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Immunoregulatory Aspects of Immunotherapy

Edited by Seyyed Shamsadin Athari

IMMUNOREGULATORY ASPECTS OF IMMUNOTHERAPY

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Meet the editor

Dr. Seyyed Shamsadin Athari (DVM, MPH, PhD) is Assistant Professor of Immunology in the Department of Immunology, School of Medicine, Zanjan, University of Medical Sciences, Zanjan, Iran. He has an allergy and asthma toxicology postdoc and an asthma management and controlling network fellowship. He has published more than 70 manuscripts in international journals and edited/coauthored more than 25 books in the fields of immunology, allergy and asthma. Dr. Athari is also on the editorial board of more than 50 international journals in medical sciences, has a number of inventions in medical sciences and has recorded gene sequences in gene banks. He has been invited to be keynote speaker at more than 30 international congresses and symposiums and has received scientific awards from different scientific societies as young top researcher and young scientist.

Contents

Foreword

Over the two decades of my experiences in medical immunology and laboratory medicine, I have been faced with many cases of difficult-to-manage and challenging problems. Some cases become more troublesome when a disease develops into a more challenging problem. This issue was becoming more problematic when I discovered that medical students, including my students and the families themselves, had very poor information concerning how to react when treating children suffering from diseases.

Worryingly, during those years, I felt that most of my efforts to relieve the suffering and pain of patients were in vain because there were neither advanced treatment strategies nor a comprehensive reference source of information on the subject. Fortunately, I have been encouraged by the game-changing progress in immunology in recent decades. Discovery of the receptors that play a crucial role in recognizing the microbial particles and antigen presenting in dendritic cells have widen our perspective of how the immune system acts, and has therefore paved the way for developing novel therapies to cure these allergies more effectively. It was my great honor to be associated with Dr. Athari and establish some of the first mechanistic-based research on the subjects of immunology and allergy in Iran, relying mostly on the recent advances in immunology. I was thinking that although we have been blessed with new knowledge in immunology there is also a crucial requirement for an updated reference book that summarizes these advances and helps students to better understand the past and current knowledge on the new strategies of immunotherapy.

This book is an enthusiastic compendium of well-organized and easy-to-read articles that simplifies the scientific topics in the field of immunotherapy. The book is well suited for both medical students and academic teachers. I believe the authors have made great efforts to provide the best scientific topics and make the book a valuable source of information in its field.

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Preface

When human beings become aware of illnesses, they attempt to prevent diseases and take care of their bodies in a safe and healthy fashion. The immune system has a main role in this sense and humankind has had to learn how to increase the power of the body and immune system against all harmful agents. Moreover, therapeutic and preventive strategies are innovative and with new diagnostic methods are leading to a revolution in medical sciences.

Frequent use of chemical and natural drugs has side effects and after the discovery of new therapeutic agents and drugs, these wide-ranging side effects can be interchangeable. In recent years, applications of the immune system and its mechanisms are improving the defense against all diseases and unsafe conditions and are at the center of medical sciences. Immune-mediated protection and cures for sickness are new important strategies. In terms of immunotherapy these methods are applicable to all communicable and non-communicable diseases. The recent advances in immunologic responses and the molecular mechanisms involved in the establishment of good health have enabled a new approach in the treatment of abnormality. Immunotherapy methods can also be used to recognize the diseases' pathophysiology, management and control, diagnosis and prevention, which can be important sources of information.

Therefore, immunotherapy as a new and valuable method in medicine has many aspects that can be used in all health and hygiene fields. This book is a result of a collaboration between eminent experts in the field of immunology, immunotherapy, medical sciences and analytic and diagnostic methods, concisely covering the mentioned topics. I organized the chapters logically from the basic to the deeply mechanistic, followed by advanced approaches, and finally the prevention, diagnostic and therapeutic strategies. I hope this book will answer these intricacies and help students (both undergraduate and postgraduate) and researchers to understand the exact immunological pathways involving immunotherapy and that it will provide advanced knowledge in the field of immunotherapy for academic researchers, immunologists, physicians and specialists.

In addition, this book benefits from providing the most recent advances so that we may have a better understanding of immunotherapy. Special thanks are due to Lada Bozic for her kind assistance for organizing and skillfully editing the text.

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Chapter 1

Immune System Disorders: Hypersensitivity and Autoimmunity

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Abstract

The immune response is known as a physiological mechanism to protect the body, providing defense to different systems that compose it and allowing its proper functioning. The ability to keep the organism free from foreign agents depends on the mechanisms of natural resistance or innate immunity, as well as the resistance that can develop over time through adaptive immunity. However, when these defense mechanisms fail, it can trigger injuries and diseases in the tissues, such as hypersensitivity, which is characterized as an excessive and undesirable reaction, produced by the immune system; as well as autoimmunity, which refers to the failure of the mechanisms of immunological tolerance, causing the reaction of the immune system against the body itself.

Keywords: innate immune response, adaptive immune response, histocompatibility, immune tolerance, hypersensitivity diseases, autoimmune diseases

1. Introduction

The immune system is characterized by both innate and adaptive immune responses. The innate response is characterized by the recognition of molecular patterns associated with damage and pathogens, whose molecules and receptors are fixed in the DNA of the germ line. Adaptive immunity is an antigen-specific response which is relatively slow, since it

requires a genetic rearrangement [1]. The main objective of the immune system is the defense against pathogens through these innate and adaptive mechanisms [2, 3]. However, dysfunction or deficiency of the immune system can lead to tissue injuries and diseases. On the one hand, there are hypersensitivity diseases, which are characterized by excessive and undesirable reactions, produced by the immune system [4]. On the other hand, autoimmune diseases refer to the failure of the immunological tolerance mechanisms, causing reactions against own cells and tissues [5].

2. Innate immune system

The innate immune system is the first line of defense against invading pathogens. It has a double role to provide initial control of the infection and initiate an adaptive immune response. The innate immune system consists of physical barriers such as epithelial layers and mucus, soluble factors such as the complement system, soluble mediators, cytokines and cells such as neutrophils, macrophages and dendritic cells [6]. These immune cells detected pathogens based on their molecules or pathogen-associated molecular patterns (PAMPs) that are recognized by multiple classes of pattern-recognition receptors (PRRs) that initiate inflammatory responses [7]. PRRs can also recognize host molecules containing damage-associated molecular patterns (DAMPs), molecules that are released from cells damaged [8]. Then, these PRRs respond by producing several soluble mediators such as the complement system and proinflammatory cytokines to kill microbes or infected cells [1].

2.1. Immune innate system cells

The cells of the innate immune system have several functions that are essential for the defense of the organism. These cells respond by producing inflammatory cytokines and some of them are responsible for removing foreign substances, pathogens or infected cells. Some of the innate immune cells include macrophages, dendritic cells, neutrophils, mast cells, basophils and eosinophils.

2.1.1. Macrophages

Macrophages function as cells that capture and degrade agents that are not recognized as belonging to the organism, in addition to being antigen-presenting cells; therefore, they are essential in both types of immunity (innate and adaptive) [9]. Macrophages are formed in the bone marrow from myeloid progenitor cells, which when stimulated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) are converted into monocytes, immature cells that are released into the bloodstream. Monocytes mature when stimulated by chemotactic substances, making them migrate to tissues as mature cells, establishing themselves for a lifetime of weeks to months. This cell type is directly related to the inflammatory response, since phagocytosis uses harmful substances that can cause acute cell injury and promote apoptosis, including reactive oxygen species (ROS), high amounts of nitric oxide and halogenating radicals. Other mechanisms that promote inflammation are through the production of cytokines such as interleukin (IL)-6

and tumor necrosis factor (TNF)-α. However, it has also been seen that macrophages modulate inflammation through the release of anti-inflammatory cytokines and growth factors such as IL-10, vascular endothelial growth factor (VEGF)-α, transforming growth factor (TGF)-β and Wnt proteins [10, 11]. Then, the macrophages can be divided into two general classes, depending on their phenotype, M1 that promote inflammation and M2 that release anti-inflammatory and pro-regenerative cytokines [12, 13].

2.1.2. Dendritic cells

The process of formation of dendritic cells (DCs) is like macrophages, being monocytes in their more immature stage. However, these cells are directed to epithelia even as immature cells and remain there for long periods (weeks or months). When they capture microorganisms or antigenic agents, they eliminate them by phagocytosis, going through the lymph to the lymph nodes, where they will perform their specialized function as antigen-presenting cells [14]. The DCs present antigens to the T lymphocytes; however, it has been proven that they are also capable of activating B lymphocytes, natural killer (NK) cells, macrophages and eosinophils. DCs participate in innate immunity; however, they regulate the adaptive immune response and are fundamental for the development of immunological memory and tolerance [15]. There are mainly two DCs subpopulations: classical and plasmacytoid DCs. On the one hand, classical DCs are specialized cells in the antigen processing and presentation, which have both high phagocytic activity and capacity for cytokine production [16]. On the other hand, plasmacytoid DCs are long-lived cells [17], which are present in the bone marrow and in all peripheral organs and are specialized to respond to viral infection with mass production of type I interferons (IFN). However, these DCs can also act as antigen-presenting cells and control the responses of T cells [18].

2.1.3. Neutrophils

Neutrophils are phagocytes that are derived from myeloid cells as well as monocytes and dendritic cells. Its morphology is very characteristic, since they present nuclear lobes of different morphologies and they are known as polymorphonuclear (PMN). It is the most abundant leukocyte in the blood (up to 70% of the total of leukocytes) and unlike the other phagocytes, neutrophils are released into the blood as mature cells; however, they have a short life time (from hours to maximum 2 days). They are the first cells of the immune system to reach the focus of infection and their function is practically phagocytosis. Although its short life has been identified that neutrophils are also involved in adaptive immunity, previously, it was known that neutrophils participated in the elimination of foreign agents by phagocytosis, dying in their function; however, it has been found that neutrophils have the ability to return to the bloodstream as antigen-presenting cells, interacting with dendritic cells, NK cells, T and B lymphocytes [19, 20].

2.1.4. Mast cells

Mast cells are derived from mesenchymal precursor cells (MCPs) in bone marrow but mature in peripheral tissues. They are distributed mainly in tissues close to the external environment such as the skin, mucous membranes, digestive tract and respiratory tract. Activation of mast

cells is practically due to the binding of immunoglobulin (Ig)-E antibodies to the high-affinity receptors for the Fc region of IgE (FcεRI) found in their plasma membrane, triggering the release of their granules containing high concentrations of histamine, tryptase, chymase, carboxypeptidase and heparin [21]. Activation of mast cells causes the activation of phospholipase A2 and breaks down membrane lipids to produce arachidonic acid, which can be metabolized in two ways: (1) the cyclooxygenase (COX) pathway, producing prostaglandins and (2) the lipoxygenase pathway (LOX), producing leukotrienes. Both prostaglandins and leukotrienes have pro-inflammatory effects, increasing vascular permeability. The mast cells boost the immune response, increasing the recruitment of specific cells against pathogens, activating different types of immune cells such as macrophages, eosinophils and lymphocytes that eliminate bacteria, fungi, some parasites and cells infected by viruses. Mast cells activate other cells of the immune system by releasing TNF-α, TGF-β, IL-4, IL-5, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), VEGF and fibroblast growth factor (FGF)-2 [22].

2.1.5. Basophils

Basophils are granulocytes derived from myeloid cells. They are the least abundant (0.5% of leukocytes) and have a nucleus in the form of S, lobed (1–3 lobes). They have many granules containing histamine, heparin, serotonin and high amounts of leukotrienes. Like mast cells, they contain histamine in their granules, being responsible for most of the early symptoms of IgE-dependent and non-dependent allergy (sneezing, pruritus, bronchospasm and edema). Basophils migrate to the site of inflammation and secrete proteases and various inflammatory mediators such as IL-4 to activate cells such as macrophages, innate lymphoid cells, fibroblasts and endothelial cells, aggravating the allergic inflammatory response [23, 24].

2.1.6. Eosinophils

Eosinophils are bilobed granulocytes originating from the bone marrow from myeloid cells, being released into the bloodstream in a mature manner and at low concentrations (3% of the total of granulocytes). An important characteristic of eosinophils is their high quantity of granules, which have different components, among which are high concentrations of leukotrienes, ROS, IL-4, IL-5, neurotoxins (EDN), main basic protein (MBP), eosinophilic cationic protein (ECP) and eosinophilic peroxidase (EPO) [25, 26]. Eosinophils play an important role in hypersensitivity since they are stimulated by IL-5 produced by mast cells and Th2 cells. Also, fibroblasts when stimulated by IL-4, release eotaxins, molecules that stimulate the function of eosinophils [27].

2.2. Pattern recognition receptors

Innate immune cells are capable of recognizing pathogens and endogenous molecules of proteins known as PRRs. These receptors recognize highly conserved motifs known as PAMPs or DAMPs. PRRs dictate the initiation of an adequate and effective innate immune response, as well as the activation of the adaptive immune response to infection or inflammation [28]. These PRRs include Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) and RIG-I-like receptors (RLRs) [29].

The TLRs family, was originally identified in Drosophila, as important genes for its ontogeny and the innate immune response in Drosophila adults [30]. The TLRs family consists of 10 highly conserved transmembrane glycoproteins in humans, which recognize a wide range of pathogens [31]. TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6 are expressed on the cell surface, while TL-3, TLR-7, TLR-8, and TLR-9 are found intracellularly in endosomes [32]. The extracellular leucine-rich repeat (LRR) regions in the TLRs mediate protein-protein or PAMP-protein interactions, while their intracellular tails mediate proinflammatory signaling through the myeloid differentiation primary response protein (MYD88) and TIR domain-containing adapter molecule 1 (TRIF; also known as TICAM1) pathways [33]. They are expressed in a wide variety of cells such as innate immune cells, T and B cells, epithelial cells, fibroblasts, and endothelial cells; however, not all cell types express every TLR [34]. Different TLRs specifically recognize distinct PAMPs and DAMPs [35]. For example, TLR2 recognizes lipoarabinomannan from mycobacteria [36]. Some TLRs detect different nucleic acids; TLR3 detects viral double-stranded RNA (dsRNA) formed during the replication of positive stranded viral RNA in the cytosol [37]; TLR7 and TLR8 both recognize viral single-stranded RNA (ssRNA) [38, 39] and TLR9 recognizes bacterial DNA [40]. TLR4 together with myeloid differentiation factor (MD)-2 recognizes lipopolysaccharide (LPS), which comes from Gram-negative bacteria [41]. Further, TLR4 is also involved in antiviral innate immunity [42, 43]. TLR5 is highly expressed DCs and detects bacterial flagellin [44, 45]. Plasmacytoid DCs express TLR7 and TLR9, and both are implicated in recognition of viral and bacterial nucleic acids [46]. TLR10 has been implicated in the recognition of *Helicobacter pylori* by gastric epithelial cells and may act as a heterodimer with TLR2 [47, 48].

The NLR family comprises 22 members in humans. Most NLRs share common structural characteristics including a C-terminal leucine-rich repeat (LRR) domain, often involved in ligand recognition, a central NOD, and a variable N-terminal effector domain [49]. Based on the type of effector domains that is either a caspase recruitment domain (CARD), a pyrin domain (PYD), or a baculoviral inhibitor of apoptosis protein repeat (BIR) domain [50], the NLR family can be categorized structurally into five subsets based on their N-terminal effector domain: NLRA, NLRB, NLRC, NLRP and NLRX [29]. The most well-defined sensors of peptidoglycan are the cytosolic NOD-like receptors (NLRs), NOD1 and NOD2, which are expressed by diverse cell types, including myeloid phagocytes and epithelial cells [51], which recognize specific ligands from various pathogens. This family is involved in increasing the proinflammatory events caused by cell death and several more proinflammatory processes [52].

The RIG-I-like receptor family consists of RNA-binding proteins that are expressed in almost all cells. Family members include RIG-1, melanoma differentiation-associated gene (MDA)-5, and laboratory of genetics and physiology (LGP)-2 [34]. They act as sensors for viral replication within human host cells necessary to mediate antiviral responses [53].

2.3. Cytokines

Cytokines are secreted proteins that can be delineated as a distinct class of signaling molecules from hormones based on two key factors. First, the kinetics of cytokine secretion (rapid and dramatic induction following specific extracellular stimuli), which is often prolonged at less dramatic concentrations to affect physiological changes. Second, cytokines can be signaling autocrine, paracrine and endocrine fashions [54, 55]. Cytokines are involved in regulating

the homeostasis of the organism, but when its production or its signaling pathway in the cell is not regulated, this homeostasis is altered, which can trigger in a pathology [56, 57].

Cytokines can be classified into five groups [57]: (1) IL-1 superfamily, there are 10 members of the IL-1 family of receptors (IL-1R1–ILR10) [58] and 11 members of the IL-1 family of cytokines (IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-38) [59]. The interleukin-1 superfamily members are closely linked to damaging inflammation; however, the same members also function to increase nonspecific resistance to infection and the development of an immune response to foreign antigens [60]. (2) TNF superfamily is composed of 19 ligands and 29 receptors [61]. This family plays a pivotal role in immunity, inflammation and controlling cell cycle (proliferation, differentiation and apoptosis) [62]. (3) The interleukin (IL)-17 cytokine family is composed of IL-17A and five other members (IL-17B, IL-17C, IL-17D, IL-17E, also referred to as IL-25, and IL17F). IL-17-related cytokines play key roles in defense against extracellular pathogen, autoimmunity. In addition, there is evidence that indicates that some of these molecules are involved in the amplification and perpetuation of pathological processes in many inflammatory diseases, such as psoriasis, rheumatoid arthritis, multiple sclerosis and allergy. However, the same cytokines can exert anti-inflammatory effects in specific settings and play key role in the control of immune homeostasis [63, 64]. (4) IL-6 superfamily is comprised by IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin (CT)-1, IL-11, cardiotrophin-like cytokine factor (CLCF)-1, viral IL-6 (vIL-6), IL-27 and IL-35 [65]. This cytokine family shows some redundant but not uniformly identical biological activity. IL-6 exerts pleiotropic effects on inflammation, immune response and hematopoiesis [66, 67]. IL-6 is produced at the inflammation site by infection or tissue damage, which induces production of acute phase proteins such as C-reactive protein (CRP), serum amyloid A, fibrinogen and hepcidin in liver. IL-6 also plays an important role in acquired immune response to induce differentiation of activated B cells in to antibody (Ab)-producing cells and to prolong survival of plasmablasts [65], while it promotes the development of Th17 cells and follicular helper T cells by naïve T cells and inhibits the differentiation into regulatory T cells (Treg) [68]. But, dysregulated excessive or persistent production of IL-6 plays a pathological role in various kinds of diseases [65]. (5) Type I superfamily, includes the common γ-chain cytokines (IL-2, IL-4, IL-7, IL-9, IL-13, IL-15 and IL-21) [69], common β-chain cytokines (IL-3, IL-5, GM-CSF) [70] and IL-12 subfamilies (IL-12, IL-23, IL-27 and IL-35), as well as similar cytokine products with unique receptor characteristics such as IL-13, IL-14, IL-32, IL-34, granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF). (6) Type II superfamily contains the interferons (type I, II and III) and the IL-10 subfamily (IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26) [54].

2.4. Inflammatory response and phagocytosis

Inflammation is a protective response to infection, tissue stress and injury [71]. This inflammatory response is characterized by its clinical signs such as redness, heat, swelling, pain and dysfunction [72]. The inflammatory response is triggered by inducers such as PAMPs derived from bacteria, viruses, fungi and parasites; and DAMPs derived from cell damage, as well as toxic cellular components or any other harmful conditions [73]. Then, these inflammatory inducers are detected by "sensors," which are present in several immune cells. These sensors are PRRs such as TLRs, NLR and RIG-like receptors [52, 74]. Subsequently, the PRRs induced

the synthesis and release of soluble mediators such as cytokines [75]. Cytokines, as optimal protection against pathogens, provide the necessary signals to initiate an inflammatory response, through the differentiation and proliferation of the immune system cells, adapting their effector functions as necessary to promote protective immunity, and once the inducers are eliminated, they suppress the inflammatory response, promoting tissue repair and return to homeostasis [54]. The inflammatory response is characterized by successive phases [76]: (1) silent phase, where cells reside in the damaged tissue releases in the first inflammatory mediators; (2) vascular phase, where vasodilation and increased vascular permeability occur; (3) cellular phase, which is characterized by the infiltration of leukocytes to the site of injury; and (4) resolution of inflammation, which is the process to return tissues to homeostasis [77–79].

Phagocytosis is the physiological process carried out by phagocytic cells to identify, digest and eliminate foreign substances or pathogens (**Figure 1**). Infection with pathogens is the most common cause to trigger this immune mechanism. The pathogens proliferate releasing small peptides with chemotactic activity, dispersing in the areas of underlying tissue and blood vessels. These chemotactic peptides come into contact with the endothelial cells that form the blood vessels and phagocytes that are found in the invaded tissue (macrophages and/or dendritic cells), as well as those found in the blood (neutrophils and monocytes). Endothelial cells initiate the synthesis of cell adhesion proteins, as do phagocytes found in the blood. The adhesion proteins allow the phagocytes of the blood to bind to the endothelial cells, causing them to roll on the surface until finding an exit between the cell junctions, migrating to the extravascular space by a process known as diapedesis. The phagocytes that were close to the area of infection and those that migrated from the blood move toward the focus of infection attracted by the chemotactic peptides. The microorganisms have structural components (the receptor for IgG (FcR) and PAMPs, among others) that are recognized by PRRs found in phagocytes [80, 81].

The interaction of these surface molecules causes the invagination of the cell membrane and the formation of cellular prolongations that end up involving the foreign pathogens in a phagocytic vacuole or phagosome. The chemical interaction of the molecules on the membrane surface of microorganisms and phagocytes activates diverse receptors, including those of Gq proteins that activate phospholipase C, an enzyme that degrades membrane phospholipids to produce inositol triphosphate (IP3) and diacylglycerol (DAG). The IP3, among many of its functions, is responsible for regulating cell movement by the cytoskeleton through the release of calcium ions by the endoplasmic reticulum. On the other hand, the DAG activates a protein kinase C (PKC), which activates the cytosolic proteins p40, p47 and p67, which, supported by ras-related protein Rap-1A (RAP1A), interact with cytochrome B558, one of the components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Activated NADPH oxidase promotes the release of ROS, molecules highly toxic to cellular components. NADPH oxidase captures high amounts of oxygen, transforming them into superoxide anions (O^{2-}), which in turn promote the formation of dangerous ROS such as hydrogen peroxide (H_2O_2), hydroxyl (OH^-) and oxygen singlet ($^1O_2$). The ROS react with the biomolecules that make up the structures of the microorganisms (lipids, polysaccharides, proteins and nucleic acids), causing their death. Simultaneously, the phagocytes fuse lysosomes to the vacuole in which the microorganism is internalized, forming the phagosome, also releasing many hydrolytic enzymes that favor the digestion of the microorganism components [82].

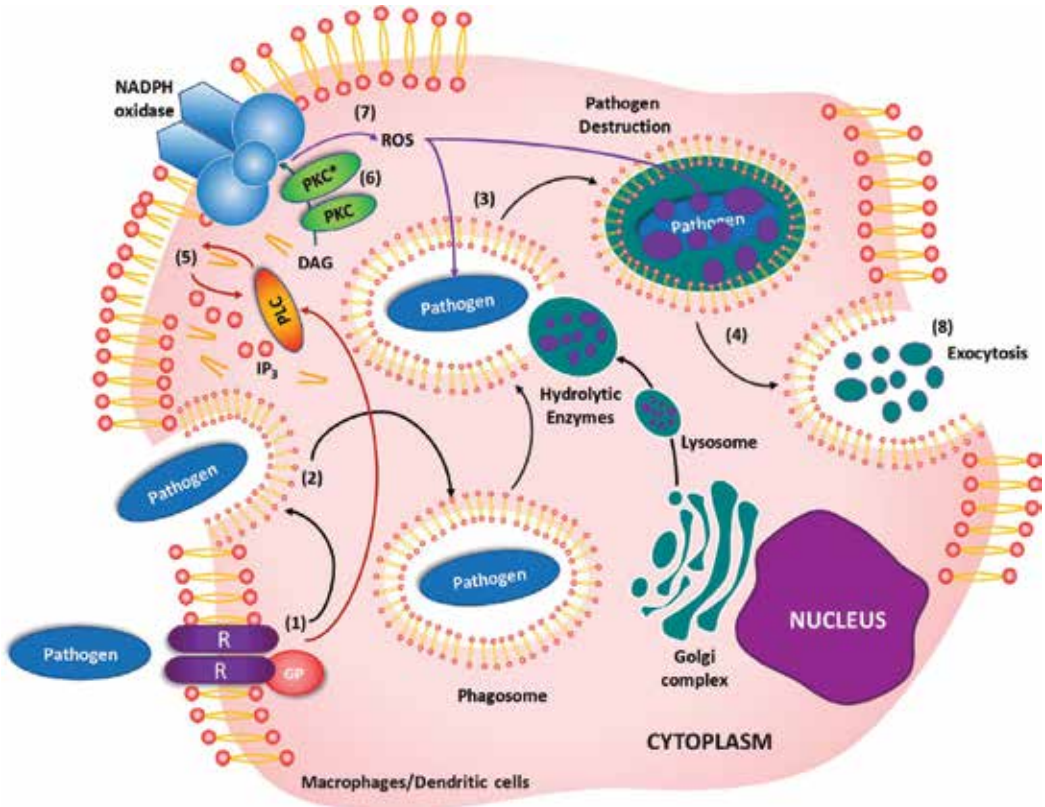

Figure 1. Phagocytosis. (1) Recognition of structural components of pathogens by the PRRs of phagocytes. (2) Invagination of the cellular plasma membrane that causes the internalization of the pathogens, forming the phagosome. (3) Fusion of the lysosomes with the phagosome, promoting the digestion of the pathogens by hydrolytic enzymes. In addition, ROS are released that contribute to the degradation of biomolecules. (4) Destruction of the pathogens. (5) The activation of phospholipase C causes the activation of PKC. (6) PKC activates NADPH oxidase. (6) ROS are produced by NADPH oxidase. (7) ROS are directed to the phagosome, contribute to the degradation of pathogens. (8) Release by exocytosis of the pathogens residual.

Lysosomes contain myeloperoxidase, an enzyme that hydrolyzes hydrogen peroxide for the formation of halogenating radicals such as hypochlorous acid, hypochlorite and hypoiodite, which increase the damage to microorganisms. Finally, cell debris has two purposes: (1) to be eliminated by exocytosis, (debris are evacuated into the bloodstream to be eliminated by renal route); and (2) to transport certain antigenic components to the cell membrane to be presented to T and B cells and be able to give the process of acquired immunity (mainly in the case of dendritic cells and macrophages) [82].

3. Adaptive immune system

The adaptive immune system has the capacity to generate a wide range of specific antigen receptors, through somatic mechanisms of gene rearrangement. These mechanisms create a

random repertoire of receptors that are clonally distributed in T and B lymphocytes. This gives it the advantage of having a wide repertoire of specific antigen receptors, which can be recognized, without these having to be encoded in the host genome, allowing the recognition of almost any antigenic structure. The activation of lymphocytes requires two types of signals: (1) a signal induced by the antigen receptor itself when recognizing its related antigen, and a costimulatory signal by professional antigen-presenting cells (APCs). Therefore, the innate immune system, as already explained earlier, determines the origin of the antigens by means of a non-clonal system of receptors, PRRs, encoded in the germ line, which controls the expression of costimulatory molecules and effector cytokines, while the adaptive immune system does it through antigenic receptors [83, 84].

3.1. T lymphocytes

During the hematopoiesis that is generated in the bone marrow, it gives rise to the precursors of all the lineages and states of differentiation of the T cells. These precursors, called thymocytes, travel through the peripheral blood and reach the thymus, where they mature in T lymphocytes. Later, they will differentiate into $CD4^+$ T lymphocytes (cooperators) or $CD8^+$ T lymphocytes (cytotoxic). Once they are differentiated, they travel through the blood circulation until they are activated by means of the surface receptor they present, when they encounter a specific antigen. This receptor, known as T cell receptor (TCR), binds to the major histocompatibility complex (MHC), a complex expressed by antigen-presenting cells, in which the antigen is presented in the form of peptides. Depending on the T cell to which the antigen is presented, MHC class I or MHC class II will be used. To present an antigen to the $CD4^+$ T lymphocyte, a presentation through the MHC-II will be required; while for the activation of a $CD8^+$ T lymphocyte, it will be necessary through the MHC-I [84, 85]. T lymphocytes are responsible for cellular adaptive immunity. The activation of $CD8^+$ T lymphocytes allows the destruction of infected cells through the release of perforins, which are proteins responsible for forming pores in the membrane of the target cell that causes the passage of water and ions, inducing an osmotic lysis of the infected cell. Similarly, $CD8^+$ T lymphocytes release toxic enzymes such as the granzyme that passes through the pores formed in the cell membrane, which causes the induction to cell death by fragmenting the DNA of the infected cell. Activation of $CD4^+$ T lymphocytes allows cooperation with other immune cells for their activation. As the case of macrophages, B lymphocytes and other T lymphocytes, through costimulatory molecules and the release of cytokines, this causes a powerful cellular activation and therefore an effective immune response. In addition to this, $CD4^+$ T lymphocytes can differentiate into cellular subpopulations with specific action. Mediated by the secretion of cytokines, they can be differentiated into Th1, Th2, Th9, Th17 and Th22 types [86].

In addition, memory T lymphocytes have a long life, functionally inactive but respond to new exposures of the same antigen quickly and efficiently. There is another population of T lymphocytes, the regulatory T lymphocytes [86]. This cellular population is responsible for eliminating autoreactive T cells that escaped the process of negative selection or central tolerance; with the purpose, to avoid the development of an autoimmune response [87]. Other lymphocytes, such as LTγ/δ, are another very rare cell type that represent about 10% of intraepithelial lymphocytes of the small intestine but increase drastically under certain

allergic or inflammatory conditions. In addition, they recognize complete proteins without needing to be processed to be presented through the MHC molecules [88].

3.2. B lymphocytes

The B lymphocytes are originated from the same precursor that gives origin to the T lymphocytes and the NK cells. However, the absence of certain cell membrane receptors in B lymphocytes leads to their differentiation in this cell line, a process that takes place in bone marrow. Up to this point, the B lymphocytes are immature, and it will be until they migrate from the bone marrow into the spleen to undergo positive and negative selection and thus produce a mature B lymphocyte [89]. B lymphocytes can be activated: (1) by a foreign agent through the TCD4$^+$ lymphocytes collaboration; (2) or in specific circumstances independent of CD4$^+$ T lymphocytes. In the case of CD4$^+$ T lymphocytes collaboration, it occurs through the MHC expressed in its cell membrane, which binds to the B cell receptor (BCR), to initiate the antigenic presentation that will end in the synthesis of antibodies [90]. B lymphocytes are cells that participate in humoral adaptive immunity, since once activated they proliferate in response to the antigen and differentiate into plasma cells to produce antibodies against the specific antigen [91]. Likewise, activated B lymphocytes can differentiate into memory cells, acquiring a capacity for survival for long periods of time, up to more than 10 years, approximately [92, 93]. However, various co-stimulatory receptors that are expressed in B cells can induce their proliferation and survival, as well as the regulation of the production of specific antibodies that contribute to a breakdown of immunological tolerance, triggering autoimmune diseases [94].

3.3. Antibodies

Antibodies, also known as immunoglobulins (Ig) are structurally composed of two heavy polypeptide chains identical to each other and two light chains also identical, joined by one or more disulfide bridges. They have a variable region with two domains (VH, VL) and a constant region with four domains (CL, CH1, CH2 and CH3) [95]. The segments of the variable region originate through a somatic recombination, which allows having the diversity in the repertoire of antibodies, since at least 1026 of different specific antibodies are generated. They have a Fab fragment (fragment antigen binding) and an Fc fragment (crystallizable fraction). The Fab portion is an antigen-binding zone, while the Fc is a constant zone where the interaction with cellular receptors and the effector part of the biological functions presented by the antibodies occurs. Among these biological functions are crossing the placental barrier, activating complement, neutralizing antigens, joining phagocytic cells and acting as opsonin; all to generate protection and eliminate pathogens or elements harmful to the host [96].

There are 5 classes recognized up to the moment of antibodies: IgA, IgG, IgM, IgE and IgD. Most are monomeric, but they can be presented pentameric as IgM and only IgA can be present in both dimeric and monomeric forms. There are 4 subclasses for IgG (IgG1-IgG4) and 2 for IgA (IgA1 and IgA2). This is due to variations in the constant regions, which causes functional differences between the antibodies of the same class [97]. Among the functions of IgG is complement activation, with subclass IgG3 having the greatest effect, whereas IgG4

cannot activate it. It is the antibody in greater amount circulating in the blood and more increases during a secondary immune response. It can cross the placenta and, in the newborn, favors its immunological protection. It helps in phagocytosis through opsonization, as well as in the neutralization of pathogens with great effectiveness [98]. IgA is found in greater concentration due to its location in epithelia, in body secretions such as saliva, tears, colostrum, respiratory, gastrointestinal and genitourinary secretions; which allows it to generate a broad protection against pathogens and allergens. In blood circulation, it is found in a monomeric way; but in mucous, it is found in a dimeric form behaving as secretory IgA [99]. The IgE antibody is found in very small concentrations in the bloodstream. The majority is bound to a surface receptor of mast cells, eosinophils and basophils, which causes it to be involved in allergic reactions in humans, since it induces the release of pro inflammatory cytokines when IgE recognizes specific antigens [100]. It also causes degranulation of the aforementioned cells, causing the release of vasoactive substances such as histamine, causing an inflammatory response. Also, it can increase the production of this antibody by the effect of allergens such as those that can be found in food, some drugs and seasonal allergens, which causes allergic reactions. This immunoglobulin is very effective in the defense against parasitic infections [101]. In the case of IgM, it is the first antibody that appears with immune response reactions. It is the first antibody that is expressed on the surface of B lymphocytes and the one that predominates in primary immune reactions. It is the largest, due to its pentameric formation, which allows it to bind several antigens (approximately, 6 antigens per IgM) and is the main activator of the complement system [102]. Finally, IgD is the immunoglobulin that is also found on the surface of B lymphocytes, being a marker of their maturity. However, at the time of contact with the antigen, IgD is lost during antigenic stimulation. It participates as an antigen receptor and signaling transmitter inside the cell and, in blood circulation, it is found in very small amounts and is not produced by plasma cells [103].

4. Histocompatibility

The molecules of the major histocompatibility complex (MHC), also called human leukocyte antigens (HLA) [104, 105], are the product of a set of genes responsible for the lymphocytes rejecting transplanted tissues and detecting foreign elements. These molecules also participate in the induction of the specific immune response, through the presentation of the antigen to the T lymphocytes [104]. In the mammalian genome and, more specifically, in the human genome, the most variable region known forms the MHC that carries a great number of different loci coding for functional genes [106]. The classical MHC encompasses approximately 3.6 megabasepairs (Mb) and is divided into three subregions: the telomeric class I, class III, and the centromeric class II regions [107]. In humans, the MHC region is approximately 4000 kb long, located on the short arm of chromosome 6 [105, 106].

Molecular markers, located on the cell surface, help to externalize the intracellular environment and give the individual a specific tissue identity, recognized by their immune system. Under normal conditions, the MHC molecules reach the cell membrane bound to their own elements, so when they are presented to the T lymphocytes, they do not activate them; when

by infection or pathological changes of the cell, they emerge, carrying a foreign molecule instead of their own, the T cell is activated and responds immediately [108]. The function of MHC molecules is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T lymphocyte. The consequences are almost always deleterious to the pathogen—virus-infected cells are killed, macrophages are activated to kill bacteria living in their intracellular vesicles, and B lymphocyte are activated to produce antibodies that eliminate or neutralize extracellular pathogens [105].

4.1. Major histocompatibility complex (MHC-I)

The genes, whether expressed, are arranged in three genomic regions or classes. The more distal region corresponds to MHC class I, which carries the genes that code for the classic (1a) class I HLA-A, -B, and -C heavy chains, all nucleated cells express class I molecules on their cell surface [109]. They present cytoplasmic or endogenous antigens (synthesized intracellularly, those of viral or tumoral origin and processed by the proteasome) to the $CD8^+$ T lymphocyte [110]. MHC-I is a molecule made up of an α polypeptide chain, with three domains (α1, α2 and α3) and the β_2 microglobulin subunit. In the cleft that is formed between α1 and α2, it is added the antigenic peptide that is going to present [108]. The classical molecules MHC-I (A, B and C) are expressed on the surface of all cells, except those of the trophoblast, erythrocytes and neurons. Its main function is the presentation of antigens to the $CD8^+$ T lymphocyte [111]. The MHC-I is formed in the endoplasmic reticulum and interacts with the chaperone molecules: calnexin and calreticulin, which help it to bind with the β_2 microglobulin and confer stability on it. A third molecule, the capsid, helps transporting antigen processing peptides (TAP)-1 and TAP2 to form the channel that allows the passage of the antigenic peptide from the cytoplasm to the endoplasmic reticulum, where it binds to the MHC-I. This complex (MHC-I-antigenic peptide) leaves the endoplasmic reticulum in a vesicle, travels through the cytoplasm and is finally exocytosed. On the cell surface, the MHC molecule and the antigenic peptide that it carries bind to the $CD8^+$ T lymphocyte receptor and it is through this union that the so-called "presentation" is made. If the presented peptide corresponds to a molecule of its own, the lymphocyte does not respond. If the presented peptide is foreign, accessory signals are transmitted through costimulatory molecules such as B7-CD28, CD40-CD40L, etc., which activate $CD8^+$ T lymphocyte. The activated cytotoxic lymphocyte, through the firing of cytolytic enzymes and the induction of apoptosis, destroys the host cell, carrier of endogenous antigens such as viruses or tumor cell elements (**Figure 2**, right) [108].

4.2. Major histocompatibility complex (MHC-II)

The MHC class II genes, coding for both chains that will form the functional heterodimer, HLA-DR, HLA-DQ, HLA-DP, HLA-DM, and HLA-DO are in the more centromeric portion of the MHC region [109]. They exhibit restricted expression, being predominantly expressed on antigen-presenting cells (APC), such as macrophages, DCs, Langerhans and Kupffer cells, as well as B lymphocytes [112], also intravesicular or exogenous antigens (synthesized extracellularly and processed by lysosomes) to $CD4^+$ T lymphocyte [110]. CMH-II is composed of two polypeptide chains: α and β, both with two domains. The antigenic peptide binding site it presents is located between α1 and β1 [105, 108]. The antigen, for its presentation, must be processed by the cell that captured it and be reduced to small peptides, since the sites to which

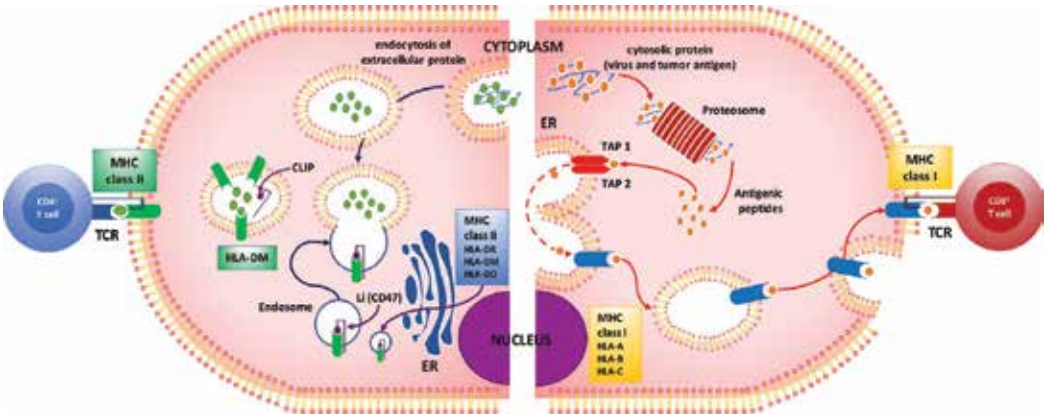

Figure 2. Processing and presentation of antigen. In the MHC class I pathway (right), the proteosomes process the protein antigens in the cytoplasm, which are transported to the endoplasmic reticulum (ER), where they bind to the MHC class I molecules. Subsequently, these are presented to the T lymphocytes, to induce a $CD8^+$ phenotype. In the MHC class II pathway (left), the extracellular protein antigens are introduced into the antigen-presenting cell by endocytosis, in vesicles, where the antigens are processed, and the peptides bound to the MHC class II molecules, which are present to the T lymphocytes to induce a $CD4^+$ phenotype.

it binds both in the MHC and in the T lymphocyte, can only host molecules with a smaller size to 25 amino acids [108]. The classical molecules MHC-II (DP, DQ and DR) are expressed, constitutively, on the surface of the cells participating in the "immune response" (phagocytes and lymphocytes), but by activation with INF-γ, they can be expressed in other cells that, like fibroblasts, keratinocytes, barley and endothelial, also participate in this response [111]. The MHC-II is synthesized in the endoplasmic reticulum and portal a molecule: the invariant chain (Li or CD74) that protects the site that the antigen will occupy, favors its exit of the reticulum and takes it to endosomes where it meets the antigenic peptides. In this place, various cathepsins break the Li chain, which leaves the site corresponding to the antigen free and allows its binding to MHC, the Li residues (CLIP) are removed by the DM molecule. Finally, the antigenic peptide emerges to the surface linked to MHC-II, a molecule through which it makes contact and is presented to the $CD4^+$ T lymphocyte. If the presented molecule is strange, the T-helper cell cytokines are activated and secreted. These cytokines can activate the host cell and lymphocytes and cells surrounding (Th1 predominant response), as well as stimulate the production of antibodies (Th2 predominant response). The class of secreted cytokines and therefore, the function that they do, depends on the type of Th cell that responds. In all cases, there is a regulation that, at the end of the Antigenic stimulus: slows the response, induces apoptosis activated cells, inhibits inflammation and initiates repair (**Figure 2**, left) [113].

5. Immune tolerance

5.1. Central tolerance of lymphocytes T and B

The "immunological tolerance" was established in 1954, as an acquired state learned during the development of the immune system by exposure to antigens in its immediate environment [114].

A single antigen can induce an immune response or tolerance depending on the context in which it occurs. Tolerance is acquired, triggered from the ontogeny of lymphocytes and there are different mechanisms to maintain it. One is carried in the primary lymphoid organs, known as central tolerance. The other is carried in the secondary lymphoid organs and is known as peripheral tolerance [115]. The central tolerance, also known as negative selection, is carried out during the development of the T and B cells, when the newly generated cells test their receptors for the recognition of antigens in their immediate environment. It consists of a clonal elimination in the bone marrow of autoreactive B lymphocytes and self-reactive T lymphocytes in the thymus. It prevents maturation of those lymphocytes capable of recognizing autoantigens through the expression of high affinity receptors and occurs through the recognition of these by the antigen-presenting cells through MHC molecules. On the other hand, peripheral tolerance allows maintenance in the control of effective immune responses against "self" [116].

5.2. Peripheral tolerance of T and B lymphocytes

After the T and B lymphocytes have passed through the control of negative selection or central tolerance and mature, they are directed by blood circulation to secondary lymphoid organs such as the spleen and lymph nodes. Lymphocytes require secondary signals to activate and generate a positive response against foreign antigens. If the lymphocytes do not generate a positive response against these antigens, the lymphocytes become anergic or die by apoptosis. Similarly, when lymphocytes are activated by antigens inappropriately (autoreactive), regulatory mechanisms are activated that correct such failures through the participation of regulatory T lymphocytes (T_{regs}) [117].

5.3. Tolerance induced by exogenous antigens

The tolerance for exogenous antigens is due to the lack of immune response against antigens from food and normal flora, as well as inhaled antigens, to avoid triggering an immune response that affects the integrity of the individual. This type of tolerance occurs mainly on mucous membranes. The participation of IgA immunoglobulin as essential component of mucosal immunity, whose function is the neutralization of antigens or immune complexes, prevents their absorption and progression of active immune response. Dendritic cells are also highly responsible for immunological tolerance toward exogenous antigens. In part, they are responsible for their ability to induce the expression of T_{regs} $FOXP3^{+}$ lymphocytes [118].

6. Immune hypersensitivity

The immune system is an integral part of human protection against disease, but the normally protective immune mechanisms can sometimes cause detrimental reactions in the host. Hypersensitivity diseases include autoimmune diseases, in which immune responses are directed against self-antigens, and diseases that result from uncontrolled or excessive responses to foreign antigens. Because these reactions tend to occur against antigens that cannot be escaped (i.e., self-antigens) and because of positive feedback systems intrinsic to

various aspects of the immune response, hypersensitivity diseases tend to manifest as chronic problems. The traditional classification for hypersensitivity reactions is that of Gell and Coombs and is currently the most commonly known classification system (**Figure 3**) [119].

6.1. Type I reactions

Immediate hypersensitivity reactions are mediated by IgE, but T and B cells play important roles in the development of these antibodies. The allergic reaction first requires sensitization to a specific allergen and occurs in genetically predisposed individuals. The allergen is either inhaled or ingested and is then processed by APC, such as a DCs, macrophage, or B-cell [120]. The APC then migrates to lymph nodes, where they prime *naïve* T cells that bear receptors for the specific antigen. After antigen priming, *naïve* T cells differentiate into Th1, Th2, or Th17 cells based upon antigen and cytokine signaling. In the case of allergen sensitization, the differentiation of *naïve* T cells is skewed toward a Th2 phenotype. These allergen-primed Th2 cells then release IL-4, IL-5, IL-9 and IL-13. IL-5 plays a role in eosinophil development, recruitment and activation. IL-9 plays a regulatory role in mast cells activation. IL-4 and IL-13 act on B cells to promote production of antigen-specific IgE antibodies. For this to occur, B cells must also bind to the allergen via allergen-specific receptors. They then internalize and process the

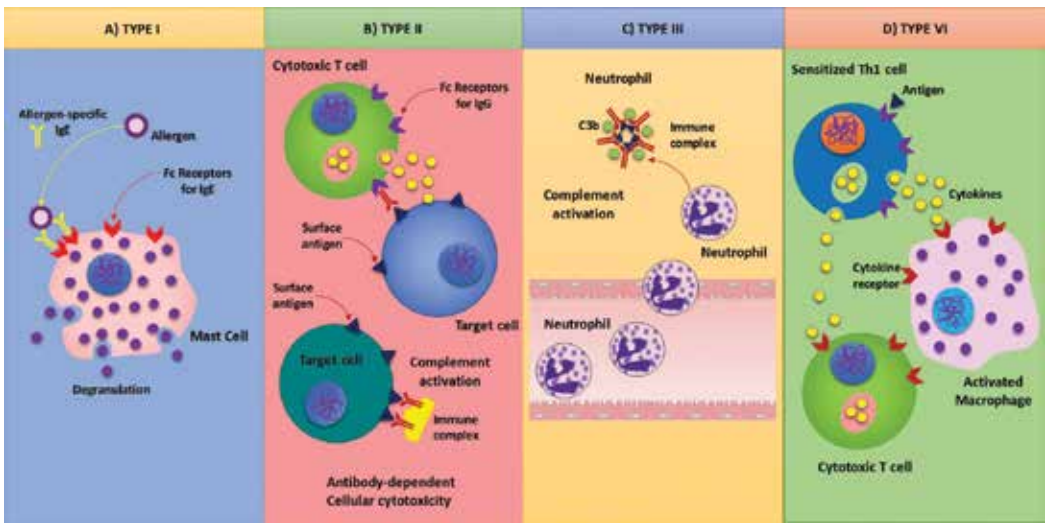

Figure 3. Hypersensitivity reactions. (A) Type I hypersensitivity. The binding of the antigen to preformed IgE antibodies bound to the surface of mast cells or basophils, causes the release of inflammatory mediators such as histamine, cytokines and metabolites of arachidonic acid, which produces clinical manifestations, such as septic shock, rhinitis allergic, allergic asthma and acute allergic reactions to drugs. (B) Type II hypersensitivity. Cytotoxic reactions involve the binding of both IgM and IgG antibodies to antigens bound to cells. The antigen–antibody binding results in the activation of the complement cascade and in the destruction of the cell to which the antigen is bound. (C) Type III hypersensitivity. Immunocomplexes are formed when the antigens bind to the antibodies. They are usually removed from the process by phagocytosis. However, the deposition of these immunocomplexes in the tissues or in the vascular endothelium can produce a tissue aggression mediated by immunocomplexes. (D) Type IV hypersensitivity. These types of reactions are not mediated by antibodies. Delayed hypersensitivity reactions are mediated primarily by T lymphocytes (cell-mediated immunity).

antigen and present peptides from it, bound to the MHC-II molecules found on B cell surfaces, to the antigen receptors on Th2 cells. Type I reactions are immediate hypersensitivity reactions involving IgE-mediated release of histamine and other mediators from mast cells and basophils (**Figure 3A**). Examples include anaphylaxis and allergic rhino conjunctivitis [121].

