunctiva, cornea, and anterior segment conditions	1 drop QID

H1, Histamine 1; AC, allergic conjunctivitis; COX, cyclooxygenase; BID, twice daily; QD, once-daily; QID, four-times-a-day

5.1 Antihistamines

Antihistamine are used in the treatment of allergy including allergic eye disease. They are H1R antagonist with first generation antihistamines having sedative effect due to its lipophilic nature that allows these drugs to cross the blood brain barrier to block histamine-mediated central neurotransmission. Second generation antihistamine has an improved side effect profile with low sedative potential [117]. Cetirizine is a piperazine derivative that possesses histamine receptor inverse agonist effect with the added feature of inhibiting chemotaxis of eosinophils. Cetirizine has a low sedative potential and it is well tolerated in patients with allergic rhinoconjunctivitis [218, 219]. Loratadine is another long acting second-generation antihistamine with low sedative potential. It is safe and efficacious in treating allergic rhinoconjunctivitis, an effect that could be attributed to its inhibitory eosinophil activation [220–222]. Topical ophthalmic antihistamine has inverse agonist effect on H1 receptors. Because of the systemic effect of oral antihistamine and reduced systemic absorption of topical antihistamines, it is preferable to treat allergic eye disease with topical antihistamines [117]. Levocabastine hydrochloride 0.05% and emedastine difumarate 0.05% are ophthalmic antihistamines with potent selective histamine type 1 receptor inhibitory effect [68, 223, 224]. These topical antihistamines are dosed one drop four times daily for relieving the symptoms and signs of AC [225].

5.2 Mast Cell Stabilizers

Topical mast cell stabilizers are anti-allergic pharmaceutical agents that block the release of mediators of allergic eye disease from mast cells by blocking the degranulation of conjunctival mast cells [223, 226]. Mast cell stabilization is achieved via the blockade of calcium influx across the cell membrane. They have no effect on allergic expression due to already released histamine [75, 195]. Nedocromil sodium 2% ophthalmic solution, a pyranoquinolone dicarboxylic acid, is a mast cell stabilizer that is safe and effective for treating AC in patients age two and up. It stabilizes the mast cell by inhibiting the influx of calcium into the mast cell [117, 133, 223, 227, 228]. It could be considered a multiple anti-allergic agent due to its inhibitory effect on eosinophil [229]. Another action of nedocromil sodium includes reducing the ability of conjunctival epithelial cells to express ICAM-1 [229, 230].

5.3 Multimodal or Dual Acting Agents

Multimodal anti- allergic pharmaceutical agents possess inverse agonist effect on H1 receptors and mast cell stabilizing effects. These multimodal anti-allergic agents provide immediate symptomatic relief from histamine-induced effects and block the

release of pro-inflammatory and pro-allergic mediators by stabilizing conjunctival mast cells [231]. Olopatadine hydrochloride (0.1%, 0.2% and 0.7%) ophthalmic solution has selective inverse agonist effects on H1 receptors and prevent the release of pro-inflammatory and pro-allergic mediators from conjunctival mast cells, which translates into a prolonged clinical effect [223, 232, 233]. Olopatadine also reduce the recruitment of inflammatory cells into the conjunctiva by inhibiting the upregulation of ICAM-1 expression on conjunctival epithelial cells [229]. Olopatadine 0.1% formulation is dosed one drop twice a day whereas olopatadine 0.2% and 0.7% formulations are dosed one drop once daily. Olopatadine is indicated for the treatment of the signs and symptoms of AC [232, 234]. It is effective and well tolerated in patients age 3 and older [68, 200]. Azelastine hydrochloride 0.05% ophthalmic solution is a phthalazinone derivative that has a rapid onset of action and prolonged clinical effect. It has inverse agonist H1 receptor effect that attenuates histamine-induced allergic expression associated with early phase allergic response and exhibits mast cell stabilizing effect [229, 230]. Azelastine can reduce the accumulation of inflammatory cells at the site of allergic reaction in the conjunctiva by downregulating the expression of ICAM-1 by conjunctival epithelial cells. It is indicated for the treatment of ocular pruritus associated with AC. It has a noticeable unpleasant or bitter taste due to passage of the drug across the lacrimal duct via the nasal cavity [223, 235–237]. Ketotifen fumarate 0.025% is a benzocycloheptathiophene derivative that possesses a potent H1 receptor antagonist effect and mast cell stabilizing properties [223, 229, 238]. Additionally, it inhibits the accumulation of eosinophil at the site of allergen-induced inflammation in the conjunctiva [68, 117, 229, 230]. Ketotifen fumarate exhibits a biphasic effect on mast cell stabilization by inhibiting histamine release at low concentration and stimulating the release of histamine at higher concentrations [229, 230]. It is indicated for preventing ocular itch associated with AC [230]. It is available over-the-counter and it is dosed one drop twice daily. Epinastine hydrochloride 0.05% has affinity for H1 and H2 receptors and it has a prolonged therapeutic effect attributed to mast cell stabilization, inverse agonist effect on histamine receptors, and inhibition of the recruitment of neutrophil and eosinophil to the site of allergic reaction in the conjunctiva [239, 240]. It is a well-tolerated anti-allergic therapeutic agent that is dosed one drop twice a day for the prevention of ocular itch associated with AC [239, 241]. Alcaftadine 0.25% is a tricyclic piperidine aldehyde with a potent inverse agonist effect on histamine receptors and mast cell stabilizing effect on mast cells. It has a high affinity for H1 and H2 receptors and low affinity for H4 receptors [242, 243]. The inhibitory effect on recruitment of immune cells such as eosinophil and mast cells could be attributed to its affinity for H4 receptors expressed on mast cells and eosinophils [242, 244, 245]. It is a well-tolerated and efficacious antiallergic agent that is dosed one drop once daily for the prevention of itching associated with AC in patients over the age of 2 [242, 243]. Bepotastine besilate 1.5% ophthalmic solution is a piperidine derivative that is dosed one drop twice daily for preventing ocular itch associated with AC in patients aged 3 and over. It has inverse agonistic effect on H1 receptors and a stabilizing effect on mast cells in the conjunctiva [246]. Bepotastine besilate can prevent the accumulation of immune cells at the site of allergic inflammation in the conjunctiva by inhibiting expression of ICAM-1 by conjunctival epithelial cells [247].

5.4 Nonsteroidal Anti-inflammatory Drug (NSAID)

NSAIDs are beneficial as adjunctive therapy in allergic eye disease, as it relieves itch and conjunctival hyperemia associated with allergic eye disease. NSAIDs are considered steroid-sparing therapy, since they inhibit the production of prostaglandin E2 (PGE2) and PGI2 that lowers the threshold of the conjunctiva to histamine-associated ocular itch [117]. Ketorolac 0.5% is a topical NSAID that has been shown to diminish ocular pruritus and conjunctival injection associated with AC via the inhibition of cyclooxygenase (COX)-1 and COX-2 enzymes without having an effect on the size of the papillae on the conjunctiva tissue [68, 176]. In patients with triad of asthma, nasal polyps, and aspirin sensitivity, use of ocular NSAIDs could result in NSAID-induced asthma [117, 228].

5.5 Corticosteroids

Corticosteroids have immunosuppressive, anti-inflammatory, and anti-proliferative effects, which are attributed to the ability of the steroid to inhibit edema, cellular infiltration, capillary dilation and permeability, fibroblast proliferation, collagen deposition, leukocyte migration, and scar formation associated with inflammation. Furthermore, corticosteroids increase the synthesis of lipocortin that blocks the enzymatic activity of phospholipase A2 required for arachidonic acid metabolism and subsequent production of prostaglandins and leukotrienes that participate in the late phase of allergic response. Moreover, corticosteroids reduce the amount of unbound histamine on the ocular surface by increasing histaminase, an enzyme that degrades histamine [248]. Corticosteroids also block the enzymatic action of histidine decarboxylase, an enzyme required for the production of histamine in mast cells [124, 223, 249, 250]. Corticosteroids inhibit the expression of cytokine, chemokine, adhesion molecules, and inflammatory enzymes by deactivating the inflammatory genes that encode these inflammatory mediators [251]. Loteprednol etabonate ophthalmic suspension is a cortienic acid-based derivative [248]. It is a highly lipophilic ester-based corticosteroid compared to ketone-based corticosteroids such as prednisolone acetate [135]. Loteprednol etabonate is a site-specific steroid, in which the active drug resides at the site of inflammation long enough to deliver its therapeutic effect with minimal adverse effects [135, 250, 252]. The rapid transformation into an inactive metabolite by esterase in the eye is responsible for the low toxicity potential of loteprednol [248]. It is of note that loteprednol etabonate 0.2% is indicated for treating signs and symptoms of AC whereas loteprednol 0.5% is indicated for treating steroid-responsive inflammation of the conjunctiva and cornea [253, 254]. Patients with AC and giant papillary conjunctivitis are responsive to loteprednol [133]. Prednisolone acetate is a drug of choice for moderate and severe VKC, if loteprednol etabonate is ineffective in controlling ocular surface inflammation [176]. Prednisolone acetate ophthalmic suspension is a corti-

costeroid that is administered one drop four times a day for the treatment of corticosteroid-responsive inflammation of the cornea and conjunctiva [255]. It is of note that long term use of topical steroidal therapy is associated with ocular adverse effects such as steroid-induced glaucoma, cataract, delayed wound repair and increased susceptibility to ocular infection [176]. Fluorometholone (FML) is a 21-deoxy-9-fluoro-6-methylprednisolone that has up to 30 times more anti-inflammatory activity than hydrocortisone. It reduces the clinical expression of VKC [133, 226]. FML 0.1% is indicated for the treatment of inflammation of the cornea and conjunctiva [256]. Because of the potency in controlling inflammation of the conjunctiva via suppression of recruitment and activation of pro-allergic and pro-inflammatory mediators in the early and late phase of allergic response, corticosteroids are considered appropriate for treating patients that present with acute flare ups and chronic allergic eye diseases [65]. It is judicious to use loteprednol for treating allergic eye disease due to its low absorption and enhanced index of therapeutic response [117]. Raised intraocular pressure, cataract, and low resistance to infection are adverse effects associated with the use of topical corticosteroids. This could be attributed to transactivating activity of corticosteroids, and as such, topical corticosteroids with potent trans-repressive effect with little or no transactivation effect, may reduce the side effects associated with currently used topical corticosteroids [251]. Because of the chronic nature of allergic eye disease requiring glucocorticoid steroids with their associated adverse effects, there is a move towards developing steroids with little or no side effects. Selective glucocorticoid receptor agonists (SEGRA) are glucocorticoid corticosteroid-based therapeutic agents with trans-repression action and little or no transactivation effect. Mapracorat is a SEGRA that selectively reduces inflammation via inhibition of pro-inflammatory cytokines and activating anti-inflammatory proteins with reduced propensity to cause adverse ocular side effects that occur with glucocorticoids. Mapracorat has a decreased ability to activate gene transcription via binding to glucocorticoid response element but primarily transrepress genes. Thus, it has potent anti-inflammatory activity with low propensity to induce adverse reactions. Furthermore, mapracorat can inhibit release of proinflammatory cytokines and chemokines from corneal epithelial cells and fibroblasts [184].

5.6 Immunomodulators

Tacrolimus and Cyclosporine are immunomodulators that inhibit calcineurin, a phosphatase that activates transcription factors required for the production of IL-2 [160, 257]. Immunomodulators are also capable of inhibiting the proliferation of mast cells and reducing the recruitment of eosinophils [226]. Tacrolimus is a potent macrolide immunosuppressant that is therapeutically beneficial for patients with atopic dermatitis and patients with AKC [117, 160, 258–260]. Tacrolimus inhibits T cell activation by binding to immunophilin FK-binding protein (FKBP-12), which in turn, blocks calcineurin, an intracytoplasmic signaling protein downstream from

calcium-dependent calmodulin activation. Inhibition of calcineurin leads to inhibition of IL-2 transcription. Tacrolimus can inhibit the release of histamine and production of lipid mediators from basophils [261]. Tacrolimus has great potential of being a steroid-sparing agent for treating patients with chronic allergic eye diseases due to its enhanced safety profile and lack of adverse effect associated with steroid use such as skin atrophy, increased intraocular pressure, and reduction in collagen synthesis [262, 263]. Cyclosporine is a cyclic undecapeptide that mediates the inhibition of calcineurin, a phosphatase that is required for dephosphorylating nuclear factor of activated T cells (NFAT), which enables NFAT to translocate into the nucleus where it induces the transcription of cytokine genes [160, 257, 263]. Cyclosporine exerts its immunosuppressant effect by blocking the production of IL-2 and IFN-γ, thus reducing T cell-mediated inflammation [176, 201, 261]. Keklikci and associates [201] showed that administration of topical cyclosporine 0.05% for 12 weeks was efficacious in improving clinical features of VKC. Spadavecchia and colleagues [188] demonstrated the efficacy of using topical cyclosporine 1.0% in treating patients with severe VKC. Topical cyclosporine 0.05% (4–6 times daily) has been effective in treating patients with VKC and AKC [117, 257, 263].

5.7 Allergen Specific Immunotherapy

This immunotherapy is based on the principle of inducing immune tolerance via the exposure to allergen and it involves administration of allergen extracts in small increments to induce allergen-specific clinical tolerance to the inciting allergen [126, 263, 264]. To induce conjunctival immune tolerance using allergen-specific immunotherapy, eye drops containing specific-allergen to the conjunctiva are administered to the eye in gradual increments over a period of time with the intent of inducing increased tolerance to the allergen. The ultimate goal is to control clinical expression of allergic response without reliance on antiallergic pharmaceutical agents [265].

5.8 Pipeline/New Pharmaceutical Agents

A number of pharmaceutical agents are in various stages of trials that may likely be added to the current therapeutic agents for treating allergic eye disease. Ocular therapeutics Inc. have started enrolling patients for the second phase 3 clinical trial with the intent of evaluating the efficacy and safety of Dextenza (sustained release dexamethasone) 0.4mg intracanalicular depot for treatment of AC [266]. However, it has been announced that the phase 3 clinical trial was successful. Nicox have completed two phase 3 trials of ocular cetirizine, a topical ophthalmic histamine receptor blocker and mast cell stabilizer for the treatment of

ocular itching associated with AC [267]. The FDA recently approved ocular cetirizine (Zerviate) 0.24% ophthalmic solution dosed twice daily for ocular itch associated with allergic conjunctivitis. Future studies could focus on assessing the potential role of monoclonal antibody against adhesion molecule such as ICAM-1, as therapy for chronic allergic eye disease [194]. Immunomodulators, monoclonal antibody-based immunotherapy, allergen specific immunotherapy, and other new drugs in the pipeline are geared towards developing therapeutic modalities that are an improvement on the current anti-allergic agents or agents that target specific mediators of inflammation or immune cells that play a major role in the immunopathogenesis and immunopathology of allergic eye diseases. In managing patients with allergic eye disease, it is important to categorize or stage the severity of the ocular condition as well as educate the patient on the nature of the allergic eye disease and various pharmacotherapeutic strategies available to treating allergic eye disease. The use of one-a-day or twice a day dosed anti-allergic agents for prophylactic and therapeutic purposes are beneficial, since it does not impact on the lifestyle of the patient because such a drug regimen promotes compliance particularly in individuals with busy life styles such as students and workers. Majority of patients with allergic eye disease benefit from supportive therapy and steroid-sparing antiallergic medications. However, short term use of steroidal therapy would be necessary when allergic eye disease does not respond to conventional antiallergic therapy [132].

5.9 Potential Role of Probiotics

It is noteworthy that the lack of exposure to microbiota during the developmental stage of the immune system is likely to tilt the Th1/Th2 immune balance to favor Th2 immunity, since allergic disorders are associated with Th2 immunity along with activation of Th2-derived cytokines. Immune cells within the conjunctiva are affected by resident ocular microbiome, and the relationship between commensal microbiome and immune cells in the ocular surface is required to modulate ocular mucosal immunity [268]. It is of note that ocular surface microbiota plays a role in strengthening the ocular surface barrier function as a constituent of the ocular innate immunity, and depletion of ocular commensal microbiome via use of topical antibiotics is likely to have an impact on the ocular surface barrier function [269]. As such, use of probiotics to induce a Th1 immune response during the early development of the immune system has the potential to shift the Th1/Th2 immune balance towards Th1 immune response [270]. There are reports in the scientific literature of reduction of ocular and nasal symptoms in patients with perennial allergic rhinoconjunctivitis following intake of yogurt supplement containing Bifidobacterium longum and fermented milk containing Lactobacillus paracasei-33. Potential benefits of probiotic as an adjunctive therapy for patients with allergic rhinoconjunctivitis needs further studies to confirm its benefits [271, 272].

6 Conclusion and Future Perspectives

Allergic eye disease is mediated mainly by IgE and/or Th2 cells along with pro-allergic and pro-inflammatory mediators participating in the immunopathogenesis and immunopathological process. Cytokines, chemokines, histamine, adhesion molecules, MMPs, and lipid mediators are important mediators that participate in allergic immune responses. Epithelial cells and fibroblasts are non-immune cells that play a role in the immunopathology of allergic eye disease when they become activated by pro-allergic and pro-inflammatory mediators. Th2 cells, mast cells, and eosinophils are the major immune cells that participate in the immunopathogenesis and immunopathology of allergic eye diseases. The major forms of allergic eye diseases that have an impact on conjunctiva and/or cornea include AC, GPC, AKC, and VKC have been discussed. AC is predominantly an IgE-mediated ocular surface disease, in which, activated mast cells undergo degranulation leading to the release of histamine and other mediators that cause the clinical features of AC [2, 123]. GPC is a multifactorial inflammatory state of the conjunctiva with immune and non-immune aspects involved in the pathogenesis. Th2 cells, mast cells, IgE, neutrophils, monocytes, and complement mediators of inflammation play a crucial role in the immunopathogenesis and immunopathology of the ocular condition [141]. AKC is a severe allergic eye disease, in which, chronic mast cell degranulation and T cell-mediated inflammation are involved in the immunopathology of the ocular condition with consequential tissue damage and remodeling of the conjunctiva and cornea [167]. VKC is a recurrent ocular surface inflammation mediated predominantly by Th2 cells with major effector cells and mediators participating in the development of fibroproliferative lesions as well as damage and remodeling of the cornea and conjunctiva [68].

Since mast cells are major effector immune cells in allergic eye diseases, histamine, lipid mediators, and cytokines released by degranulated mast cells are targets for current anti-allergic therapeutic agents. However, research and development geared towards more potent mast cell stabilizers should be considered an important future direction to focus on preventing release of mediators. Th2 cells generate cytokines that are involved in generating plasma cells that produce IgE that bind to FcεRI on conjunctival mast cells as well as cytokines that activate important immune effectors of allergic eye disease such as mast cells and eosinophils. IL-1, IL-4, and IL-5 are major cytokines that play a crucial role in immune responses in allergic eye disease. IL-4 is involved in mediating B cell proliferation and differentiation as well as in inducing fibroproliferative lesions in the conjunctiva. IL-5 is involved in activating eosinophils that have a toxic effect on the epithelial cells of the ocular surface with associated tissue damage and remodeling. IL-1 is also involved in the inflammatory process of the ocular surface via interaction with IL-1R on epithelial cells of the ocular surface. Immunotherapy that targets these cytokines and their receptors with the intent of attenuating the induced immunopathogenesis and immunopathology would be beneficial for these patients with allergic eye disease. Adhesion molecules such as ICAM-1 are involved in the immunopathology of allergic eye disease,

and they are upregulated during the inflammatory process with the intent of recruiting immune cells to the site of allergic inflammation [39]. Histamine and their receptors participate in the immunopathology of allergic eye disease. Histamine has effects on tissue and cells of the ocular surface with associated tissue changes. Activation of histamine receptor type 1, 2 and 4 are associated with various allergic expression involving the ocular surface, and histamine/H4R mediates recruitment of eosinophil and Th2 cells that exacerbate the allergic inflammation in the ocular surface with consequential tissue damage and remodeling [8, 70]. Chemokines are important for recruiting immune cells to the site of allergic inflammation, and blockade of chemokine and/or their receptors could attenuate inflammation in allergic eye diseases. Dendritic cells are important initiators of allergic eye disease, and they interact with $CD4^{+}$T cells to generate allergen-specific Th2 cells. Additionally, TSLP interacts with dendritic cells to promote the generation of allergen-specific Th2 cells [11]. As such, blockade of TSLP could be beneficial in preventing the allergic immune response from developing. A considerable understanding of cells and mediators in allergic eye disease and their respective roles in the immunopathogenesis and immunopathology of allergic eye diseases are crucial to providing significant insight on how to manage the diseases with the intent of controlling inflammation and preventing tissue damage and remodeling as well as developing pharmacotherapy and immunotherapy that would be beneficial as prophylactic and/or therapeutic agents.

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Dendritic Cell-Regulated T Cell Immunity and Tolerance against Acute Myeloid Leukemia

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Abstract Acute myeloid leukemia (AML) is the most common hematologic malignancy in adults. Although AML patients achieve a complete remission following chemotherapy, most patients relapse with residual disease. Only approximately 25% of AML patients are alive 5 years following their diagnosis. Immunotherapies, such as chimeric antigen receptor T cells, immune checkpoint inhibitors, and vaccination using dendritic cells (DCs) treated by AML cells and their-derived antigens, have emerged as promising therapeutic modalities in AML. However, for AML, in which leukemia cells disseminate in bone marrow (BM), peripheral blood and many other tissues, the mechanisms that regulate the generation and maintenance of leukemia-specific T cell responses are less clear. Growing evidence suggests that AML cells can not only directly repress antigen-specific T cell reactivity, but also induce tolerogenic DCs to reduce tumor-specific T cell responses. DC-mediated tolerogenic mechanisms include suppressing T cell proliferation, inducing T cell deletion and impairing the function of leukemia-specific T cells. Interestingly, this tolerogenic effect can be potentially reversed upon activation by Toll-like receptor agonists and ligation of CD40 in DCs. Clinical studies reveal that DC-based vaccination is potentially effective on preventing and delaying relapse of AML. In this chapter, we focus on discussing these effects of DCs on mediating immunogenicity and immune tolerance of T cells against AML cells.

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1 Introduction

Acute myeloid leukemia (AML) is characterized by infiltration of the bone marrow (BM), blood and other tissues by proliferative myeloid blast cells [1–3]. AML is the most common hematologic malignancy in adults [1, 4]. AML can be cured in 35–40% of adult patients who are 60 years of age or younger and in 5–15% of patients who are older than 60 years of age [1, 4]. While AML patients achieve a complete remission after chemotherapy; however, most patients relapse with residual disease [1, 5].

Emerging evidence indicates that immunotherapy is potentially effective strategy to prevent or delay relapse in patients with AML after standard chemotherapy [6–13]. Vaccination of AML patients in remission with leukemia antigen-treated dendritic cells (DCs) induced expansion of leukemia-specific T cells and provided protection against disease relapse [10, 12]. Long-term clinical response correlated with increased circulating frequencies of leukemia-reactive $CD8^+$ T cells and significantly improved overall survival [6–10]. However, some studies also suggest that AML cell-specific T cells may have been deleted or anergized during initial antigen priming, leading to impaired anti-leukemia activity and immune evasion [14–17]. Better understanding of how leukemia-specific T cells are induced and regulated in AML has potential ramifications for the efficacy of immune therapies for preventing or delaying relapse of the disease.

DCs are key controllers of innate and adaptive immunity (Fig. 1). DCs function as professional antigen-presenting cells (APCs) crucial for eliciting primary T cell responses [18–20]. Based on their surface phenotype, anatomical location and function, DCs at the steady state condition are broadly categorized into conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [21, 22]. Under inflammatory conditions, DCs undergo profound changes in their phenotype and functionality [22–26]. They present antigenic peptide to trigger antigen-specific T cell responses. [18–20]. DCs produce multiple molecules capable of shaping antigen-specific T cell responses. For instance, DCs may be preferentially selected to produce special types of cytokines (e.g., IL-12, IL-23) and Notch ligands (e.g., DLL1 and DLL4), which are known to instruct antigen-activated T cells to become distinct lineages of effector T cells [27–31]. Targeted deletion of a specific subset of DCs or T cells leads to selective impairment of adaptive immunity against the corresponding pathogen(s) and tumor [32–37].

Intriguingly, accumulating evidence indicate that DCs also play non-redundant roles in regulating tolerance (Fig. 1) [18, 32, 38–46]. Under immunosuppressive environments, tolerogenic DCs are prominently induced. They can promote lymphocyte tolerance through T cell deletion, T cell anergy induction, and regulatory T cell (Treg) expansion and function [47–50]. Recent studies have demonstrated that

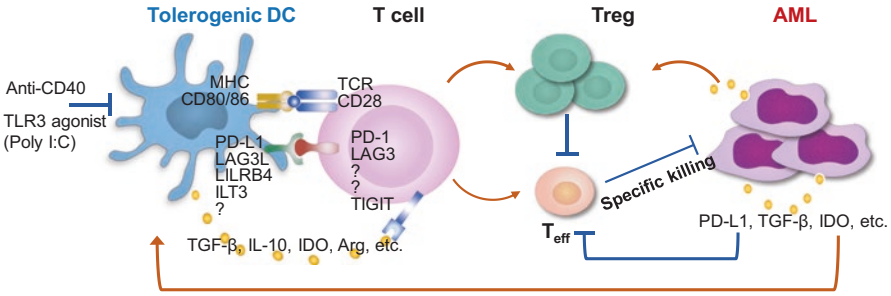

Fig. 1 DC regulation of T cell tolerance against AML. DCs play non-redundant tolerogenic roles in the regulation of T cell tolerance. These DCs produce high levels of PD-L1, ligand for LAG3, LILRB4 and ILT3 to induce abortive proliferation, exhaustion and suppression of activated T cells. In addition, these DCs produce immuno-suppressive molecules (e.g., TGF-β, IL-10, IDO, arginase) to suppress T cell responses directly or indirectly by enhancing Treg expansion and function. In addition, AML cells may induce immune evasion by producing immuo-suppressive molecules, inducing tolerogenic DCs or reducing the generation and function of immunogenic DCs. In vivo administration of anti-CD40 and TLR3 agonist (Poly I:C) can convert tolerogenic DCs to immunogenic DCs

some DC subsets induce antigen-specific T cell tolerance against AML cells and multiple myeloma (MM) as well [14, 15, 51]. In this chapter, we focus on discussing these effects of DCs on mediating immunogenicity and immune tolerance of T cells against AML cells.

