The cells of the immune system are lymphocytes (T-cells, B-cells and NK (natural killer) cells), neutrophils, eosinophils, and monocytes/macrophages. This book is an overview of some types of these cells and their role in recognizing and/or reacting against foreign material. The immune system is characterized by collaboration between cells and proteins. The development of all cells of the immune system begins in the bone marrow with a hematopoietic stem cell. Two chapters deal with neutrophils, three chapters with T-cells, four chapters with eosinophils, and other chapters review the immunomodulation of macrophages, the role of transcription factor KLF4 in regulating plasticity of myeloid-derived suppressor cells, immune reconstitution after allogeneic hematopoietic stem cell transplantation, and role of sorption detoxification in the therapy of acute radiation sickness.
Cells of the Immune System

Edited by Ota Fuchs and Seyyed Shamsadin Athari

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Cells of the Immune System

Contributors

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Preface

Neutrophils are the most abundant white blood cells in circulation. Neutrophils are a key component of innate immunity and an important regulator of adoptive immunity. Therefore, neutrophils are very important in health. The first chapter of this book deals with development of neutrophils and their role in hematopoietic microenvironment regulation. The second chapter discusses the function of neutrophils as the first line of defense and their functional activity that contributes to the high susceptibility to bacterial infections in patients with diabetes mellitus. Type 1 diabetes is associated with a reduction of circulating neutrophils. The thymus, the main organ for T lymphopoiesis, requires a permanent influx of progenitors from bone marrow or the fetal liver. Chapter 3 analyzes CD4+ T helper (TH) cells, which play a central role in the adaptive immune system by controlling a variety of cellular responses, defending the host against pathogens and tumor development. Their cytokine secretion suppresses or stimulates immune responses and leads to antibody production by B cells, immunoglobulin class switch, and macrophage activation. The various TH cell subsets can be differentiated from naive CD4+ T cells. Chapter 4 reviews tissue-resident memory T-cells, a lymphocyte lineage that occupies tissues without recirculating. They provide a first response against infections at body surfaces, where they accelerate pathogen clearance. Chapter 5 focuses on the microRNA (miRNA) from the secretome, which induces tolerogenic or proinflammatory responses. This response is in a murine model of autoimmune type 1 diabetes. This secretome miRNA approach may prove useful in treating both autoimmune diseases and cancer. Chapter 6 discusses the immunomodulation of the macrophage to better understand the effects of the physiological microenvironment factors on macrophage polarisation. Chapter 7 deals with the role of the transcription factor KLF4 (Krüppel-like factor 4) in regulating plasticity of myeloid-derived suppressor cells (MDSCs). MDSCs are bone marrow-derived cells with the ability to suppress the host immune responses, especially T-cell proliferation and cytokine production. Chapters 8-11 review eosinophils and eosinophilic disorders. Immune reconstitution after allogeneic hematopoietic stem cell transplantation is reviewed in Chapter 12. The slow T-cell reconstitution is regarded as being primarily responsible for deleterious infections with latent viruses or fungi, occurrence of graft-versus-host disease, and relapse. The role of sorption detoxification in therapy of acute ionizing radiation sickness is summarized in Chapter 13. Carbon adsorption therapy for the treatment of myelosuppression caused by acute ionizing radiation sickness and cytostatics is discussed.

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Section 1

Neutrophils
Section 1

Neutrophils
Chapter 1
Introductory Chapter: Development of Neutrophils and Their Role in Hematopoietic Microenvironment Regulation

Ota Fuchs

1. Development of neutrophils

Neutrophils are the most abundant white blood cells and play a key role in the elimination of pathogens (invading microorganisms). These specialized innate immune cells are a type of polymorphonuclear leukocyte. Humans produce about 10^{10} to 10^{11} neutrophils daily in the bone marrow from myeloid precursors in a process known as granulopoiesis. The initial precursors of neutrophils are hematopoietic stem cells (HSCs) [1]. The majority of adult blood and immune cells are derived from HSCs, which are also capable of generating new HSCs in a process called self-renewal. The interaction of HSCs with their particular microenvironments, known as niches, is important for maintaining the stem cell properties of HSCs, including cell adhesion, survival, and cell division [2].

1.1 The generation of committed proliferative neutrophil precursors

During the development, HSCs lose their self-renewal potential and produce multipotent precursors (MMPs). All blood cell lineages can be developed from MMPs (Figure 1). The differentiation of MMPs into erythro-myeloid or lympho-myeloid progenitors is directed by the antagonistic transcription factors GATA-1 and PU.1. High levels of PU.1 are important for the differentiation of MMPs into lympho-myeloid precursors (LMPs), progenitors for granulocyte-monocyte precursors (GMPs). The most important regulator of physiological granulopoiesis are granulocyte colony-stimulating factor (G-CSF) and its receptor whose effects include commitment of progenitor cells to the myeloid lineage, proliferation and differentiation of granulocytic precursors, release of mature neutrophils from the bone marrow, and modulation of their phagocyte function [3]. Neutrophils carry high levels of G-CSF receptor on their surface through their development and also in mature neutrophils. Humans deficient in G-CSF or its receptor have neutropenia. Interleukin (IL)-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3 also stimulate granulopoiesis but are not essential [3]. The transcription factor family of CCAAT enhancer-binding proteins (C/EBPs) is involved in neutrophil development. Granulocytes and macrophages differentiate from the common GMPs [4–6]. Three neutrophil subgroups were identified within the bone marrow by mass cytometry and cell cycle-based analysis [4]. Committed proliferative neutrophil precursors differentiate into nonproliferating immature neutrophils and mature neutrophils.
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Transcription factor C/EBPε is necessary for the generation of committed proliferative neutrophil precursors from the GMPs [7]. The deficiency of C/EBPε caused phenotypic and functional abnormalities of neutrophils and impaired their chemotaxis and bactericidal action [8–10]. The gene for C/EBPε knockout mice (CEBPE) displays a block in terminal granulocytic differentiation and fails to produce functional neutrophils and eosinophils. CEBPE-nul mice are susceptible to gram-negative bacterial sepsis and die from systemic infection. Loss-of-function CEBPE mutations have been found in patients with defects in neutrophil function and with neutrophil-specific granule deficiency [11–13].

Granulocyte development requires also SMARCD2 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 2), also known as BAF60b (BRG1/Brahma-associated factor 60b) [13, 14]. SMARCD2-deficient mice were not able to generate mature and functional neutrophils and eosinophils. SMARCD2 interacts with the transcription factor C/EBPε and controls the expression of neutrophil proteins.

1.2 Neutrophil terminal differentiation, granules and secretory vesicles, and release of neutrophils from the bone marrow

Neutrophil precursors differentiate into myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cells, and finally segmented neutrophils (Figure 1) [15, 16]. Three types of neutrophil granules are formed continuously starting at the promyelocyte stage during the differentiation process [16, 17]. Primary granules, also known as azurophilic granules, are generated in promyelocytes and contain myeloperoxidase (MPO). Secondary granules, also named specific granules, contain...
lactoferrin and are MPO-negative. Tertiary (gelatinase) granules contain matrix metalloproteinase 9, also known as gelatinase B.

Terminal differentiation of neutrophils is regulated by a balance between transcription factors. Runx 1 and c-myb were heavily expressed at the early stages of neutrophil differentiation and are required for the expression of azurophilic granule proteins such as MPO and elastase [18]. Runx1 and c-myb stimulate proliferation, and their downregulation after the myelocyte stage is connected with terminal neutrophil differentiation. C/EBPα is required for granulopoiesis and is found all over the neutrophil differentiation. The selective block in neutrophil differentiation was found in mice with a targeted disruption of the CEBPA [19]. On the other hand, an induced expression of the CEBPA is necessary for the induction of terminal granulocytic differentiation in 32Dcl3 myeloblasts [20]. The levels of C/EBPβ, C/EBPδ, and C/EBPζ increased substantially at the stage of metamyelocytes where proliferation terminates. However, a high expression of C/EBPγ is connected with proliferation. C/EBPγ is devoid of a transactivating domain and can inhibit other C/EBPs in their transactivating function. Cell cycle arrest and initiation of terminal neutrophil differentiation in metamyelocytes are associated with downregulation of the proliferation-stimulating factors (Runx1, c-myb, C/EBPγ) as it has been mentioned above. PU.1 transcript was found at all stages of neutrophil differentiation [18, 21]. This transcription factor is important for optimal gene expression of both early (MPO, proteinase-3, and elastase) and late (Toll-like receptors 2 and 4 (TLR2, TLR4), CD35, complement receptor 1 (CR1)) neutrophil markers [18, 21].

Neutrophils contain easily mobilized secretory vesicles (intracellular storage granules formed by endocytosis) that can transport their content to plasma membranes of human neutrophils [22–25]. Polymorphonuclear neutrophils contain multiple distinct secretory compartments that are sequentially mobilized during cell activation. Complement receptor type 1 is a marker for a readily mobilizable secretory vesicle compartment, which can undergo exocytic fusion with the plasma membrane independently of the secretion of traditional granule contents.

Release of neutrophils from the bone marrow requires the coordinate action of G-CSF and ELR-type CXC (two N-terminal cysteines separated by one amino acid) chemokines with a specific amino acid sequence (or motif) of glutamic acid-leucine-arginine (or ELR for short) such as CXCL1 and CXCL2 [26]. The interaction between CXC chemokine receptor 4 (CXCR4), a G protein-coupled receptor, and its main ligand stromal-derived factor 1 (SDF-1, also known as CXCL12) retains neutrophils within the microenvironment of the bone marrow. Efficient mobilization of neutrophils requires G-CSF-mediated disruption of the neutrophil retention mechanism and activation of neutrophil migration [26]. Deletion of CXCR4 or CXCR2 has a similar negative effect on neutrophil migration from the bone marrow to circulation. The development from a neutrophil myeloblast to a mature polymorphonuclear neutrophil lasts approximately 14 days [21]. The postmitotic phase lasts 6–7 days [21].

2. Regulation of hematopoietic stem cells in the bone marrow
microenvironment by neutrophils

2.1 Various cells influence the bone marrow microenvironment

Stem cell niches are local tissue microenvironments that promote the maintenance of stem cells and regulate their function by producing factors that act directly on stem cells [27]. The determination of niche cells can be based on the
identification of cells which synthesize HSC regulators (CXCL12, CXCL4, stem cell factor (SCF), thrombopoietin, osteopontin, transforming growth factor-β, vascular cell adhesion molecule 1 (VCAM1), glycoprotein 130, Notch ligands such as Jagged-1, fibroblast growth factor 1, angiopoietin-1, and pleiotrophin). Many further factors produced by other tissues can also affect stem cells and their microenvironment. The main site of hematopoiesis in adults is bone marrow. However, in response to severe hematopoietic stresses, extramedullary hematopoiesis was found in other niches of the liver and the spleen HSCs.

In the steady state, 90% of neutrophils reside in the bone marrow, and only 1–2% of neutrophils are present in the circulation [28]. Two neutrophil-derived proteases, cathepsin G and elastase, cleave receptors and cytokines essential for HSC retention in the bone marrow (CXCR4, CXCL12, and VCAM1) and change the HSC-supportive properties of the bone marrow [28]. Further experiments showed that neutrophil-derived proteases are not necessary for HSC mobilization. Macrophages, megakaryocytes, regulatory T cells (Tregs), and neutrophils influence HSC homeostasis and fate.

A majority of HSCs in the bone marrow localize near the sinusoidal blood vessels. Endothelial cells supply HSCs by CXCL12 and SCF. Both these factors are important for HSCs maintaining within the microenvironment of the bone marrow [29, 30]. Nestin-positive (Nes+) cells are important hematopoiesis-supporting constituents in an adult bone marrow. Studies using green fluorescent protein under the direction of nestin promoter/enhancer (Nes-GFP) revealed two groups of mesenchymal progenitor cells, one associated with arterioles (bright cells) and one associated with sinusoids (dim cells). The sympathetic nervous system also regulates hematopoietic stem cells in the bone marrow microenvironment [31].

2.2 Tumor necrosis factor α (TNF α) synthesized by activated bone marrow neutrophils and its role in the regeneration of the damaged hematopoietic stem cell microenvironment

Gr1/Ly6G (lymphocyte antigen 6 complex locus G6D) is a 21–25 kD glycosylphosphatidylinositol (GPI)-linked differentiation antigen that is expressed by myeloid-derived cells in a tightly developmentally regulated manner in the bone marrow. CD115 (M-CSF receptor) has been used to define monocytes. Bowers et al. showed that bone marrow Gr1−CD115+ neutrophils support the regeneration of the damaged vascular hematopoietic microenvironment in mice after transplantation [32]. Bone marrow Gr1−CD115+ neutrophils are a heterogenous population which contains proliferating neutrophil progenitors and immature and mature neutrophils with different transcriptional signatures in comparison with circulating neutrophils [4]. Bone marrow neutrophils are selectively recruited to the damaged sinusoidal vasculature, where they secrete TNFα. This cytokine is a potent inducer of new blood vessel growth (angiogenesis) [32]. The treatments used before transplantation for abolishment of the host hematopoietic cells destroy the bone marrow vascular microenvironment. Donor HSCs increase their proliferation and neutrophils together with other myeloid cell production. Therefore, the hematopoietic progenitor engraftment is facilitated by neutrophils [33, 34].

3. Various phenotypes and functions displayed by neutrophils

Neutrophils are innate immune cells engaged in protection against bacterial, viral, parasitic, and fungal pathogens and in tissue repair [35]. Infected tissues and tissues with sterile stress can be also damaged by the toxic activity of neutrophils [35].
Neutrophils have very efficient migratory capabilities. Neutrophils are released from the bone marrow and circulate in the blood for about 12 h and disappear with circadian frequency [35]. As neutrophils disappear from circulation, they infiltrate the vast majority of naive tissues, mainly the spleen, lungs, and liver, and have nonimmune regulatory functions together with supporting B-cell maturation and immunoglobulin production. These infiltrated neutrophils affected cell survival, migration, and susceptibility to cancer.

In addition to direct effects during injury and infections, neutrophils are also able to regulate immunity through modulation of antigen-presenting dendritic cells [36]. The cross talk between neutrophils and lymphocytes takes place at the interface between innate and adaptive immunity. Neutrophils can become involved in positive or negative regulation of interactions with various T cells such as T helper type 1 (Th1), Th2, Th17, Treg, CD8, and γδ T cells [37, 38].

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Chapter 2

Neutrophil Function Impairment Is a Host Susceptibility Factor to Bacterial Infection in Diabetes

Daniella Insuela, Diego Coutinho, Marco Martins, Maximiliano Ferrero and Vinicius Carvalho

Abstract

Diabetes mellitus is a highly prevalent noncommunicable disease globally. One of the main complications of diabetes is the increased susceptibility to bacterial infection. Neutrophils play a crucial role in inflammatory response against bacterial infections, once they are the first cells recruited to the sites of injury. In diabetes, there is a failure in the neutrophil functions, including migration, ROS production, phagocytosis, and bacterial killing, which are associated with the high incidence of bacterial infections. Herein, we point out pieces of evidence revealing the primary molecular mechanisms involved with impairment of neutrophil functions in diabetes, with relationship with high susceptibility to bacterial infections.

Keywords: diabetes, bacterial infection, neutrophils, inflammation, chemotaxis

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by a hyperglycemic condition that results in several complications, such as neuropathy, nephropathy, retinopathy, and increased risk of cardiovascular disease [1]. DM can be classified into type 1 (T1DM) and type 2 (T2DM). T1DM is common in childhood or young adulthood and is a result of autoimmune destruction of beta-cells in pancreatic islets mediated by T cells, leading to defect in insulin synthesis [2, 3]. The T2DM appears mainly in adulthood, affecting people with the most productive age. This type of diabetes is associated with insulin resistance and inadequate compensation by beta-cells, leading to a relative insulin deficiency [1, 4]. Currently, it is known that there are over 425 million people with DM globally. Worryingly, it is estimated that in 2045, this number will grow to over 600 million [5].

Hyperglycemia, a hallmark of DM, is associated with patient vulnerability to bacterial infections, such as tuberculosis and pneumonia, besides more severe sepsis of bacterial origin [5]. In fact, diabetic patients generally present microbial persistence, greater susceptibility to new infections, recurrences, and an increase in the risk of mortality if compared to nondiabetic individuals [5, 6]. This is due to the compromised immune response presented by diabetic patients, which leads to failure in leukocytes protective effects. Cyclically, infection profile in these patients can worsen glycemic control [5]. Neutrophils present an important role in host immune response to bacterial infection, once they are one of the first leukocytes
that arrive in the infected area [7]. In normal conditions, these cells act by different manners against microorganism, leading to infection control and resolution of the inflammatory process. However, the immune response in diabetic patients is characterized by impairment in neutrophil function [7, 8]. Here, we revised the mechanisms involved with the failure of neutrophil functions noted in DM and its relationship with the high susceptibility to bacterial infections.

2. Role of neutrophils in bacterial infections

2.1 Migration of neutrophils to infected sites

Neutrophils are polymorphonuclear (PMN) versatile innate effector cells essential for immune defense, which arise from hematopoietic stem cells (HSCs) in bone marrow [9]. Under normal conditions, about $5 \times 10^{10} - 10 \times 10^{10}$ new neutrophils are produced in the bone marrow daily [10, 11]. Chemokine gradients and adhesion molecules are central players that regulate neutrophil release from the bone marrow [11]. Neutrophils express CXC receptors (CXCR)-1 and CXCR2 that interact with CXC chemokines (CXCL1/KC, CXCL2/MIP-2, and CXCL8/IL-8) and result in neutrophil migration from bone marrow into the bloodstream. Neutrophils also express CXCR4, which interacts with CXCL12/SDF-1 produced by osteoblasts and other stromal cells to mediate neutrophil maintenance in the bone marrow [10, 12]. Thereby, only a small fraction of mature neutrophils is released into the blood.

Under bacterial infection, sentinel cells detect the microorganisms via pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). These receptors identify highly conserved pathogen-associated molecular patterns (PAMPs), including peptidoglycan (PGN) and lipopolysaccharide (LPS) expressed in the cell membrane surface of bacteria. They can also recognize danger-associated molecular patterns (DAMPs), such as high mobility group protein B1 (HMGB1), ATP, and uric acid, released from damaged and necrotic cells after tissue injury. Then, sentinel cells release mediators such as granulocyte colony-stimulating factor (G-CSF), which leads to neutrophil production and release from bone marrow via upregulation of CXCR2 and its ligands, and reduces expression of CXCL12/SDF-1 and CXCR4. After this event, neutrophils can be mobilized to sites of infection and combat microorganism [13–15].

Correct leukocyte recruitment requires the adhesive interactions between P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), and CD44 expressed on the neutrophil membrane surface to the P- and E-selectin which are upregulated in endothelial cells of inflamed tissue. These processes will lead to neutrophil capture and fast rolling [16, 17]. Rolling event exposes neutrophils to chemokines that are arrested on the glyocalyx of endothelial cells, such as CXCL8/IL-8. Then occurs the activation of integrin molecules such as VLA-4 (CD49D/CD29), macrophage-1 antigen (MAC-1 or CD11b/CD18), and lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) on neutrophils [10, 18]. The integrin binds to their ligands such as intercellular adhesion molecule (ICAM)-1, ICAM-2, and platelet endothelial cell adhesion molecule-1 (PECAM-1) on endothelial cells, resulting in slow rolling and firm adhesion of the neutrophil to endothelial cells [17]. Thence, the neutrophils perform diapedesis toward the tissue and migrate along a chemokine gradient until they arrive in the infected site. The long-distance recruitment is mediated by chemoattractants, including leukotriene B4 and CXCL8/IL-8, while near chemoattractants are peptides and C5a [17].
Despite the canonical neutrophil migration during infections, in sepsis occurs an inadequate migration of neutrophils even with high levels of chemokines at the infection site [12]. The decrease of CXCR2 expression on the cell surface of neutrophils is among the mechanisms leading to this failure. The prolonged exposure to CXCR2 agonists, which leads to phosphorylation of G protein-coupled receptors (GPCRs) by GPCR kinases (GRKs) and induces the desensitization and internalization of CXCR2, can explain the down-regulation of this receptor [16]. In addition, the activation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) by inflammatory products, such as C-reactive protein (CRP), bacte- rial products, apoptotic cells, or activated platelets, can also account for CXCR2 neutrophil endocytosis [12, 19].

2.2 Actions of neutrophils in infected sites

At the sites of infection, neutrophils can combat pathogenic microorganisms and clear infections by different ways including phagocytosis, degranulation of microbicidal molecules, production and secretion of reactive oxygen species (ROS), and release of neutrophil extracellular traps (NETs) [20]. For efficient bacterial phagocytosis, the microorganism needs to be covered with opsonins, such as immunoglobulins (Igs) and components of the complement system, which are recognized by neutrophil specific surface receptors. After phagocytosis of an opsonized pathogen by neutrophils, there is a mobilization of intracellular granules or lysosomes, leading to the killing of the ingested bacteria [21].

Neutrophil activation can induce the production of ROS to combat infection. It happens mainly due to the action of NADPH oxidase complex (NOX), but can also be generated by mitochondria. After neutrophil activation, NOX acts converting molecular oxygen (O₂) into superoxide anion (O₂⁻) which suffers dismutation, spontaneously or catalyzed by myeloperoxidase (MPO), generating hydrogen peroxide (H₂O₂) [22, 23]. In addition to ROS, neutrophils can also enhance the inducible nitric oxide synthase (NOS2) expression, which will convert O₂ to nitric oxide (NO), resulting in reactive nitrogen species (RNS). Both ROS and RNS contribute to microbicidal activity and are crucial for the defense against intracellular microorganisms [22]. Curiously, NO is supposed to be involved in the failure of neutrophil migration during sepsis, once it stimulates the internalization of CXCR2 on the neutrophil surface and reduces expression of adhesion molecules, leading to diminished leucocyte rolling and adhesion to the endothelium [12]. After neutrophil migration, degranulation occurs, which is the process mediated by microbial or inflammatory stimuli in which neutrophils release the granule contents, such as MPO, defensins, cathepsin G, neutrophil elastase, and collagenase. These granule contents are released by exocytosis or into the phagosome to kill microorganisms [24].

Neutrophils may also perform the antimicrobial activity directly attacking and restraining microorganisms by releasing NETs (NETosis) [25, 26]. NETs are extracellular fibrous structures composed by a network of extracellular chromatin fibers, histones, antimicrobial peptides, and enzymes, including MPO, α-defensins, cathepsin G, elastase, and lactoferrin, to capture and kill microorganisms [12, 20, 26]. NETosis occurs after neutrophil exposure to bacteria or stimulation with mediators such as interleukin CXCL8/IL-8. This neutrophil stimulation will result in activation of intracellular pro-inflammatory kinases, such as Akt, p38 MAPK, or MEK/ERK, a release of neutrophil elastase, oxidative burst, and actin polymerization [20, 26, 27]. This mechanism will result in microorganism destruction and neutrophil death [25]. NETs also limit the microorganism growth and dissemination; however, excessive formation of NETs in association with the
uncontrolled inflammatory response that occurs in sepsis can result in multiple organ damage to the host [12, 20].

2.3 Resolution of neutrophilic inflammation

Resolution phase is an essential process to interrupt the inflammatory response after the danger signal or when microorganism has been eliminated, preventing the development of chronic inflammation and fibrosis [28]. Resolution of inflammation was previously considered a passive response, associated with clearance of inflammatory stimulus, reduction of pro-inflammatory mediators, and prevention of leukocyte recruitment. Currently, it is known that resolution is an active and tightly controlled process, carried out by specialized pro-resolving mediators (SPM) such as resolvins, lipoxins, maresins, and protectins, which are produced locally from polyunsaturated fatty acids and act orchestrating the end of inflammation, but do not evoke unwanted immunosuppression [29, 30]. For a correct resolution of inflammation, the neutrophil reverse migration, lymphatic drainage, exudation to the external environment, apoptosis of activated neutrophils followed by efferocytosis, and autophagic clearance of intracellular inflammatory signals are necessary [31, 32].

The reduction in neutrophil recruitment is regulated by a class-switch from the production of pro-inflammatory to pro-resolving mediators, resulting in down-regulation of CXCR2 on neutrophils [28]. Pro-resolving lipid mediators also resolve inflammation by promoting neutrophil apoptosis [28, 32]. Apoptotic neutrophils or cell bodies are phagocytosed by professional phagocytes, mainly macrophages, in a process known as efferocytosis. This event is mediated by an interaction between phosphatidylserine expressed on the neutrophil surface and macrophage receptors, such as TIM1 and TIM4 [32, 33]. During resolution, macrophages change their profile decreasing the pro-inflammatory feature and acquiring anti-inflammatory and pro-resolving functions, acting in apoptotic cell clearance, and producing immune regulatory intracellular messengers, including cyclic adenosine monophosphate (cAMP) [28, 34]. Macrophage phagocytosis allows the complete elimination of dead neutrophils and tissue debris of the infected and inflamed area. Generally, this process is followed by macrophage autophagy [35, 36]. Together, all these processes contribute to the resolution of neutrophil inflammation and tissue homeostasis [31].

3. Impaired neutrophil migration in diabetes

The causes of increase in susceptibility to infections in DM are not yet fully known, but one of the possible and well-established explanations is that diabetics present an impairment in defense mechanisms of innate immunity, including neutrophil migration to the site of inflammation, phagocytosis, ROS production, and bactericidal activity [37].

The number of neutrophils in the circulation is also altered in DM. Older studies have shown that in T1DM patients, there is an increase in neutrophil counts compared to healthy individuals [38, 39]. Recent researches described a decrease in circulating neutrophil numbers in T1DM patients in comparison with nondiabetics [40, 41]. Impairment in neutrophil yield and maturation in bone marrow, increase in peripheral neutrophil consumption, and/or tissue sequestration could explain this reduction in blood neutrophil counts observed in T1DM [42]. This divergence between studies can be attributed to differences between ethnic groups and the discovery of the existence of various stages of DM [43]. While in T1DM, the data about circulating neutrophil counts are still controversial, most of the studies
described that in T2DM patients, there is an increase in the number of neutrophils in circulation in comparison to healthy individuals [40, 44]. This neutrophilia was related to elevation in the circulation levels of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and CRP, a known marker of inflammation [45].

Regarding migration, in vitro studies described a reduction in CXCL8/IL-8, platelet-activating factor (PAF), or N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis of neutrophils from T1DM or T2DM patients compared to cells from healthy subjects [40, 46]. In situ evaluation of chemotaxis toward fMLP using T1DM rat neutrophils also revealed a deficiency in migration. This impairment in neutrophil chemotaxis was positively related to DM severity which was characterized by glycaemia values greater than 400 mg/dL [47]. In addition, blood neutrophils of diabetic animals presented a decreased migratory response to CXCL2/MIP-2 in vitro and in vivo compared to nondiabetic animals. Despite the deficiency in CXCL2/MIP-2 induced-neutrophil migration, there was no difference between the expression of CXCR-2, a CXCL2/MIP-2 receptor, on neutrophils from diabetic animals [48, 49]. Similarity in CXCR-2 mRNA levels was also found among bone marrow neutrophils obtained from NOD mice (a strain that spontaneously develops T1DM), NOR mice (a strain that is resistant to diabetes), and control mice strain. However, CXCR-1 mRNA levels were reduced in neutrophils isolated from NOD mice in comparison to neutrophils from NOR and control mice [50]. Then, it is possible to consider that alterations in CXCR-1 expression and activity may also contribute to the impairment of neutrophil migratory activity in diabetics.

A feature well described in DM is the increase in oxidative stress which may also be related with impairment of neutrophil migration. Oxidative stress can induce glutathionylation (S-thiolation) of several proteins, including L-plastin (LPL) [51] that is expressed exclusively in leucocytes and controls polarization and migration of neutrophils through bundling of β-actin filaments [52]. Neutrophils from diabetic patients and from T2DM mice showed enhanced S-thiolation of LPL in comparison to neutrophils from nondiabetic subjects, which culminate with impaired fMLP-chemotaxis of neutrophils from diabetics. S-thiolation of LPL reduces its interaction with β-actin and this may be another mechanism involved in defective migration of neutrophils in DM [51].

In addition, T1DM rats administered with LPS by intra-tracheal route exhibited a reduction in neutrophil accumulation in the bronchoalveolar fluid (BAL), which occurred in association with a decrease in TNF-α and IL-1β levels, when compared with nondiabetic rats provoked with LPS. However, no difference was observed in relation to the expression of ICAM-1 and E-selectin in lung vascular endothelium and cytokine-induced neutrophil chemoattractant-1 (CINC-1) amount in BAL [53]. A deficiency in neutrophil migration to airways after LPS intra-tracheal injection was also observed in a spontaneous rat model of T2DM, using Goto-Kakizaki (GK) rats. This reduction in neutrophil migration to the airways in GK rats stimulated with LPS occurred despite an increase in the number of neutrophils in the blood. These data showed that there was no failure in the production of these cells by the bone marrow, but impairment in the recruitment mechanisms of these leukocytes to the lungs. Indeed, GK rats exhibited a decrease in IL1-β, IL-6, and TNF-α concentration in BAL and also a reduction in the expression of adhesion molecules, such as LFA-1 and ICAM-2, on neutrophils. All these alterations were associated with a reduction in the TLR4 expression and activation in neutrophils [54].

3.1 Failure in neutrophil migration associated with hyperglycemia

Hyperglycemia can influence various components of the immune response, including activities of inflammatory cells [55]. Incubation of human neutrophils
with supraphysiological levels of glucose decreased both chemotaxis in response to zymosan and phagocytosis/killing of the intracellular bacteria *Staphylococi in vitro*. In addition, high glucose concentrations increased neutrophil adherence *in vitro*, and this also can limit neutrophil locomotion from blood vessels toward infected tissues *in vivo* [56].

It is debated which mechanisms are involved in the benefit of insulin treatment on the immune response of diabetics. While some authors argue that the beneficial effects are dependent on the correction of hyperglycemia by insulin, others believe that the insulin may have direct actions on immune system independently of glycemic control [55]. Indeed, it has been shown that insulin *in vitro* increases human neutrophil chemotaxis induced by fMLP, calcium ionophore, or phorbol-myristyl acetate (PMA) [57, 58]. Besides, insulin presents a chemokinetics effect which required activation of tyrosine kinase and phosphatidylinositol 3-kinase (PI3K), but did not depend on protein kinase C (PKC) stimulation [59, 60]. Interestingly, in a hyperglycemic medium, the chemokinetic action of insulin in neutrophils is blocked by a mechanism that involved activation of PKC [60]. These data suggest that insulin is able to exert direct effects on neutrophils, but the maintenance of glucose levels is also important for actions of this hormone on these leukocytes. In addition to acting on neutrophils, insulin can increase expression of the PECAM-1 in endothelial HUVEC cells and thus enhance transmigration of neutrophils across these cells in response to fMLP *in vitro* [61]. Finally, *in vivo* studies showed that insulin restored neutrophil migration to the lungs in T1DM rats subjected to LPS provocation. This effect of insulin occurred in parallel to a reduction of 50% glycemia; however, the glycemic levels continued to be high in these animals compared to nondiabetic rats [53]. These data suggest that the action of insulin on LPS-induced inflammatory response was not totally dependent on its effect on blood glucose.

It is well known that chronic hyperglycemia upregulates the generation of advanced glycation end-products (AGE). AGEs are produced by a nonenzymatic reaction between reducing sugars, such as glucose, and amino acids of proteins. AGEs can induce cross-link between proteins and also can bind cellular receptors; among them, the best described is the receptor for AGE (RAGE) [62]. AGE accumulation has been associated with the development of several diabetic complications, including retinopathy, nephropathy, and neuropathy [63]. RAGE is expressed on neutrophils and its activation by AGEs, like glycated albumin, induces a transient rise in intracellular free-calcium levels and actin polymerization. Nevertheless, the dimension of increase in calcium levels induced by glycated-albumin is smaller than that induced by fMLP. In addition, glycated-albumin pre-treatment in neutrophils inhibited elevation of intracellular calcium levels promoted by fMLP, causing a defective signal processing and, consequently, a reduction in fMLP-induced transendothelial migration *in vitro* [64]. Furthermore, glycated collagen also inhibited chemotaxis in response to fMLP, and this effect was associated with the capacity of glycated collagen to increase adhesion strength of neutrophils *in vitro* [62]. In addition, the blockade of AGE formation in diabetic animals restored leucocyte rolling, adhesion, and migration in response to zymosan *in vitro* [65], and also restored neutrophil accumulation toward traumatic skin tissue induced by hot water [66]. Therefore, it is possible that in DM, AGEs promote sustained stimulation of neutrophils which decreases the responses of these cells to chemotactic stimulus.

A positive relation between hyperglycemia and serum NO levels was also described in rats [67], and some studies have reported an increase in serum or plasma NO concentrations in T1DM and T2DM patients [67, 68]. Human neutrophils treated with L-Arginine, a NO precursor, have decreased chemotaxis toward CXCL8/IL-8 *in vitro*, while treatment with NOS inhibitor increased CXCL8/
IL-8-induced chemotaxis of neutrophils in vitro [69]. In addition, a NO donor inhibited human chemotaxis promoted by fMLP in vitro and incubation with a guanylate cyclase inhibitor did not interfere with the effect of NO donor. These data suggested that the inhibitory action of NO on neutrophil chemotaxis is independent of cGMP [51]. The NO-induced impairment of neutrophil migration was confirmed using bone marrow neutrophils isolated from NOS2−/− mice stimulated with fMLP in vitro, which showed increased chemotaxis in comparison to that isolated from NOS2+/+ mice [51]. Furthermore, the pre-treatment with NOS inhibitor prevented impairment of neutrophil recruitment toward peritoneal cavity observed in severe sepsis [70]. Therefore, it is possible to hypothesize that deficiency on migration activity of neutrophils may be associated with increased serum levels of NO in diabetics.

3.2 Failure in neutrophil migration independent of hyperglycemia

DM has altered levels of several molecules in serum that are not directly related to hyperglycemia, some of which can interfere with components of immune response, including neutrophils. Alpha-1-acid glycoprotein (AGP) is one of the main acute-phase proteins in organisms; its synthesis depends mainly on liver, and during an inflammatory response, the concentration in serum increases. [71]. AGP can bind to hormones and interfere with functions of endothelial cells, platelets, and leukocytes, and in fact, it inhibits human neutrophil chemotaxis in response to fMLP in vitro [72]. In addition, intravenous administration of AGP in rats prevented migration of neutrophils to peritoneal cavity, reducing rolling and adhesion of these leukocytes on endothelium of mesenteric microcirculation induced by carrageenan [73]. DM patients present high serum levels of AGP [48], so it is feasible that AGP can mediate the impairment of neutrophil locomotion described in DM.

Furthermore, AGP-mediated neutrophil dysfunction was also demonstrated in diabetic animals upon sepsis induction by cecal ligation and perforation (CLP). Neutrophils from septic T1DM mice showed impaired rolling, adhesion, and migration from mesenteric tissue toward the peritoneal cavity, while accumulated in lung tissue. These observations were associated with an altered expression of adhesion molecules (CD62L–CD11b) and a clear reduction in CXCR2 in neutrophils from diabetic animals compared to nondiabetic, after CLP. Accordingly, neutrophils from diabetic mice presented an increased expression of GRK2, a key modulator of CXCR2 receptor desensitization, upon sepsis induction compared to control septic mice. AGP administration in septic nondiabetic mice impaired neutrophil migration to peritoneal cavity, augmenting GRK2 expression, and reducing CXCR2, which reproduced the diabetic condition. On the other hand, insulin treatment reduced GKR2 and augmented CXCR2 on neutrophils obtained from diabetic mice, while decreased AGP serum concentrations. Thus, AGP increased production is involved in neutrophil impaired migration to infection during diabetes, possibly by enhancing GRK2 expression and/or augmenting NO production in these cells [48]. Notably, CXCR2 downregulation in diabetic animals seems to depend on the presence of comorbidity since several studies showed no difference in CXCR2 expression between normal and diabetic mice.

Histamine for a long time was considered as a pro-inflammatory mediator whose main role is played in allergic inflammation. However, some evidence has shown that histamine can modulate other immunological events. Neutrophils express both histamine receptors, H1 and H2 [74] and activation of H2 inhibited human neutrophil chemotaxis in vitro [75]. Furthermore, blood neutrophils obtained after systemic or inhalatory administration of histamine in normal volunteers showed a reduction in chemotactic response to zymosan in vitro [75].
After septic stimuli, T1DM mice exhibited mast cell accumulation in the peritoneal cavity and higher plasma levels of histamine than nondiabetic mice. In addition, the augmented activation of H2 receptor promoted an increase in intracellular expression of GRK2 and cAMP levels in diabetic septic mice neutrophils, favoring CXCR2 desensitization [74].

Resistin is a cysteine-rich protein that belongs to the resistin-like molecule (RELM) family that, in humans, is released mainly by macrophages but can be also produced by adipose tissue [76]. Resistin impairs glucose tolerance and insulin action and therefore has been related to obesity–induced insulin resistance and T2DM [77]. Beyond metabolic effects, resistin can act directly in immune cells, including neutrophils. Resistin decreased fMLP-induced neutrophil chemotaxis in vitro through inhibition of PI3K pathway activation. Resistin also decreased oxidative burst in neutrophils after stimulation with PMA and Escherichia coli [78]. Since resistin directly affects neutrophil function and T2DM patients present higher serum levels of this hormone [79], it can be suggested that resistin is also involved with the deficiency of neutrophil responses in DM independently of hyperglycemia.

4. Neutrophil response to bacterial infections in diabetes

It is now generally accepted that high glucose concentrations impaired several functions of neutrophils beyond their migratory capacity, including phagocytosis and bacterial killing. Hyperglycemia hinders neutrophil activity by inducing higher concentrations of intracellular calcium and thereby reducing ATP levels, which in turn leads to reduced phagocytic ability of PMN cells. Nevertheless, under glycemic control, diabetic patients restored intracellular calcium levels and increased cellular ATP content in neutrophils, which consequently improved phagocytosis. In addition, hyperglycemia was shown to affect other immune and hemostatic responses during experimental human endotoxemia. Healthy patients submitted to high blood glucose levels presented a reduction of E. coli endotoxin-induced neutrophil degranulation and exaggerated coagulation. A reversal of these effects was observed when glucose was controlled with insulin therapy [55].

Neutrophils from diabetic patients showed increased production of inflammatory cytokines [80] and ROS without any stimulation, although neutrophil oxidative responses to certain pathogens appear to be predominantly suppressed in diabetes [64, 81, 82]. Furthermore, hyperglycemia led to decreased mRNA synthesis of different pro-inflammatory cytokines in neutrophils after LPS stimulation, compared with the euglycemic state [55]. In addition, T1DM mice showed a hyperglycemia-induced pre-activation of NOX, resulting in a significantly higher release of superoxide. Sustained hyperglycemic condition may, therefore, induce oxidative damage and the onset of diabetic complications, particularly at sites with neutrophilia [83, 84].

In DM, neutrophils increased basal ROS generation in a close-relationship to sustained hyperglycemia and the generation of AGEs [64]. On the other hand, decreased pathogen-stimulated ROS production is thought to be related to impaired glucose metabolism by the pentose-phosphate pathway, which produces NADPH that is a requirement for optimal superoxide generation by NOX [6]. Off noted, phagocytosis and NETosis were shown to depend on oxidative burst in neutrophils. Nevertheless, the relevance of the ROS production imbalance noted in neutrophils obtained from diabetics is not clear, since not all the diabetic patients with diminished ROS production presented recurrent bacterial infections [82].
4.1 Neutrophil dysfunction and sepsis

According to The Third International Consensus Definitions for Sepsis and Septic Shock, “sepsis is a life-threatening organ dysfunction secondary to a deregulated host response to an infection” [85]. During septic processes, serum inflammatory marker concentration increases in patients although innate immune response appears to be impaired. Particularly, defective neutrophil recruitment to the sites of infection was reported in animal models of sepsis [86, 87]. Clinical studies reported that the incidence of sepsis is increased in diabetic patients [5]. Accordingly, DM is associated with high severity of sepsis, likely due to compromised immune responses, such as adhesion, chemotaxis, phagocytosis, and bacterial killing by immune cells [88]. Few studies reproducing septic inflammations in the context of diabetes had been performed in animals. T1DM or T2DM animals have worse prognosis upon CLP-induced sepsis even though plasma levels of systemic pro-inflammatory cytokines, like TNF-α, CXCL2/MIP-2, and IL-6, are increased in diabetic animals compared with control animals upon sepsis induction. This situation is normally attributed to neutrophil dampened activity [48, 74, 89, 90].