6.2. Type II reactions

Type II or cytotoxic hypersensitivity [119] depends on the abnormal production of IgG or IgM directed against tissue antigens or a normal reaction to foreign antigens expressed on host cells. There are three main mechanisms of injury in type II reactions: (1) activation of complement followed by complement-mediated lysis or phagocytosis and removal by leukocytes; the IgG or IgM antibody can complex with antigens on the surface of cells or extracellular matrix and this complex then may activate complement. Complement activation will result in formation of the membrane attack complex (MAC) and cause osmotic lysis of the target cell; (2) antibody-dependent cellular cytotoxicity; the second type II reaction is called antibody-dependent cell-mediated cytotoxicity IgG antibodies that can bind FcγRIII on NK cells and macrophages, thus mediating the release of granzymes and perforin and resulting in cell death by apoptosis (ADCC); (3) inactivation of a biologically active molecule; disruption of biologically functional molecules can occur when autoantibodies bind to these molecules (**Figure 3B**). An example is antibody produced against acetylcholine receptors in myasthenia gravis resulting in increased turnover of the receptor at motor end-plates and subsequent muscular weakness or drug-induced hemolytic anemia [122, 123].

Drug-induced immune hemolytic anemia (DIIHA) is rare, and required to provide the optimal serological tests to confirm the diagnosis. The drugs most frequently associated with DIIHA at this time are cefotetan, ceftriaxone and piperacillin. DIIHA is attributed most commonly to drug-dependent antibodies that can only be detected in the presence of drug. The drug affects the immune system, causing production of red blood cell (RBC) autoantibodies; the clinical and laboratory findings are identical to autoimmune hemolytic anemia (AIHA), other than the remission associated with discontinuing the drug. Some of the mechanisms involved in DIIHA are controversial. The most acceptable one involves drugs like penicillin that covalently binds to proteins (e.g., RBC membrane proteins); RBCs become coated with drug in vivo and, a drug antibody (usually IgG) attaches to the drug-coated RBCs that are subsequently cleared by macrophages. The most controversial is the so-called immune complex mechanism, which has been revised to suggest that most drugs are capable of binding to RBC membrane proteins, but not covalently like penicillins. The combined membrane plus drug can create an immunogen; the antibodies formed can be IgM or IgG and often activate complement, leading to acute intravascular lysis and sometimes renal failure; fatalities are more common in this group. It is still unknown why and how some drugs induce RBC autoantibodies, sometimes causing AIHA [124].

6.3. Type III reactions

Type III reactions (immune-complex reactions) involve circulating antigen-antibody immune complexes that deposit in postcapillary venules, with subsequent complement fixation. An

example is serum sickness. Type III hypersensitivity is caused by circulating immunocomplexes and is typified by serum sickness (a drug reaction in which multimeric drug-antibody aggregates form in solution). Preformed immunocomplexes deposit in various vascular beds and cause injury at these sites. Multimeric antigen-antibody complexes are efficient activators of the complement cascade through its classical pathway. The vascular beds in which immunocomplexes are deposited are determined in part by the physical nature of the complexes (their aggregate size, charge, hydrophobicity, etc.), and the specificity of deposition at locations can be surprisingly precise in some diseases (**Figure 3C**). Typical sites of injury are kidney, skin, and mucous membranes. Type III hypersensitivity is common in systemic lupus erythematosus (SLE) and underlies most of the pathophysiology of this chronic autoimmune disease. Some inflammatory reactions may blend features of type II and III hypersensitivity with the formation of immunocomplexes in situ [125].

6.4. Type IV reactions

Type IV reactions (delayed hypersensitivity reactions and cell-mediated immunity) are mediated by T cells rather than by antibodies (**Figure 3D**). An example is contact dermatitis from poison ivy or nickel allergy, tuberculosis, leprosy and sarcoidosis. In tuberculosis, cellular hypersensitivity, the delayed type of allergy, may be defined as an immunological state in which lymphocytes and macrophages are directly or indirectly sensitive to tuberculin, activate macrophages [126], and can passively transfer delayed hypersensitivity to the normal host [127]. Lymphocytes, when exposed to tuberculin merely produce a toxic or irritating product affecting macrophages, whether they sensitize macrophages to tuberculin [128]. In tuberculosis, delayed hypersensitivity is both beneficial and detrimental. In low concentrations, tuberculin stimulates the development of immunity in macrophages. Therefore, the presence of hypersensitivity is an asset in preventing pulmonary tuberculosis for only small units of one to three bacilli that reach the alveolar spaces where the infections begins. In high concentrations, tuberculin kills macrophages and thus is responsible for the liquefaction of caseous foci. This process results in tremendous extracellular multiplication of tubercle bacilli followed by their spread throughout the bronchial tree and to the other people [129].

7. Pathogenesis of autoimmunity (loss of immunological tolerance)

7.1. Gene base of autoimmunity

Despite the various immunological mechanisms to maintain tolerance to itself, there are certain individuals who develop autoimmunity. In 1986, the idea was postulated that the T and B cells specific for antigens coming from infecting pathogens, also generate a cross reaction against autoantigens even though the pathogens are eliminated. This type of response is initiated by low affinity T cells that have escaped the central tolerance. In addition, there is a genetic component capable of initiating and causing a persistence of autoimmunity and, therefore, trigger an autoimmune disease. However, epigenetic factors also play an important role in their development. They have been classified as a specific organism or systemic, with

the genetic susceptibility in the alleles of class I and class II molecules, a large part of the cause of the occurrence of autoimmune diseases such as systemic lupus erythematosus and type I diabetes mellitus [90]. Thus, the appearance of polymorphisms in more than 50 genes, among which a small number has been identified that affect the expression of molecules involved in the general activation of T cells, causes a high susceptibility to type I diabetes. In the case of the presentation of systemic autoimmune diseases, genetic susceptibility occurs in the general activation of B lymphocytes, affecting the signaling and survival receptors, which allows the autoreactive B cells of higher affinity to escape from the negative selection. Also, the genetic deletion of certain TLRs, such as TLR-9, increases the susceptibility to manifest autoimmune diseases. Depositions of antigen-antibody complexes in tissues, such as kidney, have been an important factor in the manifestation of autoimmune diseases. This is due to the variation in certain genes such as those responsible for synthesizing the components of the complement and its receptors, which can initiate autoimmune pathologies. Another important factor that triggers autoimmunity is the loss of certain immunoregulatory mechanisms. Such is the case of a chronic stimulation of the TCR, by a persistent antigenic exposure that can deregulate the immune response through adaptive tolerance mechanisms. A loss of the anergy of autoreactive T lymphocytes, a failure in cell death by apoptosis of autoreactive T cells, the loss of suppression of these cells due to T_{regs} lymphocytes, polyclonal activation of autoreactive T lymphocytes, may also occur among other mechanisms that can trigger autoimmunity [130]. Finally, autoimmune diseases can affect a specific cell type, several cells or the entire organism. Its initiation will depend on the pathways by which the immunological tolerance is altered, being of great importance the genetic predisposition that certain individuals present.

7.2. Autoimmune diseases

Autoimmune diseases are a consequence of an immune reaction against an autoantigen. They can affect a single organ or cell type; however, they are usually also systemic, as is the case of the onset of rheumatoid arthritis or systemic lupus erythematosus.

Systemic lupus erythematosus (SLE) is a rare disease with a prevalence of 3.3 to 8.8 per 100,000 children. There is a high frequency reported in Asians, African Americans, Hispanics and Native Americans; the age at which it usually manifests is between 11 and 12 years of age and about 80% of adults who have SLE are women [131]. It is a multisystemic autoimmune disorder characterized by extended immunological dysregulation, formation of autoantibodies and immune complexes, resulting in inflammation and potential damage to a wide variety of organs. The clinical manifestation presented is nonspecific, such as the appearance of fever, fatigue, anorexia, alopecia and arthralgias. Symptoms such as generalized inflammation, including lymphadenopathy and hepatosplenomegaly, may manifest during the onset of SLE. However, the hallmark of this disease is the appearance of a butterfly-shaped malar rash. This condition can affect any organ of the system and its diagnosis is given through clinical manifestations and laboratory tests. Such is the case of the search for antibodies such as antinuclear antibodies (ANA), which are present in the serum of almost 98% of patients with SLE; Anti-dsDNA antibodies are present between 61 and 93% of patients with active disease; Anti-Smith antibodies are highly specific, but they can be found only in almost 50% of patients; Antibodies such as anti-Ro, anti-La, anti-U1RNP, anti-histones and rheumatoid factor, can also be used as a diagnosis of SLE. The indicated treatment is according to the activity of the disease and its severity, as well as the organs affected by

the SLE. The immunopathogenesis of this disease is mediated by the recruitment of autoreactive T cells and excessive plasma levels of proinflammatory cytokines. In addition, dendritic cells and subpopulations of T cells such as Th1, Th17 and regulatory T cells are significantly altered in function and number. However, the fundamental immunological dysfunction in the appearance of SLE is the loss of tolerance to nuclear antigens. There are defects that promote the presentation of autoantigens and the response to apoptotic residues in an immunogenic form; also, those faults that affect the signaling of the T or B cells, which causes the autoreactive abnormal stimulation of the lymphocytes; as well as those defects that promote the survival of autoreactive lymphocytes. Therefore, the loss of immunological tolerance is a factor that causes the presentation of systemic lupus erythematosus [132].

Rheumatoid arthritis (RA) is a chronic inflammatory multisystem disease characterized by destructive synovitis, in which all joints can be affected, mainly the small joints of the hands and feet. RA is a chronic progressive disease that results in decreased functional capacity and quality of life. It can manifest in individuals with genetic predisposition; however, it is of unknown etiology. It affects 0.2 to 2% of the worldwide, in a population of 40 years old, although it could happen at any age [133]. The diagnosis of RA occurs through the presentation of clinical manifestations, such as the onset of arthritis of at least 3 joints and morning stiffness of more than 30 minutes, as well as an exacerbated joint inflammation with the presence of pain. Likewise, blood concentrations of C-reactive protein and rheumatoid factor are evaluated, which will be elevated depending on the inflammatory activity of the RA. Another determinant with a high probability for the diagnosis of the disease is the evaluation of anti-CCP antibodies. The immunopathogenesis of RA results from the loss of immunological tolerance, with the consequence of an elevated secretion of proinflammatory cytokines such as IL-6, which is found in some patients, in high quantities in synovial fluid. In addition, the formations of autoantibodies that attack the joints of the entire organism are among the main causes of the presentation of RA [134].

8. Conclusion

The immune system is characterized by a network of complex mechanisms whose main objective is to protect the body. However, if there is a failure in its regulation, it can generate hypersensitivity and/or autoimmunity. For this reason, it is very important to know how our immune system works and how these pathologies originate. Currently, anaphylactic shock and skin reactions are the most frequent hypersensitivity reactions affecting organs and tissues. There are several mechanisms and factors involved which triggers hypersensitivity reactions. On the other hand, although autoimmune diseases are relatively common and our current knowledge about the mechanisms involved in their pathogenesis is very limited.

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Conflict of interest

We have no conflict of interest related to this work.

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Chapter 2

Immune Checkpoint Blockade and Immune Monitoring

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Additional information is available at the end of the chapter

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Abstract

The concept of immunological surveillance, a monitoring process in which the immune system detects and destroys by several effector mechanisms, virally infected and neoplastic transformed cells in the body, was developed more than 50 years ago. Based on current research, it is clear that the immune system can recognize and eliminate transformed cells. An increasing number of studies has investigated the immune system in cancer patients and how it is prone to immunosuppression, due in part to the decrease of lymphocyte proliferation and cytotoxic activity. Such weakened immune system is then unable to fully accomplish its role in immunological surveillance, allowing nascent transformed cells to escape the selective pressure of the immune system. The main goal of cancer immunotherapy has been to reawaken the immune system from a suppressive slumber to enable it to attack cancer cells once again. As the results from the last 10 years attest, cancer immunotherapy is the best strategy to restore the activity of the immune system and unleash its potential to destroy cancer cells in cancer patients. This chapter aims to discuss the recent findings on immune monitoring studies and the use of immune checkpoint inhibition in cancer immunotherapy.

Keywords: immune checkpoint blockade, immunotherapy, immune monitoring

1. Introduction

Throughout the evolutionary process, the immune system has developed mechanisms to protect the living beings against infections by different microorganisms, viruses, and parasites. A notorious question in immunology has been whether an immune response could also be raised against transformed cells. Researchers have indeed, for a long time, studied if cancer prevention could be a primary function of the immune system. The concept of immunological surveillance, a monitoring process in which the immune system detects and destroys virally

infected and neoplastic transformed cells, was elaborated more than 50 years ago by Lewis Thomas and Sir Frank Macfarlane Burnet [1–4]. Back in history, William B. Coley in 1893, an American bone surgeon and a pioneer in cancer immunotherapy, created a purified lysate of multiple bacteria to treat a young patient who had developed an inoperable sarcoma. As a result of the treatment, the patient had a complete remission. As head of the Bone Tumor Service at Memorial Hospital in New York, Dr. Coley would still inject more than 1000 cancer patients with bacterial products, then called Coley's toxins, which later were used by several physicians in several patients with bone and soft tissue sarcomas, reporting some excellent results [5]. In fact, the concoction initially called Coley's Toxin contained heat-killed *Streptococcus* combined with live *Serratia marcescens*. To Dr. Coley, the infection that he produced could contribute to shrinking malignant tumors. In 1909, the German biochemist Paul Ehrlich, winner of the Nobel Prize in Physiology and Medicine and father of chemotherapy, introduced the word Zauberkugel (the magic bullet) to describe antibodies. Generations of scientists interpreted the magic bullet as a compound that would target a single critical oncoprotein [6]. Unfortunately, at that time, the pioneering work led by Coley and Ehrlich could not explain the underlying mechanisms that activated the immune system to recognize and kill cancer cells. Nowadays, their ideas and the early experiments, which were aimed at fighting cancer and infectious diseases, serve to inspire new generations of researchers to develop compelling strategies of targeted therapeutics and immunotherapy. However, the role of the immune system in cancer recognition faced a shadowy period of disbelief mainly due to the difficulty in reproducing tumor regression in different types of cancer using Coley's toxin [7], the extremely toxic treatment [8], rejection of transplantable tumors [9], and the fact that thymic selection removed autoreactive T cells. These shreds of evidence led the scientists to believe that the role of the immune system as a primary strategy for recognizing cancer cells was minimal. With the advent of studies on cellular and molecular biology after the 1980s, several experiments were carried out to demonstrate that the immune system could efficiently act against cancer initiation and development. The fact that autoreactive T cells can escape from thymic selection [10], the discovery of tumor-associated antigens—TAAs [11], tumor antigen cross-presentation by dendritic cells to T lymphocytes [12], and the high frequency of cancer development in immunodeficient mice (STAT−/−, IFN−/−, RAG−/−, TCRβ−/−, TCRδ−/−, perforin−/−) [13] have considerably strengthened the concept of a protective immune system in the last decades. **Figure 1** displays a short chronological timeline of discoveries and progresses in cancer immunotherapy.

Hanahan and Weinberg on defining critical aspects of cancer development and progression, described a set of biological capabilities defined as "hallmarks of cancer." In their conceptualization, there are eight hallmark capabilities that are common to many, if not most forms of human cancer: sustained cell proliferation, evasion from growth suppressors, cell death resistance, replicative immortality, angiogenesis, tissue invasion and metastasis, deregulation of cellular energetics/metabolism, and avoidance of immune destruction [14, 15]. The primary goal of cancer immunotherapy has been to reawaken the immune system from a suppressive slumber to enable it to attack cancer cells once again. The fundamental principles that orchestrate cancer immunology and cancer immunotherapy can be described by immune surveillance, immune editing, and immune tolerance. A rapid increase in understanding the mechanistic

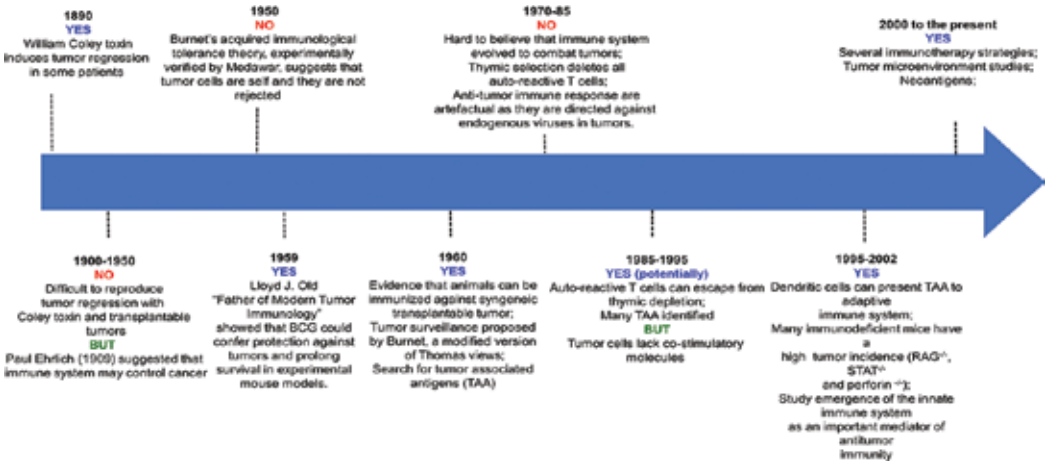

Figure 1. Is there an immune response to a malignant tumor? There was a time when the immune system was not recognized as having a protective role against developing cancer. That was until Burnet named the talent of immune system to detect tumor cells and destroy them as immune surveillance. Tumor surveillance by the immune system was, however, difficult to be practically shown.

pathways of these principles has led to clinical success in the treatment of cancer. In 2001, Robert Schreiber and Lloyd J Old (considered the father of Tumor Immunology) demonstrated that T lymphocytes and IFN-γ helped to inhibit the development of spontaneous and carcinogen-induced tumors in mice genetically deficient in RAG2 [16]. A few tumor cells escaped, however, from detection and eventually gave rise to tumors. Such selective evasion mechanism in a small number of tumor cells became known as immunoediting. In fact, the tumor cells that escaped became less immunogenic than the starting population and were no longer recognized by the immune system. At the time, the researchers wondered how these tumor cells have learned to outwit the immune attack.

Immunoediting consists of three well-established and orchestrated following processes called the "3Es" [17]. The first phase is the "elimination," in which the immune responses (innate and adaptive) can recognize and destroy tumors. Normal cells can prevent or inhibit malignant transformation through the expression of intrinsic tumor suppressors genes such as p16, p53, BRCA (breast cancer type 1 susceptibility protein), and APC (adenomatous polyposis coli). Chemical, physical, and biological factors can induce neoplastic transformation and consequently the expression of several tumor antigens, which can be captured, processed, and presented by dendritic cells and macrophages via MHC/peptides to T naïve cells. Immune cells such as CD4+, CD8+, NKT, and γδ T cells, as well as NK cells, and the cytokines released in this environment, such as IFN-y, IFN-α, IFN-β, and perforin, are responsible for tumor cell killing. The genetic instability and/or immune selection allow the transformed cells to resist to the immune response, starting the second phase, called "equilibrium," in which transformed cells that had survived to the immune surveillance phase are in dynamic equilibrium (growing cancer cells = dying cancer cells). When transformed cell variants selected in the second phase start a clonal growth in an immunologically controlled environment mainly due to the reduction of cancer immunogenicity followed by the immune exhaustion profile on T cells (PD-1, TIM-3,

LAG-3, and TIGIT), and then, the third phase of immunoediting called "escape" begins [18]. Classic mediators of immune escape include downregulation of co-stimulatory molecules (B7 molecules), antigen loss by downregulation of MHC molecules, increased resistance to apoptosis induction, and cell-mediated cytotoxicity due to the overexpression of antiapoptotic proteins such as FLIP and BCL-X, mutated Fas and TRAIL [19], expression of T cell inhibitory molecules such as PD-L1, B7-H3, HLA-G, HLA-E, and B7-x by cancer cells, tumor stromal cells, and APCs [20]. The presence of CD4 + CD25 + Foxp3 + T regulatory cells, IL-10 secreting T cells, M2 macrophages, immature dendritic cells, and myeloid-derived suppressor cells (MDSC) into the tumor or draining lymph nodes is decisive to maintain the immunosuppressive environment [21, 22]. Clearly, without a doubt, the immune system plays a dual role in the multifaceted interactions between cancer cells and the host.

Based on current immunological findings, the role of the immune system in the recognition and elimination of transformed cells is beyond any doubt. Numerous studies have investigated the immune system in cancer patients undergoing immunosuppression, mainly due to a decrease of lymphocyte proliferation and cytotoxic activity [23–27]. In this circumstance, the immune system becomes weak, inactive, or inefficient. Currently, immunotherapy includes several strategies for restoring cancer patient's immune system in an attempt to harness and to destroy cancer cells specifically. This chapter discusses the recent findings on immune checkpoint function and the immune monitoring studies in cancer immunotherapy.

2. T cell activation and immune checkpoint blockade

Currently, the pathways that preclude the complete and responsive immune response to cancer cells are better understood. Since CTLA-4 (cytotoxic T lymphocyte-associated protein 4) has a marked structural homology to CD28, and because it was unknown whether the antibodies used were agonistic or antagonistic, it was not clear whether CTLA-4 had an analogous function as a secondary co-stimulatory agent [28] or an opposing role as a dampener of T cell activation [29, 30]. Only the data from CTLA-4 knockout animals definitively revealed the inhibitory function of CTLA-4 [31, 32].

Cancer immunotherapy has been declared the breakthrough of the year, in 2013. The ecstasy is fundamentally grounded on the clinical success of antibodies that modulate immune checkpoints mainly by targeting CTLA-4 and PD-1 (programmed cell death protein 1). Immune responses are tightly regulated by a remarkable system with checkpoints that control either positively or negatively the magnitude of the immune response. Several immune checkpoint molecules expressed on T cells can promote activation of naïve T cells (stimulatory checkpoint pathway) or otherwise inhibit this activation by restraining T cell activation and extension of the immune response (inhibitory checkpoint pathway), thus regulating the homeostasis, magnitude of inflammation, and tolerance [33]. Positive co-stimulatory molecules on T cells such as CD28, 4-1BB, OX-40, ICOS, CD2, and CD226 (DNAM-1) allow for T cell activation, proliferation, and cytokine production. In contrast, negative signals, mediated by LAG-3, CTLA-4, PD-1 and PD-L1, VISTA, B7-H3, CD96, and TIGIT, downregulate T cell activation. These molecules

are critical to prevent autoimmunity and protect healthy tissues from immune activation. Finally, signals provided by pro-inflammatory cytokines mainly IL-12, IL-21, and type I interferons (IFN-α/β) are necessary for T cell response [34]. Blocking CTLA-4 and PD-1 using monoclonal antibodies represents the innovative concept in cancer therapy owing to (a) these molecules entirely ignore the tumor cells—they rely solely on the immune system; (b) they are not used to activate the immune system against a particular cancer but to neutralize inhibitory molecules that block a positive antitumor T cell response [35].

The immune system capacity to detect and destroy abnormal cells may prevent the development of many cancers. Cancer cells arise from normal cells, driven by mutations that lift brakes on cell proliferation. As an evolutionary process, tumor cells appropriate regulatory immune checkpoints to evade elimination. To keep growing, tumor cells take advantage from a sophisticated and dynamic bionetwork called the immune microenvironment. The microenvironment, in addition to tumor cells and infiltrating immune cells, contains epithelial cells, lymphatic and vascular vessels, cytokines, and chemokines [36]. Targeting the microenvironment for more efficient cancer immunotherapy in solid cancer has been a research objective in the last decade.

3. Monoclonal antibodies and novel checkpoint inhibitors in cancer immunotherapy

Monoclonal antibodies have had a considerable impact on the care of patients with cancer in the last 30 years. The initial report introducing the monoclonal antibody technology (hybridoma technique) arose from an article published by Köhler and Milstein in 1975, which caused a tremendous impact on laboratory research [37–39]. Sometime later, Kohler and Milstein won the Nobel Prize in Physiology or Medicine in 1984 awarded jointly to Niels K. Jerne. From then on, the new monoclonal antibodies aimed at cancer cell proteins such as CD20 (Rituximab, Ocrelizumab, Veltuzumab, Ofatumumab, and Obinutuzumab), HER-2 (Trastuzumab and Pertuzumab), EGFR (Cetuximab and Panitumumab), VEGF (Bevacizumab and Ramucirumab), GD2 ganglioside (Dinutuximab) or the immune cell surface inhibitors, PD-1 (Nivolumab, Pembrolizumab, and Pidilizumab), CTLA-4 (Ipilimumab and Tremelimumab), and PD-L1 (Atezolizumab, Avelumab, and Durvalumab). More effective immune responses can be achieved by modifying those monoclonal antibodies when they have failed, mainly due to the heterogeneity of epitope expression, the delivery to tumor cells, and antigenic modulation [40]. Several monoclonal antibodies have been conjugated to cytotoxic agents (mAb drug conjugates—ADCs). Some examples of ADCs include Ado-trastuzumab (anti-HER2 conjugated with emtansine), Gentuzumab ozogamicin (anti-CD33 conjugated with calicheamicin), Brentuximab vedotin (anti-CD30 conjugated with vedotin), immunotoxins (Moxetumomab pasudotox, Denileukin diftitox, DT2219, Resimmune, and SL-401), and radionuclides (131I-tositumomab and Y-ibritumomab) [41].

Presently, several efforts are being made to design effective combinations of immunotherapeutic mAbs and new agents that target particular pathways and to reach synergistic effects in the

Molecule	Expression status	Function	Cognate ligand	Mechanism of action	Tested in (Cancer types)	Blocking antibodies
CTLA-4 (CD152)	Upon activation of naïve T cells (CD4+ and CD8+); Constitutively expressed on suppressive T regulatory cells (CD4+Foxp3+ T regs; dendritic cells, monocytes, macrophages and B cells	Critical for initial activation of T cells in secondary lymphoid organs. Effector function, cell growth, survival and memory	CD80 (B7-1) CD86 (B7-2)	Outcompeting CD28 and by recruiting phosphatases to the cytoplasmic domain	Advanced and Metastatic melanoma	Ipilimumab and Tremelimumab
PD-1 (CD279)	Inducible on naïve T cells upon activation; Constitutively expressed on T regs; monocytes, macrophages, and B cells	Critical in regulating peripheral T cells tolerance. Effector function, cell growth, survival and memory	PD-L1 PD-L2	Reduces the signal downstream of TCR stimulation leading to a decreased activation and cytokine production; Induce genes that reduce T cell proliferation; decrease anti-apoptotic proteins and increase pro apoptotic	Advanced melanoma, metastatic melanoma, Prostate, Colorectal, Non–small cell lung cancer (NSCLC), Renal cell carcinoma (RCC)	Nivolumab and Pembrolizumab
TIM-3	Dysfunctional CD8+ tumor-infiltrating lymphocytes (TILs) also referred to as T cell exhaustion, intra-tumor Treg NK cells, monocytes, macrophages and dendritic cells	Effector function, cell growth, survival and memory	Galectin-9 Ceacam1 HMGB1 PtdSer	Causes negative signals on T cells resulting in apoptosis of Th1 and CD8+ cells	Tested in solid tumors and leukemia	TRS-022, LY3321367 and MBG453
LAG-3 (CD223)	T cells, NK cells, B cells, monocytes, macrophages, endothelial cells and dendritic cells Also, expressed on cancer cells	Effector function, cell growth, survival and memory	MHCII (Higher affinity than CD4)	Homologue of CD4+ Negative regulatory function on T cells; Mediate a profile of exhaustion in combination with PD-1 and TIM-3 on CD8+ T cells	Tested in Advanced renal cell carcinoma	IMP321 BMS-986016 MK-4280 GSK 2831781 LAG525
TIGIT	T cells and NK cells	Cell growth and effector function	Interacts with members of Poliovirus receptors family (PVR)	Acts as a functional ligand inducing a tolerogenic phenotype in dendritic cells, resulting in elevated IL-10 and reduced IL-12. A regulatory	Locally advanced or Metastatic solid tumors	OMP313M32 COM701

Molecule	Expression status	Function	Cognate ligand	Mechanism of action	Tested in (Cancer types)	Blocking antibodies
			DNAM-1 (CD226) CD96 PVRL 2 (CD112) PVR (CD155) Other nectins	role of TIGIT in modulating the signaling pathway, which facilitates M2-polarization, a class of immunosuppressive tumor associated macrophages that arise in response to Th2 cytokines; Like PD-1, LAG-3 and TIM-3 can be expressed by exhausted CD8+ T cells		
BTLA (CD272)	Dendritic cells, monocytes, macrophages, T cells (Th1) and B cells	Effector function, cell growth, survival and memory	HVEM	Display T cell inhibition	-	-

Data taken from: [20, 22, 30–33, 35, 47, 50, 76–78, 89–94, 100, 107, 109, 110, 112, 114, 116, 118, 124, 127, 130, 137, 145].

Table 1. Targeting potential co-inhibitory molecules which may contribute to improve the immune checkpoint blockade immunotherapy.

Molecule	Expression status	Function	Receptor	Mechanism of action	Tested in (Cancer types)	Blocking antibodies
PD-L1	NK cells, endothelial cells, stromal cells, epithelial cells and B cells	Regulates the development, maintenance and functions of T cells	PD-1	PD-L1/PD-1 on T cells provides a signal that prevents TCR-mediated activation of IL-2 production and T cell proliferation. The pathway involves inhibition of ZAP70 phosphorylation and its association with CD3ζ; PD-L1/PD-1 attenuates PKC-θ activation loop phosphorylation necessary for the activation of transcription factors NF-κB and AP-1, and for production of IL-2; PD-L1/PD-1 also	Bladder, non–small cell lung cancer, melanoma, breast, ovarian and pancreas	Atezolumab; Avelumab; Durvalumab

Molecule	Expression status	Function	Receptor	Mechanism of action	Tested in (Cancer types)	Blocking antibodies
				contributes to ligand-induced TCR down-regulation during antigen presentation to naive T cells		
PD-L2	Initially believed to be restricted to macrophages and dendritic cells; PD-L2 expression can be induced on a wide variety of other immune cells and nonimmune cells depending on microenvironmental stimuli	Regulates the development, maintenance and functions of T cells	PD-1	The same effect as above	Melanoma, Renal cell carcinoma, non-small lung cancer cell, bladder and head and neck	AMP-224 CA-170
B7-H3 (CD276)	Initially was believed to co-stimulate the immune response, but recent studies have shown that it has a co-inhibitory role on T-cells, contributing to tumor cell immune evasion; It has been found to be inducible on T cells, NK cells, and APCs; Broadly expressed on osteoblasts, fibroblasts, and epithelial cells, as well as in liver, lung, bladder, testis, prostate, breast, placenta, and lymphoid organs.	Regulates the development, maintenance and functions of T cells; Also, this molecule influences cancer development and progression beyond the immune regulatory roles	TLT-2 receptor on activated T cells	It is a member of the B7 family	Melanoma, Renal cell carcinoma, non-small lung cancer cell, bladder and head and neck, prostate, breast,	MGD009
B7-H4	mRNA is largely expressed in the peripheral tissues; Protein expression is restricted to activated B cells, T cells, and monocytes.	Regulates the development, maintenance and functions of T cells; Also, this molecule influences cancer development and	To date, the cognate receptor of B7-H4 on activated T cells remains unclear, although BTLA has been	It is a member of the B7 family	Non–small cell lung cancer, ovarian cancer, prostate cancer, breast cancer, and renal cancer	-

Molecule	Expression status	Function	Receptor	Mechanism of action	Tested in (Cancer types)	Blocking antibodies
		progression beyond the immune regulatory roles	reported as a possible receptor.			
Galectin-9	Cancer cells and MDSC	Regulates the development, maintenance and functions of T cells; Also, this molecule influences cancer development and progression beyond the immune regulatory roles	Loss of galectin-9 expression is closely associated with metastatic progression	A family of beta-galactosidase-binding proteins implicated in modulating cell-cell and cell-matrix interactions.	Several cancer cells	-

Data taken from: [20, 22, 30–33, 35, 47, 50, 76–78, 89–94, 100, 107, 109, 110, 112, 114, 116, 118, 124, 127, 130, 137, 145].

Table 2. Targeting cancer ligands which may contribute to improve the immune checkpoint blockade immunotherapy.

Molecule	Expression status	Function	Cognate ligand	Mechanism of action	Tested in (Cancer types)	Agonist antibodies
CD28	T cells	Priming, survival, cell growth and memory	CD80 (B7-1) CD86 (B7-2) ICOS-L (human)	Provide co-stimulatory signals required for T cell activation and survival. In addition to the T-cell receptor (TCR) can provide a potent signal for the production of various interleukin such IL-2, IL-4, IL-6, IL-13 and IFN-y	Solid tumors	Theralizumab (TGN1412)
CD27	T cells, NK cells and B cells	Priming, survival, cell growth, differentiation and memory	CD70	Transduces signals that promote the activation of NF-κB and MAPK8/JNK	Glioma	IMA950
ICOS (CD278)	Is not constitutively expressed on resting T	Priming, survival, cell	ICOSL	Induce the recruitment of	Advanced solid tumors	JTX-2011 GSK3359609IV

Molecule	Expression status	Function	Cognate ligand	Mechanism of action	Tested in (Cancer types)	Agonist antibodies
	cells; Rapidly induced following TCR cross-linking and/or CD28 co-stimulation on T cells and NK cells	growth, differentiation and memory		phosphatidylinositol 3-kinase (PI3K) culminating in the activation of Akt; Promotes the recruitment of p50α and p85α regulatory subunits of PI3K, in conjunction with recruitment of the p110δ catalytic subunit		
4-1BB (CD137)	Barely expressed levels on naïve T cells; Expressed by activated T cells, but to a larger extent on CD8 than on CD4 T cells.	Survival, cell growth, differentiation and memory	4-1BBL (CD137L)	A member of TNF receptor family; Delivers polyubiquitination signals via TNFR; inhibits apoptosis, enhances proliferation and effector functions; Alternative NF-κB activation	Lymphomas	PF-05082566
OX40 (CD134)	Expressed on activated CD4, T regs and CD8 T cells as well as in a number of other lymphoid and non-lymphoid cells; Low expression in naïve effector T cells, but rapidly upregulated upon TCR ligation; Additionally, suppresses the differentiation and activity of Treg	Survival, cell growth, differentiation and memory;	OX40L	Binds to TRAF2, 3 and 5 as well as PI3K; TRAF2 is required for survival via NF-κB and memory cell generation whereas TRAF5 seems to have a modulatory role (as knockouts have higher levels of cytokines and are more susceptible to Th2-meditated inflammation; Appears to be more potent costimulator of CD4+ T cells (both Teff and Treg) than for CD8 + T cells	Advanced solid tumors	PF-04518600 MEDI0562 MOXR0916
GITR	Expressed in several cells and tissues including B cells, T lymphocytes, NK cells and antigen-presenting cells (APC); It is upregulated by responder T cells ($CD4^+CD25^-$ T cells or $CD8^+CD25^-$ T cells)	Cell growth, differentiation and effector function	GITRL	It is a member of the TNFR superfamily; GITR signaling is mediated through the activation of NF-kB and members of the MAPK pathway, including ERK, p38 and Jnk; Up regulation of Bcl-XL expression on CD8+	Advanced solid tumors	MEDI1873

Molecule	Expression status	Function	Cognate ligand	Mechanism of action	Tested in (Cancer types)	Agonist antibodies
TNFRSF25 (DR3, Apo-3, LARD, TRAMP)	Expressed almost exclusively by lymphocytes (CD4+, CD8+, NK and NKT)	Survival, proliferation and effector functions	TL1A	The most recently identified TNF member; Transduces signals that promote the activation of NF-κB	Not tested yet	-

Data taken from: [20, 22, 30–33, 35, 47, 50, 76–78, 89–94, 100, 107, 109, 110, 112, 114, 116, 118, 124, 127, 130, 137, 145].

Table 3. Targeting potential co-stimulatory molecules which may contribute to improve the immune checkpoint blockade immunotherapy.

inhibition of tumor growth and development. After plenty of clinical trials and preclinical models, it is clear now that several inhibitory receptors may need to be blocked so that full T cell activation and antitumor immunity can be achieved. Blocking some T cell inhibitory receptors such as TIM-3 (T cell immunoglobulin mucin domain 3), LAG-3 (lymphocyte-activation gene 3), TIGIT (T cell immunoglobulin and ITIM domain), BTLA (B and T lymphocyte attenuator), VISTA (immunoglobulin suppressor of T cell activation), B7-H3, and B7-H4 has emerged as new target for immune checkpoint blockade strategies (**Tables 1** and **2**). In contrast, inducing T cell activation by mAbs directed to co-stimulatory molecules such as CD27, CD28, ICOS, OX-40, 4-1BB, and GITR has been successfully used as a cancer immunotherapy strategy against several types of cancer (**Table 3**) [33, 42–49].

4. Checkpoint blockade and neoantigens

The conventional treatment of patients with several cancer types involves in most cases, surgery, radiation, and chemotherapy. There is a crucial need to develop new therapies for cancer treatment. Some strategies for cancer immunotherapy including cytokines, signal transduction inhibitors, oncolytic viruses, bispecific antibodies, monoclonal antibodies, dendritic cells, engineered T cells, drug conjugates, radioimmunotherapy, angiogenesis inhibitors, and therapy with targeted toxins are currently increasing the perspectives of treating cancer patients [50]. Nevertheless, despite the recent achievements of these therapies, not every patient responds to immunotherapy and even the responders often experience toxic effects [51]. Moreover, there is a rising need to identify potential biomarkers, especially in immune cells, which could predict whether the cancer patient will respond or not to particular immunotherapy, such as immune checkpoint blockade, for example. Also, we need to improve our knowledge of the fundamental mechanisms and the elegant interface between the immune system and cancer. For example, dacarbazine has for decades been considered the gold standard for the treatment of metastatic melanoma. Immunotherapy, however, has extended the list of options available for the treatment of metastatic melanoma, and its success has been

supported by studies using immune checkpoint blockade, as with anti-CTLA-4 and anti-PD-1 [52]. Currently, the factors that preclude a completely effective immune response to cancer are better characterized. Poor immunogenicity is found in several tumors, which can be explained due to the lack of co-stimulatory factors that provide signals to fully activate T cells, mainly CD28 molecules [53]. Inhibitory molecules that repress T cell activation can also be present in the tumor microenvironment. The idea of immune checkpoint blockade and consequently the renaissance of cancer immunotherapy emerged when James Allison's group questioned why T cells were not being able to attack cancer cells effectively. Allison decided to look at a biological molecule called CTLA-4. The first evidence exhibiting the potential effect of anti-CTLA-4 arose from an article published by his group in 1996. In this article [54], the authors showed that the injection of a blocking CTLA-4 agent in tumor-bearing mice led to the rejection of pre-established tumors, including the rejection to the second exposure of tumor cells, when compared with naïve controls. The sequence of experiments published in this paper paved the route to a new perception in cancer immunotherapy—the immune checkpoint blockade [55]. Bristol Myers Squibb (BMS) sponsored the clinical trials with the anti-CTLA-4 antibody under the name Yervoy. In 2010, the results of the first phase-III clinical trial with Ipilimumab were published in the New England Journal of Medicine [56]. This paper provided evidence that Ipilimumab can significantly prolong the lives of patients with metastatic melanoma. A subset of patients under treatment exhibited permanent beneficial effects, and in some cases, their cancer was apparently "cured." Ipilimumab was the first therapy to provide durable remissions in a fraction of patients with metastatic melanoma in 30 years of exhaustive clinical research to show improved quality-of-life and overall survival (OS) [57]. The outcomes of a randomized clinical trial in patients with metastatic melanoma without BRAF mutation were reported by Robert et al. [58]. In this study, the authors compared the benefits of anti-PD-1 (Nivolumab) and dacarbazine therapy. The treatment with anti-PD-1 enhanced the overall survival, as compared with dacarbazine (objective response rate, 40 vs. 14%), in patients with advanced melanoma [58]. In a randomized, double-blind clinical trial, the results of the combination of anti-PD-1 (Nivolumab) and anti-CTLA-4 (Ipilimumab), as reported by Postow et al., achieved a considerably higher objective rate and longer progression-free survival when compared with Ipilimumab monotherapy as a first-line treatment in patients with advanced melanoma [59]. It is not new that the dual blockade using anti-CTLA-4 and anti-PD-1 improves antitumor responses by a complementary and distinctive mechanism [52]. The anti-CTLA-4 therapy acts improving the priming phase, whereas anti-PD-1 acts helping the effector phase [60]. Using a murine melanoma model, Curran et al. showed that the combination of anti-CTLA-4 and anti-PD-1 was more than twice as efficient as either therapy alone in generating an effector immune response against B16 melanomas. In this preclinical study, the authors showed that the dual immune blockade was able to expand the effector T cell infiltration and decrease the regulatory T cells and myeloid cell profile [61]. In another preclinical study, Selby et al. evaluated the dual blockade in murine colon adenocarcinoma model. The authors concluded that the concurrent therapy with anti-CTLA-4 and anti-PD-1 caused a synergic effect in the antitumor activity [62].

In the vast majority of tumors, the combination of the presence of cytotoxic lymphocytes, Th1 profile, and mature dendritic cells (DC) restrained at the tertiary lymphoid structures, are

associated with an excellent clinical outcome [63]. Nonetheless, recent findings show that the increase of CD8+ T cell infiltration does not always correlate with a good prognosis in cancer, as it could be seen in Hodgkin lymphoma, diffuse large B cell lymphoma, renal cell carcinoma (ccRCC), lung metastases from ccRCC, and non–small cell lung cancer (NSCLC) in which different densities of cytotoxic lymphocytes may or may not correlate with good prognosis [64–68]. These effects could be explained due to the expression of several negative immune checkpoints such as CTLA-4, PD-1, BTLA, TIM-3, LAG-3, VISTA, and TIGIT in infiltrating T cells or its ligands on tumor cells such as PD-L1, PD-L2, B7-H3, B7-H4 and HVEM that are fundamental to immune escape in cancer [51]. In fact, the nature of the interaction between the immune system and the tumor allows for the clinician to predict patient's prognosis and further guide immunotherapeutic strategies. One of the most meaningful challenges to the triumph of cancer immunotherapy is the relatively small percentage of responding patients. The leading causes of resistance to cancer immunotherapy, especially to the immune checkpoints blockade, could be explained by the failure of the T cells to become fully activated. Severely immune-compromised patients, low mutational neoantigen rates, inhibitory molecules, and the tumor microenvironment are considered crucial to dampening T cell activity [69]. Indeed, the resistance could also be induced by immunotherapy. After recognizing the antigen, the tumor-infiltrating lymphocytes (TILs) become activated, and then they start to produce IFN-γ. As a result of this activation, the IFN-γ released can promote the expression of PD-L1 on the tumor cells and increased IDO (indoleamine 2,3 dioxygenase) and CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) [70–72].

Starting with the comprehension that cancer is a genetic disease, the design of personalized molecularly targeted therapies, seems a rational step to endeavor. Resistance to several of these therapeutic agents such as Vemurafenib, Imatinib, Nilotinib, Erlotinib, and Trastuzumab is the main issue focused on current cancer research [73]. Transformed cells that may express, for instance, high levels of BRAF mutations, BCR-ABL, EGFR, and HER2 must be discerned from nontransformed cells. Through natural selection transformed cells, submitted to molecularly targeted therapies, have learned to escape from these therapies. Alterations in the drug target, activation of pro-survival pathways, and ineffective induction of cell death are some examples [74]. Consequently, there is a critical demand to develop new therapies for cancer treatment. The role of the immune system and its importance in conferring protection against transformed cells have been extensively discussed in this book. As the results from the last 10 years attest, cancer immunotherapy is the best strategy to restore the activity of the immune system and unleash its potential to destroy cancer cells in cancer patients. The absence of an immunocompetent system revealed the increase in the susceptibility to carcinogens induced in spontaneous cancer [75]. The genetic landscape of the antigens that allow the immune system to discriminate between cancer cells from nontransformed cells remains unclear. Not all antigens can elicit an effective immune response. A tumor rejection is defined by how satisfactory an immune response can act against a specific tumor antigen and how this response would impact on tumor growth [76].

Deep sequencing and DNA libraries have profoundly contributed to cancer immunology and immunotherapy, mainly by the characterization of neoantigens that arise from tumor-specific mutations [76]. As cancer cells divide, they accumulate mutations that result in altered or novel

peptide sequences specific to the tumor cell. Distinguished as neoantigens, these tumor-specific antigens could be the key to developing successful cancer therapies [77]. The exome-based cancer is, indeed, a crucial approach to determine the T cell reactivity against cancer neoantigens [78]. Preclinical models conducted by Castle et al. and Matsushita et al. provided the original evidence for the cancer exome-based method that could be used to identify neoantigens and interrogate about the T cell reactivity [79, 80]. One of the reasons why the immune checkpoint blockade, especially by anti-CTLA-4 and anti-PD1, successfully works on melanoma and lung cancer patients is the potential formation of a neoantigen repertoire [81]. Melanoma and lung cancer cells have a mutational rate above 10 somatic mutations per megabase (Mb) of encoding DNA, unlike astrocytoma, thyroid, medulloblastoma, neuroblastoma, glioblastoma, myeloma, ovary, thyroid, pancreas, and prostate cancers, which occasionally have one mutation per megabase [82]. That could explain why the effectiveness of the immune checkpoint blockade is not impressive in those tumors that have few somatic mutations and consequently a poor neoantigen repertoire. Such evidence together with assumptions about the tumor microenvironment, immune privilege, and the expression of negative immune checkpoints lead to an insufficient T cell activity [83, 84] and consequently cancer progression.