2 Induction of Leukemia-Specific T Cell Response and Immune Evasion by AML Cells

Clinical studies suggested that AML patients with longer survival and continuous complete remission (cCR) were usually associated with the presence of leukemia-reactive T cells. For example, stable remission of leukemia after allogeneic hematopoietic stem cell transplantation (allo-HSCT) correlated with the presence of higher proportions of leukemia associated antigen (LAA)-specific T cells. In these studies, the detection of LAA-specific $CD8^+$ T cells predicted a higher chance of long-lasting remission [6, 52, 53]. For instance, WT-1, a gene involved in Wilms tumor and present in more than 70% of AML, had been well-investigated [52]. One study examined the expression of WT1 in 226 peripheral blood and BM samples from patients with AML or myelodysplastic syndrome (MDS) before and after allo-HSCT. Patients with longer survival and cCR after HSCT showed higher and enduring frequencies of WT1-specific $CD8^+$ cytotoxic T cells (CTLs) than patients developing a relapse [6]. CTLs against WT-1 killed AML stem cells and prevented their engraftment in NOD/SCID mice [52, 53]. These observations suggest that WT1-specific $CD8^+$ T cells may contribute to the maintenance of a complete remission in AML/MDS patients.

In the setting of autologous T cell responses against leukemia, $CD8^+$ T cells specific for bona fide LAAs have been identified in the peripheral blood and BM of patients with AML (e.g., WT-1, PRAME, survivin and proteinase 3) [52, 53]. Keiholz and colleagues discovered for the first time spontaneous T cell reactivity against WT1 and proteinase 3 in AML patients [54]. These data therefore support the notion that the immunogenicity of both WT1 and proteinase 3 in AML patients can be potentially used for leukemia vaccines [52–54].

Several studies revealed the generation of functional T cells reactive to leukemia-specific antigens in AML patients. A study of 66 patients with AML shows that high percentage of total T cells in the BM was associated with increased overall survival [55]. Leukemia-reactive Vg9Vd2 T cells specifically recognize and lyse AML blasts [56]. In AML, many different mutations make the production of a vaccine to leukemia-specific antigens difficult, but CTLs against nucleophosmin (NPM1) have been reported [57]. NPM1 may be a preferable target, because of its expression in leukemia stem cells. Mutant NPM1 induces specific $CD4^+$ and $CD8^+$ T cell responses. Mutant NPM1-specific CD8 effector cells, which are characterized by secretion of IFN-γ and granzyme B, possessed potent ability to kill leukemic blasts [57]. Clinical studies suggested that patients who are capable to produce specific CD8 effector T cell responses against NPM1 peptides had better survival [58]. Upon activation of T cell receptor (TCR) signaling, T cells from AML patients showed no proliferation defect in vitro, [16] and retained the capacity to produce inflammatory cytokines (e.g., IL-12, IFN-γ and IL-10) [59].

In contrast to the observations described above, some studies suggested that T cells from AML patients showed aberrant activation phenotype compared to those of healthy donors [60, 61]. T cells derived from AML patients had significantly impaired capability to form functional immune synapses with AML cells [60]. This T-cell dysfunction in AML is thought to contribute to the failure of a host immune response against leukemia cells [60]. Van De Loosdrecht and colleagues investigated the capacity of AML patient-derived T cells to proliferate and differentiate into effector cells upon stimulation with WT1 and PRAME [62]. They found that both WT1 and PRAME failed to drive proliferation and functional differentiation of T cells derived from patients that had recently achieved a complete remission [62]. These data indicate that leukemia-specific T cell responses within AML microenvironment are subverted by immune evasion mechanisms.

Accumulating evidence indicates that leukemia cells may mediate immune evasion by multiple mechanisms. Recent studies suggested that immune suppression molecules derived from AML cells may directly repress leukemia-specific T cell responses [14, 15, 62, 63]. LILRB4, which is a marker of monocytic AML and immunoreceptor tyrosine-based inhibition motif-containing receptor, inhibited T cell responses by creating an immunosuppressive microenvironment [64]. Other studies further suggested that leukemia-specific T cells from AML patients exhibit intrinsic dysfunctional or exhausted [15, 65, 66]. T cells from relapse patients with AML expressed high levels of immune checkpoint inhibitors related to T cell exhaustion, such as PD1, TIM3 and TIGIT. These T cells exhibited functional impairment as evidenced by low production of cytokines and high susceptibility to

apoptosis [16, 65–68]. Blockade of the PD-1/PD-L1 pathway significantly enhanced T cell responses against AML cells in cultures [63]. However, immune checkpoint blockade therapy has limited effect on preventing relapse of AML in patients [13, 15].

3 Regulation of DC Development and Function

DCs are the most potent professional APCs known to elicit primary T cell responses [18–20]. DCs also play non-redundant roles in the regulation of both immunity and tolerance [18, 32, 38–46]. Under steady-state condition, DCs develop from HSPCs through successive steps of lineage commitment and differentiation [21, 22, 32, 34, 69–72]. DCs can also be induced from monocytes (named mo-DCs) [18, 19, 22, 73, 74]. In humans, mo-DCs have been widely used as vaccine adjuvants for the treatment of cancer and chronic infections [22, 73]. The analysis of gene-targeted mice has identified many critical transcription factors (TFs) in DC development, with some of them (e.g., PU.1 and STAT3) influence all DCs and others (e.g., TCF4 (also known as E2–2), ID2, IRF4, IRF8, KLF4) regulate specific subsets [32, 34, 69, 75–77].

DC subset-specifying TFs are required for the generation of functionally different DC subsets [32, 34, 69, 71]. For example, pDCs are characterized by their production of high levels of IFN-α [30, 78, 79]. pDCs are important for antiviral immune responses and autoimmune diseases [21, 30, 80]. Several TFs are known to regulate pDC differentiation, including TCF4, IRF8 and SPIB [75, 76, 81]. Among them, TCF4 is essentially required for induction of pDCs [80, 82]. In contrast, enforced expression of ID2 inhibits pDC development through a mechanism of reducing TCF4 [80–84]. This counteracting effect between TCF4 and ID2 is important for a balanced generation and function of pDCs and cDCs.

cDCs can be further classified into two classes: cDC1 ($CD8\alpha^+/CD103^+D11b^-$) and cDC2 ($CD8\alpha^-CD11b^+$) [32, 34, 85–87]. cDC1 are particularly efficient in cross-presenting exogenous antigens to $CD8^+$ CTLs. BATF3 has a non-redundant role in $CD103^+$ cDC development and partial effect on inducing $CD8\alpha^+$ DCs in secondary lymph organs [33, 34, 88]. Loss of IRF8 leads to impaired production of spleen-resident $CD8\alpha^+$ cDCs and nonlymphoid tissue $CD103^+$ cDCs [32, 34, 35]. Functional analysis shows that BATF3 is crucial for cDC1-mediated anti-tumor activity, whereas IRF8 is also important for $CD8^+$ cDC maturation and IL-12 production that regulates both TH1 and CTL responses [32, 33, 35]. TFs (e.g., IRF4, KLF4 and NOTCH2) are important to regulate cDC2 differentiation and survival [86, 89]. Among them, IRF4 is required for cDC2 to prime $CD4^+$ T cells in both lung and intestine [90, 91].

DCs express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nod-like receptors (NLRs) to respond to pathogen-associated molecular patterns (PAMPs) [92–94]. In addition, DCs are also capable of detecting certain intracellular molecules, called damage-associated molecular patterns (DAMPs),

that are released from cells stressed, damaged and/or dying in the local tissue [95]. When PAMPs or DAMPs are present, DCs are stimulated to migrate to lymphoid tissues and present both antigen and costimulatory molecules to T cells [95–97] Both PAMPs and DAMPs activate DCs through stimulating TLRs (i.e., TLR1–13) [98–102]. TLR expression among DC subsets is heterogeneous: pDC mainly express TLR1, 7 and 9; CD8α$^+$ DCs preferentially produce high levels of TLR3; whereas other cDC subsets express certain TLR subtypes but TLR9 [95–97] [103–109]. Data from our studies and others suggested that Notch ligands DLL1 and DLL4 played non-redundant roles in activating Notch signaling to drive alloreactive T cell responses [110–114]. LPS (TLR4 agonist) rapidly induces Dll4 expression in human and murine DCs [105–107, 111, 112]. Combined stimulation of human DCs with LPS with TLR7 agonist R848 further increases the expression of DLL4 [107, 111]. TLR3 is critical for presentation of viral double-stranded RNA [107, 115].

Recent studies have begun to illuminate the tolerogenic role of DCs in mediating T cell dysfunction or exhaustion in acute leukemias (Fig. 1). Kline and colleagues investigated the immune evasion mechanisms using a murine AML model. They found that AML induced abortive proliferation of CD8$^+$ T cells and their subsequent deletion. This effect is mediated, at least in part, by CD8α$^+$ DC-mediated immune tolerance [14]. Systemic activation of CD8α$^+$ DCs using TLR3 agonist Poly(I:C) dramatically improved the development of leukemia-specific T cells in vivo, leading to improved overall survival of leukemia mice [14]. Administration of agonistic anti-CD40 Ab to activate host APCs, including DCs, reversed leukemia-specific T cell tolerance in vivo [17]. Interestingly, other studies discovered that IL-10-secreting MHCII^{low} cDCs in the BM were able to promote myeloma progression [116]. Collectively, these observations suggest that targeting DCs may represent an effective strategy to prevent malignant relapse.

4 DC Differentiation and Function during AML Progression

Many studies suggested that hematopoietic malignancies impaired DC development and function (Table 1). Mohty's study of 37 AML patients showed a dramatic change in circuiting blood myeloid DCs (mDCs, also called cDCs) and pDCs. They found that 59% of patients had approximately 45-fold and 35-fold expansion in frequency of mDCs and pDCs, respectively, than healthy donors [117]. Interestingly, they also found the lack of both mDCs and pDCs in the peripheral blood of about 11% AML patients [117]. Laurent et al. has shown that CTLA-4 is constitutively expressed on the surface of AML blasts in patients at the time of diagnosis and in patients with disease resistant to chemotherapy [118]. AML cell-derived CTLA-4 were able to kill DCs through interacting with CD80 and CD86 [118]. Given the non-redundant role of DCs in the induction of T cell immunity and tolerance, altered quantity and quality of mDCs and pDCs may result in significant impact on immune evasion and exhaustion of leukemic-specific T cells.

Table 1 Strategies to activate DCs in experimental mice of AML and MM

Models	Method	Outcome	Mechanism	Reference
C1498 AML mice	Administration of agnostic anti-CD40 Ab	Enhancing accumulation of functional T cells; Prolonging animal survival	Targeting total DCs to abrogate T cell tolerance	Kline et al. [17]
C1498 AML mice	Administration of TLR3 agonist	Inducing potent anti-leukemia T cell immunity; prolonging animal survival	Activating $CD8a^+$ DCs	Kline et al. [14, 15]
Vk∗MYC MM mice	Administration of CST-1R antibody	Increasing T-cell activation; improved animal survival	Reducing production of $MHCII^{low}$ cDCs	Minnie, et al. [116]

In addition to the impact of AML cells on DC development from HSPCs, leukemic cells themselves are found to produce immature DC-like cells that were immunosuppressive. A FLT3-internal tandem duplication (ITD) mutation, which occurs in approximately 30% of AML patients, imparted a particularly poor prognosis [119]. Patients with FLT3-ITD AML often presented with more aggressive disease and had a significantly higher propensity for relapse after remission. Many studies have demonstrated that FLT3 ligand (FLT3L) induced the generation of DCs with tolerogenic effects [120]. Notably, in $FLT3\text{-}ITD^+$ AML patients, both cDCs and pDCs failed to upregulate HLA-DR and produced low levels of functional cytokines (e.g., IFN-α, TNF-α, and IL-1α) upon stimulation by CD40L and CpG [121]. Other studies suggested that $FLT3\text{-}ITD^+$ AML minimal residual disease acted directly as dysfunctional APCs or indirectly by production of factors that induced myeloid-derived suppressor cells (MDSCs), promoting immune evasion [122]. In addition, experimental studies indicated that Flt3-ITD-induced DCs promoted the expansion of Tregs while inhibiting antigen-driven T cell responses [123]. Collectively, these data suggest that effect of FLT3-ITD may not only promote proliferation of leukemia blasts, but also create a DC-mediated tolerogenic environment and impair immune surveillance by T cells.

Intriguingly, several studies suggested that leukemia-derived dendritic cells ($DC\text{-}_{leu}$) may be potentially immunogenic in the context of priming T cell responses (Fig. 2) [124–126]. Myeloid leukemia cells from AML patients can be induced to differentiate into $DC\text{-}_{leu}$ [124–126]. With the method culturing with combinations of GM-CSF, IL-4, and TNF-α, these $DC\text{-}_{leu}$ regained the stimulatory capacity of professional DCs while presenting the known/unknown leukemic antigen repertoire. After culture, blasts from AML patients exhibited morphological and immunophenotypic features of immature DCs, including expression of MHC II, CD1a, CD83, and CD86, and were potent stimulators in an allogeneic mixed lymphocyte reaction (MLR) [126]. These $DC\text{-}_{leu}$ matured upon activation by ligation of CD40 and differentiated into cells that fulfilled the phenotypic criteria of DCs comparable to their normal counterparts [127]. They were also effective stimulators in the autologous MLR, and exhibited autologous, antileukemic cytotoxicity by inducing IFN-γ-producing T help (TH)1 cells and CTLs [128, 129]. However, the capacity of

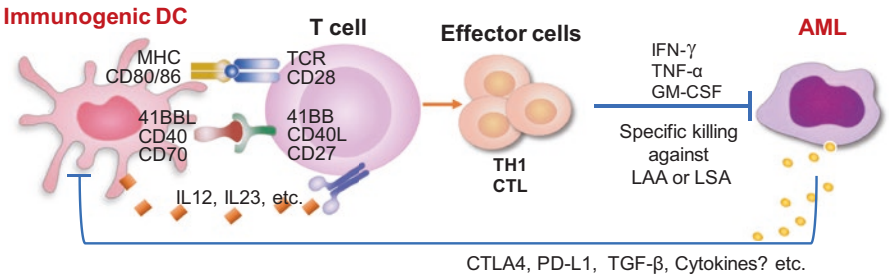

Fig. 2 DC-regulated T cell immunity against AML. Immunogenic DCs are key controlled of adaptive immunity against AML cells. They function as professional APCs to elicit primary T cell responses by presenting leukemia-associated antigens (LAA, e.g., WT-1, PR1, Survivin, PRAME) or leukemia-specific antigens (LSA, e.g., NPM1, PML-RARA, BCL-ABL) to activate naïve T cells and providing costimulatory signals (e.g., CD80/86, 41BBL and CD70) to facilitate antigen-driven T cell proliferation, survival and expansion. Upon differentiation into effector T cells that produce effector cytokines and cytolytic molecules, leukemia-reactive T cells kill AML

leukemic cells to differentiate into effective DCs highly varies among individuals. $CD34^+CD38^-$ or $CD14^+$ less immature leukemia precursors have the capacity to differentiate into DCs [130, 131]. $Flt3\text{-}ITD^+$ AML cells were negatively correlated to good DC differentiation by suppressing expression of C/EBPa and PU.1 [132].

5 Tolerogenic Role of DCs in T Cell Response Against AML

Immune checkpoint blockade therapy has been successful in treating some types of solid tumors but has not shown clinical benefits for treating leukemia [15]. It appears that leukemia may evade the immune checkpoint blockade therapy via a mechanism different from that found in solid tumors. This is supported by recent observations that AML cells produced high levels of immune suppressive molecules that are unique to leukemic cells [64]. For example, in mice with AML, blockade of LILRB4 led to $CD8^+$ T cell-dependent elimination of AML cells, leading to significantly improved overall survival of leukemia mice [64]. In addition, AML cells expressed high levels of CD47 that prevents APCs from phagocytosis of leukemia cells to process and present leukemia cell antigens [15]. In this chapter, we will focus on discussing the role of DCs in suppressing T cell immunity against leukemia.

The activation status of DCs determines their capacity to mediate T cell tolerance or immune responses against leukemia cells. In the setting of syngeneic murine leukemia model, Kline and colleagues demonstrate that antigens from circulating leukemia cells are primarily captured and cross-presented by splenic $CD8\alpha^+$ DCs [14]. Interestingly, the frequency of activated leukemia-specific $CD8^+$ T cells in mice with systemic C1498 leukemia was significantly lower than those observed in mice with localized C1498 solid tumors. Further analysis revealed that C1498 solid tumor-induced $CD8^+$ T cell responses are dependent on Baft3-lineage DCs

(i.e., CD8α$^+$ DCs and CD103$^+$ migratory DCs) [14]. However, in leukemia-challenged mice, Batf3-lineage DCs induced tolerance of CD8 T cells, leading to reduced activation and proliferation of these T cells in response to antigen priming and impaired anti-leukemia activity [14].

Tolerogenic effects of CD8α$^+$ DCs have been observed in animals undergoing allo-HSCT [43, 133]. In these studies, FLT3L treatment induced expansion of CD8α$^+$ DCs that were poor stimulators of allogeneic T cells in cultures and had great ability to suppress donor T cell responses to host antigens in vivo [43, 134]. Notably, this tolerogenic effect of CD8α$^+$ DCs could be reversed by systemic administration of TLR3 agonist Poly(I:C) [14, 15]. These findings may explain observations from clinical observations showing that in AML patients, local vaccination with leukemia antigen-pulsed DCs enhances generation of leukemia-reactive T cells and improves the overall survival of AML patients post remission (to be discussed below) [7, 9, 10, 135].

Recent studies have identified other types of DCs with immune suppression effects. For example, there was a significant expansion of MHCII^{low}CD11c^{hi}CD11b^{hi} DCs in the BM compartment of mice with relapsed multiple myeloma (MM) compared with MM controlled and MM-free mice [116]. These MHCII^{low} cDCs produced high levels of immune suppressive cytokine IL-10 and were associated with phenotypically and functionally exhausted MM cell-reactive CD8 T cells [116]. Notably, administration of CSF-1R antibody that blocks cognate cytokine signaling, prevented accumulation of MHCII^{low} cDCs and tumor-associated macrophage accumulation in the BM of MM mice, leading to increased T-cell activation [116]. Thus, MM relapse promotes IL-10 secretion by MHCII^{low} cDCs that accumulate in the BM during disease progression [116]. Other studies have found a subset of tolerogenic human DCs [136, 137]. They are characterized by high cell surface expression of the inhibitory receptor ILT3. Both ILT3-positive tolerogenic DCs and soluble ILT3 induce CD4 T cell anergy and generation of CD8 T suppressor cells [136, 137]. Recombinant ILT3-Fc protein has important immunotherapeutic potential acting directly on activated T cells and promoting the induction of immunological tolerance [136, 137].

6 DC-Based Vaccination to Prevent Relapse of AML

Through an immune-mediated graft-versus-leukemia (GVL) effect, allo-HSCT induces durable beneficial effects for many patients with hematologic malignancies [6, 8, 99]. Use of donor lymphocyte infusion (DLI) in a large cohort of 399 AML patients was associated with 21% overall survival at 2 years, compared with 9% for patients not receiving DLI, [6] underscoring the crucial role of donor lymphocytes in mediating potent anti-tumor effects. However, patients with high-risk AML often relapse, indicating the need of augmented tumor immunity in these patients. Ritz and colleagues conducted a Phase I clinical trial in which high-risk AML or myelodysplasia (MDS) patients were immunized with irradiated, autologous, GM-CSF-secreting

tumor cells early after allo-HSCT [7]. This vaccination elicited local and systemic reactions, which were qualitatively similar to those previously observed in nontransplanted, immunized solid-tumor patients, despite administration of a calcineurin inhibitor as prophylaxis against GVHD. As a result, 9 of 10 patients who completed vaccination achieved durable complete remission, with a median follow-up of 26 months [7]. Thus, combined leukemia cell vaccines and allo-HSCT may potentiate GVL effects in patients with myeloid malignancies.

Other studies revealed that broad anti-tumor responses can be induced by vaccination of autologous DCs fused with patient-derived AML cells (Table 2) [9, 10, 124, 135, 138–140]. A cohort of 17 AML patients, who achieved remission after chemotherapy, were serially vaccinated with AML cell-fused DCs. Vaccination induced a marked increase in circulating T cells recognizing whole AML cells and leukemia-specific antigens, which persisted for more than 6 months. A medium follow-up of 57 months showed that 12 of 17 (71%) vaccinated patients remained alive without recurrence [10]. Schmetzer and colleagues showed that DCs can be generated from blood samples of AML patients using methods containing different mixtures of immune-modulatory factors, including GM-CSF, IL-4, TNF-α, FLT3-L, IL-6 and PGE2 [124]. Both cytolytic and IFN-γ-producing responses to autologous myeloma were generated in 6 of 7 patients after stimulation ex vivo with DCs that had processed autologous tumor cells [138]. Experimental studies further revealed that AML patients treated with DC-$_{leu}$ induced potent T cell responses, leading to significantly reduced amount of proliferative leukemia blasts [135, 139]. Collectively, these studies provide proof-of-concept that personalized vaccination of patients with AML cell-fused DCs in remission induces the expansion of leukemia-specific T cells and may be protective against disease relapse.

A key challenge to the development of an effective AML vaccine is the selection of appropriate leukemia-reactive antigens to load on DCs (Fig. 2). Several LAAs have been identified in patients with AML, such as WT-1, Survivin, PRAME and Proteinase 3. These LAAs have been shown to elicit $CD8^+$ T cell responses that are able to eliminate AML cells [140]. A recent study investigated the vaccination effect

Table 2 Vaccination induced clinical response AML patient

Vaccine	Clinical response case (all patients)	Mechanism	Reference
Autologous GM-CSL-secreting AML cells	9(10)	Local and systemic reactions	Ho et al. [7]
DCs fused with autologous AML cells	12(17)	Expansion of leukemia-specific T cells	Rosenblatt et al. [10]
DCs fused with autologous AML cells	6(7)	Cytolytic and IFN-γ-producing responses	Dhodapkar et al. [129]
DCs derived from leukemia cells	4(5)	increase of WT1-specific CTL	Roddie et al. [135] Houtenbos et al. [139]
DCs pulsed by WT1 mRNA	13 (30)	increased WT1-specific $CD8^+$ T cells	Anguille et al. [9]

of DCs electroporated with WT1 messenger RNA as post-remission treatment [9]. There was a demonstrable anti-leukemia response in 13 of 30 patients with AML at high risk of relapse. Five-year overall survival was higher in responders than in nonresponders (54% versus 25%). Long-term clinical response was correlated with increased circulating frequencies of polypeptide WT1-specific $CD8^+$ T cells [9]. Long-term overall survival was correlated with IFN-γ^+ and TNF-α^+ WT1-specific responses in delayed-type hypersensitivity-infiltrating $CD8^+$ T cells [9]. Thus, vaccination of patients with AML with WT1 mRNA-electroporated DCs can be an effective strategy to prevent or delay relapse after standard chemotherapy.

7 Conclusion and Future Perspectives

Despite the significant progress made in defining the pathways responsible for treatment resistance, patients with refractory/relapsed AML and advanced MDS remain at high risk for eventual disease progression [1, 2, 5, 141]. Data from both clinical and pre-clinical studies indicate that therapeutic strategies that augment tumor immunity might improve the efficacy of treating AML and MDS. Among these, DC-based cancer vaccines may represent one promising approach. Improving the immune-stimulating effect of DCs might enhance leukemia-specific T cell expansion and function and promote the immune response toward leukemia cells.

Emerging evidence indicates that leukemia-specific T cell responses can be subverted by immune evasion mechanisms active within AML. Besides the direct effect of leukemia cells on repressing antigen-specific T cell responses, AML cells may induce immune tolerance by targeting DCs. AML microenvironment favors the generation of tolerogenic DCs, including immature $CD8\alpha^+$ DCs and IL-10-secreting $MHCII^{low}$ cDCs [14, 142]. Furthermore, $DC\text{-}_{leu}$ derived from $FLT3\text{-}ITD^+$ AML cells themselves might be highly tolerogenic [120]. To this regarding, further investigations are needed to define the mechanisms that regulate the development and function of DCs during leukemia progression. Identifying the molecular mechanisms that are responsible for the generation of tolerogenic DCs may lead to new strategies to improve the efficacy of immune therapy for treatment-refractory AML.

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Perspectives on the Role of T Cell Negative Immune Checkpoint Receptors in Health and Disease

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Abstract Immune inhibition orchestrated by multiple negative checkpoint receptors (NCRs) is required to control aberrant immune responses and maintain homeostasis. An imbalance between immune activation and inhibition is believed to be an important mechanism driving the development of a number of diseases. Several tumors hijack this delicate balance and are able to increase the expression of various inhibitory receptors on cytotoxic T cells (CTLs) resulting in ineffective anti-tumor CTL responses. T cell immunotherapies that blocks NCR signaling have shown remarkable clinical success and extend median progression-free survival as well as overall survival in a wide range of cancer settings. In chronic infections such as HIV and Hepatitis B and C, CTLs have been found to overexpress various NCRs resulting in an "exhausted" CTL phenotype, which is associated with disease progression and the development of co-morbidities. On the other hand, downregulation of NCRs is thought to lead to autoimmune diseases such as multiple sclerosis and other neuroinflammatory conditions. In this chapter, we primarily focus on advances in our current understanding of NCRs and the role they play in human health and disease as well as the ongoing efforts to develop novel immunotherapies that target these receptors and reverse immune perturbations impacted by NCR dysregulation.

Keywords Negative checkpoint receptors · Immune regulation · Cancer Neuroinflammation · Infectious disease · Immunotherapy

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1 Introduction to Negative Immune Checkpoint Receptors

The human immune system employs a number of mechanisms for maintaining balance between immune activation and suppression. Infection, cancers, and injury trigger inflammatory processes that need to be "checked" in order to avoid aberrant responses. This complex process involves multiple cellular components that must work in concert to achieve an appropriate level of inflammation. Under homeostatic conditions, immune cells are designed so that they are not constitutively active which could lead to damage of healthy tissues or disease. One of the ways in which the immune system moderates this balance is through the binding of ligand on antigen presenting cells (APCs) to corresponding negative immune checkpoint receptors (NCRs) on T cells [1, 2]. Specifically, these immune checkpoints are proteins that act as T cell receptor (TCR) co-signaling partners that deliver either positive or negative signals to T lymphocytes. Ligand binding results in down regulation of T cell activation and suppression of effector functions. However, in many disease states, this system is hijacked, consequently leading to T cell dysfunction and ineffective responses. Chronic infection and cancers have been shown to do just that.