On the other hand, results obtained upon CLP-induced sepsis in a mouse model of T2DM showed an increased neutrophil infiltration in the peritoneal cavity in diabetic animals compared to nondiabetic upon sepsis induction. Nevertheless, neutrophils from diabetic animals presented reduced phagocytic activity and ROS generation after sepsis induction compared to control animals in the same condition. This impairment in neutrophil functions was related to a downregulation of TAM family of receptor tyrosine kinases. The lack of an appropriated innate immune response results in deficient bacterial elimination and augmented death rate in diabetic septic animals compared to control septic animals [90].

Similar results were observed in T1DM NOD mice intraperitoneally challenged with Staphylococcus aureus. The augmented neutrophil presence in the peritoneum of diabetic mice was associated with a sustained TNF-α production which prevents apoptosis in these leukocytes. Despite it, diabetic mice were more susceptible to S. aureus infection possibly associated to neutrophil decreased oxidative burst [91]. In addition, administration of GM-CSF, a cytokine known to activate PMNs, in diabetic animals submitted to CLP was able to restore neutrophilic activity and prevent the increased mortality of the animals. These effects of GM-CSF were associated with an increased neutrophil phagocytic activity and ROS generation, which controlled bacterial proliferation in the peritoneal cavity [90].

4.2 Neutrophil counts and function in tuberculosis

Several clinical and epidemiological studies have identified DM as a risk factor for the development of pulmonary tuberculosis (TB). T2DM and TB are two of the most common co-morbid conditions in many parts of the world. In addition, DM has been associated with a greater severity of TB disease among the infected population and worse outcome in response to treatment [92]. TB-DM co-morbidity is characterized by heightened levels of bacterial loads in sputum accompanied by increased neutrophil counts in peripheral blood [93]. Neutrophilic inflammation is a central feature of TB-DM, accompanied by elevated levels of biomarkers associated with macrovascular complications.

Whole blood gene expression and plasma analyses showed that several inflammatory markers, including IL-1β, CXCL8/IL-8, IL-17A, CCL3/MIP-1, TNF-α, and VEGF, associated with neutrophilic activity and absolute neutrophil counts were highly increased in TB-DM patients compared to TB or DM patients. A higher
frequency of participants with high molecular degree of perturbation (MDP) was also noted in the TB-DM subgroup. MDP is a parameter that reflects the “distance to health,” based on molecular expression scores in comparison with a healthy population. Consequently, they suggest that epigenetic reprogramming and neutrophilic inflammation determine the pattern of plasma cytokines and growth factors in TB-DM co-morbidity, highlighting neutrophilic inflammation as the main cause of susceptibility to develop TB by DM patients. Thereby, neutrophilic inflammation may be a useful target to improve TB treatment outcomes in this growing TB-DM patient population [94]. In addition, increased levels of three of the most prominent antimicrobial peptides, cathelicidin (LL37), human β-defensin 2 (HBD2), and human neutrophil peptide 1–3 (HNP1–3), principally secreted by neutrophils were found in individuals with TB-DM and TB compared with individuals with latent TB or non-TB-infected [7]. However, neutrophils isolated from T2DM patients showed a decreased capacity to phagocyte Mycobacterium tuberculosis or other M. tuberculosis-related molecules compared to control donors [95].

There are few studies using animal models of TB-DM co-morbidity focusing on neutrophil activity. Even though it is frequently observed that diabetic animals have an increased accumulation of neutrophils within lung tissue upon infection [96, 97], T2DM animals were more vulnerable to M. tuberculosis showing a decreased survival rate compared to control infected animals. Also, diabetic animals recruited more neutrophils and express higher levels of CXCL8/IL-8 in lung tissue than control infected animals [96]. In T1DM mice, infection with M. tuberculosis led to a decreased survival rate associated with an impaired bacterial control compared to nondiabetic infected mice. This high mortality of T1DM mice was accompanied by a lung neutrophilia and IL-6 overexpression. The treatment of TB-DM animals with neutralizing anti-IL-6 antibodies reduced neutrophil numbers and controlled bacterial burden in lung tissue, improving the survival rate [97].

4.3 Neutrophil counts and function in pneumonia

DM increases the risk of patients acquiring a pneumococcal disease, and besides, adversely affects the severity and outcome of this infectious illness [98]. In fact, DM has been shown to be a significant predictor of hospitalization in patients with community-acquired pneumonia (CAP) and also, a risk factor for the development of bacteremia in patients with pneumococcal pneumonia. T2DM is frequently associated with increased mortality rate from pneumonia, which appeared to be highest in the early phase of infection where neutrophilic inflammation is more important [99]. Streptococcus pneumoniae is the most frequent cause of CAP irrespective of age and comorbidity. The phagocytosis of S. pneumonia was reduced in neutrophils recovered from eight patients with poorly controlled DM, but this defect improved with insulin treatment. Notably, control neutrophils incubated with serum taken from patients with diabetes also demonstrated a defective phagocytosis, suggesting that the inefficient bacterial opsonization might be occurring in the diabetic patient’s serum [100].

Once phagocytosed, bacterial killing by neutrophils depends on the generation of ROS. Ex-vivo studies using neutrophils from T2DM patients have demonstrated a defect in the intracellular killing of S. pneumoniae together with a reduced O₂ production, reduced MPO activity, and H₂O₂ generation. In addition, chronic hyperglycemia induces inactivation of the source of leukocyte ROS, which results in high prevalence of oral abscesses, progressive interstitial inflammation, and fibrosis in the lung of mice in the absence of an inflammatory stimulus, leading to cachexia and death. These data suggested that ROS generated by NOX is not only
beneficial but also essential to oral and respiratory health in DM, particularly when the glycemia is uncontrolled [84].

*Klebsiella pneumoniae* is emerging as an agent which induces severe CAP. DM is associated with increased susceptibility to *K. pneumoniae* and poor prognosis of infection. Streptozotocin-induced diabetic mice are more susceptible to oropharyngeal infection with *K. pneumoniae*, presenting increased mortality rate and less bacterial control. There was no difference in the antibacterial activity of neutrophils recovered from nondiabetic and diabetic mice, indicating that the higher bacterial burden in hyperglycemia is probably related to a defective inflammatory signaling and late neutrophil recruitment. In fact, *K. pneumoniae* LPS induced a fewer recruitment of neutrophils to the alveolar airspace in diabetic mice compared to nondiabetic mice. Also, diabetic mice reduced neutrophil accumulation and early production of CXCL1/KC, CXCL2/MIP-2, IL-1β, and TNF-α in lung. Additionally, TLR2 and TIRAP, a Toll receptor and adaptor protein, were under-expressed in lungs of diabetic mice following *K. pneumoniae*-LPS provocation compared to nondiabetic infected mice, while no differences were observed for TLR-4 expression. These observations suggested that the failure in neutrophil recruitment and activation during the first hours of infection with *K. pneumoniae* is a most probable mechanism for high susceptibility to pneumonia in diabetics [101].

Commonly, *K. pneumoniae* infections cause pneumonia or urinary tract infections; however, during the past two decades, a distinct invasive syndrome that causes liver abscesses (KLA) has been increasingly reported in Asia, and this syndrome is emerging as a global disease [102]. DM is the most common comorbidity in KLA patients. It was shown that DNA and MPO levels were elevated in the plasma of KLA patients compared to uninfected individuals, indicating neutrophil activation independently of diabetic status. In addition, clinical *K. pneumoniae* isolates induced phagocytosis, bacterial killing, and NETosis comparable by neutrophils from diabetic and nondiabetic patients. Notably, the IL-12-IFNγ axis and its downstream chemokines CXCL9/MIG, CXCL10/IP-10, and CCL5/RANTES were produced at lower levels by peripheral blood mononuclear cells (PBMCs) from T2DM compared to PBMCs from healthy individuals in response to *K. pneumoniae* strains. These observations indicated that although T2DM does not overtly impact on neutrophil intra- and extra-cellular killing of *K. pneumoniae*, it may influence cytokine/chemokine production and intracellular killing by PBMCs.

### 4.4 Neutrophil function in bacterial infection-induced deficiency in wound healing

Delayed wound healing is one of the main diabetes-related morbidities. Neutrophil inefficient activity has been pointed as one of the major responsible factors for the impaired wound healing in diabetes, since neutrophil depletion accelerates wound resolution independently of the presence of an infection [103]. Furthermore, increased serum elastase levels, a marker of neutrophilic inflammation, predicted delayed wound healing in diabetic patients. In addition, proteomic analyses of the diabetic patient’s foot ulcers (DFUs) showed elevated expression of NET components, including elastase, histones, neutrophil gelatinase-associated lipocalin, and proteinase-3, in nonhealing wounds as also in circulating blood. Consistently, neutrophils isolated from blood of DFU patients showed an increase of spontaneous NETosis but an impaired inducible NETosis [104]. Isolated neutrophils from T2DM patients presented higher NETosis rate than neutrophils from healthy patients in the absence of stimulation, which was associated with elevated intracellular calcium levels. Hyperglycemia is strongly related to these effects since
neutrophils derived from healthy patients produced more NETosis after pre-incubation with high glucose medium *in vitro*. In addition, large amounts of NETs were found in excisional skin sterile-wounds of streptozotocin-induced diabetic mice. Although the role of NETosis in wounds remains elusive, it has been confirmed that the inhibition of NETosis or degrading NETs improved sterile-wound healing and reduced NET-driven chronic inflammation in diabetic mice [105].

Gram-positive bacteria cause more than half of cases of diabetes-related wound infections. Especially, *Staphylococcus aureus* is a major pathogen in these infections, and its presence correlates with significant delays in wound healing [106]. Wounds induced by *S. aureus* in T2DM mice showed delayed resolution compared to nondiabetic mice. Seven days after infection, the lesions of diabetic mice presented exacerbated NETosis, while nondiabetic mice had their inflammatory process already resolved and healing was nearly completed. Although neutrophils derived from both T1DM and T2DM patients produced greater amounts of NETs compared to healthy volunteer’s neutrophils, the induction of NETosis cannot be explained just by hyperglycemia. In fact, some works showed that high glucose exposure reduced LPS- or IL-6-induced NETosis *in vitro* [105, 107, 108].

Some mechanisms that could also explain the increased neutrophil NETosis in diabetic patients are the elevated levels of zonulin and the overexpression of PAD4. Zonulin is a protein that modulates the permeability of tight junctions between cells of the digestive tract. Interestingly, the increased zonulin levels in diabetic patients revealed a strong correlation with neutrophil elastase concentration and NET formation in a glucose-independent way [109]. PAD4 is a calcium-dependent enzyme that mediates NETosis. In diabetes, PAD4 was upregulated in neutrophils from individuals with diabetes and was responsible for the unbalanced NET production by these leukocytes.

In T2DM mice, although neutrophil infiltration toward the lesion was augmented, the impaired wound healing upon surgical site infection with *S. aureus* was related to a significant reduction in phagocytic activity and bacterial killing by neutrophils. Consistently, *S. aureus*-induced phagolysosome maturation was abolished and PMA-stimulated superoxide production was decreased in neutrophils recovered from diabetic mice. In addition, treatment of neutrophils with insulin significantly restored neutrophil killing activities and increased phagocytosis. Interestingly, phagosome maturation and superoxide production restoring were dependent on glycemic control and not on a direct effect of insulin. These abnormalities in neutrophil functions were closely related with impaired wound healing in DM, once treatment with insulin restored normal wound healing in diabetic mice [110].

5. Conclusion

The increased susceptibility to bacterial infections is one of the hallmarks of diabetic complications. Under comorbidity with diabetes, the high prevalence and severity of bacterial infections, as observed in tuberculosis, pneumonia, and sepsis, is closely associated to impairment in neutrophil functions, such as migration, phagocytosis, ROS production, and NET formation. The alterations in neutrophil functions noted in diabetics occur both dependently and independently of the glycemic control. Among the mechanisms that lead to neutrophil dysfunction in diabetic conditions not related to glycemic control, some targets have been highlighted, such as AGP, H2 receptor, IL-6, PAD4, resistin, and zonulin. These potential targets should be better explored in clinical studies concerning their putative benefits to diabetic patients.
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The increased susceptibility to bacterial infections is one of the hallmarks of diabetic complications. Under comorbidity with diabetes, the high prevalence and severity of bacterial infections, as observed in tuberculosis, pneumonia, and sepsis, is closely associated to impairment in neutrophil functions, such as migration, phagocytosis, ROS production, and NET formation. The alterations in neutrophil functions noted in diabetics occur both dependently and independently of the glycemic control. Among the mechanisms that lead to neutrophil dysfunction in diabetic conditions not related to glycemic control, some targets have been highlighted, such as AGP, H2 receptor, IL-6, PAD4, resistin, and zonulin. These potential targets should be better explored in clinical studies concerning their putative benefits to diabetic patients.

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Conflict of interest

The authors declare no conflict of interest, including with the financial support agencies.

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Section 2

T Lymphocytes
Chapter 3
Immune-Mediated Inflammation: Human T CD4 Helper Lymphocyte Diversity and Plasticity in Health and Disease

Rodolfo Alberto Kölliker Frers, Matilde Otero-Losada, María Inés Herrera, Sabrina Porta, Vanessa Cosentino, Eduardo Kerzberg, Lucas Udovin and Francisco Capani

Abstract
The CD4+ T helper (Th) cells have a critical role in organizing the adaptive immune response. The emerging cells of the differentiation after the immune synapse produce helper T cell subpopulations that activate, suppress, or regulate the immune response upon interaction with varying immune cells. There are two main Th cell functional categories: the "effector cells" and the "regulatory T cells."

Classic T helper lymphocytes can also be distinguished by their lineage according to the developmental microenvironment, the expression of cell adhesion-homing receptors, the profile of cytokines they are exposed to, and the involved transcription factors. Traditionally, the CD4+ and CD8+ phenotypes have been considered as helper and cytotoxic/suppressor T lymphocytes, respectively. Currently, the distinction is little rigorous. The immune response is exceedingly complex beyond the classic Th1 and Th2 effector cells' involvement, and other populations of helper T lymphocytes like the Th17, Tfh, Th22, and Th9 lymphocytes have been phenotypically characterized. These lymphocytes also participate in the pathogenesis of several immune-mediated inflammatory disorders. Here, we revisit and discuss the essential aspects of the state of the art regarding phenotypic diversity and plasticity of TCD4 cells in the T lymphocyte repertoire frame and their potential implication in human inflammatory diseases.

Keywords: CD4+ subsets, inflammation, health, disease, plasticity, diversity, CD4+, Th17

1. Introduction
The CD4+ T cells play a key role in triggering various immunological effector and regulatory functions, promoting or attenuating inflammation. Such a diverse repertoire includes the early activation during immune synapses in the ganglion, activation of cytotoxic T cells, full activation of macrophages effector functions, maturation of B cells into plasma cells and memory B cells, antibody
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1. Introduction

The CD4+ T cells play a key role in triggering various immunological effector and regulatory functions, promoting or attenuating inflammation.

Such a diverse repertoire includes the early activation during immune synapses in the ganglion, activation of cytotoxic T cells, full activation of macrophages effector functions, maturation of B cells into plasma cells and memory B cells, antibody
production by B cells, immunoglobulin isotype switching, recruitment of PMNs, and eosinophil and basophil inflammation [1]. The Th cells promote the amplification of inflammatory leukocytes effector activity in a broad spectrum of scenarios which under physiological conditions determine protective and tolerogenic immune response. Failure in T effector and/or dysregulated regulatory functions could aid immune disorders, including immunodeficiency, autoimmunity, and cancer [2–5].

Helper T cells assist in B cells’ differentiation into antibodies-producing plasma B cells in a concerted cellular-humoral immune response. This process is triggered by specific cytokines and ligand-receptor interactions [6].

The effector T cell phenotype is driven by a specific transcriptional factor, a distinctive array of cell surface molecules, and a specific profile of cytokines, which along with microenvironmental specific conditions enable T cell subset within an arm of the immune system [7].

The Jak/Stat (Janus kinase/signal transducer and activator of transcription) pathways [8] and a specific Stat associated with one of the four main transcriptional “signature” factors, T-bet (T-box transcription factor), GATA-3, RORγt (retinoic acid receptor-related orphan receptor gamma), and Foxp3 (forkhead-box/winged-helix transcription factor P3), are essential for Th differentiation [9].

Each differentiation pathway requires specific transcription factors: T-bet and STAT-4 for Th1 and GATA 3 and STAT 6 for Th2 cells and RORyt for TH17 and Foxp3 cells for regulatory T cells (Treg) [10–13].

The T lymphocytes present a remarkable phenotypic, functional, and anatomical diversity. The T cell lineages are extraordinarily diverse and present a very broad functional repertoire (Table 1) bearing innate [14] and adaptive immunogenic or tolerogenic immune properties [15, 16]. According to the greater complexity and heterogeneity of subsets of T cells, reconsidering the pathogenicity of inflammatory diseases beyond the “classical Th1/Th2 paradigm”, Th17 effector cells and T-regulatory lymphocytes (Treg) would be appropriate. Relative increases in the number of Th9 lymphocytes, follicular helper T cells (Tfh), and Th22 subsets have been described, and even NK and NKT cells contribute to the pathogenesis of immune-mediated inflammatory diseases [17]. These helper T-cell subtypes trigger specific responses upon different tissue environments by expressing a unique set of cytokines and chemokine receptors (Figure 1).

The T cells, like the CD1d-restricted natural killer T cells (iNKT) and gamma delta T cells, and other “unconventional” T cell subsets with invariant TCRs (T-cell receptors) exhibit several characteristics that place them at the border between the innate immune system and the adaptive immune system, influencing subsequent challenges by the same antigen. Although “unconventional” T cells provide rapidly available protection and contribute to the adaptive immune system, they have no ordinary helper properties [18].

The natural killer NK (large granular lymphocytes) and NKT (T cells) cells contribute to the pathogenesis of immune-mediated inflammatory diseases (IMIDs). A

| CD4 T cell | Th1, Th2, Th3 (iTreg), Th9, Th17, Th22, ThF, nTreg, and Tr1 |
| CD8 T cell | Te1, Te2, Te9, and Tc17 |
| Gamma delta T cell | Ty91 and Ty92 |
| Natural killer T cell | NKT and NK cells |

k, helper; c, cytotoxic; reg, regulatory; n, natural; i, induced

Table 1. Representative T cell types.
large increase in these infiltrating innate immune cells has been observed in IMIDs lesions and also in blood as reported for moderate to severe skin psoriasis. In addition, NKT cells might display different cytokine profiles [19, 20].

Gamma delta T cells express a distinctive surface TCR which, unlike TCRαβ, is made up of one γ (gamma) chain and one δ (delta) chain. These cells are abundant in the mucosa and do not require antigen processing and major histocompatibility complex (MHC) presentation of peptide epitopes [21].

Gamma delta T cells, often tissue-specific, are abundant in the epithelia, orchestrating immune responses in inflammation, tumor surveillance, infectious disease, and autoimmunity.

Gamma delta 1 (Ty81) and gamma delta 2 (Ty82) cells were defined as a CD3+ cell subtype expressing γδ TCR [22]. Phenotypic analysis of gated CD3+ Ty81-positive cells has revealed that nearly 75% of them are CD4−CD8− (glycoprotein cluster of differentiation). Gamma delta T cells may be regarded as a rapidly available response to pathogens triggering the innate and adaptive immune system and a memory phenotype. Besides,
they show potent antigen-presenting properties upon translocation to the ganglion. However, the various subsets may also be considered part of the innate immunity where a restricted TCR may be used as a pattern recognition receptor, indicating the importance of these lymphocytes in immunity and tissue monitoring of pathogens [21].

In particular situations, T CD8 cells can exert helper functions and vice versa, regardless of the existing heterogeneity of CD4 and CD8 T cells. Two functionally distinctive T lymphocytes subpopulations having effector or regulatory properties were considered [23]. Currently, the traditional distinction between the CD4 phenotype as a T helper and the CD8 phenotype as a cytotoxic/suppressor T lymphocyte is relative. Both CD4+ lymphocytes with cytotoxic properties and CD8+ lymphocytes presenting a secretory profile of cytokines have been identified [24], both unable to recognize the antigen in its soluble form.

Like CD4 (+) T cells, under particular conditions, also CD8 (+) T cells express different types of interleukins or gain suppressive activity [2]. Certainly, neither the heterogeneity of T cells nor the relative cytotoxic capacity of CD8 and CD4 cells is limited to the mentioned phenotypes. Regarding CD8+ T cells, the proliferative response prevails over its cytotoxic potential against cells infected by viruses, tumors, and allogeneic cells in different situations.

Recent studies have shown differences in the effector function of memory cells depending on their localization. While memory cells in the secondary lymphoid organs are generally non-cytotoxic, the cells in peripheral tissues show intense cytolytic activity. These observations follow the concept developed by Sallusto et al. [25], stating that centrally located memory cells expressing CCR7 occupy secondary lymphoid organs, whereas effector cells lacking CCR7 remain peripheral.

Figure 2.

T helper cell plasticity in inflammation. (1) Human Th1 cells originating from virgin CD41 cells in response to the coordinated activity of IFN and IL-12 induce stable expression of the transcription factor T-bet. (2) Human Th2 cells also originate from naïve CD41 cells in response to the combined activity of IL-2 and IL-4, which induce stable expression of the GATA3 transcription factor. In the presence of IL-12, T-bet is upregulated in Th2 cells, which change to produce IFN (Tho). Human Th17 cells originate from a small subset of native CD41 cells present in the newborn thymus with receptors IL-23R, IL-1RI, and CCR6 and differentiate into mature Th17 cells in response to the combined activity of IL-1β and IL-23 in vitro. (3) In the presence of IL-12, the expression of T-bet is upregulated in Th17 cells, which change to the production of IFNγ. (4) In the presence of IL-4, GATA3 expression is upregulated in Th17/Th1 cells, which progress to nonclassical Th1 cells producing IFNγ.
No doubt that the immediate availability of effector memory cells upon infection in peripheral tissue is critical to the rapid control of pathogens. Centrally located memory cells represent a precursor T population with the ability to acquire effector properties after antigen-directed expansion.

Virgin T cells are the most homogeneous subpopulation of T lymphocytes. After activation in immunological synapses, lymphocytes differentiate into effector and memory cells with a broad phenotypic repertoire. Their properties may obey to different maturation programs, localization, and particularities in the antigenic presentation. Likewise, CD4 T cells of different lineage show phenotypic plasticity [26], eventually shifting into another T cell subset (Figure 2).

2. T-helper cell subtypes 1 and 2

The Th1 and Th2 are the most studied subtypes of helper T cells [27]. They can be distinguished by their characteristic cytokine secretion profile, Th1 classically producing IFNγ and Th2 producing IL4 and IL5, among other cytokines. Following CD4 lymphocyte characterization, different studies have found similitudes for the CD8 subpopulations called Tc1 (or type I) and Tc2 (or type II) [28]. At first sight, IL12 and IL4 appear critically involved in the differentiation to type I and II cells of the CD4 and CD8 subtypes, though the scenario is not that simple. Whereas IL21 suppresses type I differentiation and promotes type II differentiation, IL18 blocks IL4-mediated suppression of type II differentiation and promotes IL12 receptor expression and type I differentiation. Collectively, different experimental outcomes seem to support the existence of factors that stabilize, retard, or reverse Th1/Th2 polarization [27]. Likewise, as variations in the secretion profile of cytokines do not respond to the prototypical type I and II dichotomy, some authors have postulated that Th1/Th2 polarization is artefactual and may not resemble the in vivo situation [29].

Recently, transcription factors associated with lymphocyte differentiation like T-bet associated with the induction of Th1 differentiation have been characterized [30]. Certainly, TCD4 cells lacking T-bet are unable to produce IFNg but release large amounts of Th2 cytokines IL4 and IL5. Transcription factor T-bet induces the expression of the IL12 receptor and transactivates the IFNg gene. The IL12 derived from dendritic cells and macrophages triggers the release of IL12, which induces STAT 4 activation in developing Th1 cells by increasing IFNg level and IL18 receptor expression [31].

Dendritic cells are also involved in Th2 differentiation. Histamine acting on H1 and H2 receptors in dendritic cells downregulates IL12 expression and stimulates IL10 release, which with the participation of specific transcription factors (GATA-3 and STAT-6) promotes Th2 differentiation [32].

3. Follicular helper T lymphocyte

Described over a decade ago, T helper lymphocytes with follicle-positive tropism (ThF) differ from the classic Th1 and Th2 lymphocytes by expressing an array of factors essential to interact with follicular B lymphocytes [33]. The differentiation markers include CXCR5, CD25, CD69, CD95, CD57 (only in humans), OX40 (CD134), and CD40L (CD154). The homing pattern and functional characteristics of ThF have been the subject of intense investigation. The ThF interacts with B lymphocytes and modify the type of humoral response inducing long-lived memory B cells that release high-affinity antibodies [34].
Curiously, ThF cells dysfunction may induce systemic autoimmunity. The ThF cells comprise a TCD4+ subpopulation restricted to the B areas of the lymphatic organs, critically involved in the events following the interaction of dendritic cells with the virgin T lymphocytes in the secondary lymphatic organ T zone [35]. The development of follicular homing capacity by activated T cell helper is the first event in the generation of ThF cells. Virgin T cells expressing CD62L and CCR7 enter the secondary lymphatic organs in the T paracortical lymphoid region, and T-lymphocyte activation induces sub-sensitivity to lymphoid chemokines along with an increase in follicular chemokines CXCL13 (also known as B cell-attracting chemokine BCA-1 or B lymphocyte chemoattractant BLC) [36].

Activated ThF and lymphoblast B lymphocytes express the CXCR5 receptor, which confers follicle-positive tropism, and the stroma and dendritic follicular cells express the ligand CXCL13. Follicular dendritic cells supply proliferative, antiapoptotic signals, and ThF lymphocytes undergo changes increasing antigenic specificity and promote the differentiation of lymphoblasts into plasma cells or B lymphocytes with memory. The antigen-dependent T-B interaction is critical in triggering the humoral immune response [37]. The T-B collaboration is essential to generating short-lived plasma cells and inducing the germinal center where they trigger isotype change and somatic hypermutation, yielding high-affinity long-lived plasma cells and memory cells [38].

Regarding ThF relationship with Th1, Th2, and Th17 subpopulations, some authors mention that ThF cells produce IL4, IFNγ, and IL17, respectively, associated with them [39].

4. T helper subtype 9 (Th9) cells

Certain inflammatory conditions give rise to the T helper subtype 9 (Th9) cells of unknown functional contribution to the immune response [40, 41]. The in vitro development of effector cells specific to constituents of oligodendrocytes (myelin oligodendrocyte glycoprotein) Th17, Th1, Th2, and Th9 allowed evaluating the encephalitogenic activity in adoptive transfer. All Th1, Th17, and Th9 subpopulations but not Th2 successfully induced experimental allergic encephalitis [23]. The Th9 cells might express varied chemokine patterns involved in different immune responses. Their effector function balanced by regulatory T cells induces regulatory activity restoring homeostasis. This recently described Th9 subset of helper lymphocytes may escalate chronic inflammation under certain conditions independently from Th1, Th2, Th17, and regulatory T cells [42].

5. T helper subtype 22 cells

Another LT helper subpopulation, the Th22, has been recently identified in epidermic infiltrates in a variety of inflammatory skin disorders, including psoriasis [43]. They secrete IL22 and TNFα but not IFNg, IL4, or IL17, and their clones derived from psoriatic patients are stable in culture, exhibiting a distinctive transcription profile compared with the already mentioned subpopulations. Secretion profile includes fibroblast growth factors and chemokines potentially involved in angiogenesis and fibrosis [44].

6. T helper subtype 17 cells

Differentiation of Th17 cells, like Th1 and Th2 cells, requires the co-participation of CD28 and ICOS after the initial stimulus derived from antigenic recognition via
the TCR complex (TCR, CD3, ζ chains) for differentiation from virgin CD4+ T cells [45]. Ivanov and colleagues suggested that the nuclear receptor ROR gamma T is the key transcriptional factor [46] in the differentiation of the Th17 lineage. The Th17 cells producing IL17 induce inflammatory responses [47].

Differentiation to Th17 requires IL6, TGFβ, and IL23 [48], whereas IL1 and TNFα might be involved in Th17 maturation. According to this model, IL27, another member of the IL12 family, programs the TCD4+ cells to differentiate to Th1 inducing the expression of IL12Rβ2. The IL12 is required for the differentiation of the programmed cells into Th1 cells producing IFNγ. In turn, IL23, a member of the IL12 family [49], triggers the proliferation of Th1 cells from memory cells and induces the development of inflammatory Th17 cells [50]. Conversely, IL27 inhibits differentiation to Th17 cells by an unknown mechanism suppressing inflammation. The relative amount of IL6 and TGFβ in the cellular microenvironment is crucial to the severity of inflammation [51].

The proinflammatory cytokine IL-17, originally named IL-17A, has been the subject of intense research since its discovery in 1993 [52]. Interest in this cytokine increased considerably when its production by a specific subset of CD4+ T cells, the so-called Th17 cells [53], was reported. Nevertheless, Th17 lymphocytes can change their phenotype to Th1 or Th2 cells depending on the dominant cytokines [2, 54].

Figure 2 illustrates how this plasticity can influence arthritis and cardiovascular risk.

Other immune cells subsets can also synthesize and express IL-17, including CD8+ T cells (CD8+ IL17 T-cell, or Tc17). Differentiation of CD8(+) T cells depend on the antigen, co-stimulatory molecules, cytokines, and transcription factors inducing them to progress to Tc1, Tc2, Tc9, Tc17, or TCD8 [55].

Since Th17 hyperactivation is responsible for the Th17/Treg imbalance in certain pathologies, IL-17A might be considered a potential therapeutic target in modulating Th1 activity enhancing the regulatory response [56].

7. Regulatory T cells

Following Th3 cell identification and characterization based on their functions in the intestinal mucosa, many studies investigated the phenotypic characteristics of conventional Treg cells in different tissues and pathological situations. The Th3 cells (CD4+ TGFβ+) and the Foxp3+ can be induced by oral tolerance, and the TGFβ released by iTreg prevents experimental colitis [57]. Though regarded as separate lineages, the induced Treg (iTreg) and Th3 cells are substantially superimposed.

Regulatory T cells and maintenance of self-tolerance rely on natural Treg cells, typically expressing CD4, CD25, and Foxp3. They develop in the thymus and recognize specific autoantigens [58].

The Treg-induced cells (iTreg), another subset of Treg cells, are also generated in the periphery during an active immune response. In fact, CD4+ CD25− cells in the periphery can be converted, in the presence of TGFβ and IL10 into CD25+ CD4+ Foxp3+ cells. The iTreg cells induced by IL-10 are called Tr1 cells and if induced by TGFβ are called Th3. One subpopulation of nTreg expresses activation markers suggesting that it comprises autoreactive Tregs continuously activated by tissue autoantigens.

Three suppression mechanisms, not fully elucidated, have been proposed to explain the inhibitory actions of Treg cells on activated T cells. These are the contact-dependent inhibition between Treg and effector cells, the consumption and limitation of growth factors like IL-2, and the inhibition of LT effectors by the production of soluble inhibitory cytokines (TGFβ, IL-10, and IL-35) and CTL4 ligands of the Treg which interacts with the CPA molecules [59].
The T-regulatory activity (Treg) is pathologically low in both psoriasis and atherosclerosis [60]. The activity of pathogenic T cells is regulated by Treg cells activity via IL-10 and TGFβ [61]. The TGFβ inhibits Th1, and Th2 differentiation favors Th1 and Th17 hyperactivity [62] in both pathologies [60]. The increase in TGFβ [63] was reported inversely correlated with cardiovascular and psoriatic severity.

The critical role of TGFβ and Treg cells was evidenced by the finding that TGFβ-deficient mice developed multiple inflammatory diseases [64–66].

Both n'Treg cells and Tr1-induced cells are able to produce IL10. The relevance of IL10 was evidenced by the specific blocking experiments of lymphocyte IL-10 triggering protection against inflammatory processes. The Tr1 cells expressing IL-10 require the presence of TGFα [67]. Regarding Th2-mediated counterregulation, the Th2 produces anti-inflammatory IL-4, IL-5, and IL-13 which decrease Th1 cells activity. The proinflammatory, metabolic, and systemic mechanisms that operate in the pathogenesis of psoriatic disease may explain the accelerated atherosclerotic process in these patients (Figure 3). Serum level of proinflammatory cytokines can increase cell-mediated immunity, which upon decreased regulatory Th2 activity and Treg level promote endothelial infiltration of inflammatory cells and plaque formation [68].

8. Conclusions

The heterogeneity of the T cells in general and TCD4 helper, in particular, may reflect divergent pathways in response to epigenetic factors or different stages of a unique differentiation pathway. Adhesion molecules, e.g., LFA1 and ICAM, CCR and CXCR chemokine receptors, and activation molecules, among others of undetermined function, reflect the transition.

The heterogeneity may obey to a programmed developmental process or to microenvironmental stimulation. Immunosuppressive or stimulatory signals like cytokines seem crucially involved though both may participate. This information is expected to shed light on the possible pathogenic role of Th cells in human inflammatory diseases beyond the Th1/Th2 paradigm.

The relationships between the classic Th1/Th2 and the more recently defined Th17/iTreg/Tfh/Th9 cells and effector-regulatory cell interactions need clarification regarding their pathogenic role in human inflammatory diseases.
Cells of the Immune System

Figure 3. Modulation of T lymphocyte function by TGF-β factor and its importance in Th1-mediated diseases. TGFβ released by different types of regulatory cells modulates the inflammatory activity of all effector cells, Th1 cells included.

The T-regulatory activity (Treg) is pathologically low in both psoriasis and atherosclerosis [60]. The activity of pathogenic T cells is regulated by Treg cells activity via IL-10 and TGF-β [61]. The TGF-β inhibits Th1, and Th2 differentiation favors Th1 and Th17 hyperactivity [62] in both pathologies [60]. The increase in TGF-β [63] was reported inversely correlated with cardiovascular and psoriatic severity.

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Chapter 4

Resident Memory T Cells

Hasan Akbaba

Abstract

Until recently, T cells were thought to remain in circulation until recruitment of the inflammation and only a small number of T cells remained in the peripheral tissues without inflammation. However, studies have found that a group of T cells settled in the tissues and remained there for a long time. Those cells are named as tissue-resident memory T cells (TRM). TRM cells are transcriptionally, phenotypically, and functionally distinct from other T cells, which recirculate between blood, secondary lymphoid organs, and non-lymphoid tissues. They undergo a distinct proliferation that discriminates them from circulating T cells and their main cell surface markers are CD69, CD103, and CD49a. Upon exposure to the same or similar diseases, TRM cells provide a first line of adaptive cellular defense against infection in peripheral non-lymphoid tissues, such as skin, lungs, digestive, and urogenital tracts. This approach forms the basis of a novel vaccination strategy called “prime and pull”, which ensures long-term local immunity. On the other hand, abnormal activated and malignant TRM may contribute to numerous human inflammatory diseases such as psoriasis and vitiligo. Here in this chapter, we aimed to emphasize TRM cell location, migration, phenotypic structure, maintenance, and diseases associated with TRM cells.

Keywords: resident memory T cell, CD8+ TRM, CD69, CD103, CD49a, prime and pull, autoreactive human disease

1. Introduction

Tissue-resident memory T cells were discovered about a decade ago. Before the discovery of TRM cells and acceptance as a new subset of T cell, memory T cells have been subdivided into two populations: effector memory and central memory T cell [1, 2]. Traditionally, it was thought that T cells taken into the tissues during infection and leave the tissue after the pathogen clearance or undergo apoptosis [3]. However, at the beginning of this millennium, it was observed that some CD8+ T cells remain long-term in the tissues after infection.

The discovery of antigen-specific CD8+ T cells located in the lung after influenza virus infection was the first example of phenomenon [4]. Later, this finding was also observed in other non-lymphoid tissues after infections with Listeria and vesicular stomatitis virus [5, 6]. Eventually, TRM cells have been described in almost all organs and can be either CD4+ or CD8+ but tissue residency has been predominantly described for memory CD8+ T cells [7]. The term “TRM cells” were used to refer to CD8+ cells, unless otherwise specified in this chapter.

The retention of TRM cells is based on two mechanisms. First, TRM cells do not express lymph node homing molecules, which are required for tissue exit such as CD62L, CCR7, and S1PR1. Second TRM cells express adhesion molecules to their
host tissue such as CD103 and CD49a [8–12]. Not all of these markers are essential for TRM identification and function of many of them are still not fully understood.

The major function of TRM cells is to establish frontline defense against previously encountered pathogens in barrier tissues where they first encounter [13, 14]. Due to their robust systemic responses, TRM cells provide superior protection compared with circulating memory T cells in peripheral tissues [15–17]. However, dysregulation of TRM can contribute to human autoimmune and inflammatory diseases such as psoriasis, vitiligo, and multiple sclerosis [18–20].

In this chapter, we aimed to emphasize TRM cell location, migration, phenotypic structure, maintenance, and diseases associated with TRM cells. We discuss the TRM cells in a basic and perceptible form as a whole, where there is no unity due to a large number of tissue variations use. We have reviewed the subject not only on the molecular level, but also on the perspective of disease formation and therapeutic usage.

2. Location

T cells can be distinguished based to their microenvironment or their location in the host tissues and thereby it is possible to classify them as TRM cells or other T cell subsets [21–23]. TRM cells are easily identified in the tissues that have direct exposure to the pathogens such as the gut, skin, lungs, and reproductive system, where they receive signals that are required for their unique development program from these microenvironments [20, 24–27].

TRM cells have different phenotypes that show heterogeneity depending on the host tissue microenvironment. Requirement for TRM generation, proliferation, migration, and maintenance vary in different kind of tissues [9, 25, 26]. In particular, the majority of TRM cells are CD8+ memory T cells, and the TRM cell population in the skin is known as CD103+ and Cd69+. However, CD4+ TRM cell populations have been identified in the skin, lungs, reproductive tract, and salivary glands. Similar to CD8 TRM cells, they express the surface molecules CD69 but expression of CD103 is low or negative [28–31]. These requirements will be detailed below according to tissue types.

The locations of the TRM cell can be classified according to host tissues as shown in Figure 1.

The skin is one of the primary barrier tissues against infectious agents. Epidermis, dermis, and subcutaneous fatty region form a 3-layer structure of the skin and

![Figure 1. Classification of TRM cell locations according to their host tissue.](image)
TRM formation has been shown in all layers [32–34]. The skin has very complex cell populations and hosts both natural and adaptive immune system cells. These immune system cells provide a biological barrier to invasive pathogens. CD8+ TRM constitutes the majority of the memory T cell population in the epidermis. CD8+ TRM cells are commonly resident in the skin and their numbers increase rapidly when they are exposed with the infectious agents. Skin TRM cells may easily characterized due to their surface markers such as marker CD69, CD103, and CD49a [9, 27, 35]. These markers and others will be described in detail in terms of their function.

The intestinal mucosa consists of a layer of single epithelial cells and provides a barrier tissue against infectious agents. This layer is also considered as an immunological site for the maintenance of TRM cells. Following intestinal infections, a significant number of pathogen-specific TRM cells have been shown to form in the intraepithelial compartment [36, 37].