Several groups are trying to develop novel approaches so that the effect of the immune checkpoint blockade could be augmented in patients with few somatic mutations. To this end, the researchers are focusing their attention on the mechanisms involved in the antitumor response [85]. Preclinical models suggest that an effective antitumor response is obtained when Ipilimumab and Nivolumab induce lymphocyte responses to neoantigens expressed on the individual tumor [86]. If so, a therapeutic approach could be the combination of the immune checkpoint blockade with peptide vaccines. Since the majority of mutations are patient specific, this new approach could lead the way favoring personalized immunotherapy, combining immune checkpoint blockade with cancer vaccines containing a cocktail of peptides corresponding to neoantigens known to be expressed in a given patient's tumor cells.

In prostate cancer and gliomas, for example, the challenge for developing effective immunotherapy is discouraging. Although prostate cancer had the first adult solid tumor-approved vaccine (Sipuleucel-T), which prolongs survival, it was difficult to go beyond that [87]. High-grade gliomas such as DIPG (Diffuse Intrinsic Pontine Glioma) are destructive and incurable cancers, representing the main cause of pediatric brain tumor death. Growing diffusely in the ventral pons, DIPG causes disabling neurologic symptoms that gradually abolish the coordination of the face, pharynx, and body. Unfortunately, surgical resection is not a feasible option, radiation therapy results in just temporary stabilization of symptoms, and several chemotherapy trials developed for adult glioma have not been successful to date [88]. In both scenarios, the challenges that may account for this negative outcome could be (a) there are no immune-related biomarkers that can monitor efficacy in easily accessible tissues; (b) immunologic changes within the peripheral blood have been relatively unhelpful; (c) there is a disease stage; *d)* the immunotherapy efficacy may be therapy-specific (i.e., immune checkpoint therapies are more effective in cancers with high mutation rate, whereas vaccines can be more effective early in tumor progression [89]. As a basis for future research in cancer immunotherapy, immunological pathways in response to monotherapy versus combination therapy need to be assessed in the context of clinical outcome. Novel predictive and

prognostic biomarkers have been identified for immune monitoring and clinical correlation in several types of cancer [90–92].

5. Cancer immune monitoring

Immune monitoring studies have supported the hypothesis that combining immunotherapy and standard treatment or their use as monotherapy can benefit patients developing different types of cancer. Analyzes involved ligands, infiltration quality, co-stimulatory/inhibitory profile, and microenvironment. Several assays such as whole exome sequencing (WES), protein array, flow/mass cytometry (CyTOF), multicolor immunohistochemistry (IHC), Multiplexed Ion Beam Imaging (MIBI), Systematic evolution of ligands by exponential enrichment (SELEX), epigenetic modification, and B/T cell receptor repertoire sequencing have been used to pursue potential biomarkers and contribute for the future of cancer immunotherapy [93–96]. Also, these studies have the potential to elucidate immunological mechanisms of antitumor responses, monitor disease progression, evaluate the therapeutic effect, identify candidates for immunotherapy, and serve as prognostic markers of clinical outcome. As discussed above, neoantigens expressed on cancer cells can elicit cellular and humoral immune responses, and they also can be identified to develop immunotherapies [97]. Patient serum and tissue samples can be analyzed to determine candidate tumor-associated neoantigens or genes that evoke cellular and humoral immune responses in cancer patient [98]. Since fresh tumor samples from cancer patients are not always possible to obtain, several clinical studies are undertaken on peripheral blood samples. The successfulness of anti-CTLA-4 and anti-PD-1 in the clinic has stimulated further studies on other molecules that can be targeted. There are several known checkpoint molecules, and their evaluation has progressed to clinical trials. Immunophenotyping studies using the approaches quoted above, examine for instance, the activation or exhaustion of the T cell markers (CD28, CD27, ICOS, OX40, GITR, 4-1BB, PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, BTLA, and VISTA) and the tumor microenvironment ligands (PD-L1, PD-L2, ICOSL, OX-40L, 4-1BBL, Galectin, B7H3, and B7H4). T cell populations including but not limited to CD4 cells, CD8 cells, NK cells, and their subpopulations such as activated T cells, MDSCs, and Tregs have been analyzed in several immune monitoring studies [99–103]. Serum cytokines, chemokines, and angiogenic factors have also been investigated by ELISA, ELISPOT, or other relevant multiplex-based protein assay methods [104, 105]. By questioning the efficacy and even the possible failure, the potential of using immune monitoring studies in cancer prognosis, prediction of treatment efficacy, immune tolerance, and disease progression have contributed to the improvement of the immune-related response criteria (irRC) [106].

Currently, immune checkpoint blockade therapies represent the breakthrough in cancer therapy and have led to robust antitumor responses and clinical benefit in a large number of patients with cancer, but, despite the outstanding achievement of clinical applications of the checkpoint blockade, the efficacy of these therapies differ critically across individual patients and among different tumor types [107, 108]. There is an urgent need to find potential biomarkers that could predict whether cancer patients would respond to the immune checkpoint blockade [109].

Fan et al. using Ipilimumab in a cohort of patients with bladder cancer showed the ICOS molecule (Inducible T cell co-stimulator) to be selectively upregulated in intratumor CD8+ and CD4+ T effector cells [110]. This particular clinical trial indicated the ICOS/ICOSL pathway as relevant for antitumor immune responses in bladder cancer patients under Ipilimumab treatment. Liakou et al. showed that Ipilimumab therapy led to an increase in IFN-y secretion by T cells [111]. It is well-established that melanoma cells are sensitive to IFN-y and quite often some cells containing defective IFN-y signaling genes may be resistant to IFN-y mediated growth inhibition and apoptosis. In order to investigate the reasons determining responders or nonresponders to Ipilimumab therapy, Gao et al. evaluated from whole exome sequencing data the genomic alterations in the family genes of IFN-y pathways in melanoma tumors [112]. The authors encountered significantly more somatic mutations, including copy-number alterations (CNAs) and single-nucleotide variants (SNVs) in nonresponders. Since their results suggested that CNAs in genes of the IFN-y pathways in melanoma patients could predict initial resistance to Ipilimumab, the authors also evaluated data on a total of 367 patients with metastatic melanoma in the TCGA (The Cancer Genome Atlas) database. About 36% of patients had CNAs in the IFN-y pathway genes and had significantly shorter overall survival when compared with the wild-type tumor genes. In order to explain the acquired resistance to PD-1 blockade (Pembrolizumab) treatment, Zaretsky et al. compared melanoma tissues from the baseline with the tumors that had relapsed months to years. As a result, the authors found new JAK1/2 loss-of-function mutations and truncating mutations in the beta-2 microglobulin (B2M) gene. These two studies are closely related to the melanoma development, progression, and primary resistance to anti-CTLA-4 and anti PD-1 [113, 114].

Immune checkpoint blockade seems to be a promising approach for patients with orphan types of cancer like squamous cell carcinoma (SCC). This type of cancer is rare and is caused by Human Papillomavirus (HPV) infection. Until now, there is no consensus treatment for the metastatic form. Morris et al. evaluated tissues from patients who received at least one dose of Nivolumab. As a result, the authors found an objective response in 24% of patients with metastatic SCC. Immunohistochemistry and flow cytometry of baseline biopsies showed a link between the therapy responses and the presence of an activated inflammatory profile in the tumor. Tumors from the responders had more activated effector T cells at baseline than non-responders. The authors also showed a high expression of PD-1/PD-L1 and higher co-expression of inhibitory molecules such as LAG-3 and TIM-3 in baseline tissues among responders than in nonresponders [115], indicating a previous activation profile in those cells before the treatment. These results were consistent with other solid tumors such as melanoma [56]. The expression of immune molecules in pretreatment biopsies has been described to correlate with response rates in patients with melanoma and other types of cancer, but a fundamental class of biomarkers has not been identified. It seems that PD-1/PD-L1 and inhibitory molecules may serve as an indirect biomarker of acquired immune resistance in response to tumor antigen-specific T cell infiltration [116]. Gao et al. identified additional immune-inhibitory paths in the prostate tumor microenvironment in patients untreated and treated with Ipilimumab. Under the Ipilimumab therapy, there was an increase in immune cells infiltration, including macrophages expressing PD-L1 and VISTA both acting as suppressors of T cell function. Their data advocated that VISTA could represent another inhibitory

mediator after immune checkpoint blockade therapy [117]. Genomic and cellular tools to determine several immune signatures in longitudinal biopsies collected at multiple time points during anti-CTLA-4 followed by an anti-PD-1 blockade of melanoma progression were used by Chen et al. [109]. At the baseline, there was no change in any of the measured biomarkers (CD45RO, CD20, CD57, CD68, Foxp3, Granzyme B, PD-1, LAG-3, CD14, CD33, CD163, and CD206), comparing responders and nonresponders to the CTLA-4 blockade. During the treatment, however, there was a significantly higher density of CD8+ T cells in responders than in nonresponders. Furthermore, a higher expression of CD45RO, CD20, CD57, Foxp3, and Granzyme B was observed in responders versus nonresponders in the CTLA-4 blockade arm. Together, these data are relevant in the attempt to identify biomarkers of response and resistance to the immune checkpoint blockade while offering a mechanistic understanding of PD-1 blockade as associated to enhanced cytotoxic activity, antigen processing, and IFN-y pathway [109].

Anagnostou et al. performed a comprehensive study using a genome-wide sequence of protein-coding genes and T cell receptor clonotype analysis followed by functional assays of autologous T cell activation of non–small cell lung cancer in patients that demonstrated initial response and in those patients who experienced checkpoint blockade resistance (anti-CTLA/-anti-PD-1). The authors found a relationship between the acquired resistance and the loss of mutations encoding putative tumor-specific neoantigens. In the tumor samples analyzed at the time of acquired resistance, the authors also found that the majority of eliminated mutations were in genes typically expressed at high levels in lung cancer, which encoded neoantigens that were predicted to either confer high-affinity MHC binding or affect TCR contact residues [118].

TIM-3 is a co-inhibitory immune checkpoint receptor that is highly expressed in dysfunctional CD8+ tumor-infiltrating lymphocytes (TILs) also referred to as T cell exhaustion, intra-tumor Treg cells, monocytes, macrophages, and dendritic cells [119]. It is characterized as a type I transmembrane protein that was originally described in an EAE model (autoimmune encephalomyelitis). Monney et al., in an attempt to identify novel cell surface molecules that would label IFN-y producing Th1 and CD8+ stimulated naïve T cells, found the expression of TIM-3 in these cells. Furthermore, subsequent studies showed that anti-TIM-3 antibodies exacerbated EAE [120]. Galectin-9 (C-type lectin galectin-9), Ceacam 1 (carcinoembryonic antigen cell adhesion molecule 1), HMGB1 (high-mobility group box 1), and PtdSer (phosphatidylserine) have been identified as four TIM-3 ligands [121]. Interaction with TIM-3 caused negative signals on T cells resulting in apoptosis of Th1 and CD8+ cells [122].

High levels of TIM-3 on CD8+ have been correlated with poor prognosis in tumor progression [123]. Exhausted T cells were associated with PD-1+ single positive CD8+ cells [56]. In some types of cancer as lung, melanoma, and renal cancer, resistance to these therapies has gradually been observed [124–127]. To elucidate the mechanisms of adaptive resistance, Koyama et al. analyzed the tumor microenvironment in the context of anti-PD-1 therapy in two immunocompetent mouse models of lung adenocarcinoma. In the tumor progression, following response to anti-PD-1, the authors observed upregulation of TIM-3. According to the mouse model, TIM-3 upregulation was time dependent in TILs expressing PD-1. TIM-3 blockade using anti-TIM-3 overcame the acquired resistance to the PD-1 blockade. Furthermore, the same scenario could be observed in humans. Patients who developed adaptive resistance to

anti-PD-1 therapy also showed a comparable TIM-3 upregulation [119]. In patients with metastatic melanoma, Fourcade et al. found approximately 30% of NY-ESO-1–specific CD8+ T cells that expressed TIM-3 [128]. Gao et al. analyzed patients with non–small cell lung cancer (NSCLC), and approximately one-third of CD8+ tumor-infiltrating T lymphocytes (TIL) expressed TIM-3 [129]. Also, Yang et al. analyzed patients with follicular B cell non-Hodgkin lymphoma, and approximately one-third of lymph node CD4+ T and CD8+ T cells expressed TIM-3 [130]. In these three different types of cancers, TIM-3 positive T cells co-expressed PD-1 and exhibited defects in the proliferation of effector cells and cytokine production. In fact, TIM-3 labels dysfunctional T cells in multiple cancer types both in experimental models and in humans. Anti-TIM-3 antibodies have shown good results as monotherapy in some preclinical cancer models and when used in combination with anti-PD-1 antibodies [131–134]. Since TIM-3 expression has been shown to regulate Th1 and Tc1 responses negatively, Th17/T regulatory cells, innate cell activation, and T cell exhaustion, there is rational evidence for targeting TIM-3 [135]. Recently, Gefen et al. isolated an oligonucleotide aptamer ligand that blocked the interaction between TIM-3 with Galectin-9 with a high-affinity and specificity in T cells. The authors demonstrated in vitro, a reduced cell death followed by enhanced survival, proliferation, and cytokine secretion. In in vivo experiments, the aptamer postponed tumor development as monotherapy and synergized with anti-PD-1 in prolonging the survival of the tumor-bearing mice. Together, these results indicate that TIM-3 signaling exerts a secondary effect in keeping T cell immune responses in check [136].

LAG-3 (Lymphocyte-activation gene) is a reliable cancer immunotherapeutic target like TIM-3, due to its negative regulatory function on T cells and its ability to mediate a profile of exhaustion in combination with PD-1 [137]. LAG-3 is a type I membrane protein highly homologous in structure to CD4, described for the first time in 1990 as a novel protein identified on activated NK and T cells [138]. The structural motifs in CD4 and LAG-3 are highly conserved, but LAG-3 can bind to MHC class II molecules with higher affinity than CD4 [139]. As TIM-3 is a marker of IFN-producing Th1 cells, LAG-3 is a marker of IL-10 producing T regulatory cells in both mice and humans [140]. The first evidence in vitro on the role of LAG-3 inhibiting T cells was shown by Huard et al., when the authors by blocking LAG-3 increased the proliferation of human T cells [141]. Furthermore, the ectopic expression of LAG-3 on mouse CD4+ T cells reduced their proliferation [142] significantly. Unlike CTLA-4 knockout (KO) mice, which develop spontaneous lymphoproliferative diseases, mice lacking LAG-3 do not develop lymphoproliferative disorders. In the absence of LAG-3, however, T regulatory cells display a reduced activity [143]. Besides the negative regulation on T cell activation, innate cell activation, and T cell exhaustion, LAG-3 also induces the upregulation of cell surface receptors such as CD40, CD80, CD83, and CD86 in monocyte-derived dendritic cells (DCs) [144]. These facts led Quezada et al. to affirm that LAG-3 has a more complex role in immune homeostasis than just inhibiting T cell activation [145]. LAG-3 has been suggested to regulate the activity of PD-1 cells, and their co-expression has been shown in malignant mouse and human tumor cells [146]. Using murine models of B16 melanoma, MC38 colorectal adenocarcinoma, and Sa1N fibrosarcoma, Woo et al. also showed that the combinations of anti-LAG-3/anti-PD-1 antibodies inhibited tumor growth and progression besides enhancing adaptive immune responses in tumor-infiltrating lymphocytes [147].

TIGIT (T cell immunoreceptor with Ig and ITIM domains) also known as WUCAM is an inhibitory receptor, member of the poliovirus receptor (PVR/nectin family) classified as type 1 transmembrane domain, with an intracellular domain containing a canonical receptor tyrosine-based inhibitory motif (ITIM) and an immunoglobulin tyrosine tail (ITT) [148]. Yu et al [149] discovered TIGIT expressed in regulatory, memory and activated T cells. Currently, we know that TIGIT is also expressed in T regulatory and NK cells in multiple types of cancer [150]. CD155 and CD122 are TIGIT ligands, expressed in macrophages and dendritic cells [151]. TIGIT is upregulated in tumor-specific peripheral CD8+ T cells and CD8+ tumor-infiltrating lymphocytes (TILs) from patients with metastatic melanoma and TIGIT-expressing CD8+ T cells often co-express PD-1. In metastatic melanoma, Chauvin et al. showed that TILs from these patients downregulated the co-stimulatory molecule CD226. It has been shown that CD226 competes with TIGIT for the same ligand, supporting a TIGIT/CD226 imbalance in metastatic melanoma [152]. In addition to its role as a lymphocyte receptor, TIGIT acts as a functional ligand inducing a tolerogenic phenotype in dendritic cells, resulting in elevated IL-10 and reduced IL-12 [153]. A regulatory role of TIGIT in modulating the signaling pathway, which facilitates M2-polarization, a class of immunosuppressive tumor-associated macrophages that arise in response to Th2 cytokines was shown by Chen et al. [154]. The capacity of TIGIT to interfere in the tumor microenvironment by suppressing the immune response mediated by an increase of T regulatory activity, recruitment of MDSC, induction of blood vessel formation, cancer-associated fibroblasts, NK cell inhibition, and CD8+ T cell-mediated tumor killing, priming, and differentiation, suggest altogether that cancer cells upregulate TIGIT pathway to promote immunosuppression [151]. TIGIT becomes, therefore, a good candidate for the blockade in combination with anti-CTLA-4 and anti PD-1 [155–161].

VISTA (V-region Immunoglobulin-containing Suppressor of T Cell Activation) was discovered, characterized, and functionally defined as a novel hematopoietically restricted inhibitory ligand by Noelle's group. It is expressed primarily within the hematopoietic compartment (monocytes, neutrophil, and dendritic cells) with a low expression on CD4+, CD4+ Foxp3+ T regulatory cells, and CD8+ T cells [162]. VISTA is a type I transmembrane protein, with a single N-terminal immunoglobulin V domain and sharing structural similarities with PD-1, CD28, and CTLA-4 [163]. Remarkably, this molecule is at the same time a ligand and can function as a receptor. Wang et al. evaluated in vitro and in vivo the role of VISTA as a ligand. The authors conducted a range of experiments using VISTA-Ig fusion protein or VISTA expression on APC's. In both situations, VISTA was able to inhibit CD8+ T and CD4+ T cell proliferation and cytokine production at the early stage of activation mainly by suppression of CD25, CD44, CD69, and CD62L markers, IL-2, and IFN-y [164, 165]. *In vivo* experiments led the authors to conclude that VISTA expression in tumor cells can overcome protective antitumor immunity. To achieve this conclusion, mice were immunized with irradiated MCA105 fibrosarcoma tumor cells that do not express VISTA and were re-challenged with MCA105 overexpressing VISTA. Cancer cells expressing VISTA showed enhanced tumor growth compared to the VISTA negative parent MCA105. Furthermore, Lines et al. using VISTA-Ig fusion protein demonstrated *in vitro* that VISTA could increase the conversion of naïve CD4+ T into T regulatory cells in both human and mice [166]. The anti-VISTA monotherapy impaired tumor growth in several types of cancer (B16OVA melanoma, B16-BL6 melanoma, MB49 bladder

carcinoma, and PTEN/BRAF inducible melanoma) and altered the cellular composition of the tumor microenvironment enhancing T cell responses within the tumor by cytotoxic and cytokine production such as IFN-y and TNF-alpha [167].

As a receptor, VISTA molecules on T cells have been shown to regulate their activity negatively. VISTA is a co-inhibitory receptor on CD4+ T cells because it suppresses early CD4+ T cell expansion in vivo and CD4+ T VISTA−/− cells responded more strongly than wild-type (WT) CD4+ T cells to both polyclonal and antigen-specific stimulation, leading to increased proliferation and production of cytokines such IFN-γ, TNFα, and IL-17A. The anti-VISTA monotherapy impaired tumor growth in several types of cancer (B16OVA melanoma, B16-BL6 melanoma, MB49 bladder carcinoma, and PTEN/BRAF inducible melanoma) and altered the cellular composition of the tumor microenvironment enhancing T cell responses within the tumor by cytotoxic and cytokine production such as IFN-y and TNF-alpha [167]. Their results announced a new role for VISTA molecules, as a regulator of the tumor microenvironment playing an essential function in regulating protective immunity to cancer.

The exciting development of cancer treatment recently fostered the ambition of the traditional cancer therapy to increase the median of survival from a few months to definitely announce victory against cancer. Currently, we have been able to move the median survival a little longer especially with the approval by the FDA of anti-CTLA-4, anti-PD-1, and therapeutic combinations. One must be cautious, however, because currently, only about 30% patients are responders to immunotherapy. This fact has incited for the search of new molecules, new biomarkers, and new combinations such as other checkpoint blockers, co-stimulatory molecules agonists, IDO pathway inhibition, oncolytic viruses, adoptive T cell transfer, T cell engineering, therapeutic vaccines, targeted therapy, chemotherapy, and radiotherapy in the attempt to increase the number of responders and consequently of survivors. There has been much of enthusiasm on recent news about immunotherapy in the treatment of cancer patients. Int the past year, checkpoint inhibitors have become an impotant tool for treating certain types of tumor such as non-small cell lung cancer (NSCLC) and melanoma with an increase in the median survival. Novel immunotherapeutic approaches are essential to the success in the treatment of different cancer types.

6. Conclusion

The effectiveness of monoclonal antibodies, especially the immune checkpoint blocking ones, associated to other cancer therapies and consequently with the improvement of preclinical studies and the advent of screening techniques, constitutes a unique opportunity to understand and overcome drug resistance. Not only that but also by profoundly investigating predictive biomarkers related to the different immunotherapeutic agents. As discussed in this chapter, to date, there are three types of potential biomarkers that have been studied exhaustively: (a) Immune infiltrate in the tumor; (b) high mutation profile (neoantigens); and (c) expression of PD-L1 by tumor cells or tumor cell infiltrates. Data from immune monitoring studies have

provided a link between immunologic/genomic and proteomic platforms. The main goals of the immune checkpoint blockade are to either stimulate the T cells to attack cancer cells or to suspend the suppression of remaining antitumor T cells. The immune monitoring study consists in analyzing the activity of innate and adaptive cell populations like T cells, B cells, myeloid-derived suppressor cells (MDSC) and natural killer (NK) cells, which are critical in the immune response against cancer and may regulate positively or negatively T cell responses. In summary, this approach may lead to the identification of biomarkers that will predict whether immune checkpoint blockade (monotherapy or combination) would be sufficient to induce an objective response.

The most critical cell populations include the total CD4+ T and effector CD4+ T cells, T regulatory cells, total CD8+ T, naive, T central memory and T effector memory cells; MDSC (myeloid-derived suppressor cell), and B cells; and M1 and M2 macrophages have recently being studied in the context of cancer development. A great number of molecules involved in immune responses against cancer cells have been studied, such as immune checkpoint molecules on T cells, 4-1BB (CD137), CTLA-4, GITR (glucocorticoid-induced TNFR-related protein), OX-40 (CD134), TIM-3, LAG-3, PD-1, and ICOS (inducible T cell co-stimulator); cytotoxic and cytokine secreting molecules on NK cells such as 4-1BB, CD69, NKG2A, NKG2C, NKG2D, NKp30, NKp44, and NKp46; some ligands on tumor cells such as B7H3, B7H4, CD73, CD80, CD86, CD137, PD-L1, PD-L2, ICOSL, Galectin 9, MIC A/B and OX40; and the expression of transcription factors such as Bcl-6, Blimp, CD27, CD28, Eomes, Ki-67, ICOS, and c-myc. They might bring a better understanding of the immune response under immunotherapy and help us to answer why not every patient responds to immunotherapy. Immunotherapy offers at least three actions that no other modality of cancer therapy provides: specificity, memory, and adaptability. We have consistently seen that one of the principal issues of immunotherapy strategy is the enhanced proportion of responders to the immunotherapeutic agents. Combining immune checkpoint blockade with other therapies, which overcome the possible failures, may lead to synergies. That is the reason why the most broadly studied combination of checkpoint blockade agents uses the anti-CTLA-4 and anti-PD-1 monoclonal antibodies. However, a more in-depth understanding on the mechanisms of efficacy and the identification of resistance to checkpoint blockade and their agents are needed. Despite significant progress in the immune checkpoint blockade, much remains to be done. Inquiries on the responder profile, the differences between mouse models and results application to clinical studies, the relative effects on effector, T regulatory and other cells, expressing several immune checkpoints, and the comprehension regarding the differences between the immune profile in different compartments such as in the periphery versus the tumor microenvironment must be addressed. Clinical samples and immune monitoring approaches obtained at multiple time points during immune checkpoint blockade would be valuable for exploring the responsiveness and nonresponsiveness profile. Additionally, studies of immune modifications within human cancer cells and the tumor microenvironment have the potential to establish efficacy and resistance mechanisms. In this context, The Cancer Genome Atlas project (TCGA, available at https://cancergenome.nih.gov) has helped to identify some mutations in cancer cells, which increase the prospect of resistance to immunotherapy. Exciting secret waits to be unveiled.

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Conflict of interest

The authors declare there is no conflict of interest.

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Chapter 3

New Strategies to Improve Therapeutic Vaccines

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Additional information is available at the end of the chapter

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Abstract

Vaccination represents a viable and attractive strategy for therapeutic treatment of cancers by the power of a patient's own immune system. Major advances in cellular and molecular immunology have led to the approval of the first therapeutic cancer vaccine by FDA. However, the development of cancer vaccines remains infant. Maximizing the therapeutic efficacy while minimizing side effects of the therapeutic cancer vaccine remains key challenges to this field. In this review, we summarized the recently developed strategies to induce anti-tumor responses *in vivo* to improve the outcomes of cancer vaccines, with an emphasis on the guiding principles that are critical for rational design of effective and safe vaccines against cancers.

Keywords: immunotherapy, cancer vaccine, anti-tumor immunity, APCs, effector T cells

1. Introduction

Recent understanding in cancer immunology and the development of cancer immunotherapy have remarkably advanced the clinical treatment of cancer, leading to US Food and Drug Administration approvals of cell-based immunotherapies (Provenge, Kymriah, and Yescarta), and immune checkpoint inhibitors (Ipilimumab, Nivolumab, Atezolizumab, Avelumab), among others. Regardless of the progress, in most immunotherapies for cancer patients, the response is often of low frequency and moderate avidity, and does not result in objective clinical responses [1, 2]. For example, while immune checkpoint blockade therapies of various cancers yield impressive clinical outcomes, these therapies do not alter the frequency of tumor-specific T cells. Additionally, although dendritic cells (DCs) pulsed with tumor associated antigens can result in the expansion of antigen-specific T cells, the level of responses is often too low to mediate long-lasting tumor destruction [3]. This situation can be remedied with

therapeutic cancer vaccines which are designed to induce or augment the magnitude and quality of antitumor immune responses.

Currently many diverse therapeutic vaccine strategies are under development or being evaluated in clinical trials. Based on their content, they may be classified into different categories, including cell-based vaccines, subunit vaccines, and genetic vaccines. Each of these vaccine platforms targets specific immune pathways and has strengths and weaknesses detailed in our next discussion. One of the major goals for these vaccine strategies is to break the tumor-related immunosuppression. This challenge can be partially addressed by the development of new vaccine strategies, or optimization of current vaccines including the choice of antigen, the immunological adjuvants, formulations for delivery, vaccine efficacy, safety and toxicity considerations. Additionally, preclinical studies have clearly demonstrated that vaccines alone might not be sufficiently potent to overcome the complex immunosuppression within the tumor microenvironment [4]. Therefore, vaccines in combination with other immunotherapies might provide synergistic mechanisms to amplify the therapeutic outcomes. For example, the preclinical success of vaccines combined with immune checkpoint blockade have highlighted the potential to move beyond current paradigms of cancer vaccines [5, 6]. Here we summarize recent strategies to improve therapeutic vaccines for cancer.

2. Immunological background

The immune system is comprised of a network of lymphoid organs, tissues and different types of cells including lymphocytes, dendritic cells and nature killer cells. The immune system plays a crucial role in protecting the body against microbial pathogens and also in restraining the development of cancer [7–9]. Engineering the immune system to provide protective immunological memory (a procedure called vaccination) has been one of the most successful and cost effective medical interventions to date, saving millions of lives every year via pediatric and adult immunizations [9]. The process that immune system responds to foreign pathogens, allergies, self-damaged cells, and graft is called an immune response, which can be generally classified into innate response and adaptive response.

Innate response or nonspecific immune response, recognizes invading pathogens via PAMPs (pathogen associated molecular patterns) that are evolutionarily conserved molecular motifs expressed by a variety of microbes [10, 11]. PAMPs are mainly detected and recognized by innate immune cells through Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) [10]. Recognition of PAMPs by immune cells including phagocytic cells (macrophages and neutrophil) and antigen presenting cells (APCs) triggers a cascade of signaling pathways and activates these immune cells, promoting phagocytosis of pathogens and providing the first line of defense against many common pathogens. Innate response causes rapid inflammation at the site of infection which results in redness, swelling, heat, and pain. Innate response also plays a crucial role in the initiation of adaptive immune responses [10, 11].

Adaptive response, on the other hand, is referred to as a specific immune response. During adaptive response, highly specialized lymphocytes including T cells and B cells are activated by APCs engulfing and processing pathogens or antigenic molecules associated with pathogens.

Once activated, these lymphocytes undergo proliferation and differentiation into effector cells which can eliminate pathogens or inhibit their proliferation and growth. In addition to specificity, another feature that differentiates adaptive response from innate response is immunological memory which is developed after initial adaptive response to a specific pathogen and can recall specific immune response to the same pathogen in future encounters. Adaptive immune responses are tightly linked to innate immune responses [12]. For example, the TLR stimulus promote maturation of dendritic cells (DCs), the most efficient APCs and trigger the upregulation of costimulatory molecules on DCs for efficient antigen presentation.

Although it appears that adaptive response is more advanced and sophisticated than innate one, their roles in immunomodulation are inseparable and they complement each other in eliciting effective immune response to pathogens. Innate response is generally prerequisite to the activation of adaptive response which in return can enhance innate immunity by effector molecules such as cytokines and antibodies [10–12].

Cancer is one of the leading causes of death worldwide, accounting for more than 8 million death each year [13]. While traditionally cancer is treated with surgery, radiation, or chemotherapy, immunotherapy which harnesses the power of patients' own immune system has come of age over the last decades as a new treatment modality to fight against cancer, with cancer vaccine emerging as a novel approach to cancer treatment [14–18]. Unlike the traditional vaccine by which antibody responses are needed to prevent the disease from developing, therapeutic cancer vaccines heavily rely on cytotoxic T cell responses that are designed for patients with established diseases [14]. The initiation and maintenance of anti-tumor immune responses is a multi-step, complex process that involves the coordinated action of immune cells and molecular signals within the immune system [14]. For example, the induction of systemic antitumor immunity involves the priming of both $CD4^+$ and $CD8^+$ T cells specific for tumor-associated antigens. The process is initiated with antigen uptake by professional APCs especially DCs. In the presence of appropriate immune signals (e.g., TLR ligands), DCs are activated and migrate to LNs, where they present antigen fragments in the context of major histocompatibility complex (MHC) to effector T cells. In the draining LNs, $CD4^+$ and $CD8^+$ T cells recognize peptides presented via MHC class II and MHC class I on DC surface, respectively. And if DCs are properly activated, these T-cells proliferate and differentiate into effector cells that can directly kill cancer cells ($CD8^+$ "killer" T-cells) or secrete cytokines that help other cells ($CD4^+$ "helper" T-cells) [19–21]. Effector T cells traffic to tumor site, recognize tumor cells by T cell receptor (TCR), and secrete cytotoxins such as perforin and granzymes which trigger tumor cell apoptosis. An effective cancer vaccine aims to target these essential steps and reinforce tumor-specific T cells immunity to combat tumors. Adaptive immunity-dominated anti-tumor activities are illustrated in **Figure 1**.

Most vaccines in use today were developed by techniques that were pioneered more than 100 years ago and do not provide protection in many diseases. For example, although highly effective for combating acute infections such as polio, measles and diphtheria, traditional vaccination technologies have failed to elicit immune responses that provide protection against chronic infections (e.g. HIV, malaria) and have not succeeded in therapeutic settings, which are designed to harness the patient's immune system to treat an existing disease (e.g. HIV or cancer). Traditional vaccine approaches induced transient anti-tumor immunity that failed to

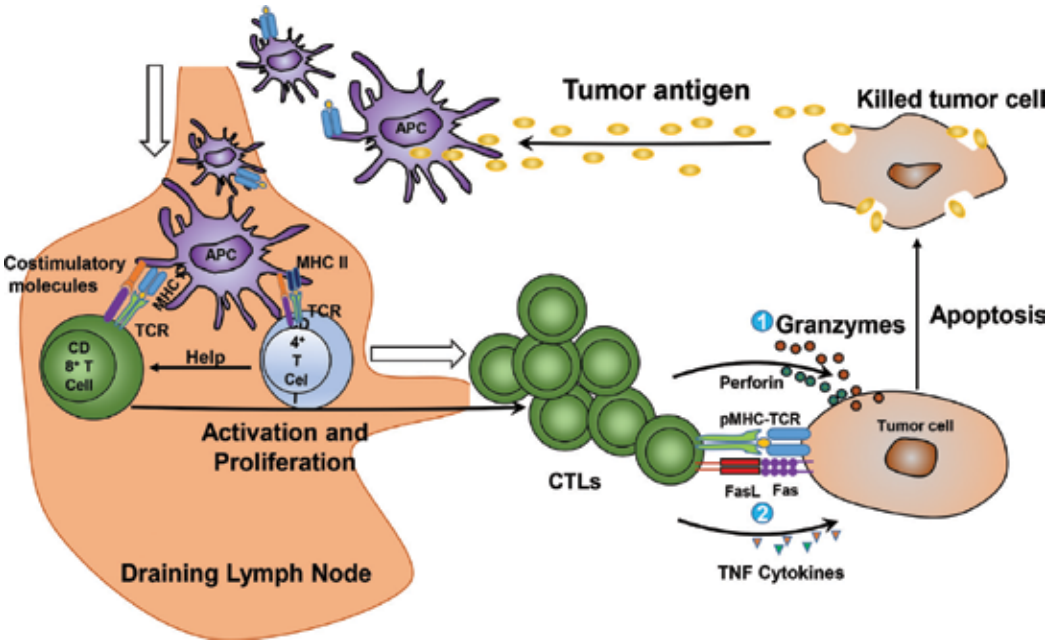

Figure 1. Immune activation of tumor-specific CTLs and the mechanisms of action of CTLs killing tumor cells. APCs acquire tumor antigen, migrate to the draining LNs, and present antigen to T cells in the context of peptide/MHC complex. Activated CTLs traffic to tumor site, trigger the programmed death of tumor cells through the perforin-granzymes pathway or FasL-Fas/TNF-TNFR death receptor pathway.

control tumor growth, primarily due to tolerance mechanisms induced by tumor cells [22]. To shield themselves from immune attack, tumor cells are able to evade the immune detection, recognition and subsequent immune attack through a variety of mechanisms [23]. First, most tumor antigens recognized by cytotoxic $CD8^+$ T cells are encoded from "self". Self-antigens expressed by solid tumors are intrinsically nonimmunogenic and do not efficiently stimulate naïve T cells. As a disease of mutations, the genetic instability or changes in cancer cells may potentially promote the generation of tumor antigen variants that are theoretically recognized as "non-self" by the immune system [24]. Thus, cancer vaccines that introduce neoantigens or tumor cell variants are promising in the induction of effective anti-tumor immune responses. Second, survived tumor cells have acquired the ability to resist immune recognition by expressing low level or defective MHC molecules, leading to insufficient antigen presentation [23]. Third, the upregulation of immune checkpoint ligand programmed death-ligand 1 (PD-L1) on tumor cells also leads to inactivation of effector T cells [23]. Accordingly, inhibitors of immune checkpoints, which target the PD-L1/PD-1 pathways, might reinforce the potency of immune response induced by cancer vaccines. In addition, tumor cells can produce suppressive cytokines including VEGF (vascular endothelial growth factor), TGF-β (transforming growth factor-β) and IL-10 (interleukin-10) to develop an immunosuppressive microenvironment, which further inhibits the activation and functions of tumor-specific effector cells [23]. These suppressive cytokines in turn recruit regulatory immune cells, especially regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [23]. Typically, Tregs and MDSCs function as major effectors of immunosuppression to inhibit host-protective anti-tumor

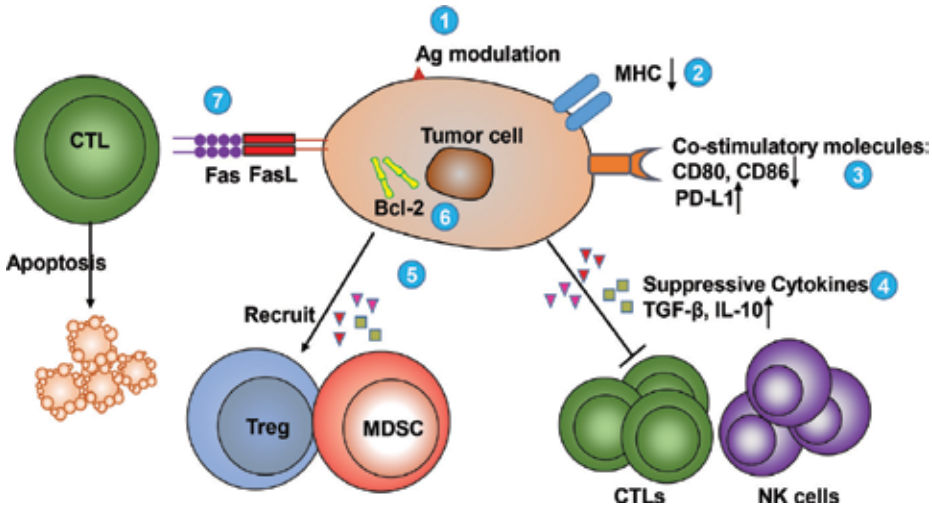

Figure 2. Mechanisms associated with immune escape of tumor cells. Fundamental Ag (antigen) modification leads to compromised immunogenicity of tumor cells (1); downregulation of MHC molecules on tumor cells also reduces the chance of tumor antigen presentation (2); abnormal expression of co-stimulatory molecules CD80, CD86 and PD-L1 leads to the inactivation or anergy of effector T cells (3); suppressive cytokines e.g., TGF-βand IL-10 produced by tumor cells inhibit the proliferation of effector CTLs and NK cells (4) but stimulate regulatory cells (Treg) and MDSC to expand, creating an immunosuppressive microenvironment (5); intracellular overexpression of anti-apoptotic molecules Bcl-2 prevents tumor cells from immune response-induced apoptosis (6); FasL expressed on tumor cells in turn induces the programmed death of CTLs through death receptor pathway (7).

immune response by secreting suppressive cytokines IL-10 and TGF- β, and by expressing high level of co-inhibitory molecules cytotoxic T-lymphocytes-associated protein 4 (CTLA-4) and PD-1 [23]. Administration of molecular adjuvants such as TLR agonists, which promote the production of proinflammatory cytokines, could be an attractive approach to neutralize the impact of suppressive cytokines modulated by tumor cells. Finally, to escape immune destruction, tumor cells cunningly overexpress anti-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2), which regulate cell death and protect themselves from immune response-induced apoptosis [25]. In parallel, FasL expressed on tumor cells binds to Fas on CTLs and directly causes the apoptosis of CTLs [26]. Collectively, as demonstrated in **Figure 2**, a combination of these underlying mechanisms ultimately contribute to the immune escape of tumor cells, which have posed challenging and complicated hurdles for the development of cancer vaccines. To improve the therapeutic efficacy of cancer vaccines and break the tolerance in tumors, the orchestration of therapeutic strategies that induce long-lasting antitumor immunity and overcome immune escape is the key for a successful treatment.

3. Cell-based cancer vaccines

Dendritic cells (DCs) are professional APCs that play a pivotal role in the regulation of cell-mediated immunity, and thus are key targets in cancer vaccine design [16–18]. The promising results from clinical trials recently have led to the approval of the first DC-based therapeutic

cancer vaccine by FDA [3]. There are generally two approaches to target DCs: *in situ* delivery of antigens via ligands that are specific for endocytic receptors expressed at the surface of DCs and ex-vivo generated antigen-loaded DCs. Though the latter approach requires laborious and expensive manipulation, immunotherapy based on *ex-vivo* tumor antigen loaded DCs bypasses the intrinsic dysfunctions of endogenous DCs in cancer patients, enabling the efficient priming of both CD4 and CD8 T cells. One of most successful examples of *ex vivo* DC-based vaccines is the use of sipuleucel-T for treating metastatic prostate cancer [27]. The FDA-approved sipuleucel-T cellular immunotherapy is comprised of autologous peripheral blood mononuclear cells (PBMCs) that are *ex vivo* pulsed with prostatic acid phosphatase (PAP) and activated with granulocyte-macrophage colony stimulating factor (GM-CSF). With sipuleucel-T treatment, the risk of death of patients was reduced by 22% in contrast with that of patients who received the placebo treatment. As a result, overall survival among male patients with metastatic castration-resistant prostate cancer was prolonged via the administration of sipuleucel-T therapy [27]. Despite the fact that DC-based vaccines can induce T cell responses, objective clinical responses are low and DC-based vaccinations have not met their expectation as an effective modality in treating other cancers [3]. Several factors might be limiting the efficacy of current DC vaccines: the types and sources of DC, the route of injection, and the migration to LNs. It has been estimated that less than 5% of the injected DCs reach the LNs [28], the anatomic sites where the immune responses are orchestrated. To overcome the insufficient migration of DCs, intranodal (IN) administration of DCs has been explored. In several clinical studies [29, 30], IN administration of mature DCs appeared to be safe, and resulted in superior T-cell sensitization.

Another challenge associated with DC vaccine is the insufficient antigen-presentation by DCs. Recent research suggests that high affinity [31, 32] and prolonged peptide–MHC presentation [33–35] of targeted epitopes are required for effective tumor eradication and tumor stroma destruction by specific T cells, presumably through the persistent T cell stimulation. However, DC pulsed with tumor associated peptides exhibits low T cell affinity and short half-lives of peptide–MHC complexes due to the clonal deletion of high affinity T cells and dissociation of peptide from MHC, respectively. In the later scenario, peptide degradation and rapid MHC turnover, leading to weak and transient T cell stimulation [36]. In addition, matured DCs loss their ability for antigen uptake and processing. This has posed a major barrier to the development of effective DC-based vaccines in clinic. Attempt to improve and stabilize MHC epitopes on DC surface has encompassed the use of altered peptide ligands (APLs) [37, 38], which incorporates mutated amino-acids in MHC anchor residues, and genetic modifications, which reprogram dendritic cells to express tumor antigens [39, 40].

Whole tumor cell vaccine is another cell-based vaccination approach currently in preclinical development and clinical trials. In this approach, tumor cells are modified to prevent replication and administered to patients to induce antitumor immune responses. The efficacy of whole-tumor cells vaccine has been investigated for more than 20 years [41]. One of the key advantages of using whole tumor cells as vaccine is that the cells provide a source of all potential antigens including neoantigens, eliminating the need for antigen identification. GVAX, by which tumor cells are genetically modified to overexpress granulocyte macrophage colony stimulating factor (GM-CSF), irradiated and adoptively transferred back to the patient,

is an early example of tumor cell vaccine. A meta-analysis about 1800 patients revealed that patients treated with whole tumor vaccines showed a more robust objective response—8.1% than those immunized with formulated tumor antigens—3.6% [42]. Prostate GVAX vaccine is an excellent example of whole tumor vaccines. In a clinical trial, administration of Prostate GVAX vaccines in patients with metastatic HRPC (hormone-refractory prostate cancer) exhibited improved survival of most patients, compared with the treatment of taxane chemotherapy alone [43–45]. Despite the promise, whole tumor cell vaccination typically requires substantial *ex vivo* genetic modification, leading to high cost, long processes, and stability, reproducibility and regulatory concerns. Additionally, immunization with whole-tumor cells has not resulted in significant long-term benefits in both preclinical models and in clinical trials. To address these issues, recently, injectable tumor cell-loaded cryogel sponges which deliver antigen-carrying tumor cells along with GM-CSF and TLR agonist was developed [46]. This biomaterials-based vaccination eliminates genetic modification, yet still delivers key DC activating factors. Immunization with cryogels in mice elicited local infiltration of DCs, which subsequently induced potent, durable T-cell responses in a melanoma model.

Apart from manipulating DCs and tumor cells to activate effector T cells, T cell-based immunotherapy provides a straightforward method to augment tumor-specific T cell immunity. One outstanding example of this therapy is adoptive T cell therapy, which involves the *ex vivo* manipulation and proliferation of antigen-specific T cells. Using this technique, two CAR (chimeric antigen receptors) T cell therapies have recently been approved by FDA. The approved therapies are targeted CD19, which is a common marker of lymphoma cells, to treat relapsed and refractory diffuse large B-cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL), respectively [47]. The CAR T cell therapy features the structural modification on autologous T cells to target virtually any tumor antigens. In general, T cells are engineered with the CAR structure which consists of a target element, scFv (single-chain variable fragments), and co-stimulatory domain and essential activation signaling domain (**Figure 3**). In the most recent approved CAR T therapy, patients with relapsed and refractory ALL were infused with autologous T cells transduced with a CD19-directed CAR, and 90% of them succeeded in complete remission [48]. Although adoptive T cell therapy has achieved remarkable efficacy in leukemia, it is less successful when this therapy is applied to solid tumor partially due to immunosuppression and rapid dysfunction of transferred effector T cells. To overcome these obstacles, a recent study demonstrated T cell surface coupling of nanoparticles loaded with IL-15 and IL-21 which fuel the T cells and boost the cell-based therapy [49]. Further study from the same group demonstrated that targeting TGF-β inhibitors to adoptive T cells via immunoliposomes greatly enhanced tumor-specific T cell immunity and significant B16F10 tumor regression in comparison to free adoptive T cells. This study suggested a complementary factor to maximize the efficacy of adoptive T cell therapy in cancer treatment [50].