Much effort has been made towards better understanding these NCRs and the role they play in health and disease. Moreover, identifying ways in which these NCRs can be blocked to restore effector function and better position T cells to fight cancerous tumors and infections has been a major focus of research. Harnessing this system in order to enhance T cell function has been groundbreaking for the field of immunology, so much so that the 2018 Nobel prize in physiology and medicine went to the two men who discovered these important immune molecules, James Allison and Tasuku Honjo. The idea of mobilizing our own immune systems to fight disease is not a new idea. However, the discovery of these immune modulatory receptors has made this tantalizing possibility feasible as a promising clinical treatment. Among the most prominent immune checkpoint receptor-ligand combinations are CTLA-4/CD80, PD-1/PD-L1, TIGIT/PVR and Tim-3/Galectin 9. In this chapter, we discuss the role of NCRs in health and disease, particularly in cancer, infection and neuroinflammatory conditions, as well as attempt to highlight advances being made in the field of immunotherapy.

2 Negative Immune Checkpoint Receptors in Cancer

Under normal homeostatic conditions, immune checkpoint receptors are critical for self-tolerance. In various cancer settings, however, these proteins are overexpressed allowing tumor cells to evade the immune response [3–5]. Blocking these receptor-ligand interactions is able to produce robust anti-tumor responses in some patients. Although several mechanisms have been proposed as to how NCR blockade leads to sustained clinical benefit, the main mechanism is thought to be by reinvigoration of cytotoxic $CD8^+$ T cells (CTLs). CTLs require co-stimulation by ligation of

co-stimulatory receptors expressed on T cells and co-stimulatory ligand on antigen presenting cells (APC) [6]. CD28 is one of the most studied co-stimulatory receptors that binds to CD80/CD86. Cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is expressed on both regulatory T cells (Tregs) and activated T cells, binds to CD80/CD86 with higher affinity than CD28 and induces trans-endocytosis of the ligands which competitively limits the availability of CD80/86 to CD28 [7, 8]. Consequently, CTLA-4 is able to limit the activation of CTLs and prevent autoimmunity and tissue damage due to hyper-inflammatory responses. However, in cancer settings, overexpression of CTLA-4 on CTLs in the tumor microenvironment limits their anti-tumor effector function and allows for immune evasion. Inhibition of CTLA-4 is thought to work through interruption of the CTLA-4-CD80/CD86 axis, which clears the path for CD28-CD80/CD86 binding and subsequent CTL activation Table 1.

Programmed cell death-1 (PD-1) is a member of the CD28 superfamily. It delivers negative signals upon interaction with its two ligands, PD-L1 and PD-L2 [9]. Known to be important in peripheral tolerance and protection from autoimmune attacks, PD-1 and its ligands are also responsible for attenuated immunity to infection and cancers. Furthermore, PD-1 has been shown to facilitate chronic infection and tumor progression. Expression of programmed PD-1 on CTLs is enhanced by TCR stimulation and cytokine stimulation including common-gamma chain cytokines and inhibition of PD-1 occurs via multiple costimulatory pathways including ZAP70, PI3K and RAS. PD-1 can also activate basic zipper transcriptional factor ATF-like (BATF), which further interrupts T cell activation [10]. In the tumor microenvironment, both PD-1 and PD-L1 expression on CTLs and tumor cells are elevated due to chronic antigen and inflammatory cytokine stimulation [5]. Furthermore, the promotor region of PD-1 is often hypomethylated, resulting in further upregulation of PD-1 expression [11]. Ligation of PD-1 by PD-L1 attenuates TCR and co-stimulatory signals resulting in increased apoptosis, reduced cytokine production and cellular proliferation, as well as decreased tumor killing activity of CTLs. PD-1 blockade is able to reinvigorate functionally “exhausted” CTLs and reverse their dysfunctional state leading to enhanced effector function. PD-1 is expressed on many cell types and its expression on melanoma cells has been shown to promote tumor growth via activation of mTOR signaling [12]. On tumor-associated macrophages, PD-1 expression is negatively correlated with phagocytic activity against tumor cells *in vivo*. PD-1/PD-L1 blockade enhances phagocytic activity and extends median survival in mouse models of cancer [13].

Since the introduction of Ipilimumab, a monoclonal antibody (mAb) that targets the CTLA-4 receptor and the first NCR inhibitor approved for clinical use, the field of cancer immunotherapy has experienced an unprecedented expansion and vastly improved cancer treatment options [14, 15]. Clinical trials using inhibitors targeting PD-1 and its cognate ligand, PD-L1, for treatment of melanoma as well as other malignancies have experienced marked success [16–22]. The number of NCR inhibitors that have successfully attained FDA approval for clinical use has grown substantially in recent years. Moreover, several of these immune checkpoint inhibitors have shown ever-growing for a wide variety of cancer types including non-small

Table 1 Summary of role of negative checkpoint receptors in various disease contexts

Negative checkpoint receptors			
Disease context	Key points	Current research/Clinical trials	Further reading
Cancer	NCR expression on tumor cells is increased in various cancers leading to immune evasion and immune cell dysfunction Ipilimumab was the first clinically approved NCR inhibitor for the treatment of metastatic melanoma (2011) PD-1 and PD-L1 inhibitors have also since been FDA-approved Progression-free survival and overall survival is greatly improved with use of NCR inhibitors but is not effective in all patients Autoimmune-like side effects are common Advances in precision medicine will likely help to improve treatment efficacy	Combination strategies targeting multiple NCRs as well as co-stimulatory molecules may be more effective than single blockade strategies for restoring immune function	Pardoll DM et al. *Cancer*. 2012 Le Mercier I et al. *Front. Immunol*. 2015 Baumeister SH et al. *Annu Rev Immunol*. 2016 Goodman A et al. *Nat. Rev. Clin. Onc*. 2016 Ni L et al. Immunol Rev. 2017 Clouthier D et al. *Science* 2017 Ribas A *et al. Science*. 2018 Patel SA et al. *Immunity*. 2018
Infection	Chronic antigen-driven immune activation and inflammation increases NCR expression on CTLs Exhausted CTL phenotype are observed in patients with chronic infection(s) Multiple NCR expression is often observed and correlates with disease progression and/or severity	Non-human primate models of infection have demonstrated promise of combination strategies	Marraco S et al. *Front. Immunol*. 2015 Chew G et al. *PLOS Pathogens*. 2016 Wykes M et al. *Nat. Rev. Immunol*. 2017
Neuroinflammation	Role of NCRs in various neuroinflmmatory diseases have been challenging to study In certain neuroinflammatory settings, NCR blockade can result in more severe neuropathy presumably due to aberrant T cell activation	NCR expression as a marker of disease progression is being studied Effect of PD-1 blockade on HIV reservoirs in the CNS currently in clinical trials	Marban C et al. *Front. Immunol*. 2016 Cuzzubbo S et al. *Eur J Cancer*. 2017

cell lung cancer, urothelial carcinoma, Hodgkin lymphoma, head and neck squamous cell carcinoma, and most recently cervical cancer.

The ability of NCR-targeted immunotherapies to extend median progression-free survival as well as overall survival in cancer patients has been remarkable [17–22]. Randomized clinical trials with treatment-naïve patients with metastatic melanoma demonstrated superior efficacy of PD-1 blockade by pembrolizumab, a first-line treatment for cancers that overexpress PD-L1, over cytotoxic dacarbazine chemotherapy with a response rate of 27% vs 10% and median overall survival of 37.5 vs 11.2 months [23, 24]. Unfortunately, responsiveness to NCR inhibitors can vary quite substantially between individuals. While complete response, defined as the disappearance of all signs of cancer after treatment, has been observed in some patients, majority of individuals exhibit only partial response. The complicated nature and relationship between the cancer microenvironment and negative checkpoint receptors are believed to be responsible, at least in part, for the observed variation.

Despite the success of clinical trials using single NCR blockade, inhibition of one NCR alone may not be sufficient enough to reinvigorate all functionally exhausted T cells. One strategy for enhancing therapeutic efficacy of NCR-targeted therapy is combinatory blockade. In a clinical trial comparing the combination of CTLA-4 and PD-1 blockade against CTLA-4 single blockade in melanoma patients, combinatory therapy exhibited a higher response rate (58% vs 19%), median progression free survival (11.5 vs 6.9 months) and 4-year survival rate (37% vs 9%) [25]. There was no statistical significance between CTLA-4/PD-1 combination and PD-1 single blockade, but descriptive analyses showed a trend toward superior outcomes. Unfortunately, treatment related side effects have been known to increase in combinatory therapy and must be taken into consideration when initiating these therapies.

CTLA-4 and PD-1, which have served as vanguard molecules in many early immunotherapy studies, are just two molecules on a growing list of NCRs that have been found to have immunomodulatory activity [3–5, 26–28]. Current research is now focused on exploring blockade of other NCRs either alone or in combination with PD-1 and/or CTLA-4 [29, 30].

3 Immunotheapeutic Targets in Preclinical Development and in Ongoing Clinical Trials

Lymphocyte Activation Gene 3 (LAG-3) is expressed on activated $CD4^+$ and $CD8^+$ T cells and polarizes $CD4^+$ T cells to a regulatory phenotype [26, 31, 32]. LAG-3 inhibits proliferation and cytokine production of effector T cells, and it is overexpressed on tumor-infiltrating CTLs [33]. Although the effect of anti-LAG-3 monotherapy has been found to be limited, dual blockade with PD-1 shows synergistic effects that results in better survival and complete response rates than either

monotherapy in mouse models of B16 melanoma and MC38 colorectal cancer [34]. The efficacy of LAG-3 blockade also has been assessed in mouse models in the context of ovarian cancer, lymphoma, and multiple myeloma [35]. Its signaling pathway is still largely unknown but it is thought to be different from that of PD-1 due to the synergetic effects observed in PD-1/LAG-3 dual-blockade [36]. LAG-3 is highly expressed on Tregs and is correlated with IL-10 production. Structurally, LAG-3 resembles CD4 and consequently binds to MHC class II with higher affinity, thus inhibiting $CD4^+$ T cell priming by dendritic cells (DCs) via competitive inhibition. Galectin-3 and LSECtin, which is highly expressed on tumor cells, are also binding partners for LAG-3 and are implicated in the disruption of effective antitumor responses [37, 38]. Several phase 1–2 clinical trials using dual blockade are currently ongoing.

B and T Lymphocyte Attenuator (BTLA), also known as CD272, shares functional similarity to PD-1 and CTLA-4 and is often co-expressed with PD-1 on tumor infiltrating CTLs [39]. Mainly expressed on B, T and mature lymphocytes, BTLA binds specifically to herpes virus entry mediator (HVEM) and inhibits T cell activation and cytokine production [40]. BTLA is downregulated with progressive differentiation of CTLs, but cancer-specific cells have been observed to maintain high levels of BTLA expression. BTLA has also been found to be a marker of a less cytotoxic T cell subset in diffuse large B-cell lymphoma [41]. Reversal of BTLA signaling is achievable *ex vivo*, which restores CTL function. Currently, no clinical trials are ongoing with regard to this molecule, but interest remains.

V-domain Immunoglobulin-containing Suppressor of T-cell Activation (VISTA) is unique due to its dual role as a receptor on T cells and a ligand on APCs [42, 43]. The highest levels of VISTA expression are found in myeloid cells. Within the CD4 compartment, VISTA expression is highest on naïve and $Foxp3^+$ Tregs [44], but is also detected at lower levels on $CD8^+$ T cells and NK cells. Molecularly, it is very similar to PD-1 but appears to have non-overlapping function. Current data shows that antagonist anti-VISTA antibodies serve to increase effector function of tumor-reactive T cells within the tumor microenvironment, as well as decrease the presence of monocytic myeloid-derived suppressor cells. Furthermore, VISTA blockade has also been shown to reduce the emergence of tumor-specific $Foxp3^+$ Tregs [44, 45]. Whether or not VISTA blockade enables expansion of the T cell repertoire, decreased exhaustion or T cell reinvigoration remains unclear. In fact, increased effector function may be an indirect consequence of VISTA blocking on myeloid cells. If so, cancers characterized by high infiltration of myeloid-derived suppressor cells may be particularly responsive to VISTA-targeted therapies. There are currently two phase 1 clinical trials that are ongoing (NCT02671955, NCT02812875).

T cell Immunoreceptor with Ig and ITIM Domains (TIGIT) is expressed on NK cells, Tregs, and $CD4^+$ and $CD8^+$ T cells, particularly activated, memory and follicular helper T cells. TIGIT is a negative regulator of the CD226-CD112/CD155 axis. CD226, which is expressed on NK cells, monocytes and activated T cells, induces activation upon ligation with CD112 or CD155, which are expressed on APCs. TIGIT binds to CD155 and CD112 with higher affinity to competitively inhibit CD226 ligation. TIGIT is highly expressed on CTLs and NK cells in the

tumor microenvironment and inhibits cytokine production and cytotoxic activity [46–48]. Blockade with anti-TIGIT mAb has been shown to partially restore normal immune function to $CD4^+$ and $CD8^+$ T cells. TIGIT signaling in Tregs has been found to stabilize the expression of signature Treg genes and promote Treg function. When TIGIT binds to CD155 on DCs IL-12 production decreases and IL-10 production increases [46–48]. TIGIT blockade could disrupt the immunosuppressive activity of Tregs and DCs, thus indirectly reinvigorating CTLs and NK. Currently, two phase 1 (NCT03119428, NCT03628677) and one phase 2 (NCT03563716) study targeting TIGIT in a melanoma model are ongoing.

T cell Immunoglobulin and Mucin-containing Domain 3 (Tim-3) is expressed on a wide variety of immune cells and binds to multiple ligands including galectin-9, phosphatidylserine, HMGB-1 and CECAM-1. Tim-3 has multifactorial roles depending on cell type and context, but in general works as an inhibitory regulator of T cell function [26]. Tim-3 is expressed on terminally exhausted T cells and $CD8^+$ T cells expressing Tim-3 have been found to accumulates in tumors. Tim-3^+ $CD8^+$ T cells have been found to secrete a lesser variety of cytokines and in reduced amounts as compared to Tim-3^- $CD8^+$ T cells. Interestingly, Tim-3 upregulation during anti-PD-1 therapy is reported to be a mechanism of adaptive resistance to therapy both in mice and humans. Consequently, dual-blockade of PD-1 and Tim-3 successfully improves survival length in a mouse model of lung cancer [49]. Tim-3 is also expressed on Tregs. Tumor-infiltrating Tregs highly express Tim-3 and exhibit superior suppressive function compared to Tim-3^- Tregs. Blockade of Tim-3 in combination with PD-L1 has been shown to markedly reduce the suppressive function of Tregs [50]. A recent study showed that Tim-3 blockade in combination with paclitaxel, a chemotherapy drug, enhanced production of chemokine CXCL9 from $CD103^+$ conventional DCs and enhanced $CD8^+$ T cell response, which ultimately slowed the progression of cancer in a murine breast cancer model [51]. In contrast, monotherapy with anti-Tim-3 has been found to be suboptimal [50, 52]. NK cells also express high level of Tim-3 and can be used as a marker of cytokine producing and cytotoxic NK cells. However, cross-linking of Tim-3 by antibody suppresses NK cell-mediated cytotoxicity [53]. Accordingly, NK cell dysfunction in melanoma patients is reversed by blockade of Tim-3 [54]. Monocytes, macrophages, and dendritic cells express Tim-3 and have been shown to exhibit both immune-enhancing and inhibitory functions depending on the disease context and ligand binding partner [51, 55, 56]. Tim-3 is known to interrupt toll-like receptor (TLR) 2/4 signaling via NF-κB inhibition on these cells [57].

Tim-3 blockade has shown promising results in multiple preclinical studies and there are several phase 1 studies are currently underway (NCT03489343, NCT02817633, NCT03099109, NCT02608268, NCT03652077, NCT03066648, NCT030680508, NCT03311412, NCT03744468, NCT03708328, NCT03446040) [58]. Most of these studies are focused on dual PD-1/Tim-3-targeted therapies. Since the expression of Tim-3 on T cell is mostly limited to terminally exhausted states, as opposed to PD-1 and CTLA-4, which are expressed on all activated T cells, the side effect of anti-Tim-3 therapy is expected to be less severe than anti-PD-1 and anti-CTLA-4 therapies.

3.1 Checkpoint Blockade in Combination with Other Immunomodulatory Agents

The targeting of positive immune checkpoint receptors in combination with NCR blockade has also been an appealing therapeutic strategy that may be able to overcome the immunosuppressive tumor microenvironment. Among these receptors is the tumor necrosis factor receptor superfamily (TNFRSF), which includes OX-40, 4-1BB, ICOS, GITR and CD40. These receptors all serve as co-stimulatory molecules for T cell activation. Some are well studied and agonistic antibodies against these molecules have been tested in multiple clinical trials. One of these studies highlighted the importance of timing in the administration of anti-PD-1 and anti-OX40 mAbs [59]. Concurrent administration of the two antibodies was expected to enhance the efficacy of PD-1 blockade by providing costimulatory signals via OX-40. However, no synergetic effect was observed. Instead, increased apoptosis of CTLs occurred. Interestingly, sequential administration of anti-OX40 followed by PD-1 blockade showed increased cell proliferation, reduced cell death and decreased expression of other NCRs on CTLs. Because NCR-mediated immune suppression is not the only mechanism by which the immune system controls for aberrant responses, combining NCR blockade together with other co-stimulatory agents may better enhance immune function.

Future Directions Immunotherapies focused on NCR blockade have shown remarkable potential in the cancer arena, though complete response to PD-1/PD-L1 blockade has been limited to a subset of patients receiving immunotherapy [60, 61]. Designing combination NCR blockades that include different mechanisms of action may improve efficacy by disrupting the immunosuppressive tumor microenvironment while boosting immune cell functioning. Furthermore, combinatorial strategies may be able to improve response rates while also curbing undesirable side effects.

4 Negative Immune Checkpoint Receptors in Infectious Diseases

Chronic antigen-driven immune activation and inflammation are known to expand NCRs on CTLs. Just as we see in many cancers, these exhausted CTLs are enriched in patients with chronic infectious diseases including viral, bacterial and parasitic infections. Persistent antigen exposure and stimulation drives gene expression that is distinct from naïve, memory and activated T cells and causes pathogen-specific T cells to become functionally inactive culminating in T cell exhaustion. Exhausted T cells is now recognized as a general feature of most chronic viral infections and the expression of inhibitory receptors is implicated in the pathogenesis of many infection-associated diseases. The focus of much research is restoring function to T cells in order to effectively clear infection.

4.1 Human Immunodeficiency Virus (HIV)

HIV is a chronic viral infection, that if left untreated leads to rapid depletion of $CD4^+$ T cells and acquired immunodeficiency syndrome (AIDS). HIV can be controlled with highly active antiretroviral treatment (HAART); however, low level viral replication and chronic inflammation are thought to cause HIV-related comorbidities even despite viral suppression. The PD-1/PDL-1 axis has been well studied in the context of HIV infection and PD-1 has been found to be highly expressed on CTLs in HIV-infected individuals [62, 63]. CTLA-4 expression, on the other hand, is not increased on CTLs in HIV infection [63, 64]. Increased numbers of PD-1-expressing CTLs correlates with higher viral loads and impaired cytotoxic and cytokine-producing functions. However, this impairment seems to be reversible via PD-1 blockade as observed in *ex vivo* studies. In addition, *in vivo* PD-1 blockade in simian models of HIV (SIV) have been shown to suppress viral load to some extent even in the absence of antiretroviral therapy (ART). It is believed that this is achieved, at least in part, through enhanced cell-mediated immunity by CTLs [65].

$CD4^+$ T helper cells in HIV have been shown to express high levels of CTLA-4 and PD-1 and expression of these receptors correlates with HIV disease progression [62, 63, 66]. In an SIV rhesus macaque model, inhibition of CTLA-4 or PD-1 reinvigorated $CD4^+$ T cells and induced antibody production. Of note, $CD4^+$ T cell subsets that are able to harbor latent HIV have been shown to express multiple NCRs. Therefore, it is thought that NCRs may serve as reliable markers of viral persistence [67–69]. Likewise, CTLA-4^+PD-1^- $CD4^+$ T cells in lymph nodes have been identified as a possible reservoir in SIV infection models. In humans, PD-1^+ follicular helper T cells found in the periphery have been proposed as one of several possible viral reservoirs [66, 70]. Consequently, CTLA-4 or PD-1 blockade has been shown to cause transient increases in viral load in simian models.

The effects of CTLA-4 or PD-1 blockade in humans has been studied in cancer patients who were also infected with HIV [71, 72]. A case report for the use of ipilimumab, a CTLA-4 inhibitory, in combination with ART reported increases in viral load followed by subsequent decline. HIV DNA, a surrogate marker of HIV reservoir size, was not shown to change during the course of treatment with ipilimumab. On the other hand, in patients who received nivolumab, a PD-1 inhibitor, HIV DNA levels decreased profoundly compared to their pre-treatment status. This is thought to be due to the reactivation of HIV reservoirs in latently infected T cells via NCR blockade and attack of these infected cells by reinvigorated immune cells, specifically virus-specific CTLs [73]. Because treatment outcome seems to fluctuate among treated individuals, further study is warranted to overcome this challenge. Furthermore, the expression of multiple NCRs, namely LAG-3, TIGIT and Tim-3 on $CD4^+$ T cells and CTLs are known to be significantly increased in HIV-infected individuals [67, 69, 74–77]. Given the increased expression of multiple NCRs on T cells, combinatorial blockade of these receptors in additional to PD-1 blockade may exhibit improved restoration of T cell function compared to single blockade and should be explored in preclinical models.

4.2 Human T Cell Lymphotropic Virus (HTLV-1)

HTLV-1 causes a wide spectrum of disease, most notably adult T cell leukemia (ATL) or HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [78]. Only a small proportion of individuals develop one of the two diseases and only after many years of chronic infection. In this section, we will discuss NCR expression in the context of ATL. HAM/TSP will be discussed in the next section.

HTLV-1 preferentially infects $CD4^+CD25^+$ Tregs but is known to also infect $CD8^+$ T cells as well as B cells and monocytes. In HTLV-1 infection, individuals who develop ATL have been found to have fewer HTLV-1-specific CTLs as compared to asymptomatic carriers (AC) [79, 80]. PD-1 expression on HTLV-1-specific CTLs did not differ between the two groups and was more commonly found on $CD25^+$ and $CD25^-$ helper T cells rather than CTLs. In contrast, PD-L1 expression on helper T cells, which includes HTLV-1 infected cells, is higher in ATL patients than in AC and blockade of PD-L1 has been shown to enhance IFN-γ production from HTLV-1 specific CTLs. Interestingly, PD-1 expression has been associated with poor proliferation and cytokine production. Therefore, the PD-1/PD-L1 axis not only facilitates immune evasion of HTLV-1 infected cells but may also play a protective role in controlling hyperproliferation of infected cells.

A phase 2 clinical trial using a PD-1 inhibitor was conducted in 3 ATL patients but resulted in rapid progression of disease after a single dose [81]. Patients exhibited leukocytosis, increased viral loads and increased peripheral atypical cells. This increase in viral load was also observed in HIV infected individuals treated with the same PD-1 inhibitory antibody, nivolumab. PD-1 expression may be a means of evading immune surveillance for virus-infected cells by limiting viral replication. Upon PD-1 blockade, virus reactivation in the case of HIV, and hyperproliferation in the case of HTLV-1 may be taking place leading to viral spread and disease progression.

4.3 Hepatitis B Virus (HBV)

Every year, nearly 650,000 people die of HBV-associated end-stage liver diseases. The pathogenic mechanism of HBV is not completely understood. However, NCRs have been implicated in HBV pathogenesis. The overall CTL response in HBV infection is weak and can be undetectable. It has been suggested that the chronicity of HBV infection and lack of CTL response may be related to increased expression of NCRs. PD-1, LAG-3, TIGIT and Tim-3 expression on CTLs has been shown to be elevated in HBV infection, and has also been shown to correlate with disease progression [82]. PD-1 blockade in HBV murine models show reinvigoration of CTLs and decreases in viral load [83]. The efficacy of PD-1 blockade in human HBV infection has been studied in cancer patients infected with HBV. However, similarly to HIV, several case reports have reported increased

HBV viral loads and acute hepatitis after treatment with PD-1 blockade [84]. BTLA expression on $CD8^+$ T cells are observed at increased levels on specific T cell subsets, particularly central memory T cells in the periphery and effector memory in the liver [85]. It is believed that this increased expression of BTLA during homing of T cells into tissues and prevents the transition of $CD8^+$ T cells from a central memory phenotype to effector phenotype further allows virus-infected cells to evade immune clearance [86]. Studies targeting BTLA would likely reveal immune mechanisms of HBV pathogenesis as well as expand treatment options for chronic HBV infection.

4.4 Hepatitis C Virus (HCV)

HCV, another chronic viral infection affecting the liver, is prone to dysregulated T cell function and subsequent pathology. Like many chronic infections, the PD-1, TIGIT, LAG-3 and Tim-3 pathways are manipulated in order to favor viral persistence [74]. PD-1 expression is found at elevated levels on virus-specific T cells and correlates directly with viremia [6]. Furthermore, T cells displayed skewed cytokine production, predominantly producing suppressive IL-10. Antiviral treatment results in normalization of cytokine production, immune reactivation and decreases in PD-1 expression. NCR blockade is less likely to be used as a curative strategy for HCV infection due to the development of drugs like Harvoni, a combination of highly effective Direct-Acting Antivirals (DAA), which is able to clear infection in up to 95% of patients. Even so, PD-1 blockade has been approved for treatment of hepatocellular carcinoma. Synergistic effects have been observed with the use of immune checkpoint inhibitors together with molecular targeted agents or local therapy. Many phase III trials are underway and researchers are awaiting the outcome of these trials with high expectations [87].

4.5 Mycobaceterium tuberculosis (TB)

Cytokines such as IFN-γ and TNF-α secreted from $CD4^+$ T cells and CTLs play a central role in the immune response against TB, which are essential for the activation of macrophages. PD-1, LAG-3 and Tim-3 levels increase during TB infection and expression of these molecules are linked to decreased production of cytokines and poorer outcome in several murine and human studies [88–90]. Blockade of these NCRs show restoration of T cell function and decreased bacterial loads. However, a study with PD-1 knockout mice showed aberrant T cell activation and high levels of inflammatory cytokine production that lead to decreased survival rate compared to wild type controls [91]. This result suggests that there exists a fine balance between immune restoration and over-activation with the use of novel therapeutic strategies that employ immunotherapies.