The female reproductive tract (FRT) is another organ that is directly exposed to external pathogens. FGT can be divided into two parts. Upper female reproductive tract consists of endometrium and endocervix and lower FRT consists of vagina and ectocervix. FRT is a variable tissue that undergoes significant cyclic changes in women. Under the control of estrogen and progesterone hormones, growth, differentiation, and degeneration occurs periodically [38, 39]. Although this suggests that anatomical sites are limited for the localization of TRM cells, it has been showed that numerous immune system cells, including memory T cells are present throughout FRT. Generation of FRT TRM cells is a promising vaccination strategy against HSV-2, and potentially against other sexually transmitted infections such as HIV and HPV [16, 39, 40].

Respiratory tract (RT) is also a structure which is directly exposed to external pathogens. RT can also be divided into two parts as upper (URT) and lower (LRT). Most common airborne pathogens in humans primarily infect URT [41, 42]. URT contains lymph nodes known as tonsils, which contain B cell follicles and T cell subsets. URT is considered a mucosal inductive region for humoral and cellular immune responses. Although the effector CD4+ T cells predominate the tonsils, the presence and the localization of TRM cells is also shown in the lungs [15, 43].

Salivary glands are exocrine epithelial tissues, which are the targets of viral infections. The presence of TRM cells in these tissues has also been shown in various studies [28, 44].

The liver is an organ, which is the member of the immune system. Through the portal vein, antigen-rich blood enters the liver and encounters the immune system cells that are resident in the tissue [22, 45, 46]. Studies have shown that CD8+ TRM cells are established in the liver especially after systemic infection or vaccination [47, 48].

Due to the presence of a blood-brain barrier, immune cells are not thought to be resident in the central nervous system [49]. However, after clearing a viral infection in the central nervous system, some of the antigen-specific CD8+ T cells maintained in the brain as TRM cells [50].

The kidney has a very high amount of blood vessels and has a very high circulating volume. This helps to eliminate toxins from the body. Therefore, healthy kidneys are not suitable tissues for the localization of immune system cells. Even so, it has been shown that a small number of resident TRM cells may be present in the kidney. White adipose tissue is another tissue in which TRM cells have been shown to be resident and they act as a reservoir of TRM cells [51–53].

CD8+ TRM cells have been reported in solid tumors [54]. Studies have shown that infiltrating T lymphocytes (TIL) are phenotypically similar to TRM cells that TRM cells from neighboring peripheral tissues could infiltrate into solid tumors [55, 56]. It was found that presence of CD8+ TRM cells is associated with good prognosis in various cancers [57].
Secondary lymphoid organs and lymph nodes are the tissues where TCM and TEM cells are more common and pass through. However, recent studies have shown that a small number of non-circulating memory T cells are present in these tissues. TRM cells in SLO show phenotypic characterization similar to those in non-lymphoid tissues [1, 58].

Primary lymphoid organs (PLO) are bone marrow and thymus. Antigen-specific TRM cells have also been found in these tissues and have been shown to facilitate long-term maintenance in the PLO. TRM cells in the PLO express CD69 and CD103 as a characteristic of TRM phenotype [59–61].

3. Migration

Circulation of T cell in the blood, secondary lymphoid organs, and non-lymphoid tissues is a complex system. Numerous receptors, ligands, chemokines, cytokines, and transcription factors have a role on this [31, 32]. T cells can be classified according to the organs or tissues in which they recirculate. Schematic illustration for migration of T cell subsets is shown in Figure 2.

- Naive T cells: recirculate in the blood, secondary lymphoid organs, and non-lymphoid tissues [62, 63].
- Effector memory T cells: recirculate in the blood, secondary lymphoid organs, and non-lymphoid tissues, same as naive T cells.
- Central memory T cells: recirculate between nonlymphoid tissues, lymph, and lymph nodes [64, 65].
- Tissue-resident memory T cells: do not recirculate between blood, secondary lymphoid organs, nonlymphoid tissues, but may migrate within the tissue it settled [8, 11, 66, 67].

CC-chemokine receptor 7 (CCR7), CD69, CD49, S1PR1, KLF2, and integrins are the main factors responsible for the migration of T cell subsets. The role of these factors may differ depending on the location of the host tissue [68, 69]. These will be further explained in more detail in phenotype and localization parts.

![Figure 2. Schematic illustration of circulation and migration of T cell subsets.](image-url)
4. TRM markers

In order to distinguish TRM cells from other T cell subsets, in most of the studies in both mice and humans, identification markers such as CD103, CD69, Cd49a, and CD44 have been widely investigated.

4.1 CD103

αEβ7 integrin (CD103) was first discovered in the late 1980s. After that several new monoclonal antibodies were produced as a specific marker for intestinal intraepithelial T cells in humans, mice, and rats, presumably contributing to their tissue-specific localization [70]. Integrins are transmembrane αβ heterodimers that bind to extracellular matrix components and to cellular counter receptors. They have important roles on cell localization, migration, and signaling and are important for T lymphocyte adhesion and stimulation [71].

Following the discovery of the ligand called E-cadherin, interest in CD103 has been increased considerably. E-cadherin is a transmembrane protein with an extracellular region containing extracellular cadherin domain repeats, which mediates cell-cell adhesion by homodimerizing in trans with E-cadherin domains of neighboring cells [72].

CD103 is important in adhesion as well as T cell activation and TGF-B induced defense in tumor microenvironment. In TGF-B environment, CD103 TRM cells have been shown to release more efficient granzyme. Although CD103 is an important marker, CD103 alone is not sufficient to detect TRM cells. CD103 negative TRM cells were found in several tissues. Furthermore, there are different types of CD103 T cells such as CD4 CD103 T cells and CD8 CD103 Treg cells.

4.2 CD69

CD8+ TRM cells can be characterized by their expression of the surface molecules CD69 and CD103. These markers are usually not expressed on circulation T cells [73]. CD69 is a type II C-lectin membrane receptor with a scarce expression in resting lymphocytes that is rapidly induced upon cell activation [74]. Because of these features, CD69 was considered as early activation antigen of immune cells. However, recent studies have shown that this molecule is an important indicator of TRM differentiation as well as activation of the immune response.

CD69 has been found to suppress the activity of sphingosine-1 phosphate receptor 1 (S1P1), helping the TRM cells that remain in peripheral tissues [75]. The S1P1 receptor/gene, originally known as endothelial differentiation gene 1, acts by binding with a bioactive signaling molecule S1P1 [76]. It was suggested that CD69 expression might help retaining TRM cells in peripheral tissues by suppressing the activity of S1P1. Decreased expression of transcription factor of KLF2 is another factor affecting S1P1 expression to remain down-regulated in TRM cells [77, 78].

Moreover, CD69 expression is not limited to TRM cells and is not essential for TRM formation. CD69 has also been shown to be expressed in cells such as natural killer cells, dendritic cells, and in the absence of CD69, TRM formation decreased but is not completely eliminated [32, 79]. Therefore, CD69 is a good TRM marker, but it is not sufficient to be the sole determinant.

4.3 CD49a

CD49a or integrin α1 paired with CD29 (integrin-β1) to form very late antigen (VLA-1). VLA-1 is a collagen-binding integrin and receptor for collagen and laminin such as CollIV and Coll [9, 80, 81].
Collagen IV enriched in the basement membrane separating epidermis and dermis. CD49a is therefore a good marker for skin TRM cells. In human skin epithelia, CD8+ CD49a+ TRM cells produced interferon-γ, whereas CD8+ CD49a- TRM cells produced interleukin-17 (IL-17). It has been reported that CD8+ T cells with a TRM phenotypes (CD103+ and CD49a+) are present in solid tumors as well as lung interstitium [9, 35, 82].

VLA1 is a receptor not only involved in adhesion but also to migration and survival. In the formation and proliferation of TRM cells, CD49a together with CD103 and CD69 are the most determinative markers of TRM presence.

4.4 CD44

The CD44 antigen is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration [83]. The most well-studied function of CD44 is as a receptor for hyaluronic acid, a component of the extracellular matrix. In regard to accessing peripheral tissues during an immune challenge, CD44 can bind hyaluronic acid expressed on vascular endothelial cells and facilitate transmigration. CD44 is a classical marker of previous activation, expressed on newly generated effector cells as well as resting memory cells [23, 84, 85].

It is important to specify that TRM cells express different markers depending on the host tissues. It should not be ignored that there may be some differences between TRM subsets in various tissue types. The results obtained by using in vivo techniques such as parabiosis, organ transplantation, using transgenic mice, and bone marrow chimera techniques were more effective in the identification TRM cell proliferation. The main factors that enable scientists to identify TRM cells as a subset of T cells have been obtained by these methods.

4.4.1 Parabiosis

Parabiosis is a surgical process that allows the sharing of blood circulation in two organisms. Bringing the skin of the two animals, in particular mice, together stimulate the capillary blood vessel formation in this region. Blood and immune cells circulate between parabiotic partners [86]. Therefore, migration or residence can be examined by investigating whether the immune system cell in one organism is in equilibrium with the other.

4.4.2 Bone marrow chimera (BMC)

BMC is another widely used technique to study donor organism, which has congenitally distinctive or labeled bone marrow, and a recipient organism, which have been irradiated, thus losing its all bone marrow-derived cells (lymphocytes) are two component of this method. Then, bone marrow cells of donor organism are transferred to the recipient organism [87, 88].

4.4.3 Organ transplantation

Transplantation is a similar approach to BMC in TRM cell studies. In this method, organ or skin graft of the donor organism is transplanted into the recipient. The equilibrium between the established T cell populations of donor and recipient organisms are examined to investigate the TRM cells. Moreover, TRM cells have important roles in organ transplantation and tissue rejection [89, 90].
### 4.4.4 Transgenic organism

Transgenic organisms are widely used in TRM studies. Numerous studies have been performed using knockout mice where proteins involved in tissue localization or tissue exit cannot be expressed [32, 57, 91, 92].

### 5. Phenotype

There is not a single phenotypic character to be used to identify TRM cells. Many researchers have examined the TRM cell phenotype in different tissues including lungs, liver, lymphoid sites, skin, and intestines both in mice and humans.

Characteristically, TRM cells express CD103 and CD69. CD49a, which binds to the extracellular collagen and laminin, can be added to these two for the skin tissue [21, 23, 93]. TRM cells do not express or express very low levels of lymph node homing molecules which are required for tissue exit such as CD62L, CCR7, and S1PR1 and it is critical for TRM cell tissue residency [1, 15, 53, 67, 69]. S1P1 is mediated by the downregulation of the transcription factor KLF2 [93].

TRM cells also express cluster of chemokines and chemokine receptors including CXCR3 and CCR6, and was able to produce chemokine ligands such as CCL19 and CCL21 [2, 93, 94].

Tissue microenvironment also promotes TRM differentiation. TRM precursors that are KLRG1 negative, are more likely to differentiate into TRM cells [53, 55].

Broad range of transcription factors is associated with TRM formation. Most common transcription factors are AHR (aryl hydrocarbon receptor), Notch, Blimbl, Hobit, Eomes, and T-bet [30, 95]. These phenotypic structures are illustrated in Figure 3 and each is described in detail in Table 1.

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**Figure 3.**
Schematic illustration of some of the most common, receptors, transcription factors, ligands, and molecules involved in differentiation and maintenance of TRM cells and their regulation for TRM formation.
Table 1. Detailed explanations of receptors, transcription factors, ligands, and molecules involved in formation and migration of TRM.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Regulation</th>
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<tbody>
<tr>
<td>CD103 (integrin αEβ7)</td>
<td>CD103 is a receptor for E-cadherin, an adherent junctional protein</td>
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<td></td>
<td>interlocking epithelial cells [96]</td>
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<tr>
<td>CD49a (integrin α1β1)</td>
<td>CD49a pairs with CD29 (integrin β1) to form the heterodimer called</td>
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<tr>
<td></td>
<td>VLA-1 which is a collagen-binding integrin [35]</td>
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<tr>
<td>CD69</td>
<td>Human transmembrane C-Type lectin protein. CD69 is a lymphoid</td>
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<td></td>
<td>activation antigen whose rapid expression makes it amenable for the</td>
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<td></td>
<td>early detection of T-cell activation and for subset activation</td>
<td></td>
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<td></td>
<td>analyses [97]</td>
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<tr>
<td>Krüppel-like Factor 2 (KLF2)</td>
<td>Klf2 also plays a role in T-cell differentiation and regulate the</td>
<td>↓</td>
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<td></td>
<td>migration of mature thymocytes from the thymus and to control the</td>
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<td></td>
<td>circulation of peripheral T cells. In the absence of Klf2, mature T</td>
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<td></td>
<td>cells exist in an activated state and are more prone to apoptosis [98]</td>
<td></td>
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<tr>
<td>Ki67</td>
<td>Function of the Ki67 protein is still unclear. Ki67 protein has been</td>
<td>↓</td>
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<td></td>
<td>widely used as a proliferation marker that is expressed by cells mitotic</td>
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<td></td>
<td>phases [99–101]</td>
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<tr>
<td>Killer cell lectin-like</td>
<td>KLRG1 is expressed by NK and T-cell subsets and recognizes</td>
<td>↑</td>
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<tr>
<td>receptor subfamily G member 1</td>
<td>members of the classical cadherin family. KLRG1 is widely used as a</td>
<td></td>
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<tr>
<td>(KLRG1)</td>
<td>lymphocyte differentiation marker in both humans and mice [102]</td>
<td></td>
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<tr>
<td>C-C chemokine receptor type 7</td>
<td>CCR7 is a chemokine receptor which regulates T cell trafficking and</td>
<td>↓</td>
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<tr>
<td>(CCR7)</td>
<td>compartmentalization within secondary lymphoid organs [103]</td>
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<tr>
<td>Sphingosine-1-phosphate receptor</td>
<td>SIPR1 was implicated in lymphocyte trafficking and it has an</td>
<td>↓</td>
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<tr>
<td>1 (SIPR1)</td>
<td>important role in regulating endothelial cell cytoskeletal structure,</td>
<td></td>
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<tr>
<td></td>
<td>migration, and T cell maturation [104]</td>
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<tr>
<td>Chemokine receptor 3 (CXCR3)</td>
<td>CXCR3 plays a role to regulate leukocyte trafficking. Ligand that binds</td>
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<td></td>
<td>to CXCR3 induces cellular responses, such as integrin</td>
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<td></td>
<td>activation, cytoskeletal changes and chemotactic migration [94]</td>
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<tr>
<td>CD62L (L-selectin)</td>
<td>L-selectin is an adhesion molecule that regulates both the migration</td>
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<td></td>
<td>of leukocytes at sites of inflammation and the recirculation of</td>
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<td></td>
<td>lymphocytes between blood and lymphoid tissues [105]</td>
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<tr>
<td>Chemokine ligand 21 (CCL21)</td>
<td>CCL21 is a high affinity functional ligand for chemokine receptor 7</td>
<td>↓</td>
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<td></td>
<td>[106]</td>
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<tr>
<td>Eomesodermin (Eomes) and T-bet</td>
<td>Downregulation of T-bet and Eomes enables increased TGF-β</td>
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<td></td>
<td>responsiveness, thereby creating a feedback loop that promotes TRM</td>
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<td></td>
<td>differentiation [30]</td>
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<tr>
<td>Blimp-1 and Hobit</td>
<td>Loss incompatible with development of tissue-resident cell types; in</td>
<td>↑</td>
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<tr>
<td></td>
<td>combination enforces tissue retention by depression of KLF2, SIPR1, and</td>
<td></td>
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<tr>
<td></td>
<td>CCR7 [69]</td>
<td></td>
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<tr>
<td>Aryl hydrocarbon receptor (Ahr)</td>
<td>is required for long-term persistence of TRM as a survival pathway for</td>
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<tr>
<td></td>
<td>T cells residing in the epidermis [33]</td>
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<tr>
<td>Notch</td>
<td>Required for maintenance of CD8 TRM; proposed to control metabolic</td>
<td>↑</td>
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<tr>
<td></td>
<td>functions in T RM and CD103 expression [23]</td>
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</table>

6. TRM and diseases

TRM cells may assume pathogenic roles if they become over-sensitized or autoreactivated. However, TRM cells are the first line protector of the immune system against the pathogen at the same time. Therefore, they play or stimulate to play an important role in effective treatment or vaccination. Figure 4 summarizes the diseases associated with TRM cells both in the perspectives of pathogenic and protective roles.
6. TRM and diseases

Migration of TRM.

Detailed explanations of receptors, transcription factors, ligands, and molecules involved in formation and role in effective treatment or vaccination.

Activated. However, TRM cells are the first line protector of the immune system against the pathogen at the same time. Therefore, they play or stimulate to play an important role in the early detection of T-cell activation and for subset activation antigen whose rapid expression makes it amenable for analyses [97]. KLF2 is widely used as a proliferation marker that is expressed by cells mitotic existing in an activated state and are more prone to apoptosis [98]. In the absence of Klf2, mature T cells exist in an activated state and are more prone to apoptosis. Klf2 also plays a role in T-cell differentiation and regulate the migration of mature thymocytes from the thymus and to control the deformation of the skin [108]. Many studies showed that the chronic inflammation observed in psoriasis arises from an uncontrolled proliferation of T cells [66, 109]. Resident T cells play a role in the formation and recurrence of psoriatic lesions. Psoriasis lesion can be triggered and sustained by the local network of skin-resident immune cells in mouse models [110].

In recent studies, TRM cells were identified in healthy skin but were increased in psoriatic lesions. And these TRM cells have been found to produce perforin and IFN-gamma and to secrete IL-17 which is responsible for unwanted symptoms [111]. Demarcated, inflamed, and hyperproliferative plaques are maintained by interleukin-23 (IL-23) and IL-17 in psoriasis [41, 112].

For the treatment of psoriasis, an autoimmune disease, various immunosuppressive drugs, neutralizing antibodies, and cytokines have been tried for the treatment [42, 113–116]. These therapies have not been fully successful nowadays due to the systemic side-effects and the presence of autoreactive resident T cells in tissues without lesions.

6.1.1 Psoriasis

Psoriasis is a common chronic inflammatory skin disease with a spectrum of clinical phenotypes and results from the interplay of genetic, environmental, and immunological factors [107]. Psoriasis can be divided into five types. The most common is plaque psoriasis, which causes itching and pain due to plaque formation. This type also maintains large areas of erythema or scaling of the skin, causing deformation of the skin [108]. Many studies showed that the chronic inflammation observed in psoriasis arises from an uncontrolled proliferation of T cells [66, 109].

Resident T cells play a role in the formation and recurrence of psoriatic lesions. Psoriasis lesion can be triggered and sustained by the local network of skin-resident immune cells in mouse models [110].

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6.1.2 Vitiligo

Another disease with several patchy appearance lesions in the skin like psoriasis is Vitiligo. These two diseases are often confused with each other. Vitiligo is an autoimmune T cell-mediated disease in which immune cells target and kill melanocytes, leading to depigmentation of the skin [73].

Vitiligo lesions recur in the same areas of the skin and this is a sign of the presence of resident autoimmune memory [117]. Recent studies have shown the presence of melanocyte-specific TRM cells in skin tissues with vitiligo. These TRM
cells are CD8+ cells secreting IFNg and TNFα and expressing common TRM markers such as CD69, CD103, and CXCR3 [19, 118]. In a mouse vitiligo model, it was showed that neutralization of the IL-15 receptor by anti-CD122 antibody decreases the IFNg production from TRM cells and leads to repigmentation of the lesion [91]. Currently, there is no FDA-approved vitiligo treatment and such studies targeting TRM cells are likely to have prosperous results in the future.

6.1.3 Multiple sclerosis

Multiple sclerosis (MS) is a chronic, immune-mediated, demyelinating disorder of the central nervous system [119]. The brain is not a frequently visited tissue for immune cells due to its barriers. In one of the few studies in this field, CD8+ TRM cells that persist within the brain after an acute systemic vesicular stomatitis virus infection were characterized [120]. These cells were not in equilibrium with circulating T cells as evidence for TRM establishment in the brain tissue [50]. However, the mechanism for the generation and maintenance of TRM cells in the brain remains unclear.

6.1.4 Asthma

Asthma, other allergic airway diseases are inflammatory lung diseases that are related to the TRM cells. Asthma is a heterogeneous disease and is characterized by chronic airway inflammation, increased susceptibility to respiratory viral infection, and altered airway microbiology [121, 122]. The lungs have been widely investigated for TRM cell formation due to their exposure to the external environment and recurrent infections. In one of those studies, house dust mite HDM-specific memory cells have been identified as central memory cells in the lymphoid organs and TRM cells in the lung [123].

The majority of T cells in the human lung are TRM cells. TRM cells provide important roles in the protection against asthma, multiple respiratory pathogens, and other allergic diseases and might be contributed for developing new therapies and vaccines [25, 26].

6.1.5 Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease which can cause cartilage and bone damage, progressive articular damage, as well as functional loss disability [124–126]. Rheumatoid arthritis, is known largely a disease of the joints, however many organs and systems are effected, including the pulmonary, cardiovascular, ocular, and cutaneous systems [127].

Recurrence of arthritis in the joints is the key for the treatment of human rheumatoid arthritis. The disease is propagated through resident cells in the synovium of the joint, resident synovial cells that interact with the infiltrating immune cells and transition from acute synovitis to chronic RA [128]. The link between recurrence and residency suggests the presence of TRM cells. Studies have shown that TRM formation was induced in the enthesis. The enthesis is the region at the junction between tendon and bone. This zone was shown to contain a unique population of resident T cells, when activated by the cytokine interleukin-23 and can cause pathogenesis [129, 130].

6.1.6 Crohn’s disease

Crohn disease (CD) is an inflammatory condition of the gastrointestinal (GI) tract, characterized by unpredictable periods of symptomatic relapses and
remissions [131]. It has been suggested that CD has clinical similarities with TRM-mediated skin diseases. The skin lesion is similar “skip lesions” in the gut seen in CD [3, 67]. The use of immunosuppressive drugs for the treatment of CD can be considered as another similarity. However, the presence of a direct link between CD and TRM formation should be investigated.

Some of the other diseases that are related to the TRM formation are mycosis fungoides, contact dermatitis, chronic eczema, and fixed drug eruption and all needs to be further investigated.

6.2 Protective roles

Autoreactive TRM cells may contribute to the pathogenesis of autoimmune, atopic, and allergic diseases as described above. In contrast, they can provide rapid and efficient protection against wide range of pathogens and various types of tumors. Malaria, HSV, and cancer must be emphasized due to the role of TRM cell mediated treatment and vaccination strategies.

Malaria is a vector-borne parasitic tropical disease found in 91 countries worldwide [132]. Plasmodium falciparum (PF) is the dominant specie that produces high levels of parasites in critical organs and cause severe anemia, especially in African children, in whom. Malaria affected an estimated 216 million people causing 445,000 deaths in 2016 [133] around the World and the vast majority of malaria deaths occur in developing countries. Over the years, extensive research has been conducted on the prevention and treatment of malaria. However, increasing drug and insecticide resistance and threatens the successes. Moreover, the results obtained from current vaccine studies have not been sufficient to prevent malaria.

Development of a broadly protective vaccine is required for the eradication of Malaria. For this purpose, TRM cell-mediated vaccination strategies can be very promising. Researchers identified that memory CD8+ T cells that expressed the gene signature of TRM cells and remained permanently within the liver [45, 48].

A recent study explored the mechanism of action of a newly developed malaria vaccine, Plasmodium falciparum sporozoites (PfSPZ), which has exhibited very promising efficacy in human clinical trials. The efficacy of this vaccine has been shown to be due to TRM formation within the liver was 100-fold higher [47]. Researcher also showed that this TRM cells within the liver can also be generated by a “prime and trap” or “prime and pull” vaccination strategy [16, 22].

This strategy has two stages. First is the conventional vaccination to obtain systemic T cell responses (prime), second is recruitment of activated T cells via topical chemokine application to the desired tissue (pull), where such TRM cells were established and mediate long-term protective immunity [16, 102, 134]. The robust protective immunity provided by memory T cells localized in peripheral tissues, together with localized memory T cells, provides hope that site-specific vaccination strategies can be developed [135].

Development of a T cells mediated vaccines are required for efficacious protection. Due to their robust systemic responses, TRM cells provide superior protection compared with circulating memory T cells in peripheral tissues [136]. Recent studies focused on TRM establishment of training to protect against infection agent where they first contact [40].

The female genital tract, which is a portal of entry for sexually transmitted infections such as HIV and HSV. In a recent study “prime and pull” strategy was used against HSV-2 infection in female genital tract. In this study, mice were infected by attenuated strain of HSV-2 subcutaneously and topical application of chemokines CXCL9 and CXCL10 have been used to recruit TRM cells in the vagina to prevent the development of clinical disease for further infections [16].
TRM-mediated vaccine development researches against infectious agents are not limited to PF HSV and HIV. Moreover, vaccine studies are being carried out in order to provide first step protection against many infectious agents such as influenza, varicella, Human papillomavirus (HPV), toxoplasma, etc. [8, 38, 43, 137, 138].

In the context of TRM cells, cancer should also be emphasized. Currently, developed cancer vaccines are generally aimed for the treatment and the number of prophylactic vaccines is relatively low. Therefore, vaccination studies for the formation of TRM against cancer are very promising.

Recent studies suggest that TRM cells also play a vital part in cancer surveillance [57, 139]. It was demonstrated in many studies that TRM cells generated by vaccines can protect against tumor challenge [10, 55, 140, 141]. Formation of CD8+ T cells is one of the main objectives in cancer vaccine development against solid tumors. The type of CD8+ T cells that can migrate and localize in tumor microenvironments are TRM cells. [55]. It was found that presence of CD8+ TRM cells is associated with good prognosis in various cancers [57]. TRM cells can act in three major ways against solid tumors [73].

- TRM cells can express cytokines: TRM cells can produce cytokines such as perforin and granzyme B, and other effector molecules such as IFNγ and TNFα and eliminate tumor cells [10, 73].
- TRM cells may promote tumor-immune equilibrium: CD8+ TRM cells can contribute tumor immunosurveillance and they prevent tumor outgrowth without completely eliminating cancerous cells [73, 142, 143].
- TRM cells express inhibitory checkpoint molecules: TRM cells also predominantly express checkpoint receptors such as programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) [55, 80].

It is becoming increasingly clear that TRM cells play an integral role in tumor surveillance in both animal models and human cancers. However, the role of TRM cells in solid human cancers should be further investigated.

7. Conclusion

The knowledge about TRM cells is at an early stage. Moreover, it has been revealed only in recent decades that TRM cells are unique subsets. It was found that TRM cells become resident by their phenotypic characteristics by adopting the microenvironment of the host tissue. TRM cells are transcriptionally, phenotypically, and functionally distinct from other circulating T cell subsets.

TRM cells have different phenotypes show heterogeneity depending on the host tissue microenvironment. Requirement for TRM generation, proliferation, migration, and maintenance vary in different kind of tissues. In order to distinguish TRM cells from other T cell subsets, in most of the studies in both mice and humans, identification markers such as CD103, CD69, and Cd49a were the most common ones.

TRM cells may assume pathogenic roles if they become over-sensitized or auto-reactivated. However, TRM cells are the first line protector of the immune system against the pathogen at the same time. Therefore, they play or stimulate to play an important role in effective treatment or vaccination. It was found that presence of CD8+ TRM cells is associated with good prognosis in various cancers.
Cells of the Immune System

TRM-mediated vaccine development researches against infectious agents are not limited to PF HSV and HIV. Moreover, vaccine studies are being carried out in order to provide first step protection against many infectious agents such as influenza, varicella, Human papillomavirus (HPV), toxoplasma, etc. [8, 38, 43, 137, 138].

In the context of TRM cells, cancer should also be emphasized. Currently, developed cancer vaccines are generally aimed for the treatment and the number of prophylactic vaccines is relatively low. Therefore, vaccination studies for the formation of TRM against cancer are very promising.

Recent studies suggest that TRM cells also play a vital part in cancer surveillance [57, 139]. It was demonstrated in many studies that TRM cells generated by vaccines can protect against tumor challenge [10, 55, 140, 141]. Formation of CD8+ T cells is one of the main objectives in cancer vaccine development against solid tumors. The type of CD8+ T cells that can migrate and localize in tumor microenvironments are TRM cells. [55]. It was found that presence of CD8+ TRM cells is associated with good prognosis in various cancers [57]. TRM cells can act in three major ways against solid tumors [73].

• TRM cells can express cytokines: TRM cells can produce cytokines such as perforin and granzyme B, and other effector molecules such as IFN\(\gamma\) and TNF\(\alpha\) and eliminate tumor cells [10, 73].
• TRM cells may promote tumor-immune equilibrium: CD8+ TRM cells can contribute tumor immunosurveillance and they prevent tumor outgrowth without completely eliminating cancerous cells [73, 142, 143].
• TRM cells express inhibitory checkpoint molecules: TRM cells also predominantly express checkpoint receptors such as programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) [55, 80].

It is becoming increasingly clear that TRM cells play an integral role in tumor surveillance in both animal models and human cancers. However, the role of TRM cells in solid human cancers should be further investigated.

7. Conclusion

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TRM cells may assume pathogenic roles if they become over-sensitized or auto-reactivated. However, TRM cells are the first line protector of the immune system against the pathogen at the same time. Therefore, they play or stimulate to play an important role in effective treatment or vaccination. It was found that presence of CD8+ TRM cells is associated with good prognosis in various cancers.

Unlike other T cell subsets, TRM cells are not present in the blood. This is one of the major logistical barriers to the study of TRM cells. Therefore, TRM studies in humans have been limited due to the need for biopsy. In human NLT tissues, TRM isolation should be performed in a small biopsy volume, they should be phenotypically redefined and distinctive surface markers should be identified for humans. However, TRM cell-mediated vaccination and effective T cell treatments against solid tumors can be achieved by overcoming these problems in the following years.

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Chapter 5
Modulating the T Lymphocyte Immune Response via Secretome Produced miRNA: From Tolerance Induction to the Enhancement of the Anticancer Response
Mark D. Scott, Duncheng Wang, Wendy M. Toyofuku and Xining Yang

Abstract
T cells are key mediators of graft tolerance/rejection, development of autoimmunity, and the anticancer response. Consequently, differentially modifying the T cell response is a major therapeutic target. Most immunomodulatory approaches have focused on cytotoxic agents, cytokine modulation, monoclonal antibodies, mitogen activation, adoptive cell therapies (including CAR-T cells). However, these approaches do not persistently reorient the systemic immune response thus necessitating continual therapy. Previous murine studies from our laboratory demonstrated that the adoptive transfer of polymer-grafted (PEGylated) allogeneic leukocytes resulted in the induction of a persistent and systemic tolerogenic state. Further analyses demonstrated that miRNA isolated from the secretome of polymer-modified or control allogeneic responses effectively induced either a tolerogenic (TA1 miRNA) or proinflammatory (IA1 miRNA) response both in vitro and in vivo that was both systemic and persistent. In a murine Type 1 diabetes autoimmune model, the tolerogenic TA1 therapeutic effectively attenuated the disease process via the systemic upregulation of regulatory T cells while simultaneously downregulating T effector cells. In contrast, the proinflammatory IA1 therapeutic enhanced the anticancer efficacy of naïve PBMC by increasing inflammatory T cells and decreasing regulatory T cells. The successful development of this secretome miRNA approach may prove useful treating both autoimmune diseases and cancer.

Keywords: T lymphocyte, miRNA, polymer, secretome, tolerance, Treg, proinflammatory, Teff, autoimmunity, cancer, adoptive cell transfer

1. Introduction
Biologically, and clinically, the concept of "self" is of crucial importance in protection against foreign biologicals (e.g., viruses and bacteria), abnormal autologous cells (e.g., cancers) and more recently developed "diseases" (i.e., the purposeful introduction of "nonself") such as enzyme-replacement therapy and transfusion
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T cells are key mediators of graft tolerance/rejection, development of autoimmunity, and the anticancer response. Consequently, differentially modifying the T cell response is a major therapeutic target. Most immunomodulatory approaches have focused on cytotoxic agents, cytokine modulation, monoclonal antibodies, mitogen activation, adoptive cell therapies (including CAR-T cells). However, these approaches do not persistently reorient the systemic immune response thus necessitating continual therapy. Previous murine studies from our laboratory demonstrated that the adoptive transfer of polymer-grafted (PEGylated) allogeneic leukocytes resulted in the induction of a persistent and systemic tolerogenic state. Further analyses demonstrated that miRNA isolated from the secretome of polymer-modified or control allogeneic responses effectively induced either a tolerogenic (TA1 miRNA) or proinflammatory (IA1 miRNA) response both in vitro and in vivo that was both systemic and persistent. In a murine Type 1 diabetes autoimmune model, the tolerogenic TA1 therapeutic effectively attenuated the disease process via the systemic upregulation of regulatory T cells while simultaneously downregulating T effector cells. In contrast, the proinflammatory IA1 therapeutic enhanced the anticancer efficacy of naïve PBMC by increasing inflammatory T cells and decreasing regulatory T cells. The successful development of this secretome miRNA approach may prove useful treating both autoimmune diseases and cancer.

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1. Introduction

Biologically, and clinically, the concept of “self” is of crucial importance in protection against foreign biologicals (e.g., viruses and bacteria), abnormal autologous cells (e.g., cancers) and more recently developed “diseases” (i.e., the purposeful introduction of “nonself”) such as enzyme-replacement therapy and transfusion.
and transplantation medicine. The immune system is tasked with preserving “self” and rejecting “nonself” and has multiple components—any of which will be of variable importance depending on the context of the immunological assault. Immunological “self” of most tissues is imparted by the major histocompatibility complex (MHC) which encodes a variety of proteins that provide a means for identifying, targeting, and eliminating foreign invaders and diseased cells while preserving normal “self” tissue. The MHC proteins themselves consist of three classes. MHC Class I molecules are expressed on virtually all nucleated cells while Class II molecules are expressed exclusively on antigen presenting cells (APC; e.g., monocytes, macrophages, dendritic cell, B lymphocytes, and endothelial cells) and activated T lymphocytes. MHC Class III genes encode components of the complement system. The human MHC is referred to as the Human Leukocyte Antigen (HLA) complex while the murine equivalent is referred to as the Histocompatibility-2 (H2) complex. In the context of MHC-mediated immune recognition, the T lymphocyte (T cell) is of particular importance. T cells themselves consist of a diverse array of subsets that fall into two general categories: 1) Regulatory T cells (Treg) which modulate the strength of an immune response and maintain “self”; and effector T

Figure 1. Immune modulation via pharmacologic and immunocamouflage therapy. (A) Current pharmacologic therapy almost exclusively targets T cell activation and proliferation consequent to allore cognition. Response to nonself is in large part mediated by cell-cell interactions between antigen presenting cells (APC; e.g., dendritic cells) and naive T cells. This cell-cell interaction is characterized by essential adhesion, allore cognition and co-stimulation events. Consequent to allore cognition, a proliferation of proinflammatory T cells (e.g., cytotoxic T lymphocyte, CTL; Th17, IL-17; Th1, IFN-γ; and IL-2+ populations) and decrease in regulatory T cells (Treg, Foxp3+ and CD25+) is observed. Current therapeutic agents are primarily cytotoxic agents preventing T cell activation (e.g., cyclosporine and rapamycin) or T cell proliferation (e.g., methotrexate, corticosteroids and azathioprine). Additionally, blocking antibodies have been investigated. Gray text indicates current techniques to prevent/limit alloimmune responses. (B) In contrast, immunocamouflage of donor cells by methoxy (polyethylene) glycol (mPEG) results in the disruption of the essential cell-cell interactions decreasing T cell proliferation and altering differentiation patterns (decreased Th1 and increased Treg). In aggregate, the polymer induced changes induces a tolerogenic/anergic state both in vitro and in vivo. Size of T cell population denotes increase or decrease in number. Size of B cell indicates antibody response. Blue text represents the consequences of polymer-mediated immunocamouflage of the allorespone. (C) As shown in photomicrographs, in a control mixed lymphocyte reaction (MLR), significant and persistent interactions (black arrows) occur between allogeneic lymphocytes (LYM) and dendritic cells (APC). The lymphocyte adhesion and antigen presentation interactions typically occur at pseudopodal extensions from the APC (white arrows). PEGylation of either allogeneic PBMC population decreases the stability and duration of initial cell-cell interactions between lymphocytes due to the global charge and steric camouflage of membrane proteins. (D) Importantly, the secretomes derived from the MLR and mPEG-MLR exert potent effects on a secondary MLR encompassing fresh PBMC from the same or different donors. The key component of the secretome are soluble (free and exosome) miRNA. Data derived from Refs [32–43].

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cells (Teff) that mediate the inflammatory response and consists, in part, of Th1, Th17 and Th2 subsets. Hence, the functional ratio of Treg to Teff (Treg:Teff) cells is critical and an imbalance of this ratio from the norm can induce either an autoimmune (excess Teff or decreased Treg) state or impaired response to “nonsensel” (e.g., cancer) consequent to biologically ill-advised tolerance (too many Treg or weak Teff response). Indeed, the T cell response plays a (the) central role in autoimmune diseases, transplant rejection, graft versus host disease (GVHD), graft versus leukemia (GVL), cancer and, more recently, cancer therapy. Hence, consequent to the central role of T cells as a key cellular component in the development of autoimmune diseases, graft tolerance or rejection, and the anticancer response, the T cell response has been a major focus in the development of clinical therapies (Figure 1A) [1].

2. Immunomodulation of the T cell response in autoimmunity and cancer

Autoimmune diseases arise when the immune system recognizes the individual’s own tissues or organs as “foreign” and targets them for destruction. Autoimmune diseases can affect virtually all tissues and organ systems and encompass such diverse diseases as Type 1 Diabetes (T1D; pancreas), Idiopathic Thrombocytopenic Purpura (ITP; platelet destruction), Crohn’s disease (CD; bowel), Multiple Sclerosis (MS; brain) and Rheumatoid Arthritis (RA; joints). Despite the diversity of tissues affected, extensive research has demonstrated that Treg are downregulated while Teff are upregulated (i.e., leading to a reduced Treg:Teff ratio) leading to a chronic proinflammatory state. Current therapeutic approaches to managing autoimmune diseases are typically focused on symptom relief and the use of immunosuppressive agents capable of inhibiting the proinflammatory response arising from “self-recognition.” Most commonly, treatment for chronic autoimmune disease is via administration of systemic steroids (e.g., dexamethasone), cytotoxic anti-proliferative/activation agents (e.g., cyclosporine) that induce a general immunosuppression, and/or IVIG (pooled, polyvalent, IgG purified from the plasma of >1000 blood donors) [2–6]. Other experimental approaches to the treatment of autoimmune diseases include blocking monoclonal antibodies directed against the TCR, CD4, costimulatory ligands and receptors, adhesion molecules, and cytokine receptors [7–9]. A more recent approach has been to interrupt the cytokine signals necessary for the activation and proliferation of autoreactive T cells. The current gold standard for this approach is Enbrel® (etanercept), a solubilized TNF-α receptor fragment that intercepts and sequesters the TNF-α cytokine thereby inhibiting the proliferation of proinflammatory T cells [10–15]. However, Enbrel® has been given a USA FDA “Black Box” warning due to significantly increased risks of serious infections that may lead to hospitalization or death [16–22]. Common to all of these approaches is an attempt to increase the Treg:Teff ratio by either directly increasing Treg or selectively decreasing Teff populations. However, despite their importance in clinical medicine, many of these agents have been plagued by both significant toxicity/adverse events and an inability to adequately eliminate or inhibit reactive T cells [8].

In contrast to autoimmune diseases, an insufficient/inefficient immune response may underlie the proliferation and dissemination of abnormal cells (i.e., cancer cells). While this may occur for a number of reasons, immunosuppression is a known risk factor. Indeed, acquired or inherited T cell defects as well as long-term therapy with immunosuppressive drugs are clearly associated with an increased risk of neoplasia. The impaired immune response to cancer cells can arise, at least in part, from an increase in the Treg:Teff ratio (too many Treg and/or insufficient Teff cell production). To address this imbalance in the Treg:Teff ratio, experimental
therapies are currently focused on the ex vivo expansion and subsequent transfusion of autologous Teff capable of killing the cancer cells [23–31]. However, these current immune enhancing methods, while promising, are expensive, complicated to accomplish (e.g., insertion of specific target cancer genes in APC) and requires weeks of tissue culture expansion to meet the threshold for cell infusion.