Although cell-based therapy is a promising and effective strategy for cancer treatment, there are still several drawbacks related to this type of therapy. For example, *ex vivo* manipulation on DCs or T cells is labor intensive and expensive, plus the safety concerns about CARs in clinical trials [51]. A promising strategy to simultaneously overcome the cost and safety limitation is to create effective CAR T cells *in vivo* without T cell isolation. Recently, nanoparticles carrying genetic materials was delivered to T cells in mice. This approach avoided the tedious and

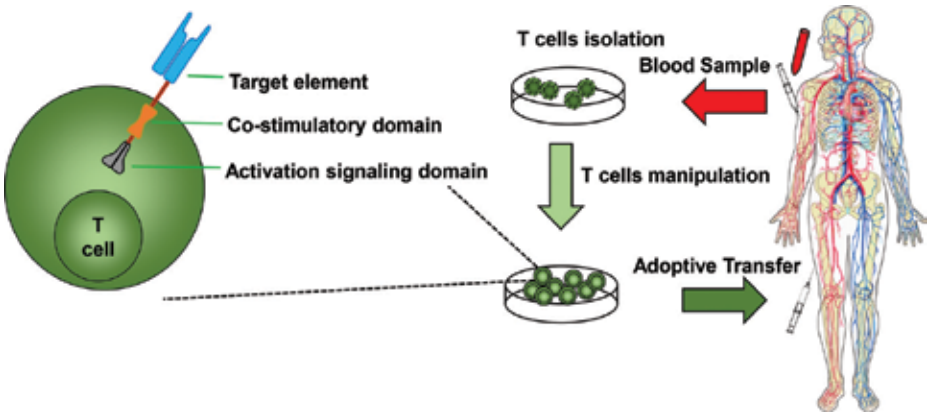

Figure 3. The general idea of CAR T cell therapy. T cells are isolated from patients' blood and subsequently engineered with a special CAR; genetically modified T cells are then expanded *ex vivo* and adoptively transferred back to patients.

expensive *ex vivo* T cell manipulation [52]. More research may be needed to demonstrate the efficacy and safety of this new *in situ* approach in humans in the future.

4. *In situ* vaccines

As some cancer therapies may fail in most patients with solid tumors, *in situ* vaccination can provide another prospect of driving a systemic anti-tumor immunity. *In situ* vaccination exploits local intratumoral treatment to simultaneously destruct tumor tissue and provides the immune system with an antigen source for the induction of antitumor immunity [53, 54]. Unlike traditional vaccines where selected tumor-associated antigens are used, *in situ* vaccination exploits complete tumor-related antigenic repertoire, including tumor-specific neoantigens derived from non-synonymous mutations [55]. Further, *in situ* vaccines can set the stage for potent antitumor immunity by inducing inflammation and facilitating the recruitment and activation of immune cells to the tumor. Thus, *in situ* vaccine approach provides opportunities for broad, more effective and less toxic treatment strategies to promote systemic antitumor immunity. This approach also bypasses the difficulties of isolating and preparing individualized vaccine *ex vivo,* providing a personalized treatment for cancer patients.

A variety of intratumoral treatments (e.g., radiation, cryotherapy) have been delivered directly to the tumors to induce tumor cell death, release tumor antigens while providing pro-inflammatory signals, which result in systemic activation of anti-tumor T cell responses, followed by inflammatory infiltration of T lymphocytes into the tumor [55–59]. While these early studies demonstrate the potential of *in situ* tumor destruction in promoting both T cell and humoral responses, the efficacy and wide-spread adoption of *in situ* vaccination have been limited. The major challenge lies in the relatively weak antitumor immunity following primary tumor destruction. For example, radiofrequency ablation or cryotherapy allows *in situ* tumor destruction and releases large amount of tumor antigens, but only induce a weak and transient immune response

which fails to prevent tumor relapse [57]. Preclinical and clinical studies combining tumor ablation with local administration of CpG-containing oligonucleotides (single-stranded oligonucleotides containing unmethylated cytosine-guanine motifs that bind TLR-9and serve as potent molecular adjuvants) can boost the induction of systemic antitumor effects [57]. Recent results of clinical trials and pre-clinical models demonstrated that intralesional treatment with cytokines, small drugs of immune checkpoint and radiation led to systemic anti-tumor immunity with limited toxicity [60, 61]. In Phase I/II clinical trial in non-Hodgkin's lymphoma, treatment of intratumoral injection of CpG and low-dose radiation safely induced objective responses at distal non-treated sites in nearly 30% of patients [62]. However, rapid dissemination of unformulated CpG from injection site often leads to systemic toxicity [63]. Conversely, immobilizing CpG ODNs or other immunostimulants [64, 65] in synthetic scaffolds at the tumor site blocks the systemic toxicity.

Overall, *in situ* vaccination represents an alternative and attractive approach to tackle the issues related with neoantigens due to gene mutations in tumor cells. By harnessing the power of nanotechnology as well as molecular adjuvants, it is possible to induce effective immune responses while at the same time overcoming the local immunosuppression at the tumor sites.

5. Nanoparticle-based vaccines

Nanoparticles have emerged as the platform of choice to improve the efficacy and safety of subunit vaccines. Nanoparticles have long served as versatile carriers and been extensively used for the delivery of therapeutic agents, including drugs, antigens, adjuvants, cytokines and other immune modulators. Nanomaterials are known interact with immune cells and carry vaccines to LNs through the interstitial flow, which exists in the lymphatic circulation with velocities of 0.1–1 μm/s [66–68]. This is because nanoparticles are able to mimic the sizes, shape, charge and surface features of virus particles, facilitating the entrance to the lymphatic capillary. Hubbell and Swartz showed that 25 nm diameters polypropylene sulfide (PPS) nanoparticles were transported and captured by APCs in the LN more efficiently than the same nanoparticles with 100 nm diameters [68]. Inorganic nanoparticles such as gold nanoparticles (AuNPs) have also successfully been shown in an animal model for localization of the sentinel LNs following intradermal injection [69–72], and have extensively used as improved vaccine carriers. Additionally, nanoparticles are ideal co-delivery platform in that multiple components can be conjugated or encapsulated in a single particle, fulfilling the requirement of co-delivery of antigens and activation signals in vaccines. We have developed a silica nanoparticle-based delivery platform (SiNPs) which targets tumor antigen and TLR-9 agonist to APCs in the LNs following subcutaneous injection [73]. Vaccine loaded SiNPs led to dramatically enhanced induction of antigen-specific B and T cell responses as compared to soluble vaccines, which in turn drove a protective antitumoral immunity in a murine tumor model [73]. Additionally, SiNPs vaccines greatly reduced the production of systemic proinflammatory cytokines and completely abrogated splenomegaly, key systemic toxicities of TLR-9 agonist that limit its advances in clinical applications. Our results

demonstrate structure-optimized silica nanocarriers can be used as an effective and safe platform for targeted delivery of subunit vaccines [73].

Liposome represents a versatile and convenient approach for vaccine delivery [74]. However, its application is limited by the in physical stability *in vivo*. To improve liposome stability, interbilayer-crosslinked multilamellar vesicles (ICMVs) was recently developed. These physically crosslinked vesicles were relatively stable but rapidly release their vaccine cargos when internalized by DCs. Results from this nanoparticle-based vaccine in mice showed striking enhancement on cellular and humoral responses, characterized by 30% antigen-specific $CD8^+$ T cell expansion and nearly 1000 times increase in antigen-specific antibody titer compared with unformulated vaccine [75]. Nanoparticles can also be used to deliver a full set of tumor associated antigens to DCs to induce anti-tumor immunity. A novel study assessed the therapeutic efficacy of PLGA (poly(lactic-co-glycolic acid)) nanoparticles (100 nm) coated with tumor cell membranes [76]. Membrane-coated PLGA nanoparticles were decorated with a TLR 4 agonist monophosphoryl lipid A (MPLA) which readily activated DCs to license the proliferation and differentiation of $CD8^+$ T cells in a melanoma model. This artificial biomimetic nanoparticle formulation proposed a unique targeting approach that could be utilized for cancer immunotherapy. But it remains to be determined whether tumor membrane-coated nanoparticles can simultaneously elicit broad T cell immune responses against various tumor associated antigens.

Another approach of using nanoparticles for cancer vaccines is artificial antigen presenting cells (aAPCs) [77]. aAPCs, functioning as direct activating units for T cells expansion, are emerging as a prominent and desirable strategy to reverse immunosuppression microenvironment in tumors and activate highly avid tumor-specific T cells. Nanoparticles-based aAPCs are a new approach to efficiently present tumor antigen while at the same time avoid the tolerogenic mechanisms associated with traditional antigen presenting cells. Nanoparticle aAPCs typically have a nanoparticle core coated with peptide/MHC and T cell stimulatory signals. Nanomaterials have been used include polymer (e.g., PLGA), inorganic particles (e.g., iron-oxide), and biomaterials (e.g., liposomes). Immune checkpoint inhibitors have also been conjugated on particle surfaces. The administration of artificial APCs coated with HLA-peptide tetrameric complexes and anti-CD28 mAb together boosted the specific activation of antigen-specific $CD4^+$ T cells [78]. *In vivo*, adoptive transfer of aAPCs obviously restrained tumor growth of a melanoma model in mice, along with IL-2 treatment [79]. While the therapeutic efficacy of these aAPCs needs more evaluation and trials, they certainly boost the development and advancement of cancer vaccine design.

Generally, nanoparticle-based vaccines hold great promise and tremendous potential in the treatment of cancers, and therapeutic efficacy generated by nanoparticle-based approach greatly promotes the development of next-generation cancer vaccines. Although some nanoparticles are commercially available and effective in cancer immunotherapy, it is still critical to physically and chemically orchestrate the design of nanoparticle-based vaccines on a structural basis. By optimizing the rationale of vaccine design and the routes of administration, we may conquer the underlying challenges associated with nanoparticles, which may include potential cytotoxicity to tissues and unexpected accumulation in local sites.

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6. Molecular vaccines

The use of nanoparticles for vaccines application has also raised safety concerns. Nanoparticles are typically encapsulated or conjugated with vaccines and their surface are modified with immune cell targeting ligands. However, it remains difficult to design nanocarriers which meet all the criteria for vaccine targeting. Most current nanoparticles do not reach a clinic application primarily due to requirements for complex designs including surface engineering to reduce host immune response, hydrophobic modification to enhance drug encapsulation, and incorporation of ligands to maintain immune cells targeting [80, 81]. Possible stability and toxicological issues including immunogenicity also greatly restrict the nanocarrier's clinical application in the short-term [80, 81]. We recently devised an 'albumin-hitchhiking' molecular approach which uniquely delivers vaccines to APCs in the LNs by binding to and transporting with endogenous albumin [63, 82]. In this approach, molecular vaccines are conjugated to a structure-optimized lipophilic albumin-binding tail linked by a solubility-promoting polar polymer and follow subcutaneous injection, bind tightly to albumin protein. Albumin binding increases the hydrodynamic size of molecular adjuvants, prevents them from rapidly flushing into the bloodstream and re-targets them to lymphatics and draining LNs, where they are filtered by APCs and accumulate. Meanwhile, because most vaccine components are trapped in the LNs, 'albumin-hitchhiking' vaccine also greatly enhances the safety profile by reducing systemic dissemination. We show that a long diacyl lipid (≥16 carbons) and a long polyethylene glycol (≥36 EG units) favors the albumin binding and LN accumulation *in vivo* [63, 82]. Subsequent immunization with the structure-optimized 'albumin-hitchhiking' vaccines exhibited massive antigen-specific T cells priming and improved anti-tumor efficacy. Administration of low dose of albumin-binding TLR-9 agonist and peptide antigens resulted in dramatically increased antigen-specific $CD8^+$ T-cell expansion relative to unmodified vaccine, as demonstrated by dramatic increases in the frequency of antigen-specific T cells measured in the peripheral. Importantly, efficient LN targeting achieved by albumin-binding vaccines also greatly reduces acute systemic side effects of TLR-9 agonist which had made it less attractive as a prophylactic vaccine adjuvant.

Although amphiphilic vaccines are prominent and excellent candidates in treating tumor-bearing mice, more study and work are required to translate this approach to clinical trials in human cancer models to validate the therapeutic and safety benefits. Additionally, the potential toxicity to LNs may be considered and addressed, and finding lipid-modified adjuvants that can function in human immune system is also urgently needed.

Another molecular vaccine which has emerged as an alternative cancer immunotherapy regimen is the DNA vaccine. DNA vaccination holds great potential in clinical translation because of their simplicity, safety and low cost [83]. In DNA vaccines, genetically engineered DNA encoding immunogenic antigens and immunostimulatory factors are injected into the host, and subsequently traffic into the cells for *in vivo* expression of therapeutic agents by using the hosts' protein expression machineries. In this way, DNA vaccines represent an innovative

strategy to induce specific anti-tumor immune response and circumvent immune escape. The injected DNA partially functions as an immunological adjuvant to stimulate the innate immune system due to its bacterial origin [84]. On the other hand, the antigens, expressed by plasmid-transfected host cells, can be processed and subsequently presented by MHC molecules which are critical to license the activation of antigen-specific $CD8^+$ T cells. One of the studies revealed that DNA vaccine encoding alphavirus replicon activated the innate immunity and induced cellular responses against self-tumor associated antigen tyrosinase related protein 1, showing impressive efficacy in reversing immunosuppression in tumor [85]. Another innovative work that elaborated the design of DNA vaccine also showed remarkable effectiveness in overcoming immune escape in tumor models [86]. In this study, the plasmid DNA was engineered to encode a secreted chimeric protein consisting of a single-chain trimer (SCT) of MHC I heavy chain, β2-microglobulin, and peptide antigen linked to IgG. The chimeric protein derived from this plasmid DNA was able to form a dimer which bound avidly to antigen-specific $CD8^+$ T cells and elicited T cell stimulation and expansion directly, bypassing antigen presenting cells. This design simplified the process of antigen presentation and potentially avoided suboptimal activation associated with traditional antigen presentation. Additionally, the IgG domain in this construct enabled chimeric proteins to target the Fc receptor on APCs which initiated the subsequent cascade of immune activation in LNs. Based on this creative design, intradermal administration of this DNA vaccine induced potent Trp2-specific $CD8^+$ T cell dominant immune responses and showed enhanced therapeutic efficacy in B16 melanoma tumor model in mice [86]. DNA vaccines have also been tested in clinical trials to evaluate their efficacy in human cancer, melanoma [87], breast cancer [88], prostate cancer [89], and cervical cancer [90].

DNA vaccination provides an innovative and attractive platform for cancer immunotherapy with additional advantages like low cost, well-defined safety. Technically, DNA vaccines can be readily customized and engineered, which makes large-scale production possible. In addition, DNA vaccines are widely recognized as safe therapeutics in both animal and human clinical trials [89, 91, 92]. Despite a variety of advantages of DNA vaccines, the intrinsic poor immunogenicity have made DNA vaccine less successful in generating desirable therapeutic efficacy in most cancers. Therefore, future development of DNA vaccines may need to focus on their rationale design to greatly improve the immune potency of DNA vaccines in cancers.

7. Combined immunotherapy

Although monotherapy of most cancer vaccines can achieve therapeutic efficacy in cancer treatment to varying extent, therapeutic benefits may be further improved if these cancer vaccines can be administrated in a combinational way to complement each other against cancer. Theoretically, when effector T cells are activated, co-inhibitory molecules CTLA-4 and PD-1 can also be expressed and up-regulated on T cell due to the suppressive microenvironment of tumors, which may compromise the efficacy of vaccine-based cancer therapy. To minimize the impact of the expression of co-inhibitory molecules, a combinational therapy of cancer vaccines and immune checkpoints inhibitors may achieve a cure to cancer treatment. The idea has been realized and supported by several preclinical studies [93, 94]. The first study revealed that breast cancer derived immunogenic multi-peptide vaccine plus anti-PD-1

antibody functioned as a combinational therapy approach and thus prolonged the vaccine-induced progression-free survival period in breast tumor-bearing mice, along with augmented expansion of Tc1 and Tc2 CD8 T cells [93]. Another study demonstrated that anti-PD-1 antibody and GVAX synergistically enhanced the anti-tumor immune responses with great therapeutic efficacy in established melanoma tumor-bearing mice. In contrast with monotherapy of vaccine or PD-1 inhibitors, only a simultaneous administration of both therapies achieved repeated expansion of antigen-specific $CD8^+$ T cells [94]. Similar strategy using GVAX and anti-CTLA-4 antibody has also been utilized for treating metastatic pancreatic cancer, giving rise to objective response in 20% of tumor-bearing patients who have been resistant to chemotherapy [95]. Cancer vaccine-based immunotherapy may weaken the resistance of some cancers to immune checkpoint inhibitors, whereas immune checkpoint inhibitors may make up the drawbacks for cancer vaccines by decreasing the possibility of immune escape in tumors and thus enhancing the efficacy of vaccination. Other combination, such as vaccines plus adoptive T cell transfer might synergistically amplify the antitumor immunity, as demonstrated in recent studies. In summary, the combinational therapy is emerging as a more powerful and comprehensive strategy to address the immune escape associated with tumors and fuel the tumor-antigen specific T cell immune responses. But more studies are needed to test the clinical efficacy of this combinational therapy and assess the potential issues related to it, such as systemic toxicity and anti-drug antibody response.

8. Conclusion and future perspective

Immunotherapies have demonstrated their potential to generate robust antitumor responses and are continuing to grow as a new treatment modality for cancers when administrated alone or as an addition for other "physical" or "chemical" therapies. Strategies based on immunotherapy mainly focus on the induction of potent immune response, especially effector T cell response, against tumor antigens and variants due to genetic mutations, and the decrease or blockade of intrinsic immunosuppression in tumors. The immune system is a sophisticated and complicated entity, which may require elaborate design and engineering of therapeutic agents to reverse tumor-induced immune imbalance. As previously discussed, each single immunotherapy may not be perfect for cancer treatment. The future work may continue improving the rational design of cancer vaccines to maximize their efficacy while minimizing side effects. To date, several immunotherapies have been approved by FDA and dozens more are under clinical evaluation. Indeed, we are at the dawn of a whole new era for cancer treatments. With the rapid technological advancement in the field, cancer vaccines, in combination with traditional cancer treatment, may ultimately lead to a miracle cure for the vast majority of cancer patients.

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Conflict of interest

The authors declare no competing financial interest.

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Chapter 4

Therapeutic Potentials of IL-10 versus IL-12

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Additional information is available at the end of the chapter

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Abstract

Cytokines are low molecular weight proteins having roles in essential biological processes, particularly for the immune system. As they have a key role to play, an abnormality in their function can lead to wide variety of diseases (clinical consequences). Thus using the cytokines as therapeutic targets has been an area of active research. Of the entire family, we would like to shed light on two major ones IL-10 and IL-12 having an array of roles in cellular response to infection and autoimmunity. IL-12 is a pro-inflammatory cytokine that has been shown to enhance IFN-γ producing T cell responses and has been widely tested as a vaccine adjuvant. Many studies have shown that IL-12 acts as a link between innate and adaptive immunity by inducing IFN-γ production and polarizing naive CD4 T cells to become Th1 cells. It also has roles in CD8 T cell differentiation. On the other hand, IL-10 is an anti-inflammatory cytokine and has role in maturation of memory CD8 T-cell. It also plays a critical role in preventing autoimmunity and also limits tissue injury by interfering with the intensity and duration of immune response. We would thus like to discuss in details about the therapeutic use of these cytokines for infections as well as diseases such as cancer, autoimmune disorders etc.

Keywords: cytokines, therapy, IL-10, IL-12, inflammation, anti-tumor activity, autoimmunity

1. Introduction

Cytokines have an array of roles in immune system. They are involved in regulation of most immune responses. They comprise of a large family of low molecular weight proteins play essential role in biological processes. They are cellular hormones which mediate cell to cell communication. It comprises of interleukins, interferons, chemokines, monokines and lymphokines. They are basically involved in signaling pathways and regulating the downstream

events. Cytokines are being studied thoroughly from past 3 decades due to the variety of roles they play in various infections, diseases etc. They have prominent roles to play when it comes to immunity, inflammation, repair and migration [1]. The idea of using them as therapeutics is as old as more than 20 years. It evolves from two very different strategies, the first one being administration of recombinant purified versions of cytokines and the second in which therapeutics are designed against them inhibiting their harmful effects and limiting excessive upregulation. Interferons and CSF (colony stimulating factor) are the ones which form the success stories as therapeutics. Their major role seems to be in maintaining the Th1-Th2 paradigm [2, 3].

The basic classes of cytokines fall under the category of pro-inflammatory, promoting inflammation and anti-inflammatory, resolving inflammation. As the name suggest, the functions are opposing and hence a very fine line separates the two, striking a balance is thus extremely important. A slight dysregulation can lead to immunopathology, at times fatal to the host. Immune homeostasis is attributed via signaling through these small molecules. Thus they are prone to dysregulation by any microbial invasion or injury. Micro-organisms have found ways to fool the immune system and leading to imbalance in various regulatory pathways.

IL-10 and IL-12 have opposing roles. Owing to their pleotropic properties, they have been widely considered for therapy. Various viral infections, tumor models and autoimmune diseases are being targeted through them. Most prominent issue in translating all therapeutic approaches to clinic is their opposite effects. Since they are not constitutively expressed, perfect timing and cellular location are of paramount importance in designing the right class of therapeutics. Here we attempt to shed light on their roles in immune responses and the way of their use in therapy. A deeper understanding of their functions, mode of action, involvement of other cytokines, and link between cellular processes would serve as a key feature in developing successful modalities.

2. IL-10: structural features

IL-10 family of cytokines falls under the umbrella of class II cytokine family. IL-10 is known to limit the overt inflammation preventing tissue damage and acts mainly upon leucocytes. It has a major role in maintaining tissue homeostasis. IL-10 was first identified in 1989. It was described as a protein involved in inhibition of IFN-γ secretion from Th2 type cells. IL-10 is expressed by almost all immune cells, both of innate and adaptive immune system. It is expressed by dendritic cells (DCs), macrophages, NK cells, mast cells, T cells and B cells etc. [4–8]. Recent evidences suggest that it is also secreted by regulatory T cells (Tregs). Structurally it has a stretch of 160 amino acids forming non-covalently linked homodimers. It binds to R1 and R2 receptor chains. R1 is shown to have structural similarity with IFNR. IL-10 functions via activating the JAK-STAT signaling pathway, STAT3 being the downstream transcription factor involved [4, 9]. Various studies have shown that immune cells such as macrophages lacking STAT3 escape the suppressive effects of IL-10 on pro-inflammatory cytokine production.

2.1. IL-10 secretion

Besides immune cells several non-immune cells also secrete IL-10. Leucocytes, as well as epithelial cells both falling under different categories of cell types, secrete this anti-inflammatory cytokine highlighting its important and major role in homeostasis [10]. Initially, only Th2 type T cells were known to secrete IL-10, but more recently Th1, Th9, Th22, CD8 T cells and even regulatory T cells have been reported to secrete IL-10 [5, 6]. Pathogen induced IL-10 production occurs mainly in APCs such as DC's and macrophages [11, 12]. TLRs have a major role to play in pathogen induced secretion. Pathogen Associated Molecular Patterns (PAMPs) act as TLR ligands for the receptors. This in turn initiates a signaling cascade termed as the TLR signaling pathway. Signaling through TIR domain conjugated to adapter molecules, either TRIFF or MyD88 leads to IL-10 secretion and other cytokines [13]. TLR 2 ligand has been studied extensively and is considered as a major inducer of macrophage derived IL-10. Macrophages are the only cells also producing IL-10 via the TLR3 signaling pathway [14, 15]. LPS is also an inducer of IL-10 via common TRIF and MyD88 signaling via type I IFNs. Signaling through the MyD88 leads to activation of NF-κβ and MAPK activation. MAPK functions via ERK group of kinases. The differential levels of IL-10 secreted/expressed are dependent upon ERK activation strength in DC's and macrophages. Signaling via this route is often exploited for finding new targets of anti-inflammatory drugs. Regulatory T cells (Tregs) are very recent and major producers of IL-10. Tregs are characterized by expression of transcription factor Foxhead box P3 (FOXP3). Tregs must receive in-vivo signals to induce IL-10 expression. A proper understanding through these cell types however remains elusive.

2.2. Regulation of immune responses through IL-10

Microbial sensing through the innate immune system initiates a signaling cascade terminating into generation of pro-inflammatory cytokines. This creates an inflammatory environment favorable for the activation of adaptive immune cells, however higher levels of inflammation can give rise to systemic and metabolic imbalances having deleterious effects to the host. Therefore the immune system has developed anti-inflammatory mechanisms to limit the production of pro-inflammatory molecules thus limiting tissue damage and maintaining a state of homeostasis. Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that plays a crucial and essential role in averting inflammatory and autoimmune pathologies. It has also role in limiting antiviral, antibacterial responses, remodeling damaged tissues and wound healing. Deficiency or aberrant expression of IL-10 can enhance inflammatory response to microbial challenge but also lead to development of inflammatory bowel disease and a number of autoimmune diseases. Mice studies have shown that IL-10 deficiency leads to exacerbate immune responses to microbial or bacterial challenge. Thus, impaired IL-10 expression or signaling can enhance clearance of pathogens during an acute infection leading to exaggerated inflammatory responses, ultimately leading to immunopathology and tissue damage. Conversely, some pathogens can harness the immunosuppressive capacity of IL-10 to limit host immune response, leading to persistent infection characterized by unaffected pathogen load. In all, IL-10 plays an indispensable role in mediating host anti-inflammatory response and hence identifying the cellular sources of IL-10. The molecular mechanisms

that regulate IL-10 expression are extremely important in developing therapeutic strategies directed against pathology-associated impaired IL-10 production [16].

IL-10 is considered to have multiple roles when it comes to immune regulation. It has been shown to inhibit production of many inflammatory molecules such as IL-12, MHC and other costimulatory molecules from dendritic cells and macrophages [17]. It is also shown to have role in B cell survival, proliferation and antibody production. More recently, its role in tumor immunity has been elucidated [18].

2.3. IL-10 as therapy

Owing to its unique properties, IL-10 is considered as a potential candidate for use in therapy against inflammatory diseases, chronic infections, cancer and autoimmunity. IL-10 is involved in feedback regulation of Th1 and Th2 responses. It has been reported that the levels of secretion of Il-10 and IFN-γ through Th-1 is the deciding factor between clearance and persistence of infection. Studies in IL-10 deficient mice show that some intracellular pathogens could be cleared but is often accompanied by immunopathology. This clears the role of IL-10 in preventing host damage and maintaining a balance [19].

IL-10 is widely tested as a therapeutic for inflammatory diseases. Administering recombinant IL-10 has been tested in many clinical trials for rheumatoid arthritis, Crohn's disease and psoriasis [20]. All these clinical studies show the role of IL-10 in immune stimulatory as well as anti-inflammatory abilities. Because IL-10 has potential to prevent T cell-mediated tissue injury, it is often considered as a therapeutic for diseases involving autoimmune inflammation, the most studied model being autoimmune encephalomyelitis (EAE). The presence of IL-10 within the target organ is linked to its role in CNS inflammation and also in a null mouse model of acute CNS inflammation. The effectiveness of the therapy, however, depends upon the timing of IL-10 administration as well as target/route/localization because peripheral administration of IL-10 shows an exacerbated disease condition of EAE in mice [16, 19]. In case of various other infections, it is shown that a delicate balance of pro-inflammatory and anti-inflammatory environment is required for tackling the disease. Temporal and spatial IL-10 induction is critical for resolving any infection. Excessive IL-10 production can inhibit the pro-inflammatory response to a number of pathogens, including Leishmania spp., *T. cruzi*, Mycobacterium, Plasmodium spp., and Lymphocytic choriomeningitis virus, to the extent that pathogens can escape immune control, resulting in either persistent or chronic non-treatable infections [21–23]. In cases of viral infections such as HIV and HCV, elevated IL-10 signaling can inhibit pro-inflammatory cytokine production mainly via two processes, first is targeting of immune effector types directly, and second by indirectly modulating immune function eventually inhibiting maturation of different types of APCs such macrophage and dendritic cells, thus limiting co-stimulatory, chemokine secretion and antigen presentation capacity of the host. In the case of HIV, it has been reported that IL-10 hampers APC maturation, limiting antigen presentation from these cells, and initiates T cell-dependent suppression of anti-viral responses [10, 24, 25].

We can thus say that IL-10 therapy can only be successful only if everything is just right from signaling to secretion. Dysregulation of IL-10 can lead to autoimmunity or severe immunopathology due to extended inflammation. Also there are pathogens that have

found ways to promote chronic establishment by hijacking the IL-10 regulation pathways. Hence IL-10 works differently in different environments, thus having a deeper knowledge of the intermediates involved in its functioning is necessary. Promising and most studied candidate seem to be type I interferons, IFN-γ as reviewed by many, but IL-12 can also act as a potential player.

Work from Cheng G. lab and others have shed light on the role of type I IFNs, IL-27, and IL-10 as suppressors of neurodegenerative diseases like EAE. Such diseases are induced as a result of impaired functioning of the immune system, wherein the system generates response against its own cells. These studies were important contributions highlighting potential efficacy of IFNβ therapy against multiple sclerosis, which shares many of the clinical symptoms and features with EAE animal models. In case of viral infections, most pathology to the host is prevented via type I IFN. In case of acute respiratory influenza infection it is known that type I IFN exerts temporal control over excessive inflammation through the infiltration of IL-10-producing lymphocytes at the infection site. However, type I IFN signaling is also known to worsen the pathology in context of chronic or persistent viral infections. Prolonged host-derived IL-10 production can actively suppress pro-inflammatory T-cell responses, giving an opportunity to the virus to persist, as in case of LCMV infection. However, considerable success is achieved in re-establishment of T cell function by using molecules acting as IL-10 antibody block. Theoretically elevated IL-10 expression could be initiated as well as sustained through type I IFN host response generated during the primary viral infection or initial phase of infection. Thus, type I IFN signaling could play double roles firstly by promoting robust clearance of acute viral infection via host directed anti-viral response, and secondly by creating an immunosuppressive environment that paves way for persistent or chronic infection to establish. IL-10 also modulates this excessively and hence understanding its potential from infection point of view is equally important.

3. IL-12

IL-12 is a pro-inflammatory cytokine that has been shown to enhance IFN-γ producing T cell responses and has been widely tested as a vaccine adjuvant. It is produced by phagocytes in response to microbial stimulation and is an important early mediator in host defense. IL-12 is known as a conjugate between innate and adaptive immunity as it induces IFN-γ production and thereby helps in polarizing naive CD4 T cells to become Th1 cells. Over and above this, IL-12 has also been shown to enhance CD8 T cell homeostasis and provide a third signal that promotes full activation and survival of activated CD8 T cells. Collectively, many studies have documented IL-12 as a potent inducer of effector T cells, and this property has led to its testing as commercial vaccine adjuvant.

3.1. IL-12 signaling

IL-12 is primarily produced by all professional APC types such as DC's, monocytes and macrophages. IL-12 is composed of two chains *p*-35 and *p*-40 encoded by IL-12a and IL-12b respectively, activating NK cells and induce CD4 T cells to become IFN-γ producing Th1 type cells.

IFN-γ, in turn, acts on APCs to promote IL-12 secretion in a positive feedback loop. IL-12 signals via the IL-12 receptor (IL-12R) consisting of two subunits namely IL-12Rβ1 and IL-12Rβ2 known to be expressed on DCs, T cells and NK cells. IL-12 functions via non-receptor Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) activities, leading to the phosphorylation of signal transducers and activators of transcription (STATs), mainly, STAT4 homodimers. IL-12 is also involved in secretion of IL-2, TNF-α and GM-CSF apart from the main one IFN-γ.

3.2. Regulation of IL-12 production

Biologically active IL-12 is produced when both Il12a and Il12b genes are expressed coordinately in the same cells. Contradictory to the notion, mRNA of Il12a is widely expressed in many cell types, albeit at low levels in some cells, most of which do not even produce IL-12. The Il12b mRNA is restricted to cells that can produce biologically active heterodimer. A rate-limiting step for IL-12 production is synthesis of the p35 chain linking to limited availability of its transcripts in cells under homeostatic conditions. Over the past 2 decades, a huge plethora of molecular analyses have identified numerous transcription factors that bind to the promoter regions of Il12a and Il12b. The promoters of Il12a have been shown to bind transcription factors such as nuclear factor kappa B (NFκB), c-Rel (in DCs), c-Maf, and IFN regulatory factor 1 (IRF-1) in activated macrophages. TLR signaling in DCs through LPS and other ligands is also reported as contributor in IL-12 production.

3.3. IL-12 in therapy

Owing to its pleotropic properties IL-12 is widely used in therapy against tumors and infections. Most effects of IL-12 are mediated via IFN-γ. IL-12 regulates inflammation via linking the innate and adaptive arms of the immune system (**Figure 1a**). It thus emerges as an early pro-inflammatory cytokine in immune response to pathogens. However, complete functioning requires signals from IFN-γ, CD40-CD40L interactions and IL-15 [26–29]. Role of IL-12 in tumor regression and chemotherapy is most studied. Many pre-clinical studies have demonstrated the potent anti-tumor activity of IL-12. Extensive research is being carried out to deliver recombinant IL-12 directly to tumor site. The challenge here again is to understand at mechanistic level, the involvement of other pathways which could be immunosuppressive, administering IL-12 in a way which makes it less toxic, targeted tissue delivery and generating tailor made responses depending on the type of tumor.

To study the mechanism of protection of IL-12 in tumors, it is generally overexpressed in tumor cell lines. Subcutaneous inoculation of this cytokine in C26 colon carcinoma cells, B16 melanoma cells and few others have shown to induce tumor suppression. In melanoma cells protection is mediated by ILCs (Innate Lymphoid Cells) and in colon carcinomas it is independent of IFN-γ and rather dependent upon CD4 T cells and NK cells [30]. This sheds light on location dependence and also upon the role of various cell types involved in the process. Thus, the entire tumor microenvironment is affected by IL-12 in a variety of ways [31]. In B-16 melanoma, IL-12 showed to remodel the vasculature by upregulating adhesion molecules having role in leucocyte migration to the tissue and also by inhibiting angiogenesis through IFN-γ secretion.

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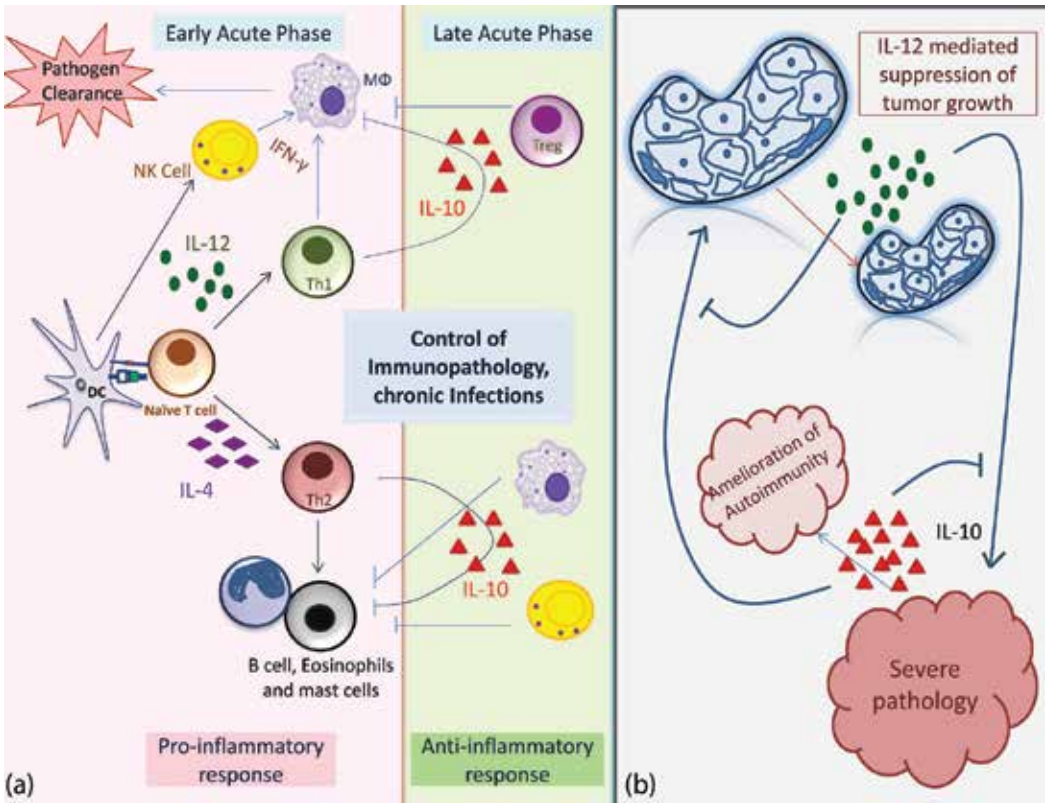

Figure 1. (a) During acute infection settings, a pro-inflammatory environment is created mainly via secretion of IL-12 through DC-T cell interaction, promoting Th1 responses which are IFN-γ promoting. APCs promote pathogen clearance and control by activating adequate effectors of adaptive immune system. On the other hand Th2 response is involved in B cell, mast cell activation under the influence of other cytokines. During late stages of infection, an anti-inflammatory environment is created by IL-10 secretion by Th1 & 2 cells, Tregs, NK cells and macrophages. (b) IL-10 and IL-12 both antagonize each other under different conditions such as tumor and auto-immunity suggesting a delicate balance between the two is necessary to prevent host from adverse effects.

Various genetically modified mice models have been used to explore the therapeutic potentials of IL-12. It is well known that IL-12 therapy is dose and context dependent; hence various routes of immunization have been tested in different localized tumor models. Delivery models used and studied include infusion of recombinant proteins, electroporation, gene therapy using non-viral and viral vectors, nanoparticles and microspheres containing IL-12 and immune cells or non-immune cells expressing IL-12. IL-12 affects a series of events involved in tumor immunity. IL-12 acts on NK cells and CD8 T cells to trigger effector functions via perforin and granzyme secretions. It also stimulates B cells to secrete anti-tumor antibodies. It acts upon CD4 T cells polarizing them to Th1 phenotype in turn secreting cytokines and IFN-γ. It in turn acts upon APCs promoting antigen and cross presentation enhancing cytotoxic activity. Owing to its activity, IL-12 is considered in combinational therapy with other cytokines, chemotherapeutic agents, peptide vaccines and monoclonal antibodies. This has also been tested in melanoma and mammary carcinoma models [32, 33].

The main issue however with these therapies is excessive systemic IFN-γ production leading to toxicity. In combination to chemotherapy modest success was observed only if IL-12 was administered at early stages. Hence the limitations lead to exploration of other modes of delivery and again based upon the type of tumor, suitable delivery methods need to be adapted. Several studies have evaluated the use of IL-12 for therapy by delivering this cytokine within the tumor site specifically. Even after most of these approaches resulted in impressive antitumor responses, the translation into the clinics was not satisfactory. There are many questions still remaining unanswered in the oncology field. Firstly, the schedule optimization for therapeutic IL-12 delivery in clinical trials has proved to be challenging. Various treatment schedules have been evaluated such as subcutaneous versus intravenous vs. intra-tumor in daily as well as consecutive injections. Even though the most successful way to administer IL-12 appeared to be in cycles of twice weekly injections, repeated administration of the cytokine leads to increased immunosuppressive properties of the tumor by the induction of IL-10 [34].

IL-12 also has major role in shaping immunological memory to viral infections. During a typical viral infection, T cells undergo via phases of T cell activation and differentiation, governed majorly by the cytokine micro-environment. IL-12 along with other cytokines such as IL-15 assists in generating appropriate T cell responses. Of the two types of T cells, CD4 and CD8, the effects of type I IFNs and IL-12 on CD8 T cell differentiation are seen to be intersecting as they both directly provide signals to the responding cells. Also they act in co-ordination with antigenic and costimulatory signals thereby promoting the development as well as expansion of short-lived effector cells [35, 36]. These cell types are eliminated from the response once there job of limiting the pathogen is complete. The T cells which survive the entire process culminate into T cell memory. IL-12 signaling induces expression of T-box expressed in T cells (T-bet), a transcription factor which determines the differentiation state of the T cells. High levels of T-bet directly correlate with the terminal differentiation of short-lived effector T cells, while lower levels of T-bet are linked to the development of memory precursor T cells which can add up to the long-lived memory pool. It has been reported that CD8 T cells lacking the IL-12 receptor, IFN receptor (IFNAR), or both inflammatory cytokine receptors, are defective in the formation of short-lived cells following infection with LCMV, VSV, or the intracellular bacteria *Listeria monocytogenes* (LM) [37]. These receptor deficient CD8 T cells are known to express lower levels of T-bet and higher levels of Eomes, the related T-box transcription factor Eomesodermin. Although these transcription factors possess overlapping roles, in terms of initiating IFN-γ, the expression of Eomes is preferentially associated with the formation of memory CD8 T cells. Eomes may operate to recruit cells into the memory pool by upregulating expression of CD122, the b-chain of the IL-2 receptor, which is also required for IL-15 signaling. Co-operation between cytokines shapes both short-term and long-lived anti-viral CD8 T cell development. Often the induction of type I IFN or IL-12 following infection boosts the expansion of highly cytolytic short-lived effector cells. Curtailing the inflammatory conditions via investigating the exact roles of IL-10 surrounding CD8 T cells might permit the formation of long-lived memory populations attributing protection against re-exposure to the infection [38].

4. Striking the IL-10/IL-12 balance for better therapeutic value

The potency of IL-12 and IL-10 in host defense makes them a target for precise regulation. Indeed, the temporal, spatial, and quantitative expression of these two cytokines during an immune response in a specific tissue microenvironment contributes to the determination of the type, extent, and resolution of the response in major ways. Disturbing the intricate control and balance frequently leads to immunologic disorders and pathologies. One of the most important and well-studied negative regulators of TLR-induced IL-12 production is IL-10. IL-10 repression of both IL12a and Il12b genes is primarily seen at the transcriptional level; however the inductions of the two genes have different requirements for de novo protein synthesis. IL-10 suppression Il12a transcription is not completely known. IL-10 acts upon the enhancer 10 kb upstream of the Il12b transcriptional start site, bound by nuclear factor, interleukin 3-regulated (NFIL3), which is a B-ZIP transcription factor. It has been reported that myeloid cells lacking NFIL3 produce excessive IL-12p40 and increased IL-12p70. This indicates that STAT3-dependent expression of NFIL3 is a key component of the negative feedback mechanism in myeloid cells that suppresses pro-inflammatory responses. Quite a bit of elegant studies have focused on the Il12b promoter transiently associated to acetylated histone H4 in WT bone marrow-derived macrophages (BMDMs), whereas association of these factors was seen to be prolonged in $Il10^{-/-}$ BMDMs. Experiments incorporating histone deacetylase (HDAC) inhibitors and HDAC3 short hairpin RNA have shown data to indicate that HDAC3 is involved in histone deacetylation of the Il12b promoter by IL-10. This means the histone deacetylation on the Il12b promoter by HDAC3 mediates the homeostatic effect of IL-10 in macrophages. More details clearly need to be worked out to understand the important homeostatic regulation of IL-12 production by IL-10, in terms of cellular pathways, different mechanisms might be taking place in a tissue specific manner etc. In this context, the IL-4-inducing transcription factor c-Maf is an interesting molecule that can directly and conversely regulate IL-12 and IL-10 gene expression in activated macrophages. Again, IRF-5 is considered as a factor leading to the "M1" polarization of macrophages thereby promoting Th1 and Th17 activities with activated transcription of inflammatory genes, including Il12a, Il12b, and Il23a, and repressed Il10 transcription [39].

A classic study by Lopez MV et al. showed the first evidence of synergistic anti-tumor effects of IL-12 and IL-10 in a novel combination cancer immunotherapy [40]. They demonstrated the eradication of established primary colon and mammary tumors by administering the Th1 and Th2 cytokine together. They observed an increased expression of IP-10, MCP-1 and TCA-3 at day 7 after administration of the combined immunotherapy. An interesting result was also the persistent expression of IFN-γ locally and the abrogation of IL-4 increase following the combined immunotherapy, indicating that infiltrating cells were expressing a Th1 phenotype. Simultaneous and timely activation of Th1 and Th2 responses is thus necessary for appropriate responses.

Autoimmunity is a condition in which host generates immune responses against its own healthy cells. Experimental allergic encephalomyelitis (EAE), a demyelinating disease of

the central nervous system, is widely used as an animal model for multiple sclerosis. Segal et al., in 1988 demonstrate that IL-12 is essential for the generation of autoreactive Th1 cells that induce EAE, in the presence as well as absence of IFN-γ. The disease-promoting effects of IL-12 are often antagonized by IL-10 having its origin from antigen nonspecific CD4 T cell which, in turn, is regulated by the endogenous production of IL-12 (**Figure 1b**). This unique immune-regulatory circuit appears to play a non-ambiguous role in controlling Th cell differentiation. It also provides a mechanism through which microbial triggers of the innate immune system can harmonize autoimmune disease [41]. IL-12 has an EAE promoting potential, it triggers formation of autoimmune effectors. Anti-IL-12 therapy helps in regression of disease through prolonged administration. The innate immune system would always trigger inflammatory responses towards autoimmune cells; a delayed IL-10 secretion by a different subset of immune cells, the adaptive ones in turn suppresses the activity of autoimmune cells. This has been established in *L. major* and *T. gondii* mice infection models. This suggests that manipulating the cytokine milieu, balancing IL-12/IL-10 ratio via the innate immune system can surmount establishment of autoimmune disorders. This may not hold true for chronic autoimmune diseases and more intervention is therefore required.

Generating CD8 T cell memory for infections due to intracellular pathogen has been an active area of research. IL-10 has been shown to promote resolution of infection, thereby promoting memory formation. The source of IL-10 is considered to be CD4 Tregs in acute LCMV model [42]. The probable reason for memory maturation of CD8 T cells could be their insulation to the pro-inflammatory effects of IL-12 driving them towards terminal effector differentiation. Pathogenic insult to the host triggers innate immune responses via NK cells and other innate immune cells. Microbial sensing occurs through PRRs (pattern recognition receptors) and the most studied of them being the TLRs (toll like receptors). TLR signaling pathway in turn leads to secretion of cytokines, pro-inflammatory ones, activating the adaptive arm. Innate immune cells together control the infection from spreading further. Upon activation of antigen specific T cells, a specific course follows which is dynamically in sync with increasing pathogen load. Upon clearing the pathogen, the antigen specific effector T cell population contracts to form a small pool of memory T cells. Several cytokines affect the phenomena of T cell maturation. The resolution phase has the role of IL-10 acting as anti-inflammatory and promoting T cell maturation which can add to the memory T cell pool. Thus this property of cytokines can be explored in vaccination strategies or designing new vaccine adjuvants. Therapeutic intervention during specific stages of T cell maturation can help in generation of protective long lived immunological memory.

5. Conclusion

Maintaining homeostasis requires an intricate interplay among various functioning systems of the body (**Figure 2**). Cytokines form an important class of such system. Understanding the system properly and deeply would allow meaningful interventions and restoring the steady state.