4.6 Malaria

Malaria is a global problem with over half a million people dying each year, most of these individuals being children and pregnant women. With no vaccine available and resistance to antimalarial drugs on the rise, new therapeutic approaches are much needed [92]. During malaria infection, $CD4^+$ T cell activation and subsequent antibody production from B cells are responsible for developing protective immunity. However, it has been shown that *Plasmodium*-specific T cells show features of T cell exhaustion [93]. The identification of exhausted T cells in malaria, therefore, provides an avenue for novel therapeutic approaches. Therefore, new therapeutic approaches and prevention strategies need to be explored. Expression of CTLA-4, PD-1, LAG-3, TIGIT and Tim-3 on $CD4^+$ T cells increases in infected individuals and correlates with disease severity in both human and murine studies [93–96]. Blockade of PD-1 results in increased cytokine production from $CD4^+$ T cells and decreased parasite burdens in murine models. Dual blockade of PD-L1 and LAG-3 has been shown to increase levels of protective IgG antibodies, follicular helper T cells, and *Plasmodium*-specific $CD4^+$ and $CD8^+$ T cells resulting in enhanced parasite clearance in mouse models [93]. PD-1/LAG-3 dual-blockade may be able to attribute its synergy to the upregulation of IFN-γ upon PD-L1 blockade, which in turn leads to higher expression of MHC class II, the ligand for LAG-3, and consequently increased LAG-3 inhibitory signaling.

4.7 Future Directions

T cell exhaustion driven by negative immune checkpoint receptor signaling is a common mechanism of immune evasion in chronic infection and cancer. The approval of inhibitory blockade therapies for various cancers opens the door for these strategies to be used in the treatment of chronic infections. Blockade of these pathways alone or in combination offers great hope for better treating and perhaps curing many infection-associated diseases.

5 Negative Immune Checkpoint Receptors in Neuroinflammation

As discussed earlier, expression of NCRs is linked to impaired tumor suppression and T cell exhaustion. Under steady-state conditions, these inhibitory signals play an important role in preventing aberrant cellular activation and the development of autoimmune diseases [26, 97]. Furthermore, the CNS can be a target of many acute infections, as well as a reservoir of latent and persistent pathogens. The brain is considered an immune-privileged tissue site, therefore swift resolution of infection

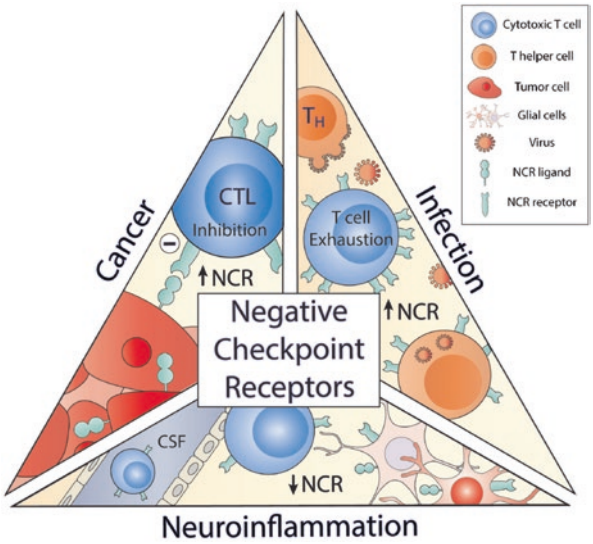

Fig. 1. Schematic overview of the role NCRs play in the settings of cancer, infection and neuroinflammation

Under homeostatic conditions, engagement of NCRs with cognate ligand results in decreased T cell activation and is one of the ways in which the body naturally curbs aberrant immune responses. However, persistent immune activation, as seen in cancer and chronic infection, can lead to increased expression of these NCRs resulting in inhibition of effective CTL function and "exhausted" T cell phenotypes. In neuroinflammatory settings, decreased NCR expression leads to dysregulated T cell responses, further inflammation and disease progression

and associated proinflammatory responses, such as through NCR signaling, is ideal to limit CNS complications. Conversely, premature termination of the immune response through NCR signaling prior to rectifying the mediator of neuroinflammation could be detrimental. Here, we discuss current studies that implicate NCRs as important players in neuroinflammation, particularly in the context of neurodegenerative (multiple sclerosis and Alzheimer's disease) and infectious diseases (HIV, HTLV-1 and viral encephalitis) Fig. 1.

5.1 Multiple Sclerosis (MS)

MS is a chronic inflammatory neurodegenerative disease and pathogenesis is characterized by blood brain barrier (BBB) impairment and infiltration of peripheral immune cells into the CNS, particularly $CD4^+$ effector T cells [98]. The protective function of NCRs has been well investigated using various mouse models of experimental autoimmune encephalitis (EAE), which are widely used in MS studies. Deletion or blockade of CTLA-4, PD-1, VISTA, TIGIT or Tim-3 causes exaggera-

tion of EAE or other autoimmune-related diseases, which may be a result of the accumulation of activated T cells [43, 99–102].

In humans, NCRs expression has been observed at higher levels on T cells in cerebrospinal fluid (CSF) relative to peripheral blood [79]. This is most likely the result of tighter control of T cell activation in the CNS as a means of controlling inflammation in the brain. Accordingly, polymorphisms in CTLA-4, PD-1, LAG-3 or Tim-3 have been shown to correlate with the progression of MS [103–106]. Unlike what we see in HIV, lower expression of these receptors is observed on T cells in both the CSF and in the periphery of MS patients compared to healthy donors. Furthermore, individuals with MS exhibit enhanced T cell function following stimulation *ex vivo*. For instance, the *ex vivo* stimulation of TIGIT on Tregs from MS patients has been shown to reverse T cell suppression and reduce the production of IFN-γ in effector T cells [107]. Of note, stimulation with IFN-ß or glatiramer acetate, which are both used for treatment of MS, induces mRNA expression of these negative checkpoint receptors [106, 108]. *In vitro* experiments show that human brain endothelial cells maintain the integrity of the BBB and can express PD-L1 or PD-L2 to modulate T cell transmigration and immune responses, but this protective function might be impaired in MS due to the decreased levels of PD-L2 on brain endothelium [109].

5.2 Alzheimer's Disease (AD)

AD, an age-related neurodegenerative disease, is the most common form of dementia. Evidence shows that the pathogenesis of AD is not restricted to neuronal dysfunction but strongly linked to inflammation and alterations in immunological mechanisms in the brain [110]. The production of proinflammatory cytokines and other inflammatory mediators in the CNS have been observed in AD, which leads to the recruitment of peripheral immune cells, further promoting neuroinflammation [111]. The PD1/PD-L1 pathway has been implicated as an important means of regulating neuroinflammatory responses in AD. It has been shown that PD-1 and PD-L1 expression is decreased on $CD4^+$ T cells and $CD14^+$ monocytes and macrophages, respectively. An increase in the frequency of PD-1^+ Tregs has also been observed in AD patients [112, 113]. Interestingly, PD-1 blockade was found to reduce disease in mouse models of AD through the mobilization of monocyte-derived macrophages to the brain [114].

5.3 Viral Encephalitis

Viral encephalitis is characterized by severe acute inflammation of the brain parenchyma. Often times, inflammation extends to the meninges as well. Serious cases of encephalitis can result in death. Some examples of viral infections that can result in

encephalitis are herpes simplex viruses (HSV), varicella-zoster (VSV), cytomegalovirus (CMV), Epstein-Barr (EBV), influenza A, a number of flaviviruses (West Nile, Dengue, Yellow Fever), paramyxoviruses (rubella, measles), and polyomaviruses [115]. Although studies investigating NCRs in viral encephalitis are limited, those that are available found associations between decreased NCR responses and favorable outcomes as measured as by decreased neuroinflammation and CNS damage. For example, in a polyomavirus CNS infection model in mice, the absence or blockade of PD-1 signaling was shown to limit the severity of neuroinflammation during persistent infection and increase the number of virus-specific, tissue-resident $CD8^+$ memory T cells in the brain [116]. Additionally, in a murine study of CMV CNS infection, it was demonstrated that the PD-1/PD-L1 pathway plays a role in the generation of CNS-resident memory T cells following viral infection [117].

5.4 HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

HAM/TSP is a demyelinating disease caused by HTLV-1 infection, which resembles MS in several ways. Like MS, HAM/TSP is associated with over-activation of the immune system [100, 118]. It is reported that PD-1 expression on HTLV-1-specific CTLs from HAM/TSP patients is comparable to levels seen in asymptomatic carriers (AC). However, PD-L1 induction on HTLV-1-infected helper T cells after antigen-specific stimulation is profoundly reduced in HAM/TSP patients as compared to AC. Furthermore, T cells from HAM/TSP patients exhibit increased cytokine production following peptide stimulation *ex vivo* [100]. In contrast to PD-1, Tim-3 is expressed to a lesser extent on HTLV-1 specific CTLs in HAM/TSP patients compared to AC. However, these CTLs display greater cytokine production upon peptide stimulation [118]. With no vaccine or treatments available for HAM/TSP, NCR blockade may prove to be an effective means of reinvigorating immune cells to better combat and clear infection.

5.5 HIV-Associated Neurocognitive Disorders (HAND)

Complications regarding cognitive performance is a concern for many individuals living with HIV in the era of highly active antiretroviral therapy (HAART) [119]. The disease pathogenesis HAND has not been fully elucidated; however, chronic CNS inflammation is regarded as a key component of the development of this disease. The brain harbors HIV in macrophages and microglia cells and low levels of HIV RNA is detected even after long-term administration of HAART [120]. Low-level viral replication may induce chronic inflammation of the brain and consequently lead to neurocognitive impairment. However, the relationship between

NCR expression and HAND severity still remains unclear. The brain is an immune privileged site and because HIV infected cells likely serve as reservoirs in the CNS, therapies that overcome the physical barriers that exist to protect this site are currently being developed. A clinical trial of pembrolizumab treatment in HIV-infected adults on suppressive ART is currently ongoing (NCT03239899) to investigate the safety and efficacy of PD-1 blockade, particularly measuring outcomes impacting HIV-1 biology and immune function in the CNS.

5.6 Other CNS Infections

In contrast to the previously discussed neuroinflammatory diseases, upregulation of NCRs is observed in some cerebral infections. Peripheral helper T cells from patients with cerebral malaria showed higher expression of CTLA-4 and PD-1 compared to those from patients with uncomplicated malarial infection [96]. In malaria infected mice, soluble PD-L2 treatment that inhibits PD-1 and PD-L1 ligation ameliorates cerebral malaria symptoms and increases survival [121]. In a mouse model of *Toxoplasma gondii* infection, CTL in the brain exhibited high levels of PD-1 expression. Blockade of PD-1 was found to restore CTL function, diminish brain cysts and improve survival rates [122, 123]. Furthermore, upregulation of the Tim-3 NCR has been observed in the brain of mice infected with *Toxoplasma gondii* and is hypothesized to potentially regulate Th1-biased responses [124].

It is known that various cells of the CNS including astrocytes, microglia and neurons express PD-1. Chen et al. recently showed that PD-1 expression on neurons suppressed nociceptive neuronal activity during acute and chronic pain stimulation [125]. However, the role of PD-1 in these cells is still largely unknown. However, engagement of these inhibitory receptors could be utilized for the treatment of immune-mediated neuroinflammatory episodes.

5.7 Future Directions

The process of neuroinflammation is complex and an increased understanding of contributing mechanisms will lead to improved means of intervention. Examination of recent literature shows that NCRs likely play a role in neuroinflammation, driven either by neurodegenerative or infectious disease processes. While results from animal studies and patient cohort studies are promising, further investigation, particularly in the safety and efficacy of manipulating these pathways, is warranted to posit NCRs and their respective ligands as additional therapeutic targets in the context of neuroinflammation.

6 Summary

Advances in the fields of immunology and immunotherapy have yielded promising immunotherapeutic strategies for previously difficult-to-treat diseases. Specifically, research focused on immune checkpoint blockade in various cancer settings and in chronic infections are showing great promise in restoring functionality to exhausted T cells. Further research that characterizes the expression of NCRs and the dynamics of their expression in various disease states is crucial for developing novel therapeutic strategies and improving treatment outcomes. As new immune checkpoints are discovered and our understanding of these important molecules advances, the prospect of curing cancers, viral infections and neurological diseases will continue to push the field of immunotherapy forward and serve as an impetus for continued research.

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Chimeric Antigen Receptor T Cells: Clinical Applications, Advances and Challenges

Margaret H. O'Connor, Kiran Madugula, and Melody Smith

Abstract Chimeric antigen receptor (CAR) T cells have emerged as a potential groundbreaking treatment for patients with advanced B-cell and other hematologic malignancies. CAR T cells recognize and eliminate tumor cells via cytotoxic killing, independent of the major histocompatibility complex. They are predominantly used in the treatment of many leukemias and lymphomas, such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, Non-Hodgkin lymphoma, and multiple myeloma, via the administration of CD19-targeted or BCMA-targeted CAR T cells respectively. Although there is strong clinical data to support the efficacy of this therapy, toxicity, relapse, and a lack of its broad application for solid tumors have emerged as challenges. In this section, we will highlight the application of CAR T cells in treating hematologic malignancies, as well as their application in solid tumors. Here, we will review the engineering of CAR T cells, clinical data on CD19 and BCMA CAR T cells, and limitations of these therapies. Additionally, we will discuss the development of novel approaches to engineer CAR T cells, identify target antigens, increase their effectiveness and mitigate toxicity. These advances will allow for progress of this therapy and help to overcome the hurdles that are currently present in the use of CAR T cells.

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1 T Cell Engineering With CAR T Cells

The genetic engineering of T cells with a chimeric antigen receptor (CAR) enables adoptively transferred T cells [1–5] to recognize specific tumor targets. These synthetic receptors have a structure that is analogous to the canonical components that are essential for T-cell signaling. CARs have an antigen-binding domain, the single chain variable fragment (scFV), which consists of the immunoglobulin VH and VL [6]. The CD3 zeta chain mediates the activating property of CARs, whereas the costimulatory properties are executed by co-receptors such as CD28 and 4-1BB. Hence, the CAR mediates antigen recognition, T-cell activation, and costimulation [4]. Of note, CARs are distinct from physiologic T-cell receptors in that these molecules do not need peptide processing or HLA expression for antigen binding. CAR T-cell engineering has evolved over time and there are now products that are denoted as fourth generation CAR T cells. The generations of CAR T cells are outlined in Fig. 1. These CART-cell products may utilize costimulatory receptors, such as CD28, 4-1BB, CD134, or CD137. Of note, the fourth generation CARs are the most novel iteration and use a domain referred to as TRUCK or T-cell Redirected to Universal Cytokine Killing. This specific generation is supported by activated T-cell nuclear transcriptional signals, which allows them to secrete specific cytokines such as IL-12 into the tumor microenvironment. This signaling also aids in the recruitment and activation of other immune cells to ensure a robust immune response [7].

Most CAR T-cell studies have utilized retroviral or lentiviral vectors as a mechanism to incorporate CAR cDNA into the T-cell genome [4]. Here we review the use of autologous CAR T cells, which are generated from the patient's peripheral blood T cells, engineered to express the CAR, and re-infused following the administration of conditioning chemotherapy [8–12] as illustrated in Fig. 2. The use of donor-derived or alternative cell sources for CAR T cells is outside the scope of this section.

2 CD19 CARs Targeting B-Cell Malignancies

CD19, an antigen expressed on normal B-cell as well as several B-cell malignancies, is the most common CAR target. Clinical studies of CD19-targeted CAR T cells have demonstrated that they are effective against CD19 malignancies [13–21]. For patients with B-cell malignancies who relapse following chemotherapy, treatment options and the potential for cure are limited. The three main B-cell malignancies that are treated with CD19 CAR T cells include, B-cell Acute Lymphocytic Leukemia (B-ALL), Chronic Lymphocytic Leukemia (CLL), and Non-Hodgkin Lymphoma (NHL). In B-ALL, there is a rapid outgrowth of cancerous immature B-cells that take over the bone marrow and blood stream [18]. CLL, on the other hand, is the most common leukemia in adults, and the main form is a slowly

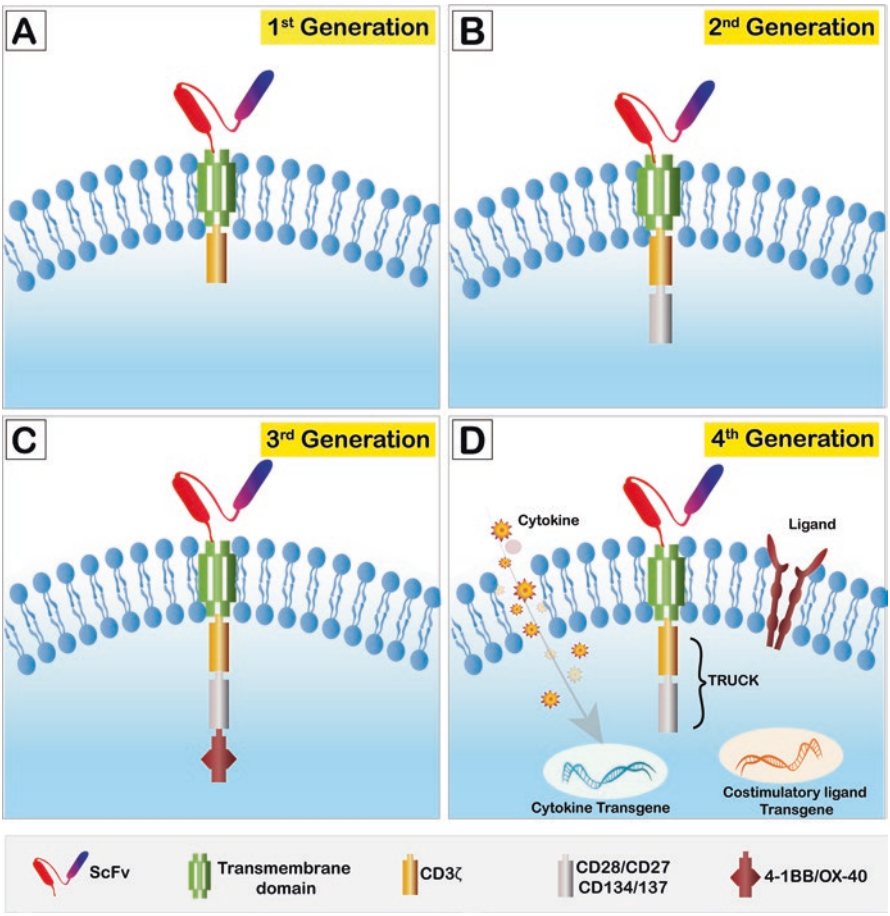

Fig. 1 Progressive generations of CAR T cells. (**A**) The first generation of CARs have a CD3ζ chain along with the scFV with linkers and a transmembrane domain. This generation of CAR T cells lack a costimulatory domain. (**B**, **C**) The second and third generations have one or two co-stimulatory domains, respectively, that induce enhanced proliferation, decreased terminal differentiation and higher activation of the T cells. (**D**) The fourth generation of CAR T cells are engineered with T cells redirected to Universal Cytokine Killing (TRUCK). These cells are designed with an inducible cytokine transgene cassette and additional receptors for the co-stimulatory ligand transgene

progressing outgrowth of more mature cancerous B cells [22]. Finally, NHLs are malignancies of B, T and Natural Killer Cells (NK) that typically infiltrate lymphoid and hematopoietic tissues. The cancerous cells of NHL can arise from either immature or mature lymphocytes. Several CD19 CAR T-cell clinical trials have focused on targeting the mature B-cell NHL neoplasms, which include Diffuse Large B-cell Lymphoma (DLBCL), Primary Mediastinal B-cell Lymphoma, and Follicular Lymphoma (FL). As noted in Table 1, the clinical outcomes of recipients

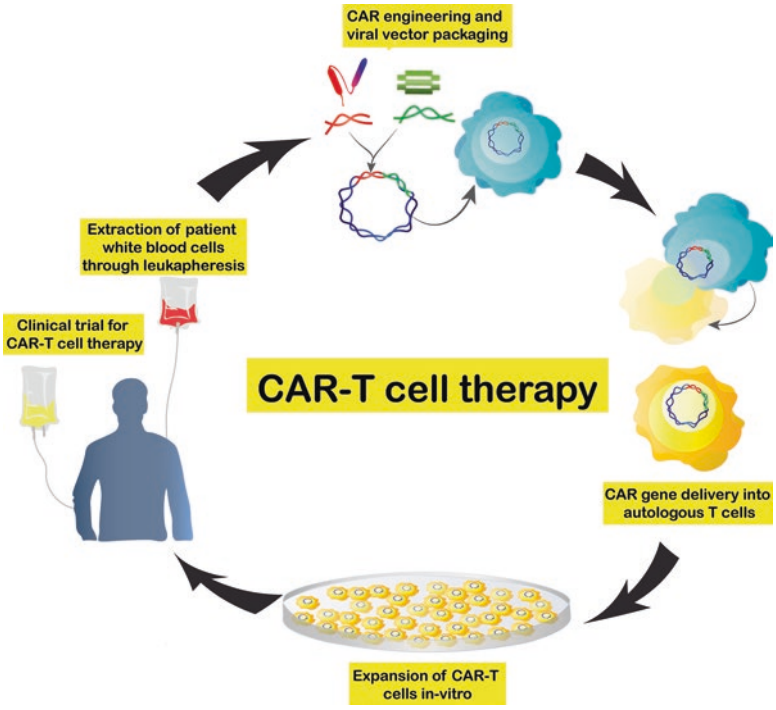

Fig. 2 The workflow of engineering CAR T-cell therapy. The treatment begins with isolating patient T cells by a process called leukapheresis. Once the T cells are isolated from the patient's blood, they are enriched and activated. The selected antigen CARs are transduced using a lentiviral or retroviral vector and introduced into the autologous patient's T cells for reprogramming. These newly engineered CAR T cells expand in vitro in the laboratory. Following adequate expansion, the CAR T cells are re-introduced into the patient's blood stream intravenously

Table 1 Summary of CD19 CAR T cells

Disease	Clinical Outcome (%)
Relapsed B-cell Acute Lymphocytic Leukemia [18, 20, 21, 23–26]	CR: 70–90
Relapsed Chronic Lymphocytic Leukemia [22, 26]	CR: 25–50 ORR: 50–80
Relapsed Non-Hodgkin Lymphoma [13, 26, 27]	CR: 33–60 ORR: 60–70

of CD19 CAR T cells vary by disease. Patients with B-cell ALL who receive CD19 CAR T cells have the highest complete response (CR), followed by patients with Non-Hodgkin lymphoma and chronic lymphocytic leukemia.

The clinical efficacy of CD19 CAR T cells ultimately led to the FDA approval of two CD19-targeted CAR T-cell products. Tisagenlecleucel (Kymriah™) is approved for patients up to 25 years of age with B-cell precursor acute lymphoblastic leukemia who are refractory or in second or later relapse. This cellular product uses

4-1BB for its co-stimulatory domain and is delivered via a lentiviral vector [26]. Axicabtagene ciloleucel (Yescarta™) is approved for adult patients with specific types of relapsed or refractory non-Hodgkin lymphoma (DLBL, primary mediastinal, and FL) after two or more lines of systemic therapy. This cell therapy uses CD28 for its co-stimulatory domain and is delivered by a retroviral vector [27].

3 CD19 CAR T-Cell Toxicity

Despite the therapeutic efficacy of CD19 CAR T-cell therapy, there are several potential toxicities to consider. First, CD19 CAR T-cell therapy presents with on-target off-tumor toxicity that presents as B-cell aplasia. B cell aplasia causes hypogammaglobulinemia that can be treated with intravenous immunoglobulin replacement therapy. Second, cytokine release syndrome (CRS) is the most frequent life-threatening complication that may occur due to the release of cytokines from CAR tumor killing [4]. Classical CRS presents with symptoms including, fever, fatigue, nausea, vomiting, diarrhea, rashes, acute kidney injury, delirium, hallucinations, hypotension, and even severe multiple organ failure [28]. Finally, neurotoxicity or immune effector cell (IEC)-associated neurotoxicity syndrome (ICANS) is another potential serious toxicity that may occur following CAR T-cell therapy [29]. ICANS may present with delirium, headache, encephalopathy, aphasia, lethargy, difficulty concentrating, agitation, tremor, seizures, and, rarely, cerebral edema [30]. Both CRS and ICANS are assessed clinically with a Grade of 1 to 4 depending upon the severity of the patient's symptoms and the symptoms are treated based upon the corresponding grade [31].

4 Challenges With CD19 CAR T-Cell Therapy

Although the introduction of T-cell engineering has created new strategies to target malignancies that have failed 2 or more other treatment regimens, relapse rates remain high. In B-ALL, approximately 30–60% of patients relapse after CAR treatment, and among those, 10–20% are CD19-negative, suggesting antigen escape by the tumor cells [32]. With regards to CD19-positive relapse, the key mechanism for CAR failure is poor persistence of the CAR T cells [33]. Several approaches to overcome these challenges have been investigated, including the use of dual-targeting CAR T therapy and the development of CAR constructs with the capacity for increased persistence in patients. These CAR construct advances have included alterations to the transmembrane, extracellular and intracellular signaling domains [32].

5 BCMA CAR T Cells for Multiple Myeloma

Multiple Myeloma (MM) is the second most common hematologic malignancy in the United States. This disease is characterized by the expansion of malignant plasma cells in the bone marrow and associated with excessive production of monoclonal antibodies in the blood and urine of patients. Additional clinical findings include osteolytic bone lesions and immunodeficiency both of which limit the length and quality of life [34]. Treatment with proteasome inhibitors (PI) and immunomodulatory drugs (anti-CD38 and anti-SLAMF7 drugs) has significantly increased progression free and overall survival in MM patients in the newly diagnosed and relapsed/refractory setting. Both of these immunomodulatory drug targets, however, are highly expressed on normal tissues especially hematopoietic lineages and immune effector cells [35, 36]. Thus, other MM specific targets must be explored for long-term usage. Additionally, overall survival of patients with relapsed disease after PI and immunomodulatory drug treatments is quite low. Accordingly, more efficacious therapies and novel strategies are urgently needed in order to develop curative therapies.