Perhaps most importantly, current tolerogenic or proinflammatory therapeutic approaches fail to persistently reorient the systemic T cell immune response thus necessitating continual therapy. Moreover, despite the importance of the Treg:Teff ratio, in both autoimmune diseases and cancer, there are a paucity of pharmacologic tools that can directly, and in tandem, target the regulation of both the Treg and Teff subsets. Hence, to diminish or overcome the need for chronic administration of immunotherapeutic agents, new approaches capable of persistently reorienting the endogenous immune (Treg:Teff) response would be of value.

3. Immunomodulation via immunocamouflage and differential miRNA production

Previous studies from our laboratory demonstrated that a persistent and systemic reorientation of the animal (murine; or in vitro human) immune response towards a tolerogenic response could be induced via the adoptive transfer of immunocamouflaged allogeneic leukocytes to a recipient animal [32–43]. Immunocamouflage of cells is mediated by the covalent grafting of methoxypoly(ethylene glycol) (mPEG) to the leukocyte membrane surface. Consequent to mPEG-grafting (PEGylation), MHC-mediated T cell alloproliferation is dramatically inhibited due to consequent to impaired cell:cell interaction and weak allostimulation (Figure 1A and B). These studies demonstrated that the PEGylated allogeneic leukocytes diminished intracellular communication preventing a Teff response while simultaneously inducing the generation of Treg cells skewing the Treg:Teff ratio towards a tolerogenic state (Figure 1B and C) [36, 38–43]. Further in vitro and in vivo studies demonstrated that, using MLR-based secretome biomanufacturing systems, distinct acellular microRNA (miRNA) based therapeutics could be manufactured from control and PEGylated allore cognition reactions that systemically and persistently reorient the immune response to either a proinflammatory (IA1) or tolerogenic (TA1) state (Figure 1) [40, 43]. In this chapter, we will demonstrate how these miRNA-based therapeutics can inhibit the progression of T cell mediated autoimmune diseases (TA1) or conversely enhance the proinflammatory anticancer T cell response (IA1).

4. Production of miRNA therapeutics via the alloreponse pathway

Since their discovery in 1996, the role of circulating (cell-free) miRNA in disease processes has become an active research area and recent findings suggest that they may be biomarkers, or possibly mediators, of cancers as well as autoimmune diseases such as T1D [44–46]. To understand mechanistically how the TA1 and IA1 miRNA biologics function, an appreciation of the biological role and regulatory complexity of miRNA is needed. Recent studies have demonstrated that miRNA are key epigenetic regulators of cellular processes including immune responses, inflammation, proliferation, survival, and cellular differentiation [47, 48]. miRNA are short (~22 nucleotides) single-stranded RNA molecules found in all eukaryotes and it is estimated that ~60% of mammalian genes are targeted by one or more miRNA [49, 50]. Moreover, because of their evolutionary importance in gene regulation, miRNA and their
sequence and processing are highly conserved between mammalian species (e.g., mouse and human) [49]. While miRNA are most commonly found intracellularly, significant amounts of stable miRNA are also found in the serum of mammals suggesting an important messenger/regulatory role. While the nomenclature of miRNAs is relatively straightforward it is important to note that similarities in miRNA number designation is not indicative of similarity in functionality (Figure 2). Moreover, the literature is replete with conflicting claims for the specific actions of a single miRNA.

Indeed, there is a significant lack of clarity regarding the function of a single miRNA. This lack of functional clarity likely arises consequent to the complexity and low fidelity of the miRNA bioregulatory process. Of note, a single miRNA can potentially affect tens to hundreds of genes and individual genes can be regulated by multiple miRNA [50]. Hence, the effect of modifying the expression of a single miRNA on protein regulation and bioregulatory networks is unpredictable. Because of this regulatory complexity, most studies have focused on miRNA as disease biomarkers, not as therapeutic agents as there is a low probability that altered expression of a single, or even a few, miRNA would exert a potent and definitive biological response [51–54]. From a bioregulatory approach, it is more probable that multiple miRNA control protein expression, proliferation and differentiation and it is this “pattern of miRNA expression” (encompassing increased, decreased and static levels) that must be mimicked to achieve pharmacologically effective miRNA-based therapeutics. To achieve this goal our laboratory approach has been purposefully chosen to biologically manufacture relatively complex miRNA preparations mimicking normal biology in order to achieve maximal biological functionality.

Using a Mixed Lymphocyte Reaction (MLR) production model the T cell centric proinflammatory IA1 and tolerogenic TA1 therapeutics can be reproducibly manufactured using the control-MLR and mPEG-MLR (respectively; Figure 1). As demonstrated, the allogeneic PBMC populations within the control- and mPEG-MLR express significantly different patterns of miRNA expression relative to resting

**Understanding MicroRNA Nomenclature**

![MicroRNA Nomenclature](image)

*Figure 2.* miRNA nomenclature explained. An important concept to understand is that the miRNA number (e.g., 123 as shown) has no relationship to function or structure. For example, “hsa-miR-123” has no implied structural or functional similarity to “hsa-miR-128.” However, because of the highly conserved nature of miRNA, human “hsa-miR-123” has very similar structure and function to murine “mmu-miR-123.”
PBMC as evidenced via clustergram (Figure 3A), volcano plot (Figure 3B) and Log2 Fold (Figure 3C) miRNA expression analyses. Importantly, as shown in Figure 3C, the control- and mPEG-MLRs show unique patterns of expression. While there are some similarities in the pattern of expression there are significant disparity in miRNAs expressed as well (not shown are the miRNA unchanged from resting cells).

Importantly, the differences in miRNA expression between the Control- and mPEG-MLR leukocyte yield secretomes that exert dramatically different effects when used to treat resting human PBMC or murine splenocytes. Collection of the secretome produced (Figure 4A) during the control and polymer modified allorrecognition-based MLR yields a reproducible, acellular, miRNA-rich, material that is stable and can be frozen and thawed with minimal decrement to its activity. As schematically presented (Figure 4B), TA1 upregulates regulatory T cell populations (e.g., Treg) while simultaneously downregulating Teff (e.g., Th17 and Th1) cells. In contrast, the proinflammatory IA1 increases Teff while decreasing Treg cells. Of note, the secretome from resting cells (SYN) has minimal to no effect on human or mouse immune cells. Moreover, due to the conserved nature of mammalian miRNA, cross species efficacy is observed with both TA1 and IA1. As shown in Figure 4C, murine splenocyte produced TA1 and IA1 exerted dose-dependent effects on a human MLR with murine-sourced TA1 reducing CD3+CD4+ T cell proliferation and the murine IA1 enhancing CD3+CD4+ T cell proliferation. Hence, a polymer-based, alloreponse manufacturing system may provide a unique avenue for more effectively, and safely, modulating the Treg:Teff cell ratio via the production of therapeutically effective TA1 and IA1 miRNA-based therapeutics [32–43]. Importantly, the effects of TA1 and IA1 immunotherapy was persistent. In murine studies, a single dosing of TA1 to mice resulted in significant increase in Treg cells within the spleen of normal mice that persisted to ≥270 days post treatment (Figure 4D).

**Figure 3.**
Partial qPCR characterization of the miRNA expression in the Control- and mPEG-MLR. (A–C) Clustergram (A), Volcano Plot (B) and Log2 Fold (C) analyses of the miRNA expression in the mPEG-MLR and Control-MLR relative to resting cells. (C) Because of the complexity of miRNA regulation of genes, we have consciously chosen to produce a relatively complex miRNA preparations mimicking normal biology in order to achieve maximal biological functionality. Multiple miRNA changes are noted in the hTA1 miRNA compared to either resting cells (green dashed line = 0) or the proinflammatory hIA1 miRNA preparation. Using miRNA expressing a net ΔLog2 Fold change, significantly different “patterns of expression” are noted between the hTA1 and hIA1 miRNA. This pattern of expression, comprising both INCREASED and DECREASED miRNA species is essential for effective immunomodulation of recipient animals. Values derived from a minimum of 3 independent biological replicates. Unpublished data.
5. Tolerogenic TA1: immunomodulation of autoimmune disease

Autoimmune destruction of pancreatic islets gives rise to T1D and occurs via T cell dependent pathways [55–57]. Elucidation of the role of T cells in T1D has been most effectively examined in the nonobese diabetic (NOD) mouse model. In the NOD mouse, evidence suggests that a deficit in Treg control over diabetogenic Teff cells leads to the development of insulitis and disease [56–66]. Indeed, changes in the Treg:Teff ratio (i.e., balance) can be observed as early as 3–4 weeks of age and becomes more pronounced with disease progression (Figure 5) [56]. Human studies have similarly demonstrated that T1D Treg exhibit an impaired ability to suppress Teff [67]. Thus, the emergence of an aggressive diabetogenic lymphocyte response in NOD mice, and likely humans, is dependent upon a change in the Treg:Teff ratio.

As demonstrated in Figure 5, the Treg:Teff ratio (defined as the ratio of Foxp3+ to Th17+ T cells) in control (saline treated) NOD mice decreased with disease progression from 103 in nondiabetic 7 week old mice to only 4.7 in diabetic mice at time of sacrifice (15–30 week). Moreover, control NOD mice exhibited a rapid onset of diabetes with 75% (12 of 16) of the mice becoming diabetic by week 19. Subsequent to week 19, no additional mice became diabetic. In contrast, a single dosing (3 injections at 2 days intervals) of the TA1 therapeutic at 7 weeks of age dramatically altered both the incidence and rate of progression of the T1D in the NOD mouse. By week 19 only 13% (2 of 15) of the TA1 treated mice became diabetic with an additional 4 mice becoming diabetic between weeks 21 and 23 (total diabetic 6/15; 40%). Mechanistically, these findings were associated with...
a systemic alteration of the immune system as noted in Figure 5. In control NOD mice, the progression to diabetes was characterized by significantly elevated levels of most proinflammatory Teff (e.g., INF-γ’, Th17’, and IL-2’) lymphocytes and a corresponding decrease in regulatory subsets. In contrast, TA1 therapy dramatically and significantly blunted the expansion of Teff cells (as exemplified by INF-γ’, Th17’, and IL-2’ lymphocytes; Figure 5A) relative to diabetic or nondiabetic control NOD mice coupled with a simultaneous increase in a broad range of tolerogenic/anergic regulatory T cell subsets (e.g., foxp3+, IL-10+, TGF-β’; Figure 5B) in the pancreatic lymph node. These studies also demonstrated that TA1 treated NOD mice had significant numbers of histologically normal pancreatic islets while no normal islets were identified in the untreated mice [40]. It is worth noting that all diabetic mice (control and TA1-treated) exhibited significantly lower levels of these tolerogenic cells than did the 30 week old nondiabetic (control or TA1) mice. Moreover, the effects of TA1-miRNA therapy were not localized to the pancreatic lymph node microenvironment. Analyses of the T cell subsets present in the spleen and brachial lymph node of control and TA1 treated NOD mice (diabetic and nondiabetic) similarly demonstrated dramatic changes in the

![Figure 5](image_url)

*Figure 5. The autoimmune disease of T1D process is mediated by a decrease in the Treg:Teff ratio and can be prevented by TA1 administration (top). Treatment with the TA1 miRNA product prevents the decrease and, in fact, significantly increases the Treg:Teff ratio. The increased Treg:Teff ratio is protective as evidenced by the finding that the majority of TA1 treated animals remained normoglycemic. Shown in the blue bars are the Treg:Teff ratio for TA1 treated mice who were diabetic (ratio of 70) and nondiabetic (ratio of 255). Mechanistically, TA1 immunotherapeutic significantly altered the expression of multiple proinflammatory (A) and tolerogenic/anergic (B) T cell subsets. These changes were systemic in nature as shown by changes in not only the pancreatic lymph node but in other immune tissues (spleen and brachial lymph node). Diabetic tissues were harvested at time of conversion, nondiabetic tissues were harvested at week 30. Diabetic values are the mean ± SD of 12 saline and 6 TA1 treated NOD mice. Nondiabetic results are the mean ± SD of 4 saline and 9 TA1 treated NOD mice. Derived from Ref. [40].*
Teff cell populations (Figure 5A, right) and tolerogenic T cells (Figure 5B, right). These findings demonstrate that miRNA-based TA1 therapeutic, directly targets the Treg:Teff ratio yielding a systemic protolerogenic state both in vivo (mouse) and in vitro (human and mouse) suggesting that this approach would be of utility in a broad range of autoimmune diseases. Furthermore, due to the persistence of the immunomodulatory activity in mice (Figure 4), TA1-like drugs could, potentially, dramatically reduce the need for chronic administration of drugs.

6. Proinflammatory IA1: enhancing the immune response to cancer

T cells plays a critical role in the anticancer inflammatory responses. An effective anticancer proinflammatory T cell response is dependent upon the activation of Teff cells. Normally, T cells are activated upon ligation of their antigen receptors with specific cognate antigens [68]. However, because of the low frequency of cancer antigen-specific lymphocytes, the immune response to cancers can be initially, and all too often remains, weak. While previous studies have attempted to enhance the anticancer T cell response using pan T cell mitogens (e.g., phytohemagglutinin; PHA), cytokines (e.g., IL-2), or monoclonal antibodies (e.g., anti-CD3 and anti-CD28) the overly robust T cell response arising from these approaches often induced significant systemic toxicity leading to the suspension or abrogation of multiple clinical trials [69–74]. In contrast, in an allore cognition response only 1–10% of T cells are alloreactive [75]. Hence, the IA1 therapeutic, derived from a bioreactor allore cognition response (MLR), is expected to activate endogenous T cells in a more controlled manner, with less toxicity.

To assess IA1's ability to enhance the anticancer activity of resting PBMC, cells were treated for 24 hours with IA1 and overlaid on HeLa and SH-4 cancers cells. Cancer cell proliferation was then followed for 168 hours. Importantly, IA1 exerted no toxicity to resting PBMC but, as shown in Figure 4, induced significant activation (e.g., proliferation) of resting CD3+ (CD4+ and CD8+) skewed towards proinflammatory subsets thus decreasing the Teff:Treg ratio. However, as predicted by the biology of the alloresponse, IA1-mediated T cell proliferation was much more restrained than that induced by the anti-CD3/anti-CD28 or PHA stimulation [43]. This finding suggests that the systemic toxicity, relative to pan T cell activators, should be greatly reduced. Crucially, IA1-activated PBMC demonstrated a potent inhibition of cancer cell (HeLa and SH-4 melanoma) proliferation relative to the resting PBMC (Figure 6). The anti-proliferation effect of IA1-activated PBMC was noted within ~12 hours vs. 4–5 days for resting cells. These findings demonstrate that miRNA-enriched therapeutics can be biomanufactured from the secretome and can induce a potent proinflammatory, anticancer, effect on resting lymphocytes.

The potential utility and use of IA1 in Adoptive Cell Therapy (ACT) is diagrammatically shown in Figure 6. The bioproduction of IA1 is both inexpensive and rapid (5 days) and the IA1 can be stored for long periods (several months frozen in the laboratory; data not shown). Moreover, neither IA1 or TA1 production actually requires donor specific tissues (PBMC) making these secretome-based therapeutics an “off-the-shelf” immune adjuvant. Most importantly for patient care, ex vivo activation of lymphocytes is rapid (24 hours). The rapidity of this approach is in stark contrast to the weeks to months necessary for production and expansion of CAR-T cells. Hence, IA1 activation of autologous PBMC could be employed as a first line therapy or, potentially, be used as an immunotherapeutic bridge while CAR-T cells are produced. Due to the simplicity and low cost of the approach, multiple rounds could be used as necessary with large numbers of autologous PBMC employed. Indeed, due to the ability to infuse large numbers of IA1 treated autologous cells, enhanced recognition

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of not only the primary tumor but metastatic sites as well could be achieved thus improving long-term survival. Of note, similarly to our use of the tolerogenic TA1 in NOD mice (Figures 4 and 5), IA1 could be directly injected into the recipient yielding a systemic proinflammatory reset of the immune system [40].

7. Conclusions

The immunomodulation of the endogenous immune system has become a major focus in treating a broad range of clinical conditions ranging from tissue/organ engraftment, autoimmune disease and cancer therapy. While significant clinical advancements have been made in immunotherapy, substantial challenges remain. One target of interest is the biologic/clinical desire to induce a persistent systemic immunological reset that could reduce both the need for chronic therapy and reduce the potential toxicities associated with current immunomodulatory approaches. Recent studies have demonstrated that miRNA are key regulators of cellular processes involved in both tolerogenic and proinflammatory immune responses and mediate immune cell proliferation and differentiation. Using an alloreponse bioreactor secretome system we have demonstrated that miRNA-based therapeutics can be reproducibly manufactured that can systemically reorient the immune system to either a tolerogenic or proinflammatory state by simultaneously...
modulating both regulatory and effector T cell subsets thus skewing the Treg:Teff cell ratio to favor tolerance or inflammation. The tolerogenic TA1 therapeutic is derived from polymer-mediated immunocamouflage of the alloresponse reaction while the inflammatory IA1 preparation is derived from the alloresponse itself. The secretomes from these reactions are processed to maintain the miRNA within the secretome. In contrast to most miRNA therapeutic tactics, our approach has been to mimic the “complex pattern of miRNA expression” seen in protolerogenic or proinflammatory states. This “complex” approach was predicated by the inherent nature of miRNA bioregulation in that there is a low probability that altered expression of a single, or even a few, miRNA would exert a potent and definitive biological response. As shown, this approach successfully results in significant and, in mice, systemic and persistent changes to the immune system. The tolerogenic TA1 proved useful in reducing the onset and incidence of autoimmune diabetes in the NOD mouse while the proinflammatory IA1 therapeutic greatly enhanced the efficacy of human T cells to recognize and kill cancer cells without inducing the systemic inflammatory response seen with mitogens or monoclonal antibody (e.g., anti-CD3/CD28) therapies. Moreover, this approach can simultaneously modulate both regulatory and effect T cell subtype. The successful development of this miRNA-immunomodulatory approach may prove useful in facilitating organ engraftment, treating autoimmune disease and enhancing the endogenous anticancer response.

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### Conflict of interest

Canadian Blood Services is pursuing patents related to the production and utilization of the described acellular immunomodulatory agents. Canadian Blood Services, a not-for-profit organization responsible for collecting, manufacturing and distributing blood and blood products to all Canadians (except Quebec), is the assignee for relevant patents. MDS, DW and WMT are inventors on these patents. XY has no conflicts of interest beyond being paid by Canadian Blood Services.
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Section 3

Macrophages
Chapter 6

Mucosal Macrophage Polarization
Role in the Immune Modulation

Tsung-Meng Wu, Shiu-Nan Chen and Yu-Sheng Wu

Abstract

Immunotherapy has advantages including few side effects and low probability of abuse by patients. Recently, functional materials with immunomodulatory functions, which act through reduction of free radicals, have been developed for cancer and anti-inflammatory therapy. However, the therapeutic application of natural functional materials involves a complex mechanism along with various organic factors. These substances, including polysaccharides and triterpenoids, have immunomodulatory effects. However, to our knowledge, the mechanism underlying the action of such substances in the physiological immunity of animals remains unclear.

Immune cells, particularly macrophages, are crucial in the modulation of immune response. Macrophages polarise into two types, namely, M1 and M2, from the M0 form, based on the physiological microenvironment factors. M1 macrophages have functions in pathogen elimination through phagocytosis, oxidative damage, and complement system activation. M2 macrophages are involved in tissue recovery and tumour tissues containing ample M2 macrophages that release growth factors, which promote angiogenesis. In this study, we focus on the immunomodulation of the macrophage to further understand the effects of the physiological microenvironment factors on macrophage polarisation.

Keywords: macrophage, polarisation, immune modulation

1. Introduction: immune cells in the mucosal system

The mucosal system is ubiquitous throughout the body; the mucosal tissue is typically present in association with various organ systems, including the gastrointestinal tract, respiratory tract, and genitourinary tract, as well as the exocrine glands associated with these systems, such as the pancreas, lacrimal glands, salivary glands, and breasts. According to their location and function, mucosal tissues can be divided into nasopharynx-associated lymphoid tissue (NALT) [1], bronchus-associated lymphoid tissue (BALT) [2], and gut-associated lymphoid tissue (GALT) [3]. The surface area of the mucosal system is very broad; its physiological functions include gas exchange, food absorption, and sensory function. The mucus on the mucosal surface acts as a protective barrier inside the body to protect the body from foreign pathogenic infections [4]. Because of the distribution of mucus over a large surface area, the probability of mucosal tissues coming in contact with pathogens is higher than that of other tissues in the body. Nevertheless, these tissues are responsible for the evasion of the pathogens. Adhesion molecules, expressed by tissues and organs, enable the binding of lymphocyte receptors that attract lymphocytes towards the mucosal surface.
Chapter 6

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GALT, the largest lymphoid organ in the human body, contains 70–80% of the lymphoid tissues of the human body. The main GALT components include lamina propria (LP), Peyer’s patches (PP), and mesenteric lymph node (MLN).

A mucosal immune response involves various cells, particularly macrophages. Macrophages are present in almost all tissues and have distinct location-specific phenotypes; their gene expression profiles demonstrate considerable functional diversity in innate immune response, tissue development, and tissue homoeostasis [5, 6]. Resident macrophages in different organ tissues are named differently. For instance, microglia cells have pathogenetic significance regarding perivascular inflammatory phenomena in the brain [7, 8], Kupffer cells have a major role in the homoeostatic function of the liver and are associated with the tissue damage [9], and alveolar macrophages (AMs) are a key determinant of pulmonary immune responses and thus have a role in lung inflammation (e.g. asthma) [10]. Previously, tissue-resident macrophages were considered to be recruited from circulating blood monocytes. However, recent studies have demonstrated that tissue-resident macrophages, such as microglial, Kupffer, and Langerhans cells, are established prenatally; they arise independently from the haematopoietic transcription factor [11, 12], which is required for the development of haematopoietic stem cells (HSCs) and all CD11b\textsuperscript{high} monocytes and macrophages, but is not required for yolk sac (YS) macrophages and for YS-derived F4/80\textsuperscript{bright} macrophages in several tissues, which can all persist in adult mice independently of HSCs [12]. Kupffer cells and other resident macrophages (e.g. microglia) originate from the YS in a colony-stimulating factor-1 receptor (CSF-1R)-dependent and Myb-independent manner and may be maintained through local proliferation, resulting in extensive mitosis after stress or an exchanged tissue microenvironment [13, 14].

2. Phenomenon of macrophage and its importance in the immune response

Macrophages are primarily divided into two types based on function and differentiation: classically activated (M1) and alternatively activated (M2) macrophages (Figure 1). Both have roles in innate resistance and constitute a link between inflammation and autoimmune disease. In mouse models, macrophages contain CD11b, F4/80, and CSF-1R, where F4/80 is the surface protein for M1 and M2 macrophages [15, 16]; these are circulating monocytes (present in the peripheral blood), which are secreted in response to chemokines produced in response to exposure to an antigen (e.g. pathogens entering the organism from the portal vein of the intestines). When interacting with pattern recognition receptors, antigens may lead to M1- or M2-polarising activities, depending on the secreted Th1 cytokine [interferon (IFN)-\(\gamma\)], Th2 cytokines [interleukin (IL)-4 and IL-13], and other immune factors [17–19]. Macrophage is also role in the antigen presenting, to induce the B cell active and response to the antibody production. The antibody production is from the plasma cell (active B cell), where there is a molecule material expression on its surface. A part of these receptors is named B-cell receptors (BCRs).

B-cell receptor is a B-cell membrane-bound surface protein that acts as a cellular receptor. During B-cell differentiation, differentiated B cells, transferred as plasma cells, secrete immunoglobulins (Igs). Structurally, Igs are similar to the BCRs and are called antibodies [20].

The main functions of antibodies include neutralising the antigen, activating complement reaction, and participating in the adaptive immunity. An antibody comprises two heavy and two light chains, is Y shaped, and is divided into variable and constant regions. The variable region contains the antigen-binding sites [21],...
Macrophages are present in almost all tissues and have distinct location-specific functions. They play a crucial role in innate and adaptive immune responses, tissue development, and maintenance of tissue homoeostasis. Macrophages are primarily divided into two types based on function and location: M1 and M2.

The inactive macrophage is differentiated into M1 or M2 macrophage by various stress and stimulants in the host microenvironment. M1 macrophage is participating in the inflammatory response, a major function in the pathogen clearance. M2 macrophage is in contrast to the M1 macrophage.

2.1 B-1 cells

B-1 cells are called natural antibodies and include the IgG, IgA, and IgM isotypes [27, 28]. Natural antibodies are secreted when B-1 cells are not stimulated by foreign pathogens, which can bind to different pathogens but have low affinity.

2.2 Ig isotype switching (class switching)

B cells can express different C-region genes during cell maturation and proliferate during the reaction [29]. It simultaneously expresses IgM and IgD through RNA modification [30]. As the immune function continues to respond, antibodies in the same variant region can be expressed as IgG, IgA, and IgE. This process is called homotypic conversion or class switching, which protects all parts of the body at the appropriate time by using specific antibodies produced by the same antigen.
2.3 Ig isotype

According to the structural differences of the heavy chain, Igs are divided into five isotypes [25]:

i. IgG, a major Ig in the blood, exists in a monomeric form and thus easily spreads throughout the body through peripheral blood. For instance, in humans, IgG enters the foetus through the placenta, providing passive immunity to the foetus. IgG has a modulating function, which efficiently promotes the phagocytosis of pathogens [31].

ii. IgA, present in serum as a monomer, can diffuse outside blood vessels. In most of the body, IgA is present as a dimer, mainly in mucus and particularly the intestinal and respiratory mucosa. The main function of IgA is antigen neutralisation [32]. Transforming growth factor (TGF)-β and IL-4 can effectively promote IgA isotype switching [33].

iii. IgM is mainly distributed in the blood and less in the lymph. IgM is effective in activating the complement system and is important for controlling infection [34].

iv. IgE is the immunoglobulin that the body mainly produces when the allergens invade. IgE is relatively low in blood and body fluids and strongly binds to the Fc receptors of the mast cells under the blood vessels, submucosa, and connective tissue [35].

v. IgD is the least among other types of immunoglobulins in the body, and there is no clear biological function discovery [36].

Class switching is mainly caused through the influence of cytokines or antigen secretion, which stimulates B cells to express different Igs. For instance, IL-4, IL-5, IL-10, and TGF, present in GALT, enable B cells to secrete IgA after isotype switching [37, 38]. When B cells isolated from mice were exposed to TGF-β in an in vitro culture, the proportion of IgA secreted by the TGF-β-treated B cells was significantly higher than that secreted by the untreated cells [33]. Through homologous switching, B cells secrete antibodies specific for an antigen and supply it to the appropriate body part in a timely manner. The vertebrate intestinal mucosal immune system secretes a large amount of secretory IgA (sIgA) [39]. In mucus, the proportion of slgA is higher than that of other antibody isotypes. SlgA mainly neutralises pathogens and limits the entry of pathogens into the body. Mcghee et al. found that coculture of B cells of PP with either IL-5 or IL-6 can promote the differentiation of B cells into IgA-secreting plasma cells [40]. Plasma cells release intact J chain-linked IgA dimers, which bind to the endothelial Ig receptors expressed by intestinal epithelial cells and undergo transcytosis [41]. Piskurich et al. cocultured human colonic cell line (HT-29) with IFN-γ and found that IFN-γ stimulated the expression of the poly-Ig receptor gene in a concentration-dependent manner, as detected through immunofluorescence [42]. In other words, IFN-γ can stimulate the expression of poly-Ig receptors. In mice with poly-Ig receptor gene deficiency, IgA expression in serum is significantly higher than that in normal mice, whereas slgA expression in the mucosal sites is significantly lower than that in normal mice; taken together, poly-Ig receptor gene defects cause IgA to accumulate in the serum of mice. Thus, poly-Ig receptors are crucial for slgA expression in the mucosal sites [43–45].
3. Immunomodulation in the mucosal system

In the mucosal system, the immune response is an important reaction regulating the physiological homoeostasis, including immunomodulation, in the whole body. The major mucosal systems controlling the immune response are as follows:

3.1 LP of the intestinal mucosa

The LP of the intestinal mucosa is located below the intestinal epithelial cells and includes various cell types, including Ig-derived plasma cells, T cells, dendritic cells, macrophages, and various cytokines [46]. Under normal conditions, the LP of the intestinal mucosa exhibits high levels of TGF-β [47] and IL-10 [48], which promotes antigen-activated B-cell isoforms. The pathogen enters the LP of the intestinal mucosa from the intestines. The pathogen is recognised by the immune system, and it stimulates B cells to undergo isotype switching to secrete IgA, IgG, and IgM. In a study, rats were administered inactivated *Entamoeba histolytica* through feeding, and IgA, IgG, and IgM were detected in serum and faeces on postfeeding days 2, 4, 6, 8, and 10. IgG and IgM expression in rat serum increased, and IgG and IgA expression in faeces also increased [49].

3.2 PP

PP, located below intestinal epithelial cells, is also the induction sites of the intestinal mucosal immune response [50] and has a high number of B and T cells compared with other lymph nodes [51]. PP contains numerous cytokines, including TGF-β, IL-4, IL-6, and IL-10, which stimulate B cells to secrete IgGs [52, 53]. The upper part of PP includes specialised epithelial cells called microfold (M) cells [54]. The antigen in the intestine can enter the lymphoid tissue of the subsequent layer through M cells and initiate an immune reaction. However, the proportion of M cells in the intestine is not high; thus, the ability of M cells to deliver antigens is limited [55]. PP is an indispensable immunotolerance-related tissue, particularly in mice [56].

3.3 MLN

Lymph nodes are tissues located at the junction of the lymphatic system and higher organs [57]. The vast lymphatic vasculature collects lymph from tissues and returns it to the blood. MLNs are the lymph nodes in the intestinal mucosal immune system [58]. When an antigen enters the body through the intestinal mucosal system, it encounters the lymphatic system and is recognised; the antigen-presenting cells are then activated. These cells carry the antigen to the MLN, perform the antigen presentation reaction, and finally activate appropriate T and B cells [59–61].

3.4 Relationship between intestinal immune response and Igs

GALT macrophages have different characteristics from macrophages in other parts of the body; that is, they have good phagocytic and bactericidal abilities [62]. CD4+ FOXP3+ regulatory T (T_{Reg}) cells are located in the regulatory layer of the intestinal mucosa. T cells differentiate into T_{Reg} cells in the presence of TGF-β. The balance between functional T and T_{Reg} cells highly affects the homoeostasis of intestinal mucosal immune response [63].
4. Immunomodulation of macrophages in the immune system

Polysaccharides extracted from mushrooms or algae have immunomodulatory functions, such as increasing macrophage activity, regardless of whether innate or adaptive immunity is activated [64]. For instance, the phagocytic activity of cells, the killing ability of natural killer cells, and the promotion of immune cells to secrete cytokines activate the immune system. In our previous laboratory studies, mushroom polysaccharide administration could enhance the tumour-suppressive and anti-allergic ability in mice, along with significant enhancement in the wound-healing ability in rats. Immune cells of the innate immune response, such as macrophages and dendritic cells, or other nonimmune cells, such as epithelial cells, have many nonspecific recognition receptors associated with antigens that evade pathogens. Based on molecular identification and binding, complement receptor type 3 (CR3) on these cells can identify polysaccharides [65]. When polysaccharides bind to CR3, it triggers a series of signalling to activate transcription factors. Cells secrete a cytokine that triggers an inflammatory response, and the antigen exhibits the major histocompatibility complex of the cell, thereby activating other immune cells to achieve immunomodulatory functions [66, 67]. Dectin-1 belongs to the c-type lectin receptor family and is expressed on the cell membranes of macrophages, dendritic cells, neutrophils, and T and B cells [68]. Dectin-1 binds to polysaccharides to promote macrophage phagocytosis and respiratory burst; it also promotes the degranulation of neutrophils and secretion of cytokines and chemokines from immune cells [69–72]. Polysaccharides from Antrodia camphorata were cocultured with immature dendritic and T cells isolated from healthy human blood, and the polysaccharides could promote dendritic cell maturation and stimulate T-cell proliferation and IFN-γ performance [73, 74]. Coculture of polysaccharides with macrophages can promote the secretion of immune-related factors and cytokine gene expression, such as nitric oxide (NO), tumour necrosis factor (TNF)-α, IL-1β, and IL-6, to promote macrophage activity [75–78].

Based on our teams’ experimental results, the functional polysaccharide can stimulate macrophages and further activate cytokines TNF-α, IL-12, IFN-γ, IL-2, IL-4, IL-10, and IL-17, which are associated with apoptosis and cell cycle. Growth hormone, a multipeptide hormone regulator, promotes growth and cell proliferation [75, 78, 79]. Polysaccharides can reduce CCl4-induced liver damage by regulating related antioxidant enzymes and effectively reducing oxidative damage in liver tissue [80, 81]. In mice, intraperitoneal polysaccharide injection could effectively prevent lipid peroxidation and inhibit the production of reactive oxygen species in the liver [82, 83]. Taken together, the immunomodulation function of polysaccharides may effectively regulate cellular immune response [84].

5. Immunomodulation of macrophage differentiation

Immune cells are crucial in immune response modulation. As mentioned, macrophages polarise into M1 and M2 macrophages, which have distinct functions and are affected by the physiological microenvironment factors. M1 macrophages perform pathogen elimination through phagocytosis, inflict oxidative damage, and complement system activation. M2 macrophages have tissue recovery functions. Tumour tissues contain considerable amounts of M2 macrophages that release angiogenesis-promoting growth factors.

Inflammatory reactions can induce chronic diseases; thus, reducing inflammation is important for inhibiting chronic disease. To achieve anti-inflammatory effects, immunotherapy is a novel therapeutic approach without known side effects.
and drug resistance problems. However, anti-inflammatory processes involve complex reactions; for instance, cellular ROS production for eliminating pathogens can also induce cellular apoptosis [85, 86]. Thus, the balance between inflammatory and anti-inflammatory processes is essential. In case of any imbalance, natural functional materials, such as triterpenoids and polysaccharides, can be applied for immunomodulation.

In summary, polysaccharides can regulate macrophage differentiation to modulate host physiological response through cytokine secretion. Polysaccharides, such as beta-glucan, are known biological response modifiers that can activate leukocytes, monocytes, and macrophages [87, 88]. The activation mechanism involves the polysaccharides binding to the receptors, such as Toll-like receptor, expressed on AMs or Kupffer, Langerhans, mesangial, or microglial cells. After the binding, the immune cells are activated via Toll-like receptor four-mediated signalling pathways to modulate the immune capacity. The activated immune cells then produce IFN-γ, TNF-α, ILs, and other cytokines to modulate the anti-inflammatory process.

In conclusion, the use of the functional materials as alternative medicines in clinical therapy is feasible; however, before implementation, the substance's immunomodulatory mechanism should be clearly realised, particularly in the immune cell signal transduction.

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Conflict of interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Section 4

Myeloid-Derived Suppressor Cells
Section 4

Myeloid-Derived Suppressor Cells
Chapter 7

KLF4-Mediated Plasticity of Myeloid-Derived Suppressor Cells (MDSCs)

Daping Fan, Samir Raychoudhury and Walden Ai

Abstract

Robustness of tissues refers to their capability to maintain normal functions despite perturbation such as injuries. Recent studies suggest a key role of the immune system in injury repair. In this process, several immune cell lineages exhibit considerable plasticity as they migrate toward the site of damage and contribute to repair. For example, myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature cells and possess phenotypic plasticity in cancer, a pathological status that is considered as “wounds that do not heal.” They are characterized by their potent ability to suppress immune responses. In cutaneous wound healing, MDSCs not only execute their immunosuppressive function to inhibit inflammation but also stimulate cell proliferation once they adopt a fate of a totally different cell type. At a molecular level, we found that Krüppel-like factor 4 (KLF4), a transcription factor with multiple roles in homeostasis and disease development plays a critical role in regulating MDSCs. In this review, KLF4-mediated plasticity of MDSCs and the underlying mechanisms are discussed.

Keywords: KLF4, FSP-1, myeloid-derived suppressor cells (MDSCs), plasticity, cancer, wound healing

1. Introduction

KLF4 is a member of the Krüppel-like factor family, a group of zinc finger-containing transcription factors that are highly homologous with the Drosophila Krüppel protein [1–4]. It has important functions in a variety of cellular processes that include cell proliferation, differentiation, development, and maintenance of normal tissue homeostasis [5]. KLF4 has also been shown to act either as a tumor suppressor or an oncoprotein in a context-dependent manner [6–8]. Moreover, KLF4 is critical to barrier function of the skin and promotes physiological and pathological wound healing [9–11].

MDSCs are bone marrow-derived cells present in bone marrow, spleen, and circulation. They are a heterogeneous collection of immature myeloid cells. These immature cells possess typical CD11b+Ly6G+ markers in mice with a wider range of markers in humans. The main function of MDSCs is their potent ability to suppress the host immune responses, especially T-cell proliferation and cytokine production [12]. They possess phenotypic plasticity in cancer [13, 14], a pathological status that is considered as “wounds that do not heal.” However, while the involvement of MDSCs in wound healing has been shown by their recruitment to the wound sites [15], the
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role of their plasticity in wound healing has not been fully examined. On the other hand, two immune cell lineages closely related to MDSCs, namely neutrophils and macrophages, demonstrated their phenotypical and functional plasticity in wound repair [16]. In addition, we showed that in wound healing MDSCs not only execute their immunosuppressive function to inhibit inflammation, but also stimulate cell proliferation once they adopt a fibrocyte fate [11]. Collectively, these observations support a key role of MDCS plasticity in wound healing leading to tissue robustness, though the underlying cellular and molecular mechanisms are not clear.

We recently reported that KLF4 promotes cancer development by regulating the recruitment and function of MDSCs [8, 17, 18]. In addition, we found that KLF4 regulates generation of fibrocytes, emerging effector cells in chronic inflammation [19, 20], from MDSCs in cancer [8], wound healing [11], allergic asthma [21]. Given the importance of plasticity of macrophages, a highly relevant cell type to MDSCs, in tissue repair and regeneration [22], we postulate that KLF4 also regulates myeloid plasticity in wound healing. In this review, the role of KLF4 in regulating plasticity of MDSCs in wound healing and the underlying molecular mechanisms will be discussed.

2. Plasticity of MDSCs in cancer and wound healing

MDSCs represent a group of heterogeneous monocytes during myeloid cell development with a major attribute of immunosuppressive activities. The population of these cells increases in a number of conditions associated with chronic inflammation, autoimmune diseases, and cancer. These heterogeneous cells are now further divided into two major subgroups including polymorphonuclear (PMN) and monocytic (M)-MDSCs [23]. Although non-immunosuppressive MDSCs exist in tumor-bearing hosts or in conditions of chronic inflammation [24], in which MDSCs can be classified as MDSC-like cells (MDSC-LC), demonstration of immunosuppressive activities is required to accurately define MDSCs after the initial phenotypical characterization by cell surface markers. In term of immunosuppressive activities of MDSCs, different mediators were reported, such as arginases, nitric oxide (NO), reactive oxygen species (ROS), indoleamine 2,3-dioxygenase (IDO), transforming growth factor-β1 (TGF-β1), and prostaglandin E2 (PGE2) among others, depending on specific conditions. As MDSCs are heterogeneous and suppress immune functions with different mechanisms, it is not surprising that they possess phenotypical and functional plasticity [25], reflecting their adaptation to varied environmental conditions. Note that immune cell plasticity could be understood from two different and important senses [16]. The first one is intra-lineage cell plasticity, that is, changes in cell function within a given cell lineage. This is also known as functional plasticity. The second sense is trans-lineage cell plasticity, that is, the switch from one lineage to another. Alternatively, this can be called “transdifferentiation” or “phenotypical plasticity.” We will mainly use “phenotypical plasticity” and “functional plasticity” to discuss MDSC functions in this chapter.