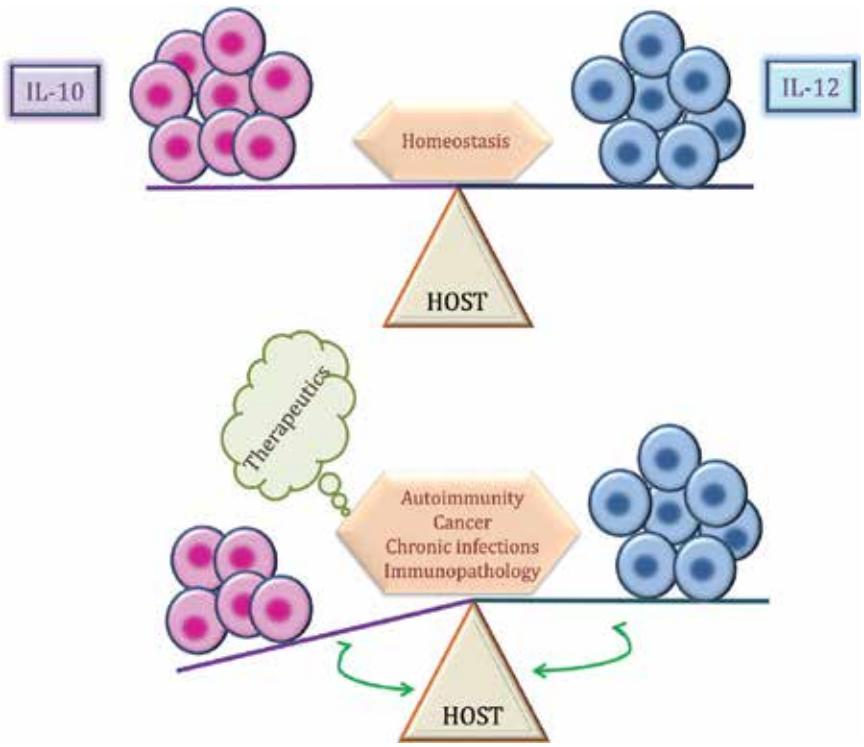

Figure 2. A balance between IL-10 and IL-12 secretion by host cells helps in maintaining homeostatic state. A slight imbalance in any of the processes of their function can lead to immunopathology, cancer, autoimmunity and chronic established infections. Targeting therapeutics towards maintain the balance could help the host restore the equilibrium.

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Chapter 5

Immune System Modulation Produced by Ultraviolet Radiation

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Additional information is available at the end of the chapter

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Abstract

Exposure to ultraviolet radiation (UVr) contained in sunlight is a major cause of skin illness such as sunburn, aging and cancer. UVr triggers local effects on the skin, which involve local inflammation, tissue remodeling, regulatory cytokines release and migration of dendritic cells (DCs). However, these localized effects on the exposed area are not the only ones that take place after sun or UVr exposure. A less known effect of UVr is the modulation of systemic immunity, through the generation of specific regulatory cells. These cells are induced, at least in part, by skin-migrating tolerogenic DCs. Moreover, bone marrow cell precursors can also be biased to a tolerogenic or suppressor phenotype. The sunlight- or UVr-induced immune system modulation can cause skin disorders like skin cancer and cutaneous photosensitivity in Lupus, but it also may be useful to treat cutaneous pathologies such as psoriasis and vitiligo. Moreover, the systemic immunosuppressor effect of UVr exposure may also be useful to treat autoimmunity of internal organs. Finally, as an inducer of cutaneous inflammatory response, UV phototherapy may also be useful in the treatment of cutaneous infections. Overall, UVr has profound immunomodulatory capacity that can be beneficial or deleterious for human health.

Keywords: skin, sunlight, immunosuppression, cancer, autoimmunity

1. Introduction

Sunlight is essential for life on the Earth, since there will be no nourishment provision for all the earth life forms without it. In addition to its central role in the production of large macromolecules (carbohydrates) from carbon dioxide by photosynthetic organisms, it also plays an important role in promoting an adequate life environment through heat generation: our

planet's average temperature is around 15°C, comparing with 482°C in Venus and −63°C in Mars (the two nearest planets of our system). Unfortunately, global temperature is increasing during the last years due to global warming, a problem that exceeds the scope of this chapter. Sunlight is an electromagnetic field composed of radiations with different wavelengths, ranging from X-rays to infrared radiation. Some of these radiations are absorbed in the space and in our atmosphere. Due to this absorption, sunlight spectrum that reaches the Earth's surface is composed of ultraviolet (UV) radiation (280–400 nm), visible light (400–720 nm) and near infrared radiation (720–2500 nm). The UV-radiation at the Earth surface is subdivided into UVB (280–320 nm) and UVA (320–400 nm), which have different characteristics in their effects on biological systems.

But sunlight not only provides warmth and food to the planet but also to the human beings. The different radiations that constitute the sunlight have impacts on mammalian cells, particularly on skin cells, since these are the naturally exposed cells. These effects are multiple and include DNA damage [1], reactive oxygen species (ROS) production [2], mitochondrial alterations [3, 4], matrix metalloproteases expression [5] and complex immune system changes, which are discussed in detail in this chapter. Many of these effects are specifically mediated through UV radiation, but visible light and infrared radiation also mediate cellular alterations [6].

Human skin exposure to sunlight has a range of effects on health, both beneficial and detrimental and not only cutaneous but also systemic.

On the side of the beneficial effects, the bright side, it is very well known that sun exposure is required to provide adequate amounts of circulatory Vitamin D, since this vitamin level depends on a step of UVB-induced photoisomerization of 7-dehydrocholesterol to previtamin D_3 [7]. Sunlight has also been used to treat skin diseases. In ancient India and Egypt, there was a treatment for vitiligo that consisted of the consumption of an herbs extract and a subsequent exposure to the Sun, a treatment that is used in our days known as photodynamic therapy or photochemotherapy [8]. In 1901, Niels Ryberg Finsen published his work in which he treated Lupus vulgaris, a cutaneous infection caused by *Mycobacterium tuberculosis*, using an artificial source of ultraviolet light, during the years where the antibiotics were still undiscovered [9]. For his investigations, Finsen was awarded with the Nobel Prize in 1903, and he still remains as the only dermatologist in winning this prize, more than a century later. It is not surprising that sunlight has been employed as a treatment of different diseases. In 1903, the first hospital specialized in heliotherapy opened its doors in Switzerland. In that hospital patients with tuberculosis and rickets were treated with a precise schedule of sunbathing during several weeks [10]. Moreover, during the First World War, heliotherapy was used to treat ulcers and wounds in the absence of antibiotics.

However, there is also a dark side of sunlight exposure. It is also very well known that sunlight has the ability to promote skin cancer, both melanoma and non-melanoma ones [11]. This ability to induce malignant transformation of skin cells and their subsequent progression to form a tumor is based on its capacity to induce DNA damage and mutations to the exposed cells. This damage, described in 1958, consists of the formation of pyrimidine dimers, a covalent bond between adjacent DNA bases, being the thymidine dimers the most frequent lesions [1]. Even though mammalian cells have a specialized enzymatic machinery to detect

and correct DNA lesions, these mechanisms can be overwhelmed leading to DNA mutations. Damaged cells can arrest their growth in order to detect and correct DNA damage, being p53 a key regulator of the process. If the damage is too extensive to be repaired, apoptotic cell death is initiated, through the intrinsic pathway [12]. Besides nuclear damage, UV radiation also promotes mitochondrial alterations, leading to electron transport chain uncoupling, loosing of mitochondrial membrane polarization and increasing superoxide anion production [13, 14].

UV radiation effects on the biology of exposed cells are very well known, but it also has profound effects on immune system that may affect the local and systemic immune responses. These effects are studied in the field of photoimmunology and are presented in this chapter.

2. Ultraviolet radiation effects on skin immune system

As it was mentioned, sunlight has an important impact on human health. A vast majority of the effects initiated by sunlight are triggered by UV radiation, being UVB the most relevant in terms of induction of DNA damage and detrimental effects on immune system.

The UV-induced immune system alterations can be divided into direct effects and indirect effects. The first ones are produced on the exposed organs (most likely the skin, but also the eyes) by specific responses of UV-exposed cells, which have the ability to produce several molecular mediators and, in the case of dendritic cells, to migrate. The second ones are induced in distant organs, both primary and secondary lymphoid organs, by the skin-produced molecules and migrating cells.

2.1. Direct effects on cutaneous immune system

The energy contained in the UV radiation can be transferred to different molecules within skin cells. This energy transference can modify the target molecules, such as DNA, trans-urocanic acid (UCA) and L-tryptophan, leading to molecular changes and a downstream cascade of complex cellular responses.

Trans-UCA absorbs energy from UVB radiation and isomerizes to cis-UCA, which interacts with the serotonin receptor [15], activating gene transcription and promoting reactive oxygen species (ROS) production [16], which may act as intracellular second messengers activating different kinases such as Erk1/2 in UVB-exposed keratinocytes [17]. But not only ROS can activate cellular kinases. After UV exposure, many intracellular pathways are activated, including NF-κB and MAPK ones, leading to the transcription of many inflammation-related genes [18, 19].

As keratinocytes are the most abundant cell type in epidermis, around 95% of total cells, they are the most frequent target of UV radiation. This is particularly important considering that UVB radiation only reaches the epidermis, and UVA is the only one which can penetrate deeper into the skin to reach the dermis. For these reasons, keratinocytes are central players in the establishment of the UV-induced inflammatory response. These cells are able to sense and react to different stimuli (including UV radiation) by producing pro-inflammatory

cytokines (TNF-α, IL-1 α and IL-1β, IL-6, IL-18, INF-γ), chemokines (IL-8, CCL-20), growth factors (GM-CSF, VEGF-α) and antimicrobial peptides (α and β-defensins, cathelicidin, S100 proteins and ribonucleases). These molecules create an environment that promotes vasodilatation and an increase in vascular permeability, leading to edema formation and recruitment of different immune cells, such as neutrophils, macrophages and lymphocytes to the exposed area, reinforcing the inflammatory response. But keratinocytes are not the only cell type directly affected by UV radiation. Langerhans cells (LCs), the specific dendritic cell subtype present in the epidermis, are also affected. It has been very well described that after UV exposure, the epidermis is depleted of LCs [20], but it has also been established that in vitro UV-exposed LCs promotes defective T cell activation due to UV-induced LCs apoptosis and co-stimulatory molecules alterations [21, 22].

Besides keratinocytes and LCs, dermal cells also play an important role in the UV-induced cutaneous inflammation. Dermal fibroblasts, mast cells, macrophages and dermal dendritic cells can be stimulated both directly by UV radiation (mainly UVA, since UVB does not reach the dermis) and indirectly by epidermal produced soluble mediators [23–27]. As a consequence of repetitive acute exposures to UV radiation, there is a degeneration of skin cells, a destruction of collagen fibers and blood vessels, which, in turn, leads to premature aging, photodermatoses and actinic keratosis. These alterations are consequence of the production of ROS by exposed keratinocytes and fibroblast and the increased production of metalloproteases, which finally ends in the extracellular matrix degradation.

Besides the abovementioned pro-inflammatory mediators, when the skin is exposed to UV radiation, keratinocytes and other immune cells, such as mast cells, neutrophils and monocytes, also produce regulatory soluble mediators such as IL-10, IL-4, prostaglandin E_2 (PGE_2) and platelet-activating factor (PAF). PAF induces the expression of cyclooxygenase-2 (COX-2, the inducible form of the COX enzyme), which is necessary to produce PGE_2. At the same time, cis-UCA induces keratinocyte's production of neuropeptides that stimulates mast cells to release histamine, which, in turn, induces the production of PGE_2 leading to a retro alimentation system. PGE_2 induces the production of IL-4 by lymphocytes and monocytes, potentiating the release of IL-10 by keratinocytes. In this way, all the described mechanisms converge in the production of IL-10.

UV-induced cutaneous inflammation can be controlled by specific regulation of the immune system. It has been recently described that both epidermal LCs and apoptotic keratinocytes are essential for the correct control of UV-induced cutaneous inflammation, through the phagocytosis of apoptotic keratinocytes by LCs [28].

2.2. Systemic effects on immune responses

As it was mentioned in the introduction, skin cancer development is one of the major health problems associated with UV exposure. This carcinogenic effect was first demonstrated in a mice model by Dr. George Marshall Finley in 1928 [29]. More than four decades later, Dr. Margaret Kripke observed that UV-induced skin carcinomas were highly immunogenic and were rejected once transplanted on naïve mice [30]. Dr. Kripke realized that there must had been something else than mutagenic effects on UV-radiation, and she proved that this

extra effect was a marked systemic immunosuppression that impeded the immune system to attack the tumor [31, 32]. Since Dr. Kripke pioneering work, photoimmunologists from all over the world have elucidated many mechanisms involved in UV-induced systemic immunosuppression [33].

One of the most employed models to study this specific suppression of immune responses is the contact hypersensitivity reaction (CHS) to different molecules (the most commonly used are oxazolone and dinitrofluorobenzene). The reaction consists of a first contact between the antigen and the skin, named sensitization, where specific T cell activation takes place in draining lymph nodes, and a second contact in the ear skin of sensitized animals, named challenge. After the challenge, a specific T cell-driven inflammatory response is established and can be measured as the increase in ear thickness. Using CHS reaction, it was demonstrated that several immune cells are involved in the UV-induced immunosuppression:

- The DCs that migrate from the skin to lymph nodes to present antigens to T cells expose a tolerogenic phenotype after UV irradiation. This leads to the promotion of T cell differentiation to a regulatory phenotype. The exact role of LCs in this process is controversial, since it was demonstrated that these cells are not essential to establish the UV-induced immunosuppression [27, 34], but it was also observed in other experiments that LCs are required to produce the phenomena [35]. These events were demonstrated applying the sensitizer onto the irradiated skin.
- During T cell activation by tolerogenic DCs in skin draining lymph nodes, a differentiation to regulatory T cells (Tregs) is produced [36]. These Tregs are antigen specific and may transfer the immunosuppressive estate when injected to naïve mice. Moreover, these Tregs can modulate new immature DCs to turn them into tolerogenic, reinforcing the suppression on the immune response [37].
- Skin mast cells can also migrate to the lymph nodes after irradiation. This migration is essential to establish the immunosuppression [38], and these mast cells are necessary for the generation of regulatory B cells (Bregs), which are other important regulators of the immune response that are involved in the cutaneous response after UV exposure [39].
- Regulatory B cells, as well as Tregs, can modulate DCs' induction of immunity [40], favoring a vicious circle of specific immunosuppression that is set up after skin exposure to UV radiation.

The abovementioned cells produce their effects by sensing and releasing soluble mediators. A pivotal cytokine involved in this suppression is IL-10, since its blockade by specific antibodies [41, 42] or using knock-out mice [43] leads to normal immune responses even after UV exposure. This cytokine is produced by many cells, described earlier in this chapter, such as keratinocytes [44], DCs [45], Tregs, mast cells [46] and Bregs. Vitamin D, whose synthesis in the skin is dependent on UV exposure, also plays an important role as a soluble mediator of UV-induced immunosuppression. This vitamin induces a tolerogenic phenotype on DCs in vitro [47] and can mimic the effect of the radiation in vivo [48]. Another important soluble mediator with systemic effects which is produced in the skin after UV exposure is

prostaglandin E_2. This eicosanoid is a main product of cyclooxygenase-2 (COX-2), which is up-regulated in irradiated skin and whose drug-mediated blockade decreases the UV-induced immunosuppression [18]. Other soluble mediators have been implicated specifically in UVA-induced suppression such as the alternative complement component factor B [49] and serotonin [50]. Platelet-activating factor, TNF-α, IL-4 and histamine also play a role in that process.

Besides its effects on mature T and B cells during their activation in skin draining lymph nodes, UV radiation can also modulate the differentiation of immune cells in primary lymphoid organs. In particular, bone marrow cells are affected in UV-exposed animals. DCs (CD11c + cells) differentiated in vitro from the bone marrow of UV-exposed animals were less competent than the control cells (differentiated from non-exposed animals). The defective bone marrow precursor phenotype can be restored by treating the exposed animals with a COX-2 inhibitor, demonstrating the role of PGE_2 in affecting bone marrow cells [51].

2.3. Other indirect effects on immune system

The effects of microbiota on immune system has been vastly described and is a topic of growing interest during the last years. Even though the most important efforts are directed to study and to explain the interaction of immune system with gut microbiota, this is not the only important microenvironment that may affect the human health. Skin microbiome is indeed an important stimulus for cutaneous immunity. It is composed of a complex group of microorganisms, including bacteria, virus and fungus, which has their particular equilibrium. A disbalance in the commensal microbial community may impact on skin health, as is the case of *Staphylococcus aureus* role in atopic dermatitis [52]. It is not the aim of this chapter to discuss in detail the role of skin microbiome in health and disease, but what is certain is that it can modulate the skin immune system.

Skin exposure to the Sun and UV radiation not only impact skin cells, but also may affect microorganisms living on the skin surface. The effect of the radiation on the microorganisms depends on the type of microbe, its life cycle (spores tend to be more resistant that other forms) and the location (microbes can penetrate deep into the skin appendages). However, UV radiation can definitely affect skin microbiota, leading to different interactions with both adaptive and innate immunity [53]. The exact role of UV radiation on skin microbiome and its effects on immune system need to be studied in detail during the next years, in order to elucidate their implications in skin diseases.

3. Role of UVradiation exposure and immune system modulation on skin diseases

As it was mentioned earlier in this chapter, the most important effect of UV radiation on human health is the induction of skin cancer and the establishment of an immunosuppressive environment which allows the growth of the tumors. However, skin cells malignant transformation is not the only affection produced by UV radiation on the skin. Photosensitivity in Lupus erythematosus is also produced by UV exposure. Moreover, skin infections may be favored by skin UV irradiation. The mechanisms of these skin malignancies are discussed in the following sections.

3.1. UV-induced immune effects implicated with skin cancer development

The skin exposure to sunlight, as well as to artificial sources of UV radiation, is the main etiological factor in basal cell carcinoma, squamous cell carcinoma, cutaneous malignant melanoma, actinic keratosis and melanocytic nevi [11]. The mutagenic role of UV radiation was already mentioned, but it is important to mention the most common mutated genes in skin cancer. Even though the mutagenic effect is directed to DNA sites with adjacent pyrimidines, there are some target genes more frequently affected, as is the case of p53. The protein codified by this gene is crucial in the regulation of cell cycle, favoring cell arrest in order to allow enzymatic machinery, another important protective factor, to repair the UV-induced DNA damage. More than 60% of aggressive basal cell carcinoma was found to have mutations on p53 gene [54]. These mutations were also found in normal sun-exposed skin of skin cancer patients [55]. The importance of cellular DNA repair machinery can be seen in patients with xeroderma pigmentosum. These patients present an autosomal recessive deficiency of DNA repair enzymes, developing basal cell carcinoma and other skin cancers at a very young age [56]. These patients are highly susceptible to sunlight, and they need, in some cases, to completely avoid sun exposure, being known as "children of the moon" [57].

Besides cell biology affections produced by UV radiation, this carcinogen promotes systemic immunosuppression. The relevance of the immune system alterations in skin cancer can be weighted through different experimental data:

- As it was mentioned, IL-10 is a pivotal cytokine in UV-induced suppression, which is secreted by several cell types after skin irradiation. In IL-10 knock-out mice, it has been reported the absence of skin tumors after chronic UV irradiation, besides the mutagenic effect of the radiation [58]. Moreover, in human studies, it has been demonstrated that the presence of a polymorphism in the IL-10 promoter that leads to a deficient transcription of the gene is inversely correlated with the development of skin cancer in UV-exposed skin areas [59].
- Prostaglandin E2 is also a very important molecule implicated in the UV-induced skin carcinogenesis. The pharmacological blockade of its production, by oral or topical administration of the drugs, along chronic irradiation protocols in mice, lead to a decrease in the number and size of the tumors [60, 61]. The selective inhibition of the inducible isoform of the enzyme cyclooxygenase (COX-2) by drugs, like Celecoxib, as well as with nonselective drugs (COX-1 and COX-2 inhibitors), like indomethacin or naproxen, both show the same antitumoral effect [62]. However, even though the number of tumors is decreased by these treatments, there is still a high frequency of mice with at least one tumor. The role of COX-2 in UV-induced skin tumors is described in detail in [18], but it is important to mention that plenty of natural compounds derived from plants are effective in decreasing the expression or the activity of this enzyme.
- The relevance of IL-12 to the immune response against UV-induced tumors was reported by Meeran et al. [63]. They observed that the animals which were deficient in this cytokine were more sensitive to the UV-induced carcinogenesis, developing a greater number of tumors and also generating them in a shorter period of chronic irradiation.

But the modulation of the immune system to allow transformed cells to growth is not the only effect of UV radiation. It has been recently reported that UV promotes angiogenesis and metastasis of melanoma, once this tumor is developed [64]. Using a genetically engineered mice model, Bald et al. demonstrated that, once the tumor is established, the chronic exposure to UV radiation lead to the release of DAMPs (damage associated molecular patterns) from keratinocytes, promoting an TLR-4-dependent inflammatory response. This inflammation recruits neutrophils and promotes vascular activation, allowing melanoma cells to invade blood vessels and to migrate to distant organs, like the lungs.

3.2. Role of UV exposure on cutaneous photosensitivity in systemic lupus erythematosus patients

Lupus (word that means wolf in Latin) is a widely known autoimmune disease, since Hippocrates times [65]. Its name was due to the characteristic destructive injuries that resemble the bites of the animal. One of SLE patients' main symptoms is a cutaneous rush on the face, butterfly-shaped, due to skin exposure to the Sun.

The immune mechanism involved in this symptom is supposed to be mediated by specific autoantibodies directed against nuclear proteins, Ro/SS-A 52, Ro/SS-A 60 and La/SS-B [66]. During the apoptotic cell death of irradiated keratinocytes, the nuclear antigens are relocated to the cellular membrane and exposed to the immune system [67, 68]. This exposure of nuclear antigens is supposed to be involved in two different processes:

- The induction of the specific autoantibodies, which involves the recognition of the apoptotic bodies, with the nuclear antigens exposed on their membranes, by specific B cells. This recognition promotes the production and release of the specific autoantibodies anti-Ro/SS-A and anti-La/SS-B, a common laboratory finding in these patients.
- Once the autoantibodies are produced, they can reach the irradiated skin, recognize the apoptotic cells that expose the antigens on their surface and lead to an attack to the apoptotic bodies by complement factors. This can ultimately produce the lysis of apoptotic cells, releasing their cellular content to the extracellular space. The release of the cellular content produces a strong inflammatory reaction where, in a normal condition, there will be no inflammation.

However, this theory does not explain why patients with other pathologies who are also positive for anti-Ro/SS-A and anti-La/SS-B autoantibodies, such as Sjögren's syndrome patients, do not show cutaneous photosensitivity. This controversy is still present in the bibliography [69], and the molecular mechanism underlying the butterfly-shaped rush is still unknown.

3.3. Other skin conditions

As skin exposure to UV radiation affects DCs function, T and B cell activation, macrophage phagocytic activity and other immune mechanisms, it is expected to find alterations in responses against pathogens, including virus, bacteria, parasites and fungus [70]. In different

infectious diseases in mice models, it has been described the alteration of T cell function by exposing the animals to UV light before or after the infectious challenge [71]. It has been recently published that different procedures of irradiation, which include a single high UV dose and repetitive low UV doses, differentially affect the evolution of a *Staphylococcus aureus* cutaneous infection [72]. However, in humans, this effect is more elusive. There are just a few infections, mainly viral infections that have been observed to be affected by exposure to UV radiation.

4. Immune system modulation by UV radiation as a therapy

Due to the profound effects that UV radiation promotes on exposed cells and on local and systemic immune system, the use of this radiation for human therapy is widely spread. Besides all the knowledge on the molecular effects of UV radiation described earlier, the first therapeutic use of this radiation was Finsen's work, mentioned in the introduction of this chapter, where he treated Lupus vulgaris using a carbon arc lamp, more than a century ago [73]. Currently, UV phototherapy is mainly employed in different dermatological disorders, including psoriasis, vitiligo, atopic dermatitis and cutaneous T cell lymphomas [74]. However, the usage of phototherapy has also been proposed to treat different systemic conditions like autoimmune diseases. Phototherapy procedures include exposure to broad-band UVB (BB-UVB), narrow-band UVB (NB-UVB) and psoralen + UVA (PUVA), but the analysis of their differences exceeds the purpose of this chapter [75].

4.1. Phototherapy effects in psoriasis

Psoriasis is a chronic inflammatory skin disease. It is characterized by cutaneous lesions, produced by hyperproliferation of keratinocytes under the control of T cells. In the pathogenesis of the disease, different inflammatory cytokines are involved, like TNF-α and IL-17. UVB phototherapy has been widely used for psoriasis treatment during decades, evolving from BB-UVB to NB-UVB. The role of phototherapy in these patients can be inferred from the abovementioned molecular mechanisms induced by UV radiation. Basically, it promotes several changes in the affected skin, including the direct effect on keratinocytes preventing their proliferation and indirect effects on immune system. The T cell activity is unbalanced by UV radiation, increasing IL-4 production that affects Th-17 and Th-1 profiles, both implicated in the perpetuation of the inflammatory state in the patients. In this way, UV phototherapy produces a decrease in the production of IFNγ, TNFα, IL-17 and IL-22 and an increment in IL-10 secretion. Moreover, an augmented migration of Tregs to the irradiated psoriatic skin has been observed, which may also contribute to control this disease [76].

UVB phototherapy must be rationally employed, since the chronic exposure to this treatment may increase the risk of skin cancer development. Patients under this therapy may have to replace it by any other available therapy, such as topical and systemic drugs or blocking antibodies, after a prolonged period of use.

4.2. Phototherapy in vitiligo

Vitiligo is an autoimmune disease characterized by the absence of melanin in particular zones of the skin, which remains unpigmented. This lack of pigmentation is due to deficient activity of melanocytes, the epidermal cells with the ability and the enzymatic machinery to synthesize this pigment [77].

Phototherapy in vitiligo promotes two main cellular effects such as the activation of melanocytes and the promotion of Tregs. The first effect is directly related to the lack of pigmentation, and melanocytes are sensitive to UV radiation as a signal that induces melanin synthesis. In this way, UV phototherapy directly promotes the melanin synthesis. The second effect is associated to the main topic of this chapter, the modulation of the immune system by UV radiation. The mechanisms are the same that have been discussed in this chapter, but the goal is to induce immunological tolerance once it has been lost [78, 79]. The exact antigens that trigger vitiligo are unknown, but the mechanisms that lead to suppression of immunity after UV irradiation seem to be effective to counteract this cutaneous autoimmune disease.

4.3. Experimental phototherapy for cutaneous infections

As it was mentioned, UV irradiation may promote several changes in skin and systemic immune system. These changes are dependent on the dose and the periodicity of the exposure, and include local inflammation, activation of innate immune response, generation of tolerogenic DCs and Tregs [80]. Many of these changes can positively impact on the progression of cutaneous infections produced by different types of pathogens.

The parasite from the genus *Leishmania*, an endemic pathogen in some South American, African and Asian countries, promotes characteristic cutaneous lesions on infected individuals. Using mice models, it has been demonstrated that the exposure of animals to low dose UVB radiation (exposures on 4 consecutive days) prior to the challenge with *Leishmania amazonensis* promastigotes protects the animals against the cutaneous lesions. Moreover, UV-exposed animals presented higher levels of serum IFN-γ and TNF-α, compared to nonirradiated control infected mice [81]. These results demonstrate that cutaneous leishmaniasis can be modulated by UV radiation. However, the exposure to the radiation was performed prior to the parasite challenge, and the results cannot be extrapolated to a treatment for infected individuals. It is worth to notice that there is a research vacancy on experimental phototherapy for cutaneous leishmaniasis, but there are a few reports that have demonstrated the effectiveness of this treatment in mice models, which received UV or sunlight irradiation prior to the infection as well as during the long-term observation of the lesions. In the works of Giannini and Hoseinipoor et al., it can be observed that irradiated animals can recover from the infection faster than nonirradiated control animals [82, 83].

As UV radiation, especially UVC, present a direct bactericidal effect, it was directly used for the treatment of a cutaneous infection caused by antibiotic-resistant bacteria. The case reported by Aleem et al. shows the use of an UVC germicidal lamp to treat a poly-microbial burn wound infection. The infection was caused by *Staphylococcus aureus*, *Klebsiella pneumoniae*

and *Pseudomonas aeruginosa*, and the antibiotic therapy was unsuccessful. The physicians decided then to use UVC phototherapy and, finally, the 9-year-old patient recovered from the infection [84]. The success obtained with this treatment may be due to a direct germicidal effect or by immune system modulation. More studies need to be performed to clarify this point and to allow the establishment of new treatments for cutaneous infections produced by antibiotic-resistant bacteria.

4.4. Experimental phototherapy for autoimmune diseases

As it was mentioned earlier, skin exposure to UV radiation leads to an important systemic immunosuppression. The influence of this tolerogenic effect on an internal organ autoimmune disease was deeply studied by Dr. Scott Byrne's group using a mice model of multiple sclerosis (MS), the experimental autoimmune encephalitis (EAE). They showed that exposing the animals to UV radiation prior to the antigenic challenge, and also during the evolution of the disease, it was possible to decrease the severity of the EAE, including the demyelination process [85]. This effect was dependent on B cells, since the protection against the pathology can be obtained by transferring B cells from irradiated animals to naïve mice. Moreover, pharmacological depletion of B cell abolished the UV-mediated protection against EAE.

Being aware of these effects on mice, it seems promising to treat MS patients using phototherapy. Dr. Prue Hart faced this challenge, and set a clinical trial to treat patients suffering clinically isolated syndrome, who have a high susceptibility to develop MS [86]. The results of this clinical trial may open a new era on the treatment of autoimmune diseases using cutaneous phototherapy.

4.5. Extracorporeal blood irradiation

Skin exposure to UV radiation is not the only therapeutic procedure that employs artificial sources of this radiation. There are two procedures based on the extraction of blood from the patients and its subsequent exposure to a source of UV radiation: extracorporeal photopheresis (ECP) and ultraviolet blood irradiation (UBI). These two procedures base their efficacy on the modulation of the immune system. However, while ECP promotes a downregulation of immune effector mechanisms, UBI produces the opposite effect.

ECP consists of blood extraction, buffy coat separation, its subsequent mixture with a sensitizer (psoralens) and an exposure to a UVA source. The irradiated white blood cells are then reinfused to the patient. This procedure has shown to be not only effective but also safe, and it has mainly mild and transient side effects. It was first described to treat cutaneous T cell lymphoma and chronic graft versus host disease, but it can also be employed in different autoimmune pathologies such as scleroderma, multiple sclerosis, Type 1 diabetes mellitus, rheumatoid arthritis or Crohn's disease [87]. Its mechanism of action is not completely understood, but it has been shown that the PUVA treatment produces the apoptosis of exposed cells, which are phagocyted by DCs once they are reinfused. These DCs acquire a tolerogenic phenotype, promoting the generation of regulatory T cells that ultimately lead to the control of the pathologic T cells [88].

On the other hand, UBI is an old and almost forgotten technique, whose use was extensive in the 1940s and 1950s. Similarly to ECP, it consists of blood extraction with citrate (around 5–7% of total blood) and its subsequent irradiation using UVC or UVB radiation, without any cellular separation. It was employed to treat many infectious diseases such as septicemia, tuberculosis and pneumonia, and other pathologies like arthritis and asthma [89]. The exact mechanisms of action are not fully understood, but it is known that an activation of antigen presenting cells is produced during the procedure. Even though this treatment has almost been abandoned, it may be a therapeutic option against multi-resistant bacterial infections.

5. Conclusions

Skin exposure to sunlight, specifically to UV radiation, triggers very well-known mechanisms that may ultimately promote a profound modulation of the immune system, including both innate and adaptive immunity. This modulation of the immunological response leads to a defective control of tumor cells and pathogens. Moreover, as it affects systemic immunity, it can also alter the response to vaccines. On the other hand, the knowledge of these detrimental effects has led to multiple options to treat immune-based pathologies. In this way, the potentiality of cutaneous and systemic immunomodulation by different types of phototherapy is yet far to be completely explored.

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Conflict of interest

The authors have no financial conflict of interest.

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Chapter 6

Toll-Like Receptors: The Key of Immunotherapy in MSCs

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Additional information is available at the end of the chapter

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Abstract

Human mesenchymal stem cells (MSCs) are potential candidates for various applications in the fields of immunotherapy. Their multilineage differentiation capability and immune modulatory features allow their prospective application for the management of different immunological circumstances. However, the local microenvironment, in addition to the source of the MSCs can control diverse biological features of the cells. Indeed, throughout their therapeutic application, MSCs may interact with their microenvironment through their expressed toll-like-receptors (TLRs), producing immune modulating reactions. Stimulation of MSCs before or within the potential treatment procedures with distinct TLR ligands may assist as an effective step controlling the biological function of the MSCs as needed in different therapeutic stages of the disease.

Keywords: TLR, immunotherapy, immunomodulation, mesenchymal stem cells

1. Introduction

Human mesenchymal stem cells (MSCs) are potential candidates for various applications in the fields of immunotherapy [1, 2]. Their multilineage differentiation capability and immune modulatory features allow their prospective application for the management of different immunological circumstances [1, 2]. However, the local microenvironment, in addition to the source of the MSCs can control diverse biological features of the cells [1, 2]. Indeed, throughout their therapeutic application, MSCs may interact with their microenvironment through their expressed toll-like-receptors (TLRs), producing immune modulating reactions of the

cells. Thus, by comprehending these TLR-promoted properties on immune regulating functions of MSCs, potential therapeutic applications of these cells can be optimized [1, 2].

Toll-like receptors (TLRs), major molecules connecting the innate and adaptive immune responses, are germ line-encoded pattern-recognition receptors (PRRs), identifying specific pathogen-associated molecular patterns (PAMPs), thus supporting the activation of immune cells [3, 4]. They work as sensors for different pathogens and play an important role in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases [5]. So far, 10 functional human TLRs have been categorized [2, 6]. Depending on their PAMP ligands and their cellular localization, TLRs are divided into intracellular and extracellular receptors. Extracellular TLRs are expressed on the cell surface and generally identify constituents of microbial membranes as lipids and lipoproteins (TLR1, TLR2, and TLR6), lipopolysaccharide (LPS) (TLR4), and flagellin (TLR5). The intracellular group is expressed inside the cells, where they recognize double-stranded RNA (TLR3), single-stranded viral RNA (TLR7 and TLR8) and unmethylated CpG DNA of viruses and bacteria (TLR9) [7].

Multipotent stromal cells (MSCs) of diverse origin have been presented to express functional TLRs in definite patterns [2, 8], turning them selectively sensitive to specific microbial compounds. When activated by these compounds, TLRs can control MSCs' proliferative, immunomodulatory and differentiation potentials [9]. Differential expression profiles of functional TLRs 1–10 were described on MSCs from various tissues of the human body [10]. Results displayed that the specific profile of expressed TLRs differs according to the tissue origin of the MSCs, which endorses different immunomodulatory and therapeutic potentials of these cells during transplantation in infectious and inflammatory environments *in-vivo* [1, 10].

2. The immune system

Defending the human body against potential threats of invading pathogens depends on a number of natural mediators which are capable of recovering the homeostasis and preserving it [11]. This biological process of protection involves cells and molecules opposing the microorganisms detected by the immune system, originally developed in the human embryo. This mechanism starts with hematopoietic stem cells that differentiate into the key players of the immune reaction of our bodies (granulocytes, monocytes, and lymphocytes). Through the various activities of these major units of immunity, the immune response holds two chief divisions, the innate and the adaptive immune reactions. The innate immunity includes different protective walls of microbiological, as well as chemical and physical nature but also delivers the immune components responsible for abrupt actions against the invading threats. Though this defensive response is fast, it lacks specificity and could damage some normal tissues. On the contrary, the adaptive immune response provides a higher accuracy in its defensive process, nevertheless it takes several days or weeks to develop. This can be clarified by the development of an immunological memory through the adaptive immunity, which permits specific reactions against the pathogens with less harm to the normal tissues than the innate response.

2.1. The innate immune system

The innate branch of the immune system refers to none- or partially-specific defensive mechanisms starting directly or after a short interval of a pathogen's invasion of the body [12–14]. In this immune reaction, the genetic memory of the germline-encoded receptors enables the detection of certain molecular patterns of common pathogens [12, 15]. It is responsible for primary steps of protection against microbial threats. Simple chemical and physical barriers as epithelial layers and mucous secretions lining numerous tracts, such as the oral mucosa or the gastrointestinal tract, contribute to this defensive first line [16–18]. Furthermore, soluble proteins and bioactive molecules within biological body fluids as cellular secretions of cytokines or complement proteins are able to weaken a varied spectrum of invading pathogens [16]. Cellular constituents of the innate reaction consist of dendritic cells, macrophages and natural killer cells [16–18]. In order to confirm its role restoring the homeostasis and clearing the invading microorganisms the innate immune response has to accomplish the fundamental mechanism of early pathogen recognition. This mechanism primarily is initiated by a group of receptors termed the Pattern Recognition Receptors (PRR). These receptors are able to detect conserved microbial patterns known as Pathogen-Associated Molecular Patterns (PAMPs) [19]. One of the most important PRRs are TLRs, as they are able to recognize bacteria, fungi and viruses [20, 21]. Following PAMP-PRR detection, a reaction cascade is introduced by cells of the innate immunity creating antimicrobial mediators as reactive oxygen. In conjunction with that reaction, produced chemokines and cytokines enable recruitment of immune cells favoring the clearance of pathogens. Likewise, the ligation of PRR promotes the synthesis of antimicrobial acute phase proteins, such as complement factors. The initiated innate immune response is essential for the inception of the adaptive response as both divisions of the immunity do not function separately, but depend on their inter-reliant activities [7, 22].

2.2. The adaptive immune system

Consecutive to the innate immune reaction the second branch of immunity begins. This adaptive or acquired reaction is unalike the innate response considered highly specific against certain microbes. This is endorsed by a special capability of the cells of this arm of immunity to perform a recombination of their antigen receptors, creating the immunological memory, by which pathogens can be identified specifically [17]. As this mechanism may need 3–5 days, the innate response has to coordinate to fulfill its functions, creating the first line of defense in the body [23]. The adaptive immune response is composed of a number of specialized cells that originate during hematopoiesis from lymphoid cell lineage. Among these cells are $CD4^+$ and $CD8^+$ T-cells as well as B-lymphocytes, which are accountable for antibody production [24]. The antibodies deliver the humoral immunity, a main defense against pathogenic invasion. After pathogen detection, the antibodies bind to the microbes, triggering their neutralization and averting the pathogenic access into the host cells. Other functions of antibodies implement an incitement of phagocytic immune cells including macrophages and neutrophils, as well as natural killer cells. Through forming the first step of complement cascade activation by antigen-antibody complexes, they also allow the phagocytosis of unrecognized bacteria. This is enabled through the opsonisation mechanism to microbial pathogens.

Additionally, killing infected cells is operated by T cells, a second cellular constituent of the adaptive immune response [17].

3. Toll-like receptors

3.1. Discovery and description

The TLRs family is considered the first PRR group of to be discovered. The Toll protein was identified in 1985 and categorized as being substantial for embryonic growth of the fruit fly, *Drosophila melanogaster* [25]. An alternative function described in auxiliary studies is facilitating host responses to fungal infections and encouraging the release of antimicrobial mediators [26]. In 1997 a human toll like homolog was described [27]. This protein was named the TLR and displayed an important function in interplay between innate and acquired immunity [28]. TLRs are extracellular and intracellular proteins, which differentiate classes of various molecules. This allows the innate immune reaction to utilize the TLRs for detecting microbial pathogens [29]. By recognizing definite microbial products or patterns by the TLRs the early immune response can be commenced [30]. Among PAMPs activating TLRs are, peptidoglycan, lipoproteins, lipopolysaccharide, bacterial DNA, as well as double-stranded RNA [30]. Resulting from this TLR-PAMP complex, expressions of defensive or pro-inflammatory genes are induced within the cells. Simultaneously, signaling pathways are initiated promoting NF-κβ and MAPK pathways, along with supporting cytokine production, leading to the instigation of the adaptive immune response [31].

3.2. Identification of TLRs

The first reported mammalian TLR was TLR4 [28, 32]. The PRR-PAMP complex associated with TLR4 presented a critical function in identifying the bacterial element LPS [33]. Further studies reported a family of 13 TLRs in mammalian species [34], with functional TLRs 1–10 in human cells [6]. TLRs show a resemblance to IL-1 receptor family in their cytoplasmic fragment. Due to this correspondence, the intracellular areas of TLRs were called Toll/IL-1 receptors (TIRs). Extracellularly the TLRs display leucine-rich replications, while IL-1 receptors show immunoglobulin-like domains [27, 35]. Due to their functionality, most reliable investigations have been pointing attention to TLRs 1–10 in humans, as wells as other mammalian species.

3.3. TLR activating PAMPs and their signaling pathways

Investigations have presented different molecular components and patterns of pathogenic microorganisms, comprising combinations of nucleic acids, lipids, proteins, and carbohydrates functioning as ligands (PAMPs) stimulating the TLRs. Among the these PAMPs, bacterial lipoproteins, lipopolysaccharides, flagellin and viral RNA are considered significant components detected by TLRs [30, 36, 37]. By triggering TLRs through their specific ligands, signaling pathways are initiated, promoting elements as MyD88 and NF-κβ within the cells.

MyD88, a structurally related molecule to the IL-1R family, was reported as one of the major factors employed to initiate the signaling pathway of most TLRs, producing the transcription factor NF-κβ [38]. This nuclear factor can induce both pro- and anti-inflammatory reactions and promotes the expression of different genes, as cytokines, chemokines and adhesion molecules [39]. Through these intracellular responses, the innate immune reaction is commenced and a signaling cascade is provided to resist the pathogenic invasion. This important step is the first defensive tool of the cells against the pathogens, leading to the adaptive response as a second stage defense to defend the cells by specific means [31]. Corresponding to their specific PAMPs, TLRs can be categorized into subfamilies. Studies have displayed the detection of lipids by (TLRs 1, 2 and 6), nucleic acids by (TLRs 7, 8 and 9) and different ligands by TLR4 [30, 31]. TLRs can also be classified regarding their cellular expression, as TLRs 1, 2, 4, 5, 6 and 11 are existent on the cell surface, while the rest are expressed inside the cells [30].

3.4. TLR subgroups

3.4.1. TLR1, TLR2, TLR6 and TLR10

TLR2 has the ability to recognize a wide range of PAMPs. These include pathogenic lipoproteins, gram-positive bacterial lipoteichoic acid and peptidoglycans, *Porphyromonas gingivalis* fimbriae and fungal zymosan, as well as mycobacterial lipoarabinomannan [30, 40]. Additionally, bacterial LPS originating from *Porphyromonas gingivalis*, *Capnocytophaga ochracea* and *Bacteroides fragilis* can also be identified by TLR2 [30]. Two possible processes have been suggested considering the TLR2 identification of different pathogenic components. In the first mechanism, TLR2 produces heterophilic dimers with other TLRs that show structural similarity to it, as TLR1, TLR6 and TLR10. Therefore, TLR1, TLR6 and TLR10 are considered associative in their function with TLR2, being able to identify correlated types of PAMPs as diacyl and triacyl lipopeptides [20, 29]. The second model proposes the TLR2 mediated recognition of fungal proteins. This feature explains why TLR2 associates with dectin-1, a fungal cell wall constituent [41]. Through this functional coordination with different types of proteins, TLR2 gains its aptitude to detect various pathogenic invasions at early stages, activating the immune reactions.

3.4.2. TLR3

TLR3 primarily identifies dsRNA formed in the replication phase of most viruses. It activates the formation of NF-κβ and type I Interferon [42]. TLR3 can also homodimerize with TLR4 and TLR9 creating an intercommunicative response against invading pathogens [43, 44].

3.4.3. TLR4

TLR4 is a significant receptor identifying PAMPs as LPS from different bacterial species [30]. This LPS pattern shows structural variances from the LPS detected by TLR2, seen in the number of acyl chains of the bacterial protein [45]. Other molecules of endogenous nature, as heat shock proteins (HSP60 and HSP70), also showed activation of TLR4 in higher concentrations [46].

3.4.4. TLR5

TLR5 can detect flagellin through a process of physical interaction with the pathogens [30, 46]. Its expression has mainly been reported on epithelial cells of mucosal surfaces of the lung [47] and the intestine [48], promoting the detection of microbes at these surfaces.

3.4.5. TLR7 and TLR8

TLR7 and TLR8 both show the capability to distinguish similar ligands in certain conditions. Studies have reported that the two are stimulated by organic materials as Imidazoquinoline [30] and viral ssRNA [49–51], whereas host ssRNA is not identified by them [29]. The recognition process initiates by internalization and replication of the virus releasing its viral RNA into the cellular endosomes. The interaction mechanism between the viral ssRNA and TLR7/8 triggers the recruitment of MyD88 and production of NF-κβ, as well as proinflammatory cytokines [52].

3.4.6. TLR9

TLR9 is capable of distinguishing bacterial DNA [30]. This DNA contains unmethylated CpG promoting immunostimulation dissimilar to the vertebrate DNA that contains methylated CpG only [53]. By triggering of TLR9 by bacterial DNA the production of cytokines as IL-12, IFN-α and TNF-α is potentiated [54]. The proficiency of TLR9 to induce IFN-α production and to identify unmethylated CpG designates that it may also play a role in processes of viral pathogen identification [55].

4. Human mesenchymal stem cells

4.1. History and description

Human mesenchymal stem cells were defined originally by Friedenstein et al. [56] and designated as bone marrow isolated, non-hematopoietic and plastic-adherent cells, holding the abilities of self-renewal and multipotent differentiation *in vitro* [57–59]. These undifferentiated cells arise from different niches of the human body [60].

The multilinenage ability and the potential of self-renewal both describe the main characteristics of MSCs [61]. Self-renewal is the mechanism through which stem cells can expand their number throughout development. This capacity is essential for MSCs to allow their expansion within the tissues and plays a very important role in stem cell related therapies [62]. Investigations on this characteristic displayed its dependence on the life span of the cells. Most human MSCs are limited to a maximum of 44 weeks [63] or 55 population doublings *in vitro* [64].

Multilineage potential, or multipotency of MSCs forms the exceptional capacity of the cells for differentiating into other mesodermal lineage cells, as osteocytes, chondrocytes and adipocytes. Nevertheless, they can correspondingly differentiate forming cells of other embryonic lineages [65].

These exceptional features of MSCs as well as their communication with specific signals and mediators of the human body display great therapeutic prospectives and may develop into possible treatments for different diseases in the future [66, 67].

4.2. Identification of mesenchymal stem cells

The identification of MSC populations and the verification of their "stemness" have been confronting researchers in recent years. Without an ability to recognize MSCs among mixed cell populations' cultures of MSCs of higher purity would be very effortful to achieve.

Considering this, numerous studies investigated different characteristics of MSCs identification. In 2006 the plastic adherence of MSCs maintained under basic culture conditions was defined [68]. In addition, the multilineage differentiation potential of MSCs *in-vivo* or *in-vitro* after stimulation by specialized media was postulated by a number of studies [68–70].