Excitingly, B-cell maturation antigen (BCMA), a transmembrane glycoprotein in the tumor necrosis factor receptor superfamily 17 (TNFRSF17), is expressed at significantly higher levels in all MM malignant cells but not on other normal tissues besides mature plasma cells (PC) [37]. BCMA itself is only induced in late memory B-cells committed to the PC lineage and is present on all PCs [38, 39]. Consequently, BCMA-targeted CAR T cells were developed to treat patients with MM. Early clinical trials have already shown significant clinical activity in patients with relapsed/refractory MM who have undergone at least three prior treatments, including a proteasome inhibitor and an immunomodulatory agent treatment. As of 2019, four Phase 1 dose-escalation clinical studies were completed, three are open and recruiting and two are still in the preclinical stages. Of the four completed Phase 1 trials, of which all use lentiviral delivery of the vector, three utilize 4-1BB as their co-stimulation domain and one uses CD28 for co-stimulation. One Juno-sponsored trial, has a construct, EGFRt/BCMA-41BBz, that incorporates the suicide gene EGFRt [40]. Many of the newer BMCA CAR T-cell constructs in the preclinical phases have begun to include suicide genes or inactivation switches. The details of these Phase 1/2 and preclinical trials are summarized in Table 2.

6 Challenges With BCMA CAR T-Cell Therapy

The application of CAR T-cell therapy in cancer treatment for MM still faces several challenges and clinical limitations including the persistence and survival of CAR T cells, toxicity of conditional chemotherapy or the CAR T-cell therapy itself, and disease progress due to antigen escape. There is limited data to assess the duration of the benefits of BCMA CAR T-cell therapy. To overcome some of these

Table 2 Summary of BCMA CAR T cells

BCMA CAR T	Clinical Development Phase	Clinical or Preclinical Details	Toxicities
Anti-BCMA chimeric antigen receptor National Cancer Institute [41, 42]	Phase 1 completed	24 patients, 3 dose escalations ORR: 81% CR: 8% VGPR: 33% PR: 13%	15/16: Grade 4 toxicities CRS, pancytopenia
CART-BCMA Novartis [43]	Phase 1 completed	25 patients, 3 cohorts ORR: 48% CR: 8% PR: 20% VGPR: 20%	CRS, neurotoxicity, Grade 3: 8 (32%) Grade 4: 3 (12%)
LCAR-B38M Nanjing Legend Biotech [44]	Phase 1 completed	57 patients ORR: 88% CR: 68% VGPR: 5% PR: 14%	Grade ≥ 3 toxicities 37/57 patients (65%) CRS: 51 (90%) Grade 3: 4 (7%) 1 patient: neurotoxicity
bb2121 Bluebird Bio Celgene [45]	Phase 1 completed	33 patients, 4 dose cohorts plus a expansion phase ORR: 85%, CR: 45% 6/15 of CRR relapsed	Grade 3 pancytopenia CRS: 76% Grade 1/2: 70% Grade 3: 6% Neurologic toxicities: 42% 3% reversibleGrade 4 neurologic toxicity
EGFRt/ BCMA-41BBz Juno [46]	Phase 1-open/ recruiting	Includes a suicide gene EGFRt	
P-BCMA-101 Poseida Therapeutics [47]	Combined Phase 1/2-open/ recruiting	No transfection, uses mRNA and plasmid DNA for CAR T engineering of T stem cell memory CART A Phase 1, open-label, single ascending dose (SAD), 18 patients ORR: 83% CR: 73% VGPR 5% PR: 17% Safety switch activated by rimiducid	Toxicity: Grade 2 CRS 1 patient

(continued)

Table 2 (continued)

BCMA CAR T	Clinical Development Phase	Clinical or Preclinical Details	Toxicities
Descartes-08 Cartesian Therapeutics [46]	Combined Phase 1/2-open/ recruiting	30 patients $CD8^+$ anti-BCMA CAR T modified by mRNA not transfection Phase 1: dose escalation of the $CD8^+$ BCMA CART Phase 2: treatment with fludarabine and cyclophosphamide	
BCMA CAR Pfizer [48]	Preclinical	Inactivates the TCR alpha chain Contains an intra-CAR rituximab recognition domain to deplete CAR T	
P-BCMA-ALLO1 Poseida Therapeutics [46]	Preclinical	Uses CRISPR to disrupt both the TCR and MHC I expression	

challenges, the field is investigating several strategies to utilize conditioning and combination therapies to aid CAR efficacy and persistence [49]. The combination of BCMA CAR with another antigen targeting CAR T-cell or with other immunomodulatory agents may reduce the risk of relapse due to tumor antigen escape. Additionally, pre-conditioning may deplete T regulatory cells, leading to enhancement of CAR T-cell therapy [50]. Given that one-third of newly diagnosed MM patients are older than 75 years and more than 30% of them are frail, these factors could be barriers to the use of CAR T-cell therapy or the incorporation of the necessary conditioning chemotherapy prior to CAR T-cell therapy [51]. Another issue that needs to be addressed is whether CAR T-cell therapy should move to an earlier line of therapy to avoid only treating MM patients who have more advanced disease and antigen-altered disease states.

7 CARs for Solid Tumors

Although immunotherapy with CAR T cells has achieved success in the treatment of hematological malignancies, the treatment of solid tumors with CAR T cells has been challenging due to the intricacies of solid tumor microenvironments and tumor locations. T cells trafficking to and infiltrating into tumor sites are oftentimes greatly limited by the immunosuppressive microenvironment created by the tumor cells themselves. This limits the ability of the CAR T cells to access the solid tumor milieu and execute their function of killing tumor cells. Furthermore, solid tumors tend to display a large degree of antigen heterogeneity. Many tumors may contain only a subset of cells that express the CAR T target antigen. Even in the setting of a

uniformly expressed tumor antigen, such as the B-cell leukemias and lymphomas discussed above, there is still the possibility of antigen loss or escape [52]. Given these obstacles, strategies have been employed to overcome them, including knock-out of PD-1 in the CAR T-cell, engineering the simultaneous secretion of cytokines or chemokines, and combining CAR T cells with other pharmacologic treatment strategies [53, 54].

8 Challenges With CAR T-Cell Therapy for Solid Tumors

Another complication that limits solid tumor-directed CAR T-cell therapy is immune-related adverse events. These toxicities may occur upon binding of the CAR to antigens on target tumor cells, resulting in the activation of the CAR and the subsequent release of a large quantity of inflammatory cytokines causing CRS which is detailed symptomatically in the CD19 CAR section. Unlike hematological malignancies, most solid tumors share many antigens with normal tissues. This may lead to off-target effects and the destruction of healthy organs by the infused CAR T cells. In order to reduce the risk of this toxicity, more specific antigens for the tumor should be selected. Tumor killing may be improved by utilizing dual-antigen CAR T-cell targeting and modulating the sensitivity of the scFv that comprises the CAR T-cell itself [55].

Despite these efforts, there are still no CAR T cells clinically approved for solid tumor treatment. Excitingly, as of 2019, there were more than forty ongoing CAR T-cell clinical trials for the treatment of solid tumors registered in China alone [56]. The antigen targets of these CAR T cells vary, some of which target EGFR (gliomas, colorectal cancers), EpCAM (hepatic, gastric, esophageal, colorectal, prostate cancers), GPC3 (hepatocellular carcinoma, squamous cell lung carcinoma), MSLN (pancreatic, ovarian, endometrial and other mesothelin positive cancers) and MUC1 (pancreatic, hepatocellular, glioma, gastric, colorectal, non-small cell lung cancer and triple negative breast cancer) [57]. A MUC1-targeted CAR T-cell, manufactured by Minerva Biotechnologies, and initiated in September 2019, is a first in human clinical trial in the United States conducted at the Fred Hutchinson Cancer Center. This CAR was engineered to target a truncated form of MUC1 that is highly expressed on breast cancer cells and not as highly expressed on normal tissues. Results from this trial will demonstrate the advancement of solid tumor CAR T-cell technology as we seek to have more clinical studies for other solid tumor antigens.

Furthermore, pre-clinical studies are ongoing to investigate other therapeutic approaches for the treatment of solid tumor with CAR T cells. Tumor heterogeneity in malignancies, such as glioblastoma multiforme (GBM), has proven challenging for treatment with CARs. Peptide-based CARs are being evaluated in order to harness the binding potential of chlorotoxin (CLTX) to tumor cells, given that CLTX binds with higher affinity to tumor cells than any other antigen. CAR T cells bearing the CLTX as the targeting domain demonstrate higher anti-tumor activity both *in vitro* and *in vivo* with minimal off-target effects, which supports this strategy as a

potential treatment for GBM and other solid tumors [58]. Yet another approach has targeted Glypican-3 (GPC3), which is over-expressed in hepatocellular carcinoma (HCC) but not in normal tissues. GPC3-specific CAR T cells are designed with the PiggyBac (PB) transposon-transposase system as opposed to conventional viral vectors. Upon stimulation with the GPC3 antigen, the GPC3 CARs undergo activation and proliferation. Investigators have found that the administration of GPC3 CAR-T cells to HCC xenograft mice results in higher cytokines, such as interferon-γ, and increased cytotoxicity in comparison to mice injected with mock T cells and vehicle controls [59].

9 Future Directions of CAR Therapy

Ongoing research with CAR T cells is focused on strategies to (1) improve CAR T-cell persistence, (2) decrease antigen loss as a mechanism of disease relapse, (3) develop CARs for a wider range of hematologic malignancies as well as solid tumors and, (4) decrease costs of the therapy. Along with the modifications to the engineering of the CAR construct and improvements to the domains of the receptor, CRISPR/Cas9 editing of the CAR has begun to further improve signaling of the CAR. The CRISPR/Cas9 system has been employed to target the genes of inhibitory receptors, such as PD-1 [54], Fas, and HLA-I, to simultaneously delete these genes and limit protein expression of these immune system inhibitors on the CAR itself. Pre-clinical in vivo and in vitro studies with the Fas/HLA-I/CD3 triple deletion CAR have shown that this strategy allows for increased CAR persistence and enhanced immunologic activity with improved cytotoxicity and cytokine secretion from the CAR T cells [60]. Dual targeting strategies for CAR T cells aims to decrease the potential for relapse due to antigen loss by simultaneously targeting multiple antigens, such as CD19 and CD22 [61]. Investigators are actively working to investigate potential antigens to successfully treat diseases ranging from AML [62] and pancreatic cancer [63]. Finally, others are also utilizing CRISPR/Cas9 to develop off-the-shelf CAR T-cell therapies that have the potential to decrease the costs needed for the generation of personalized CAR T cells [63].

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Neuroprotective Immunity for Neurodegenerative and Neuroinfectious Diseases

Katherine E. Olson, R. Lee Mosley, and Howard E. Gendelman

Abstract The interplay between innate and adaptive immunity strongly influences the pathobiology of neurodegenerative, neuroinflammatory, and neuroinfectious diseases. Specific and sustained immune responses can induce disease by affecting neuronal injury and death. Disease progression parallels glial proliferation, proinflammatory cytokine production and adaptive immune responses against the inciting misfolded protein or infectious agent. All affect neuronal demise. Neuroprotective immune transformation remains a therapeutic avenue being developed by several research groups towards the shared goal of sustaining a nourishing brain microenvironment.

Keywords Human immunodeficiency virus · Alzheimer's disease · Adaptive immunity · Parkinson's disease · Innate immunity · Neuroprotection · Neurodegeneration

1 Introduction

Multifaceted disease mechanisms characterize the pathobiology of neurodegenerative and neuroinfectious disorders. One common pathway affecting neuronal vitality in all diseases states is disordered innate and adaptive immunity [1]. Innate microglial and astrocyte responses are considered early signs of disease as is antigen-driven T cell proliferative responses. Such immune responses affect multiple disease components including neuronal loss, peripheral blood cell extravasation across the blood brain barrier (BBB) and lymphocyte surveillance of pathogenic proteins or microbes [2, 3]. During disease, both innate and T cell responses be

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come operative and are considered to be detrimental for a spectrum of diseases. These include, but are not limited to, Alzheimer's and Parkinson's diseases (AD and PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and infectious diseases including human immunodeficiency virus type one (HIV-1) and its associated neurodegeneration [4, 5]. Furthermore, immune-incited neurodegeneration can affect both disease onset and progression [6, 7]. Indeed, mounting evidence shows that the interplay between the peripheral immune system and resident central nervous system (CNS) immune cells amplifies neuroinflammatory responses and exacerbates neurodegeneration [8]. This chapter examines the role of immunity in neurodegenerative and neuroinfectious disorders. Particular focus rests in the interactions between the innate and adaptive immune responses that affect neurodegenerative and neuroprotective responses.

2 Immune Interplay for Neurodegenerative Diseases

Neurodestructive immune responses can be harnessed or even transformed to control disease onset and progression [9]. Our laboratories and others have investigated the role of immunity in affecting the onset and progression of Alzheimer's and Parkinson's disease (AD and PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Huntington's disease, stroke, traumatic brain and spinal cord injuries, and drug-related nervous system damage [10–16]. With this in mind, the pathobiology of neurodegeneration is required [17]. Neurodegeneration is a pathological condition in which the nervous system loses structure or function characterized by synaptic loss and neuronal death. Clinically, this leads to progressive cognitive decline and motor dysfunction [18]. While the precise cause(s) have not yet been fully elucidated for each disease, there is no cure, and disease progression is unavoidable. While neurodegenerative diseases affect the nervous system differently [19] common disease mechanisms do exist. First, all are associated with the death of specific neuronal cell subpopulations, resulting in the degeneration of specific brain regions often leading to disease-specific manifestations [18]. Second, neuronal loss is linked to the formation and spread of protein aggregates. These occur during advanced age but can also be present sporadically or due to defined genetic mutations [20]. Each neurodegenerative disease is further classified based on the kind and type of protein deposition seen in brain sub regions. Third, neurodegenerative disorders are linked, in measure, to immune responses that trigger overt neuroinflammatory responses that can affect disease [21]. For most neurodegenerative disorders, the pathways of neuronal demise are similar. Common mechanisms include oxidative stress, mitochondrial damage, excitotoxicity, and misfolded or post-translationally modified protein aggregation [20, 22–24]. To counteract these events, therapies have been developed to elicit neuroprotective responses with the intent to preserve already damaged neuronal and synaptic structure [25]. Such treatments serve to attenuate inflammation, oxidative stress, and excitotoxicity [9, 26, 27].

2.1 Innate and Adaptive Immunity and Neurodegeneration

Both innate and adaptive immune responses are important for mounting the body's defense against a pathogen or foreign microorganism [28]. The innate response is the first line of defense. It is rapid, does not require immune memory, and is characterized by phagocytic activity of macrophages, dendritic cells, or microglia. While serving as a first line of defense against microbial infections and injuries, it also perpetuates tissue and wound healing and repair [28]. Within the brain, microglia are the resident innate immune cell with similar functions to macrophages [29]. Apart from cell ontogeny, both brain macrophages and microglia maintain CNS homeostasis. Morphologically, microglia have long, branched processes that are constantly surveying the environment for homeostatic changes [30]. They are in contact with neurons, astrocytes, endothelial cells, and other surrounding microglia. When a change in the CNS microenvironment occurs, microglia become amoeboid and rounded in appearance [31, 32]. This morphological change reflects a reaction to injury or infection with increased phagocytic capacity and production of proinflammatory cytokines. As a result of aging and/or neurodegeneration, microglia become functionally impaired leading to an overactive neuroinflammatory response that further contributes to neural injuries [17]. In the aged brain, there is evidence for increased number of reactive microglia and increased proinflammatory microglial function [33]. Likewise, evaluation of cerebral spinal fluid (CSF), serum, and brains of individuals suffering from neurodegeneration also indicate increased levels of tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 [29, 34]. These secretory products are from resident microglia themselves [35] and display a link between disease progression and microglial immunity.

The adaptive immune response is specific [36]. To mount an immune response, the innate arm of the immune system must be activated [37]. Antigen is taken up by antigen presenting mononuclear phagocytes (MP) such as macrophages, dendritic cells or microglia, processed, and then presented to cells of the adaptive immune system generating an effective, robust, and specific immune response. Because of this, antigen presenting cells (APCs) are the bridge between the innate and adaptive immune system [38]. They directly activate T cells during antigen presentation, causing them to proliferate and migrate to areas of injury or infection [39]. Specifically, APCs activate T cells through presentation of antigen in conjunction with major histocompatibility complex (MHC) molecules and interaction with T cell receptors (TCRs) and co-stimulatory molecules such as CD80, CD86, CD70, CD40, and CD200 [8]. Because of the ability to recognize specific antigens, T cells comprise the cell population that is responsible for unique immune specificity. Once activated, T cells undergo clonal expansion to increase their cell number and potential to eliminate pathogens [8, 39]. Such activation causes T cell differentiation, expansion, and proliferation with associated cytokine production within a surrounding environment. Likewise, APCs themselves deliver many cytokine signals including IL-12, IL-4, IL-6, and transforming growth factor beta (TGF-β) to polarize naïve T cells into activated T cells with specific effector functions [40].

There are major T cell subsets that can be generated from both lymphoid tissues such as thymus, spleen, and lymph node, or in the periphery [40]. Upon activation by innate immunity, CD4+ T cells differentiate into different subsets such as T helper 1 (Th1), Th2, Th17, and regulatory T cells (Tregs) [41]. Classically, Th1 and Th17 cells mount active immune responses through the secretion of proinflammatory cytokines and mediators, including interferon gamma (IFN-γ) and IL-17A [42, 43]. On the other hand, Th2 and Tregs are responsible for anti-inflammatory responses [44]. Specifically, Tregs maintain suppression of an immune response [45]. Tregs mediate this function by diminishing antigen presentation and secreting anti-inflammatory cytokines including IL-10, IL-35, and TGF-β. These cause suppression of activated MP and T effector cells (Teffs) [46]. Each of these T cell subsets play crucial yet independent roles in mounting a robust and effective adaptive immune response. Following activation, T cells are recruited to sites of disease and promote inflammation [47]. To enter sites of disease, cells undergo extravasation. This process allows circulating lymphocytes to migrate across cell barriers such as the BBB to gain entry to sites of inflammation [48]. Once inside the brain, cell-mediated immune responses can affect neurodegeneration. The cross-talk between T cells and glia mediate effector functions by either cell-cell contact or cytokine-mediated mechanisms, including direct cytotoxicity by proinflammatory cytokines, activation of microglia or diminished suppressive function of Tregs [49].

This interplay between the innate and adaptive immune arms is essential for the development of neuroinflammation as it affects neurodegeneration or neuroprotection. Findings from multiple neurological disorders have provided insight into common disease outcomes [50]. Although neuroinflammation and T cell interactions play a prominent role in disease progression or protection against disease, it should be noted that the type of immune response are commonly specific [8].

2.2 Immunity in Alzheimer's Diseae (AD)

Recent research findings in studies of human and animal models of neurodegenerative disorders have shown direct involvement of T cells in disease initiation and progression [51]. An example of such immune-linked disease effects is linked to the pathobiology of AD. AD is notable as it is the most common neurodegenerative disorder affecting anywhere from 10–30% of individuals over 65 years of age [52]. Cognitive loss is associated with impairment in short term memory that eventually leads to profound cognitive and memory deficits. Pathologically, the disease is characterized by loss of neurons in the hippocampus and cortical regions. The key neuropathological features include senile plaques containing beta-amyloid (Aβ) protein and the formation of neurofibrillary tangles (NFT) containing tau protein [53]. Aβ is processed by the sequential cleavage of amyloid precursor protein (APP) into smaller peptides [54]. The majority of the processed peptides consist of either Aβ40 or Aβ42 forms. These peptide forms can cluster into monomers, oligomers, protofibrils, or fibrils resulting in the formation of protein aggregates [55, 56]. Normally,

extracellular Aβ peptides are removed from the brain and drained into the CSF, where they are degraded by microglia within the parenchyma [55, 57]. However, in a diseased state degradation is impaired. Tau is a microtubule-associated protein that can be phosphorylated at multiple serine, tyrosine, or threonine residues [58]. The mechanism of tau aggregation is thought to be mediated through abnormal phosphorylation leading to atypical conformations that can aggregate together [59]. Therefore, the loss of functional peptide clearance is proposed as a disease inciting event [60].

Post-mortem evaluation of AD brains reveals a relationship between neuron loss and memory [61]. This finding is associated with brain inflammation characterized by microgliosis, astrocyte activation, edema, and infiltration of MP across the BBB [62]. Activated microglia are shown to integrate deep into senile plaques, along with the detection of increased levels of proinflammatory cytokines [63, 64]. The associated glial activation and neurotoxicity is due to the formation of reactive nitrogen and oxygen species, increased proinflammatory cytokine production, and changes in excitatory amino acids in a diseased microenvironment [65]. The enhanced proinflammatory state decreases phagocytosis of Aβ plaques and inhibits intracellular Aβ degradation [66]. The resulting Aβ aggregates preferentially activate surrounding microglia launching signaling cascades needed to initiate clearance [55]. Resident microglia mediate such Aβ clearance, displaying the ability to phagocytize and ingest Aβ through a range of surface receptors. These pattern recognition receptors include CD14, TLRs, and CD47 [67–69]. Immune stimulation with Aβ enhances microglial phagocytosis. Microglia internalize Aβ through interactions with Aβ-scavenging receptors such as SR-A, CD36, and RAGE [70, 71]. However, even with this uptake, studies show that phagocytized Aβ can remain within the activated microglia for up to one month [72]. Aβ protein accumulation results from the failure of microglia to successfully remove the aggregated protein [73].

Post-mortem assessment of AD brains shows microglia surrounding Aβ plaques [74, 75]. These microglia were determined to be functionally impaired, lacking the capacity to properly uptake Aβ. Furthermore, Aβ can induce inflammatory responses involving inflammasome activation, resulting in increased proinflammatory cytokine production, including IL-1β and IL-18 [76]. These cytokines, along with IL-12, TGF-β, TNF-α, and IL-6, have been implicated in the progression of AD [77]. Increased IL-1β in serum is linked to cognitive impairment, and IL-12 is important for regulating the innate and adaptive immune response [78, 79]. Likewise, increased TGF-β levels have been noted in senile plaques, as well as in the CSF of individuals with AD [80, 81]. Presence of TGF-β is also associated with NFT formation [82]. Similarly, there is evidence showing that IL-1β and IL-6 can lead to hyperphosphorylation of tau, further contributing to tangle formation [83]. Apart from microglial cytokine production, there are also increases in reactive nitrogen and oxygen species, leading to direct neuron cytotoxicity [65]. Therefore, to assess the neuroinflammatory condition within the living AD patient, positron emission tomography (PET) scans have been utilized [77, 84, 85]. Scans indicate that, when compared to age-matched controls, there are increased numbers of activated microglia near

primary disease areas [77]. Similarly, microglia that were collected post-mortem were biased toward a proinflammatory phenotype following immunological challenge.

In the brain, Aβ also interacts with resident astrocytes. Astrocytes uptake and remove Aβ in a CCL2-dependent manner [86]. This primary innate immune response is mediated through a variety of inflammatory factors including proinflammatory cytokines, proinflammatory chemokines, acute phase proteins, and complement factors [87]. Upregulating these systems results in enhanced cytokine production, including increases in IFN-γ, IL-1β, IL-6, TNF-α, CD40L, and macrophage inflammatory protein 1-alpha (MIP-1α). In response to the enhanced neuroinflammatory state, increased APP production occurs in surrounding neurons, causing overall Aβ production to be upregulated [88]. Resulting Aβ deposits can form, which may be the cause of plaque formation [89]. It has also been shown that autoantibodies bound to neurons can induce Aβ internalization and deposition, leading to further neuronal damage [90–92].

Under normal physiological conditions, few T lymphocytes cross the BBB and survey the brain [3]. In AD patients, there is an increase in the number of T lymphocytes within the hippocampus and cortex [88, 91]. This infiltration arises due to chemoattractants originating from activated microglia and astrocytes within injured brain sub regions. The ensuing immune cross-talk can influence immune cell populations and their mediators in the periphery. Therefore, peripheral changes in the function of immune populations may have an effect on the CNS microenvironment. Notably, there are a variety of changes in lymphocyte distribution, signature and specific cytokine levels and signatures within whole blood and plasma of AD patients [93–95]. However, the exact peripheral immune dysregulation observed varies. For instance, peripheral blood mononuclear cells (PBMCs) from AD patients produce increased levels of IL-1β, when compared to controls [96, 97]. Other studies, however, show decreased amounts of naïve T cells, increased memory T cells, increased CD4+ T cells, reduced CD4+CD25+ Treg populations, and decreased total B cell populations [93, 98]. Other studies indicated a significant reduction in CD3+ T cells, but CD4+ and CD8+ levels remained unchanged [99]. A fourth evaluation confirmed the decrease in CD3+ populations, but also observed a decrease in CD8+ populations and a modest increase in CD4+ T cells [100]. Along with decreased Treg numbers, one investigative group noted a decrease in CD8+ suppressor cells and a decrease in IL-10, suggesting that the immunosuppressive capacity is diminished during AD [101]. This immune dysfunction decreases the ability to control detrimental Teff responses. Such Teff responses are characterized by increased activities of Th17 and Th9 subsets in AD [102]. Saresella and colleagues observed increased levels of proinflammatory cytokines associated with Th17 and Th9 subsets, including IL-21, IL-6, and IL-23, and the Th17-associated transcription factor, RORγ, in lymphocytes isolated from AD patients. Similarly, PBMCs recovered from AD patients, and consequently activated, exhibit significantly increased production of IL-1β, IL-6, TNF-α, and IFN-γ [102]. Even though a consensus has not been reached, these immune profiling studies do indicate significant aberrations in adaptive immune populations associated with disease that may result

in decreased ability to regulate immune responses. Taken together, this data may suggest a profound skewing of systemic immune populations affecting the brain microenvironment.

2.3 Immunity in Parkinson's Disease (PD)

PD is the second most common form of neurodegeneration, yet it is the most common movement disorder [103]. It is characterized by the formation of proteinaceous inclusions termed Lewy bodies. Lewy bodies contain modified and misfolded forms of alpha-synuclein (α-syn) along with ubiquitin [104]. The main clinical features include resting tremor, postural instability, rigidity, and bradykinesia [104]. Most often, the clinical presentation of PD is sporadic, with a small fraction of individuals actually inheriting the disease. The clinical manifestations of the disease are preceded by a loss of dopaminergic neuronal cell bodies within the substantia nigra pars compacta along with their projections into the striatum [105]. Although postmortem investigations indicate that other ascending dopaminergic pathways within PD brains are affected, they are not affected as profoundly as the nigrostriatal pathway [106]. Apart from neuronal loss and the formation of proteinaceous inclusions, there is also an immune imbalance and proinflammatory response associated with disease and disease progression [107].