2.1 MDSC plasticity in cancer

Immunotherapies against cancer rely on activated T cells or NK cells to recognize and eliminate tumor cells. However, the effector cells in the tumor microenvironment encounter a wide array of factors that limit their activities. MDSC-mediated immune suppression represents one of the major mechanisms by which the functions of immune effector cells are blocked in cancer. In addition, MDSCs are implicated not only in regulating tumor immune response, but also in tumor angiogenesis, tumor cell invasion, and formation of pre-metastatic niches [26].
Phenotypical plasticity of MDSCs in cancer could be first understood from the capacity of myeloid regulatory cells to convert from each other under certain conditions. Such plasticity could explain confusing observations on the role of MDSCs in tumor growth or tumor inhibition [13]. For example, while MDSCs are well known for their tumor promoting function because of their immunosuppressive activities against T cells, they can be converted to dendritic cells (DCs) in the presence of nature killer T (NKT) cells and α-galactosylceramide, leading to an anti-tumor immune response against HER2/CT26 tumor [27]. Mechanistically, it was proposed that NKT cells interact with MDSCs. This interaction leads to the conversion of MDSCs to DCs by increasing gene expression of CD80, CD86 and CD70. Consequently, interactions of CD80 and CD70 on newly converted DCs with CD28 and CD27 on T cells support these T cell responses to the tumor cells resulting in elimination of MDSC-mediated immune suppression [13].

Phenotypical plasticity of MDSCs could also be understood from the existence of MDSC subtypes and their differentiation into macrophages under normal and abnormal conditions. Because PMN-MDSCs are short lived, M-MDSCs have been studied in a more detail. In addition, most studies did not correlate M-MDSCs with monocytes expressing high levels of Ly-6C (Ly-6C^hi cells). These Ly-6C^hi cells are frequently referred to inflammatory monocytes. Given their elevated function at the tumor site and their potent immunosuppressive activities, Ly-6C^hi monocytes in the tumor microenvironment most likely represent bona fide M-MDSCs [14]. M-MDSCs have been shown to differentiate into tumor-associated macrophages (TAMS) after they are recruited to the tumor site [28]. It was shown that the CD45-mediated inhibition of STAT3 in MDSCs promotes TAM differentiation [29]. Besides TAMs and DCs as we discussed earlier, MDSCs differentiate into fibrocytes, an emerging group of cells with multiple functions in inflammation and cancer [19, 20, 30, 31].

Functional plasticity of MDSCs could be understood by their intrinsic features especially their immunosuppressive activities. It is known that immunosuppressive activities of MDSCs are mainly detected in tumors, but rarely in other tissues or organs including bone marrow or spleen. However, MDSCs in tumor and other chronic inflammatory conditions may not always be immunosuppressive. For example, in the initiation stage of chronic inflammation or early stage tumors, there are cells with MDSC phenotypical markers but without potent immunosuppressive activities. Moreover, even in advanced stage tumors, not all cells with a MDSC phenotype possess immune suppressive activity. For example, recent studies showed that in chronic inflammation, cells with an MDSC phenotype lacking suppressive activity actually contribute to the early stages of tumor inflammation [32]. However, the exact nature and the mechanism of how MDSCs acquire their immune suppressive activities are not entirely clear.

2.2 Potential role of MDSC plasticity in tissue repair

Though immunologists generally consider the immune system as a system of defense, recent studies suggest a key role of the system in tissue robustness, the capability of an organism to maintain its function and performance despite perturbations [33, 34]. One of the major ways by which the immune system contributes to robustness is through immune cell plasticity. Most studies of tissue repair have focused on the innate immune system, which may reflect the evolutionary conservation of the repair-mediated robustness. Although plasticity of γδT cells [35, 36], innate lymphoid cells [37], and regulatory T cells [38] is also involved in tissue repair, we will mainly discuss the role of neutrophils, macrophages, and MDSCs in the process.

Neutrophils are the major innate cells recruited to the damage site and are considered as the first line of defense against infection [39]. However, these cells
can switch phenotypes, display distinct subpopulations, and produce a large variety of cytokines and chemokines [40]. In tissue repair, neutrophils can show their intra-lineage or functional plasticity by pro- or anti-inflammation, during the early stage of a typical wound repair. In addition, in an inflammatory and pro-type 2 microenvironment of a lesion, neutrophils transdifferentiate into antigen presenting cells (APCs) [41]. Such transdifferentiation into APCs has also been studied in rheumatism, where it could drive sustained inflammation, thereby preventing normal repair [42]. Besides neutrophils, macrophages fulfill roles that change over the duration of wound healing [43]. Initially they are bactericidal, and voraciously phagocytose cell and matrix debris, particularly red blood cells and any spent neutrophils at the wound site. These early stage macrophages are called M1 macrophages, and they are pro-inflammatory. Later in the repair process, macrophages develop the pro-repair capacity. These macrophages are called M2 macrophages, and they are anti-inflammatory and pro-reparative. The resting macrophages are called M0 macrophages. Not surprisingly, the plasticity of macrophages, namely the changeable cellular phenotypes and the range of differentiation and activation states, helps to explain the pleiotropic nature of these cells and their complex functions in wound repair [22, 44]. Beside their role in the early inflammatory stage of wound healing, macrophages contribute to tissue remodeling in wound healing by transdifferentiation, notably into endothelial cells [45, 46], a phenotypical plasticity.

When compared to those of neutrophils and macrophages, the role of MDSCs and their plasticity in wound healing are less studied [47]. However, there is ample evidence supporting a critical role of MDSC plasticity in repair. For example, as a heterogeneous and immature population of myeloid cells, recruited MDSCs at wound sites can differentiate into macrophages, DCs, and neutrophils [25]. In addition, because of their immunosuppressive function, MDSCs appear to dampen inflammation at the early stage but then promote healing after inflammation wanes by adopting a fate of fibrocytes [11], a cell type that can further differentiate into myofibroblasts that produce extracellular matrix in wound closure [48, 49]. In cancer, a pathological condition considered as “wounds that do not heal,” fibrocytes are viewed as a subpopulation of MDSCs [50, 51], further highlighting a dynamic and plastic nature of MDSCs in wound healing.

3. KLF4-mediated plasticity of MDSCs

3.1 KLF4 promotes cancer development through regulating plasticity of M-MDSCs

KLF4 is expressed in many tissues and cells types. Besides in epithelial cells, it is also expressed in bone marrow-derived cells and is key to inflammation [52, 53] and monocyte differentiation [54, 55]. However, it was not clear whether and how immune cell-expressing KLF4 is involved in the development of tumor. It is our hypothesis that the overall function of KLF4 depends on its expression in immune cells and in the resident epithelial cells. In the following discussion, we will focus on the role of MDSC-expressing KLF4 in cancer.

To study the function of KLF4 in MDSCs, we used a 4T1 mammary tumor model. This model is unique due to its similar characteristics with human breast cancer, particularly the ability to spontaneously metastasize to lungs. Based on 4T1 cells, we generated stable KLF4 knockdown cells and control cells using siRNA technology. They were designated as siKLF4 and siCon, respectively. We found that in siCon cell-inoculated BALB/c mice tumors were observed as early as Day 9 and the tumor size reached to 18.2 ± 1.6 mm in diameter. However, in siKLF4 cell-inoculated
mice the primary mammary tumors became visible on Day 14 and the tumor size was only 11.3 ± 1.4 mm in diameter [18]. These data were in agreement with our previous results showing that KLF4 knockdown delayed the onset of mammary tumor development and inhibited lung metastasis in immunocompromised NOD/SCID mice inoculated with MDA-MB-231 human breast cancer cells [56]. We then tested whether MDSCs were involved in KLF4-mediated tumor development. We examined MDSCs in bone marrow, spleen, and tumor by flow cytometry. We found that after implantation of 4T1 cells, KLF4 knockdown significantly reduced the numbers of MDSCs in bone marrow and spleen when compared to siCon counterparts [18]. As a critical control, we examined the immunosuppressive activities of MDSCs from control cell- and KLF4 knockdown cell-inoculated mice [57, 58]. As expected, MDSCs from siKLF4 cell-inoculated mouse inhibited proliferation of CD4+ and CD8+ T-cell significantly less than their siCon counterparts. The same assay using MDSCs purified from mouse tumors confirmed this observation. Moreover, consistent with higher T cell proliferation upon KLF4 knockdown, the arginase activities in MDSCs from siKLF4 cell-inoculated mice were lower when compared to those in siCon counterparts. Furthermore, we examined the infiltration of T cells into tumor sites by CD3 immunofluorescence staining. We found that there were more T cells accumulated in siKLF4 cell-inoculated mice than in siCon group.

Consistently, in a mouse B16-F10 implantation melanoma model, we showed that KLF4 deficiency in bone marrow drastically reduced lung metastasis accompanied by decreased recruitment of monocyteic CCR2+ MDSCs (M-MDSCs) in the lungs. Interestingly, bone marrow KLF4 deficiency was linked with significantly reduced numbers of fibrocytes and myofibroblasts in metastatic lungs [8]. We further performed a cause-effect study to exclude the effect of KLF4-mediated development of MDSCs and to test the direct effect of KLF4-regulated fibrocyte generation from M-MDSCs on tumor metastasis. We sorted M-MDSC subset from the lungs of mice bearing B16-F10 melanoma. They were mixed with B16-F10 tumor cells and then injected wild-type mice with the mixture intravenously. We then induced KLF4 knockout in these mice by tamoxifen injection. In the control mice, they only received B16-F10 tumor cells, but were still injected with tamoxifen or sunflower seed oil as controls. Mice were sacrificed at Day 7 after tumor cell inoculation. We found that no difference was observed in the incidence of lung metastasis between the mice administrated with tamoxifen or sunflower seed oil. However, in the KLF4−−/− and control groups, metastatic nodules in the pulmonary were drastically fewer than those in the KLF4+/+ group. The results strongly suggest that KLF4 controls the process in which M-MDSCs facilitate the seeding and growth of pulmonary metastatic nodules. We also took advantage of the EGFP marker in the transplanted M-MDSCs. We examined MDSC differentiation in the lung by immunofluorescence using COL1A1 and α-SMA antibodies. We found that although there was no difference in the total number of EGFP+ cells between the KLF4+/+ and KLF4−−/− group, in KLF4 deficient mice the number of COL1A1/EGFP+ cells decreased significantly when compared to that in the KLF4+/+ mice. Similarly, α-SMA ‘EGFP’ cells also decreased in KLF4−−/− mice, further supporting our speculation that KLF4 regulates the differentiation of M-MDSCs into fibrocytes and myofibroblasts after they are recruited to the lungs in vivo.

3.2 KLF4 deficiency compromised cutaneous wound healing depending on functional MDSCs

A pressure ulcer (PU) is defined as an injury caused by unrelieved pressure that results in damage to the skin and underlying tissue [59, 60]. They are thought to be caused by local tissue ischemia, interstitial and lymphatic blockage, reperfusion injury,
and mechanical deformation of cells by compressive forces [61]. PUs are detrimental to the patients by prolonging their hospital stay, affecting social life-styles, and contributing to negative psychological consequences [62, 63]. Generally, wound healing includes the early inflammatory phase and the later proliferative and remodeling phases [64–66]. However, this process in PU is frequently stalled in the inflammatory stage [67]. This is the reason why PU has been considered a chronic wound [68].

We have reported that KLF4 ablation delayed cutaneous wound healing in KLF4-CreER/KLF4(flox) [69] and RosaCreER/KLF4(flox) double transgenic mice [11], in which KLF4 was knocked out upon tamoxifen induction. To further test the possibility that KLF4 deficiency-induced delay of cutaneous wound healing may be attributed to bone marrow cells, we transplanted bone marrow cells from RosaCreER/KLF4(flox)/β-actin-EGFP triple transgenic mice into wild type C57BL/6 mice and used these chimeric mice to perform full-thickness wound healing experiments. The wound-closure kinetics showed that wound healing was significantly delayed upon KLF4 knockout in bone marrow. In addition, M-MDSCs in the skin wounding bed significantly decreased in the KLF4−/− group compared to those in the KLF4+/+/ group. By flow cytometric analysis, after we gated EGFP+ cells and analyzed COL1A1+/CD45−/CD11b+ populations to examine bone marrow-derived fibrocytes in the skin wounding bed, we showed that fibrocytes decreased in KLF4−/− group compared to those in KLF4+/+/ group. This finding was further confirmed by immunofluorescent staining of the wounding bed, as demonstrated by significantly reduced numbers of COL1A1/EGFP and α-SMA/EGFP co-expressing cells in KLF4−/− group. Moreover, we transplanted bone marrow cells from KLF4/EGFP transgenic mice, in which KLF4-expressing fibrocytes are very good candidates for carrying the expression/function of FSP-1, also known as S100A4, a marker of myofibroblasts that play a critical role in wound healing [70, 71].

KLF4 was highly expressed in M-MDSCs, and we postulated that KLF4 in M-MDSCs may directly regulate the cutaneous wound healing. Because of the highest expression level of FSP-1 in M-MDSCs among all MDSC subpopulations, to test our hypothesis, we used FSP-1-Cre/KLF4(flox) mice to produce PUs [72]. The dorsal skin of WT and FSP-1-Cre/KLF4(flox) (KLF4 null) mice were shaved, gently pulled up and placed between two cylinders of magnets (12 mm in diameter and 5 mm in thickness), producing a compressive pressure of 50 mmHg between the two magnets according to the established PU model [72–74]. A single ischemia-reperfusion cycle (I/R) consisted of a period of magnet placement for 16 h followed by a release or rest of 8 h. Three I/R cycles were used in each animal to initiate decubitus ulcer formation. Ulcers were typically formed at Day 3 (at the end of third I/R cycle) accompanied by full-thickness loss of skin. To assess the wound healing of PU, the detached full-thickness skin (ulcered skin) was removed at Day 3 right after the third I/R cycle, and the closure of open ulcer area in each mouse was monitored and photographed consecutively for 10 days. We found that 1 day after the ulcered skin was removed, the opening areas were increased in both WT and KLF4 null mice, probably because of the acute responses. From Day 2 to Day 10, wounds were gradually healed in WT mice, but the healing was delayed in KLF4 null mice as also indicated by an unclosed wound at Day 10. H&E staining showed an increased suprabasal layer of the skin and decreased hair follicle densities. The infiltrated lymphocytes were almost doubled in granule tissue of the skin in KLF4 null mice. These results suggest an elevated inflammatory status in KLF4 null mice. In agreement with reduced numbers of M-MDSCs and fibrocytes upon KLF4 knockout in
bone marrow in our full-thickness wound healing model, these populations were also decreased in FSP-1-Cre/KLF4(flox) mice in the PU model. Interestingly, we found that the populations of CD11b+Ly6G++ cells, which may represent inflammatory monocytes [75], in both blood and skin wounding beds were increased when compared to those in wild type mice. This observation is consistent with the increased inflammation in KLF4 null mice.

3.3 Mechanisms of KLF4-mediated MDSC plasticity

MDSC plasticity, and in general, myeloid plasticity, is regulated by the local microenvironment. These cells are environmental sensors and adapters [25]. In tumor, myeloid cells are the most abundant immune cells, and signals within the tumor microenvironment instruct these cells to change their dynamics and plasticity. There are many potential factors/mechanisms in these processes, including hypoxia, tumor ER stress, exosomes, and tumor-derived soluble factors [76]. In the following discussion, we will focus on KLF4-mediated plasticity of MDSCs in cancer and wound healing based on our recent studies.

3.3.1 KLF4 regulates FSP-1 in fibrocyte generation from MDSCs

FSP-1, also known as S100A4, is widely accepted as a fibroblast-specific marker [77, 78]. Given the fact that FSP-1 is expressed in more than 90% of monocytes of the host immune system [79] and that it has a “specific” expression in fibroblasts, it is challenging to reconcile the function of FSP-1 at the cellular level between these two very different cell types. On the other hand, fibrocytes are bone marrow-derived progenitor cells that can differentiate into myofibroblasts and promote cutaneous wound healing and cancer development [20, 51, 80, 81]. Therefore, fibrocytes are very good candidates for carrying the expression/function of FSP-1 from the host immune cells such as MDSCs to fibroblasts.

It has been reported that fibrocytes can be generated from bone marrow-derived cells such as MDSCs [82]. We postulated that KLF4 controls MDSC-mediated generation of fibrocytes. To test this hypothesis and to examine the underlying mechanisms, we isolated spleen cells from KLF4 inducible knockout Rosa26CreER/KLF4(flox) mice and examined fibrocyte differentiation using an ex vivo assay with murine IL-13 and M-CSF [83]. We found that the application of IL-13 and M-CSF resulted in 58 ± 7 fibrocytes per 1 x 10^5 cells (Figure 1A) in the control group. However, the same treatment decreased the number of fibrocytes to 5 ± 2 cells per 1 x 10^5 splenocytes when KLF4 was knocked out by induction of 5 μM 4-OH tamoxifen (Figure 1B). Furthermore, we examined KLF4 and FSP-1 expression in the process of fibrocyte generation by quantitative RT-PCR analysis. As shown in Figure 1C, both KLF4 and FSP-1 mRNA levels were significantly elevated after the application of IL-13 and M-CSF, which was consistent with ex vivo generation of fibrocytes. The induction of KLF4 deficiency by 4-OH tamoxifen correlates with a significant decrease in FSP-1 expression, suggesting a KLF4-mediated regulation of FSP-1 in the process. Since splenocytes are a mixed group of cells, we proceeded to examine KLF4 and FSP-1 expression in different subsets of MDSCs from the wild type mouse splenic tissues (Figure 1D). Highest levels of KLF4, FSP-1, and CCR2 expression were found in the CD11b^+Ly6G^int subpopulation of MDSCs (P2 in Figure 1D and E), known as M-MDSCs [84, 85]. Note that these M-MDSCs had the highest potential for fibrocyte generation (Figure 1F), thus supporting the observation that KLF4 deficiency led to significant decrease in FSP-1 expression and fibrocyte generation (Figure 1A–C) in the MDSC pool. To test whether KLF4 directly regulates FSP-1 gene expression, we first using two different KLF4 antibodies to perform a chromatin
Figure 1.
KLF4 regulates FSP-1 gene expression in fibrocyte generation. (A) Representative photographs of morphological fibrocyte generation from splenocytes in the absence and presence are indicated by red arrows. KLF4 deficiency was induced by 4-OH tamoxifen (TAM). (B) Quantification of the data from (A). (C) Relative levels of KLF4 and FSP-1 mRNA in fibrocyte generation as assessed by qRT-PCR. (D) Different MDSC subsets in mouse splenocytes measured by flow cytometry. (E) Relative levels of KLF4, FSP-1 and CCR2 mRNA in different MDSC subsets by qRT-PCR. (F) Potential of fibrocyte generation from MDSC subsets in mouse spleen. (G) Left—binding of KLF4 to the FSP-1 promoter as assessed by chromatin immunoprecipitation assay using two KLF4 antibodies (KLF4-1 and KLF4-2). IgG was used as a negative control. Right—the effect of KLF4 overexpression on FSP-1 promoter activities, as examined by transient transfection and dual luciferase assays, *P < 0.05, **P < 0.01.
immunoprecipitation (CHIP) assay. We found that KLF4 directly bound to the FSP-1 proximal promoter region (Figure 1G left). Then we constructed a FSP-1 promoter luciferase reporter containing ~2.3 kb of the FSP-1 promoter region. By transient transfection and dual luciferase assays, we found that KLF4 overexpression resulted in three fold increase of the FSP1 promoter activity (Figure 1G right), suggesting a direct regulation of FSP-1 by KLF4 at the transcriptional level.

3.3.2 Epigenetic control of MDSC plasticity

The studies of epigenetics, heritable changes to gene expression without changes to DNA, are significantly advancing our knowledge of the inflammatory conditions [86]. They include DNA modifications mainly methylation, histone tail modifications, and non-coding RNA-mediated gene regulation. Recent data revealed that epigenetic mechanisms could provide novel strategies for modulating wound healing [87–89].

Critical functions of KLF4 have been shown in the generation of induced pluripotent stem cells and in cancer development through epigenetic mechanisms [90, 91]. In addition, there are numerous reports showing that microRNAs regulate KLF4 [92–94] or KLF4 regulate microRNAs [95, 96] in varied pathological conditions. KLF4-mediated DNA methylation have also been reported in hTert promoter [97] and methylation of KLF4 promoter is associated with urothelial cancer progression and early recurrence [98]. Moreover, the correlation of KLF4 and histone modifications has also been reported. For example, histone methyltransferase KMT2D, a frequently aberrant epigenetic modifier in various cancer, sustains prostate carcinogenesis and metastasis via epigenetically activating KLF4 [99]. From the perspective of MDSCs, epigenetic regulation of their differentiation and function is not completely understood. However, there is evidence to indicate the importance of epigenetic regulation. Shang et al. showed that long non-coding RNA retinal non-coding RNA3 (RNCR3) promotes C/EBP homologous protein (Chop) expression by sponging microRNA 185-5p during MDSC differentiation [100]. In addition, although histone modifications related to myeloid differentiation have been extensively studied [101], currently there is no clear indication about epigenetic markers that can discriminate specific MDSC subsets. Given the role of KLF4 in epigenetic regulation and the importance of MDSC plasticity in cancer and wound healing, it will be very interesting to examine how KLF4 is involved in epigenetic control of MDSC subsets or plasticity.

3.3.3 Is there potential molecular plasticity of KLF4 in cancer and wound healing?

KLF4 is a transcription factor with multiple functions in different physiological and pathological conditions, notably in cancer development. For example, KLF4 is well known for its tumor suppressive effect on tumor development in the gastrointestinal tract [102]. However, high expression of KLF4 is associated with skin cancer and breast cancer development [56, 103, 104], suggesting a tumor promoting function of KLF4 in these tissues. Recently, a tumor suppressive function of KLF4 was also reported in breast cancer [105]. These contradictory reports suggest context-dependent functions of KLF4 in cancer development [106]. At a molecular level, different KLF4 transcripts were found in testis [107], and alternative splicing of KLF4 has been proposed to explain context-dependent functions of KLF4 [108]. Consistently, an oncogenic KLF4 isoform, named KLF4α, has been found in both pancreatic cancer [109] and breast cancer [110]. In line with these observations, there is dynamic expression of KLF4 isoforms in mouse embryogenesis [111].
Interestingly, another human KLF4 isoform with an additional 34 amino acid-fragment in the C-terminal region has been reported in leukemia patients [112] and in myeloid cells [113], which further supports the importance of differential expression of KLF4 in different conditions.

We speculate that the existence of different isoforms of KLF4 and possibly relative ratios of these isoforms may explain different functions of KLF4 in cancer development and even in wound healing. Because KLF4 is a transcription factor that regulates gene expression, different isoforms of KLF4 will have different patterns of gene regulation of the downstream targets. In analogy to MDSC dynamics and plasticity, we propose a concept of KLF4 plasticity, which reflects the dynamic nature of KLF4 expression under different conditions. It is likely that under one condition, a major isoform of KLF4 regulates a group of genes that are responsible for one signaling transduction pathway. This pathway may be linked to one functional or phenotypical MDSC group. Under a different condition, another KLF4 isoform dominates and regulates a different group of genes and a different signaling pathway. This kind of differential regulation may cause the plastic change of MDSCs in cancer or wound healing. To confirm our hypothesis, future experiments will be needed to characterize the different KLF4 isoforms during the dynamic change of MDSCs. Validation of our hypothesis will not only reveal novel molecular mechanisms whereby KLF4 regulates MDSC plasticity, but also help design KLF4-based therapeutic strategies to manipulate MDSC plasticity in the treatment of cancer and wound healing.

4. Conclusion remarks

Studies of immune cell plasticity have recently gained momentum due to their novel functions in tissue repair and robustness beside their well-known functions in system defense. MDSCs, as a myeloid population with unique functions in tumor and tissue repair, are less studied regarding their phenotypical and functional plasticity, compared to macrophages and neutrophils. Given the ample evidence showing MDSC plasticity in cancer and wound healing, it is essential to elucidate the underlying molecular mechanisms in order to harness MDSCs in tissue repair and cancer treatment. In the meantime, we have shown KLF4 as a key molecule to regulate MDSC plasticity in cancer, wound healing, and allergic asthma. KLF4-controlled FSP-1 expression and possible epigenetic alterations are two possible mechanisms underlying MDSC plasticity. In addition, the existence of different KLF4 isoforms prompts us to hypothesize that KLF4 isoforms control gene expression of different signaling pathways that may contribute to MDSC dynamics and plasticity in both cancer and wound healing. In this regard, future studies to characterize different KLF4 isoforms during MDSC plastic changes and the relevant signaling pathways will pave the way to harness MDSC plasticity in the treatment of cancer and wound healing.

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Conflict of interest

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Section 5

Immune Reconstitution after Transplantation
Section 5

Immune Reconstitution after Transplantation
Eosinophilic Phenotype: The Lesson from Research Models to Severe Asthma

Guida Giuseppe and Antonelli Andrea

Abstract

Eosinophilic airway inflammation is a hallmark in the pathophysiologic and clinical definition of asthma. In the last decades, asthma evolved in the recognition of different phenotypes identified by natural history, clinical and physiological characteristics, and the underlying immune mechanisms. Among these phenotypes, many have been associated with eosinophilic-driven inflammation. This is the case of either early-onset allergic Th2 asthma or late-onset persistent eosinophilic asthma. Both animal models and analysis from human samples have contributed to elucidate the role of eosinophils in the asthmatic inflammatory response and the synergic role of Th2 cytokines. In severe asthma, high numbers of eosinophils can persist despite treatment with inhaled and oral corticosteroids leading to the definition of severe refractory eosinophilic asthma. The combined role of IL-4-, IL-13- and IL-5-associated pathways has focused the view over the T2-type endotypes, wherein a specific biological pathway explains the observable properties of different phenotypes and the identifiable biomarkers can predict response to monoclonal antibodies directed against a selected immune target. In the era of precision medicine and personalized therapy, both the identification of Th2 molecules and eosinophils as targets and biomarkers have become the best clue for treating and monitoring severe asthma.

Keywords: severe asthma, eosinophilic phenotypes, T2-type inflammation, eosinophilic refractory asthma, anti-IL5 treatment

1. Introduction

Asthma and chronic rhinosinusitis (CRS) are chronic inflammatory disorders involving the lower and upper airways. According to the definition by Global Initiative for Asthma (GINA) documents, asthma is a heterogeneous disease characterized by chronic airway inflammation associated with a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough and evidence of variable expiratory airflow limitation [1]. Airway inflammation is usually present and persists even when symptoms are absent or lung function is normal.

In the last decades, the role of chronic airway inflammation has been central in the definition of asthma that was recognized as a chronic inflammatory
Chapter 8

Eosinophilic Phenotype: The Lesson from Research Models to Severe Asthma

Guida Giuseppe and Antonelli Andrea

Abstract

Eosinophilic airway inflammation is a hallmark in the pathophysiological and clinical definition of asthma. In the last decades, asthma evolved in the recognition of different phenotypes identified by natural history, clinical and physiological characteristics, and the underlying immune mechanisms. Among these phenotypes, many have been associated with eosinophilic-driven inflammation. This is the case of either early-onset allergic Th2 asthma or late-onset persistent eosinophilic asthma. Both animal models and analysis from human samples have contributed to elucidate the role of eosinophils in the asthmatic inflammatory response and the synergic role of Th2 cytokines. In severe asthma, high numbers of eosinophils can persist despite treatment with inhaled and oral corticosteroids leading to the definition of severe refractory eosinophilic asthma. The combined role of IL-4-, IL-13- and IL-5-associated pathways has focused the view over the T2-type endotypes, wherein a specific biological pathway explains the observable properties of different phenotypes and the identifiable biomarkers can predict response to monoclonal antibodies directed against a selected immune target. In the era of precision medicine and personalized therapy, both the identification of Th2 molecules and eosinophils as targets and biomarkers have become the best clue for treating and monitoring severe asthma.

Keywords: severe asthma, eosinophilic phenotypes, T2-type inflammation, eosinophilic refractory asthma, anti-IL5 treatment

1. Introduction

Asthma and chronic rhinosinusitis (CRS) are chronic inflammatory disorders involving the lower and upper airways. According to the definition by Global Initiative for Asthma (GINA) documents, asthma is a heterogeneous disease characterized by chronic airway inflammation associated with a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough and evidence of variable expiratory airflow limitation [1]. Airway inflammation is usually present and persists even when symptoms are absent or lung function is normal.

In the last decades, the role of chronic airway inflammation has been central in the definition of asthma that was recognized as a chronic inflammatory
disorder in which many cells and cellular mediators play a role and result in the characteristic pathophysiological changes [2]. The inflammation involves all the airways from the main bronchi to the peripheral small airways. A characteristic pattern of inflammation has been described in asthma involving inflammatory cells mainly mast cells, eosinophils, T lymphocytes, dendritic cells, macrophages and neutrophils, which release mediators that induce symptoms. Both animal models and analysis from human samples have contributed to elucidate the type of inflammation involved in asthma [3]. The most common phenotype of asthma is characterized by eosinophilic airway inflammation and the role of eosinophils as a key player in the pathophysiology of asthma is well documented. Eosinophils emerged as leading cells from the first post-mortem studies of asthmatic lungs, passing through the finding of increased in number and activation status of eosinophils in asthmatic airways [4] and of increased eosinophil surrogates as fractional exhaled nitric oxide (FENO) [5]. Nowadays, the focus is on the definition of the forms of uncontrolled or severe eosinophilic asthma in which airways, sputum and blood eosinophils are consistently increased and represent a biomarker of the eosinophilic endotype of asthma and a guide for biologic target therapies [6, 7].

2. Eosinophils and allergic asthma

Allergen challenge models have been conceived to reproduce many features of clinical asthma [8]. Actually, atopy, which is the production of allergen-specific IgE antibodies, is a predisposing factor for asthma development, and birth cohort studies have shown that sensitization to allergens such as house dust mite, cat and dog dander and Aspergillus is independent risk factors for wheezing in children [9]. Moreover, exposure to allergens is one of the most recognized environmental factors that trigger asthma symptoms. The term allergic asthma has been used to define the presence of sensitization to environmental allergens and the clinical correlation between exposure and symptoms, both indoor and outdoor allergens being well-known triggers of asthma exacerbations [10].

Both allergen challenged animal models of asthma and allergic asthma in humans are associated with a T-lymphocyte CD4+ Th2-polarized response as the main feature of airway inflammation. The allergic response is characterized by immediate and late inflammatory responses in which Th2 cells govern the inflammatory cell recruitment and activation by the release of the signature cytokines IL-4, IL-5 and IL-13 as well as IgE antibody synthesis.

2.1 Mouse models of allergic asthma

In acute allergen challenged mouse models of asthma, after the sensitization period (usually 14–21 days), the animal is challenged with the allergen via the airway and this causes many key features of clinical asthma. The analysis of bronchoalveolar lavage (BAL) and biopic samples of airway walls has supported the hypothesis that asthma is a Th2-mediated disease. A dominating influx of eosinophils has been demonstrated and related to the development of AHR [11]. Moreover, the adoptive transfer of Th2 cells into recipient mice was able to reproduce airway eosinophilia, mucus hypersecretion and AHR after allergen inhalation [12].

However, some of these effects resulted in transient changes and do not involve structural changes. Through chronic allergen exposure in mice, allergen-dependent sensitization, Th2-dependent allergic inflammation, eosinophilic influx into the
airway mucosa, mucus overproduction and AHR have been reproduced [11, 13]. Generally, acute and chronically treated mice had similar early and late asthmatic responses; however, the acute model had higher levels of eosinophilia, whereas the chronic model showed hyperresponsiveness to lower doses of methacholine and had higher total IgE. On the other hand, many of the lesions observed in chronic human asthma, such as chronic inflammation of the airway wall and airway remodeling changes, are absent.

Moreover, transgenic mice that overexpress the Th2 cytokines—IL-4, IL-5, IL-13 and IL-9—in the airway epithelium exhibit the same inflammatory features. IL5 is a Th2 cytokine essential for differentiation, maturation and survival of eosinophils. A key role in allergen-induced inflammatory responses has been shown in murine IL-5-deficient model chronically challenged with an allergen in which the eosinophilia, lung damage and airway hyperreactivity were abolished. The reconstitution of IL-5 production using recombinant vaccinia virus that expressed IL-5 restored eosinophilia and airway dysfunction [14]. Using a clinically relevant model of chronic allergic asthma in mice, Kumar RK et al. showed that anti–IL-5 inhibited inflammation in terms of accumulation of eosinophils in the tracheal epithelium and inflammatory cells in the lamina propria, but had no effect on airway responsiveness to methacholine [15].

Many studies have demonstrated the significant role of IL/4IL-13 pathway in asthma. Through the agonization of IL-4R, both IL4 and IL13 activate a tyrosine kinase-dependent signal that after phosphorylation of STAT6 regulates the transcription of Th2-involved genes. Models of IL-4−/− mice were protected from the development of AHR and aspects of remodeling, while the administration of soluble IL-4 receptor reduced inflammation and mucus hypersecretion, but had no effect on AHR [8]. Similarly, soluble IL-13 suppressed pulmonary inflammation but had a limited effect on AHR [15].

Limitations evidenced in mouse models are that inflammation is not restricted to the conducting airways, but extended to vascular and parenchymal parts of the lung; moreover, some of the clues of asthma inflammation such as the large increases in airway smooth muscle and MC infiltration are not generally observed.

2.2 Human models of allergic asthma

In humans, the role of Th2 cytokines and eosinophils in allergic asthma comes from many experimental data that in part differ from the mice models.

Sensitizations to environmental allergens in allergic subjects are documented by positive skin prick test reactions and elevated allergen-specific IgE serum levels. Activation of FcεRII on mast cells and basophils by allergen-bound IgE induces the release of preformed vasoactive mediators, which rapidly elicit edema of the bronchial mucosa, mucus production and smooth muscle constriction. This mechanism is confirmed by the increased numbers of cells expressing the high-affinity receptor for IgE (FcεRI) in allergic asthmatic tissues [16].

Biopsies from bronchial mucosa show CD4+ cell infiltrates and enhanced expression of Th2-type cytokines and chemokines. IL-4 and IL-5 mRNA were localized in activated T cells (CD3+), mast cells (tryptase +) and activated eosinophils (EG2+) both in BAL and bronchial biopsies from mild atopic asthmatic patients [17], and the number of activated CD4+ T cells and IL-5 mRNA positive cells is increased in asthmatic airways following antigen challenge. This skewed cytokine involvement is reflected by the expression of the transcriptional regulators GATA-3 (GATA binding protein 3) after segmental allergen challenge in asthmatics [18]. GATA-3 is a transcription factor that finds its binding site in the IL-5 promoter and induces Th2 cytokine gene expression.
by biasing Th1/Th2 balance. The increase in GATA-3 expression in the asthmatic subjects correlated significantly with IL-5 expression and AHR [19]. In summary, CD4+ Th2 cells are believed to initiate and perpetuate the inflammatory response in allergic asthma.

IL-5 expression is increased 18–48 h after allergen challenge in BAL samples in mite-associated bronchial asthma when they were stimulated with Dermatophagoides farinae [20]. The levels of IL-5 mRNA-positive cells and IL-5 correlate with the number of eosinophils infiltrating the bronchial mucosa and BAL of asthmatic subjects, with pulmonary function and symptom severity [21]. Biopsies from the respiratory mucosa of allergic asthmatics show the enhanced expression of other Th2-type cytokines and chemokines such as IL-4, IL-6, IL-9, IL-10 and IL-13. Allergen challenge induces in patients with asthma IL13 and IL4 release in BAL and sputum eosinophils that positively correlate with IL-13 expression in asthmatic bronchial submucosa [22]. IL13 is thus involved in the regulation of allergen-induced late-phase inflammatory responses. IL-13, indeed, can modulate the production of IgE through the isotype class switching of B cells; therefore, it is involved in the early phase of allergic reactions.

### 2.3 Recruitment of eosinophils in allergic asthma

Eosinophils are recruited from progenitors after allergen exposure. Levels of Eo progenitors arise in the peripheral blood after seasonal allergen exposure, during controlled exacerbations of atopic asthma and after single allergen challenge to the airways in atopic asthmatics and animal models. Trafficking of these cells from the bone marrow, where they are produced, to the airways was also demonstrated. In fact, these CD34+ CD45+ progenitors express the IL-5 receptor alpha and are recruited by IL5 and GM-CSF produced in asthmatic airways, subsequently acquiring an activating form that reaches the inflamed airways [23]. Eosinophiloipoiesis develops after 24 h from allergen challenges and is followed by the accumulation of eosinophils in the airways.

### 2.4 Eosinophils in different phases of allergic asthma

The sensitization phase is supposed to be determined by the differentiation of Th naïve cells into Th2 lymphocytes. Dendritic cells (DCs) in response to allergen stimulation drive a Th2-oriented response. DC subsets have been described to respond to various stimuli coming from the inflammatory milieu generated after the allergenic encounter. Myeloid CD1c+ DCs respond to thymic stromal lymphopoietin (TSLP) produced by the epithelium after allergen encounter by activating allergen-specific memory CD4+ cells [24]. Eosinophils also contribute to the initiation phase of Th2 response by suppressing the Th1/Th17 pathway.

The main role of eosinophils in asthmatic response is yet related to the effector phase of the inflammatory response. After allergen challenge, asthmatics generally develop immediate bronchoconstriction, the so-called early asthmatic response, which is maximized within 30 min and resolves between 1 and 3 h. A proportion of subjects develop a second, delayed bronchoconstrictor response, named the late asthmatic response, which is characterized by prolonged AHR and pronounced airway eosinophilia [25]. So it can be assumed that in isolated early responders a significant or sustained eosinophilic response does not develop. On the other hand, the so-called dual responders develop a sustained IL-5-dependent eosinophilic response in terms of both bone marrow recruitment and sputum accumulation. This response is accompanied by increases in circulating eosinophils, greater
increases of activated eosinophils in the airways, and the development of airway hyperresponsiveness [26].

Recruited eosinophils in the airways release a variety of toxic products, oxygen radicals, granule-associated cytotoxic proteins and membrane-derived proinflammatory mediators that damage the bronchial epithelium and increase AHR.

IL-5 is the most important constituent increasing eosinophil survival, recruitment, degranulation and lung injury following inhalation of antigen, as demonstrated in a segmental antigen lung challenge model [20], and the levels of eosinophils and their cationic proteins in the BAL fluid following allergen challenge correlate with the magnitude of the late phase response. Moreover, a positive correlation between the percentage of BAL eosinophils and the ECP was demonstrated at baseline but not after 4–6 h after allergen inhalation, thus suggesting that eosinophil recruitment and activation seem to follow different temporal kinetics [27].

The effect of IL-5 on eosinophils is demonstrated by the finding of increased expression of the alpha chain of IL-5R mRNA in the bronchial biopsies of atopic and nonatopic asthmatic subjects; the membrane-bound α2IL-5R is coexpressed with EG2 in the eosinophils within the bronchial mucosa of asthmatics and inversely correlated with FEV1 [28].

2.5 Eosinophilic chemokines in allergic asthma

IL-5 acts as chemotactic factors for eosinophils, promoting eosinophil-endothelial adhesion by inducing the expression of VCAM-1 on endothelial cells. In turn, VCAM-1 may bind to integrins on the eosinophils leading to the migration of eosinophils to sites of airway inflammation. Blood eosinophils stimulated with IL-5 adhere to VCAM-1 via the integrins α4β1 and αMβ2 that are the major eosinophil integrin-mediating cell adhesion [29]. Eosinophils obtained from BAL after segmental antigen challenge have both β1 and β2 integrins in a high-activity conformation and adhere to VCAM-1 to a higher degree than blood eosinophils [30]. It seems, therefore, that blood eosinophils are primed by IL-5 or P-selectin (expressed by platelets) to an integrin activation status and are consequently arrested in vessels of inflamed bronchi and move into lung tissue. It is remarkable that the administration of anti–IL-5 can lower β2 integrin activation [31]. IL-5 not only has got the ability to prime eosinophils for subsequent activation but also enhances their survival at sites of allergic inflammation.