Another widely reported method for MSCs' recognition is the analysis of the expression of specific surface markers of the cells by flow cytometry. Markers as CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120, CD124, CD166 and Stro-1 show positive expressions on the cell surface, while markers as CD11, CD14, CD18, CD31, CD34, CD40, CD45, CD56, CD80 and CD86 are missing or weakly expressed [59, 68, 71]. Colony forming units (CFUs), which are cellular colonies formed by the MSCs after isolation, were also reported as a method of MSC recognition by CFU assays [71, 72].

4.3. Sources of adult mesenchymal stem cells

Hazards and morbidity risks of stem cell based therapies have turned into one of the most debated subjects in the latest years. These discussions led to multiple studies regarding the carcinogenic potential of embryonic stem cells [73–75]. Simultaneously, ethical discussions about the use of these cells have raised many disagreements within the scientific society, promoting a large number of investigators to discover potential sources for safer adult (somatic) stem cells. Although bone marrow has been established to be the primary source of adult mesenchymal stem cells [76, 77], several efforts and investigations are being prepared to establish new stem cell bases that could deliver large quantities of MSCs with less risks and donor site morbidity. Among these niches, umbilical cord blood (UCB) [78, 79], placental tissue (PT) [80, 81], adipose tissue (AD) [82, 83] and Wharton jelly (WJ) [84] have been described as possible sources of MSCs. Additionally, MSCs can be extracted from oral tissues as gingiva [72, 85–87], alveolar bone proper [88, 89], periodontal ligament [90], dental follicle [91] and dental pulp [92]. Despite the phenotypic resemblance of MSCs isolated from various niches of the body, differences in their actions and functions of have been reported, emphasizing the individuality of MSCs derived from every source [10, 93].

4.4. Immunobiology of MSCs

4.4.1. MSCs mediated immunomodulation

Among the MSCs characteristics presented recently, their therapeutic ability to modulate immune inflammatory reactions by various means has been noticeably highlighted [94]. This

communication between active MSCs and different immunological aspects in the human body presents a significant role played by them for restoring damaged tissues, as well as protecting them during inflammatory conditions [95].

Tissue injuries endorse the stimulation of inflammatory cells, as $CD4^+$ T cells and $CD8^+$ T cells, the macrophages and neutrophils, promoting the release of specific factors, as IL-1β and TNF-α [94]. These inflammatory alterations lead to a differentiation and organization of MSCs to repair the damaged tissue. In an inflammatory environment produced mediators as IL-1, TNF-α and IFN-γ, besides the tissue hypoxia trigger MSCs to release growth factors like epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEG), playing an important role in regeneration and repair of damaged tissues [96–98]. In some studies, even myocardial infarction was reported to be recovered by MSCs related factors [99]. Furthermore, MSCs can release a number of other molecules as stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), and angiopoietin-1 (Ang-1), promoting the repair mechanism intrinsically [96, 100, 101].

Supplementary to the tissue repairing ability, immunomodulatory effects of the MSCs were further demonstrated [94]. Recently, the immunosuppressive capacity of MSCs has been reported in combination with an environment containing IFN-γ and inflammatory cytokines as TNF-α, IL-1α or IL-1β [94]. In such inflammatory higher expressions of adhesion molecules and chemokines are promoted, bringing the immune cells closer to the MSCs and enhancing their effectiveness of immunosuppression [102, 103]. Nevertheless, in other reports, MSCs showed the ability to raise the immune reactions and support the pro-inflammatory milieu [104]. This designates the immunomodulatory flexibility of MSCs, depending on many factors, as the source of the MSCs, or the level of inflammation surrounding them [1, 2].

4.4.2. MSCs and expression of TLRs

TLRs are considered one of the most significant factors directing the immunomodulatory role of MCSs into pro- or anti-inflammatory reactions. Observing these results, the profiles of TLRs expression and their effects on immunomodulation have become a central field of scientific investigations to understand possible interplay of TLR ligands with MSCs in inflammatory and non-inflammatory sites. Multiple studies have been implemented on TLR expression profiles in human MSCs. The reported outcomes displayed different expressions of TLRs depending on the tissue origin of these cells. Although bone marrow-derived MSCs displayed an expression of TLRs 1, 2, 3, 4, 5, 6, 8, 9 and 10 [10, 105, 106], MSCs isolated from the umbilical cord blood and Wharton jelly presented the same outcomes with an exclusion of TLR8, TLR10 [107, 108] and TLR4 [10, 109]. Studies on oral tissue related MSCs, showed an expression of all TLRs except TLR7 in periodontal ligament MSCs [106], in addition to TLRs 2, 3 and 4 in MSCs derived from dental follicle [110, 111] and dental pulp [110, 112]. On the other hand, MSCs isolated from the free gingiva showed an expression of all TLRs 1–10 [2]. Moreover, the evidence has revealed the potential modulation of this pattern of expression by microenvironmental factors surrounding the MSCs. Inflammatory conditions have been suggested to upregulate the expression of TLR2 [105, 113], TLR4 [105, 114] and TLR7 [113]. On the other

hand, TLR6 was downregulated under the same conditions [2, 105] . Likewise, in human bone marrow MSCs (BM-MSCs) viral infections [115, 116] and hypoxia [115] encouraged an augmented expression of TLRs 1, 2 and 3 and TLRs 1, 2, 5, 9 and 10 respectively.

In many circumstances the results of TLRs' activation on MSCs and the delivered immune response appears to be linked to the origin of cells, as well as the type of TLR triggered. Recent studies have shown no significant alteration by TLR activation on human adipose tissue MSCs (AD-MSCs) [117], BM-MSCs [10], UCB-MSCs [107], as well as Wharton jelly MSCs' [10] immunosuppressive effect. However, other scientific results confirmed the BM-MSCs mediated immunosuppression by TLR ligands explained by different mechanisms. Regarding TLR3 and TLR4, some groups detected the increased immunosuppressive effect after TLR activation without association with IDO activity or PGE2 levels [9]. Others presented different results showing the indirect induction of IDO1 production leading to a similar effect by TLRs on BM-MSCs [118]. In another study TLR3 and TLR4 ligands were reported to have reducing effects on human BM-MSCs facilitated suppression of T-cell proliferation [6], while other examinations reported the opposite result by stimulated TLR3 and TLR4 in the same type of MSCs [118]. Furthermore, TLR3 activation enhanced the suppressive role of DF-MSCs and DP-MSCs and G-MSCs to the local immune response, while activated TLR4 promoted the immunosuppression in DF-MSCs and decreased it in DP-MSCs and G-MSCs [1, 2, 110].

Furthermore, TLRs of MSCs have presented the aptitude to elicit the production of pro- and anti-inflammatory cytokines modulating the immune response [119]. The kinetics of TLR stimulation, besides the concentration and timing of the active ligand, have been reported as the main factors controlling this cytokine and mediator release [119]. This function also appears to be contingent on the TLR type and the MSC niche. TLR4 activation endorsed the production of pro-inflammatory mediators as IL-6 or IL-8. TLR3 activation on the contrary enhanced anti-inflammatory responses by triggering molecules as IL-4, IDO, or PGE2. These cytokines act in concert together, directing the immune reaction against the invading microorganisms. While pro-inflammatory immune modulating responses increase the production and stimulation of immune cells and cytokines, this mechanism is counter-regulated at the same time by the anti-inflammatory mediators on cellular and humoral levels [120]. Correspondingly, a pro- and anti- inflammatory influence was reported in relation to MSC TLR3 and TLR4 activation on the level of lymphocyte proliferation [119]. MSC induced secretion of mediators by TLR activation has also shown a modulating effect on neutrophils as another mechanism of their immune regulating function. TLRs of BM-MSCs delayed neutrophil apoptosis by triggering the production of cytokines as IL-6 and IFN-γ. This outcome was reported to be similar in MSCs originating from adipose tissue, thymus and spleen [121].

Studies also presented the possible effect of active TLRs on the differentiation potential of MSCs. Adipogenic differentiation presented no changes following UCB-MSCs and AD-MSCs'TLR3 and TLR4 activation [108, 117, 122]. Otherwise, osteogenic differentiation potential of BM-MSCs, AD-MSCs and UCB-MSCs was strengthened after activation of TLRs 2, 3 and 4 [108, 117, 122] and repressed with TLR9 ligands [6, 117, 123]. Chondrogenic differentiation displayed only an improvement with TLR2 stimulation [108], while TLRs 3, 4 and 7 activation had no obvious effect [6].

MSC proliferation rate and migration was reported to be influenced by TLRs, as inhibition of proliferation was detected with TLR9 activation [122]. Studies implemented on MSC migration to injury sites after TLR activation displayed no amplification of the MSCs' movement [124, 125], except for TLR3 activated human BM-MSCs [126]

Regarding the TLR triggering in MSCs and its potential therapeutic benefits *in vivo* diverse results have been issued so far. Many studies described therapeutic benefits of LPS triggered MSCs for the treatment of induced lung injuries in animal models [127–129]. Other surveys about MSCs engraftment for cardiac protection and its inflection by TLRs exhibited varying results. Positive effects of TLR4 triggered MSCs in the treatment of acute myocardial infarctions were reported in rats [130]. A contrasting outcome was shown by a different study [131]. This concludes that different modulations promoted by TLR stimulation on MSCs originating from various niches of the body need further investigations to explain the prominence of these factors and their possible administrations in MSC related therapies.

4.4.3. MSC immunomodulation through TLR activation

One of the special abilities of MSCs is presented in sensing the microenvironment surrounding the cells and accordingly adjusting the biologic functions of various immune cells and responses [132]. Therefore, MSCs can display immune interactions performing their immunomodulating effects. Triggering of BM-MSC TLRs initiate pathways of downstream signaling particularly for TLR3. Accordingly, this activation promotes the production of cytokines mainly active in cell migration mechanisms [126]. Indeed, migration of MSCs was endorsed by exposure with TLR3 ligand as a primary mediator of MSC stress migration responses compared to TLR2 and TLR9. TLR3 (Poly I:C) and TLR4 (LPS) activation have consequently transformed BM-MSCs into special chemotactic cells proficient of improving the inflammatory immune cell recruitment by promoting the production of IL-6, IL-1β, IL-8, IP10, monocyte chemotactic protein (MCP)-1 and CCL5 (RANTES) by NF-κβ signaling activation [113]. Analogous outcomes have been attained in AD-MSC, as TLR ligands for TLR2 and for TLR4 promoted mRNA synthesis of MCP-1 and -2, IL-1β, granulocyte chemotactic protein-2 (GCP-2) and macrophage inflammatory protein-3α (MIP-3α) [115]. Human turbinated MSC (hTMSC) were reported expressing high percentages of TLR3 and TLR4. Nevertheless, hTMSCs were only responsive to TLR4 as displayed by the significant changes in their cytokine profiles [133]. Macrophage-activating ligand-2 (MALP-2), an agonist of TLR6, as well as its heterodimer partner TLR2, initiated the activation of NF-κβ pathway leading AMC to obtain a pro-inflammatory profile by highly secreting cytokines as IL-4, IL-8 and IL-6 [134].

Dissimilar to TLR3, ligation of TLR4 significantly encouraged expression of cytokines as IL-6, IL-12, IL-8, RANTES (CCL5), IP-10 (CXCL10), TNF-α and GM-CSF. Furthermore, it was reported that TLR3 activation by Poly(I:C) a Janus kinase (JAK) 2/signal transducer and activator of transcription (STAT) 1 pathway is triggered with an increased simultaneous expression of suppressor of cytokine signaling (SOCS) proteins [135]. These outcomes further showed that SOCS1 and SOCS3 can perform a distinct function in modulating TLR3, JAK/STAT, and CXCR4/CXCR7 signaling pathways in BM-MSCs. These results propose that as negative regulation mediators, SOCS proteins can influence the way MSCs react to signals *in vivo*, thus manipulating TLR signaling pathways to elevate the distribution of infused MSCs at injury sites [135].

Immune cell binding and migration to MSCs surrounding milieus has been presented to be a main stage for inaugurating immunomodulation [136]. Under TLR3 activation, tonsillar mesenchymal (T-MSCs) obtain a chemoattractant character permitting the migration of immune cells into the environment surrounding the MSCs. This is achieved by an augmented secretion of CXCL5, CXCL1, CXCL6, CXCL10 and CXCL8 active chemokines [137]. Regarding the leukocyte binding ability of MSCs after TLR activation, TLR3 triggering of BM-MSCs elevated the leukocyte number binding to MSCs, through hyaluronic acid structures while TLR4 activation raised VCAM-1 and ICAM-1 promoted binding of leukocytes to MSCs [138].

B cell activating factor (BAFF), known for its prominent stimulating action on B cells was also investigated in human BM-MSCs and displayed a higher expression after TLR4 activation by LPS, while other TLR agonists had no significant outcome. This proposed that TLR4 in human MSCs could play an important role in the regulation of B lymphocyte-associated immune responses [139].

Once the MSCs are in the area of injury or inflammation, surrounded by immune cells and different regulatory mechanisms, multiple factors can play a role in the process of immunomodulation. To date, results of TLR activation and immune modulatory responses by MSCs are discrepantly reported in different studies.

The secretion and differential expression of immune regulatory mediators was described to be controlled mainly by two elements; specifically the tissue origin of the MSCs and the TLR triggered [140]. TLR stimulation in MSCs has been presented to start the intracellular pathways of MAPK, AKT and NF-κβ [6, 118, 126] and to influence other biologic functions of MSCs promoting the secretion of pro-inflammatory, or/and anti-inflammatory mediators [9, 141, 142]. In one investigation, a new pattern for MSC immunomodulation was explained, as MSCs could be polarized by different TLR agonists into pro-or anti-inflammatory phenotypes. TLR4-activated BM-MSCs (MSC1 phenotype), mostly produced pro-inflammatory mediators and were able to trigger T-lymphocyte stimulation, whereas TLR3-activated BM-MSCs (MSC2 phenotype), mainly expressed immunosuppressive factors as IDO (indoleamine-2,3-dioxygenase) and (prostaglandin E2) leading to T-cell inhibition [119]. In another study on G-MSCs outcomes were in accordance to the same paradigm, as a distinct pro-inflammatory phenotype of G-MSCs (G-MSC1) was triggered by all TLR agonists except TLR3, which promoted the immunosuppressive phenotype of G-MSCs (G-MSC2) [1]. This was also in confirmed by different studies, presenting an immunosuppressive character of MSCs created by TLR3 triggering in MSCs originating from human umbilical cord [143, 144], human bone marrow [113, 118], human dental pulp and dental follicle [145], as TLR3 agonist Poly (I:C) significantly raised the expression of anti-inflammatory cytokine IDO in these investigations [1].

While different investigations described no significant outcome of TLR triggering on BM-MSC, AD-MSC and T-MSC-mediated immunosuppressive responses [117, 133], other studies reported decreased responses. TLR3 and TLR4 activated MSCs originating from human nasal mucosa (nmMSCs) preserved their capability of leukocyte suppression, partially mediated by prostaglandin secretion. Nevertheless, another study described an impairment of leukocyte suppression after TLR3 and TLR4 activation in BM-MSCs [6]. These mechanisms were associated mainly with jagged-1 down-regulation initiated by TLR3 or TLR4 activation.

Contrasting to these outcomes, TLR3 and TLR4 triggering promoted the immunosuppressive ability of BM-MSCs presented by the increase of regulatory molecules of kynurenines by the enzyme IDO1 [118]. In a comparative investigation, activation of TLR3 by Poly(I:C) and TLR4 by LPS differentially influenced the suppressive ability of BM-MSCs, as well as WJ- and AD-MSCs [10]. While BM-MSC displayed decreased inhibition of lymphocyte activation, the immunosuppressive function of WJ- and AD-MSC was scarcely changed.

Furthermore, alterations in the amounts of HGF and PGE2 secreted after TLR triggering in MSCs have been also postulated to emphasize these immunomodulatory changes. One of the studies reported that, TLR treated CB-MSCs significantly increased their abilities of immunosuppression only after TLR3 triggering by Poly(I:C). This was explained by an increased expression of cyclooxygenase-2 (COX-2) [143]. Later outcomes showed that miR-143 regulates the influence of Poly(I:C) on the immunosuppressive function of MSCs by targeting COX-2 gene.

Investigations have previously underlined that MSC-facilitated T-cell suppression arises through the discharge of galectins. After TLR2 triggering, galectin-3, a main modulator of T-cell biology, was elevated at both protein and mRNA levels in BM-MSCs, but showed no change in immunomodulation [146]. Moreover, galectin-9 expression was differentially induced by TLR activated BM-MSCs [147]. While TLR2, TLR3 and TLR4 triggering promoted the expression of galectin-9, activated TLR5 and TLR7/8 did not present significant changes on galectin-9 expression. Consequently, in the occurrence of particular infectious incitements through TLR activation, BM-MSCs can preserve or enhance their immunosuppressive ability by increased galectin-9 expression.

Another immunomodulatory effect of MSCs can be directed toward the cellular component of the innate immune response. TLR3- and TLR4-activated MSCs were presented to differently prolong the function and survival rate of neutrophils (PMN) [121]. TLR3 triggered BM-MSCs had higher anti-apoptotic effects on PMN than TLR4 activated ones. Both TLR ligands could in addition augment the respiratory burst ability and CD11b expression by PMN. These biological functions exerted on PMN by TLR3 triggered BM-MSCs were mediated by the action of secreted mediators as IL-6, IFN-β, and GM-CSF, while TLR4-triggered BM-MSCs depended on GM-CSF in their PMN regulating mechanism. In addition, MSCs and NK cells were reported to interact in complex mechanisms with bidirectional regulation. This was described as TLR3 and TLR4-activated MSC enhanced their suppressive functions against NK cell proliferation and cytotoxicity, which may provide a potential stroma-targeted therapy of tumors [148].

5. Conclusion

MSCs are exceptional applicants for use in cellular treatments which can possibly transform the current field of immunotherapy. Although MSCs display pronounced potentials in the therapy of many immune conditions, the wide inconsistency in the quality of cells isolated from various donors and tissue sources, varying protocols, fluctuating measures and changing patterns of transfusion may decrease their therapeutic advantage. Therefore, a watchful assessments of suitable cell sources and tissues, more consistent scientific results, as well as better understanding of immunomodulation mechanisms of MSCs are required. Factors as, standardized cell culture protocols for cell expansion, differentiation and cryopreservation

need to be applied to allow better controlled therapeutic results. Another factor as comprehending the influence of TLR triggering on the immunobiology of MSCs plays a major role to allow correct and efficient therapeutic application of the cells. Despite the great amount of information obtained about that subject, there are many conflicts of the outcomes among the investigations. These may be related to the variety of experimental situations used to investigate the influence of TLR triggering on MSCs. Especially, the effect of specific culture conditions or the MSC source, as well as the TLR triggered seem to be the most influential factors among the studies. Therefore, this topic has to be studied in a more critical manner in standardized and well-designed investigations. Stimulation of MSCs before or within the potential treatment procedures with distinct TLR ligands may assist as an effective step controlling the biological function of the MSCs as needed in different therapeutic stages of the disease.

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Chapter 7

Immunotherapeutic Approaches of Rheumatoid Arthritis and the Implication on Novel Interventions for Refractoriness

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Additional information is available at the end of the chapter

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Abstract

Rheumatoid arthritis is an autoimmune disorder involving the chronic inflammation of affected joints which lead to the distortion and eventually destruction of the articular tissues. Clinically, many therapeutic methods are being used for RA treatment. Non-steroidal anti-inflammatory drugs (NSAIDs), steroid, and disease-modifying anti-rheumatic drugs (DMARDs) are the three main categories of intervention approaches. Among which DMARDs, targeting mainly the release of pro-inflammatory cytokines, demonstrated high efficacy because of its direct drug action that alter the underlying disease mechanisms rather than simply to mediate symptoms relieve. However, the use of DMARDs also accompanying some unwanted adverse side effects, in particular, the development of refractoriness, which hampers the successful rate of treatment. In this chapter, the conventional RA drugs will be reviewed, focusing on the currently used and latest development of DMARDs. Novel methods that could improve RA pathogenesis will also be introduced. Because of the critical role of refractory RA, the progress of the disease to develop resistance to standard drug treatment will also be described. Finally, innovative RA therapeutic methods inspired by researches concerning the pathogenesis and contemporary treatments of RA will be discussed.

Keywords: rheumatoid arthritis, DMARDs, refractory, immunotherapy, immunosuppressive

1. Introduction

Around 0.5–1% of the world population is challenged by rheumatoid arthritis (RA) with patients afflicted by progressive articular destruction accounting for the commonest chronic systemic autoimmune disorder [1, 2]. RA is demonstrating prevalence in developed countries or urban areas [3] and is around three times more commonly found in female [4]. Apart from persistent synovitis, structural bone damages, and the eventual deformity of affected joints, 40% of the RA patients are also accompanied with extra-articular manifestations in multiple organs including kidney (glomerulonephritis), heart (atherosclerosis), and skin (small vessel vasculitis) [5, 6] critically compromising the quality of life [7]. Owing to the comparatively early disease onset (at the age between 30 and 50) [8], RA also implied increased individual and socioeconomic impact as a result of reduced work capacity and early unemployment [9–11].

Thus far, the etiology of RA is unclear and curing strategy is lacked. Environmental and genetic factors are considered as the main causes of increased risk of RA. Smoking is the strongest environmental stimuli that trigger the onset of RA. Both cohort and case-control studies demonstrated that the number and duration of cigarette consumption is positively correlated to RA risk in a dose dependent and irreversible manner [12, 13]. By contrast, the use of alcohol significantly reduced RA susceptibility in habitual drinkers when compared with non-drinkers or individuals consuming low level of alcohol [14, 15]. In the case of smoking-induced RA, the involvement of polymorphisms in genes mechanistically regulating the immunity, such as the human leukocyte antigen-DRB1 (*HLA-DRB-1*)-encoded type II major histocompatibility complex (MHC), is critical to more than half of such condition [7]. Of note, some of the disease-associated polymorphic variants of HLA-DRB-1 are specific to severe disease phenotypes, for example, more aggressive erosive disease and higher mortality [16]. Genetic involvement in RA is also suggested by the existence of shared epitope of RA-associated circulatory autoantibodies such as the IgG rheumatoid factor (RF) and antibodies recognizing the citrullinated peptide [7]. Although not all RA patients are positive for IgG- and citrullinated peptide-recognizing autoantibodies, the heritability rate of RA is approximately 40–65% for seropositive rheumatoid arthritis, and 20% for seronegative disease [17, 18].

The pathogenesis of RA is as complicated as the causation of the disorder, conventional therapeutic methods is not confined to the application of single pharmaceutical intervention. Instead, personalized treatment algorithms with the combinational use of different RA medications are required, which is highly dependent on individual patient and the stage of disease progression. Generally the drug used for RA can be classified into three categories: (1) non-steroidal anti-inflammatory drugs (NSAIDs); (2) glucocorticoid (steroid); (3) non-biological (synthetic origin)/biological disease-modifying anti-rheumatic drugs (DMARDs). The therapeutic effects of NASIDs like aspirin and Coxibs targeting the cyclooxygenase pathways, together with glucocorticoid acting via the cortisol receptor, are rapid which effectively alleviate the analgesic, pyretic, and inflammatory symptoms associated with RA [19, 20]. However, the effects of NASIDs are limited to symptoms relief and demonstrated no significant delaying effect on disease progression [21]. Glucocorticoids can slow down the progress of bone erosion resulted from cytokines-induced imbalance of local bone turnover under long-term treatment

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with low dosage [22]. Unfortunately, the extensive side effects affecting the different organs outweighed such beneficial immunosuppressive property. As such, therapeutic compounds, for example the treat-to-target DMARDs, which control the inflammatory and destructive processes of RA is inevitable to the maintenance of persistent remission. Since, DMARDs is slow-acting drug which take 1 month to a year to be effective [23], NSAIDs/glucocorticoid are usually applied together with DMARDs to serve initially as moderator of pain and stiffness before the drug action of DMARDs commence.

Many DMARDs have previously been reported targeting various cellular signaling molecules or receptors related to the immunoregulatory machinery of RA. In this chapter, the currently employed, as well as newly exploited DMARDs will be described. The latest therapeutic strategies that can potentially be applied to RA intervention will also be updated. Eventually, insight provided by such methods that could innovate novel RA therapeutic development will be discussed.

2. Innate and adaptive immunity in the pathogenesis of RA

Inflammation and swelling of the synovial membrane, or synovitis, are the pathological features of RA. Both the innate and adaptive immunity are participating the disease pathogenesis through the orchestration of cellular communication between the two systems. The pathogenesis of RA is heterogeneous (**Figure 1**) and the exact triggering factors of the inflammatory

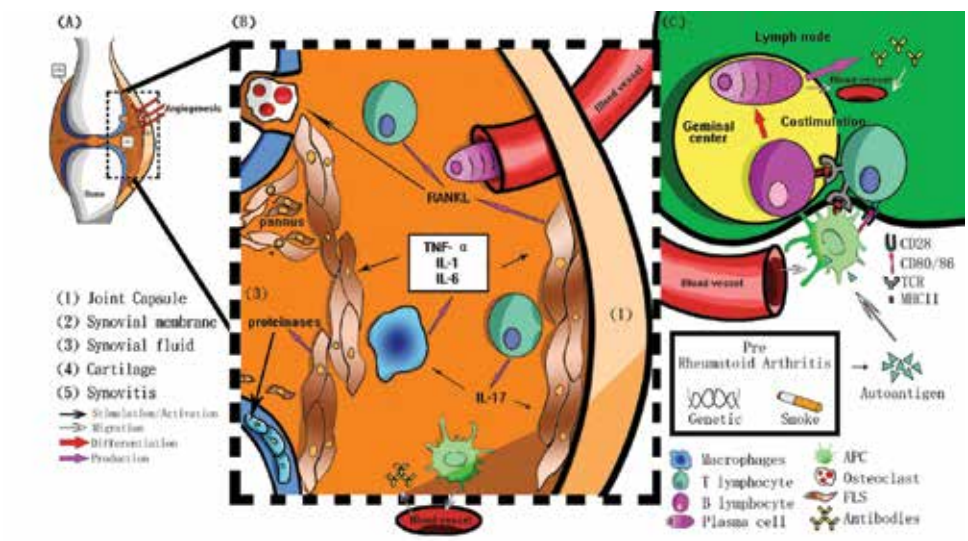

Figure 1. Cellular and molecular pathogenesis of RA: (A) Gross anatomical structure of the inflamed joint of RA. (B) The inflammatory synovium with cytokine signaling and cellular interactions underlying the RA pathogenesis indicated. (C) The involvement of lymph node in orchestrating the activation of immunocellular components involved in RA progression. TCR: T cell receptor; APC: Antigen presenting cells; FLS: Fibroblast-like synoviocyte; MHCII: Major hoistocompatibility complex class II.

response at the diseased joint are still unclear. In the inflammatory milieus, the synovial macrophages (SM) positioned at the cartilage–pannus junction is activated to secret the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1, and 6 (IL-1/IL-6). In fact, findings suggested that the SM are the key regulator of RA inflammation [24]. These cytokines then activate another important residential cell type, the fibroblast-like synoviocytes (FLS), in the synovium leading to hyperproliferation and the consequential formation of an abnormal layer of fibrovascular tissue called pannus. The pannus expresses the cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) which together with the macrophage-released cytokines, stimulate the differentiation of osteoclast [25] to resorb the calcified bone matrix (i.e. bone erosion) via the secretion of acid and hydrolytic enzymes such as cathepsin K and matrix metalloproteinase-13 (MMP-13). In addition, the activation of FLS in pannus generates proteinases that are correlated to the destruction of cartilage [1, 26]. The activated FLS also migrate to other unaffected joints and induce inflammatory responses and bone and cartilage destruction [27].

Infiltrated antigen-driven $CD4^+$ lymphocytes and plasma cells (c.a. 50% and 5% of lymphocytes found in synovium, respectively) are also found in the inflamed joints. The RA joints are highly vascularized due to the inflammation-induced angiogenesis, and that the newly generated blood vessels facilitate the infiltration of the lymphocytes from the periphery. In the synovium, $CD4^+$ lymphocytes secreted a repertoire of cytokines, including IL-17, which further stimulate the expression of inflammatory mediators from SM and FLS supporting the persistent inflammatory environment of the affected joints. The plasma cells also involve in such process by the secretion of different cytokines. One of the activation routes of the infiltrated $CD4^+$ lymphocytes and plasma cells is via the cell-cell interaction with the antigen presenting cells (APC), for example dendritic cells (DC). Environmental factors, like smoking and genetic abnormalities and chronic inflamed RA tissues can induce the modification, including citrullination, of autoantigens, such as citrullinated self-proteins of vimentin, alpha-enolase, fibronectin, and type II collagen [28], which activated the APC to generate the surface MHC-peptide complex. The autoantigen-activated APC migrate to the secondary lymphoid organs (SLO) and stimulate the maturation of $CD4^+$ T lymphocytes via the MHC-peptide complex and co-stimulatory molecules, such as CD80/CD86, on the cellular surface [29]. B lymphocytes in the germinal center are then co-stimulated by the mature $CD4^+$ T lymphocytes to become plasma cells which produce the destructive autoantibodies, such as RF and anti-citrullinated protein antibodies (ACPAs) [30, 31]. These $CD4^+$ T lymphocytes, plasma cells, and autoantibodies eventually return to the RA joints mediating the destructive process of bone and cartilage and underpinning the chronic inflammatory response.

3. Conventional and current development of DMARDs for RA treatment

The intricate interaction between the immunocellular components during RA progression suggested that the effective regulation targeting the signaling mediators of such cellular communication or the physiology of the involved immune cells appeared as the key to successful RA therapy. Several FDA-approved DMARDs, for example Methotrexate, TNF antagonists,

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Rituximab, and Tocilizumab, which modify the biologic responses are extensively used as immunotherapy for preventing immune attacks associated with RA in clinical settings.

3.1. Methotrexate (MTX)

MTX is among the quickest-acting DMARDs and represent the mainstay of DMARDs therapy. As a non-biologic DMARDs, MTX is produced through chemical synthesis which is structurally analogous to folate inhibitory targeting the dihydrofolate reductase (DHFR) [32] which is critical to the *de novo* synthesis of purine and pyrimidine, therefore, down-regulate cellular proliferation and induce apoptosis. MTX-induced inhibition of DHFR also leads to the generation of resultant polyamines which is accumulated and converted into toxic ammonia and hydrogen peroxide by synovial mononuclear cells in RA patients functionally suppressing the stimulated T lymphocytes [33]. As expected, MTX suppress the proliferation and inhibit the turnover of inflammatory cells during RA treatment [34]. However, the mechanism of action of MTX is complex which is not merely mediated through limiting the growth and survival of the immunocellular components. MTX also target and inhibit 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase leading eventually to the increased intra and extra-cellular levels of adenosine [35, 36], which contributed significant to adenosine-induced immunosuppression [37] as demonstrated by the inhibition of phagocytosis, secretion of TNF, IFN, IL2, IL12, expression of HLA expression, etc. [38–43]. Findings also evident the indirect anti-inflammatory and inhibited neutrophil chemotaxis effects of MTX signaling via the cyclooxygenases-2 (COX-2) and lipoxygenase pathways [44–46]. In the synovial tissue, MTX also indirectly inhibit the synthesis of metalloproteinase (MMP) and activate tissue inhibitor of MMP (TIMP) via cytokines (IL-1) modulation [47].

3.2. Tocilizumab (TCZ)

The monoclonal antibody TCZ, together with TNF and CD20 antagonists [48] which will be discussed later, represented the subcategory of biologic DMARDs, is produced through genetic engineering as contrast to the chemically synthesized non-biologic counterpart. The biologic DMARDs exhibit their therapeutic effect in a treat-to-target manner by modulating the various molecular pathways signaling the dysregulated immunity. As the first of its kind, TCZ specifically target the IL-6 receptor (IL-6R) by functioned as an antagonist [49] and is usually used when conventional treatment (e.g. MTX) [50, 51] and other biologic DMARDs (e.g. TNF antagonists) become irresponsive or tolerated [52]. However, TCZ also works with high efficacy when applied as monotherapy in early disease [53]. In the course of RA pathogenesis, serum and synovial tissue levels of IL-6 is elevated and is positively correlated to RA disease severity and radiological joint damage [54–57]. Mechanistically, IL-6 coupled with IL-6R triggering the Janus kinase (JAK) pathways [58] and activates the downstream effector functions of the different RA-associated immune cells. For example, IL-6 stimulates the plasmablasts to produce autoantibodies [59] and significantly regulates the differentiation process of T lymphocytes by enhancing T helper 17 (Th17)/regulatory T lymphocytes (Treg) balance skewed toward the Th17 profile [60]. In addition, IL-6 are involve in the proliferation and

production of MMP of FLS [56, 61] and differentiation of osteoclast [62], which lead to cartilage damages and bone resorption. TCZ can downregulate these RA-associated cellular responses by interacting with the IL-6R. New findings suggested that the therapeutic effects of TCZ can be mediated by lowering the serum level of IL-6, however, the underpinning mechanisms are yet to be defined [63].

3.3. TNF antagonists

Infliximab, etanercept, and adalimumab are the currently approved TNF antagonists being used clinically in RA treatment [23]. These type of DMARDs are sharing similar efficacy as MTX exhibiting rapid drug response [64, 65]. When compared with MTX, the mechanism of action of these inhibitors are more specific which target to the tumor necrosis factor receptor type 1 and 2 (TNFR1/TNFR2) signaling pathways mediated by the cytokine tumor necrosis factor-alpha (TNF-α) [64, 65]. Cells with monocytic origin are the main cellular components producing TNF-α [66], however, other immune cell types , for example lymphoid and mast cells, has also been reported to participate the secretory process [23]. During the course of RA pathogenesis, TNF-α serves as a strong chemoattractant for the tethering of neutrophils to the endothelial cells [67]. Also, TNF-α is the central regulator of the immunity which autocrinally and paracrinally repress the expression of other cytokines, such as IL-1, IL-6, IL-8, and granulocyte colony-stimulating factor (G-CSF) [68]. Therefore, the blockage of TNF signaling with the use of TNF antagonists can molecularly suppress the pro-inflammatory progression of RA in a systemic sense. The three TNF antagonists are recombinant monoclonal antibodies that function by binding either to TNF-α (infliximab) or the corresponding receptor (etanercept and adalimumab). Clinically, the use of TNF inhibitors in conjugation with MTX at the initial stage of RA is associated with rapid disease remission [69].

3.4. Rituximab (RTX)

Similar to TCZ and TNF antagonists, RTX is a chimeric monoclonal antibody recognizing the antigen CD20 which is a transmembrane receptor expressed on the surface B lymphocytes during differentiation from the pre-B cell to memory B cell stages [70, 71]. As previously mentioned, B lymphocytes are contributing to the development of RA via the production of autoantibodies. The B lymphocytes also produce a spectrum of pro-inflammatory cytokines, including IL-6, IL-16, TNF-α, and lymphotoxin-β (LT-beta) [72, 73], which are pivotal to the perpetuation of inflammatory environment aggravating joint damage. Therefore, the use of RTX to deplete $CD20^+$ B lymphocytes suggested an effective therapeutic strategy for RA. The antibody binds to the cell surface protein CD20 and induces cell death toward the targeted B lymphocytes via three potential pathways: (1) Antibody dependent cell-mediated cytotoxicity (ADCC) which stimulates phagocytosis and cell lysis of $CD20^+$ lymphocytes by the binding of immunocellular components, such as macrophages, monocytes, and natural killer cells, via the Fcγ region of RTX [74, 75]; (2) Complement dependent cytotoxicity (CDC) by inducing membrane attack toward $CD20^+$ B lymphocytes resulted in cell lysis via the formation of rituximab-complement (C1q) complexes [76]; (3) Direct promotion of $CD20^+$ B cell apoptosis [77]. It is worth noting that, CD20 neither present on stem cells nor the antibody-secreting plasma cells which enhance the safety of the practical application of RTX. Although the monoclonal antibody

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is approved for use in combination with MTX, current study reported that RTX is efficient and safe for managing RA patients without the presence of MTX [78].

3.5. The development of novel DMARDs

The mentioned FDA-approved DMARDs (both biologic and non-biologic) are demonstrating profound effects in RA intervention. Owing to the chronic nature of RA, however, patients are subjected to prolonged exposure of DMARDs resulted in the development of side effects and drug resistance. For the use of MTX, hepatic fibrosis or cirrhosis is one of the most severe side effects affecting the patients [33]. Skin and soft tissue infections and abnormal liver function are observed in patients treated with TCZ [53]. TNF antagonists have been reported to associate with adverse responses such as serious bacterial and opportunistic infections, and lymphoma [79–81]. RTX treatment is also plagued by severe skin reaction and infection [23]. Accordingly, researchers have been making efforts to improve the toxicity of classical DMARDs and to exploit of newer anti-rheumatic agents.

In 2007, a polypeptide-based novel TNF antagonist with amino acid sequence isolated from part of the pre-ligand assembly domain (PLAD) has been patented [82]. In the extra-cellular region of TNFR, PLAD is located at the position NH_2-terminal to the ligand binding domain essential for inducing receptors trimerization. The binding of the synthetic polypeptide to PLAD elicit inhibition to the formation of functional receptors complex and repress the down-stream signaling thereof. Such therapeutic peptide inhibitor is recognized by the immune system as "totally-self" and is highly specific instead of blocking immunocomponents in a global sense, therefore, avoiding undesirable immune reactions minimizing the appearance of potent adverse side effects [83]. A patent application of another 18-residue peptide targeted the secretory phospholipase A2 (sPLA2) has also been filed [84]. As a pro-inflammatory mediator, sPLA2 is found to be related to the onset and severity of RA in animal [85] and patients [86], respectively. The neovascularization process which is critical to the RA-associated hyperplasia of synovial tissue has been suggested as therapeutic target for the development of novel DMARDs as well. The cytokines that are significantly responsible for new blood vessel out-growth is vascular endothelial growth factor (VEGF) which also participates to vascular leakage [87]. VEGF inhibitory compounds, such as derivatives of quinazoline [88, 89], have been synthesized and can potentially be used for RA treatment. There are still many others new DMARDs, for example, the colony-stimulating factor (CSF) inhibitors, which target the key regulator of neutrophil production [90, 91]. All these compounds are potentially new therapeutic strategy with high efficacy and improved adverse effects. Investigation of the combinational use of these compounds with the classical DMARDs will also encourage the discovery of formulation against refractory RA for patients who respond poorly to conventional interventions.

4. Next-generation immunosuppressive strategies

In general, the described DMARDs directly inhibit the pro-inflammatory signaling molecules and their receptors. The ligand-receptor interaction is circumvented by the use of monoclonal

antibodies, synthetic peptides, or natural compounds. These therapeutic strategies tune down the cytokines machinery of the targeted cells or manipulate the cells that produce the cytokines lead eventually to RA remission. Theoretically, any immunosuppressive methods, not limited to interfering the ligand-receptor interaction process, can result in therapeutic effects toward RA and innovate the development of RA treatment.

4.1. Sphingosine-1-phosphate receptors (S1PRs) agonist

Antagonizing the recruitment of immunocellular components to reach the site of inflammation provided a clue to RA treatment. Chemokine receptors such as CCR5 and CXCR3 are playing significant roles in RA pathogenesis via the regulation of monocyte and T lymphocyte chemotaxis [92, 93]. The use of inhibitors against these receptors has shown therapeutic efficacy toward RA treatments [94, 95]. Instead of blockage of immune cells from entering the inflammatory site, to enhance the homing of these cells could be another approach for controlling the inflammatory status of RA joints. Fingolimod (FTY720), an analog of sphingosine-1-phosphate (S1P) extracted from a vegetative wasp composed of the fruiting bodies of *Isaria sinclairii* and its parasitic host larva [96], is able to deplete lymphocytes from the circulatory and lymphatic systems [97, 98]. The mechanism of action involves the formation of phosphorylated FTY720 (FTY720-P) by sphingosine kinase 2 which agonistically stimulates the sphingosine-1-phosphate receptors (S1PRs). Located on the surface of lymphocytic cells, S1PR is internalized upon interaction with FTY720-P inhibiting lymphocytes egress from the secondary lymphoid tissues and thymus (lymphopenia). As such, the FTY720-treated lymphocytes are sequestrated in the lymph nodes, spleen, and thymus, which are not able to recirculate to peripheral inflammatory tissues. In fact, the migration of other immune cells are also regulated by the S1P-S1PRs axis, for example, dendritic cells (DCs) [99] and macrophages [100]. FTY720 is originally used for allotransportation to induce long-term graft acceptance [101] reflecting the pharmaceutical value of the immunosuppressive property of the compound. In fact, FTY720-induced lymphopenia has been proposed as a new therapeutic approach for RA as demonstrated in the adjuvant-induced arthritis (AIA) rat [102]. In a recent animal experiment using the collagen-induced arthritis (CIA) model, it was revealed that the migration profile of DCs is also modulated by FTY720 and is responsible for the beneficial effects of the treatment [103]. Most importantly, the normal function of the immune cells is preserved after FTY720 treatment [100, 104], suggesting the safety and practicality in the application of the compound.

4.2. Dendritic cell (DCs)-targeted therapeutics

As a key regulator of both the innate and adaptive immunity, DCs in the inflamed synovial tissues of RA plays a significant role in the pathomechanism. In RA patients, DCs are activated in response to pro-inflammatory cytokines stimulation, with up-regulated co-stimulatory molecule expression [105]. DCs also induce the differentiation of Th1 and Th17 cells via production of IL-12 and IL-23 [106]. Also, treatment with TNF-α inhibitors could reduce the number of activated DCs and inhibits its maturation, leading to improvement of the clinical symptoms of RA [107]. These observations support the strategy of targeting DCs for the treatment of RA. Accordingly, DCs with tolerogenic function has been proposed as therapeutic tool for RA

treatment, which specifically targets the pathogenic autoimmune response and simultaneously maintain the integrity of protective immunity [108]. In a randomized, unblinded, placebo-controlled, dose-escalation phase I study, tolerogenic DC therapy demonstrates promising results in RA patients without major adverse effects. However, administration of tolerogenic DC therapy should ideally be given to RA patient as early as possible, to avoid the establishment of autoimmunity desensitizing the RA treatment [109].

4.3. Manipulation of neuroimmune communication

The reciprocal effects of the nervous system on immunity have attracted high focus. The nervous system regulates inflammation via a variety of neurotransmitters, neuropeptides, and peripheral nerves. In general, activation of sympathetic nervous system may exhibit both pro-inflammatory and anti-inflammatory properties, whereas the para-sympathetic nervous system via the vagus nerve, exerts anti-inflammatory actions [110]. Subsequent research has identified the neuronal type α7-acetylcholine (Ach) receptor is necessary to regulate the anti-inflammatory effects mediated by the para-sympathetic nervous system [111]. Interestingly, the α7-ACh receptors are also widely expressed in immune cells and FLS [112, 113]. TNF-α expressed by the residential macrophages in spleen located in the red pulp and marginal zone can be repressed via the stimulation of vagus nerve mediated by nicotinic acetylcholine receptor subunit α7 [114]. Accordingly, administration of a specific α7-ACh receptor agonist showed effective inhibition of systemic inflammatory responses in CIA models [115, 116]. In animal models of neurological disorders, peripheral denervation suppressed joint inflammation in mice with AIA has also been demonstrated [117]. Recently a clinical trial consolidated that vagal nerve stimulation (VNS) could be therapeutically feasible in RA. VNS refers to the technique of manually or electrically stimulates the vagus nerve, which has been approved more than a decade ago by the FDA for the treatments of severe and recurrent unipolar and bipolar depression [118], as well as pharmaco-resistant epilepsy [119]. The potential use of VNS for insomnia, anxiety, etc., have also been reported [120]. Clinical relevance of VNS in RA was debuted in 2012 in which a volunteer patient with surgically implanted pacemaker-like nerve stimulator successfully halted the joints attack with remarkable symptoms recovery [121]. It was proved to be a result of VNS-stimulated inhibition of peripheral blood production of TNF, IL-1β, and IL-6 in a later clinical study [122]. Such achievement posited the alternative use of computerized device in RA treatment as compared to the traditional biological or chemical pharmaceutics

4.4. Cell-based therapy

The modulation of disease pathogenesis by the delivery of cellular materials to patients has been proposed as promising intervention method for many incurable conditions [123]. The application of mesenchymal stem cells (MSCs) is the prototype among the various types of cell-based therapy, which illustrated another possibility of managing RA without using the biological or chemical pharmaceutics. MSCs are capable of bypassing the sanction of immune system upon transplantation [124, 125] conferring the practicality *per se* to act as potential allograft by abolishing the concomitant requirement of immunosuppressant drugs. A recent

randomized, single-blind, placebo-controlled phase Ib/IIa clinical trial has been established to evaluate the safety and tolerability of intravenously administrating the Cx611, a preparation of allogeneic expanded adipose-derived MSCs (eASCs), in patients with refractory RA [126]. Results demonstrated that patients were having good response and generally well tolerated the infused Cx611 without evidence of dose-related toxicity at the dose range and time period studied. The precise mechanisms mediating the beneficial effects of Cx611 is not clear, it could be associated with the interruption of T lymphocytes and SLF pathophysiology including inhibited cellular proliferation and inflammatory cytokines production, enhanced generation of anti-inflammatory cytokines, and antigen-specific T lymphocytes [127–129]. The allogeneic nature of Cx611 in this study circumvents the limitation of using patient-specific clinical grade stem cell product with unstable availability during manufacture making itself an "off-the-shelf" therapeutic product [130]. Provided that, the isolation and expansion procedures of MSCs are comparatively easy, which further secured their supply during immediate need [123]. On top of that, the limited replicative lifespan of MSCs provided additional safety by avoiding the formation of unwanted malignancy [123].

5. From intervention to innovation: Implication of conventional and emerging RA therapies on the exploitation of novel immunosuppressive strategies against refractoriness

Conventional DMARDs, both the non-biologic and biologic types, are able to achieve clinical remission or reduce disease activity status for RA. Nevertheless, the unmet need in the treatment of RA remains high because of substantial number of RA patients do not response sufficiently to the currently available regimens. Therefore, remission of disease is not always achieved and refractory cases are very common [131, 132]. The causes of refractory RA are multifaceted varying depend on individual, patient-tailored management approach is presented to determine whether persistence of signs and symptoms is based on the inflammatory disease activity, and the role of comorbidities [133]. Owing to the complexity in etiology of refractory RA, it is difficult to summarize a single picture that can comprehensively depict the underpinning cellular responses and molecular pathways. Here in this section, some of the possible causative factors that may responsible for the development of resistance against standard RA treatment are to be listed. Ingenious intervention methods for refractory RA as inspired by the increasingly understanding of the disorders will also be discussed.

5.1. P-glycoprotein (P-gp)-mediated drug resistance

P-gp, also known as multidrug resistance protein 1 (MDR1) or CD243, is an important membrane pumps for the cellular removal of foreign substances. In patients with refractory RA and high disease activity, overexpression of P-gp on lymphocytes can cause resistance to anti-rheumatic drugs through efflux of intracellular drugs [134]. Recent studies further showed that high expression level of P-gp found in FLS of refractory RA patients is the potential mechanism for multidrug resistance in RA treatment [135]. Also, activated B lymphocytes with elevated P-gp expression seems to be associated with drug resistance, disease activity, and

destructive arthritis with extra-articular involvement in RA [136]. Overcoming drug resistance by using P-gp inhibitor could sensitize the response to DMARDs in patients with refractory RA further support the role of P-gp in the development of refractoriness [137].