PD progression is linked, in measure, to neuroinflammation [108]. Loss of dopaminergic neurons is associated with both microgliosis and astrogliosis. Morphologically, microglia within affected brain regions are reactive, exhibiting ameboid cell bodies and thick, elongated processes and altered immune control [109, 110]. Likewise, the number of reactive microglia is much greater in PD than in age-matched controls [111]. Diffuse microglial activation is located near dead or dying neurons within the substantia nigra, as well as within the striatum [109]. This indicates the possibility that microglial activation could be initiated by a change in the neuronal state. This change triggers the release of soluble factors or mediators into the surrounding microenvironment. For instance, release of cyclooxygenase-2 or neuromelanin from neurons can activate microglia [109, 112, 113]. It is also hypothesized and suggested that misfolded, aggregated, and post-translationally modified proteins, such as nitrated alpha-synuclein, are released from dying neurons [17]. Biochemically, PD brains show increased levels of post-translationally modified proteins, lipid peroxidation, DNA damage, and reduced glutathione levels, all indicative of an aberrant response and neurotoxic milieu [114–117]. There are also elevated levels of nitrated proteins in both the brain and CSF of PD patients [118]. The most prevalent form is comprised of a 3-nitrotyrosine modification [119, 120]. Similarly, the expression of markers of reactive microglia correlates with the deposition of α-syn within the substantia nigra of PD patients [121]. The resulting reactive microglia become potent generators of reactive oxygen and nitrogen species, proinflammatory cytokines, and prostaglandins, all contributing to the inflammatory state and continued neuronal death. Nitric oxide, NAPDH-oxidase, TNF-α,

and IL-1β are some of the major oxidative and inflammatory mediators released by reactive glia [122–125]. All are increased in the substantia nigra and CSF of PD patients [123]. Resulting interactions with cytokine receptors trigger intracellular death-related pathways, involving translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Interestingly, PD patients display 70 times more NF-κB than controls within dopaminergic neurons, suggesting the presence of neuronal death activation [126]. Collectively, these observations indicate an aberrant innate immune response that is associated with disease progression and PD pathology.

Besides chronic innate immune activation, there is compelling evidence that cell-mediated adaptive immune responses also play a role in PD progression. While T cells generally remain outside the CNS, the neuroinflammatory response results in the recruitment and extravasation of lymphocytes from the periphery to sites of active neurodegeneration [3]. The response is associated with disruption of the BBB due to the secretion of toxic mediators into the environment [127]. This dysfunction allows peripheral immune populations to readily enter the normally "immune-privileged" brain. Within the substantia nigra of PD patients, there are increased numbers of CD8+ and CD4+ T cells at levels exceeding 10-fold when compared to controls [128]. These peripheral T cell populations are found in close proximity to reactive microglia and degenerating neurons. Of note, the increased levels of T cells are not detected in any non-lesioned brain regions, suggesting that infiltration is site-specific and related to the neuronal injury itself. Upon microarray analysis of infiltrating T cell populations, it was determined that cells displayed gene changes associated with Th17-mediated immune reactions, indicating that PD may be a Teff-mediated immune disorder. Additionally, increased levels of Th17 cells have been noted in newly diagnosed PD patients, further suggesting their involvement in disease initiation [129]. A second group confirmed higher frequencies of Th17 cells in the blood of PD patients, as well as an increase in the number of CD3+ T lymphocytes within the midbrain of PD brains [130]. The study confirmed that infiltrating lymphocytes induce neuronal death through IL-17 receptor ligand interactions. The observed increased infiltration could potentially be due to increased BBB permeability. In vivo evidence for this phenomenon is observed using PET scans in PD patients [131, 132]. Scans indicate increased BBB permeability through the detection of albumin within the CSF. However, whether T cell infiltration occurs prior to neuronal cell death or after degeneration has occurred is not yet defined.

Apart from direct immune cell infiltration into the brain, peripheral immune populations and mediators are affected in PD patients as well. Compared to controls, levels of total lymphocytes, both B and T cells, are decreased in PD patients [133]. Specifically, CD19+, CD3+, and CD4+ levels are significantly reduced, whereas CD8+ levels remain relatively unchanged. Likewise, a correlation study indicates a decrease in CD3+ and CD4+ T lymphocytes within peripheral blood isolated from PD patients [134]. Work from our own group also indicates a shift in T cell phenotypes [135, 136]. Our cohort of PD patients had increased effector memory T cell subsets and decreased CD4+CD25+FoxP3+ Treg numbers. Similarly,

the Tregs that were present were functionally inadequate in suppressing the proliferation of other Teff immune populations [135]. This deficit correlated with an increase in disease severity, which indicates that Treg dysfunction leads to an unbalanced and overactive immune response that ultimately speeds disease progression. These findings were verified in numerous animal studies using neurotoxin models of PD [15, 137–139]. A second recent study noted that PD patients have a Th1-biased immune response [140]. This study indicates increased levels of IFN-γ-producing cells within the periphery, with an overall decrease in CD4+ T cells in total. Along the same vein, increased proinflammatory cytokine levels including, IL-1β, TGF-β, IFN-γ, and IL-6, are detected in the substantia nigra and the CSF following post-mortem analyses [123–125, 141, 142]. Increased levels of IL-6 and TNF-α within the serum of PD patients is also correlated with increased disease severity based on Hohn and Yarr staging [143]. Increases in complement proteins are also observed, indicating an overall immune dysfunction both inside and outside of the brain. Importantly, it is shown that dopaminergic neurons exhibit enhanced sensitivity to cytokines such as TNF-α and IFN-γ, so increases within the periphery may be indirectly affecting neuronal survival within the brain [65]. Together, the majority of human observations suggest a clear pathogenic role of inflammation on disease severity, indicating that neuroinflammation could be targeted to modify disease progression.

2.4 Neuroimmunity in HIV-1 Infection

HIV-1-associated neural dysfunction is characterized by chronic CNS infection [144]. Infection results in notable cognitive impairments, leading to HIV-associated neurocognitive disorders (HAND) [145]. HAND can affect the frontal cortex, subcortical regions, hippocampus, and putamen of the brain [146]. Development of cognitive impairment is accompanied by motor and behavioral impairments including slowed movement, decreased motor coordination, decreased learning, and impaired memory [147]. Overt and unregulated viral infection leads to brain inflammation termed HIV encephalitis (HIVE). Neuropathology of viral encephalitis is characterized by the presence of HIV-1-infected macrophages within the brain, resulting in enhanced microgliosis and reactive microglia formation [148, 149]. Likewise, there is an increased occurrence of multi-nucleated giant cells and astrogliosis. Both macrophages and microglia are the primary viral targets; however, astrocytes have been shown to be infected, but at much lower levels [150]. Clinical manifestations correlate to the number of activated microglia and macrophages within the CNS, implicating them in disease pathogenesis [151]. Virus is thought to enter the brain through the “trojan-horse” method. Infected monocytes, macrophages, and/or lymphocytes crossing the BBB carry the virus into the CNS with them since virus does not readily cross the barrier itself [152]. This viral entry occurs relatively early after primary infection and maintains itself at low levels within the CNS due to the general immune privileged nature of the brain. However,

there is a significant correlation between the amount of viral burden in the brain and the neuro-cognitive deficit [146]. Once inside the brain, the small number of infiltrating cells still secrete viral factors and neurotoxins, leading to neuronal damage by direct and indirect methods. Multiple studies indicate that virus-infected macrophages and microglia secrete neurotoxic metabolites such as arachidonic acid, TNF-α, IL-1β, nitric oxide, glutamate, and viral particles such as tat and gp120 [153–156].

Initial control of viral infection is mediated by cytotoxic CD8+ T lymphocytes (CTLs) [157, 158]. CTLs mediate their immune function by selectively targeting virus-infected cells through interaction with viral particles presented on infected cells [159]. There is a strong association with the lack of effective T cell responses and HIVE development [158]. Analysis of brain tissue from HIVE individuals reveals increased numbers of CD8+ CTLs near virus-infected mononuclear phagocytes when compared to brain tissue of diseased patients that did not succumb to HIVE [160, 161]. Here, CTLs release perforins and granzymes into the microenvironment that may contribute to the neurological insult resulting from HIV infection itself. Infiltrating CD8+ CTLs are also shown to be a source of CD40L and IFN-γ, further activating mononuclear phagocytes within the brain [160]. Individuals suffering from this disease have a profound loss in peripheral lymphocyte populations as well, making it hard to fight against the virus. Not only is viremia inhibited by CD8+ T cells, but HIV-1-specific CD4+ T cells appear to play a role too [162]. However, limited attention has been paid to CD4+ T cell control of viral replication due to the fact that they are major viral targets [163]. During primary HIV-1 infection, there is a massive infection of both resting and activated CD4+ T cells, reaching levels as high as 60% [164]. Initially, there is a rise in CD4+ T cell numbers; however, after a few months of infection, these numbers begin to decrease. This may be due to a natural contraction following viral infection or due to preferential infection and death of this cell type.

In the early stages of infection, there is a Th1-predominant profile, characterized by a high production of IL-2 and IFN-γ [165]. Late stage HIV infection is generally regarded as a Th2- predominant profile, indicated by increased production of IL-4 and IL-10 [166–168]. The exact role of CD4+ T cell subsets and their ability to control infection and viral replication is still under debate. For instance, Th17 cells have been implicated as being proinflammatory and immune activating in this disease [169]. However, similar to their role observed in AD and PD, this immune activation may not be beneficial in the context of HIV-1 infection. On the other hand, several studies have linked a protective role to HIV-specific CD4+ T cells with regard to viremia and disease progression [170–172]. These studies indicate that gag-specific CD4+ T cells and granzyme-producing CD4+ T cells are important for viral inhibition [170, 171]. Similarly, lack of these types of cell responses can be associated with disease progression [172]. In a contradictory human study, levels of CD4+ T cell activation correlated directly to viral load [173]. Characterization of these activated cells indicated an effector memory phenotype that was inversely associated with Treg phenotypes, and this dysregulation was found to drive the pathological immune activation in HIV-1 infection. Nonetheless, the growing body

of evidence does support a specific role for CD4+ cells in HIV infection. Conversely, it still remains unclear how viral replication and peripheral immune activation shape CD4+ T cell responses and whether or not these responses may actually contribute to early immune activation with infection.

Similar to shifting immune cell phenotypes, cytokine alterations can be observed over the course of HIV disease progression [165]. Dysregulation is thought to contribute to HIV-associated immune deficiency. Increases in soluble factors and cytokines such as TNF-RII, neopterin, and β_2-microglobulin are observed with HIV infection and indicate cellular activation [174]. They are also associated with disease progression and viral load measurements. When compared to uninfected controls, HIV-infected individuals have significantly higher levels of IL-2, IL-6, and IFN-γ [175]. Increases in IL-1β and TNF-α levels within HIV-infected brains and CSF are also been reported [176]. Their presence and mechanism of action can be detrimental on surrounding neurons, implicating these cytokines in the development of HAD. For instance, TNF-α and IL-1β increase the permeability of the BBB and induce an over-stimulation of NMDA-receptors on neurons resulting in fatal increases of Ca2+ [176]. TNF-α is also reported to induce translocation of NF-κB to the nucleus, causing upregulation of many other potent inflammatory cytokines, further contributing to disease progression [177]. Likewise, exposure of microglia to gp120 viral particles results in the upregulation of IL-1β and reactive oxygen species [178, 179]. Together, these findings indicate the presence of an overactive peripheral and central immune response occurring with disease, justifying the need for neuroprotective targets in this disease.

3 Neuroprotective Immune Responses

As discussed, activated microglia and Teffs are thought to be the main mediators of neuroinflammatory processes in these disease states. Left uncontrolled, these mediators support an inflammatory cascade that affects the tempo of disease. However, there are neuroprotective immune responses available that counterbalance the inflammatory milieu observed with disease progression. Current neuroprotective strategies are focused on modulation of microglial responses, alteration of Teff responses, induction of immunosuppressive cell populations, formation of antibodies, and enhancement of misfolded protein or viral clearance [9, 180–183]. Targeting the immune response to elicit a protective mechanism would diminish the extent of neuroinflammation and therefore increase the number of surviving neurons in the CNS of patients with neurodegenerative disorders. Here, we discuss the role of neuroprotective immunity and the current clinical and preclinical strategies being utilized to modulate the inflammatory immune response into one that is neurotrophic and protective.

Healing in response to injury is orchestrated by numerous factors and processes working together or sequentially. Therefore, it involves specific interactions between resident immune populations and peripheral immune cells [184]. Outside of the

brain, tissue damage triggers infiltration of circulating immune cells to the site of injury. Initially, this immune population is mainly comprised of circulating monocytes that become activated and converted into macrophages. The primary job of these activated cells is wound healing and debris clean up [185]. This is done through the secretion of cytokines and growth factors. Without the function of this cell type, wound healing occurs much slower [186]. However, in the CNS, invading monocytes are not as prevalent. Macrophage infiltration is also delayed so resident microglia become the major phagocytic populations at the injury site. As discussed previously, once activated, microglia can become over-reactive [187]. This results in a neurodestructive cascade furthering damage [188]. In vitro work indicates that production of proinflammatory cytokines and growth factors can decrease the ability of astrocytes to support neuronal survival and increase the formation of tissue scarring [189]. Other studies suggest that these factors have a cytotoxic effect on oligodendrocytes as well [190–194]. Therefore, shifting microglial phenotype from proinflammatory to anti-inflammatory would potentially decrease these cytotoxic effects.

Microglia are a unique cell type that maintain two main functions within the CNS. Microglia are both supportive glial populations and immunocompetent defense cells [195]. During an infection with foreign antigen, microglia act as potent generators of proinflammatory mediators and reactive oxygen species that help drive the immune response needed to clear the brain of foreign invaders [196]. On the other hand, many studies indicate that microglia can support a neuroprotective and potentially proregenerative role in the injured CNS environment depending on their activation state [195, 197, 198]. Microglia have been found on or near the cell surface of neurons that do not undergo cell death but eventually regenerate axons [199]. This data suggests that microglia may be enhancing and supporting the recovery and regeneration of damaged neurons. Upon activation, microglia are also shown to upregulate their release of neurotrophic molecules and protective cytokines and/or chemokines [196]. Increased production of protective mediators into the microenvironment results in recruitment of neural progenitor cells to help regenerate previously lost neurons [200, 201]. Mediators can also act on surviving neurons, resident astrocytes, and other reactive microglia, shifting the brain microenvironment to one that is anti-inflammatory and restorative rather than proinflammatory and destructive [17]. For instance, early downregulation of TNF and increased levels of IL-10 have been linked to decreased scarring, decreased tissue and cell loss, and increased functional capacity following CNS injury [202, 203]. The exact mechanism in which this occurs is still under debate. However, some investigators propose the idea of "protective autoimmunity," in which having a controlled and localized proinflammatory immune response may be required for neuronal repair [204].

Classically, microglia can exhibit an activated inflammatory and neurotoxic phenotype called M1, but they can also acquire a neuroprotective phenotype termed M2 (Fig. 1) [17]. The M1 phenotype is generated in response to harmful stimuli and inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IFN-γ [205]. Generally, Th1 cells produce the cytokines necessary for this polarization, but microglia have

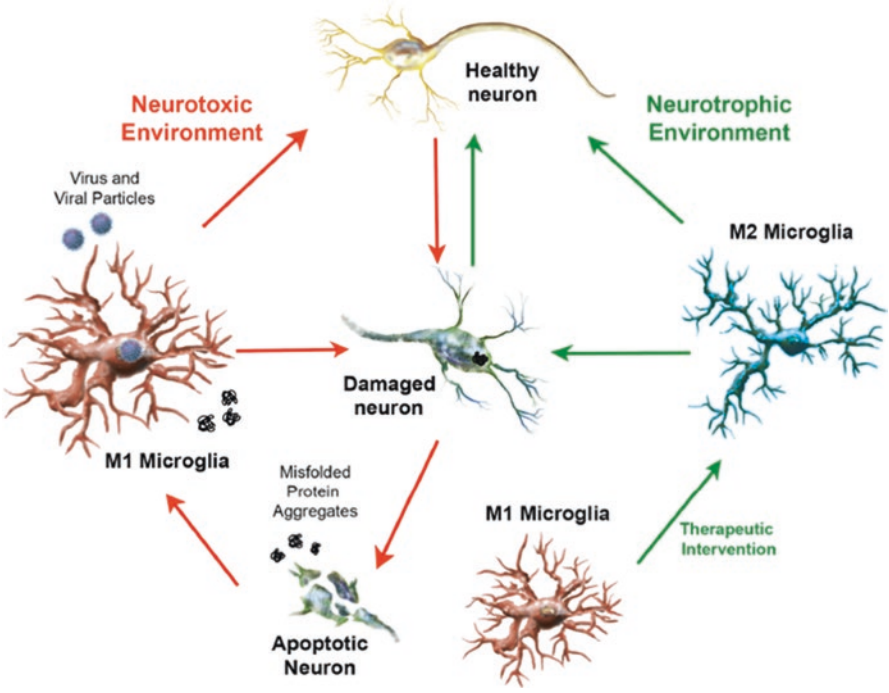

Fig. 1 Immune modulation in neurodegenerative disease. In neurodegenerative diseases, neuronal death occurs through either environmental or genetic insult. Damaged neurons undergo apoptosis, leading to microglial activation and phenotypic shift into proinflammatory M1 microglia (red arrows). This activation occurs through events such as ingestion of misfolded protein aggregates containing beta-amyloid or alpha-synuclein or through direct viral infection or ingestion of viral particles. Either way, activation leads to production of proinflammatory and neurotoxic mediators, resulting in additional neuronal death and damage of healthy neurons in the surrounding area. Therapeutic intervention through the use of immune-modulating agents can shift the M1 phenotype into a neurosupportive and neuroprotective M2 phenotype (green arrows). M2 microglia can act on damaged neurons and support neuronal growth and regeneration through the production of neurotrophic and anti-inflammatory mediators. The presence of M2 microglia also provide a neuroprotective microenvironment allowing healthy neurons to remain viable. Modulating microglial phenotypes ultimately shifts the microenvironment from neurotoxic to neurotrophic

been shown to secrete them as well, allowing them to regulate in an autocrine fashion [206]. In most cases, this response is downregulated once the damage has been cleared but in many neurodegenerative diseases, this does not occur. This leads to an uncontrolled and prolonged immune activation further exacerbating disease. The neurosupportive and protective phenotype is characterized by the production of anti-inflammatory mediators and neurotrophic factors such as insulin-like growth factor 1 (IGF-1), brain-derived neurotrophic factor (BDNF), and glial cell-derived neurotrophic factor (GDNF) [207, 208]. Therefore, in order to shift microglial populations into an M2-like anti-inflammatory and proregenerative phenotype, researchers are focusing on agents known to directly modulate these responses [209–211].

To do so, studies have been focused on utilizing M2-inducing molecules such as IL-10, resolvin D, peroxisome proliferator-activated receptor (PPAR-y) agonists, and minocycline to directly modulate microglial responses [9]. Their exact protective effects and mechanisms are discussed later in "Neurotrophic mediators, endogenous neuropeptides, and cytokines as immunomodulators." However, these two separate states may be an oversimplification. Microglia within the brain are plastic, resulting in a range of microglial phenotypes [206]. For instance, two-photon microscopy indicates that microglia within the CNS are constantly sampling the environment in order to maintain homeostasis, suggesting that they are never truly resting [206, 212]. A second target and source of neuroprotective immunity lies in modulating the adaptive immune response associated with disease initiation and progression. Currently, research is focused on the induction of immunosuppressive cell types within the periphery, such as regulatory T cells and/or tolerogenic dendritic cells. Researchers are also focused on vaccination strategies and antibody formation against proteins of interest in order to help clear protein plaques or virus associated with neuronal loss. Lastly, there have been numerous studies concentrated on the use of anti-inflammatory drugs, known immune modulators, neuropeptides, and cytokines as neuroprotective agents for the treatment of neurodegenerative and neuroinflammatory diseases. These neuroprotective targets are outlined below.

Both CD4+ and CD8+ T cells can play a dual role in neurodegeneration and neuroprotection during CNS disorders depending on their phenotype and environmental signals [8]. Therefore, targeting this portion of the adaptive immune system would provide a potential strategy to halt neurodegenerative disease progression. Treg are potent modulators of the immune system, have distinct immunosuppressive capabilities, and are characterized by the positive expression of CD4, CD25, and FoxP3 and negative expression of CD127 [213]. They maintain the ability to suppress inflammation through multiple mechanisms including inhibition of Teff differentiation and proliferation, secretion of anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β, direct killing of Teff subsets through granzyme and perforin release, blockade of T cell co-stimulation, and metabolic disruption of Teffs and APCs via uptake of IL-2 and use of CTLA-4 [44, 137] (Fig. 2). Anti-inflammatory cytokines produced by Tregs, such as IL-4, IL-10, and TGFβ, are crucial anti-inflammatory mediators that diminish neuroinflammation and increase neuroprotection [214]. Induction of Tregs contributes to development of M2 anti-inflammatory microglial phenotypes, leading to the release of neurotrophic factors, including IGF-1 and BDNF, ultimately promoting neuronal protection [206]. Similarly, from our own animal studies, we demonstrated that Tregs elicit neuroprotection of dopaminergic neurons along the nigrostriatal pathway in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesions and in hippocampal neuron populations within an AD mouse model [12, 14, 15, 138]. Other analyses indicate that Tregs have the capacity to act directly on activated microglia, resulting in an attenuation of reactivity, decreased phagocytosis and migration, and decreased production of neurotoxic factors [138, 215]. In vitro studies suggest that Tregs can mediate inhibition of proinflammatory microglial functions through the suppression of NF-κB pathways via direct cell-cell contact [215]. Specifically, Tregs elicit a

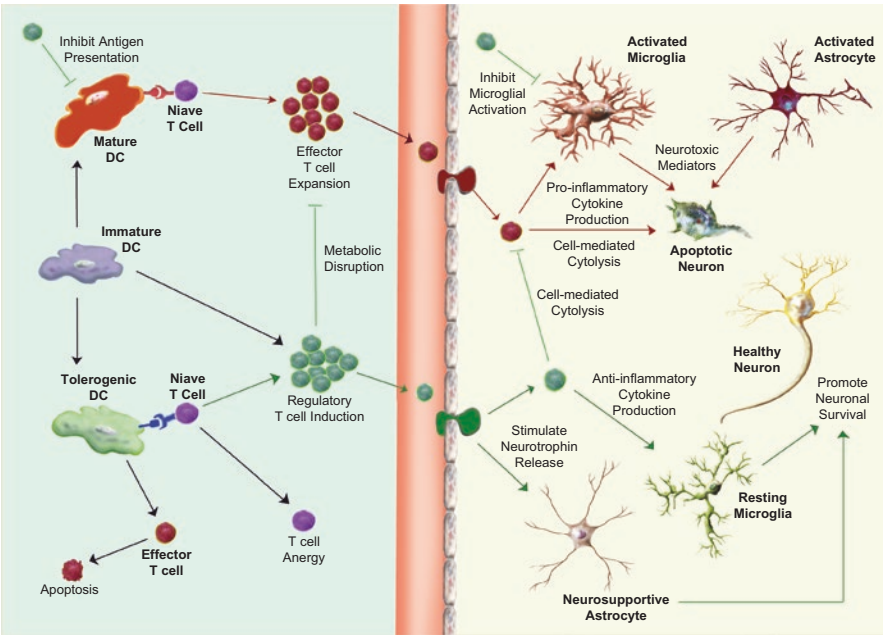

Fig. 2 Immune-mediated neuroprotection. Within the periphery, immature dendritic cells will differentiate into fully mature dendritic cells and elicit an immune response. Naïve T cells interacting with mature dendritic cells undergo clonal expansion and proliferation in response to antigen. Once activated, the effector T cell population will cross the blood-brain barrier and enter the central nervous system. Effector T cells enter the brain and secrete pro-inflammatory cytokines causing resident microglia and astrocytes to become activated. Upon activation, glia cells secrete neurotoxic and proinflammatory mediators, resulting in neuronal cell death. Effector T cells can also mediate cytolysis of neurons directly. Induction of tolerogenic dendritic cells and regulatory T cell populations can counteract this inflammatory milieu. Immature dendritic cells are also differentiated and shifted into tolerogenic dendritic cells in order to regulate immune responses. Tolerogenic dendritic cells can interact with T cells in various ways, resulting in three different end-points. First, tolerogenic dendritic cells can induce apoptosis in activated, effector T cell populations. Second, when interacting with a naïve T cell, tolerogenic dendritic cells can induce T cell anergy. Third, tolerogenic dendritic cells are potent inducers of regulatory T cell populations. Induction of regulatory T cells leads to overall immune suppression in both the periphery and the central nervous system. Regulatory T cells carry out their immunomodulatory cascade through a number of mechanisms, indicated by the green lines and arrows. These include inhibition of antigen presentation, metabolic disruption, inhibition of reactive microglial and astrocytic activation, stimulation of neurotrophin release from neurosupportive astrocytes, cell-mediated cytolysis of effector T cell subsets, and production of anti-inflammatory cytokines and suppressive molecules. Each of these mechanisms provides support for overall neuronal survival and an anti-inflammatory and neuroprotective microenvironment

potent down-regulation of proinflammatory mediators such as iNOS, TNF-α, IL-1β, IFN-γ [216]. Coincidently, this was associated with decreased levels of ROS production and NF-κB activation [217]. Utilization of this regulatory population would shift microglial responses from a neurotoxic M1 response to a neurotrophic M2

response [138]. In further support, adoptive transfer of CD3-activated Treg resulted in the attenuation of both astrogliosis and microgliosis in HIV-1-associated neurodegeneration [218]. This attenuation was associated with neuroprotection mediated by upregulation of BDNF and GDNF and downregulation of proinflammatory mediators.