The role of other chemokines in allergic asthma is sustained by different pieces of evidence. Eotaxin and regulated on activation, normal T-cell expressed and secreted (RANTES) act on eosinophils inducing chemotaxis as well as specifically activation. In human challenges with the HDM allergen, the peak of eosinophils immunopositive for eotaxin, RANTES and IL-5 occurs at 7 h after allergen inhalation, but persisting eosinophilic airway inflammation and AHR remained for 7 days after allergen inhalation [32].

These chemokines are released by several cell types in the lung: endothelial cells, epithelial cells, fibroblasts, DCs and smooth muscle cells. Eotaxin creates a chemotactic gradient so that eosinophils pass the endothelium of the blood vessels and migrate to the site of inflammation [33]. Eotaxin has the potential to mobilize eosinophils and their progenitors from bone marrow and this effect is potentiating with that of IL5. Second, in atopic asthmatic patients, high concentrations of eotaxin in BAL fluid are detected as well as an increased expression of eotaxin mRNA and protein in the epithelium and submucosa of their airways. In the airways of allergic asthmatics, eotaxin is in sufficient concentrations to exert chemotactic activity on eosinophils in vitro and this effect is enhanced by IL-5 [34].
RANTES is also found in high concentrations in the sera in allergic asthma, as well as monocyte chemoattractant protein-1 and -3 (MCP). These chemokines play a role in ongoing lung inflammation, lung leukocyte infiltration, bronchial hyper-responsiveness and the recruitment of eosinophils.

Eotaxins and RANTES bind to the CCR3 receptor expressed on Th2 cells, eosinophils and basophils. Eosinophils in CCR3R knockout mice reach the blood vessels and the endothelium but fail to migrate into lung tissue. Indeed, these mice are protected from AHR after allergen challenges [35]. After antigen challenge, the percentage of CCR3+ eosinophils is downregulated on BAL eosinophils compared with peripheral blood eosinophils, while other chemokine receptors like CCR4, CCR9 and CXCR3 do not, being predominantly involved in activation of eosinophil effector responses [36].

The relationships between the levels of eosinophilic chemokines and AHR or bronchoconstriction are not documented in the same way. Some data suggest that mediators released by cells other than eosinophils, similar to MCs or basophils, can contribute to AHR. In addition, chemokine receptors might be involved in the activation of airway eosinophils for degranulation or prolonged survival. Even if antagonists derived from peptides and small molecules exist to block the chemokine receptor CCR3, the in vivo effect on airway inflammation is not sufficiently proved [33].

Once activated, eosinophils may produce effector molecules like eosinophil major basic protein and eosinophil-derived neurotoxin and degranulate at the site of injury contributing to tissue damage in the asthmatic lung. These molecules have cytotoxic effects on respiratory epithelium, facilitate the entry of other toxic molecules and trigger the degranulation of mast cells and basophils. In asthmatic airways, eosinophils also take part in respiratory-burst–oxidase reactions and generate large amounts of cysteinyl leukotrienes that contribute to increased vascular permeability, mucus secretion and smooth muscle contraction [37].

2.6 Local eosinophilopoiesis

It has been proposed that CD34+ IL-5Ra+ progenitors after mobilization from the BM during allergen challenge are able to undergo in situ differentiation at the site of allergic inflammation. Actually, CD34+45+IL-5Ra+ progenitors are increased in BAL in mouse models after allergen challenge and precede an increase in BAL eosinophils through a local differentiation via an IL-5-dependent mechanism [38]. Moreover, the CD34+ eosinophil committed pool is maintained within the airways via autocrine IL-5 release and IL-5-induced upregulation of IL-5R. CD34+/IL-5Rα mRNA+ cell number is increased in the airways of asthmatic subjects and related to asthma severity [39]. Surprisingly, eosinophilic precursors persist in the sputum of severe asthmatics that are prednisone resistant after anti-IL-5 treatment [40] and it has been documented that anti-CCR3 strategies do not suppress circulating and airway eosinophils in moderate-to-severe asthmatics. Consequently, it can be hypothesized that blocking local differentiation and expansion of CD34+/IL-5Rα+ cells may reduce eosinophilic inflammation in the airway in asthma.

2.7 Other mechanisms of eosinophil activity into allergic asthmatic airways

Allergic inflammation is locally perpetuated in the airway by the cross-talk between eosinophils and other resident cells. MCs are activated by MPB and stem cell factor (SCF), both released by eosinophils, contributing, by their direct effects on mast cells, to the perpetuation of allergic inflammation [41].

Eosinophils can also affect fibroblast properties, modulating the process of tissue remodeling. First, eosinophils are the main source in asthma of transforming
growth factor-beta (TGF-β) that induces proliferation and regulates fibroblast function as well as controls the production of proteins of the extracellular matrix (ECM). In turn, tumor necrosis factor-α (TNF-α) derived from mast cells enhances TGF-β synthesis from eosinophils as well as fibroblasts promote survival of MCs and eosinophils by releasing SCF and granulocyte–macrophage-colony stimulating factor (GM-CSF) [42]. Anti-IL-5 humanized monoclonal antibody has been shown to decrease the deposition of many ECM proteins such as collagen III in the RBM of mild atopnic asthmatics as well as the number of eosinophils and the degree of TGF-α in the BAL fluid [43].

In addition, eosinophils express basic fibroblast growth factor (β-FGF) and VEGF in the submucosa of asthmatic subjects and release many pro-angiogenic cytokines such as IL-8, IL-6, TGF-β and GM-CSF.

The effect on T-cell immune modulation of eosinophils is more controversial. Cytokine produced by eosinophils may directly influence T-cell selection by DCs determining T-cell tolerance or activation. One example is the induction by IFN-γ of indoleamine 2,3-dioxygenase (IDO) in eosinophils that in turn converts tryptophan (TRYP) to kynurenine (KYN) inducing apoptosis in Th1 cells, while Th2 cells are spared from KYN-induced apoptosis by IL-4 [44].

3. Eosinophils in nonallergic asthma

The increase of the number of activated Th2 lymphocytes and eosinophils, as well as IL-5 levels, in both BAL fluid and bronchial biopsies from intrinsic asthmatics, has been extensively reported [45]. No difference between atopic and intrinsic asthmatics have been observed in studies examining the expression of high-affinity IgE receptor, IL-5 and IL-4 mRNA and protein expression in bronchial biopsies [16]. Actually, total serum IgE levels have been noted to be increased in the serum of patients with intrinsic asthma. This reflects the increases in Iå and Cå RNA+ cells in the bronchial mucosa and provides evidence for a local IgE synthesis even in the absence of a known antigen or allergen trigger.

Eosinophilic infiltration in nonallergic asthma can be even much more than in allergic asthma and this fact is reflected by the finding of a larger amount of RANTES in the bronchoalveolar lavage fluid of patients with nonallergic asthma compared with patients with allergic asthma [46].

Attempts to differentiate the inflammatory cascade between allergic and nonallergic asthma have proposed a different signal in the Th2 pathway of nonallergic asthma attributed to reduced signal transducer and activator of transcription 6 (STAT6) expression and consequently reduced IL-4R signaling in nonallergic asthma [47]. Another peculiar finding was the increased expression of GM-CSF receptor alpha expression in the macrophages detected in mucosa and BAL. Peripheral blood eosinophilia is present both in allergic and nonallergic asthma, in some studies being higher in the former compared to the latter group [48].

4. The eosinophilic phenotype of asthma

Different attempts have been found in order to identify an eosinophilic phenotype of asthma. Eosinophilic asthma is reported to account for approximately 50–60% of the total asthma population. The definition of eosinophilic asthma implies that eosinophils are the dominant cells responsible for the pathophysiological changes of the disease. The pathogenic role of eosinophils in these patients is
demonstrated by their increased number and status of activation in the airways, and consequently, they are detected in sputum, bronchoalveolar lavage, or bronchial mucosa or submucosa. These findings may be persistent and associated with severe or uncontrolled asthma [7].

Eosinophils may be demonstrated in the airways in the bronchial mucosa or submucosa or in the lumen (in the bronchial wash, BAL, or sputum). Bronchial biopsy is not routinely used as it is an invasive procedure and practicable only far from exacerbations to avoid dangerous complications and the quantification of eosinophils in BAL is not standardized and generally reflects samples of the peripheral airways.

Sputum examination is currently the most comprehensive and noninvasive method for measuring airway inflammation, processing and analysis being standardized and reliability, validity, and responsiveness proven [49].

The definition of “eosinophilic asthma” implies the existence of noneosinophilic asthma. A large cohort of patients with mild-to-moderate asthma in longitudinal studies resulted in approximately 50% of them with the absence of eosinophilic airway inflammation. The cellular pattern in noneosinophilic asthma may result in either predominant neutrophilic inflammation or normal sputum cell count. Within eosinophilic asthma, eosinophilia may result in persistent (22%) or on at least 1 occasion (intermittent eosinophilia, 31%) under multiple sputum examinations [50]. Sputum inflammatory granulocytes may identify phenotypic subgroups of differing pathology and clinical characteristics within asthmatics. Within the Severe Asthma Research Program (SARP), which included a population of severe and none severe patients with and without corticosteroid treatment, the stratification in four groups by granulocyte % in sputum showed significant clinical differences. Patients were divided combined for stratification by granulocytes in <2%Eos + <40%Neu, <2%Eos + ≥ 40%Neu, ≥2%Eos + <40% Neu, and ≥ 2%Eos + ≥40%Neu. In this study, eosinophilic asthma, indicated by ≥2%Eos, accounted for 31% of patients, those being with combined increased sputum eosinophils and neutrophils the most severe patients in terms of lowest lung function measures, worse asthma control, greatest symptoms and use of healthcare resources [51]. In another retrospective series of 508 asthmatics, the proportion of patients with raised sputum eosinophil counts ≥3% was 42% independent of the exclusion of steroid-treated patients. Eosinophilic phenotype exhibited higher atopy, levels of IgE, bronchial hyperresponsiveness to methacholine, FENO levels and lower asthma control, while the mixed granulocytic phenotype, with both eosinophilic and neutrophilic inflammation, had the lowest lung function and the highest degree of bronchial hyperresponsiveness to methacholine [52].

In most mild-to-moderate asthma patients untreated with steroids, sputum eosinophilia >2% was significantly and inversely associated with PC20 methacholine identifying 69% of the asthma group [53]. Sputum eosinophils correlate, in addition, with symptom score and FEV% and, as previously reported, are increased by exposure to common allergens. The association between asthma exacerbations and sputum eosinophilia is suggested by different pieces of evidence. First, sputum eosinophil count is able to predict asthma deterioration after cessation of ICS treatment in mild-to-moderate asthma, while it is decreased by treatment with corticosteroids [54]. Sputum eosinophilia may be a good additional predictor of FEV1, PC20 methacholine or quality of life of response to inhaled steroids [55].

Consequently, a clinical strategy, based on re-administration of ICSs when a change in sputum eosinophil percentage by using the 0.8% threshold was reached, could lower the rates of asthma deteriorations and the number of individuals treated with ICSs by 48%. In addition, an increase in sputum eosinophils is detected up to 3 months before the development of a clinical exacerbation [56]. The usefulness of sputum cell count to improve treatment has been shown by Green et al. that
showed the efficacy of reducing exacerbations when treatment was guided according to the sputum eosinophils (to achieve a sputum eosinophil count of less than 3%) [54]. A different study used sputum cell counts to guide corticosteroid therapy to keep eosinophils <2% in moderate-to-severe asthma resulting in the sputum strategy group lower number and milder exacerbations (overall risk of exacerbations by 49%, it reduced the number of severe exacerbations) that were prevalently noneosinophilic [57].

Management of asthma—inhaled corticosteroid treatment based on sputum eosinophil levels has been the object of a Cochrane review that concluded that actually the risk of exacerbations is significantly reduced compared to that based on clinical symptoms with or without lung function, as well as the rate and severity of exacerbations defined by requirement for use of oral corticosteroids and hospitalizations [58]. Sputum eosinophilia may, therefore, be considered a modifiable risk factor to reduce exacerbations. Small studies in selected populations have suggested increasing ICS dose independent of the level of symptom control.

In this context, the eosinophilic subtype of asthma may be defined as symptomatic asthma in the presence of airway eosinophilia and that is characterized generally by a good response to glucocorticosteroids.

5. Eosinophilic refractory severe asthma

When eosinophilic inflammation in asthma leads to uncontrolled disease, the patient is at risk of exacerbations. In a proportion of patients, asthma becomes difficult to be treated despite the adequate use of high-dose corticosteroid treatment. Once the management of modifiable factors such as incorrect inhaler technique, poor adherence, smoking or comorbidities is optimized but asthma remains still uncontrolled, the diagnosis of severe asthma can be formulated [59].

In a subgroup of patients with severe asthma, eosinophilic inflammation is still active despite the high-dose ICS treatment or oral corticosteroid intake. The use of sputum cell counts was thus defined as a marker allowing the identification of a subgroup of subjects with severe eosinophilic asthma who were at risk of more frequent asthma exacerbations [60].

In patients with severe eosinophilic asthma, sputum eosinophils may be suppressed by using increasing doses of inhaled steroids reducing the number of subsequent exacerbations [54, 57]. Yet, the persistence of airway eosinophilia in these subjects reflects a failure of usually adequate doses of corticosteroid to suppress inflammation [61]. Corticosteroid insensitivity is therefore intrinsic in the definition of severe asthma resulting in persistent lack of control despite corticosteroid therapy or worsening of asthma control on reduction or discontinuation of corticosteroid therapy [62]. A majority of severe asthmatics are corticosteroid dependent, refractory or insensitive and require oral corticosteroids (OCS) in addition to ICS to maintain some degree of asthma control. Only a small portion of severe asthmatics can be considered completely “corticosteroid unresponsive” or resistant [63]. The proportion of asthmatics with corticosteroid insensitivity is confirmed from the fact that one-third of the current SARP cohort were on regular OCS, with over half needing more than three bursts of OCS in the previous year [64].

A dose-response relationship between the use of OCS in asthmatic patients and the risk of many adverse events has been documented. Long-term exposure to OCS leads to increased risk of osteoporosis, arterial hypertension, diabetes, metabolic syndrome, cataracts, gastrointestinal bleeding and neuropsychiatric diseases such as depression [65]. The negative effect of systemic corticosteroid is associated not only to its maintenance or use but also to cumulative prescriptions of OCS burst [66].
In the global strategy for asthma management and prevention (GINA) 2019 update, sputum eosinophilia \(\geq 2\%\) is presented as a criteria to identify patients with severe asthma with refractory type 2 inflammation despite high-dose ICS or daily OCS treatment [1, 59].

Type 2 high asthma was initially used to identify the eosinophilic phenotype of asthma. The current concept of type 2 asthma includes a phenotype, characterized by the release of signature cytokines like interleukin IL-4, IL-5 and IL-13 from cells of both the innate and the adaptive immune systems. Th2 cells and type 2 innate lymphoid cells (ILC2s) are primarily responsible for the production of high levels of T2 cytokines in the airways. The demonstration of this cytokine pathway from cellular to molecular and transcriptomic levels represents the signature for type 2 (T2) asthma [67]. The importance of identifying different phenotypes of asthma has been addressed by hypothesis-based and unbiased analyses that showed different characteristics of asthma phenotype in terms of severity, functional and clinical features, comorbidities, prognosis and response to treatment [68].

5.1 Severe eosinophilic asthma in cluster analysis

Asthma phenotyping has involved biased and unbiased approaches with the aim of grouping clinical, physiological and genetic characteristics.

In the TENOR study (the epidemiological and natural history of asthma: outcome and treatment regiments), a severe allergic asthma phenotype emerged as a high-risk group of patients for severe exacerbations with early-onset, IgE and allergen sensitization [69]. The existence of this population was confirmed in the cluster analysis by Haldar P and coworkers who found an early-onset atopic asthma cluster in which a concordance between symptom expression and eosinophilic airway inflammation is present and a symptom-based approach to therapy titration may be sufficient. On the other side, a marked discordant cluster with late-onset active predominant eosinophilic inflammation emerged as a refractory phenotype of severe asthma [70].

The predominance of sputum eosinophilia in the inflammatory patterns of severe asthmatic subphenotypes is confirmed in the unsupervised hierarchical cluster analysis of the Severe Asthma Research Program cohort where cluster 4 of severe asthmatics was associated to atopic disease and reversible severe reductions in pulmonary function, while cluster 5 was characterized mainly by later-onset disease and airflow limitations that remain with a FEV1 < 80% predicted [71].

The expansion of this analysis using a supervised learning approach that included blood, bronchoscopic, exhaled nitric oxide and clinical data gave a further focus on severe asthma phenotypes. Therefore, while cluster 4 resembled that previously described with early-onset allergic asthma with low lung function and eosinophilic inflammation, the eosinophilic refractory asthma could be further split into cluster 5, characterized by late-onset severe asthma with nasal polyps and eosinophilia and cluster 6 with persistent inflammation in blood and bronchoalveolar lavage fluid, increased FENO levels and exacerbations despite high systemic corticosteroid use and side effects. Consequently, cluster 5 was characterized as more prone to respond to corticosteroid treatment, even if rapidly deteriorated after discontinuation (corticosteroid dependent), while cluster 6 was characterized to be corticosteroid complete insensitivity [72].

5.2 Blood eosinophilia as a biomarker of severe eosinophilic asthma

The question of whether blood eosinophilia may be considered, in this contest, a surrogate marker of airway eosinophilia, is debated. The measurement of
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eosinophil counts in blood is inexpensive and widely available. However, blood eosinophilia is nonspecific for asthma and often asthmatic patients have normal levels of eosinophils. In asthmatics with increased blood eosinophilia, there exists a direct correlation with symptom scores and an inverse correlation with FEV1 in both children and adults, independently of atopy [73].

Blood eosinophilic counts have been reported to exhibit a moderate-to-good correlation with sputum eosinophils in asthma in large cohorts of asthmatics. A high blood eosinophil count >220/mm³ resulted in good predictors of sputum eosinophilia ≥3% as revealed by an AUC of ROC curves of 79% that yielded 77% sensitivity and 70% specificity and an independent factors associated with the presence of sputum eosinophilic inflammation in multiple logistic regression models [52]. Other studies confirmed that blood eosinophils are an accurate biomarker of eosinophilic airway inflammation comparing two independent cohorts, mild-to-moderate asthma versus moderate-to-severe asthma. The authors used a cut-off point of ≥0.27 × 10⁹/L blood eosinophils that were able to differentiate eosinophilic inflammation of ≥3% with a sensitivity of 78% and specificity of 91% [74].

In a multiple clinical variable analysis within the SARP cohort, the sensitivity and specificity of blood eosinophil counts of greater than 300/mL to detect an “eosinophil phenotype” based on sputum eosinophil counts of greater than 2% were 59% and 65%, respectively. This means that a blood eosinophil count of less than 300/L yields a 41% false-negative rate that has yet a sputum eosinophil percentage of greater than 2%, and likewise, many subjects with sputum eosinophil count of less than 2% would also be misclassified with a false-positive rate of 35%. Therefore, although statistically significant, the AUC of the ROC curve for predicting sputum eosinophil percentages of less than 2% or 2% and greater shows fair-to-poor accuracy and positive predictive values. These results are not improved when the cut-off of sputum eosinophil counts is more than 3% or whether the analysis is restricted to subjects with severe asthma only [51]. The stratification of SARP subjects based on blood eosinophil counts of less than 300 or 300/mL and greater showed significant differences only in methacholine bronchial hyperresponsiveness (log PC20), FEV1 percent predicted and FEV1/FVC ratio, neither in any variable related to overall asthma health care use or frequency and severity of exacerbations. This not-enthusiastic result has been confirmed both in patients with mild-to-moderate or in those with severe asthma who entered a clinical trial for mepolizumab for severe eosinophilic asthma [6].

In a study that evaluated 75 uncontrolled asthmatic patients, a significant positive relationship between the percentage of sputum eosinophils and the percentage of blood eosinophils (r = 0.3647; p = 0.0013) was demonstrated. An important limits of this study were the cut-off point of blood eosinophils of 2% of WBC and again the low accuracy of ROC curves [75].

Increasing the peripheral blood eosinophil cut-off percentage (2.7% or 0.26 × 10⁹/L) yielded a significant higher sensitivity and specificity and AUC as a diagnostic biomarker of sputum eosinophilia (≥3%) in a population of uncontrolled asthmatics [76] suggesting that blood eosinophils can be used in the clinic for detecting airway eosinophilia in uncontrolled asthma. These data are confirmed when looking at the population selected for treatment with reslizumab, another anti-IL-5 mAb, in which blood eosinophil counts of greater than 400/mL might be able to improve the prediction of sputum eosinophil counts of greater than 3% [77].

A systematic review and meta-analysis estimated the diagnostic accuracy of markers for airway eosinophilia in patients with asthma. Looking at the 14 studies that investigated blood eosinophils as a predictor marker, an overall modest ability to distinguish between patients with or without airway eosinophilia was reported
with a summary estimate of AUC of 0.78 [78]. To be noticed that among the different studies, five different definitions of airway eosinophilia had been used, but either eosinophils ≥2% or 3% was used and this did not affect the accuracy of the test. Moreover, a subanalysis of the study showed the forest plots for blood eosinophilia in detecting sputum eosinophilia in subgroup populations of asthmatics. Smoking habit, steroid-treated or untreated and asthma severity revealed a considerable variability of positive thresholds of the marker. In severe asthma, only groups with the cut-point between 275 and 315 μL gave the highest sensibility and specificity [79]. As the most robust clinical value of sputum eosinophilia is tailoring inhaled corticosteroids and reducing the frequency of asthma exacerbations, it is expected that blood eosinophilia to replace induced sputum in this context should yield a sensitivity and specificity of at least 90%, so that only a small portion of patients will be misclassified. One of the most evident limits in the role of blood eosinophilia as a biomarker comes for the cross-sectional nature of the study populations. Significant variability of blood eosinophil count in the same patient over time and according to treatment status must be taken into account.

5.3 Treatment of severe eosinophilic asthma

Asthma guidelines are recommending the use of sputum eosinophil count in severe asthma. The international ERS/ATS guidelines on the definition, evaluation and treatment of severe asthma addressed the phenotypic management of severe asthma and evaluated the utility use of sputum eosinophilia to guide treatment. The document suggested that treatment guided by clinical criteria and sputum eosinophil counts should be performed in centers with experience in this procedure and in selected patients, allowing avoidance of inappropriate escalation of treatment and waste of resources [62]. In the global strategy for asthma management and prevention (GINA) 2019 update, this concept is reinforced by claiming that treatment guided by sputum eosinophil count has the best benefits in patients with moderate-to-severe asthma requiring secondary care [1]. Within step 5 of treatment scale, adults with persistent symptoms or exacerbations despite high-dose ICS or ICS/LABA are advised to adjust treatment based on sputum eosinophilia >3%.

When a refractory or underline type 2 inflammation is proven in severe asthma, add-on biologic type 2 target treatment must be considered for patients with exacerbations or poor symptom control [1, 59]. Actually, sputum eosinophil count also provides an effective method to identify patients who will benefit from targeted therapy with anti–IL-5 monoclonal antibodies (mAbs). In patients with refractory eosinophilic asthma that had a sputum eosinophilia >3% DCC, despite high dose of inhaled corticosteroids, and at least 2 exacerbations in the last 2 years, with the need to make a short course of systemic corticosteroids, mepolizumab therapy reduces exacerbations and improves AQLQ scores [80]. Other studies confirmed the efficacy of anti-IL-5 mAb therapy in patients with asthma who had consistently increased eosinophil counts in sputum of greater than 2.5–3% on at least two occasions [81].

Yet, the measurement of eosinophils in sputum or airway fluids may not truly reflect the contributions of airway tissue eosinophils. Actually, a study was assessed to understand whether induced sputum has the ability to distinguish the eosinophilic and noneosinophilic phenotypes compared to bronchial biopsies in moderate and severe asthma. This study showed that among patients with severe asthma could identify a BrEos+ group with high mucosal eosinophils and a BrEos– group. Even if there was no a correlation between sputum eosinophil count and eosinophils found in the bronchial mucosa, there was a significant correlation between
the number of asthma exacerbations reported by the subjects with severe asthma during the year preceding the study and the percentage of sputum eosinophils [82]. This result is reflected by the fact that on one hand mepolizumab depleted <50% of bronchial tissue and bone marrow eosinophils in spite of its effect in reducing blood, BAL fluid and sputum eosinophils abolishing established airway eosinophil infiltration [83]. Among the explanation to this phenomenon, it can be supposed that eosinophils in the airway lumen may be in a different state of activation than in the bronchial mucosa or may reflect greater concentrations of intraluminal chemokines such as eotaxin and RANTES or epithelial activation.

Another possible consequence of the supposed partial effect of mepolizumab over all the aspects of eosinophilic inflammation is that FEV1, symptoms and FENO levels were not affected [80]. On one hand, this means that these therapeutic strategies may not be sufficient to reverse remodeling changes of severe asthma even if mepolizumab has been shown to decrease the deposition of tenascin, lumican and collagen III in the basal membrane of mild atopic asthmatics as well as the degree of TGF-β in the bronchoalveolar lavage fluid [43]. Accordingly, lung function was not expected to be positively modified by anti-IL-5 treatments in severe asthma and a meta-analysis of nine randomized, placebo-controlled, clinical trials including mepolizumab or reslizumab reported a mild absolute difference of FEV1 in favor of the anti-IL-5 treatment compared to placebo [84].

On the other hand, the persistently high level of FENO can guess, in a proportion of eosinophilic refractory severe asthmatics, that the IL-5 pathway is not in these patients the predominant. This fact can explain why targeting the type 2 phenotype on the IL4/IL13 pathway with dupilumab, a humanized MoAb to IL4-Ra, gave partially different results. When type 2 severe asthmatics with sputum eosinophilia >3% had been enrolled to be treated with dupilumab, the endpoints consisting of improvement of control (ACQ), symptoms and FEV1 were reached. These clinical and functional results were coupled with decreasing FENO, eotaxin 3 and IgE levels [85].

Another question is whether blood eosinophils are a good predictor of response to mepolizumab in patients with severe eosinophilic asthma. The DREAM study identified a blood eosinophil count of 300/mL or greater as a high predictive biomarker of response to mepolizumab [86].

In systemic corticosteroid severe asthma with persistent blood eosinophilia, at least 150 cells, the goal of reducing >75% of oral corticosteroid dose was reached in more than 40% of patients, confirming the role of persistent blood eosinophilia as predictor marker [87].

Benralizumab binds with high affinity to the alpha-chain of human IL-5R, blocking its activation and transduction and determining a neutralizing activity. Moreover, it is able to induce antibody-dependent cell-mediated cytotoxicity (ADCC) on NK cells that release cytotoxic mediators and cause eosinophil apoptosis [88]. A significant clinical efficacy in terms of reduction of annual exacerbations, improvement of FEV1 and steroid-sparing effect was demonstrated in the clinical trials [89]. A threshold of >300 cells per mcl represents a useful marker for quantifying the advantage of this treatment in patients with steroid-dependent asthma. It has been supposed that benralizumab results in a complete depletion of eosinophils in the airway lumen and this can in part explain why in the registrative studies pre-bronchodilator FEV1 improved in the treatment groups. Actually, benralizumab is highly selective on eosinophil and basophil protein and gene-related immune signaling pathways [90] and not only reaches almost complete eosinophil eliminations at plasma levels but also determines the reduction of blood eosinophil precursors [91].
6. Conclusions

The definition of eosinophilic asthma engages different models according to different contests [92]. However, a single common thread can be glimpsed in the ability of eosinophils to catch biological and clinical features that are crucial in each contest. Mouse and human allergic asthma models teach us that as the eosinophilic cascade can be dominant after acute exposition to triggers but only within the chronic stimulation, it contributes to deeper structural changes of the airways [11, 17]. The role of eosinophils in different phases of allergic asthma as well as the involving of Th2 cells, cytokines including IL5, IL4 and IL13 and chemokines has been smartly showed in the majority of the experimental studies. In addition, the mechanisms leading to AHR or persistent inflammation imply the need of sharing of different pathways of the Th2 cascade and the cross-talk between eosinophils and other immune cells [41]. The contribution of either IL-5–independent ways or the regulation of local or systemic eosinophilopoiesis has been addressed [40]. In real life, these phenomena can explain the ability of eosinophilic inflammation to be controlled by corticosteroid treatments, and, under certain circumstances, it becomes insensitive to this treatment.

Accordingly, in the context of severe asthma, eosinophilic airway inflammation becomes exceptionally deregulated and needs a biological approach to be controlled. The eosinophilic phenotype of asthma is currently defined by sputum examination that reveals eosinophilic airway inflammation. Generally, eosinophilic subtype of asthma may be defined as symptomatic asthma in the presence of airway eosinophilia and that is characterized by a good response to glucocorticosteroids. The efficacy of reducing exacerbations when corticosteroid treatment was guided according to the sputum eosinophils has addressed the point of eosinophilic target therapy in a subgroup of patients who encounter worse asthma control, higher use of healthcare resources, higher risk of exacerbations and the need of high-dose ICS or systemic corticosteroid treatment to be controlled. Continuous or bust oral corticosteroid exposure is associated to significant adverse effects that significantly impact on the patients’ outcome [66], highlighting the urgent need of sparing corticosteroid approaches. Even if limits in accuracy have been evidenced, blood eosinophils can be used in the clinic for detecting airway eosinophilia in uncontrolled severe asthma [78] and as eligibility criteria for anti–IL-5 target therapy. Therefore, new add-on therapies for severe asthma have showed to reduce both asthma exacerbation rate compared to standard of care and daily OCS use. Five biologicals have been now approved for severe eosinophilic asthma and can be applied depending on asthma phenotype and endotype [93]. As a consequence, the precision medicine and personalized therapy have become the best clue for treatment and monitoring the response by identification of suitable biomarkers in patients with more severe and refractory forms of asthma [94].
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Chapter 9

Eosinophilic Disorders: Extrinsic and Intrinsic Immune Response, New Diagnostic Perspectives, and Therapeutic Alternatives

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Abstract

Eosinophils are immune response cells located in the peripheral blood, bone marrow, and lymph nodes, among others; an increase in the number of eosinophils in the peripheral blood above 5000/mm³ is associated with conditions ranging from infections (bacterial and parasitic) and allergy (asthma, rhinitis, or drugs), even neoplasms. Various study groups have classified them according to their etiology, thus facilitating their diagnosis and treatment. The WHO divides them as primary and secondary and also considers the number of eosinophils/mm³ and the involvement of white organs, while others have divided them into intrinsic and extrinsic. The former include mutations in the pluripotential hematopoietic cells, which lead to chronic myeloid leukemias with clonal expansion of eosinophils and extrinsic ones where the changes are related to a TH2 response activated by different cytokines such as IL-5. Current treatments are specifically aimed at modifying the clonal expansion of eosinophils with corticosteroids, hydroxyurea, interferon (peg) alpha, imatinib, among others, and bone marrow transplantation, while in extrinsic alterations corticosteroids and IL inhibitors are used —5 (mepolizumab).

Keywords: eosinophilia, hypereosinophilic syndrome, interleukin-5, diagnosis, treatment

1. Introduction

1.1 Characteristics: morphological, physiological, origin, immunological regulation, and distribution of eosinophil

Eosinophils are leukocytes (white cells) found in the peripheral blood, hematopoietic, lymphatic organs, the bone marrow, spleen, and thymus, and can migrate to connective tissues and digestive tract; they are part of the group of leukocytes called granulocytes, along with basophils and neutrophils. They were described
by P. Ehrlich in 1879 calling them eosinophils because their acidic granules in the cytoplasm were stained by their affinity dye aniline-eosin giving them the form of red-orange ammunition observed by optical microscopy: They are rounded cells from 8 to 15 μm in diameter, with a bilobed core with a fine nuclear bridge joining both lobes [1].

Identification and quantification.
Methodology: Manual count in Neubauer chamber and automatic hematology analyzer using impedance and colorimetry and flow cytometry CD16 (FcγRIII-CD16). Under normal conditions peripheral blood eosinophils represent 1–5% of total leukocytes, with an upper limit of 0.4 × 10⁹ L⁻¹, the absolute eosinophilic count (AEC) of 350–500/mm³ and in children is greater than 0.75 × 10⁹ L⁻¹, increasing the number of eosinophils (eosinophilia) to more than 3–5 times which is indicative of an activity of infectious, parasitic, allergic, and eosinophilic and hypereosinophilic disorders [1–5].

They originate in the bone marrow, by a process of maturation and differentiation that lasts approximately 8 days (hematopoiesis) from a pluripotential precursor cell (stem cell) differentiating itself as myeloid granulocytic line, under the influence of IL-3, IL-4 - granulocytic colony stimulation factor (GM-CSF) of eotaxin; evolving toward a mixed eosinophil-basophilic precursor and then differentiating toward eosinophils by action of IL-3, GM-CSF, and especially IL-5, they have a survival of 6–12 hours before moving to tissues where they remain between 2 and 5 days; once there is a stimulus, they respond by exercising their multiple functions regulated by T lymphocytes (Figure 1) [1, 2, 6].

The text begins with: Its main functions are the defense against parasites, helminths, nematodes, participate in allergic responses, inflammatory processes, restoration, and tissue repair; since they have specific chemotactic receptors on their membrane, eotaxin, cytokines (IL-3 -IL-5 and GM-CSF), eosinophil chemo-tactic factor of anaphylaxis (ECF-A); and nonspecific such as f MLP (from the wall of bacteria), complement activation products (C3a, C5a, C6, and C7), platelet-activating factor (PAF), leukotrienes (LTB 4 and LTD 4), histamine and IL-8. Diapedesis is mainly performed by integrins to adhere to the vascular endothelium.
(e.g., LFA-1-ICAM-1, the VLA-VCAM-1) and other multiple antibody receptors: IgA (FcεRI CD89), (FcεRIII-CD23-IgE), (FcγRI-degranulation), (FcγRI-C64-IgG1, IgG3 respiratory burst induction of microbial death), (FcγRIA-CD32-IgG1-degranulation), (FcγRIIB-CD32-IgG1-No Phagocytosis, inhibition of cellular activity) (Figure 1) [2, 6, 8].

Granular content: Eosinophil mature contains in its cytoplasm primary granules rich in phospholipase A, rich in crystalline proteins of Charcot-Leyden-specific secondary granules containing the major or main basic protein (MBP), the eosinophilic peroxidase (EPO), eosinophilic protein (ECP), and eosinophil-derived neurotoxin (EDN) that also appears in basophils and neutrophils; its response capacity is less than 1 hour, small granules containing arylsulfatase B and acid phosphatase and five lipid bodies main source of arachidonic acid, can be presenting cells, proliferation of T lymphocytes and basophils are capable of deliberating more than 35 cytokines, chemokines, and growth factors (Figure 1) [5, 9].

2. Diseases and classification

The severity of eosinophilia has been arbitrarily divided into mild (AEC from the upper limit of normal to 1500/mm³), moderate (AEC 1500–5000/mm³), and severe (AEC >5000/mm³).

The classification of eosinophilic diseases was revised in 2008 and reaffirmed in 2016. In 2017 its diagnosis, risk stratification (prognosis), and management (treatment) proposed by the World Health Organization were covered [10].

Eosinophilic diseases can be classified in two types: primary, intrinsic hematology due to clonal disorders, and secondary, extrinsic or reactive disorders to an external cause that cause damage to different organs. Primary eosinophilias or clonal disorders can be diagnosed by studying the blood and bone marrow by the following methods: standard cytogenetics, molecular biology with monoclonal antibodies, flow cytometry, in situ hybridization, and evaluation of T cell clonality.

The major category of primary diseases corresponds to myeloid/lymphoid neoplasms with eosinophilia and rearrangements PDGFRA, PDGFRB, or FGR1; with PCM/JAK2 and MPN, a subtype of chronic eosinophilic leukemia or not specified by CEL-NOS, there is another lymphoid-eosinophilic variant of aberrant T cell clone.

The modern definition of hypereosinophilic syndrome (HES) is a vestige of the historical criteria outlined by Chusid and colleagues in 1975: The absolute eosinophil count is >1500/mm³ for more than 6 months, and tissue damage is present [10, 11].

The Working Conference on Eosinophil Disorders and Syndromes proposed a new terminology for eosinophilic syndromes. Hypereosinophilia (HE) for persistent and marked eosinophilia (AEC >1500/mm³) in turn, HE subtypes were divided into a hereditary (familiar) variant (HEfa); HE of undetermined significance (HEus), primary (clonal-neoplastic), HE produced by clonal/neoplastic eosinophils (HEn), and secondary (reactive) (HER) can be considered a provisional diagnosis until a primary or secondary cause of eosinophilia is ascertained [12].

To have to a better understanding of the pathogenetic aspects of eosinophilia, other classifications of eosinophilic diseases were generated according to the site of eosinophilic infiltration associated with organ damage and dysfunction. The primary cause of eosinophilia located within the eosinophils (and/or eosinophil precursors) themselves or in other cells, similar to allergic diseases, can be divided in IgE-mediated (extrinsic) and non-IgE-mediated (intrinsic) diseases; the terms extrinsic and intrinsic eosinophilic disorders indicate whether the primary cause of eosinophilia is inside or outside the eosinophil lineage [11].
2.1 Eosinophilic intrinsic disorders

Chronic eosinophilic leukemias belong to a special group of chronic myeloid leukemias, in which eosinophil differentiation is dominant, resulting in blood eosinophil counts of greater than 1500/mm³. However, other lineages are also affected, because the disease is the result of a mutation in a pluripotent hematopoietic stem cell. The chromosomal translocations related to breakpoints on chromosome 8p11 result in fibroblast growth factor receptor 1 fusion genes with increased kinase activity causing the so-called 8p11 syndrome. The increase in tyrosine kinase activity is caused by gene 1 and the growth factor, and this leukemia has a worse prognosis, which transforms chronic leukemia to an acute, 1–2 years. Another type of cause may be the increase in tyrosine kinase by fusion of the platelet growth factor alpha receptor genes (PDGFRA). PDGFRA is fused by the Fip1-like 1 (FIP1L1) gene as a result of a 4q12.9 chromosome damage. This is both in eosinophils and in other hematopoietic lineages such as neutrophils, monocytes, lymphocytes, and mast cells. This type of leukemia is pluripotent hematopoietic stem cell which responds to the tyrosine kinase inhibitor (imatinib) [10, 11].

Mutations in multipotent myeloid stem cells: In the chronic myeloid leukemias with eosinophilia, eosinophils are part of the clone. This is because eosinophil differentiation is often not as prominent as other myeloid cells, such as monocytes, which also show increased differentiation. Chromosomal translocations related to breakpoints on chromosome 5q33 are common and represent the basis for the formation of platelet-derived growth factor receptor b (PDGFRB) fusion genes; this result increases the tyrosine kinase activity. There are patients with positive Philadelphia chromosome who can develop chronic leukemia with eosinophilia due to two factors: fusion by breakpoint cluster region-Abelson (ABL) and fusion of transcription gene 6 (ETV6). Marked eosinophilia often associated with a cytogenetic evolution and other accelerated phases of ABL can occur during an acute transformation; ABL may be fused with the transcription factor E26 by means of variant ETV6 triggering chronic leukemia [10].

Myelodysplastic syndromes: During hematopoiesis there may be an inefficient process in the differentiation of stem cell by mutations, malignant clones producing myelodysplastic syndromes that lead to myeloproliferative diseases such as polycythemia vera, essential thrombocytopenia, and agnogenic myeloid metaplasia. The exact molecular genetic abnormalities resulting in eosinophilia in these disorders remain to be determined [10, 11].