5.2. Autoantibodies-mediated refractoriness

Although the direct association of autoantibodies and refractory RA is enigmatic, RA patients with more severe disease and a worse prognosis are seropositive for RF and ACPAs [138, 139]. Patients with unsatisfactory responsiveness toward conventional DMARs, in particular MTX, resumed clinical improvement after TNF antagonists treatment through the manipulation of autoantibodies level, which represent a promising therapeutic method for refractory RA to other treatment options [140–142]. In a one-year prospective study with the use of adalimumab (monoclonal anti-TNF-α antibody), MTX-resistant patients are clinically benefited from the treatment in terms of decreased tender/swollen joint counts, erythrocyte sedimentation rate, and C-reactive protein values associated with RF and ACPAs titer reduction [143]. Treatment using another monoclonal antibody infliximab against TNF-α also demonstrated clinical efficacy toward patients who do not respond to DMARDs by decreasing the serum levels of RF and ACPAs [144]. Of note, the efficacy of TNF antagonists is correlated to the titers of both RF and ACPAs. The higher the autoantibodies titer, the lower is the clinical response of the anti-TNF-agents [145]. These findings strongly suggested that autoantibodies generation during the course of RA pathogenesis may lead to the progression to refractoriness.

5.3. Role of cytokines in refractory RA development

Regardless the profound effects of TNF antagonists in refractory RA treatment, many patients either do not respond or relapse after initially responding to these agents [146]. Recent study revealed that the responsiveness of anti-TNF-α agents in RA patients depend on high blood level of granulocyte–monocyte colony-stimulating factor (GM-CSF) and low blood level of IL-17. Circulatory lymphocytes from most anti-TNF-α responder patients produced higher levels of GM-CSF than non-responder patients, whereas non-responsiveness to anti-TNF-α is associated with high IL-17 levels suggest that the responsiveness of TNF-α inhibitors is likely to be driven by different inflammatory pathways [146].

5.4. Current drugs and therapeutic approaches for refractory RA

As MTX is the first-line and frequently used DMARD, it is one of the most studied compounds for RA therapy. Information concerning the development of and the method to overcome drug resistance of MTX is well documented. Only about 40-60% of RA patients compromise with the MTX monotherapy [147]. Combination therapeutic approaches are commonly adopted and found effective in clinic, especially with MTX combined with other anti-arthritic agents. For instance, CsA, sulfasalazine, LEF, doxycycline, and HCQ individually combined with MTX demonstrated good efficacy in clinic, whereas triple DMARD therapy (MTX-sulfasalazine-chloroquine) and step-up combination of four DMARDs (MTX-CsA-HCQ-prednisone) are also applied in clinical treatment of RA [147–149]. However, a new synthetic small-molecule DMARD called iguratimod was recommended to treat RA patients who showed inadequate response to

MTX-CsA-HCQ-prednisone treatment [147]. Another immunosuppressive drug, tacrolimus (TAC) was found to be a promising therapeutic option for refractory RA patients despite treatment with anti-TNF therapy combined with methotrexate [150]. Later study further demonstrated the new oral triple combination therapy using TAC with MTX and mizoribine (MZR), this oral triple therapy might be safe and economical for clinical practice in effective against refractory RA [151]. Unfortunately, combination therapy is not always working and drug-resistant cases are still commonly found in refractory RA patients [147]. Therefore, the search of novel therapeutic strategies to combat refractoriness of RA is still urged.

5.5. Innovative therapeutic strategies for refractory RA with clinical potential

As mentioned in the earlier section, the use of monoclonal anti-CD20 antibody RTX is one of the approved conventional therapeutic methods for RA for the co-treatment with MTX. A randomized, double-blind controlled clinical trial indicated the potent efficacy of low dose RTX in RA refractory to first-line DMARD, MTX [148]. In addition, a recent clinical study has evaluated the impact of RTX on patient-reported outcomes (PROs) in a US-based observational cohort of patients with active RA refractory to TNF-α antagonist. Results demonstrated that patients with long-standing refractory RA experienced improvements in PROs 1 year after initiating RTX [152]. Another clinical study further revealed that RTX-based B cell depletion therapy is effective in refractory RA and systemic lupus erythematosus (SLE) [153]. Together with the role of $CD20^+$ plasma cells in autoantibodies production, deletion of B lymphocytes with specific identity of surface antigen appeared to be a prominent therapy for tackling refractory RA. In 2017, a new kind of immunotherapy, namely chimeric antigen receptor (CAR)-T cell therapy, has been approved by FDA for the cancers of acute lymphoblastic leukemia and advanced lymphomas [154], which provide insight to the exploitation of novel tool for B lymphocytes removal. The concept of CAR-T cell therapy involves the genetically engineering of autologous T lymphocytes to express a chimeric antigen receptor of target. Accordingly, expressing the CAR that recognized CD20 for RA therapy, in principal, can preserve the same effects as monoclonal anti-CD20 antibody. Another surface antigen CD19 has also been suggested as a promising marker for targeting B lymphocyte in RA [155] , which can also serve as another CAR-T cell therapeutic target. The humoral responses of B lymphocytes isolated from RA patients are repressed by the administration of the monoclonal anti-19 antibody XmAb5871 which facilitate the engagement of CD19, B cell antigen receptor, and Fcγ receptor IIb inhibitory receptor [156]. When compared with the passive administration of monoclonal antibody, CAR-T cells are benefited from its tissue biodistribution property because of their extravasate capacity [157], active responses to chemokine signaling [158], and the secretory ability of proteolytic enzymes [159]. Also, the self-amplification property of CAR-T cells enhance their *in vivo* persistence after adoptive transfer [160, 161].

On the other hand, the therapeutic effects toward RA by S1P-induced lymphopenia and VNS suggested the development of intervention methods by means of systemic regulation of the immunity. Intriguingly, the gut-associated lymphoid tissue (GALT) represent the largest mass of lymphoid tissue in the human body, which is an immune hub intimately communicating with

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residential microbiome to maintain the homeostatsis of different organs [162]. The involvement of gut microbiome in the systemic dysregulation of host immunity in different disease as a result of disturbed phyletic distribution or amount in the gut environment has aroused tremendous attention. Evidence associating gut microbiome and RA has recently been documented based on the characterization of the expansion of rare lineage intestinal microbes in RA [163]. Also, a metagenome-wide association study (MGWAS) suggested that the dysbiosis of gut microbial detected in RA is associated with clinical indices, which can be partly normalized after DMARDs treatment [164]. These information tempted us to ask if the application of pharmaceutic or health care supplement which manipulate the microbiome physiology could be potential RA therapeutic approach for providing more effective treatment with fewer side effects [165]. Chinese herbal medicine (CHM) appeared to be an ideal pharmaceutical method accordingly, since the philosophy of traditional Chinese medicine (TCM) emphasized on the maintenance of holistic balance of the human body which is best fit to the idea of managing local inflammation via the regulation of systemic immune system. In addition, CHM is natural constituents of herbal plant, and have been used for centuries, which is comparatively safe for clinical trial. In China, the country with wide application of CHM, medicinal compounds extracted from herbal plant has been using as folk remedy to manage RA for a long time. Many herbal formulated drugs targeting RA are prescribed in clinics, for example, Qingfu Guanjiesu capsule and Zhengqing Fengtongning tablet exploited by our group are demonstrating good clinical efficacy. The single molecule sinomenine is the main bioactive component constituting the above two pharmaceutics, which can successfully inhibit the proliferation of activated lymphocytes [166]. Our recent studies further suggested that the mechanism of action of sinomenine is cell type-oriented and is targeting multiple signaling pathways. Sinomenine suppresses the proliferation of FLS by regulating α7nAChR expression [167]. It also downregulates the expression of mPGES-1, which lead to the alleviation of arthritic inflammation [168]. Of note, sinomenine inhibits mPGES-1 transcription without affecting prostacyclin (PG)I2 and thromboxane (TX)A2 synthesis, therefore, lowering the risk of cardiovascular complication upon treatment. As yet, report documented CHM usage in RA by targeting gut microbiome is scarce. However, it is suggested that CHM can maintain the homeostasis of the gut ecosystem mainly via two processes [169]: (1) metabolic manipulation of the administrated CHM by gut microbiota; (2) gut microflora-targeted modulation of physiological conditions. The possibility of practising CHM to manipulate gut microbiota has been demonstrated in a recent case study using the decoction Du-Shen-Tang containing ginseng polysaccharides and ginsenosides [170]. The administration of Du-Shen-Tang, after gut microbiota-involved metabolism, was able to recover the pathologically ablated gut microbiota and specifically stimulated the growth of the commensal *Lactobacillus spp*. and *Bacteroides spp*. As such, CHM that could similarly modulate the gut-microbes as polysaccharides and ginsenosides decotion, may enhance the immune system and ultimately intervent the refractory condition.

6. Conclusion

Although a lot of intervention methods are available for the treatment of RA, they often come along with drug resistance upon unavoidable persistent administration. In addition, the

long-term usage of RA drugs is often associated with various side effects which complicated the treatment performance. Therefore, the search for novel therapeutic approaches is needed so as to explore more effective RA medication. Such new drugs could enlarge the pharmaceutical scope of choices for refractory cases by acting as monotherapeutic agent or being used in combination with other conventional RA drugs. Of note, the novel findings related to the control, or more precisely, the regulation of local joint inflammatory profile via systemic immune system deserved more attention and in-depth investigation. Since, researches in such orientation could provide information not just for the cure of RA, but also, hopefully, could help to achieve the goal of disease prevention.

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Conflict of interest

Vincent Kam Wai Wong and Liang Liu are co-corresponding authors of this chapter. We request to indicate this information in the final version.

Dedication

This chapter is dedicated to adorable Gwyneth and her gorgeous mother for contributing to the manuscript with their laughter.

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Chapter 8

Present and Future Therapies for Alzheimer's Disease

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Additional information is available at the end of the chapter

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Abstract

Alzheimer's disease (AD) is an incurable, progressive neurodegenerative disorder and the most common type of dementia. Although four kinds of drugs are currently available for AD, these are symptomatic treatments and do not halt disease progression. Therefore, there is an urgent need for development of curative drugs for AD. Amyloid plaques are the main disease hallmark observed in AD brains. As amyloid-β (Aβ) is a major constituent of amyloid plaques, Aβ has been supposed to be pathogenic for AD (amyloid hypothesis). Thus, current, mainstream AD drug development is based around this hypothesis. In particular, both active and passive immunotherapies are aggressively employed. However, most clinical trials based on this hypothesis, including immunotherapies, failed to improve cognitive impairment in AD. Therefore, it is likely that AD onset is caused by factors besides Aβ. We have previously demonstrated that the intracellular domain of amyloid precursor protein (AICD) induces dynamic changes in gene expression and neuron-specific apoptosis, probably related to AD pathogenesis. Therefore, AICD may be a favorable target for AD therapies. In this chapter, current trials for AD therapies, especially immunotherapies targeting Aβ, are summarized. In addition, therapies targeting tau, another possible pathogenic molecule, are also described. Furthermore, we discuss the possibility of AICD as a novel therapeutic target for AD.

Keywords: Alzheimer's disease (AD), immunotherapy, amyloid precursor protein (APP), the intracellular domain of APP (AICD), tau, signaling

1. Introduction

Alzheimer's disease (AD) is an age-onset, incurable, neurodegenerative disorder and the most common form of dementia in elderly people worldwide. This disease presents as progressive memory loss, accompanied by cognitive impairment and behavioral abnormalities, caused by neuronal dysfunction and cell death of neurons. At present, only four kinds of drugs are

approved for the treatment of AD, all of which act as modulators of abnormal neurotransmitter systems: three cholinesterase inhibitors (donepezil, rivastigmine and galantamine) [1] and one N-methyl-D-aspartic (NMDA) glutamate receptor antagonist (memantine) [2]. Low levels of acetylcholine and dysfunction and loss of cholinergic neurons are observed in AD brains [3]; therefore, these cholinesterase inhibitors are thought to inhibit acetylcholine degradation, maintain it at high levels and restore the function of cholinergic neurons. NMDA glutamate receptors are excessively and continuously activated in AD brains, which may lead to memory disorder and excitotoxicity. Memantine, a NMDA receptor antagonist, suppresses this excessive activation and may improve cognitive function. These drugs are temporarily effective in improving cognitive function; however, they are symptomatic and unable to halt disease progression in AD. Therefore, there are currently many trials of curative treatments for AD underway.

Although current understanding of AD etiology is not sufficient for development of AD therapies, two pathological hallmarks are commonly observed in AD brains and thought to be related to AD pathogenesis: extracellular amyloid plaques, consisting of amyloid-β (Aβ) and intracellular neurofibrillary tangles, consisting of hyperphosphorylated tau proteins. Aβ peptide, a major constituent of amyloid plaques in AD, is generated from amyloid precursor protein (APP) through stepwise, proteolytic cleavage by β- and γ-secretases. Mutations not only in APP [4], but also in presenilin (PS) 1 [5] and PS2 [6, 7], both of which are catalytic subunits of γ-secretase complex, are known to be responsible for early onset familial AD (FAD). These mutations accelerate proteolysis of APP and accumulation of Aβ, leading to the formation of amyloid plaques. Therefore, it is generally believed that Aβ is a causative factor in AD, called the amyloid hypothesis [8, 9], and this forms the basis for current, mainstream AD drug development. Additionally, tau, a microtubule-associated protein, is thought to be another pathogenic agent and potential therapeutic target for AD. Tau proteins stabilize microtubules, and hyperphosphorylated tau disperses and destabilizes microtubules, potentially resulting in neurodegeneration.

Immunotherapy is a disease treatment designed to modulate an immune response against certain target molecule(s) and is widely used in a variety of diseases, such as cancers, autoimmune diseases and allergies [10]. In the field of AD, immunotherapies, especially those designed based on amyloid hypothesis, have been aggressively employed [11]. Other therapeutic approaches, including inhibition of β- and/or γ-secretases to reduce Aβ production, have mostly failed, so immunotherapies targeting Aβ have been much anticipated as curative treatments, and many clinical trials have been attempted. Although both active and passive immunotherapies, againsnt various species of Aβ, have been designed and tested in clinical trials, most of these trials have not met endpoints in terms of improving survival or cognitive impairment, even in the cases with marked clearance of amyloid plaques [12]. Recently, immunotherapies against tau have also been attempted [13]. Although neurofibrillary tangles are formed within neurons, and the mechanisms by which intracellular pathogenic tau proteins can be excluded/neutralyzed by immunotherapies are not fully elucidated, these therapies are also expected to improve AD symptoms.

As outlined above, current mainstream AD drug development is based on the amyloid hypothesis. However, almost all trials targeting Aβ, including immunotherapies, have

failed to show efficacy in AD patients. Although genetic linkage analyses in FAD indicate that mutations in APP, PS1 and PS2 genes are linked to increased APP proteolysis and Aβ secretion, other fragments are also generated during the proteolysis of APP. For instance, extracellular fragments of APP are produced by β-secretase cleavage, and subsequent γ-secretase cleavage generates the intracellular domain (ICD) of APP (AICD) at the same time as Aβ. It is likely that these APP-derived fragments other than Aβ, contribute to AD pathogenesis [14].

γ-Secretase was originally identified as an APP-cleaving enzyme [15]. Although its biological roles are not fully characterized, γ-secretase is known to play a critical role in the regulation of Notch signaling [16, 17]. In canonical Notch signaling, γ-secretase cleaves Notch within its transmembrane domain to release the ICD of Notch (NICD), which immediately translocates to the nucleus and modulates expression of target genes through transcription factor binding. It was recently reported that many type-1 transmembrane proteins, besides APP and Notch, are substrates for γ-secretase, [18, 19] and their resultant ICDs can be detected in the nucleus. These observations suggest that the role of γ-secretase is to generate ICDs from its substrates to act as transcription factors [20–23]. Indeed, we have demonstrated that AICD alters the expression of a broad range of genes, leading to neuron-specific apoptosis, probably associated with AD pathogenesis [24, 25]. Based on these results, we propose that AICD may be a novel therapeutic target for AD [26].

In this chapter, current trials for AD therapies based on the amyloid hypothesis, especially immunotherapies against Aβ, are summarized. Other therapies, including immunotherapies against tau, are also described. Furthermore, we discuss the possibility of AICD as a novel therapeutic target for AD.

2. Aβ and amyloid hypothesis

APP plays a key role in AD pathogenesis [27, 28], although its physiclogical function has not been fully elucidated [29]. As previously described, because of the formation of amyloid plaques as one of the major hallmarks in AD, their main constituent, Aβ, is believed to be pathogenic.

Aβ is generated from APP through two stepwise enzymatic cleavages (**Figure 1(a)**). During the proteolytic process, APP is initially cleaved at either the α-site or the β-site, within its juxtamembrane domain, by α-secretase or β-secretase, respectively. Subsequently, both resultant membrane-tethered stubs are further cleaved at the γ-site and ε-site within the transmembrane domain by γ-secretase, resulting in the secretion of a non-amyloidogenic p3 fragment (in combination with α-secretase) or an amyloidogenic Aβ fragment (in combination with β-secretase). In both scenarios, AICD is simultaneously released from the cell membrane into the cytoplasm by γ-secretase cleavage. As mutation of APP, PS1 or PS2 genes in FAD accelerates proteolytic processing of APP and increases levels of Aβ, the amyloid hypothesis is believed to best explain the pathogenic mechanism of AD, and is the basis for most AD drug development.

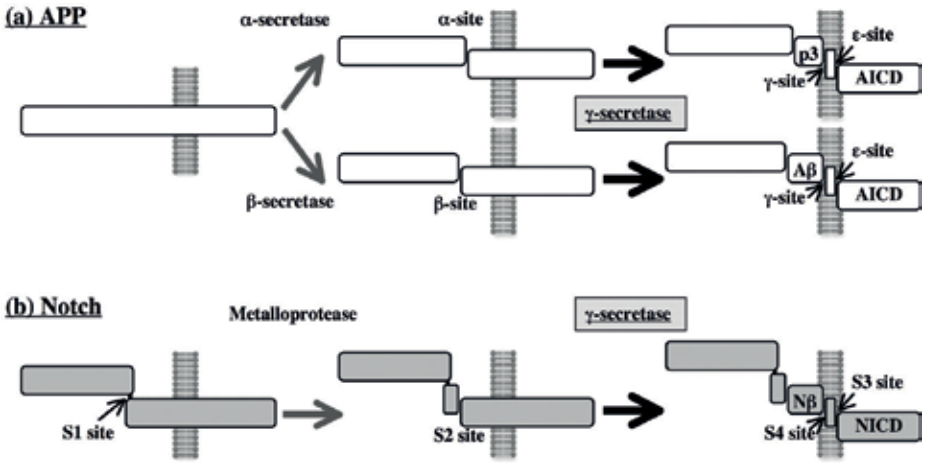

Figure 1. Proteolytic processing of APP and Notch. (a) After the cleavage of APP at the α-site or the β-site within its juxtamembrane domain by α-secretase or β-secretase, respectively, both remaining membrane-tethered stubs are further cleaved at the γ- and ε-sites within their transmembrane domains by γ-secretase. These sequential proteolytic reactions result in the secretion of either a non-amyloidogenic p3 fragment (α- and γ-secretase combination) or an amyloidogenic aβ peptide (β- and γ-secretase combination), and AICD (both combinations). (b) Notch is expressed on the cell surface as a heterodimer after cleavage at the S1 site by furin-like convertase. After the cleavage of Notch at the S2 site within its juxtamembrane domain by a metalloprotease, its remaining stub is further cleaved at the S3 and S4 sites within its transmembrane domain by γ-secretase, resulting in the production of NICD and Nβ fragment.

3. Secretase inhibitors

Several drugs to decrease and/or remove pathogenic Aβ and amyloid plaques have been developed. As Aβ is generated through proteolysis by β-secretase and γ-secretase, inhibitors for these enzymes were developed to decrease Aβ production [30].

β-Secretase is a type 1 transmembrane aspartic acid protease, also known as β-site APP cleaving enzyme 1 (BACE1). As human BACE1 knock-in mice showed accumulation of Aβ and, in an early study, BACE1-deficient mice did not display any abnormal phenotype, BACE1 inhibitors were expected to be safe therapeutic drugs for AD [31, 32]. LY2886721 was the first BACE1 inhibitor to reach Phase 2 clinical trials, but it was terminated due to liver toxicity [33]. Although other BACE1 inhibitors, such as MK-8931 and E2609, have been developed, and some are currently in clinical trials [34], recent studies have demonstrated that there are putative substrates for β-secretase besides APP [35], and adverse effects due to the inhibition of cleavage of these substrates should be of concern. In fact, further analyses of BACE1 knockout mice revealed several abnormal phenotypes such as axon guidance defects and hypomyelination [36]. Thus, it is likely that it will be difficult to inhibit the activity of β-secretase without side effects.

Inhibitors of γ-secretase (GSI) have also been developed to reduce levels of Aβ. Semagacestat was the first γ-secretase inhibitor to be taken into Phase 3 clinical trials; however, these trials were discontinued because patients showed worsened clinical measures of cognition and side effects such as increased risk of skin cancer and infections [37]. There are a large number of

membrane proteins that are substrates for γ-secretase [18, 19], and it is likely that GSIs will induce various adverse effects due to the inhibition of cleavage of other substrates, especially Notch. To date, GSIs that are selective for APP but not Notch (Notch-sparing GSIs) such as avagacestat, have been developed. Disappointedly, avagacestat failed to improve cognitive function and caused gastrointestinal and dermatological side effects [38].

γ-Secretase modulators (GSMs) are drugs that shift the γ-secretase cleaving site of APP and impair the production of the most toxic Aβ42 fragments, but do not affect other substrate cleavage. One such GSMs, tarenflurbil, was tested in Phase 3 clinical trials, but did not show beneficial effects in AD patients [39]. Recently, meta-analysis of pharmacological agents targeting γ-secretase have shown that these drugs increase risk of cancer and cognitive decline in AD patients, indicating that γ-secretase may not be an appropriate target for clinical treatment of AD [40].

4. Immunotherapies targeting Aβ

Besides the development of inhibitors for β- and γ-secretases, alternative attempts based on amyloid hypothesis have been made to remove soluble and/or insoluble Aβ utilizing immune responses. The first clinically relevant trial of immunotherapy directed towards Aβ was an active immunization using PDAPP transgenic mice, which overexpress mutant human APP with a valine to phenylalanine mutation at position 717 (V717F) under the control of a platelet-derived growth factor (PDGF) promoter. These transgenic mice, which exhibit accumulation of Aβ deposits at the age of 8–10 months, were immunized with Aβ, inducing high titers of Aβ-specific antibodies, resulting in the reduction and prevention of Aβ deposits [41, 42]. Based on these results, similar effects and clinical benefits were expected in human AD patients.

Active immunization with Aβ was the first immunotherapy performed in AD patients, who received full-length synthetic Aβ42 with QS-21 as the adjuvant (AN1792) [43–46]. A Phase 1 study demonstrated good safety and tolerability of this immunization. However, in Phase 2a, aseptic meningoencephalitis occurred in approximately 6% of AD patients treated with AN1792, probably due to a strong Th1 response, resulting in the termination of this trial [47]. Furthermore, follow-up assessments of the Phase 1 AN1792 study 6 years after immunization revealed that there was no evident difference in cognitive decline between the treatment group and the placebo group, although postmodern pathological studies of some of the treated patients showed marked clearance of amyloid plaques in the brain [48]. In addition, follow-up of the Phase 2a trial 4.6 years after immunization reported no significant difference in the majority of cognitive assessments between AN1792-treated and placebo groups [49].

To avoid an inflammatory T cell response, Aβ1–6 peptide, which is derived from the N-terminal Aβ-specific B cell epitope, coupled to a bacteriophage QB protein coat, was employed as an immunogen for active immunotherapy (CAD106). Phase 1 trials showed induction of anti-Aβ antibodies, safety and tolerability of this immunization, and no incidence of meningoencephalitis [50]. The Phase 2/3 clinical trial of CAD106 is currently ongoing.

ACC-001 is a conjugate of multiple short N-terminal Aβ fragments (Aβ1–7), coupled to inactivated diphtheria toxin as a carrier. In two Phase 2 trials, ACC-001 was administered with or without QS-21 adjuvant to patients with mild to moderate AD [51]. ACC-001 administered with QS-21 elicited higher peak and sustained anti-Aβ IgG titers compared with ACC-001 alone. Exploratory cognitive evaluations did not show any difference or trends between treatment groups and placebo groups, and this immunotherapy was discontinued from clinical development.

A number of other active immunotherapies have being developed, such as ACI-24, UB-311, ABvac40, and Lu AF20513 [52]. ACI-24 is a liposome vaccine against tetra-palmitoylated Aβ1–15, which favors β-sheet structure, to induce antibodies specific to its conformation. UB-311 is a vaccine against Aβ1–14, formulated with CpG oligonucleotides and aluminum salt, which preferentially stimulates a Th2 regulatory response over a Th1 proinflammatory response. ABvac40 is a vaccine against multiple repeats of short Aβ C-terminus that is conjugated to keyhole limpet hemocyanin (KLH) and formulated with aluminum hydroxide. Lu AF20513 is a vaccine developed against three repeats of Aβ1–12 peptides interspersed with P30 and P2 T-cell epitopes of tetanus toxin to overcome weak immune response in the elderly by utilizing immunological memory generated by tetanus vaccination in childhood. These active immunotherapies are currently in the process of undergoing their respective clinical trials.

Although active immunotherapies are potentially cost-effective for long-term treatments, there are some limitations to this type of treatment. In elderly individuals over 65 years old, immune responses are generally weaker than in younger individuals, meaning active immunization may not induce sufficient production of antibodies specific for Aβ. In addition, active immunization could potentially induce autoreactive and/or unintended immune responses associated with severe adverse events, including the aseptic meningoencephalitis observed in AD patients treated with AN1792. Direct administration of passive immunotherapies, utilizing ready-made antibodies specific for Aβ, may be able to overcome these problems, if the Aβ-specific antibodies have no, or reduced, cross-reactivity to self-antigens. To this end, several human/humanized monoclonal antibodies (mAb) against various epitopes of Aβ have been developed for passive immunization. Generally, the N-terminus of Aβ is exposed when it forms aggregates, while its middle and C-terminal regions are not accessible for antibodies. Thus, it is likely that mAbs specific for Aβ N-terminus are effective for the removal of Aβ aggregates, and those specific for Aβ mid-regions or C-terminus are effective for prevention of aggregation and exclusion of monomeric Aβ. Two mechanisms have been suggested to explain the reduction of Aβ induced by mAbs [53, 54]: microglia activation through Fc receptors and the peripheral sink effect. Microglia are activated by administration of the mAb, through binding to the Fc receptor, and can recognize and clear amyloid plaques. The peripheral sink effect hypothesizes that there is a decrease of soluble Aβ in the circulation due to binding of mAb to Aβ that alters the equilibrium of Aβ between the brain and the periphery and might draw Aβ out of the brain.

Bapineuzumab is a humanized immunoglobulin (Ig) G1 of murine mAb 3D6, which recognizes the N-terminus of Aβ (Aβ1–5). This mAb binds fibrillar and soluble Aβ, and activates Fc receptor-mediated, microglial phagocytosis of Aβ deposits. Although some clearance of fibrillar Aβ was observed upon analysis with [^{11}C]-Pittsburgh compound B and positron emission tomography (PET) in a Phase 2 study [55], two large Phase 3 studies of bapineuzumab showed no clinical benefits [56], and these trials were terminated.

Solanezumab is a humanized monoclonal IgG1 of a murine mAb clone, m266, which binds the middle region of Aβ (Aβ16–26). This mAb recognizes soluble monomeric Aβ, not fibrillar Aβ. In two Phase 3 trials, solanezumab was administered to patients with mild to moderate AD, and no improvement on the primary endpoints was detected in these trials overall [57]. In contrast, subgroup analyses based on disease severity (mild or moderate) showed slowing of cognitive and functional decline in pooled mild AD patients [58]. However, in the next Phase 3 trial in patients with mild AD, solanezumab did not meet the primary endpoint and was abandoned as a treatment for mild AD [59].

Gantenerumab is a fully human IgG1, recognizing a conformational epitope on Aβ fibrils which encompasses both the N-terminal and middle regions of Aβ. Gantenerumab is thought to act mainly to disassemble and degrade amyloid plaques by supporting phagocytosis of microglia. In a Phase 3 study, no differences in efficacy measures were observed, and this clinical trial was stopped based on interim analysis for futility [60]. Despite the lack of a clinical benefit in subjects overall, gantenerumab showed a beneficial trend in the fastest progressors, especially with higher serum levels of gantenerumab, on post hoc subgroup analysis. Further Phase 3 clinical trials employing dose titration schemes are planned, and the degree of amyloid reduction and cognitive improvement by administration of high doses of gantenerumab will be assessed.

Crenezumab is a humanized mAb that recognizes multiple aggregated forms of Aβ, including its oligomers, fibrils and amyloid plaques. Its affinity for Aβ monomers is lower. This mAb uses an IgG4 backbone to activate microglial phagocytosis and to minimize inflammatory responses related to side effects, such as vasogenic edema. Phase 2 trials of crenezumab showed no clinical benefits at its endpoints. However, a post hoc subgroup analysis of the high dose group suggested that treatment with crenezumab attenuated cognitive decline in the milder subgroups. The Phase 3 trial of crenezumab in prodromal to mild AD patients at higher doses is in progress [61].

Aducanumab is a fully human IgG1 mAb generated from memory B cell libraries of healthy, aged individuals by screening for reactivity against aggregated Aβ. It is thought that immune systems of these donors without AD symptoms may be able to prevent AD, and that their antibodies can assist in removal of amyloid plaques. Aducanumab interacts with the N-terminal region (Aβ3–6) of aggregated Aβ, including soluble oligomers and insoluble fibrils, but not monomers. In a Phase 1b clinical trial, florbetapir (^{18}F) PET scans showed marked reduction of brain fibrillar Aβ in a dose- and time-dependent manner [62]. In addition, although this Phase 1b trial was not sufficient to detect clinical changes, exploratory analyses of clinical assessments revealed dose-dependent slowing of disease progression at 1 year. Based on these interim analyses, two identical Phase 3 trials were launched to evaluate the efficacy of aducanumab for slowing cognitive decline in patients with prodromal to mild AD.

BAN2401 is a humanized IgG1 mAb that selectively recognizes a specific conformation of large, insoluble Aβ protofibrils. A Phase 1 study of BAN2401 demonstrated its safety and tolerability [63], initiating a Phase 2 trial to evaluate its efficacy against cognitive impairment.

Ponezumab is a humanized IgG2 mAb that binds to the C-terminal region of Aβ (Aβ33–40). Although Phase 1 studies of ponezumab showed sufficient safety [64, 65], its Phase 2 trials revealed no significant cognitive improvement in patients with mild to moderate AD [66]. The development of ponezumab for AD was discontinued, although it is still in Phase 2 trials for cerebral amyloid angiopathy (CAA).

Several other mAbs for Aβ have also been developed, with some of their clinical trials currently in progress. In addition to these passive immunotherapies, another strategy of passive immunization is currently being investigated. Pooled human plasma antibodies are prepared from donated blood and administered intravenously to AD patients (IVIg). These preparations contain a small fraction of naturally occurring polyclonal anti-Aβ antibodies and are expected to result in Aβ clearance and/or prevention of Aβ aggregation. Several of these IVIg preparations, such as gammagard and gamunex, are currently being administered to AD patients in clinical trials. A Phase 3 study of gammagard, observed no beneficial effects despite a significant decrease in plasma Aβ levels [67]. Clinical trials of other IVIgs are still in progress.

Although most passive immunotherapies targeting soluble and/or insoluble Aβ have entirely failed to show clear beneficial effects in patients with mild to moderate AD, Phase 3 clinical trials of solanezumab suggested that earlier intervention with this drug during the disease course may provide beneficial effects [58]. Although Phase 3 trials of solanezumab in mild AD patients did end in failure, administration of solanezumab and gantenerumab in asymptomatic and very mildly symptomatic carriers of autosomal-dominant mutations in APP, PS1 or PS2 genes, as well as cognitively healthy subjects at risk of developing sporadic AD, is being tested as a secondary prevention method [68]. In addition, crenezumab is also being tested in presymptomatic carriers of the E280A mutation in the PS1 gene [69].

Thus, there are no drugs based on amyloid hypothesis, including immunotherapies targeting Aβ, that show clear efficacy in AD patients to date, although some clinical and prevention trials are still in progress. These results may suggest the necessity of approaches other than targeting Aβ to develop efficacious treatments for AD.

5. Therapies against tau

In addition to extracellular amyloid plaques, intracellular neurofibrillary tangles are generally observed in AD patients as another histopathological hallmark of AD brains, and may be linked to AD pathogenesis. Pathological neurofibrillary tangles are aggregates of paired helical filaments composed of hyperphosphorylated tau. Tau is a microtubule-associated protein and binds to microtubules through its C-terminal assembly domain, thereby stabilizing microtubules and promoting microtubule polymerization. In the brains of AD patients, tau proteins are abnormally hyperphosphorylated by several protein kinases, such as glycogen synthetic kinase-3 β (GSK3β). Hyperphosphorylated tau proteins are detached from microtubules due to their reduced affinity for tubulins, resulting in destabilization of cytoskeletal microtubules and the formation of neurofibrillary tangles. Thus, the hyperphosphorylated tau protein is thought to be another favorable target molecule for AD therapy.

Several compounds to inhibit hyperphosphorylation or aggregation of tau have been developed. Tideglusib is a small molecule that acts as an inhibitor of GSK3β and counteracts tau phosphorylation. In a Phase 2 trial tideglusib was reported to produce no clinical benefit in patients with mild to moderate AD [70]. Rember is a first generation drug designed to prevent tau aggregation and/or dissolve existing aggregates. It is a formulation of methylthioninium

chloride, known as methylene blue. Its second generation, TRx0237, a stabilized, reduced form of methylthioninium chloride, was developed to improve drug absorption. Phase 3 trials of TRx0237 are currently underway [71]. Several microtubule stabilizers, such as epothiline D and TPI287, have also been developed as AD drugs to counteract microtubule destabilization by tau hyperphosphorylation. Epothiline D has been discontinued for AD and TPI287 is in Phase 1 trials [72].

6. Immunotherapies targeting tau

Various forms of pathogenic tau proteins may also become targets for immunotherapies. Two mechanisms for recognition of intracellular tau proteins/neurofibrillary tangles by anti-tau antibodies have been proposed. First, pathogenic tau proteins are supposed to be propagated from one cell to the next. Experiments with P301S transgenic mice, which express human mutant P301S tau, causing inherited frontotemporal dementia and development of filamentous tau inclusions, revealed that injection of brain extracts from P301S mice into the brains of wild-type human tau-expressing mice induced assembly of wild-type tau into filaments [73]. This pathological abnormality spread from the injection site to adjacent regions, suggesting extracellular transmission of tauopathy between cells. If certain pathological forms of tau can spread and induce abnormal assembly of normal tau in AD brains, it is therefore expected that anti-tau antibodies could bind to tau outside of cells and prevent the spread of tauopathy, resulting in the inhibition of disease progression. Second, anti-tau antibodies may be translocated inside neurons. It has previously been shown that neurons can take up anti-tau antibodies via clathrin-dependent low affinity FcγII/III receptors-mediated endocytosis and that these now-intracellular antibodies can bind to pathological tau within the endosomal-lysosomal system, promoting the clearance of pathological tau [74]. Thus, immunotherapies against pathological tau are hoped to be efficacious as AD treatments, and several active and passive immunotherapies, targeting various forms of pathological tau, have been developed.

AADvac-1 is an active vaccine consisting of a synthetic tau peptide (amino acids 294–305) coupled to KLH that is a carrier, with aluminum hydroxide as an adjuvant. The short tau domain synthesized for this vaccine is essential for pathological tau-tau interaction. A Phase 1 trial of AADvac-1 showed a good safety profile and excellent antibody response [75]. A Phase 2 trial is currently in progress.

In another Phase 1 study, ACI-35, which is a liposome-based vaccine consisting of a synthetic tau peptide (amino acids 393–408) phosphorylated at S396 and S404, is being tested in patients with mild to moderate AD [76].

Several humanized anti-tau mAbs are currently being tested in clinical studies as passive immunotherapies against tau. RG7345 is a humanized mAb specific for the tau phospho-epitope pS422, which is critical for binding to microtubules. Tau phosphorylated at S422 is prominent in early stages of AD and persists until late-stage disease, making it an attractive target for antibody therapeutics. RG7345 was tested in a Phase 1 trial but was subsequently discontinued [77].

BIIB092 is a humanized IgG4 mAb specific to extracellular, N-terminal fragments of tau (eTau), originally isolated from neuronal cultures using induced pluripotent stem cells derived from FAD patients. Since eTau is supposed to be involved in the spread of tauopathy, BIIB092 is expected to neutralize eTau propagation. Phase 1 trials of this mAb showed a dose-dependent accumulation in serum and CSF and marked reduction of CSF eTau, and a Phase 2 trial is planned [76].

ABBV-8E12 is a humanized mAb that recognizes an aggregated, extracellular form of pathological tau, and, thus, has the potential to stop or slow the propagation of tau pathology observed in AD and other tauopathies. Since a Phase 1 study showed an acceptable safety and tolerability profile of single doses, two Phase 2 trials to assess the efficacy and safety of multiple doses are in progress [76].

Although there have not yet been any successful clinical trials of immunotherapies targeting tau, many researchers have recently focused on the tau protein as a target molecule for AD treatment because of the failure of most clinical trials based on the amyloid hypothesis. However, since both the mechanism of transmission of tauopathies among cells, and that of antibody-uptake in neurons, have not fully been characterized, further studies to elucidate these mechanisms will be required in order successfully to design immunotherapies targeting tau.

7. γ-Secretase-regulated signaling and AICD

Since amyloid plaques, a major histopathological hallmark observed in AD, are thought to be a pathogenic factor in AD, Aβ, a main constituent of amyloid plaques, has long been thought to be a prime therapeutic target for AD. However, as mentioned, no clinical trials targeting Aβ have shown any efficacy in terms of cognitive improvement in progressing AD to date, and it is likely that there may be another mechanism that leads to the onset of AD.

As described above, genetic mutations in genes coding APP, PS1 and PS2 cause early onset of FAD. Since these genes are all associated with APP processing and Aβ production, and amyloid plaques are observed in the brains of both FAD and sporadic AD patients, the amyloid hypothesis, that Aβ is toxic for neurons and pathogenic in AD, has been widely accepted. Although several clinical trials of drugs targeting Aβ, including immunotherapies, are still in progress, no trials to date have succeeded in improving cognitive decline and/or behavioral abnormalities, even in the cases with marked reduction of soluble and insoluble Aβ. In addition to the failures of these clinical trials, several questions regarding amyloid hypothesis in AD have been raised. For example, amyloid plaques are often detected in the brains of healthy elderly people with no AD symptoms [78]. In addition, Aβ-overexpressing transgenic mice did not show any neurodegeneration although these mice exhibited Aβ deposition mimicking amyloid plaques observed in AD brains [79]. These observations suggest that Aβ has no neurotoxicity or pathogenicity in AD. Based on the FAD genetic analysis described above, it still seems likely that APP itself and/or its proteolysis contributes to AD pathogenesis, and it is possible that an APP-derived fragment other than Aβ, is the cause of AD [14].

γ-Secretase has been primarily characterized as an APP cleavage enzyme, and is thought to play a role in AD pathogenesis [15]. Although the physiological functions of this enzyme have not been

fully elucidated, it is well known that γ-secretase plays a central role in the regulation of Notch signaling (**Figure 2**) [16, 17]. In the canonical Notch signaling pathway, Notch is cleaved at its S2 site within the juxtamembrane domain by metalloproteases after the binding of ligands on neighboring cells to Notch. Subsequently, the remaining membrane-associated, C-terminal fragment is further cleaved at S3 and S4 sites within the transmembrane domain by γ-secretase, resulting in the release of the ICD of Notch (NICD) into the cytoplasm. NICD immediately translocates to the nucleus where it binds to a CSL transcription factor (CBF-1/RBP-jκ in mammals, Suppressor of Hairless in *Drosophila*, Lag-1 in *Caenorhabditis elegans*) [16, 80] and a mastermind-like (MAML) protein [81] to form a complex. In this process, a co-repressor in this complex is substituted with a co-activator, such as p300 and P/CAF [82], and the resultant complex induces expression of certain target genes such as Hes [83]. In this way, γ-secretase plays a regulatory role in Notch signaling.

Recently, many type-1 transmembrane proteins have been reported as substrates for γ-secretase [18, 19]. Interestingly, the proteolytic processing of some of these substrates, including APP, is very similar to that of Notch, and several of these ICDs have been detected in the nucleus. These observations suggest that some of these substrate proteins possess γ-secretase-regulated signaling mechanisms similar to Notch signaling [20–23]. Indeed, we have previously demonstrated that Delta, a ligand of Notch, possesses a γ-secretase-regulated signaling mechanism similar to Notch [84]. The ICD of Delta acts as a transcription factor through binding to Smad, a transcription factor in the TGF-β/activin signaling pathway, and modulates expression of target genes. These findings indicate that γ-secretase functions as a signaling regulator by generating ICDs of substrate membrane proteins, which then act as transcription factors in the nucleus.

AICD is generated through a proteolytic process similar to NICD (**Figure 1 (a)** and **(b)**), and it is possible that APP has a γ-secretase-regulated signaling mechanism similar to Notch [20–23]. We have previously demonstrated that AICD may act as a transcriptional factor, leading to neurodegeneration potentially related to AD pathogenesis. To examine the function of AICD, we used a teratocarcinoma P19 cell line, which can differentiate into neurons through stimulation with all-*trans* retinoic acids (RA), overexpressing AICD (AICD/P19). Although undifferentiated AICD/P19 cells were morphologically the same as control P19 cells, induction of AICD/P19 cells into neurons caused neuronal cell death with characteristic features of apoptosis, while control P19 cells differentiated normally into neurons [24]. In addition, DNA

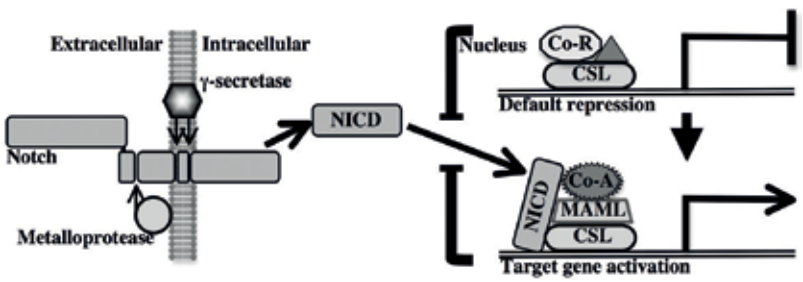

Figure 2. Notch signaling. When Notch binds to its ligand, Notch is cleaved at the S2 site by metalloproteases. Then, its remaining stub on the membrane is further cleaved at the S3 and S4 sites by γ-secretase, resulting in the release of NICD into cytoplasm. Immediately, NICD translocates to the nucleus and binds to the CSL transcription factor and MAML. During this process, the co-repressor(s) (Co-R) dissociate from this complex and the co-activator(s) (Co-A) is recruited. Finally, the resultant NICD-complex promotes the transcription of target genes as an activator.

microarray analyses with these cells revealed that AICD dramatically altered expression of numerous genes, possibly due to function of AICD as a transcription factor [25]. Although genes with altered expression were not directly related to the apoptotic cascade, it is likely that such dynamic alteration in the expression of many genes would disturb homeostasis in neurons, leading to neuron-specific apoptosis. Thus, AICD has a γ-secretase-regulated mechanism similar to Notch and may have potential as a therapeutic target in AD.

Figure 3 shows a diagram of APP signaling model. To prevent AICD-induced neurodegeneration, several strategies are possible: (1) reduction of AICD generation, (2) prevention of AICD translocation to the nucleus and (3) removal of AICD in the cytoplasm.

To reduce AICD generation, inhibitors of β- and/or γ-secretases would be an obvious choice. However, these enzymes have many substrates, and it is highly possible that inhibition of these other proteolytic reactions may induce an adverse reaction [18, 19, 35].

To translocate to the nucleus, AICD must bind to the adaptor protein Fe65 [85, 86], and it is likely that this binding is regulated by phosphorylation/dephosphorylation at T668 within AICD [87]. Phosphorylation of T668 interferes with the interaction between AICD and Fe65, and AICD is constitutively phosphorylated at T668, impairing its translocation to the nucleus. Phosphorylated AICD remains in the cytoplasm and is rapidly degraded by the proteasome and/or insulin-degrading enzyme (IDE) [88], suggesting that AICD is not toxic in the brains of healthy individuals. When AICD is dephosphorylated by phosphatases, or kinase phosphorylation of AICD is decreased, non-phosphorylated AICD can bind to Fe65 and translocate to the nucleus where it may act as a transcriptional factor, leading to neuronal cell death [89]. Therefore, it is thought that drugs that can modulate AICD phosphorylation by either decreasing dephosphorylation activity or restoring/increasing phosphorylation activity on AICD, preventing its translocation into the nucleus, are potential future AD treatments. Alternatively, removal of cytoplasmic AICD prior to its nuclear translocation is another potential treatment avenue, and, in this regard, drugs that upregulate the activity of AICD degrading enzymes, such as IDE, may also be efficacious.

From an immunotherapy perspective, if anti-AICD antibodies can be taken up into neurons as described in the section of 'Immunotherapies targeting tau' [74], immunotherapies targeting AICD may also act to remove intracellular AICD and/or prevent its translocation to the nucleus.

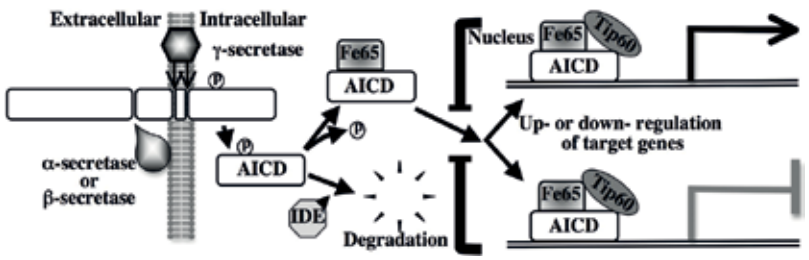

Figure 3. APP signaling model. Most APP proteins on the membrane are phosphorylated at T668 within AICD in neurons. After the removal of APP ectodomain by α-secrease or β-secretase, AICD is released by γ-secretase from the cell membrane into the cytoplasm. Then, phosphorylated AICD stay in the cytoplasm and quickly degraded by the proteasome and/or insulin-degrading enzyme (IDE). Dephosphorylated AICD can bind to Fe65 and then translocate into the nucleus. In the nucleus, AICD/Fe65 complex further binds to the histone acetyltransferase Tip60, and acts as a transcriptional regulator for up- or downregulation of target genes.