On the other hand, in many neurodegenerative diseases, there is a dysregulation in the number and/or function of this suppressive cell type. For instance, in preclinical and clinical studies, we found that individuals suffering from PD have decreased levels of Tregs with a decreased ability to suppress Teff proliferation [135]. Likewise, this dysregulation was associated with increased movement disorder, indicating that the induction or enhancement of this cell population is worth investigation. This can be done through the use of immunomodulatory agents such as granulocyte-macrophage colony-stimulating factor (GM-CSF), vasoactive intestinal peptide (VIP), copolymer -1 (Cop-1), or vaccine strategies targeting Treg populations. Many of these agents are being tested in the preclinical and clinical setting. Adoptive transfer of VIP- or GM-CSF-induced Tregs following MPTP intoxication leads to significant dopaminergic neuronal sparing with a parallel decrease in microglial activation [14–16]. These findings prompted a phase I clinical trial utilizing sargramostim, a form of human recombinant GM-CSF, in patients suffering from PD [136]. This study supported the notion that Treg populations are decreased and dysfunctional in PD and that modulation and induction of this population is beneficial. Patients receiving treatment displayed increased Treg numbers, increased suppressive T cell function, and decreased motor deficits, when compared to both baseline and placebo-treated controls. Similarly, Cop-1 immunization in a model of HIVE resulted in anti-inflammatory and neuroprotective effects [219]. This immunization strategy yielded the development of T cells secreting IL-10 and IL-4, as well as an increase in the number of Treg. It was later determined that in HIVE, Tregs readily crossed the BBB and migrated to sites of infection and neuroinflammation while still maintaining phenotype and immunosuppressive function [220]. However, other studies have suggested that breaking immune tolerance through Treg targeting can actually mitigate disease-related pathologies, suggesting that the time and extent of induction may play a role in whether the result is either protective or more detrimental [221].

4 Induction of Tolerogenic Dendritic Cells

Dendritic cells (DCs) are a heterogeneous population of APCs that contribute to innate immunity and initiate the adaptive immune response associated with inflammation and autoimmunity [222]. However, apart from this, DCs also play an important role in maintaining immune homeostasis and immune tolerance [223] (Fig. 2). Unlike classical DC function, tolerogenic DCs should not stimulate T cell proliferation or inflammatory cytokine production. Instead, they act by suppressing the immune response and the effector populations required for the response. Their anti-

inflammatory response involves roles in tolerance induction and silencing the immune response. This function is mainly carried out through the induction of regulatory T cells, T effector cell apoptosis, and T cell anergy [224]. The ability of DCs to promote tolerogenic and/or inflammatory responses is related to their maturation state [225]. Generally, immature DCs expressing low levels of MHC class II and co-stimulatory molecules are responsible for generating immunosuppressive responses, whereas inflammatory adaptive immune responses are achieved by mature DCs [226]. Immature DCs have the capacity to induce and expand regulatory T cells; however, some studies have also linked mature DCs to the induction of this cell type [225]. Immature DCs can be defined by their surface marker expression. Phenotypic analysis indicates that this suppressive and regulatory population is $CD11c^{low}CD11b^{high}MHCII^{low}CD86^{low}$ and has the capacity to produce high levels of IL-10, ultimately inhibiting Teff proliferation and promoting Treg function [227]. This cell type is now considered to be tolerogenic. Along with the secretion of IL-10, tolerogenic DCs play a significant role in maintaining peripheral and central tolerance through the secretion of TGF-β, indoleamine 2,3-dioxygenase (IDO), and retinoic acid (RA) [228–230]. Tregs that come in contact with this subset exhibit parallel tolerogenic functions and anti-inflammatory functions [231]. On the other hand, tolerogenic DC interaction with activated Teff populations results in an inhibitory effect by decreasing CD4+ T cell proliferation and increasing IL-10 production.

In order to maintain the tolerogenic environment, studies show that there are reciprocal interactions between induced Tregs and tolerogenic DCs [231]. Crosstalk between both populations is needed to induce and maintain immune tolerance. Tregs are shown to modulate both the phenotype and function of DCs [232]. For instance, tolerogenic DCs promote the expansion of Tregs through the expression of PDL-1 while Tregs maintain the tolerogenic population through the production of TGF-β and IL-10 [233]. IL-10 producing Tregs can inhibit DC maturation, maintaining an immature and immunosuppressive state [234]. Furthermore, when FoxP3+ Tregs are depleted, DCs have trouble interacting with CD4+ Teffs, indicating that FoxP3+ Tregs are essential for maintaining the immune tolerant and suppressive state of tolerogenic DCs [235]. Therefore, generation of tolerogenic DCs, either naturally or pharmaceutically, would be beneficial in chronic and progressive neuroinflammatory diseases, such as PD, AD, and HIV-1-associated neurodegeneration.

In support of this, treatments with immunomodulatory agents such as VIP, rapamycin, and GM-CSF have been shown to induce tolerogenic DCs and promote immune suppression [10, 232, 236]. VIP treatment regulates DC differentiation by inducing an upregulation of CD86 in immature DCs and a downregulation of CD80 and CD86 in LPS-stimulated DCs [237]. The induced CD4+ T cells generated via VIP-treated immature DCs exhibit an anti-inflammatory Th2 phenotype as well. Similarly, another study reported that VIP induces tolerogenic DCs that cause surrounding CD4+ T cells to release anti-inflammatory cytokines such as IL-10 and TGF-β, indicating the formation of a regulatory subset rather than an effector population [238]. Likewise, in human studies, VIP treatment generated tolerogenic DCs that induced both CD4+ Tregs and CD8+ Tregs, further supporting the idea that signaling via VIP receptors (VIPRs) is involved in the generation of multiple

immunosuppressive subsets [239]. Similarly, in our own studies, treatment with GM-CSF resulted in the generation of tolerogenic DCs, as indicated by an alteration of co-stimulatory molecules and the ability to convert naïve CD4+ T cells into a Treg population [10]. Adoptive transfer of the induced tolerogenic DCs attenuated the neuroinflammatory response and spared dopaminergic neurons in a PD model.

These insights may yield potential clinical targets for the treatment of neuroinflammatory conditions. The role of DCs as an immunotherapy has been confirmed in AD and PD studies utilizing mouse models [240–243]. Administration of DCs tolerized to Aβ peptide slowed the rate of cognitive decline, increased levels of anti-Aβ antibodies, reduced Aβ plaques within the CNS, and increased spatial learning and memory [240, 241]. Intravenous injections of DCs sensitized against α-syn results in the generation of antibodies against the protein coincident with improved motor function and decreased inflammatory response associated with disease progression [242, 243]. However, translating these findings for clinical use may be challenging due to the varying phenotypes of human DCs and the ability to maintain a stable tolerogenic DC population [244]. Secondly, the tolerogenic response must be maintained for a prolonged amount of time. Due to these factors, clinical trials targeting DCs are not as common.

5 Vaccination Strategies

Modulation of the humoral immune response is a vaccination strategy directed at targeting immunogenic and pathogenic epitopes [245]. Ultimately, this therapeutic strategy focuses on ameliorating neuroinflammation by utilizing the immune system to target misfolded or aggregated proteins and/or viral particles. For instance, immunization of transgenic mice containing human α-syn with misfolded α-syn results in the production of high affinity anti-α-syn antibodies [246]. This antibody formation was associated with decreases in α-syn inclusions in neuronal cell bodies and at neuronal synapses [247]. It also results in decreased neuronal loss and overall neurodegeneration. Also, anti-α-syn antibodies supported the active degradation of α-syn aggregates. Another recent study utilizing an AAV-α-syn rat model of PD indicates that formation of anti-human α-syn N-terminal peptide antibodies can elicit neuroprotection and decrease microglial activation [246]. Vaccination led to increased production of circulating IgGs, increased MHCII expression, and augmented CD4+ T cell infiltration into the CNS [246]. Administration of monoclonal antibodies against the C-terminal region of α-syn reduces levels of protein aggregation, improving PD pathology. Monoclonal antibody treatment attenuated dopaminergic neuronal cell death and decreased motor deficits associated with disease [248–250]. Based on these findings, Roche and Prothena commercialized this approach and utilized PRX002 to specifically target α-syn (NCT02095171). Analysis from the phase I clinical trial indicated that the vaccine was safe and tolerable and ultimately prompted a second trial assessing dose, immunogenicity, and pharmacokinetics (NCT02157714). Similarly, several other studies entered clinical

trials, showing promise in the use of vaccines for the treatment of PD by demonstrating Treg recruitment, increased levels of neurotrophins, and increased antibody formation [251–253]. Collectively, these studies show that α-syn-targeted vaccine strategies have been successful and display the potential to delay dopaminergic neurodegeneration and decrease neuroinflammation.

Similarly, vaccination strategies have been pioneered for the treatment of AD. Anti-Aβ antibodies prevent formation of new Aβ plaques and help dissociate existing plaques [254–258]. The presence of these antibodies also improved learning and protected transgenic mice from developing memory loss. Moreover, the presence of naturally occurring antibodies against Aβ is reported in the CSF of AD patients, but levels are significantly lower than healthy controls, suggesting a dysfunction in the ability of AD patients to induce the desired protective humoral immune response [94]. Therefore, active and passive immunization strategies have been researched and explored for the treatment of AD [259–265]. For example, active immunization with Aβ1-42 peptide (AN1792) was tested in the clinical setting; however, the trial was halted due to unexpected meningoencephalitis and death associated with vaccine [259–262]. Post-mortem analysis showed a significant drop in the number of plaques, but vaccination did not continue due to the active neuroinflammatory response that ensued with vaccination [259, 261, 263]. Still, those that did not succumb to adverse events were monitored and appeared to benefit from the vaccine [264, 265]. Individuals with the highest antibody titers remained cognitively stable for up to 2 years post-vaccination. Because of the potential adverse events associated with this vaccination strategy, there have been a number of alternative vaccine approaches to enhance the formation of antibodies against Aβ. For instance, a synthetic and truncated form of Aβ, UB-311, is utilized as a vaccine strategy in order to break self-tolerance and limit the possibility of developing a similar T cell reaction as seen with AN1792 vaccination [266]. Other approaches include production of B cell epitopes against Aβ, DNA-based vaccines, and use of monoclonal antibodies as therapeutic options [267, 268]. Likewise, passive immunization using monoclonal antibodies against Aβ is also effective in reducing amyloid deposits in the CNS [269, 270].

Similar vaccination strategies have been utilized for the treatment of HIV-infection. Antibodies against HIV-associated proteins, such as Tat, are found in the brain and spinal fluid of infected individuals [271, 272]. Anti-Tat antibodies are also detected in the CSF of individuals suffering from HAND [273]. Recent work indicates that antibodies generated against Tat results in the suppression of Tat-induced viral replication and HIV-associated cytotoxic effects [274]. It is suggested that antibodies against Tat are also protective against NMDA-mediated excitotoxicity [275]. Taken together, it is clear that vaccination strategies may hold promise in clearing disease-causing protein inclusions and viral particles.

6 Immunomodulators and Neurotrophins

Progressive neurodegenerative disorders, such as those discussed above, present a challenge for developing treatments because of unknown time and mechanism of disease onset. As noted previously, therapies aimed at targeting neuroinflammation either directly or indirectly are now front and center. Among these therapies, use of non-steroidal anti-inflammatory drugs (NSAIDS), specifically ibuprofen, is associated with a lower risk of PD development, and is protective in MPTP and 6-hydroxydopamine (6-OHDA) induced lesions [276]. These findings suggest that there is an association with anti-inflammatory use and decreasing the probability of being diagnosed with PD. Therefore, many anti-inflammatory agents have been explored, such as minocycline and natural or endogenous compounds including resveratrol, silymarin, resolvins, and apocynin [9]. These compounds act by downregulating glial activation, decreasing proinflammatory cytokine production, suppressing M1 microglial phenotypes, reducing NF-κB activation, and decreasing amounts of reactive oxygen species present in the brain. Additionally, PPAR agonists, such as pioglitazone and rosiglitazone, also possess neuroprotective and anti-inflammatory activities both in vitro and in vivo [277–279]. These agents selectively act on decreasing the amount of reactive microglia and their secreted neurotoxic factors.

A second therapeutic target is found by modulating T cell phenotypes and functions with pharmacologic agents. Ideally, enhancing phenotypes that shut down the inflammatory response within the brain microenvironment through the use of potent immune modulating agents such as VIP or GM-CSF would be of benefit [12, 14, 16, 280, 281]. Such therapeutic interventions have been effective in PD and AD, along with other chronic inflammatory conditions and as such, support their ability to restore immune homeostasis and repair tissue injuries. Similarly, due to the wide variety of biological targets and effects of VIP, previous studies have utilized the native peptide for neuroprotection from HIV neurotoxicity [282–285]. Various studies have shown that VIP treatment prevents HIV-1 induced neuronal death [283, 284]. This protection is mediated through VIP-associated signaling within astrocytes. When astrocyte and cortical neuron cultures are treated with VIP, there is an increase in MIP-1α, beta-chemokine, and RANTES [282, 283]. This chemokine upregulation blocks the receptor interactions that are needed for viral entry and toxicity, resulting in neuronal survival. Likewise, when VIP binds to the VIPR2 on astrocytes, it induces changes in activity-dependent neuroprotective protein (ADNP), which is associated with cell survival and development, further supporting the neuroprotective effects of VIP-targeting [285].

Apart from anti-inflammatory and immune modulating therapies, researchers are also seeking to utilize neurotrophic factors within damaged brain regions [286]. Neurotrophic factors are a family of molecules that support growth, survival, synaptic plasticity, and differentiation of developing and mature neurons [287]. Thus, their use in diseases in which there is neuronal loss is intriguing. Amongst these factors are GDNF, BDNF, neurturin, and neurotrophin [286]. GDNF is

neuroprotective and restorative in the dopaminergic neuron system and has been demonstrated in multiple experimental models including rodents and primates [288, 289]. Some of these studies indicated that the degree of neuroprotection observed correlates with the amount of neurotrophin, specifically GDNF, levels present within the brain region [290]. Similarly, neurturin, a homolog of GDNF, has also shown neuroprotective efficacy with no side effects observed within a large margin of doses [291, 292]. A study using BDNF-treated neural stem cells in an AD model indicated an improved transplant effect resulting in increased memory and learning and increased overall cell survival [293]. A study utilizing neurotrophin-3 (NT3) in an ex vivo PD model showed that NT3 treatment led to an increase in cell survival, an overall neuroprotective response, and an increase in dopamine production [294]. Taken together, use of neurotrophic factors in brain diseases has shown promise as a potential clinical therapy.

7 Summary

Alzheimer's disease, Parkinson's disease, and HIV-1-associated neurodegeneration are devastating disorders of the CNS with few therapeutic avenues. Collectively, these diseases are linked to neuroinflammation and aberrant immune responses. Each involves altered innate and adaptive immune responses leading to increased glial reactivity associated with altered frequencies of T effector and T regulatory populations. Since both of these populations play an important role in maintaining a successful and healthy immune response, it is likely that their dysfunction controls the tempo of disease progression. Due to this, many laboratories have focused on harnessing the immune system for therapeutic gain. Current strategies aim to shift the neurodestructive immune phenotypes into those that are neuroprotective. The universal goal of such strategies is to suppress neuroinflammation in order to spare neuronal populations normally lost or affected during the course of disease. Throughout this chapter, we have discussed many neuroprotective strategies, including modulation of the innate glial immune response and transformation of the peripheral adaptive immune response through inhibition of proinflammatory cytokine production, induction of regulatory T cells, induction of tolerogenic dendritic cells, increased production of circulating antibodies, and various vaccination strategies. We have also discussed the protective role of anti-inflammatory agents, neurotrophins, and cytokines in diseases of the brain. Overall, researchers utilizing these strategies are attempting to modify the diseased CNS microenvironment by targeting proinflammatory glial populations directly to decrease proinflammatory and neurotoxic mediator production or by targeting them indirectly through the induction of immunosuppressive populations such as regulatory T cells and tolerogenic dendritic cells. The potential neuroprotective effects of these cell types would certainly restore the harmful inflammatory response to its normal homeostatic state. However, the immune system is also needed to clear debris and repair cellular and tissue damage, which would serve to restore homeostasis and lead to neuronal

survival and repair. Therefore, it is likely that a timed control of regulating and shifting the immune response is needed in diseases of the brain in order to maintain the highest level of therapeutic gain.

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Polycystic Ovary Syndrome: An Overview of a Complex, Heterogenous Genetic Condition

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Abstract Polycystic Ovary Syndrome (PCOS) is a heterogenous, complex, genetic trait of unclear etiology, comprising of ovarian hyperandrogenism and hyperinsulinemia. It is the most common endocrine abnormality in women of reproductive age and the most common cause of anovulatory infertility. PCOS has been shown to be associated with certain autoimmune diseases like Autoimmune Thyroid Disease (AITD) and Systemic Lupus Erythematosus (SLE). Furthermore, over a hundred candidate genes have been linked to PCOS and Genome Wide Association Studies on these are ongoing. Two among these, are the most promising chromosome 9p33.3 DENND1A (DENN/MADD domain-containing protein 1A) and 2–21 THADA (Thyroid adenoma-associated) susceptibility loci. In the majority of PCOS patients, the fundamental defect is intrinsic androgenic dysfunction termed Primary Ovarian Hyperandrogenism. Primary Ovarian Hyperandrogenism is believed to be due to the rapid, high-amplitude pulsation of Gonadotropin-Releasing Hormone (GnRH) from the hypothalamus, which causes preferential release of Luteinizing Hormone (LH) over Follicle Stimulating Hormone (FSH) from the anterior pituitary gland. Hyperandrogenemia may present as hirsutism, acne or alopecia. The pathophysiology of PCOS is multifactorial but is related to insulin resistance in many cases. Hyperinsulinemia may manifest as obesity, difficulty losing weight, prediabetes or Diabetes Mellitus Type II. Many PCOS women also have irregular and anovulatory cycles, and some have polycystic ovaries on transvaginal ultrasound. Overall, PCOS encompasses a wide range of metabolic and reproductive disorders ranging from prediabetes, to infertility, endometrial hyperplasia and endometrial cancer. Treatment of PCOS is multifaceted and aims at targeting the underlying hyperinsulinemia, hyperandrogenemia and menstrual irregularity. Biguanides (i.e., Metformin) and Glucagon-like peptide 1 (GLP-1) receptor agonists are insulin sensitizers that have been studied in the treatment of PCOS.

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1 Introduction

Polycystic Ovary Syndrome (PCOS) is a complex genetic trait of unclear etiology. It is the most common endocrine abnormality in women of reproductive age and the most common cause of anovulatory infertility [1]. An autoimmune basis of etiology has been suggested and studied but has not been substantiated with studies. Some reports link PCOS with Autoimmune Thyroid Disease (AITD) and Systemic Lupus Erythematosus (SLE) [2, 3]. AITD has been reported in 18–40% of PCOS patients. Several autoantibodies have also been linked with PCOS. There are ongoing studies looking into the association between autoimmunity and PCOS. American Gynecologists Irving F Stein Sr. and Michael L Leventhal first described this syndrome in 1935, as a triad of amenorrhea, hirsutism and polycystic ovaries [4]. They studied seven women with amenorrhea, hirsutism, and enlarged ovaries with multiple cysts, five of whom had hirsutism and acne and four had obesity. The syndrome was called Stein-Leventhal syndrome. However, the presence of sclerotic ovaries had been identified over 90 years prior to this. This syndrome was also known by other names, such as functional ovarian hyperandrogenism, ovarian hyperthecosis and sclerotic ovary syndrome. Currently this syndrome is known as Polycystic Ovary Syndrome which is a misnomer as it does not accurately reflect the features of this disorder and the presence of polycystic ovaries is not essential for the diagnosis of PCOS. Therefore, the Evidence Based Methodology Workshop by NIH in 2012 suggested renaming this syndrome [5]. One in ten women have PCOS, but prevalence may vary 5–15% based on ethnic predilection. Thus, PCOS is seen in roughly 4.8% Caucasians, 13% Latina/Hispanics and 8% African Americans. Twin studies have demonstrated a genetic basis of inheritance for PCOS [6]. Many PCOS adolescents have a mother with polycystic ovaries who may not manifest symptoms of PCOS, although maternal PCOS is a risk factor in daughters. 40% of PCOS patients may have an affected sister with PCOS. Familial factors associated with PCOS include metabolic syndrome, insulin resistance, and obesity, which may be seen in either parents, but more commonly in the father [6]. Over one hundred genes have been found to be associated with PCOS, but the vast majority of these have not been replicated in multiple studies. Sixteen of these genes have shown supporting evidence of replication from multiple reports. Two among these that are the most promising are chromosome 9p33.3 DENND1A (DENN/MADD domain-containing protein 1A) and 2–21 THADA (Thyroid adenoma-associated) susceptibility loci [6]. As of now, there is no genetic testing for the diagnosis of PCOS, but ongoing Genome Wide Association Studies may hold promise for future.

2 Criteria for Diagnosis

There are three diagnostic criteria for PCOS each suggested by a different group. The NICH/NICHD criteria was defined in 1992, the ESHRE/ASRM (Rotterdam Criteria) in 2004 and the Androgen Excess criteria in 2006 [6]. PCOS is a diagnosis of exclusion, and all three groups agree that other hyperandrogenic disorders like non-classical Congenital Adrenal Hyperplasia (NC-CAH), thyroid disorders, hyperprolactinemia, etc., must be excluded before a diagnosis of PCOS can be established [7]. The prevalence of PCOS varies based on the diagnostic criteria used, and according to a meta-analysis published in the Journal of Epidemiology in 2014, prevalence varied 6–9% based on the NIH criteria, 8–15% based on the Androgen excess criteria and 15–20% based on the Rotterdam Criteria [5]. The Rotterdam criteria is the most inclusive and hence the 2012 Evidence Based Methodology Workshop on PCOS by NIH suggested using this for the diagnosis of PCOS.

NIH/NICHD 1992	ESHRE/ASRM (Rotterdam criteria) 2004	Androgen excess society 2006
Exclusion of other androgen excess or related disorders	Exclusion of other androgen excess or related disorders	Exclusion of other androgen excess or related disorders
Includes all the following: Clinical and/or biochemical hyperandrogenism	Includes two of the following: Clinical and/or biochemical hyperandrogenism	Includes all the following: Clinical and/or biochemical hyperandrogenism
Menstrual dysfunction	Oligo-ovulation or anovulation	Ovarian dysfunction and/or polycystic ovaries
	Polycystic ovaries	

Adult Phenotypes Specification of PCOS phenotypes was proposed by the 2012 Evidence Based Methodology Workshop by NIH for research and clinical purposes. There are four adult phenotypes listed in decreasing order of specificity and severity [8, 9]:

Phenotype 1 (Classic PCOS)- most severe

- Clinical and/or biochemical evidence of hyperandrogenism
- Evidence of Oligo-anovulation
- Ultrasound evidence of polycystic ovary

Phenotype 2 (Hyperandrogenic anovulation)

- Clinical and/or biochemical evidence of hyperandrogenism
- Evidence of oligo-anovulation

Phenotype 3 (Ovulatory PCOS)

- Clinical and/or biochemical evidence of hyperandrogenism
- Ultrasound evidence of polycystic ovary

Phenotype 4 (Non-hyperandrogenic PCOS)

- Evidence of oligo-anovulation
- Ultrasound evidence of polycystic ovary

3 Pathophysiology

In the majority of PCOS patients, the fundamental defect is intrinsic androgenic dysfunction termed as a Primary Ovarian Hyperandrogenism. The high intraovarian androgen concentration stimulates the ovaries, causing excessive growth of the small ovarian follicles and inhibiting follicular maturation and development of a dominant follicle. It also causes premature luteinization of the follicles and hyperplasia of the thecal, stromal and cortical cells which results in anovulation and the polycystic appearance of ovaries [10]. Primary Ovarian Hyperandrogenism is believed to be due to the rapid, high-amplitude pulsation of Gonadotropin -Releasing Hormone (GnRH) from the hypothalamus, which causes preferential release of Luteinizing Hormone (LH) over Follicle Stimulating Hormone (FSH) from the anterior pituitary gland. The high LH acts on the theca cells resulting in release of high levels of androstenedione and testosterone. The testosterone is then converted in the granulosa cells by the aromatase enzyme to estradiol. The high estradiol level paradoxically inhibits follicular maturation. There is also peripheral conversion of androstenedione to estrone and testosterone, and these hormones further stimulate the LH release from the anterior pituitary. In women without PCOS, increase in LH level above steady state causes desensitization of theca cells by down regulating the LH receptors on the theca cells. This in turn inhibits ovarian steroidogenesis. In PCOS, there is partial escape from LH receptor downregulation, and ovarian steroids are hyperresponsive to LH [10, 11] (Fig. 1).

Overall, 50–70% of women with PCOS demonstrate clinically measurable insulin resistance in vivo, above and beyond what can be expected for their body weight [7]. Insulin directly stimulates the rapid high-amplitude pulsation of GnRH from hypothalamus. There are Insulin-like growth factor-1 (IGF-1) receptors on the ovarian theca cells, and insulin directly stimulates the theca cell to secrete androgens. Insulin also inhibits the production of hepatic sex-hormone binding globulin (SHBG), resulting in an increase in the level of free testosterone. Hypersensitivity to insulin also exists in lean women with PCOS who are not insulin-resistant [7, 10]. All treatments that aim at lowering insulin levels including weight loss, will improve ovarian androgen excess and promotes ovulation. 30–40% of PCOS patients have adrenal hyperandrogenemia in addition to ovarian hyperandrogenemia. The rapid high-amplitude pulsation of GnRH causes the release of Adrenocorticotropic Hormone (ACTH) from the pituitary gland, which in turn stimulates the adrenal glands to release dehydroepiandrosterone sulfate (DHEAS), androstenedione, and testosterone LH [10, 12, 13].