2.2 Eosinophilic extrinsic disorders

T cell-mediated eosinophilias: The common diseases are allergic rhinoconjunctivitis, bronchial asthma, drug allergic, eosinophilic esophagitis, and atopic dermatitis. Eosinophilia and IgE production due to the polarization of TH2 cells whose causes are extrinsic or external by stimulation of environmental immunogens or chemical compounds, which are presented by APC-MHC, stimulating the release of pro-inflammatory cytokines (IL4, IL5, and IL13), induce the increase in eosinophils of IgE survival, high affinity receptors with PKC activation, cross-linking and signaling for histamine release, as well as vasoactive amines that produce inflammatory processes and organ damage [10, 11].

Infectious diseases: TH2 inflammatory responses are induced by helminths; these responses are characterized by IgE antibody production and eosinophilia; both have been implicated in mediating protective immunity to the parasites. In contrast, there is little doubt that eosinophils contribute to tissue damage and therefore to the pathogenesis of these infections.
Viral infections are not common; however, when virus-specific T cells are generated in a TH2 environment, they can also release IL-5 and therefore trigger eosinophilia. In chronic rhinosinusitis, eosinophilia is related to fungal infections with certain molds (e.g., Alternaria) which is present in the nasal and paranasal cavities [5, 10, 11].

Autoimmune diseases: Because these diseases are often associated with a TH1-associated inflammatory response, eosinophilia is not frequent, but in systemic sclerosis, levels of major basic protein and extracellular major basic protein deposits were observed in skin and lung tissues. In primary biliary cirrhosis, eosinophilia is a distinctive feature that might be useful in the diagnosis of the disease [10, 11, 13].

Graft-versus-host diseases: When an allogeneic bone marrow transplant is carried out and there are differences in MHC molecule polymorphism, these can be recognized by the immune system, and responses can be made against the alloantigens, producing graft-versus-host-disease (GVHDs), carrying out a reaction antigen antibody, cellular or cytotoxic that produces lysis and destruction in specific organs (skin, liver, and gastrointestinal tract mainly).

Drug-induced diseases: Hypersensitivity drug reactions may present in some cases increased eosinophils. The manifestations range from maculopapular rashes of the skin to severe life-threatening drug reactions with eosinophilia and systemic symptoms (DRESS). Drugs and their metabolites can produce hypersensitivity by means of mechanisms mediated by APC-MHC TCR π concept, generating TH2 polarity or TH1 with memory T cells [10, 11, 13].

There are other subgroups of this syndrome as episodic angioedema and hereditary eosinophilia. Where there is evidence of mechanism mediated by IL-5-producing T cells [5].

![Figure 2. Diagnostic algorithm for patients with hypereosinophilia. Due to the fact that eosinophilia can occur in different pathologies, an exclusion of the unlikely causes for hypereosinophilia is performed, in addition to a three-step follow-up treatment with imatinib due to mutation processes that is considered. Laboratory tests would be at the discretion of the doctor according to the medical history and the search according to the type of response to the genes involved [12].](image-url)
Severe primary (IL-5) and secondary immunodeficiencies (HIV) are associated with eosinophilia when there is polarization of TH2 by the immunogen (allergen) or drug (antiretroviral); infections such as tuberculosis are the cause of infections and resistance to treatment (Figure 2) [11].

2.3 Treatment of HES and CEL-NOS

Corticosteroids should be considered a first-line treatment, which are potent anti-eosinophil agents, effective in producing rapid reductions. Maximal dose was 1 mg x kg 2 months, with symptom control and reduction of the eosinophil count to below 1500/mm³ after 1 month of treatment.

Hydroxyurea is an effective first-line agent for HES which may be used in conjunction with corticosteroids or in steroid nonresponders. A typical starting dose is 500–1000 mg daily which can serve as effective palliative to control leukocytosis and eosinophilia but with no proven role in favorably altering the natural history of HES or CEL-NOS (Figure 2) [10, 12, 14].

IFN-a has demonstrated hematologic responses and reversion of organ injury in patients with HES and CEL-NOS refractory to therapies including prednisone and/or hydroxyurea. Remissions have been associated with improvement in clinical symptoms and organ disease, including hepatosplenomegaly, cardiac and thromboembolic complications, mucosal ulcers, and skin involvement [5, 10–12].

![Diagnostic and treatment algorithm based on revised 2016 WHO classification of eosinophilic disorders. According to the algorithm, the type of eosinophilia can be monitored according to the cases where other drugs other than imatinib should be used, with three pathological options being present: chronic leukemia with eosinophilia, idiopathic hypereosinophilia, and lymphocyte variant, all share the administration of imatinib and corticosteroids (idiopathic hypereosinophilia and lymphocyte variant) [10].](image-url)
Mepolizumab anti-IL-5 antibody is a fully monoclonal IgG antibody that inhibits binding of IL-5 chain of the IL-5 receptor expressed on eosinophils [5, 14]. Alemtuzumab is an anti-CD52 monoclonal antibody that has been evaluated in idiopathic HES based on expression of the CD52 antigen on eosinophils. In patients with refractory HES, alemtuzumab was administered intravenously at a dose of 5–30 mg once to thrice weekly.

Bone marrow/peripheral blood stem cell allogeneic transplantation has been attempted in patients with aggressive disease; a disease-free survival ranging from 8 months to 5 years has been reported.

Imatinib is a small-molecule tyrosine kinase inhibitor 100 mg per day; it also shows activity against platelet-derived growth factor receptor (PDGF-R), c-Kit, Abl-related gene (ARG), and their fusion proteins while sparing other kinases (Figure 3) [10].

3. Hematologic and neoplastic diseases

Mastocytosis: Develops from a neoplastic proliferation of mast cells. It develops from a neoplastic clonal proliferation of mastocytes that accumulate in one or more organ systems and are organize as compact cohesive aggregate groups or multifocal groups of abnormal mastocytes. This disorder is diverse; it can be found as cutaneous lesions that may naturally recede, to highly aggressive neoplasias related with multiple organ failure and short outliving. Mastocytosis subtypes are principally characterized by the clinical manifestations and the spread of the disease. When cutaneous mastocytosis (CM) occurs, mastocyte infiltration is restricted to the skin, whereas systemic mastocytosis (SM) includes at least one extracutaneous organ, with or without skin lesions. Mastocytosis must be distinguished from mastocyte hyperplasia or from the mastocyte activation states, without the morphological or molecular abnormalities that characterize neoplastic proliferation [15]. The WHO classification includes seven types:

a. Cutaneous mastocytosis

b. Indolent systemic mastocytosis (ISM)

c. Systemic mastocytosis with associated clonal, hematologic non-mast cell lineage disease (SM-AHNMD)

d. Aggressive systemic mastocytosis (ASM)

e. Mast cell leukemia (MCL)

f. Mast cell sarcoma (MCS)

g. Extracutaneous mastocytoma

Hypereosinophilic syndrome (HES): It has been described as a condition associated with persistent eosinophilia in the peripheral blood, organ damage, and exclusion of any other underlying disease or condition that may explain eosinophilia or organ damage [4, 16–18]. The diagnostic algorithm must begin with the evaluation of peripheral blood hypereosinophilia (HE), defined as a persistent increase of blood eosinophils, above 1.5 X 109/L blood [4, 16–18]. The term “tissue HE” has also been proposed, and it may be useful in the evaluation and the classification of the disorders related to HES [16, 19]. The establishment of an HES diagnosis must be
considered: (a) the existence of an underlying disease or condition and (b) the presence of clinical signs and symptoms or laboratory abnormalities that show organ damage induced by HE (HES) [19]. There are four important groups of underlying disorders in patients with documented HES:

1. Hematopoietic neoplasias

2. Other neoplasias (non-hematopoietic) (paraneoplastic HE)

3. Common allergic, reactive, or immunological conditions

4. Infrequent clinical syndromes that present HE, including rare hereditary disorders [19]

Lymphoid and myeloid leukemias: Many hematologic disorders may present eosinophilia, but only a few present clonal (primary) neoplasias, and just a small number of neoplasms present HE and organ damage. Myeloid neoplasias that present HE include rare acute eosinophilic leukemia types. The most common type of chronic leukemia is chronic eosinophilic leukemia (CEL), which is frequently associated with the FIP1L1-PDGFRα rearrangement in endomyocardial fibrosis/thrombosis and other myeloid neoplasias with rearrangements, such as the 8p11 syndrome [19, 20]. Clonal eosinophilia is frequently observed in advanced cases of systemic mastocytosis [19, 21, 22].

Lymphoid neoplasms may present HE, and in most cases, a T cell lymphoma is diagnosed. Nevertheless, in such patients with 8p11 syndrome and other rare entities, both eosinophils and lymphocytes may be involved in the neoplastic clonal processes [19, 21].

Paraneoplastic conditions associated with hypereosinophilia. Different types of cancers may be preceded or accompanied by eosinophilia. Cancers associated with HE include lung, gastrointestinal tract, pancreas, and thyroid adenocarcinomas, gynecologic tumors, and skin cancer. Although pathogenesis is unclear, there is a widely accepted hypothesis stating that carcinogenic cells or cancer or the cancer microenvironment around fibroblasts produce eosinophilopoietic cytokines [19, 23].

Identification and quantification.

Classic methodology: Clinical manifestations and diagnosis depend on the type of disease and other factors, where different organs may be involved in patients with HES, for example, skin, gastrointestinal tract, heart, and central nervous system.

In order to establish an HES diagnosis, it is recommended to include clinical and laboratory parameters, such as:

a. Physical exam of organs and body systems

b. Laboratory exams: white blood cell count (eosinophils, basophils, neutrophils), hemoglobin, platelet count, B12 vitamin, hepatic enzymes, kidney function tests, and urinalysis

c. Organic functional tests: electrocardiogram, echocardiogram, pulmonary function tests, chest computed tomography and radiography, abdominal ultrasound, and normal endoscopic study [19]

d. Molecular detection of some translocations, such as TCR, BCR/ABL1, JAK2 V617F, KITD816V, PDGFRA/PDGFBR, and FGR1
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considered: (a) the existence of an underlying disease or condition and (b) the presence of clinical signs and symptoms or laboratory abnormalities that show organ damage induced by HE (HES) [19]. There are four important groups of underlying disorders in patients with documented HES:

1. Hematopoietic neoplasias
2. Other neoplasias (non-hematopoietic) (paraneoplastic HE)
3. Common allergic, reactive, or immunological conditions
4. Infrequent clinical syndromes that present HE, including rare hereditary disorders [19]

Lymphoid and myeloid leukemias: Many hematologic disorders may present eosinophilia, but only a few present clonal (primary) neoplasias, and just a small number of neoplasms present HE and organ damage. Myeloid neoplasias that present HE include rare acute eosinophilic leukemia types. The most common type of chronic leukemia is chronic eosinophilic leukemia (CEL), which is frequently associated with the FIP1L1-PDGFRA rearrangement in endomyocardial fibrosis/thrombosis and other myeloid neoplasias with rearrangements, such as the 8p11 syndrome [19, 20]. Clonal eosinophilia is frequently observed in advanced cases of systemic mastocytosis [19, 21, 22].

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d. Molecular detection of some translocations, such as TCR, BCR/ABL1, JAK2 V617F, KITD816V, PDGFRA/PDGFRB, and FGR1. The most recommended bone marrow exams are cytogenetic assays and fluorescence in situ hybridization (FISH)—other studies which do not include molecular detection are tissue immunohistochemistry and histology (Figure 4) [16].

3.1 Laboratory diagnosis by molecular parameters

Immunoglobulins rearrangements are detected by real-time polymerase chain reaction with TaqMan molecular probes, such as TCR, BCR/ABL1, JAK2 V617F, KITD816V, PDGFRA/PDGFRB, and FGR1. The most recommended bone marrow exams are cytogenetic assays and fluorescence in situ hybridization (FISH)—other studies which do not include molecular detection are tissue immunohistochemistry and histology (Figure 4) [16].

4. Allergy and hypersensitivity to drugs (DHRs)

The WHO defines an ADR to any predictable noxious reaction that appears at therapeutic doses, depends on the doses, and is related to pharmacological actions. Other unpredictable reactions: hypersensitivity or allergic (DHRs) associated with immunological mechanisms, susceptibility (atopy), and polymorphism (pharmacogenetic, MHC-HLA) [24–27].

It is considered as a public health problem due to its high morbidity and mortality being 20%; hence, the importance of its clinical diagnosis and laboratory tests is being considered at all stages of life (prenatal, postnatal, childhood, adolescence, adult, and older adult).

4.1 Immune response to drugs in DHRs: haptens, pro-haptens, and TCR pi

Medications are usually non-immunogenic haptens of different types:

Pro-haptens. Drugs are generally non-immunogenic haptens of different types: Pro-haptens (non-active reagents) low molecular weight chemicals of less than 1000 D; examples aromatic, heterocyclic, sulfonamides, OH, halogens, resonance, and beta-lactam are processed and presented in the CPA-MHC context and produce a humoral response, IgE, IgG and IgM or cellular.

Active reagents: aromatic, polar, with nitrogen, to induce an immune response CPA-MHC.
Inert TCR pi (pharmacological interaction with immune receptors): Some drugs are able to bind non-covalently to TCR pi receptors pre-developed by a previous immune response to a non-covalently reversible drug and signaling toward a response of hypersensitivity and explain the rapid appearance of symptoms, some cross reactions to the drug, or its metabolites.

Pi concept and HLA restriction in hypersensitivity: In the pi concept, drugs primarily activate TCR, for example, abacavir associated with the HLA-B * 5701 allele in whites, Stevens-Johnson syndrome (SJS) with carbamazepine treatment in Chinese associated in patients with the HLA-B * 1502, and HLA-B * 5801 allele in allopurinol-induced adverse reactions such as SJS and toxic epidermal necrolysis (TEN) [28–31].

4.2 Hypersensitivity and diagnosis

Hypersensitivity is an exacerbated immune response, which produces a clinical picture with dermal, systemic disorders, and sometimes sudden death. In 1930 Coombs systematized these reactions according to the period of time in which the symptoms appear, and the dose of challenge has been fundamental to guide the diagnosis, treatment, and monitoring. It has many points in common with autoimmunity, where the antigens are their own; in the case of allergies to medications, the antigens are allergens: drugs or metabolic derivatives. Hypersensitivity reactions require that the individual has been previously sensitized or exposed to at least the antigens in question. The classification of allergic or hypersensitivity reactions into four types (I, II, III, and IV) and subsequently Pichler in 2003 proposed the subdivision of type IV into IVa, IVb, IVc, and IVd (Table 1) [28, 29].

4.3 In vitro tests associated with drug and drug eosinophilia: antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs) anticonvulsants, and antidiuretics

Modified basophil degranulation (MBD): The test is a basophil activation test (BAT) which consists of incubating the basophils in vitro with the suspected drug to be carried out: epitope-paratope binding, activating the basophils and causing degranulation and release of the aforementioned content (specificity 100%, sensitivity 84.0%) [28, 29].

CD63 flow cytometry: Basophils with specific IgE when incubated with the suspected drug are activated by Fce I receptors; high affinity and low affinity cause cross-linking and protein kinase signal transduction (MAP, PKC) that stimulate expression of the receptor (CD63) -gp53 (lysosomal-transmembrane protein tetraspanin LAMP-31) on the surface of the basophil while the eosinophilic expresses CD23 [30].

Modified leukocyte migration inhibition factor (MLIF) type IV a, b, and c. Associated with anaphylactic degranulation: It has been reported that leukocytes including basophils (BAT-Chemotaxis) also play a role in directional chemotaxis; therefore, when microhematocrits are incubated in Bloom chambers with medications in two dilutions (1 and 0.1 mg/mL) in an RPMI medium, with negative and positive controls, at 37°C, the first (20 min at 2 hours) and delayed migration can be measured (4, 6, and 18 hours); the % of MLIF can also be calculated against the negative control, as well as the reference values (RV) for MLIF (0–25% inhibition of leukocyte migration) [29].

Eosinophilia in the peripheral blood is a common cause in patients who consume medications, especially in developed countries, who are monitored and can restrict their consumption without changes. However, for the doctor, concern may arise in cases of impending hypersensitivity reaction (HSR). Severe HSRs associated with peripheral blood may include specific reactions of organs (heart, kidney, liver,
Eosinophilic Disorders: Extrinsic and Intrinsic Immune Response, New Diagnostic Perspectives...
DOI: http://dx.doi.org/10.5772/intechopen.89229

lungs, joints, central nervous system, and skin) and adverse skin reactions (SCAR) where SJS, TEN, and DRESS are included [32, 33].

<table>
<thead>
<tr>
<th>Type</th>
<th>Type of immune response</th>
<th>Clinical symptoms</th>
<th>In vitro diagnostics</th>
<th>In vivo diagnostics</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Measured by IgE eosinophils, mast cells, and basophils (immediate)</td>
<td>Urticaria Angioedema Rhinitis Bronchospasm Anaphylaxis</td>
<td>IgE specific Serum tryptase Cell stimulation test (CAST) BAT (MDB, CD63)</td>
<td>Cutaneous tests (prick, intradermal) Challenge tests Proving tests [Coombs]</td>
</tr>
<tr>
<td>II</td>
<td>Cytotoxicity dependent on IgG and IGM antibodies (not immediate) and complement</td>
<td>Hemolytic Anemia Thrombocytopenia Neutropenia Autoimmunity</td>
<td>Coombs test Ab vs. platelets Ab vs. neutrophils</td>
<td>Only challenges to the drug can make diagnosis but are high risk [Coombs]</td>
</tr>
<tr>
<td>III</td>
<td>Deposit of immunocomplexes [IgG and IGM] (not immediate) Complement or FcR</td>
<td>Serum disease Vasculitis, LES-like by medications Glomerulonephritis drug</td>
<td>C3, C4, ANA, ANCA, CCP, antithyroid, etc. Liver and kidney function tests Pathological anatomy</td>
<td>Biopsies with immunofluorescence [Coombs]</td>
</tr>
<tr>
<td>IVa</td>
<td>TH1 (IFNγ), TNFα, IL12, and macrophages (late)</td>
<td>Contact dermatitis</td>
<td>Lymphocyte transformation test (LT or BT), MLIF, cytotoxic T lymphocyte precursors (CTLp), cytokines (ELISA, PCR)</td>
<td>Patch tests [Pichler]</td>
</tr>
<tr>
<td>IVb</td>
<td>TH2 (IL-4, IL5, IL13) eosinophils</td>
<td>Maculopapular eruptions (MPE) with eosinophilia (DRESS)</td>
<td>CBC with check eosinophil cellularity, atypical lymphocytes MLIF, BT, LT</td>
<td>Patch tests [Pichler]</td>
</tr>
<tr>
<td>IVc</td>
<td>CLT, CD4/CD8 (perforin, granzyme B, Fas L)</td>
<td>Contact dermatitis, maculopapular, and bullous diseases (SJS), TEN</td>
<td>MLIF, liver function tests, CD4/CD8 (death keratinocytes) Activity of IgM vs. herpes virus, Epstein-Barr, and cytomegalovirus (CMV)</td>
<td>Patch tests [Pichler]</td>
</tr>
<tr>
<td>IVd</td>
<td>T cells, IL8, CXCL8 cells Neutrophils Inflammation</td>
<td>Acute generalized exanthemtic pustulosis (AGEP) pharmacodermias associated with neutrophilia</td>
<td>CBC T cells CD4/CD8</td>
<td>Patch tests [Pichler]</td>
</tr>
</tbody>
</table>

Hypersensitivity reactions require that the individual has been previously sensitized or exposed at least once to the antigens in question. The classification of allergic or hypersensitivity reactions into four types (I, II, III, and IV) and subsequently Pichler in 2003 proposed the subdivision of type IV into IVa, IVb, IVc, and IVd [27–29].

Table 1. Hypersensitivity classification according to the Gell and Coombs modified by Sell, Pichler, and ICON.
The prolongation of eosinophilia can cause tissue damage, although without being clarified specifically, adding to the condition infections as another factor that preserves eosinophilia (parasitic and fungal infestations) or decreases (eosinopenia due to bacterial and viral infections). The diagnosis can be complicated because of the presence of the drug which worsens a preexisting eosinophilia, particularly in atopic patients [33].

DRESS is more common in adult patients than in children, with approximately 50 drugs being described, highlighting anticonvulsants (phenytoin, phenobarbital, and carbamazepine) and antibiotics as the main causes of the syndrome and, to a lesser extent, sulfate derivatives, antidepressants, NSAIDs, and antidiuretics [34]. There is no clear association between variability of the type of drug and the affected organ with the degree of eosinophilia, which can be mild or self-limited and severe when multisystemic complications are generated due to the presence of symptoms that are not appreciated in the mild form [32, 33].

Other proposals that lead to the pathogenesis of DRESS include detoxification defects at the time of the formation of reactive metabolites, slow acetylation, and reactivation of the human herpes virus (HHV-6-7) or EBV [34].

In general, the diagnostic algorithm for eosinophilia linked to SCAR can be visualized as a hypersensitivity response type IVb (SJS and NET) and type IVc (DRESS), which in some way can highlight the pathogenesis proposals previously mentioned not only by DRESS but identify an atopic patient (Table 1).

5. Conclusions

Eosinophils are leukocytes (white blood cells) found in the peripheral blood, hematopoietic, lymphatic organs, thymus, connective tissue, and digestive tract. They are identified and quantified by manual counting (Neubauer chamber), automated count with autoanalyzer hemocytometers (impedance, colorimetry, and differential in optical microscope), flow cytometry after the advent of monoclonal antibodies, currently the most used to identify surface markers and immunoenzymatic methods (ELISA, RAST, IMMUNOCAP) for cytoplasmic granules.

The classification of eosinophilic diseases “eosinophilic disorders” was revised in 2008 and confirmed in 2016; its study focused on external (extrinsic) and internal (intrinsic) causes (optimized) and optimized and failed diagnosis by precise and timely diagnosis. The algorithms are used and started with the main pillar: The clinical history (clinical criteria, anamnesis, and exploitative maneuvers leading to clinical laboratory algorithms, with initial, basic, and special tests including imaging, tomography, and X-rays to finally improve the prognosis and modify the natural history. The intrinsic and extrinsic disorder algorithm planting is different; this is due to the recognition of molecular altered T cell clones, bone marrow studies, and markers of apoptotic genes, PCM1-JAK2, Fas L, and bcl2.

Some allergies to medications with symptomatology related to specific organ and severe cutaneous against antiepileptics (phenytoin, phenobarbital, carbamazepine) as well as other medications (antibiotics, NSAIDs, antidiuretics) can be related, which rethinks the proposed immunological response algorithm not only in basophil evaluation but also the search for eosinophils in flow cytometry or optical microscopy to assess not only damage but neutralization (eosinophil histaminase).

Corticosteroids are considered the first line of treatment because of their potent anti-eosinophilic effect for disease control, prognosis, and prevention. So the new
treatment alternatives could displace steroids with monoclonal antibodies such as the IL-5 inhibitor that show less long-term toxicity.

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Conflict of interest

There is no conflict of interest.

Appendices and nomenclature

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AEC</td>
<td>absolute eosinophil count</td>
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<tr>
<td>HSR</td>
<td>hypersensitivity reaction</td>
</tr>
<tr>
<td>SCAR</td>
<td>severe cutaneous adverse reaction</td>
</tr>
<tr>
<td>SJS</td>
<td>Stevens-Johnson's syndrome</td>
</tr>
<tr>
<td>TEN</td>
<td>toxic epidermal necrolysis</td>
</tr>
<tr>
<td>DRESS</td>
<td>drug rash eosinophilia and systemic symptoms</td>
</tr>
<tr>
<td>CBC</td>
<td>complete blood count</td>
</tr>
<tr>
<td>DHRs</td>
<td>drug hypersensitivity reaction</td>
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</tbody>
</table>
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patients receiving outpatient parenteral antibiotics. The Journal of Allergy and Clinical Immunology. 2015;136(5):1288-1294. DOI: 10.1016/j.jaci.2015.04.005


Chapter 10
Eosinophilic Cholangitis
Gilles Jadd Hoilat, Judie Noemie Hoilat, Mohamad Fekredeen Ayas, Sana Riaz and Divey Manocha

Abstract
A variety of benign etiologies of biliary stricture may initially be mistaken for hilar cholangiocarcinoma. Consequently, many patients undergo surgery for a benign disease that could have been treated medically. Eosinophilic cholangitis (EC) is an uncommon, benign, self-limiting disease that should be considered when approaching a case of obstructive jaundice since it causes biliary stricture formation. Transmural eosinophilic infiltration of the biliary tree is characteristic of EC. It may initially be indistinguishable from hilar cholangiocarcinoma. We worked on a case of a patient who was referred to our hospital for jaundice and abdominal mass investigation with the provisional diagnosis of cholangiocarcinoma. During the workup, the index of suspicion for malignancy remained high as the typical laboratory and radiological findings for benign causes of biliary stricture were not present. Hence, the patient underwent left hepatectomy with caudate lobe resection and received a retrograde diagnosis of EC. The case demonstrates that EC could present in the elderly with cardinal signs of cancer and absence of the typical findings of EC which was not previously reported. Furthermore, this disorder has been reported to respond well to steroid therapy, hence, diagnostic criteria for EC would provide another treatment option for elderly and/or those who are not fit for surgery.

Keywords: bile duct diseases, cholangitis, constriction, pathologic, stricture

1. Introduction
While approaching a patient with jaundice, it is important to understand the different types and approach towards jaundice in order to reach the correct diagnosis. Similarly, when approaching a biliary stricture, one must consider benign as well as malignant etiologies since they can be clinically identical. Eosinophilic cholangitis (EC) is a rare benign disorder of the biliary tract which can cause biliary obstruction. This is the first book that has a specific chapter for a very rare disease, eosinophilic cholangitis. In this chapter, we will briefly discuss the approach towards jaundice before jumping to the main topic of eosinophilic cholangitis. We hope that you will find the information provided informative.

2. Approach to jaundice
Jaundice (i.e., icterus) is the buildup of bilirubin; a waste product stemmed from the metabolism of aging/destruction of red blood cells (RBCs), causing a yellowish
Eosinophilic Cholangitis

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Jaundice (i.e., icterus) is the buildup of bilirubin; a waste product stemmed from the metabolism of aging/destruction of red blood cells (RBCs), causing a yellowish
discoloration in tissues that are filled with elastic collagen, such as skin, sclera, and mucus membranes, etc. [1].

Let us take some cases here to further understand how jaundice can present.

### 2.1 Case-1

A 14-year-old (y/o) African-American male presented to the emergency department (ED) with severe abdominal pain, swelling of both hands and jaundice. Family history includes two uncles that suffer from “blood problems.” Blood tests showed normal alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin was 3.2 mg/dL (2.8 indirect). On peripheral blood smear, sickling was found. Hemoglobin electrophoresis confirmed SS hemoglobin and patient was diagnosed with sickle cell anemia (SCA).

### 2.2 Case-2

A 42-year-old Caucasian male presented to the office with 2 months of jaundice and abdominal pain. The patient had a blood transfusion in 1988 at the age of 15.

<table>
<thead>
<tr>
<th>Type of jaundice</th>
<th>Differential diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-hepatic (hemolytic)</td>
<td>• Excess aged/destroyed RBCs (e.g., hemolysis, blood transfusion)</td>
</tr>
<tr>
<td></td>
<td>• Decreased hepatic uptake (e.g., portosystemic shunt, drugs)</td>
</tr>
<tr>
<td></td>
<td>• Decreased conjugation (e.g., Gilbert’s syndrome)</td>
</tr>
<tr>
<td>Hepatic (hepatocellular)</td>
<td>• Excretion defect (Dubin-Johnson syndrome, Rotor syndrome)</td>
</tr>
<tr>
<td></td>
<td>• Viral hepatitis</td>
</tr>
<tr>
<td></td>
<td>• Hepatic steatosis</td>
</tr>
<tr>
<td></td>
<td>• Alcoholic hepatitis</td>
</tr>
<tr>
<td></td>
<td>• Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td></td>
<td>• Autoimmune hepatitis</td>
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<tr>
<td></td>
<td>• Ischemic hepatitis</td>
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<tr>
<td></td>
<td>• Drug-induced hepatitis</td>
</tr>
<tr>
<td></td>
<td>• Hemochromatosis</td>
</tr>
<tr>
<td></td>
<td>• Wilson’s disease</td>
</tr>
<tr>
<td>Post-hepatic (obstructive)</td>
<td>• Biliary tract disease (primary sclerosing cholangitis, primary biliary cirrhosis, eosinophilic cholangitis, etc.)</td>
</tr>
<tr>
<td></td>
<td>• Biliary tract obstruction (gallstones, cholangiocarcinoma, pancreatic or liver cancer, pancreatic pseudocyst, etc.)</td>
</tr>
</tbody>
</table>

**Table 1.**

Types of Jaundice [2, 3].

<table>
<thead>
<tr>
<th>Lab findings</th>
<th>AST + ALT</th>
<th>ALP</th>
<th>Conjugated bilirubin (CB)</th>
<th>Unconjugated bilirubin (UCB)</th>
<th>Total bilirubin</th>
<th>Conjugated bilirubin in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-hepatic (hemolytic)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal/↑</td>
<td>Normal/↑</td>
<td>Negative</td>
</tr>
<tr>
<td>Hepatic (hepatocellular)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>Positive</td>
</tr>
<tr>
<td>Post-hepatic (obstructive)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>Normal</td>
<td>↑</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Table 2.**

Lab findings depending on type of jaundice [4].

**Figure 1.**

Approach to Jaundice.
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discoloration in tissues that are filled with elastic collagen, such as skin, sclera, and mucus membranes, etc. [1].

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<table>
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<tr>
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<th>Pre-hepatic (hemolytic)</th>
<th>Hepatic (hepatocellular)</th>
<th>Post-hepatic (obstructive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab findings</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 1.
Types of Jaundice [2, 3].

Physical exam showed typical signs of cirrhosis. Labs showed an ALP of 200, ALT 2810, AST 2670 U/L, normal gamma-glutamyl transferase (GGT), bilirubin of 3.3 mg/dL (2 direct, 1.3 indirect), and normal iron and copper levels. Patient was positive for hepatitis C, attributed to his previous blood transfusion. Alpha fetoprotein (AFP) levels were elevated and patient was scheduled for a liver ultrasound (US) and biopsy to rule out hepatocellular carcinoma (HCC).

Table 3.
Clinical findings depending on type of jaundice [4].

Physical exam showed typical signs of cirrhosis. Labs showed an ALP of 200, ALT 2810, AST 2670 U/L, normal gamma-glutamyl transferase (GGT), bilirubin of 3.3 mg/dL (2 direct, 1.3 indirect), and normal iron and copper levels. Patient was positive for hepatitis C, attributed to his previous blood transfusion. Alpha fetoprotein (AFP) levels were elevated and patient was scheduled for a liver ultrasound (US) and biopsy to rule out hepatocellular carcinoma (HCC).

Figure 1.
Approach to Jaundice.
2.3 Case-3

A 39-year-old Caucasian female with a body mass index (BMI) of 36 and a history of hamishoto's thyroiditis, presented to the ED with worsening itching and jaundice. Patient’s thyroid function tests were within normal range. Cholesterol was 310 mg/dL, ALP 318, ALT 24, AST 21, and GGT 1120 U/L. Liver US showed no signs of bile duct dilation. Anti-mitochondrial antibody (AMA) titers were elevated. Patient was diagnosed with primary biliary cholangitis (PBC) and was started on ursodiol.

Now what do we take from these three different cases, all of whom presented or were seen to have jaundice? To understand the different presentations of jaundice, let us classify it.

The causes of jaundice can be classified in different ways such as pre-hepatic (hemolytic), hepatic (hepatocellular), and post-hepatic (obstructive) (see Table 1). Now, we can differentiate between them in many laboratory and clinical findings (see Tables 2 and 3) and know how to approach it (see Figure 1).

Understanding the classification, differentiating lab results and approach towards jaundice is important. Figure 1 can help you as a guide in term of what to do and what to expect. It is helpful to keep it in mind as we go through the chapter.

3. Approach to eosinophilic cholangitis

3.1 Introduction

Now that we have established the approach towards obstructive jaundice. We will dig deeper into eosinophilic cholangitis.

EC is an uncommon, benign, self-limiting cause of biliary structure characterized by transmural eosinophilic infiltration of the biliary tree which may result in obstructive jaundice. The severity and prognosis vary considerably and may affect part or the entire biliary tree mimicking malignancy [5].

3.2 Pathogenesis

The exact pathogenesis is poorly understood.

The cause of eosinophilic cholangitis is unknown. In some reports, hypereosinophilic syndrome (HES) has been mentioned as possible cause. The diagnosis of hypereosinophilic syndrome is based on the following criteria [6]:

1. Sustained eosinophilia (more than 1500 eosinophils per cubic millimeter) for more than 6 months.
2. The absence of other causes of eosinophilia, including parasitic infections and allergies.
3. Signs and symptoms of organ involvement.

Since all reported cases do not appear to have completely met the criteria for HES, the relationship between eosinophilic cholangitis and HES is uncertain.

An allergic mechanism is thought to play a key role in the development of eosinophilic cholangitis, hence the name. In most reported cases, there was an increased level of IgE, interleukin 5, or eosinophil cationic protein. The latter is one of the major cationic granules released by activated eosinophils and is the most
widely used clinical biomarker of eosinophil in atopic diseases. Furthermore, it has been it has been demonstrated that eosinophils produce transforming growth factor-beta, a cytokine known to stimulate fibrosis, a devastating effect that may leave liver transplantation as the only cure [7, 8].

3.3 Clinical presentation

Nash et al. conducted a study where they collected around 23 cases of EC revealing that this disease [9]:

- Slightly more prevalent in men than women (1,6:1).
- The most common presenting symptoms were abdominal pain followed by jaundice.
- Around 69.6% of patients demonstrated peripheral eosinophilia and 30.4% had normal eosinophilic count.

One of the challenges that accompanies eosinophilic cholangitis is the fact that it can present with a multitude of nonspecific signs and symptoms that makes it hard to differentiate from malignancy such as:

- Abdominal mass
- Abdominal pain
- Jaundice
- Generalized fatigue
- Nausea and vomiting
- Weight loss

At this point, it can be anything and a more in-depth investigation is required. So where do we go from there?

3.4 Investigations

The issue with eosinophilic cholangitis is that it mimics malignancy very closely so what are the options that we have that can help differentiate it from cancer?

Normal routine labs, taking into account the presenting symptoms should be done.

- CBC with differential to look at the eosinophilic count.
- A liver function test:
  - Bilirubin (total and direct) may be elevated.
  - ALT and AST may or may not be increased.
○ ALP and GGT are usually increased like any other diseases involving the biliary tree.

○ Amylase and lipase to rule out a pancreatic cause.

○ Since eosinophilic cholangitis can mimic cholangiocarcinoma, tumors markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) can be ordered and surprisingly may be elevated making the diagnosis even more challenging.

Again, looking at the laboratory investigation, it is still hard to pinpoint the diagnosis, the next step would be to move on into imaging modalities.

3.5 Imaging modalities

There are many available imaging modalities that are helpful in visualizing and evaluating the biliary system. Noninvasive imaging modalities can demonstrate common nonspecific findings of EC such as bile duct wall thickening (segmental or diffuse) on US (see Figure 2) and contrast enhanced CT and MRCP with or

![Figure 2](image)

This contrast enhanced ultrasound (CEUS) shows thickened wall of intrahepatic bile ducts (from hilar to peripheral) with dilation, and the lesion was well enhanced.

![Figure 3](image)

(a) Computed tomography scan (CT scan) of abdomen and pelvis; (b) magnetic resonance cholangiopancreatography (MRCP); (c) endoscopic retrograde cholangiopancreatography showing a focal dilation of the biliary tree to the left lobe through the suggestion of subtle ill-defined enhancing mass lesion at the level of liver hilum.
without biliary dilation (see Figure 3). These findings can also be seen in malignant processes, hence the need to obtain a brush cytology and tissue biopsy by means of performing invasive imaging modalities such as ERCP.

While MRCP is useful in demonstrating an irregular narrowing of the bile duct, ERCP and percutaneous transhepatic cholangiography (PTC) provide additional information such as irregularities of the common bile duct and the intrahepatic ducts as well as the length and site of biliary stricture.

ERCP with brush biopsy, PET-CT (see Figure 4) and an endoscopic guided fine needle aspiration (EUS guided FNA) are also used to try to differentiate a benign from a malignant cause of biliary tree dilation. As you can see, the CT scan shows an ill-defined enhancing mass lesion at the level of liver hilum suggesting cholangiocarcinoma.

ERCP with brush biopsy may not show malignant cells.
EUS-guided FNA may show a background of mixed inflammation including many eosinophils.

Sometimes the diagnosis can be made, and targeted treatment can be started but most of the time, the index of suspicion for malignancy remains high.

3.6 Proposed diagnostic criteria

Matsumoto et al. revealed a characteristic feature of EC that helped rule out malignancy: staining of a parenchymal echo in the bile duct wall on
contrast-enhanced ultrasound (CEUS). However, they suggested the following requirements to accurately diagnose EC [6]:

1. Thickening of the biliary wall or narrowing of the biliary tree;

2. Eosinophilic infiltration on histopathology;

3. Regression of the stricture or resolution of other biliary abnormalities in the absence of treatment or subsequent steroid therapy.

3.7 Treatment

Even though EC is a self-limiting disease, it has a variable course, making precise treatment recommendations difficult. The challenge remains to exclude malignancy, which is not always possible with various imaging modalities and biopsies. Hence, mandatory surgical intervention is an effective and definitive measure of treating EC if there is diagnostic uncertainty.

According to the literature, two cases of EC described a stricture in the common hepatic duct that regressed spontaneously without any medical intervention within 3 weeks, but most of the published cases of EC were treated surgically and received a retrograde diagnosis (see Figure 5) [10].

Seow-En et al. suggested that the best option to simultaneously treat a stricture, exclude malignancy, and attain a definite diagnosis of EC is surgical intervention. They also described the advantages of surgery over medical therapy, indicating that medical treatment does not eradicate the chance of recurrence and that it could put patients at risk of complications of repeated steroid therapy [11].

![Figure 5](image)

(a) Severe degree of periductal onion skin fibrosis (hematoxylin and eosin stain displaying 2× magnification).
(b and c) The inflammatory infiltrates around the partially damaged bile duct are mostly eosinophilic cells (hematoxylin and eosin stain displaying 10× magnification). (d) The eosinophilic count exceeds 40 cells per HPF (hematoxylin and eosin stain displaying 40× magnification).
A diagnostic trial of oral corticosteroid can be tried to see if any resolution occurs, however the dose and duration of treatment are yet to be determined due to the poor understanding of the diseased natural course.

4. Conclusion

In conclusion, EC is an uncommon, benign, and self-limiting cause of biliary stricture. Although this disease has a good response to corticosteroid therapy, it often mimics cholangiocarcinoma which makes reaching a definite diagnosis by clinical and radiological findings difficult. Hence most cases are treated surgically and receive a retrograde diagnosis.

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Chapter 11

Eosinophilic Granulomatosis with Polyangiitis: The Beginning of a New Era

Carlos Melero Moreno, Marta Corral Blanco and Rocío Magdalena Díaz Campos

Abstract

Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare type of anti-neutrophil cytoplasm antibody-associated vasculitis (AAV) with unique features, such as involvement of eosinophils in the pathogenesis, which requires different therapies from those used for other AAV. Conventional treatment includes glucocorticoids (GC) and immunosuppressants. GC are the cornerstone of the initial treatment of EGPA, but relapses are frequent. Cyclophosphamide is typically used in combination with GC for patients with life- and/or organ-threatening disease manifestations. Azathioprine and methotrexate are recommended to maintain remission after induction with cyclophosphamide or as a GC-sparing agent. Nowadays, a better comprehension of the physiopathology of EGPA has opened new therapeutic targets, such as interleukin-5, which has a key role in the refractory disease, relapses, and GC dependence, especially for asthma manifestations. Mepolizumab is the first anti-IL5 antibody approved to treat EGPA. Another anti-IL5 monoclonal antibody, reslizumab, and an anti-IL5 receptor monoclonal antibody, benralizumab, are now being investigated for EGPA.