Thus, AICD may be a pathogenic agent in AD and has potential as a novel therapeutic target.

8. Conclusion

AD is an age-related, incurable, neurodegenerative disease and the most common type of dementia in elderly people. Increasing numbers of people suffer from this disease, but very few treatments are available, all of which are only symptomatic. Therefore, there is urgent need for development of curative AD therapies.

Although the precise mechanism underlying AD pathogenesis has not been elucidated, Aβ, a major constituent of amyloid plaques commonly observed in AD brains, is considered to be pathogenic. Most AD drugs have been designed in accordance with this 'amyloid hypothesis'.

Based on the amyloid hypothesis, immunotherapy was expected to be a powerful approach to remove and decrease pathogenic Aβ, and many trials of both active and passive immunotherapies targeting Aβ have been attempted. However, these immunotherapies targeting Aβ have totally failed to show efficacy, even in cases with marked clearance of amyloid plaques. Therefore, Aβ may not be the pathogenic entity in AD. At present, several immunotherapies targeting another possible pathogenic agent, tau, are also being tested.

Since it is highly possible that an APP-derived fragment, probably one other than Aβ, is responsible for AD pathogenesis, we have focused on AICD. According to our observations, AICD induces neuron-specific apoptosis, and has potential as a therapeutic target in AD. Based on our findings, the most important step in designing a drug against AICD, is likely preventing its translocation to the nucleus. This step may also help to remove pathogenic intracellular AICD. Taken together, it is hoped that AICD, and other promising target molecules, as well as Aβ and tau, will be further explored, and that efficacious treatments for AD will be established in the near future.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Chapter 9

Serological Biomarkers for the Prediction and Detection of Human Papillomavirus Associated Cancers

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Abstract

High-risk human papillomavirus (HPV) types are not only associated to uterine cervical cancer, but also to a fraction of cancers of the vulva, vagina, penis, anus, head and neck. An HPV infection generates a protective humoral immune response against the capsid proteins L1 and L2; however, an immune response against other HPV early proteins is also generated. This latter is not a protective response, but those antibodies can be useful as biomarkers of the status of the infection and/or the stage of the cancer lesion. Until now, there are no conclusive results regarding the use of anti-HPV antibodies as biomarkers in diagnosis. In this review, we hereby summarized the actual panorama of the humoral immune response against different HPV early proteins during the development of the disease as possible biomarkers for the prediction and detection of HPV-associated cancers.

Keywords: serological biomarkers, human papillomavirus, humoral immune response, HPV-associated cancers, cancer diagnostic

1. Introduction

Prevention of cervical cancer (CC) and other related human papillomavirus (HPV) diseases constitutes a public health priority worldwide [1]. Primary prevention has been achieved through the introduction of the prophylactic HPV vaccines, but the target groups are only adolescent girls and young women (up to 25 years old) [2]. Secondary prevention has been

implemented through screening methods to prevent precancerous lesions from progressing to cancer [3]. The CC prevention programs in the world are based on cervical cytology and colposcopy to detect precancerous lesions, which have helped to reduce significantly the incidence of this illness in countries with well-organized programs and good coverage of the target population, but this is not the case in developing countries [4, 5]. The main problems are the lack of qualified personnel, the poor quality of the screening tests, lack of follow-up colposcopy and treatment, and over-saturation of the health system facilities, estimating that less than 20% of the CC cases are detected opportunely in these countries [6]. The HPV has been the target for the new molecular diagnostic technologies to detect high-risk (HR)-HPV DNA in cervical cells, but these tests have not been sufficient to discriminate women with precancerous lesions in progression to cancer, from those that eliminate the infection and the lesion. Thus, the increasing incidence of HPV-related cancers worldwide, the inefficacy of the cancer prevention programs in developing countries, and the lack of efficient HPV diagnostic tests, make this a priority health problem worldwide [7].

For this reason, it is important to develop new screening methods, which should achieve high sensitivity, specificity, and should be inexpensive for developing countries. These new diagnostic methods could be used in triage with the cytology or HPV-screening tests to detect opportunely women at risk to develop CC. In addition, it would be important to develop new technologies and to identify new biomarkers that allow the early detection of other HPV-related cancers. In this sense, antibodies against HPV antigens have become the new biomarkers that can be used to detect persistent HPV infection that in combination with other molecular tests could be useful for early detection of HPV-associated cancers.

2. Differential expression of human papillomavirus proteins during the viral cycle

The HPV is a non-enveloped icosahedral virus of approximately 50 nm in diameter that contains a double-stranded circular DNA genome of around 8 Kb, which is divided into three regions: the long control region (LCR) that regulates the viral DNA transcription and replication; the early region (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7* genes) that controls the transcription and replication of the viral genome as well as to control the carcinogenesis; the late region (*L1* and *L2* genes) that contains the genes that expresses the viral capsid proteins [8, 9]. Differential expression of HPV proteins during the viral cycle is important for virus replication and evasion of the host immune response. In new infected cells, the HPV replication starts with the expression of low levels of HPV E6 and E7 viral oncoproteins that generates cellular proliferation and genome instability [10, 11]. First, the major viral oncoprotein E7 binds to the retinoblastoma tumor suppressor protein (pRb), which allows the cell to continue into the cell cycle [12]. Simultaneously, the E6 oncoprotein is expressed, and binds to the cellular ubiquitin ligase E6AP, which in turn results in degradation of the p53 protein, a transcription factor for cell cycle arrest, and in extreme situations, for induction of apoptosis [12]. As the infected basal cells migrate to the upper layers and differentiate, viral DNA replication is favored by the binding of E1 and E2 proteins to the LCR to regulate viral proteins expression [13, 14].

Once the viral DNA replication ends, the E2 protein represses the expression of the E6 and E7 oncogenes to allow the continuation of the viral cycle [15]. In the middle of this process, the E5 oncoprotein is expressed to maintain for a longer time the S-phase of the cell cycle and to delay the differentiation process to allow the complete expression of the viral proteins and the viral DNA replication [16, 17]. In the upper layers, E4 protein interacts with the cytoskeleton collapsing the cytokeratin filaments and enhancing the liberation of viral particles [18, 19], and it is also involved in the viral DNA replication [20]. Finally, the two viral capsid proteins L1 and L2 are expressed in terminally differentiated cells, once the replication of the viral genome has been completed, and ending with the release of the viral particles [21–23].

On the other hand, during a HPV persistent infection, there is a gradual loss of regulation of the E6 and E7 expression genes, which allows the development of early cervical lesions (CIN 1–3; cervical intraepithelial neoplasia grade 1–3) [24]. However, more than 70% of the CIN lesions are eliminated by the immune system. Progression to CC occurs due to an over-expression of E6/E7 oncoproteins, as a result of integration of the viral genome, which leads to the loss of the regulator *E2* gene [15]. This is an important event in the carcinogenesis of CC, as the over-expression of E6 and E7 oncoproteins generates cellular immortalization [25], stop cellular differentiation that generates dysplasia, the cells became anergic for TNF-α (tumor necrosis factor) and TGF-β (transforming growth factor) [25, 26], and chromosomal rearrangements could occur, as has been observed with the *c-myc* gene [27].

During a normal viral cycle, all the early HPV proteins carry out their functions inside the cells, and the viral antigens are poorly exposed to the immune system of the host. However, persistence of HPV infection allows the production of antibodies. Although the antibodies generated against the early HPV proteins are not of neutralizing type, these are suitable for their study as possible biomarkers, which recently is under investigation.

3. Cancers associated with HPV infection

Among all human cancers, 15% are caused by viral infections. HPV infection is recognized as one of the major causes of infection-related cancers in both men and women. Generally, HPV has been associated with more than 90% of anal and cervical cancers, about 70% of vaginal and vulvar cancers, 70% of oropharyngeal cancers, and more than 60% of penile cancers [28].

The HPV is the most common sexually transmitted virus and the HR-HPV types 16 and 18 are more prevalent in CC (approximately 70%) [28]. This type of cancer has been a major public health problem among adult women in developing countries. The last worldwide report for CC identified more than 440,000 incident cases and over 230,000 deaths due to this disease [1]. HR-HPV infection is necessary but not sufficient to cause this cancer, which develops over a long period of time through precursor lesions at the squamocolumnar junction cells near the transformation zone [29]. These cells shown to be multipotent residual embryonic cells have also been identified at the anorectal junction similar to the cervix [29]. Although, the cellular origin and the HPV-DNA prevalence are similar in the anus and in the cervix, the incidence ratio of cervical/anal cancer is quite different (17:1) [30]. The majority of low-grade squamous

intraepithelial lesions (LSIL) do not progress to high-grade lesions (HSILs) or carcinoma, which suggests that the HPV infection alone is not sufficient to generate cancer, as other cofactors such as immune deficiency, host genetic factors, among others are involved [30].

In anal cancer (AC), the HPV infection is detected in 80–90% of the cases, and HPV16 is the predominant type (80%) [31, 32] with a frequency higher than in other anatomical sites [32, 33]. This high frequency of HPV16 may reflect a differential tropism of this type that leads to malignant transformation in the anal mucosa. The prevalence of HPV in AC differs by geographic region, with the highest prevalence in North America and Europe and the lowest in Africa [31]. From the gastrointestinal tract malignancies, the prevalence of AC is around 2–3%, with 27,000 new cases reported worldwide in 2008 [31].

Vulvar cancer (VC) is originated from a precursor in intraepithelial lesions named vulvar intraepithelial neoplasia (VIN) and this type of cancer accounts for >90% of the malignant tumors in the vulva [34]. Recently, there is increasing evidence that suggests two different etiopathogenic pathways for the development of VC, one that is associated to HR-HPV and the second that is HPV independent. The prevalence of HPV-DNA in VIN lesions varied from 52 to 100%, but it is over 90% in VC [32, 34]. Over the last decade, the incidence of HPV-associated VC has increased mainly in young women, probably because of high-risk sexual behavior and a better recognition of these lesions due to HPV-DNA detection [32, 35].

On the other hand, penile cancer (PC) has been considered a relatively rare malignancy in the western world, although recent reports indicate an increase in incidence rates in developing countries (from 0.8 to 1.4/100,000) [36]. The etiology of PC is multifactorial with multiple established risk factors including infection with HPV. Epidemiological studies found that 48% of evaluated PC samples were positive for HPV-DNA and the type 16 or 18 was implicated in approximately 31% of these tumors, with HPV16 being the predominant type [37, 38]. In men, HPV infection can result in a spectrum of genitourinary manifestations that can cause genital warts, penile intraepithelial neoplasia (PIN), and PC. However, most HPV infections are asymptomatic, and up to 70% are cleared within 1 year [38].

The final group of the HPV-associated cancers is the one related to the head and neck cancer (HNC) that is the fifth most common cancer in the world [1]. Every year, there are more than 640,000 cases of this cancer reported and it causes over 350,000 deaths [1]. Squamous cell carcinoma is the most frequent type of neoplasia lesions affecting the head and neck area [39]. The laryngeal cancer (LC) is the most common among head and neck neoplasia and it accounts for about 60% of all cancers in the head and neck area [39]. LC may result from late complications of squamous cell papilloma (SCP), although most of those malignant changes develop without papillomas. Generally, squamous laryngeal cancer development begins with dysplastic changes within the epithelium of mucosa membrane lining the organ, this is followed by an intraepithelial neoplasia and finally the development of the pre-invasive cancer (carcinoma in situ) [39, 40]. However, HPV involvement in LC etiology has not yet been fully evaluated [41].

Within this group of cancers, oropharyngeal carcinomas (OPC) are the most dependent on HPV. The incidence of HPV-positive OPC has been markedly increasing in North America and Europe, with a higher rate in men than in women in North America [30], and HPV16 has been detected in the majority of these cancer cases [40]. Until now, little is known about the transmission and immunogenicity of HR-HPVs within the oropharynx. There is a strong

association with having performed oral sex and the number of lifetime partners [42], suggesting that initial infection of HPV in the oropharynx is related to high-risk sexual activity. HPV nucleic acid examination in rinse and gargle samples showed a prevalence of 4.7% of HR-HPV infection in the general population among the ages 45–65 years old. However, it is still unclear the implications of the viral infection in the development of OPC [42].

Moreover, esophageal cancer (EC) is the leading cause of cancer mortality worldwide, with approximately 500,000 incident cases and more than 400,000 deaths each year [1]. There are two types of EC; the most common is the squamous cell carcinoma (ESCC), which is highly prevalent in Eastern countries and in developing countries. The second type is the adenocarcinoma (EAC), which is associated with Barrett's esophagus, and its incidence has raised by 5–10% each year, in developed (Western) countries [43].

4. Immune response to the HPV infection

Mucosal HPV infections frequently arise in the anogenital tract and in the head and neck region, and these sites of infection have high threshold of immune tolerance [44].

The infection and replication of HPV is restricted to differentiating epithelial cells, where there is a limiting presentation of viral antigens to the host immune system. As a result, there is a low but detectable humoral immune response in most infected individuals [45]. HR-HPV types 16 and 18 mainly induce persistent infections without frequent serious complications for the host; they are also highly successful in releasing viral particles transmissible to others [46]. This virus takes the host to a point of balance where the infection does not represent a serious drawback, and viral replication is not limited by the host immune response [46], because the virus does not have a blood-borne phase or viremia. The HPV infection does not induce necrosis, cytolysis, or inflammation, and as a result, there is little or no release of pro-inflammatory cytokines in the local environment [47]. The HPV viral cycle occurs in cells that are destined for death by anoikis (detachment), and because of this, there are no danger signals to alert the immune system to generate an efficient response to eliminate the infection [48].

It is well documented that more than 80% of the genital lesions caused by HPV infections are cleared as a result of a successful cell-mediated immune response, during which cells of the innate immune system such as keratinocytes, dendritic cells (DCs), Langerhans cells (LgCs), macrophages, natural killer (NK), and NKT cells, may play an important role in clearing the infection by promoting a pro-inflammatory process [49]. In the female genital track, the natural host of the HPV infection, there are keratinocytes that could act as immune sentinels, as it has been shown in skin [50]. These cells express Toll-like receptors (TLRs, belonging to the pathogen recognition receptors (PRR) family) on the cell surface (TLR-1, -2, -4, -5 and -6) and in the endosomes (TLR-3 and -9). Specifically, TLR-9 is activated by unmethylated double-stranded CpG-rich DNA [51], allowing the secretion of interferons (IFNs) to activate the NK cells [52], which in turn kill the HPV-infected cells [53]. However, if the HPV infection becomes persistent, there is a downregulation of the innate immune response, which facilitates the virus to escape from the immune system. This mechanism could be through the downregulation of the IFN response by the oncoproteins E6 and E7 that interfere with different molecules involved in the signal transduction pathways of these cytokines [54].

Conversely, the LgCs and DCs (antigen-presenting cells) initiate the adaptive immune response to eliminate the HPV infection, through antigen-specific presentation to B and T cells in the lymph node. In this process, there is a generation of a Th1-type microenvironment by secretion of pro-inflammatory cytokines, which helps to activate Tc cells (directed against the early HPV proteins E2, E6 and E7) to kill the infected cells [55]. This immunological response is complemented by the generation of neutralizing antibodies against the L1 capsid protein to further inhibit the spread of the viral infection, but the virus uses several strategies to evade this adaptive immune response [56]. Recently, it was demonstrated that E2, E6, and E7 proteins upregulate the expression of IL-10 and TGF-β by interacting with their promoters, which allows an immunosuppressive environment [56]. Additionally, the E5 oncoprotein regulates the antigen presentation to the Tc cells by retaining the MHC-I in the Golgi apparatus, and preventing the MHC-I complex transportation to the cell membrane [57]. Besides, the antigen presentation to the Th cells is also regulated by E5, since this protein prevents the acidification of the endosomes, where the MHC-II restricted antigen is processed [58]. At the end, the immune system fails to clear the HPV infection as a result of an imbalance between Th1 (pro-inflammatory) and Th2 (anti-inflammatory) cytokines, which allows a persistent infection with a high risk for the development of CC [56, 59].

Finally, the humoral immune response against the HPV infection is driven through the activation of the B-cell receptor by recognition of HPV antigens and stimulation by the CD40 Th cells receptor that allows the differentiation into plasma cells to produce antibodies against HPV proteins [48]. In this way, a differential antibody response against different HPV antigens (E1, E2, E4, E5, E6, E7, L1, and L2) is generated and detected in the sera of HPV-infected women [60], and specifically, anti-E7 antibodies have been identified and associated to CC, and suggested as a possible markers for late stages of this disease [61–65].

5. Humoral immune response against human papillomavirus antigens

For several decades, the humoral immune response against HPV proteins has been used to study the viral cycle, and more recently as markers of HPV-associated cancers at different anatomical sites [66]. In this regard, some studies showed that seroconversion and the presence of anti-HPV antibodies were associated to the occurrence of precancerous lesions at different anogenital sites such as CC, AC, and from other sites like oral cancer (OC) [66, 67].

The presence of anti-HPV antibodies has been investigated through several epidemiological studies in several populations with different exposures to the virus and found a great variety in the prevalence and kinetics of these antibodies. The variations observed in the antibody response could depend on the population type, anatomical site of infection, viral antigens present, among others, but also the detection method may influence the antibody results observed (**Tables 1** and **2**). The immune response to the HPV proteome (or lack thereof) may provide some biological clues required to answer some of these questions. The HPV oncoproteins E6 and E7 are early viral proteins that drive neoplastic transformation, are reliable indicators of an HPV-associated neoplasia, and can lead to detectable serum antibodies prior

to and at time of diagnosis, as well as post treatment [68]. To evaluate the serum antibodies against HPV antigens, generated during infection, precancerous lesions and cancer, several laboratory technics have been used, such as the ELISA (enzyme linked immunosorbent assay), western blot, radioimmunoassay (RIA) and more recently, Luminex multiplex. These methods use different antigens such as L1 virus like particles (VLPs) from different HPV types; synthetic peptides from L1, L2, E2, E4, and E7, bacterial recombinant proteins, or *in vitro*-translated viral antigens [7, 69].

A large number of epidemiological and clinical studies have been carried out to search for the presence of antibodies against HPV proteins and to identify associations of these serological markers with different types of anogenital cancers. In this sense, antibodies against proteins L1, E4, E6, and E7 from HPV16 are the most explored and are most frequently associated with different HPV-associated cancers (**Table 1**) [62, 66, 69–89].

Until now, the anti-L1 antibodies are more commonly associated to anal, penile, vulvar, vaginal, and cervical cancer, but the results are contradictory. Some researchers found similar prevalence of anti-L1 antibodies in controls and in penile, vulvar, and vaginal cancer cases (17-38%) [66, 79], while others have shown higher prevalence of anti-L1 antibodies in AC (~54%) and CC (~68%) than in controls [79, 82]. These variations could be the result of different sensitivities in the tests used, as well as the purity and the origin of the viral antigens [55, 90]. Even though there are anti-L1 antibodies present in different types of cancer, these antibodies do not differentiate the anatomical site of the HPV infection or the lesion site. Still some differences have been identified, as anti-E7 antibodies are good markers for CC and anti-E6 antibodies for OC [62, 91]. In this way, several studies have been carried out and showed that antibodies against E7 have been commonly associated with AC and CC, with prevalence that goes from 45% in the anus [78] and up to 75% in the cervix [62, 69], while in the penis, vulva, and vagina, the antibody prevalence was under 15% [79]. In contrast, although serological antibodies against E6 prevalence were high in CC patients by using different tests (from 37 to 44%) [70, 86], the association of anti-E6 antibodies with this type of cancer has not been very well defined [70, 74, 75, 88].

One of the most studied HPV proteins is E4, and this is probably due to its abundance (20–30% of total protein in condylomas) and to its differential production along the viral cycle [92]. During HPV DNA replication in low-grade lesions, high expression levels of E4 protein are observed, while in high-grade lesions, this protein is almost absent [24, 92, 93]. As a result of these observations, the E4 protein is proposed as a marker of viral replication [92, 93]. However, the methodology to detect E4 protein relies on biopsy samples, which are difficult to obtain. For that reason, the detection of HPV antibodies has become a more sensitive system to indirectly follow-up the expression of viral proteins. Several epidemiological studies have shown higher prevalence of anti-E4 antibodies is observed in women with premalignant lesions than among CC cases or in the general population [62, 75, 94, 95]. Previously in our laboratory, we showed that anti-E4 antibodies were in low prevalence in healthy women (11%), but the prevalence increased in subjects with CIN1-3 lesions (70%), and slightly decreased in CC (60%), which suggests an early recognition of this protein by the immune system [61, 62], and postulated as early markers of the disease (**Table 1**).

Cancer type	Method	Population	Serum antibodies (%)						Ref.
			E1	E2	E4	E6	E7	L1	
Anal	ELISA	AC cases		70			45	50	[78]
	ELISA	Hospital controls and AC cases						25–52	[71, 72]
	Multiplex GST	Anogenital cancer cases	21	13	29	29	33	54	[79]
Penile	ELISA VLPs	Hospital controls and PC cases						63	[84]
	ELISA L1	Hospital controls and PC cases					11	24–38	[66, 71, 72]
	Multiplex GST	Anogenital cancer cases	8	13	17	8	13	17	[79]
Vulvar	ELISA VLPs	Hospital controls and VC cases						27	[84]
	ELISA L1	Hospital controls and VC cases						43	[72]
	Multiplex GST	Anogenital cancer cases	5	8	16	2	8	27	[79]
Vaginal	ELISA VLPs	Hospital controls and cancer cases						27	[84]
	ELISA L1	Healthy controls and cancer cases						44	[72]
	ELISA	Hospital controls and cancer cases					0	26	[66]
	Multiplex GST	Anogenital cancer cases	0	0	0	8	0	25	[79]
Cervical cancer	Multiplex GST	Healthy controls and CC	10	12	17	32–37	28–42	19–44	[70, 74, 75, 88]
	Western blot	Healthy controls and CC			60		75		[62]
	ELISA	Healthy controls and CC				19–54	13–53	28–68	[63, 70, 76, 77, 81–83, 86, 87]
	RIA	Healthy controls and CC				50–51	33–39	56	[76, 80]
	Luminex multiplex	Healthy controls and CC				11–44	14–61	21–35	[70, 73, 89]
	Slot blot	Healthy controls and CC			73		80	40	[69]

AC, anal cancer; PC, penile cancer; VC, vulvar cancer; CC, cervical cancer.

Table 1. Antibodies against HPV16 antigens in different types of anogenital cancers.

In contrast, little is known about the presence of anti-E4 antibodies in other anogenital HPV-associated cancers. The study of Kreimer and coworkers [79] carried out in AC, VC, and PC patients identified prevalence of anti-E4 antibodies under 30%, but they did not look for these

Cancer type	Method	Population	Serum antibodies (%)						Ref.
			E1	E2	E4	E6	E7	L1	
Tongue	ELISA	Healthy controls and TC					25	4–21	[66, 84]
Oral cavity	ELISA	Hospital controls and OC						12–25	[84]
	ELISA GST	Hospital controls and OC				9	10		[100]
	Multiplex GST	Hospital controls and OC	8	6	8	1	6–14	5–23	[101, 102]
Oropharyngeal	ELISA GST	Healthy controls and OPC	63–74	36–72	24–42	42–90	12–80	6–33	[66, 100, 103–105]
	Luminex GST	Healthy controls OPC and Partners	73	80–83	43	50	63	23	[106]
	Multiplex GST	Healthy and Hospital controls and ADC	16–21	24–25	11–13	30–35	20–25	14–42	[101, 102]
		OPC and Non-OPC	37–70	45–77	34–45	61–85	47–72	55–61	[107, 108]
		OPC	64	84	36	90	71	70	[91]
Laryngeal	ELISA	Healthy controls and LC					12	20	[66]
	Multiplex GST	Hospital controls and ADC	9	5	12	1–2	6–12	3–24	[101, 102]
Esophageal	ELISA	Hospital controls and EC cases				17	0	8–31	[66, 84, 109–111]
	Multiplex GST	Hospital controls and EC cases	6	5	8	0.3–3	5–9	2–23	[101, 102]

TC, tongue cancer; OC, oral cancer; ADC, aero-digestive cancer; OPC, oropharyngeal cancer; LC, laryngeal cancer; EC, esophageal cancer.

Table 2. Antibodies against HPV16 antigens in different types of head and neck cancers.

antibodies in early lesions of these anogenital cancers, where this early HPV marker should be the prevalent, as it was observed for CC (**Table 1**).

Antibodies against E1 and E2 have been analyzed in anogenital cancers and only in AC, the anti-E2 antibodies have been observed with high prevalence (70%), which is through the use of peptides in ELISA [78]. However, a more recent study using a multiplex assay showed that the prevalence of these anti-E2 antibodies was fewer than 15% [79]. In the case of CC, the observed prevalence of anti-E1 and anti-E2 antibodies was under 15% [75] (**Table 1**), but the prevalence of anti-E2 in different degrees of cervical lesion by ELISA was high in CIN1-2 lesions (64%), and it decreased with the increasing severity of the cervical lesion (CIN3, 31%) [96, 97]. Overall, these data suggest that anti-E2 antibodies could constitute a good biomarker for CIN lesions, but these need further studies.

The presence of anti-E5 antibodies in cervical lesions has been described in only one report by using a microarray assay, but no associations were identified with any stage of the uterine cervical lesions [98]. It is necessary to characterize better anti-E5 antibody response, with a more sensitive and specific assay, as this could be an interesting serological marker, since by looking for the presence of E5 mRNA, this was associated with low-grade anogenital lesions [99].

Studies of anti-HPV antibodies in other HPV-associated cancers are underway, but the most recently studied are those localized in the head and neck sites, where different antibodies against E1, E2, E4, E5, E6, E7, and L1 viral proteins have been studied to try to identify associations with the presence of some type of cancer lesion in the oral cavity, in the tongue, pharynx, larynx, and even esophagus (**Table 2**) [66, 84, 91, 100–111].

The study of anti-HPV antibodies in sites such as the mouth and the esophagus began in the late 1990s, but more recently, the study of these antibodies has focused on lesions caused in the oropharyngeal area. In TC, low prevalence of anti-E7 antibodies (25%) and anti-VLPs (4–21%) antibodies were observed [66], while in OC, the prevalence of anti-E7 was under 15%, but it was very variable for anti-VLPs antibodies (from 5 to 25%), differences that could depend on the methodology of antibody detection used (**Table 2**) [79, 84, 101].

One of the cancers in which the presence of anti-HPV antibodies has been analyzed in more detail is the OPC, in which ELISA and multiplex assays have been used with different HPV antigens. These studies strongly showed that anti-E6 antibodies are highly prevalent in OPC (from 30 to 85%), but this prevalence increased up to 90% when the OPC cases were HPV16 positives [91, 102, 106]. However, this was not the case for anti-E7 antibodies, where the prevalence varied from 12 up to 80% [66, 103]. Also, some studies measured the antibodies presence before and after cancer treatment, and they showed that seropositivity to E6 and E7 significantly decreased after treatment. However, only anti-E6 antibodies showed an increased risk of disease recurrence, making these anti-E6 antibodies good biomarkers for disease prognostic [100, 103–105].

The other anti-HPV antibodies with high prevalence in OPC have been those against E1 (~74%) and E2 (~77%) viral proteins [66, 100, 103–105, 107, 108], but this is not the case for other head and neck cancers, where the highest prevalence of antibodies against these viral antigens was under 10% (**Table 2**) [101]. Although the E4 protein is proposed as a marker of viral replication, in the case of OPC, the prevalence of anti-E4 antibodies was under 45%, and this was using the ELISA-GST system that is a highly sensitive method (**Table 2**) [104]. These studies suggest that the same serological markers are not present at the different anatomical site where the HPV-associated cancers appear. These results are very promising in the search for serological biomarkers, which in combination, they generate profiles that could differentiate the anatomical sites where the HPV-associated cancer is present, as it has been the case for the profile anti-E1/E2 + anti-E6 that has been associated to OPC [91], and for CC, the suggested antibody profile is anti-E4 + anti-E7 [69].

There are few studies that have analyzed anti-HPV antibodies in LC and showed low prevalence of the antibodies that varied from 2% for anti-E6 antibodies to a maximum of 24% for anti-L1 antibodies (**Table 2**) [66, 68, 101, 102]. Similar results have been reported for EC, and

the prevalence of anti-HPV antibodies fluctuated from 31% for anti-L1 antibodies and were under 10% for the rest of the anti-HPV antibodies [66, 68, 84, 109, 111]. At this moment, the identification of serological markers for LC and EC are inconclusive, and more studies need to be carried out with more sensitive methodologies such as the slot-blot system, but also a restriction to HPV positive cancer cases should be considered, as a low proportion of these two types of cancers are associated to HPV.

In addition, most of the studies of anti-HPV antibodies in different HPV-associated cancers have been carried out in the late stages of these cancers. It would be of great interest to study early stages of the HPV-associated cancers as some of these anti-HPV antibodies seem to be important in early diagnostic and during follow-up as possible prognostic markers. In the case of CC, there are several studies of anti-HPV antibodies carried out with precancerous uterine cervical lesions, where it has been suggested that anti-E4 antibodies are important markers for CIN1-2 [61, 62], while the profile anti-E4 + anti-E7 is a good marker for CC [69]. In the case of other HPV-associated cancers, there are only few studies that measured anti-HPV antibodies in early stages of the disease, as it is the case of anus and oral cavity lesions, where anti-VLPs antibodies presented the highest prevalence (43 and 30%, respectively) [111–115]. However, more studies are necessary to characterize the humoral immune response in the different HPV-associated cancers and their related precancerous lesions.

It is important to mention that differences in methodology and concerns about HPV misclassification, aside from the heterogeneous responses in the antibody patterns seen in the various studies, and in the different HPV-associated cancers require further evaluation. Besides, there are other confounding variables such as HPV type, viral load, viral exposure history, host immune system factors, and clinical risk factors. Therefore, prospective evaluations of anti-HPV serum antibodies should be controlled for as many of these factors. In addition, it is likely that an antibody signature consisting of a panel rather than a single antibody may provide the highest yield to be able to differentiate anatomical site, as well as early detection of these HPV-associated cancers.

6. Diagnostic and prognostic tests for HPV-associated cancers

Essentially all cervical cancers, most anal and oropharyngeal cancers, and some vaginal, vulvar, and penile cancers are caused by HR-HPVs, but until now, there are no general guidelines for screening of all these HPV-associated cancers. Recently, the availability of new tests and ongoing research are changing the approach to screening and diagnosis of these types of cancers. However, most of the studies have been carried out in CC.

6.1. Cervical cancer

For CC, there are specific guidelines that have been modified in the last years, which include the introduction of new testing technologies, which have improved the early diagnostic of this disease. The cytology is the primary screening system for precancerous lesions and can

be done using Papanicolaou-stained smears (Pap), although this test has a low sensitivity (50–80%) [116, 117], and a high percentage of false positives, due to the fact that inflammatory cytology is considered abnormal [7, 117]. To confirm the presence of a uterine cervical lesion, a colposcopy test is required, which has a high sensitivity (80–95%) and a low specificity (23–63%), because the test becomes positive in the presence of an inflammatory process, and it is not useful to detect early uterine cervical lesions [118].

The introduction of molecular tests to detect DNA from HR-HPVs has shown to be highly sensitive and makes them good screening systems. Recently, there are different HPV molecular technics that are FDA (Food and Drug Administration, USA) approved to use in conjunction with cytology in CC screening programs. Among these tests are the Hybrid Capture® 2 (HC2) (Qiagen, GmbH, Hilden Ger) and Cervista® HR-HPV Test (Hologic, Inc., MA, USA) that amplify the positive hybridization signal and allow the detection of multiple HR-HPV types in one step [119, 120]. The Cervista® HR-HPV and Cervista® HPV types 16/18 tests have shown to be complementary with a 100% of sensitivity for detection of CIN3+ and of 98% for CIN2+ in women with diagnostic of ASC-US (atypical squamous of undetermined significance) by cytology and HR-HPV positive [121], system that has been approved by FDA.

On the other hand, HC2 has been tested at the general population for the detection of HPV worldwide. This system detects 13 HR-HPV types (-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68) and 5 of low risk (LR) (-6, -11, -42, -43, and -44), and it is semi-quantitative (detects up to 1 pg HPV-DNA/ mL). The HC2 was FDA approved to detect women who have been referred to colposcopy with an ambiguous cytology of ASC-US and for the screening of women over 30 years old in conjunction with the cytology test [120]. Several epidemiological studies have shown the high predictive value of the HPV test for early detection of CC. This test is highly sensitive (93–98%) and specific (60–85%) to detect high-grade lesions, which makes the HC2 ideal for screening, and in combination with the cytology test increases the possibility to identified correctly women in risk to develop CC [3, 122, 123].

There are other systems to detect HPV-DNA by using reverse hybridization, some of which are INNO-LiPA® (Line Probe Assay, Innogenetics, Ghent, Belgium), CLART® HPV2 (Genómica, Madrid, Esp), Clinical Arrays® HPV (Genómica, Madrid, Esp) and the Linear Array® (Roche Mol Diagnostics, CA, USA), and this last one detects 37 HR- and LR-HPV types [119, 124]. The difference between these tests is the sensitivity as the LiPA detects from 10 to 100 DNA viral copies. These genotyping methods have a high sensitivity (95%) to detect CIN2+ lesions, making these tests suitable for screening the general population [119].

The detection of the HPV-DNA as indicator of the viral presence does not determine the presence of an active infection, and because of this, a complementary diagnostic test is necessary. Evaluation of viral load has been used as a biomarker of persistent infection, because cytological abnormalities are more frequently observed in CIN2-3 and CC with high viral load [125]. The viral load is determined by Real-time PCR and this system is highly sensitive and specific, and genotyping can be carried out in the same assay. Two diagnostic tests have been developed that use this technology, the Abbott Real-Time HR-HPV (Abbott Mol GmbH & Co. KG, Germany) and the COBAS® 4800 HPV (Roche Mol Diagnostics, CA, USA). These systems detect 14 HR-HPV types in one reaction, and use a colorimetric detection system to differentiate

the types 16 and 18; however, the sensitivity of COBAS® 4800 to detect CIN2 is higher (97%) than the one from Abbott (95%) [123, 126], and just recently have been FDA approved.

In the case of CC, the integration of HPV to the cellular genome seems to be an important part of the carcinogenesis, as this generates an abortive infection with high-level production of the E6 and E7 oncoproteins due to the loss of the *E2* gene expression. This event causes the high expression of mRNA of the *E6* and *E7* oncogenes in the upper layer of the stratified epithelium in CIN2-3 and CC [127]. Thus, the E6/E7 mRNAs have been suggested as specific markers for precancerous lesions. In this way, several commercial tests have been developed to detect mRNAs from E6/E7 such as PreTect® HPV Proofer (NorChip AS, Norway) and APTIMA® HPV (Gen-Probe CA, USA). Both tests detect E6/E7 mRNAs by Real-time PCR, but PreTect® detects only 5 HPV types while APTIMA® detects 14 HR-HPVs, and this is carried on samples from liquid-based cytology. The APTIMA® assay has a similar sensitivity (98%), but higher specificity for HR-HPVs than HC2 (90% vs. 85%, respectively) to detect CIN2-3, making these molecules potential markers for the detection of high-grade lesions, but this is still under investigation [119, 128].

For more than 15 years, it was established that the treatment of early lesions (CIN1, ASC-US, and LSIL) was through follow-up patients with cytology and colposcopy for up to 2 years. However, this procedure is costly, and several visits to the medical office are required, and saturation of the colposcopy system, and loss of patients during the follow-up have made unable to give adequate treatment to those women at risk for developing CC. Because of these problems, it has been necessary to look for new biomarkers that allow the early identification of uterine cervical lesions that could progress to CC. From these efforts, some biomarkers have been identified and are described below.

The $p16^{INK4a}$ is an inhibitor of the cell cycle by inhibiting the hyperphosphorylation of pRb, through blocking the activity of the cyclin D-CDK4/6 complex. It has been observed that the inactivation of pRb by E7 results in the over-expression and accumulation of $p16^{INK4a}$ in the cells, making this protein a surrogated marker of E7 expression, which is associated to the CC development [129, 130]. The detection of $p16^{INK4a}$ is carried out in liquid-based cytology samples and in tissue biopsies by immunohistochemistry (IHC). Different epidemiological studies have demonstrated that $p16^{INK4a}$ is a good marker to identify HPV positive women with CIN 2-3 [129, 130] and it is a useful marker to clarify 90% of ambiguous histopathological diagnostics [131, 132]. The disadvantage of this biomarker is that in other non-associated HPV cancers also it is over-expressed [133].

The Ki-67 proliferation nuclear antigen (cell proliferation marker) is localized in the parabasal layer in the normal stratify uterine cervical epithelium, but during the development of CIN lesions, the expression of Ki-67 is extended all along the cervical epithelium. This marker is ideal to identify tumor cells, which correlates with the clinical stage of the lesion and the development of cancer. The detection of this Ki-67 marker is carried out in liquid-based cytology samples by IHC, and it is useful to detect CIN 2-3-associated HPV lesions [131]. Epidemiological studies in Europe and USA showed that Ki-67 can be used in combination with $p16^{INK4a}$ and helps to increase the sensitivity to a similar level as for HPV detection, but research is still under way to better characterize and validate these biomarkers [131, 132, 134].

6.2. Anal cancer

The AC has similar features with CC, and natural history studies showed that high-grade anal intraepithelial neoplasia (AIN) is a precursor to invasive anal cancer. Because of these similarities, several features of the CC screening program have been taken for routine screening to detect precancerous anal lesions. In the general population, the Pap test to screening for precancerous anal lesions is carried out by taking a sample from the anal canal and the lesions are classified by using the Bethesda nomenclature. At the moment, anal cytology seems to be useful for screening of high-risk individuals, including HIV-positive patients [135].

In contrast, HPV testing has limited utility for AC screening because of the high prevalence of the infection and multiple HPV types in the anal canal of women and HIV-infected men. Only when the test is restricted to HPV16/18, the specificity of the test increased (77%), but the sensitivity dropped (62%), making this diagnostic system not suitable to detect individuals at risk to develop AC [136].

In AC, the high-resolution anoscopy is the standard procedure to diagnose this type of cancer, although several biomarkers such as Ki-67, $p16^{INK4a}$, and others have been investigated to improve the accuracy of histologic diagnosis. Up to date, only the $p16^{INK4a}$ IHC test has been very well documented to increase the predictive positive value of the histopathology test to identify correctly AC cases [137].

In the case of other anogenital HPV-associated cancers such as vulvar, vaginal, or penile, the diagnostic is carried out by identifying a lesion by visual examination from the genital site to the perianal area, and confirming by performing a biopsy. Screening tests are not available and there are no recommended screening methods to detect HPV infections in these types of cancers. From the surrogated markers of HPV infection, $p16^{INK4a}$ was the only marker with sufficient data to support its utility in the evaluation of lower anogenital tract lesions [138].

6.3. Head and neck cancers

HNCs are often detected at late stages, as conventional visual and tactile examination is a way to diagnose this type of cancer. Substantial efforts to develop oral lesion detection systems such as those based on autofluorescence or tissue reflectance (e.g., the Dentlight Oral Exam Light kit, Microlux DL, Orascoptic DK, Sapphire Plus, Trimira Identafi 3000, and ViziLite-Blue and VELscope) have been developed. However, the ability of these tests to discriminate between cancer and benign mucosal lesions is limited and because of this, the OC screening guidelines still do not recommend these diagnostic tests for routine screening of asymptomatic adults for HNCs [139]. The presence of HPV DNA in saliva was thought to be promising for early detection, but the test showed to have low sensitivity and specificity. For this reason, more studies are required to define the populations where the HPV test could have a positive impact and to evaluate the clinical value of this test [140].

On the other hand, the variable prevalence reported for HPV in HNCs could be attributed to the anatomical site where the sample is taken, but approximately 25% of all HNCs are HPV-DNA positive, and type 16 is the most prevalent. The variations in viral prevalence among different studies may be due to a combination of lesions of the different head and

neck anatomical subsites, sample sizes, sampling techniques (frozen, formalin-fixed or paraffin-embedded sections, scraping or oral rinses), and the methodologies used to detect HPV. Because of this, rigorous criteria should be considered for the separation of samples from the various anatomical subsites of the head and neck, as well as to increase sensitivity, specificity, accuracy, and reproducibility of the HPV tests for this type of cancer [140, 141].

Recently, it was suggested that HPV-DNA status in HNC should be analyzed together with other specific markers of active infection such as E6/E7 mRNAs transcription or $p16^{INK4a}$ expression, thus to better characterize these types of HPV-associated cancers. In this sense, the presence of E6/E7 mRNAs and $p16^{INK4a}$ expression was detected mostly in OPC [142]. Numerous other markers such as Ki-67, over-expression of epidermal growth factor receptor, p53, and others, have been studied in HNCs, but none of them have been consistently reliable [140, 141].

6.4. Serological biomarkers for the detection of HPV-associated cancers

The diagnostic of HPV infections for the detection of HPV-associated cancers has been difficult as there is no general screening test that alone or in combination with others allows the early identification of the disease. Because of this, it is necessary to look for tests that would be highly sensitive, specific, less invasive, and inexpensive, and that could be implemented at the general population.

It has been very well described that during the viral cycle, there is a sequential expression of the HPV proteins, which has been associated to different infection stages such as replication (associated to E4 protein), transformation (associated to E6/E7 proteins), and past infections (associated to L1 protein). Thus, the organism generates an antibody response against the viral antigens, in the same way as they are expressed during the viral cycle, letting the identification of different infection stages. Therefore, the humoral immune response naturally amplified the reaction against HPV antigens so that this becomes a good source of new biomarkers to detect HPV-associated premalignant lesions at risk of developing cancer. As a result, the presence of antibodies against E4 protein has been related to viral replication, whereas anti-E7 antibodies are considered markers of a current HPV-associated malignancy [7, 63, 143]. In this context, the use of serological markers has been constantly studied to identify patients with different types of cancer associated with HPV. At present, most of the studies have focused mainly on CC and HNCs.

To study these HPV serological biomarkers, different techniques and reagents have been developed; as for instance, recombinant fusion proteins have been used as antigens in Western blot; synthetic peptides with important immunogenic epitopes for B cell are used in ELISA tests and modifications of this technique have been used to increase the specificity and sensitivity of the assay (**Tables 1** and **2**). Other systems developed to detect HPV antibodies involve the *in vitro* protein transcription and translation, and this is used for radioimmunoassays and also for a novel slot-blot system [7, 61, 62, 69, 143]. The use of these tests to measure anti-HPV antibodies in different populations have shown the utility of these as biological markers of different types of lesions not only at the uterine cervix, but also at other anatomical sites.

In the uterine cervix, antibodies against HPV16 E6 and E7 were detected late during the development of CC with a new streptavidin-biotin capture ELISA, and were pointed as bad prognosis markers [81]. In a retrospective study, by using an ELISA-GST, the presence of anti-E6 and anti-E7 IgG antibodies were identified between 0.5 and 5 years before CC diagnosis, suggesting the usefulness of these antibodies as disease predictors [144]. More recently, in a study conducted by Salazar-Piña and coworkers, by using a novel slot-blot system, they showed that anti-E4 + anti-E7 antibodies discriminate CC from CIN 2-3 lesions with high sensitivity (80%) and a low false negative rate (20%), which corroborate the usefulness of these antibodies as markers for early detection of CC. In this study, they also observed that anti-E4 antibodies alone could be useful as HPV exposure markers at early stages of the disease [69].

Similar results were also observed with OPC, where a bead-based multiplex serology method (multiplex-GST) was used to detect different anti-HPV antibodies and showed a strong association between HPV16 E6 seropositivity and the disease, which suggests that these antibodies can be predictive markers of the disease as they were present more than 10 years before the cancer diagnosis [101, 102]. The sensitivity and specificity of this multiplex-GST system for anti-E6 antibodies was close to 100%, and because of that these antibodies have been proposed as a tool for diagnosis and prognosis of HPV-OPC [91, 145]. This multiplex-GST system also showed a high sensitivity (82%) and specificity (100%) for anti-E2 antibodies in the diagnosis of HPV-OPC. However, low sensitivity and specificity were observed for anti-E7, anti-E1, anti-E4, and anti-L1 antibodies [145].

All this together suggests that anti-HPV antibodies are promising diagnostic, prognostic, and potentially screening markers of HPV-associated cancers, as the presence of anti-HPV serum antibodies can vary according to the anatomical site where the cancer is generated by the HPV infection, however, more studies of anti-HPV antibodies are needed to validate them as serological markers for HPV-associated cancers.

7. Conclusions

This review of serological biomarkers is not intended to be an exhausted one, but rather to bring together the most important findings in the field and to point out the usefulness of these biomarkers in the diagnostic and prognostic of the different HPV-associated cancers. Numerous methods are being developed to detect HPV and related biomarkers that alone or in combination can be used to improve the positive predictive value of current screening methods, and to be able to identify precancerous lesions with a high risk of progression to cancer.

At present, serological anti-HPV antibodies are promising diagnostic, prognostic, and potentially screening markers of HPV-associated cancers. It is likely that a combination of anti-HPV antibodies will generate profiles that could discriminate precancerous lesions in progression to cancer, and also to differentiate the presence of HPV-associated cancers at different anatomical sites. For instance, it was shown that anti-E4 antibodies are associated to CIN1-2 lesions and that the profile anti-E4 + anti-E7 antibodies are useful for early detection of CC, while the presence of anti-E1/E2 + anti-E6 antibodies are prognostic of OPC. Besides, the

immunoglobulin isotype also seems to play an important role in differentiating the site where the HPV infection develops, as it was shown in CC. It is clear that the presence of anti-HPV serum antibodies can vary according to the anatomical site where the cancer is generated by the HPV infection. These results are very promising, however, more studies with larger populations, different anatomical sites, evaluation in precancerous lesions, and the development of new and more sensitive methodologies are required to better characterize the humoral immune response against HPV and to validate these anti-HPV antibodies as serological markers of different HPV-associated cancers.

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Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish.

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Immunotherapy is an innovative, leading and valuable approach to the treatment and control of many diseases. It can solve many problems of public health worldwide. Many people in numerous countries are suffering from a wide range of diseases (communicable and non-communicable) that can be cured or controlled by the immune system and immunotherapy. Some immunological diseases (i.e. allergic reactions and asthma, autoimmune disease, immunodeficiency disease, hypersensitivity reactions, etc.) have immune response pathophysiology and by controlling immune system mechanisms, these diseases can be controlled and cured.

Immunoregulatory Aspects of Immunotherapy focuses on immune system mechanism, diagnosis, treatment and other related problems. The chapters have applicable and scientific data in immunotherapeutic approaches based on medical sciences, and would be of benefit to all researchers in immunology, allergy and asthma fields. The book discusses the prevention, diagnosis, treatment and follow-up of patients who have dangerous diseases. We hope this book will be a new approach to the immunotherapy of diseases and will improve public health and wellbeing.

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