The association of autoimmunity and inflammation in PCOS patients has been extensively studied. The following autoantibodies have been linked to PCOS: Anti-Nuclear Antibodies (ANA) were linked to PCOS in a study showing that the number of ANA positive cases increased from 8.6% to 28.6% in patients with PCOS following electrocauterization [14]. This same study reported zero cases of ANA in the control group. This result suggests there is some association between the disease and autoimmunity [14]. Another study showed significantly higher serum levels of Anti-dsDNA in patients with PCOS compared to a control group [15]. Anti-Ro

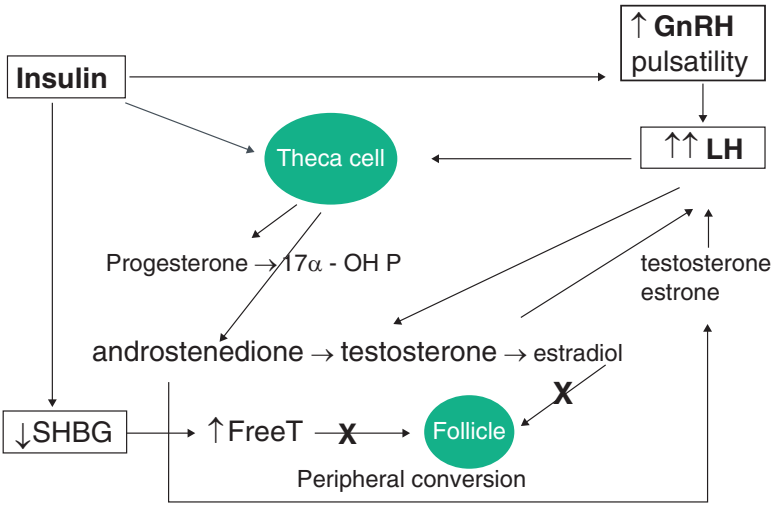

Fig. 1 Pathophysiology of PCOS. Overall, 50–70% of women with PCOS demonstrate clinically measurable insulin resistance in vivo, above and beyond what can be expected for their body weight. Insulin directly stimulates the rapid high-amplitude pulsation of GnRH from hypothalamus. There are Insulin-like growth factor-1 (IGF-1) receptors on the ovarian theca cells, and insulin directly stimulates the theca cell to secrete androgens. Insulin also inhibits the production of hepatic sex-hormone binding globulin (SHBG), resulting in an increase in the level of free testosterone. Hypersensitivity to insulin also exists in lean women with PCOS who are not insulin-resistant. All treatments that aim at lowering insulin levels including weight loss, will improve ovarian androgen excess and promotes ovulation

(SSA) was the main subtype identified of ANAs in positive cases of PCOS [14]. Anti-thyroglobulin levels were not found to be significantly different between patients with PCOS and control groups. However, Kachuei et al. reports greater levels of anti-thyroglobulin in patients with PCOS [16]. Anti-TPO was found to be significantly higher in patients with PCOS when compared with patients in a control group [16]. This finding supports the assessment of thyroid function and autoimmunity in patients with PCOS. Antibody to protein tyrosine phosphatase was shown to be associated with a low risk of progression to type 1 diabetes [17]. Anti-histone antibody was shown to be higher in patients with PCOS than patients with unexplained fertility and healthy fertile subjects [15]. Anti-carbonic anhydrase-1 mean serum levels were found to be significantly higher in women with PCOS compared with control subjects [18]. Anti-spermatic antibody was shown to be significantly correlated with higher scores of Hirsutisms in patients with PCOS. Hirsutism is a well-known symptom of PCOS. Islet cell antibodies were associated with a low risk of progression to type 1 diabetes [17]. In the presence of other islet autoantibodies, a high risk of progression to diabetes was observed. GAD was shown to be associated with a low risk of progression to type 1 diabetes. Insulin autoantibodies was shown to be associated with a low risk of progression to type 1 diabetes [17]. However, autoimmunity as the etiological cause of PCOS has not been supported by

several studies. One possibility that has been suggested that the non-organ-specific autoantibodies lead to systemic immune activation in PCOS women. This could explain the frequent association between PCOS and autoimmune diseases, especially Autoimmune Thyroid Diseases (AITD) [2].

4 Clinical Features

PCOS usually presents in adolescence, and hyperandrogenism is one of the most common presenting complaints. PCOS is responsible for 85% of androgen excess in adolescent females [19]. Hyperandrogenemia may present as hirsutism, acne or alopecia. Hirsutism is seen in 70% of women with PCOS. In hirsutism there is conversion of the female pattern vellus hair to the male pattern terminal hair and this is seen commonly on upper lip, chin, around nipple, and along the linea alba of lower abdomen. Figure 2 Some PCOs females with high testosterone level may not present with hirsutism as they lack testosterone receptors on the skin. This can be seen especially in some Asian females. Alternatively, hirsutism may occur without elevated testosterone level, which can be referred as an idiopathic hirsutism and these patients should not be labelled as having PCOS. Therefore, elevated testosterone level along with hirsutism is a more reliable indicator of hyperandrogenism. Alopecia may be seen in 10% of PCOS females [4, 6]. PCOS patients with alopecia may present with male pattern hair loss with fronto-temporal-occipital baldness or female pattern hair loss typically affecting the crown and manifesting early as a widening midline parting in a 'Christmas tree' pattern. Figure 3 PCOS patients are

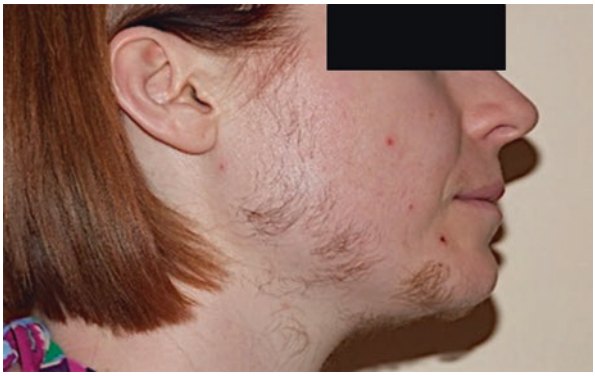

Fig. 2 Hirsutism in PCOS. Hirsutism is seen in 70% of women with PCOS. In hirsutism there is conversion of the female pattern vellus hair to the male pattern terminal hair and this is seen commonly on upper lip, chin, around nipple and along the linea alba of lower abdomen. Some PCOS females with high testosterone level may not present with hirsutism as they lack testosterone receptors on the skin. This can be seen especially in some Asian females. Alternatively, hirsutism may occur without elevated testosterone level and this is idiopathic hirsutism and these patients should not be labelled as having PCOS. Therefore, elevated testosterone level along with hirsutism is a more reliable indicator of hyperandrogenism

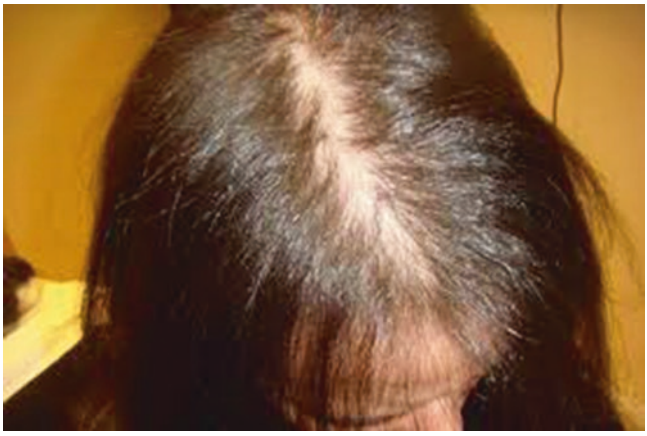

Fig. 3 Female pattern hair loss in PCOS. Alopecia may be seen in 10% of PCOS females. PCOS patients with alopecia may present with male pattern hair loss with fronto-temporal-occipital baldness or female pattern hair loss typically affecting the crown and manifesting early as a widening midline parting in a 'Christmas tree' pattern

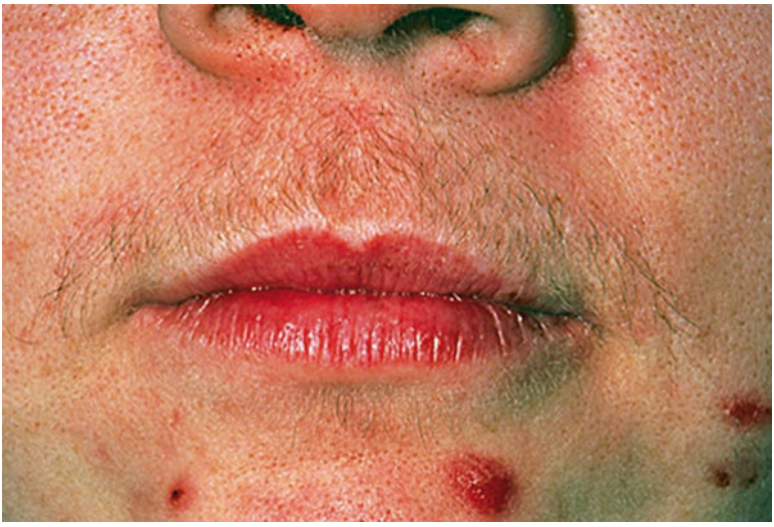

Fig. 4 Pustular acne in PCOS. PCOS patients are prone to develop moderate to severe inflammatory acne in unusual locations, especially in the anterior chest and back. One paper estimated the incidence of acne to be 20–40% in PCOS patients

also prone to develop moderate to severe inflammatory acne in unusual locations, especially in the anterior chest and back. Figure 4 it has been estimated the incidence of acne to be 20–40% in PCOS patients [4]. Alternate cutaneous manifestations of hyperandrogenism include seborrhea (may manifest as white flaky skin in eyebrow and face), hyperhidrosis or hidradenitis suppurativa [20, 21].

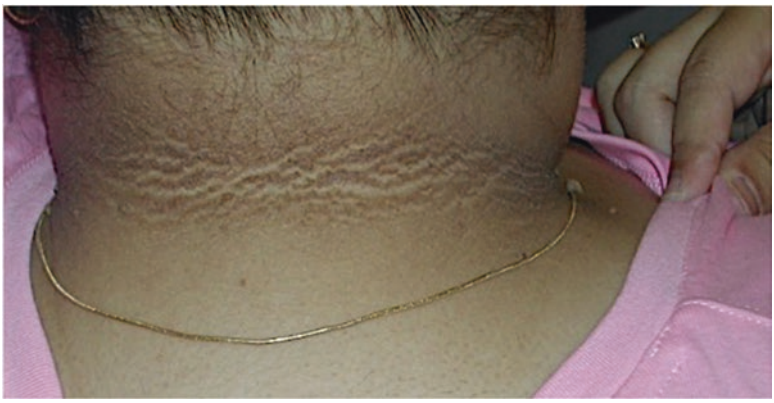

Fig. 5 Acanthosis nigricans in PCOS. PCOS women may also present with acanthosis nigricans which is hyperpigmented, thick, velvety areas in skin creases, and folds. Acanthosis nigricans is most commonly seen in the elbows, knuckles, back of the neck, or knees and is a sign of insulin resistance. Insulin resistance may be cause development of skin tags in some women

PCOS patients are usually obese and have a difficult time losing weight due to the underlying insulin resistance. Rapid weight gain and obesity are seen in 35–50% of PCOS females. Gestational Diabetes Mellitus is two and a half times more common in PCOS than in normal females. 10% of PCOS women develop Diabetes Mellitus Type II by age forty [22]. PCOS women may also present with acanthosis nigricans which is hyperpigmented, thick, velvety areas in skin creases, and folds. Figure 5 acanthosis nigricans is most commonly seen in the elbows, knuckles, back of the neck, or knees and is a sign of insulin resistance. Insulin resistance may cause development of skin tags in some women [20, 23].

Abnormal menstrual cycles may be seen in 60–70% of PCOS patients and is one of the most frequent complaints of PCOS women and the reason why they seek evaluation and treatment. Abnormal menses is common during puberty, due to the immaturity of the Hypothalamic-Pituitary-Ovarian (HPO) axis. Therefore, consensus groups have urged caution before labelling hyperandrogenic adolescents as having PCOS if the menstrual abnormality has not persisted for 2 years or more. Many PCOS adolescents have delayed menarche followed by irregular menstrual cycles or they may have normal cycles at menarche which becomes abnormal with weight gain [5]. Interestingly 85–90% of women with oligomenorrhea on further work-up turn out to have PCOS and 30–40% of women with amenorrhea have PCOS. Females with PCOS may also present with menorrhagia and metrorrhagia due to the unopposed estrogen action. PCOS patients also exhibit anovulation or oligo-ovulation which leads to subfertility or infertility. Even if they do conceive, chances of multiple miscarriages are 20–40% higher than in the general obstetric population. There is also a much higher incidence of preterm birth and stillbirths in pregnant PCOS women. Many PCOS females have a higher incidence of cardiovascular risk factors like hypertension or hyperlipidemia, at a younger age [6].

5 Co-morbidities

Due to the underlying insulin resistance seen in PCOS patients, gestational diabetes is two and a half times more common in PCOS patients. Ten percent of PCOS patients develop Diabetes Mellitus Type II by age 40. DM type II is 3–5 times more common in PCOS patients. Twenty five precent of PCOS patients have metabolic syndrome and this is three times more common in PCOS patients. Sleep apnea/disordered breathing is 30–40 times more common in PCOS patients. Interestingly sleep apnea in PCOS patients is not related to the patient's weight or androgen level but is related to the underlying insulin resistance [24]. Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH) are seen in 6.7% of PCOS patients [25]. Estrogen excess of PCOS increases the risk of developing endometrial hyperplasia, atypia and cancer. One study found that PCOS patients were three times more likely to develop endometrial cancer [26, 27]. The risk of PCOS patients developing ovarian and breast cancer has not been substantiated. Depression is four times more common in PCOS patients. One study found that the depression in PCOS patients was not related to obesity or symptoms of hyperandrogenemia [28].

The data on the direct effects of PCOS on cardiovascular disease is conflicting. However, we do know that women with PCOS are more likely to have a higher level of small dense low-density lipoprotein (LDL) particles when compared to women of similar Body Mass Index (BMI) and insulin resistance without PCOS [6]. Small dense LDL particles are strongly associated with an increased risk of coronary heart disease. There is higher incidence of coronary calcification, aortic calcification, and increased carotid intima media thickness (CIMT) in PCOS women compared to controls. Women with PCOS may also have more extensive coronary disease on angiography when compared to normal women [29]. This was illustrated in a report of women younger than 60 years of age, who were undergoing coronary angiography for assessment of chest pain or valvular disease. Dyslipidemia is highly prevalent in patients with PCOS [30] PCOS patients are also at higher risk of developing hypertension at a younger age [31]. PCOS patients who have been treated with high dose of metformin for a long period of time have an increased risk of developing vitamin B12 deficiency and therefore should have their vitamin B12 level checked at least yearly [32, 33]. A meta-analysis on pregnancy outcomes in women with PCOS demonstrated a significantly higher risk of developing gestational diabetes mellitus, pregnancy-induced hypertension, preeclampsia, and preterm birth in PCOS patients [31].

6 Diagnosis

PCOS is a diagnosis of exclusion, therefore other causes of amenorrhea and hyperandrogenemia must be excluded, before establishing the diagnosis. In patients who have signs of virilization (rapid development of deepening of voice, muscle development and clitoromegaly) adrenal or ovarian androgen secreting tumor must be

considered and appropriate evaluation for these must be done. In patients without signs of virilization and with PCOS phenotype, the following work-up is recommended. In women who present with symptoms consistent with PCOS, serum total testosterone concentration provides the best overall estimate of androgen production. Even though free testosterone measurement is the single most sensitive test to diagnose hyperandrogenemia, the currently available radio immune assays are not accurate. If free testosterone test is ordered, the method used should be equilibrium dialysis. If the total testosterone level is >200 ng/dl, virializing ovarian tumor must be considered and a pelvic MRI should be ordered. Mild elevation of DHEAS is seen in 30–40% females because in addition to ovarian hyperandrogenemia, many PCOS women also have a component of adrenal hyperandrogenemia. However, if the DHEAS level is more than 700, adrenal tumor must be considered, and CT scan of the adrenal glands must be checked for evaluation [4, 6]. The dexamethasone androgen-suppression test (DAST) helps in delineating the ovarian and adrenal dysfunction of polycystic ovary syndrome (PCOS) and will help differentiate other disorders that mimic PCOS. The response of serum androgens (testosterone and DHEAS) and serum cortisol to dexamethasone are the primary outcomes measured. Serum FSH, LH and estradiol levels must be checked in women who present with amenorrhea. If FSH level is high and the estradiol level is low, this indicates premature ovarian failure (POF). If the FSH level is low and the estradiol levels is low to normal, this indicates hypothalamic amenorrhea, which is common in adolescent females. A LH/FSH ratio of 2 or 3:1 is also diagnostic of PCOS. It is also important to check Thyroid Stimulating Hormone (TSH) and prolactin levels to rule out hypothyroidism and hyperprolactinemia respectively, as the cause of amenorrhea. 17-hydroxy progesterone (17-OHP) level is used to rule out non-classical CAH, which resembles PCOS. Patients with non-classical CAH may have a combination of amenorrhea, hyperandrogenemia and polycystic ovaries (PCO) [34]. This is however prevalent in certain ethnic populations, especially in women of Eastern European Jewish (1:27 prevalence), Hispanic, Slavic or Italian descent. It is important to measure an early morning sample of 17-OHP as there is diurnal variation of this hormone. It is also essential to check an early follicular sample of 17-OHP level. A 17-OHP value of >200 ng/dL is suggestive of nonclassical CAH in an anovulatory cycle but is also compatible with recent ovulation. The high dose cosyntropin (ACTH) stimulation test is recommended to confirm the diagnosis of CAH [35]. If the serum 17-OHP is <1000 ng/dl post ACTH simulation, this excludes the diagnosis of CAH because most CAH patients will have a level of >1500 ng/dl [36].

In women who have cushingoid appearance, 24-hour urine cortisol or urine free cortisol level must be measured to rule out Cushing syndrome. In women with acromegalic phenotype, insulin-like growth factor 1 (IGF-1) level and Oral Glucose Tolerance Test (OGTT) should be checked. Transvaginal ultrasound is the best test for diagnosis of polycystic ovaries (PCO). However, this is not essential for the diagnosis of PCOS if the patient meets the other diagnostic criteria for PCOS. PCO can be seen in around 20% of females without PCOS. This includes women with hypothalamic amenorrhea, hyperprolactinemia, and in normal adolescent females [5]. Hence, consensus groups have urged against using PCO as a criterion for diagnosis of PCOS especially in adolescent females.

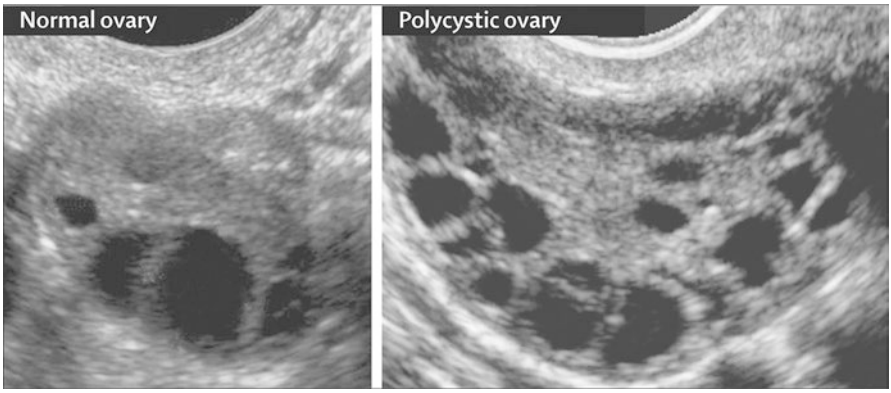

Fig. 6 Polycystic ovary and String of pearl appearance in PCOS. Follicles are arranged in the rim of the ovary in a string of pearl fashion. This appearance is diagnostic of PCOS

Sonogram Morphology for diagnosis of PCO includes the following [37]:

- 12 or more follicles in each ovary measuring 2–9 mm in diameter (25 or more follicles when using newer ultrasound machines)
- ± Increased ovarian volume of >10 mm
- ± String of Pearls (Follicles arranged in the rim of the ovary in a string of pearl fashion. This appearance is diagnostic of PCOS)
- The presence of these findings in a single ovary is sufficient for the diagnosis of PCO (Fig. 6).

7 Treatment

Treatment of PCOS is multifaceted and aims at targeting the underlying hyperinsulinemia, hyperandrogenemia, and menstrual irregularity [6]. Lifestyle modification is a key factor in the treatment of PCOS patients [38]. It is imperative to educate patients on the importance of low carbohydrate, low glycemic diet and the need for regular exercise. For PCOS patients, consistent daily low to moderate intensity exercise is probably more beneficial than high intensity exercise done 2 or 3 days per week. Even a 5–10% weight loss will significantly help in normalization of most biochemical abnormalities of PCOS and can cause resumption of cycles. Biguanides and Glucagon-like peptide 1 (GLP-1) receptor agonists are insulin sensitizers that have been studied in the treatment of PCOS. Metformin (Biguanide) improves insulin sensitivity, the endocrine and metabolic profiles, hyperandrogenemia and in addition helps with weight loss in obese PCOS patients, according to data from multiple clinical studies [39]. The treatment is usually initiated with 500 mg dose of metformin. This dose is gradually increased by 500 mg on a weekly basis as tolerated, to a maximum dose of 2 gm/day, which is usually achieved in

'four weeks' time. This is the dose at which most patients experience maximum benefit. Metformin has been also been shown to increase ovulation rates when compared to placebo, in six Randomized Control Trials (RCT) [40]. The GLP-1 agonist, liraglutide has been shown to be beneficial in treating PCOS patients in several studies [41]. They are very effective with weight loss and in reducing insulin resistance in PCOS patients. One RCT showed that combination of liraglutide and metformin improved the androgen profile beyond weight reduction and was associated with better tolerability [42]. A review of the available clinical trials of GLP-1 use in PCOS, showed that exenatide and liraglutide are effective in weight reduction, reducing androgen levels, increasing menstrual frequency, and improving the glucose parameters and eating behavior [43, 44]. Hyperandrogenic features especially alopecia, acne, and hirsutism can be extremely bothersome to women with PCOS. Oral Contraceptive Pills (OCP's) are very effective in suppressing testosterone levels and are helpful in addressing the hyperandrogenic symptoms, but the concern is that these may worsen insulin resistance in PCOS and may also increase the risk of venous thromboembolism (VTE) in obese women [45]. A good option when choosing OCP's would be to start one containing 20 mcg of ethinyl estradiol combined with a progestin with minimal androgenicity and with less likelihood of causing VTE, like norgestimate, norethindrone or norethindrone acetate [46]. Drospirenone and desogestrel are great options for progestin with anti -androgenic properties, but are associated with increased risk of VTE, so are not recommended for PCOS patients [47]. Spironolactone can be used to treat hyperandrogenic symptoms in PCOS females. Spironolactone, an aldosterone antagonist, primarily acts by binding to the androgen receptor as an antagonist. It also inhibits adrenal and ovarian steroidogenesis, competes for androgen receptors in the hair follicles and directly inhibits 5-α-reductase activity. It may typically take around 6–8 weeks for the effects of spironolactone to become apparent [45]. Finasteride is another anti-androgenic agent that may be used to treat alopecia in PCOS. Finasteride competitively inhibits tissue and hepatic 5-α-reductase, thus preventing the conversion of testosterone to dihydrotestosterone and thereby suppressing serum dihydrotestosterone level [48]. Vaniqa (eflornithine hydrochloride cream 13.9%) is a topical drug that inhibits hair growth and is used to treat hirsutism in PCOS patients. Eflornithin acts by inhibiting the enzyme ornithine decarboxylase in the skin, which inhibits cell division and synthetic functions, and therefore reduces the rate of hair growth. It must however be used indefinitely to prevent regrowth of hair [49]. Electrolysis or laser treatment can be used to treat hirsutism in PCOS, but it is important to ensure that the testosterone level is low before starting treatment. Otherwise hirsutism may recur following expensive laser treatment [45]. Hormonal contraception and androgen receptor blockers are effective in improving menstrual irregularity, reducing serum androgens and improving hirsutism, but do not improve insulin sensitivity [6]. The chronic anovulation and hyperestrogenemia seen in PCOS are associated with an increased risk of endometrial hyperplasia and possibly endometrial cancer [26, 50]. To prevent endometrial hyperplasia, it is important to induce cyclic bleeding at least every 2 months in PCOS patients. This may be achieved by either using combination estrogen and progesterone OCP's or proges-

terone only pill like minipill daily, to induce cyclic monthly bleeding. Alternatively, patients may be advised to try progesterone either as medroxyprogesterone acetate 10 mg daily or prometrium 200 mg daily, for 10–14 days each cycle to induce a withdrawal bleeding [4]. Progesterone based IUD like mirena are also effective in preventing endometrial hyperplasia. Thiazolidinediones (TZD) have been tried in the treatment of PCOS. They improve insulin resistance and menstrual frequency but have no effect on the serum testosterone level. They also have limited efficacy and there is concern for weight gain and toxicity. Statins have also been studied in the treatment of PCOS. The rationale behind using this was that it helps reduce adrenal hyperandrogenemia in PCOS [6, 45, 51, 52]. Statins do improve low-density lipoprotein cholesterol and lowers the serum testosterone slightly, but has little impact on insulin resistance, improvement in menses, hirsutism or acne [53]. There have been some clinical studies looking into the effects of using two inositol isomers, myo-inositol (MI) and D-chiro-inositol (DCI) in treatment of PCOS [54]. These have inconsistent effect on PCOS ovarian function, including improvement in insulin resistance and serum androgen levels. Some studies have investigated using fenugreek Seed Extract (Trigonella foenum-graecum, Furocyst). While fenugreek may help with resumption of cycles and reduction of ovarian volume in some women, it has no consistent effect on PCOS [55]. Therefore, consensus groups have recommended against using thiazolidinediones, statin, inositol isomers or fenugreek seed extract in the treatment of PCOS. For PCOS women who have difficulty conceiving, referral to a fertility specialist is recommended. Many PCOS patients may require ovulation induction with letrozole or clomiphene to achieve successful pregnancy [56].

8 Conclusions

PCOS is a heterogenous, complex, genetic trait of unclear etiology, comprising of ovarian hyperandrogenism and hyperinsulinemia. Over a hundred candidate genes have been linked to PCOS, and Genome Wide Association Studies on these are ongoing. The pathophysiology of PCOS is multifactorial but is related to insulin resistance in many cases. Most PCOS women demonstrate clinical and biochemical signs and symptoms of hyperinsulinemia and hyperandrogenemia. Hyperinsulinemia may manifest as obesity, difficulty losing weight, prediabetes or Diabetes Mellitus Type II. Hyperandrogenemia may manifest as acne, hirsutism or alopecia. Many PCOS women also have irregular and anovulatory cycles, and some have polycystic ovaries on transvaginal ultrasound. The complications of PCOS encompasses a wide range of metabolic and reproductive disorders ranging from prediabetes, metabolic syndrome and Diabetes Mellitus Type II, to infertility, endometrial hyperplasia and endometrial cancer. Studies have also shown a link between PCOS and Obstructive Sleep Apnea, nonalcoholic steatohepatitis and cardiovascular disease. The treatment of PCOS is multifaceted and aims to reduce insulin resistance, regulate cycles and address patient's concern of acne, hirsutism and alopecia.

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