Keywords: eosinophilic granulomatosis with polyangiitis, Churg-Strauss syndrome, vasculitis, eosinophilia, anti-IL5 therapy, glucocorticoids, cyclophosphamide, mepolizumab

1. Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA), formerly Churg-Strauss syndrome, is a rare necrotizing vasculitis, with an annual incidence and prevalence of 0.9–2.4 per million [1–4] and 10.7–17.8 per million [5–8], respectively, depending on geographic regions and applied criteria. The disease is now recognized as one form of anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV) characterized by eosinophil-rich granulomatous inflammation and small to medium size vessel vasculitis associated with asthma and eosinophilia [9].

ANCA occur only in about 30–70% of patients with newly diagnosed untreated EGPA, and organ involvement can be different depending on the presence or not of ANCA [10], probably being different subgroups with specific characteristics. In this way, Matsumoto et al. [11] reported that AAV patients could be divided into three subgroups according to peripheral immune cell numbers: antibody production...
related, cytotoxic activity related, and neutrocytosis/lymphocytopenia. These
subgroups could have different prognosis and treatment.
EGPA has been excluded from most of randomized controlled trials for AAV
because of its rarity and unique features, such as involvement of eosinophils in the
pathogenesis. Reliable evidence of treatment for EGPA is limited, and there are no
strong recommendations for treatment of EGPA at the moment [12].
Treatment has been based on the use of glucocorticoids (GC) and immunosup-
pressants. Cyclophosphamide is typically used in combination with GC for patients
with poor prognostic factors (assessed by the Five Factor Score). Azathioprine
and methotrexate are recommended to maintain remission after induction with
cyclophosphamide or as a GC-sparing agent. All these medications have many and
deleterious adverse effects.
Fortunately, a better comprehension of the physiopathology of EGPA has opened
new therapeutic targets, such as interleukin-5, which has a key role in the disease.

2. Assessing vasculitis severity

The French Vasculitis Study Group conducted two randomized controlled clinical
trials to develop a score to assess the severity of vasculitis disease: the Five Factor
Score (FFS) [13].
The FFS is a prognostic tool used to quantify the extent of the disease and guide
therapy. It consists of five items. Age ≥65 years old, cardiac insufficiency, severe
gastrointestinal involvement, and renal insufficiency [stabilized peak creatinine
1.7 mg/dL (150 μmol/L)] are associated with poor prognoses, each scores +1 point,
while the fifth factor (ear, nose, and throat [ENT] manifestations) is associated
with better outcome and its absence is scored +1 point.

3. Conventional therapy for EGPA

3.1 Systemic glucocorticoids

Glucocorticoids are the cornerstone of the initial treatment of EGPA. They act
quickly against vasculitis and normalize the value of peripheral eosinophils within
few days of treatment.
A multicenter retrospective study, done by Cottin et al. [14] in 2016, showed that
treatment with systemic GC was associated to a decrease in peripheral eosinophilia
(with a mean cell count <1.0 × 10⁹ L⁻¹ over the long-term).
The initial management of EGPA includes high doses of GC, usually 1 mg/kg/day of
prednisone or its equivalent. Methylprednisolone pulses (75–15 mg/kg intravenously,
repeated at 24 h intervals, for 3 days) can be used in the presence of life-threatening
symptoms. When clinical response is obtained and inflammation reactants return to
normal values, usually within 3–4 weeks, GC can be tapered slowly to the minimal
effective dose or, when possible, until withdrawal. However, most patients need to
maintain GC to prevent relapses of systemic manifestations and control asthma. The
optimal minimal dose should be 7.5 mg/day to limit GC-induced side effects [15, 16].
In the French Vasculitis Study Group cohort [17], which included 383 EGPA
patients, approximately 85% required long-term prednisone (mean dose
12.9 ± 12.5 mg/day) to control asthma, rhinitis, and/or arthralgias.
GC as monotherapy may be suitable for most EGPA patients. In a study, which
included 72 EGPA patients without poor prognosis factors, 93% achieved remission
with systemic GC therapy alone [18]. However, additional immunosuppression can
be considered for patients with life- and/or organ-threatening disease, when the prednisone dose cannot be tapered to 7.5 mg/day after 3–4 months of therapy or for patients with recurrent disease [16, 19].

Samson et al. [20] assessed the outcomes of 118 EGPA patients (with or without FFS) enrolled in two prospective, randomized, open-label clinical trials from 1994 to 2005. Forty-four patients with poor prognosis (FFS ≥ 1) were assigned to receive 6 or 12 cyclophosphamide (CPh) pulses plus GC, and 74 without poor prognosis factors (FFS = 0) received GC alone (with immunosuppressant [IS] adjunction when GC failed). Follow-up was done from 2005 to 2011. Twenty-nine percent achieved long-term remission, while 41% had ≥1 relapse at 26 months after treatment onset. More than half of the relapses occurred when GC tapering reached <10 mg/day, especially in patients with anti-myeloperoxidase antibodies and baseline eosinophilia <3000/mm³.

### 3.2 Immunosuppressants

#### 3.2.1 Cyclophosphamide

Cyclophosphamide is typically used in combination with GC for patients with life- and/or organ-threatening disease manifestations (i.e., heart, gastrointestinal involvement, central nervous system, severe peripheral neuropathy, severe ocular disease, alveolar hemorrhage, and/or glomerulonephritis) [14].

In a retrospective study of 595 patients with severe necrotizing vasculitides (including EGPA), treatment had no significant impact on early death, except for patients with FFS ≥ 2 for whom GC monotherapy showed association (p < 0.05). The principal cause of early death was uncontrolled vasculitis (58%), followed by infection (26%) [21]. A study of 278 patients with polyarteritis nodosa, microscopic polyangiitis, and EGPA showed that survival was significantly higher in patients with FFS > 2 treated with GC and CPh rather than GC alone [22].

Cyclophosphamide can be administered as continuous oral therapy or intravenous (IV) pulses. The doses should be adjusted according to age and renal function. Cyclophosphamide pulses are usually preferred to oral administration because of the lower cumulative dosage. The frequency of relapses, however, can be higher with pulses, and it has been shown that oral CPh can be effective when pulses have failed [15]. Sodium 2-mercaptopethanesulfonate is recommended to reduce bladder toxicity.

Regarding the duration of CPh therapy, Cohen et al. [23] conducted a prospective, multicenter trial to compare first-line treatment with systemic GC and 6 or 12 pulses of adjunctive CPh for the treatment of severe EGPA. Forty-eight patients were included, 42 (87.5%) achieved complete remission, 21 (91.3%) in the 6-pulse regimen, and 21 (84%) in the 12-pulse regimen. Severe side effects were similar in both groups. However, a too-short duration of CPh administration was associated with more relapses, so the authors concluded that a 12-pulse regimen was better to control severe EGPA than a 6-pulse regimen. Other less toxic IS, as azathioprine (AZA) or methotrexate (MTX), were not tested for maintenance so further data is needed to clarify the optimal duration of therapy.

#### 3.2.2 Azathioprine and methotrexate

AZA and MTX are recommended to maintain remission after induction with CPh or as a GC-sparing agent in patients requiring long-term therapy with prednisone at doses >10 mg/day. Maintenance therapy with an IS usually begins 2–3 weeks after the last CPh pulse, or a few days after oral CPh, and continues for 12–18 months [16].

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Pagnoux et al. [24] conducted a prospective, open-label, multicenter trial to evaluate the safety and efficacy of AZA and MTX in ANCA-associated vasculitis. One hundred twenty-six patients in remission with IV CPh and GC were randomly assigned to receive AZA (at a dose of 2.0 mg/kg/day) or MTX (at a dose of 0.3 mg/kg/week, progressively increased to 25 mg per week) as maintenance therapy for 12 months. Adverse events occurred in 29 AZA recipients and 35 MTX recipients. There was one death in the MTX group. Twenty-three patients who received AZA and twenty-one patients who received MTX had a relapse. The results suggested that none of the drugs were more effective at maintaining remission, but severe adverse events were more frequent in the MTX group.

4. New therapeutic strategies

4.1 Role of IL-5 in EGPA

EGPA is characterized by elevated peripheral eosinophilia with different degrees of activation. Eosinophilia is secondary to more synthesis, enhanced extravasation, and its prolonged survival in target tissues. Histological features of EGPA are small-vessel angiitis and extravascular necrotizing granulomas, usually containing eosinophilic infiltrate [25, 26].

An initial Th2-mediated immune response provokes the migration of eosinophils to tissues. IL-5, produced by TH-2 lymphocytes, plays an active role in chemotaxis, activation, degranulation, and survival of eosinophils [26]. IL-5 is not the only mediator of eosinophilic tissue infiltration; IL-4 and IL-13 are two other cytokines of Th2-mediated immune response that play an important role in tissue infiltration and degranulation of eosinophils [27, 28]. Epithelial and endothelial cells, when activated by Th2 cytokines, secrete eosinophil-specific chemokines like eotaxin 3 (CCL26), CCL17, and CCL22 that facilitate recruitment of eosinophils and effector Th2 cells in target organs, amplifying the immune response [29, 30].

A better comprehension of the physiopathology of EGPA highlights the role of eosinophils and IL-5. It has been observed that serum level of IL-5 correlates with disease activity and that it decreases with the initiation of immunosuppressive therapy [30–32].

4.2 Anti-IL-5 antibodies in EGPA

Interleukin-5 is well known as a key mediator in eosinophil activation. Thus, the efficacy of mepolizumab, a humanized monoclonal antibody against interleukin-5, was evaluated in EGPA patients.

Kahn et al. [33] published the first case of refractory EGPA treated with mepolizumab. The patient had many relapses despite treatment with GC, MTX, interferon alpha, CPh, IV immunoglobulin, AZA, and etoposide. Mepolizumab 750 mg IV monthly was started. After the first dose, asthma significantly improved, the eosinophil count decreased (reaching normal values), and the chest computed tomography (CT) showed complete regression of parenchymal findings. After 6 months of treatment, asthma symptoms disappeared, and chest CT did not show infiltrates, so an attempt to increase the intervals between mepolizumab infusions to 2 months was done which resulted in relapse with reappearance of asthma symptoms, interstitial lung infiltrates, and increase of peripheral eosinophilia. All parameters normalized with transient increase of prednisone and reintroduction of mepolizumab monthly infusions.
A single-center, phase 2, uncontrolled, investigator-initiated trial [34] included 10 consecutive patients with refractory or relapsing EGPA. Relapse was defined by a Birmingham Vasculitis Activity Score (BVAS) >3 despite treatment with IS plus GC at a dosage of 12.5 mg/day or higher. After stopping previous IS, the patients received nine infusions of mepolizumab, 750 mg monthly. Then it was switched to MTX maintenance therapy and a tapered dosage of GC as tolerated. Eight patients reached remission (BVAS = 0 and GC dosage <7.5 mg/day) after two or three mepolizumab infusions. One patient had a partial response (BVAS = 0 but did not achieve a GC dosage <7.5 mg/day), and another patient reached remission but was excluded owing to nonadherence. During mepolizumab treatment, no relapse occurred, the daily GC dose was reduced in all patients, and eosinophil count decreased after the first infusion. After switching mepolizumab to MTX, seven relapses occurred, over a median follow-up of 10 months. The same authors, in a later work [35], followed up these nine patients during a median of 22 months. Only three of them were still in remission at the end of the study. So, a high relapse rate after stopping mepolizumab was observed, which suggests that patients with EGPA could need a continuous treatment with mepolizumab.

An open-label pilot study [36] treated seven patients with four monthly doses of mepolizumab 750 mg IV to assess its steroid-sparing effect in GC-dependent EGPA patients. Mepolizumab allowed for safe GC reduction in all patients. The GC mean dose at baseline was 12.9 mg/day and after 12 weeks of therapy was 4.6 mg/day (64% reduction). On cessation of mepolizumab, EGPA manifestations recurred, needing steroid bursts. Mepolizumab was well tolerated, and the most frequent adverse events were headache, pruritus, and loose stools.

In 2017, a multicenter, double-blind, parallel-group, phase three trial was published [37]. It included 136 patients with relapsing or refractory EGPA, who had received treatment for at least 4 weeks and were taking a stable prednisolone or prednisone dose. They were randomized to receive 300 mg of mepolizumab (n = 68) or placebo (n = 68), administered subcutaneously (SC) every 4 weeks, plus standard care, for 52 weeks. Mepolizumab treatment led to significantly more accrued weeks of remission (defined as BVAS = 0 and prednisolone or prednisone ≤4 mg/day) than placebo (28 vs. 3% of the participants had ≥24 weeks of accrued remission) and a higher percentage of participants in remission at both weeks 36 and 48 (32 vs. 3%). Remission did not occur in 47% of the participants in the mepolizumab group vs. 81% of those in the placebo group. A total of 44% of the participants in the mepolizumab group, as compared with 7% of those in the placebo group, had an average daily dose of prednisolone or prednisone of ≤4 mg/day during weeks 48 through 52. Eighteen percent of the patients receiving mepolizumab were able to discontinue prednisolone or prednisone completely, as compared with 3% receiving placebo. Also, time to first relapse was longer, and annualized relapse rate was lower in the participants in the mepolizumab group. The most commonly adverse events with mepolizumab were headache, nasopharyngitis, arthralgia, sinusitis, and upper respiratory tract infection. A post hoc analysis [38] of the results according to peripheral eosinophilia (<150 cells/ul), GC dosage (>20 mg/day), and weight (>85 kg) was done. It showed that those patients treated with mepolizumab, with peripheral eosinophilia <150 cell/ul and weight >85 kg, had greater clinical benefit (BVAS = 0 and GC dosage ≤4 mg/day) than placebo. Although no significant differences were found in patients treated with GC dosage >20 mg/day, results favored mepolizumab treatment, but it must be considered that the study include few cases (n = 21).

Recently, Shiroshita et al. [39] published a case report of a 61-year-old man with refractory EGPA despite treatment with GC, CPh, and plasmapheresis who developed a diffuse alveolar hemorrhage. Rituximab and methylprednisolone...
pulses were administered, and remission was obtained. Then mepolizumab 100 mg SC monthly was started that kept remission until now. To our knowledge, this is the first published paper where the authors used, in an EGPA patient, the same dosage and way of administration of mepolizumab used in severe asthma.

The Food and Drug Administration (FDA) approved the use of mepolizumab in adult patients with EGPA in the United States (USA), in December 2017, based on Wechsler [5] results, being the first biological treatment approved with this indication in data sheet [40–42]. The dosage of 300 mg, three times the recommended dose in severe eosinophilic asthma, is based on observations done in asthma, but no specific dose evaluation has been done in EGPA [42].

Another anti-IL5 monoclonal antibody, reslizumab, and an anti-IL5 receptor monoclonal antibody, benralizumab, are now being investigated for EGPA (NCT02947945 and NCT03010436, respectively) [43, 44].

5. Conclusions

The pathogenesis and role of ANCA in EGPA are mostly unknown, although it has been reported that patients with positive ANCA usually present renal involvement (glomerulonephritis), while those with negative ANCA usually have cardiac involvement (heart failure), possibly corresponding to two different subgroups with different characteristics still to be determined which will provide information and facilitate specific treatments. GC and IS are effective in EGPA, but relapses are frequent, and there is no standard therapy based on the results of randomized clinical trials. However, there is new data that shows mepolizumab as a good treatment option due to its clinical benefit, and its use in EGPA has recently been approved in the United States.

Advances in the knowledge of EGPA pathophysiology together with the appearance of new drugs, such as mepolizumab, seems to be a solution to the unmet needs in this disease.

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Chapter 12

Assessment of Immune Reconstitution Following Hematopoietic Stem Cell Transplantation

Meenakshi Singh, Selma Z. D’Silva and Abhishweta Saxena

Abstract

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potential curative treatment for both congenital and hematological malignancies. Immune reconstitution after allogeneic hematopoietic stem cell transplantation is implicated in successful transplant outcomes such as overall survival and relapse-free survival. The reconstitution of immune cell subsets after HSCT occurs in different phases at different time points encompassing pre-engraftment, engraftment, and post-engraftment. The recovery of innate cellular immunity with the appearance of monocytes, dendritic cells, and natural killer cells in peripheral blood correlates with initiation of cellular engraftment. The cellular adaptive immunity is characterized by both thymic-independent expansion of T cells infused with graft and thymus-dependent expansion of naïve T cells derived from donor stem cells. The humoral immunity consists of B-cell reconstitution, which consists primarily of transitional and naïve subsets with the recovery of memory B cells that occur much later. In this review, we highlight the factors affecting immune reconstitution, the reconstitution of innate and adaptive immunity, techniques to assess immune reconstitution, and ways to enhance it.

Keywords: immune reconstitution, hematopoietic stem cell transplantation, innate immunity, adaptive immunity

1. Introduction

Hematopoietic stem cell transplantation (HSCT) is a choice of treatment for thousands of leukemic patients. The main outcome expected from HSCT is the lifetime engraftment of the donor graft. The preferred donor is a HLA matched-related donor; however, this is available in about 25% of the patients. Other options such as matched unrelated, matched cord blood units, and haploidentical-related donor also do exist. The success of HSCT is marred by conditions such as graft-versus-host disease (GvHD), relapse, treatment-related toxicity, and infection, which lead to higher morbidity and mortality [1]. The effectiveness of HSCT is dependent on the immune reconstitution in the host, which is linked to the number of active T and NK cells present in the graft. Delayed immune reconstitution results in unfavorable transplant outcomes; hence, faster immune reconstitution of donor origin is required for long-term survival of patients.
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1. Introduction

Hematopoietic stem cell transplantation (HSCT) is a choice of treatment for thousands of leukemic patients. The main outcome expected from HSCT is the lifetime engraftment of the donor graft. The preferred donor is a HLA matched-related donor; however, this is available in about 25% of the patients. Other options such as matched unrelated, matched cord blood units, and haploidentical-related donor also do exist. The success of HSCT is marred by conditions such as graft-versus-host disease (GvHD), relapse, treatment-related toxicity, and infection, which lead to higher morbidity and mortality [1]. The effectiveness of HSCT is dependent on the immune reconstitution in the host, which is linked to the number of active T and NK cells present in the graft. Delayed immune reconstitution results in unfavorable transplant outcomes; hence, faster immune reconstitution of donor origin is required for long-term survival of patients.
Soon after HSCT using myeloablative conditioning, the patient experiences a period of pancytopenia. It takes several months or years for immune reconstitution and for patients to regain immunocompetence after transplant. The immune cells start re-appearing in the following order: neutrophils (0.5 months), monocytes (1 month), NK cells (1 month), T cells (2 months), and B cells (3 months); however, the normal levels are reached much later (Figure 1) [2].

There are various factors affecting immune reconstitution after transplant such as

1. thymic damage (age-related or pre-transplant conditioning regimens)
2. source of stem cells
3. HLA disparity between donor and host
4. post-transplant immune suppressant
5. occurrence of graft-versus-host disease.

Age or pre transplant chemotherapy or radiation leads to thymic damage. The severity of the damage caused to the thymus depends on the dose of the drugs used and also on the age of the patients, which in turn affect the immune recovery. In younger patients (<18 years), there is faster thymic regeneration after chemotherapy than older patients [3]. The age of the donor also affects the engraftment and reconstitution potential of hematopoietic stem cells as shown in mouse models [4]. Moreover, the thymic recovery is faster and is associated with faster T-cell reconstitution and recovery of normal T-cell repertoire in autologous (9 months) than allo-HSCT (12 months) [5]. This delayed thymic-dependent immune reconstitution is further reduced by the occurrence of aGvHD after allogeneic HSCT [6, 7].

The source of stem cells used as graft could be either bone marrow, peripheral blood, or cord blood. Source of stem cells used predicts the rate of immune reconstitution. It has been observed that platelet (20 × 109/L) reconstitution is faster in peripheral blood (11–18 days) than bone marrow (17–25 days) HSCT. Similarly, neutrophil (>0.5 × 109/L) reconstitution is also faster in peripheral blood (12–19 days) than bone marrow (15–23 days) HSCT. This is because of the presence
of long-term HSCs and more committed multipotent progenitors in the peripheral blood than bone marrow [8]. Further as compared to transplantation using in vivo or ex vivo T-cell depleted graft, faster immune reconstitution is seen in unmodified graft transplantation [9]. Using peripheral blood graft, faster reconstitution of CMV-specific cytotoxic T cells and CD4+ T cells is observed than stem cells from bone marrow source [10, 11]. The advantages of using umbilical cord blood units are its ready availability and its ability to cross the HLA barrier. The rates of engraftment and post-transplant outcomes are dependent on the number of total nucleated cells (TNCs) and CD34+ cell dose present in the graft source. Martin et al. [12] previously reported high TNC dose in association with positive transplant outcomes such as improved overall survival (OS), lower relapse rate (RR), and increased risk of chronic GvHD. Since there is a higher number of TNCs in the bone marrow and peripheral blood, there is faster engraftment (~14–21 days) after HSCT using this source of graft than umbilical cord blood source (~30 days) [13, 14]. Remberger et al. [15] reported faster engraftment but poor survival and higher relapse after HSCT using high CD34+ cell dose peripheral blood as graft source. Various researchers have reported immune cell reconstitution using different cell sources (Table 1).

Graft manipulations such as T-cell depletion (TCD) have resulted in lower chances of GvHD and graft rejection in unrelated and HLA mismatched transplants. However, T-cell depletion results in delayed immune reconstitution and increased morbidity and mortality due to infection [19–21]. An advantage of using T-cell depletion is that in case of malignancies it also leads to better GVL effect depending on the malignant disease being treated. For example, in CML, TCD is related to increased relapse rate [22], whereas in AML and AML cohorts, lower rate of relapse has been observed in TCD transplantation [23–25].

The degree of HLA mismatch is an important factor in immune reconstitution. It has been observed that the outcomes from matched unrelated transplantation are at par with that of matched related transplantation [1]. Chang et al. reported similar reconstitution of T-cell subsets, except for CD4+ cells and CD4+ naïve T cells, in haploidentical and HLA-matched transplantation [16]. Various researchers have reported reconstitution of immune cells following different transplant strategies. It has been observed that the immune reconstitution is best in matched sibling related followed by matched unrelated donor, haploidentical donor, T-cell replete, and T-cell depleted transplants.

Conditioning regimens deplete host immune system, eliminate the leukemic cells, and create space for engraftment of the donor cells. Although this eliminates the patient’s leukemic cells, it also reduces the alloreactivity between host and donor cells after HSCT and further results in severe depletion of all immune cells. The use of drugs such as ATG or alemtuzumab depletes the host T cells further and results in

<table>
<thead>
<tr>
<th>Cells/L type of transplant</th>
<th>NK cells</th>
<th>CD4+ T cells</th>
<th>CD8+ T cells</th>
<th>B cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matched sibling donor</td>
<td>—</td>
<td>220</td>
<td>645</td>
<td>33</td>
<td>[16]</td>
</tr>
<tr>
<td>Matched unrelated donor</td>
<td>253</td>
<td>198</td>
<td>447</td>
<td>43</td>
<td>[17]</td>
</tr>
<tr>
<td>Haploidentical donor</td>
<td>—</td>
<td>152</td>
<td>672</td>
<td>23</td>
<td>[16]</td>
</tr>
<tr>
<td>T-cell depleted</td>
<td>357</td>
<td>7</td>
<td>7</td>
<td>55</td>
<td>[18]</td>
</tr>
<tr>
<td>T-cell replete</td>
<td>183</td>
<td>127</td>
<td>181</td>
<td>64</td>
<td>[18]</td>
</tr>
</tbody>
</table>

Table 1. Reconstitution of various immune subsets in different types of HSCT.
a delayed recovery of donor-derived T cells. Increase in the severity of the conditioning regimen results in prolonged immune deficiency after transplant [26].

Both thymus-dependent and thymus-independent T-cell reconstitutions are affected by the increase in HLA mismatch between the patient and the donor, probably because of higher risk of GvHD [27]. Clave et al. [28] reported higher reconstitution of both CD4+ and CD8+ T cells in transplants involving unrelated cord blood grafts (190 cells × 10^3/μL for CD4+ and 280 cells × 10^3/μL for CD8+) than CD34 selected peripheral blood haploidentical donor grafts (68 cells × 103/μL for CD4+ and 80 cells × 103/μL for CD8+). Mehta et al. [29] showed lower reconstitution of absolute CD4+ and CD8+ T cells at 3 months and higher B-cell counts (6 months) after unrelated cord blood HSCT than HLA matched HSCT (121.53 vs. 261.18 for CD4+, 36.03 vs. 190.56 for CD8+, and 210 vs. 31.2 for B cells). There was similar reconstitution of B cells but lower CD4+ and CD8+ T-cell reconstitution in single unit umbilical cord blood transplantation than HLA mismatched donor HSCT (11 vs. 9 for B cells, 15 vs. 21 for CD4+ cells, and 14 vs. 21 for CD8+ cells) [30].

Acute graft-versus-host disease occurs when donor lymphocytes react against normal host tissue to cause serious complications after allogeneic HSCT. Although there is faster recovery of the innate immune system after allo-HSCT, lymphocyte recovery is delayed due to aGvHD [3, 31]. The recovery of T cells depends on the thymic efficiency as well as the peripheral niche, which provides resources for T-cell survival. As GvHD targets the bone marrow, in patients with graft-versus-host disease, the peripheral resources are reduced because of which there is increased immunosuppression leading to delayed T-cell reconstitution in allogeneic HSCT as compared to autologous HSCT. The options to increase the efficiency of T-cell reconstitution must be selected in a manner so as to not aggravate the already present GvHD [32, 33]. Similarly, the drugs used to treat GvHD can also result in delayed immune reconstitution. Drugs such as cyclosporine A and methotrexate interfere with the T-cell receptor signaling and hence result in alteration of peripheral T-cell survival and B-cell differentiation [34, 35]. Tyrosine kinase inhibitors like imatinib mesylate used for controlling refractory cGvHD also lower T-cell survival by interfering with T-cell receptor (TCR) or IL7 signaling [36, 37]. Reconstitution of dendritic cells is decreased in GvHD [38]. Conversely, it has been suggested that depletion or inactivation of the host dendritic cells before allogeneic HSCT reduces the occurrence of GvHD [39–41].

2. Reconstitution of innate immunity

After HSCT, the first cells to engraft are the monocytes, followed by granulocytes, platelets, and NK cells [42]. Monocytes are primarily involved in phagocytosis and release of cytokines. They are classified into classical (CD14++CD16−), intermediate (CD14++CD16+), and nonclassical (CD14+CD16++) based on the expression of CD14 and CD16 [43, 44]. Monocytes remain below the normal levels for up to a year [45, 46].

The conditioning regimen used prior transplant results in a neutropenic phase till the neutrophils reconstitute, which takes approximately 11–12 days in T-cell depleted haploidentical HSCT [47, 48]. Although neutrophil counts rise to normal numbers within 2 weeks after transplant [49], they become functionally competent only after 2 months [50, 51]. The type of graft affects the reconstitution of neutrophils: 2 weeks in case of GCSF mobilized grafts, 3 weeks in case of bone marrow, and around 4 weeks in umbilical cord blood [1]. Use of peripheral blood has decreased the neutrophil recovery time from an average of 16 to 12 days [52].
NK cells recover in both number and function within the first few weeks after transplant [53], and functional reconstitution of NK cells is reached within 2 months [1]. The time taken for NK-cell reconstitution is dependent on the occurrence of GvHD [47, 54] and does not differ if the source of stem cells is peripheral blood or bone marrow [55]. However, the number of functional NK cells is higher when the transplant involves T-cell replete grafts than T-cell depleted grafts [56]. The most prominent functional NK cells after transplant are CD56brightCD16dim [57, 58]. Also, higher overall survival is seen in patients with high CD56bright NK cells at day 14 after unmanipulated haploidentical HSCT. The cytolytic function of NK cells is regulated by the interaction of inhibitory/activating killer immunoglobulin like receptors (KIRs) present on their surface and their specific HLA class I ligands. The reconstitution of the inhibitory and activating KIRs is dependent on factors such as conditioning regimen, T-cell deplete/replete graft, and immunosuppression used after transplant.

In a study evaluating NK-cell reconstitution after matched related/unrelated donor HSCT, it has been reported that the NK-cell counts are lower for longer period (2–3 months) after MUD (156/μL) than MRD (265/μL). The most frequent immature NK cells were CD56bright and NKG2A+CD57-CD56dim NK cells [59]. Russo et al. [60] reported that in haploidentical HSCT using after transplant cyclophosphamide, the immature NK cell starts appearing at 2 weeks; however, the mature NK cells expressing CD16 and CD56 and NKG2A appear at about a year.

Host dendritic cells that escape chemotherapy/radiation activate alloantigenic T cells in the donor and hence play an important role in GvHD. Since host dendritic cells present MHC antigens to donor CD8+ T cells after transplant, depleting these cells could result in lower risk of GvHD [61, 62]. Lower reconstitution of lymphoid dendritic cells has been associated with inferior overall survival [63].

Gamma delta T cells make up ~5% of the T-cell population, and their receptors are composed to gamma and delta chains. These T cells have been reported to enhance engraftment and graft-versus-leukemia effect without an increase in GvHD [64]. Gamma delta T cells reconstitute faster in patients in whom bone marrow (60 days) is used as the graft source than peripheral blood (200 days) [65].

3. Reconstitution of adaptive immunity

T-cell reconstitution is faster in transplantation with peripheral blood as graft source than bone marrow due to higher number of T cells present in the graft [55]. Ciurea et al. [18] reported better T-cell reconstitution in recipients of T-cell replete haploidentical HSCT than recipients of T-cell depleted haploidentical HSCT at 6 months after transplant. Use of ATG for T-cell depletion also affects the rate of immune reconstitution. This effect is more prominent in umbilical cord blood transplantation than bone marrow transplantation. T-cell reconstitution in allo-HSCT without the use of ATG is seen in about 7–12 months when using bone marrow and umbilical cord as stem cell source as compared to 6–24 months when using peripheral blood as stem cell source [66]. T cells recover primarily via peripheral expansion of memory T cells or endogenous T-cell development. Hence, functional thymus is required for effective reconstitution of T cells [67]. This is an issue in aging patients where there is thymus atrophy [68]. Due to this, although full immune recovery is possible in middle-aged patients, it is not possible in older patients and is a cause of morbidity and mortality [69]. Reconstitution of T cells is slow probably due to the prolonged depletion and reduced function of naïve T cells [70]. T cells that reconstitute are primarily from the donor origin in case of T-cell replete transplant or host T cells that have escaped the conditioning
regimen in case of T-cell depleted transplant. Naïve T cells/T-cell receptor excision circles (TRECs) are lower for approximately 10–30 years after transplant [71, 72]. Reconstitution of functional T cells as observed by their ability to secrete interferon gamma and interleukin-4 to normal levels returns in 30 days after haploidentical HSCT for patients in whom acute GvHD is not observed [73]. Recipients of T-cell depleted haploidentical HSCT show higher CD31+ naïve CD4+ T cells than their donors at approximately 4–6 years [74]. Homeostatic peripheral expansion is induced by various homeostatic cytokines such as IL7 and IL15, inflammatory cytokines, and viral exposure. Peripheral homeostatic expansion leads to an inverse CD4/CD8 ratio in patients for several months after transplant. CD4 counts are considered as the best predictive marker for the recovery of immune competence after HSCT, and its recovery has also been associated with lower risk of infections and improved transplant outcomes [1]. CD4+ T-cell counts are as low as <200 cells/μL in the first 3 months and reach levels of 450 cells/μL at about 5 years after transplant [55, 75]. CD8+ T-cell counts increase rapidly during the first 3 months after transplant possibly due to the expansion of herpesvirus-specific CD8 T cells [55, 76]. GvHD reduces the number of CD4+ T cells by inhibiting the thymic output, whereas CD8+ cells increase in number during GvHD or CMV reactivation [77, 78]. The reconstituting CD4+ T cells have a higher expression of CD11a, CD29, CD45RO, and HLA-DR and a lower expression of CD28, CD45RA, and CD62L than normal individuals [79, 80]. The early reconstituting CD8+ T cells are mostly memory or effector cells. Naïve or TREC+CD8+ T cells recover at a slower rate [77, 81]. The number of regulatory T cells (Tregs) is much higher after transplant than normal individuals and may contribute to remission [82, 83]. A Treg:CD4+ T cell ratio of less than 9% has been associated with higher risk of aGvHD [84]. Chang et al. [16] reported lower CD4+ T cells, dendritic cells, and higher CD28 expression on CD4+ and CD8+ T cells in patients receiving haploidentical HSCT than patients receiving HLA matched HSCT.

B-cell reconstitution is also delayed after HSCT: ~6 months for autologous and ~9 months after allogeneic transplantation and is mainly due to GvHD or its treatment. In the first 2 months after transplant, B-cell counts are low but rise higher than the normal levels in approximately 1–2 years [55, 85]. Since restoration of full humoral immune functioning requires both naïve and memory B cells, all patients who have undergone HSCT remain susceptible to infections for at least a year after transplant [1]. The reconstituted B cells express higher levels of CD1c, CD38, CD5, membrane IgM, and membrane IgD and lower levels of CD25 and CD26L than normal individuals [86].

A number of studies have reported comparisons between reconstitution of different immune cells depending on the graft source. Faster reconstitution of

<table>
<thead>
<tr>
<th>Cell type and numbers</th>
<th>Bone marrow</th>
<th>Peripheral blood</th>
<th>Unrelated cord blood</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils (&gt;0.5 × 10⁹/L)</td>
<td>16 days</td>
<td>15 days</td>
<td>19 days</td>
<td>[87]</td>
</tr>
<tr>
<td>Natural killer cells (&gt;0.1 × 10⁹/L)</td>
<td>1.5 months</td>
<td>4 months</td>
<td>4 months</td>
<td>[16, 87]</td>
</tr>
<tr>
<td>T cells (&gt;0.5 × 10⁹/L) CD4</td>
<td>2–3 months</td>
<td>6 months</td>
<td>3 months</td>
<td>[28, 88]</td>
</tr>
<tr>
<td>Naïve T cells (&gt;0.5 × 10⁹/L)</td>
<td>9 months</td>
<td>24 months</td>
<td>12 months</td>
<td>[87, 89]</td>
</tr>
<tr>
<td>Cytotoxic T cells (&gt;0.25 × 10⁹/L)</td>
<td>3 months</td>
<td>9 months</td>
<td>8 months</td>
<td>[65, 90]</td>
</tr>
<tr>
<td>T helper cells (&gt;0.2 × 10⁹/L)</td>
<td>4 months</td>
<td>10 months</td>
<td>1 months</td>
<td>[65, 90]</td>
</tr>
</tbody>
</table>

Table 2. Reconstitution of different immune cells depending on the graft source.
different immune cells was observed when bone marrow was used as graft source as compared to peripheral blood or cord blood (Table 2).

4. Assessment of post-transplant immune recovery

There are different methods to assess the immune recovery after transplant, such as estimation of absolute lymphocyte count (ALC), levels of immune cell subsets (NK cells, B cells, and T cells), and antibody titers to assays for T- and B-cell repertoires [91].

ALC levels have been reported in association with overall survival and rate of relapse. ALC >500 cells/μL on day 15 is linked with better OS and lower relapse after autologous as well as allogeneic transplantation [92, 93]. An increase in the levels of CD16+ monocytes has been associated with aGVHD [94].

Early recovery of CD4+ T cells is associated with overall survival, nonrelapse mortality, and risk of infections [95, 96]. Admiral et al. [97] reported the time taken by circulating CD4+ T cells to reach 0.5× 10⁹/L as a strong marker for probability of relapse. In myeloablatative allogeneic HSCT, higher levels of CD3+, CD8+ T cells, regulatory T cells, and myeloid dendritic cells are correlated with relapse-free survival [98].

Recently, flow cytometric analysis has been used to differentiate between the T, B, and NK-cell subpopulations. Low levels of NK cells within the first few weeks after transplant have been associated with poor transplant outcomes like lower overall survival and higher risk of infection [99, 100]. Surface markers such as CD45RA, CD28, CD27, CD62L, and CCR7 can be used to differentiate naïve, effector, effector memory, and central memory CD4+ and CD8+ subsets [101, 102]. The surface markers expressed by naïve T cells are CD45RA+,CCR7+; central memory T cells are CD45RA-CCR7+; effector memory T cells are CD45RA-CCR7--; and effector T cells are CD45RA+CCR7+ [91]. CD4+ T cells also include regulatory T cells (CD25+FoxP3+) and Th17 cells [103, 104]. The expression of CD27, IgM, and IgD helps in distinguishing between naïve B cells (CD27−IgD+), memory B cells (CD27+IgD+), and isotype switched memory B cells (CD27+IgD−) [105]. Myeloid and plasmacytoid dendritic cells can be distinguished based on the expression of CD123 and CD11c: CD123low CD11c+(myeloid) and CD123bright CD11c− (plasmacytoid) [106].

TRECs have been suggested as a marker for reconstitution of naïve T cells (CD4+CD45RA+) derived from the thymus. TRECs, however, remain low up to 6 months after HSCT [107]. Due to thymic atrophy with age, older patients have T cells with low TCR repertoire, which leads to higher risk of infections leading to lower transplant outcomes [108, 109]. Thymopoiesis can also be evaluated by measuring the number of TRECs by real-time quantitative in purified CD4+ and CD8+ T cells [110]. Lewin et al. [111] reported faster recovery of TRECs in younger patients and patients who received conventional grafts as compared to T-cell depleted grafts. Lower levels of TRECs are associated with GvHD and opportunistic infections [77, 112].

Certain cytokines can also be used as predictive markers for transplant outcomes. One such marker is IL7, which can be used to evaluate successful T-cell recovery. Increased IL7 is associated with delayed reconstitution and increased mortality and aGVHD [113]. High levels of IL6, GCSF, and IL2α have also been indicated in association with risk of aGVHD [96, 114]. For assessing chronic GvHD, high levels of IL8 and low levels of IL17A have been suggested [103, 115]. Min et al. [104] have also correlated high levels of IL6 and IL10 with poor transplant-related outcomes.
Further, T- and B-cell receptor repertoire gene arrangements can be evaluated by molecular techniques such as next generation sequencing [116, 117]. Michalek et al. [118] have demonstrated β chain sequencing of the T-cell receptor in order to identify the T-cell clones that could mediate either graft-versus-host disease or graft-versus-leukemia effect. Brink et al. [9] reported higher diversity in CD4+ T cells than CD8+ T cells following allogeneic HSCT. Greater diversity was observed in cord blood grafts, followed by unmanipulated grafts and T-cell depleted grafts.

5. Strategies to improve immune reconstitution

Many strategies, such as administration of recombinant cytokines, adoptive cell therapy, and hormone-based therapies, have recently been used to improve immune reconstitution after transplantation.

IL7 cytokine has been shown to effectively enhance reconstitution of T and B lymphoid cells by enabling thymopoiesis [105, 119]. It has been demonstrated that IL7 increased the CD3+, CD4+, and CD8+ T-cell levels to more than four folds and also leads to increase in functional and diverse T cells [120]. Administering IL-7 predominantly increases the naïve CD8+ T cells. The timing of administering is, however, important, as administering early after transplant aggravates GvHD [116, 121], whereas administering it at a later stage after HSCT results in lower risk of GvHD. This is contributed by the activation of alloreactive T cells that express lower IL-7Rα levels [32, 38]. Other cytokines that enable immune reconstitution are insulin-like growth factor 1 (IGF-1), IL22, IL15, and IL12 [122–124]. IL15 has been shown to significantly increase the reconstitution of CD8+ T cells and NK cells and improve the GvL effect in haploidentical murine models [125]. Sauter et al. [126] reported better lymphocyte reconstitution after IL-15 administration in T-cell depleted allogeneic HSCT; however, it has been shown to worsen GvHD.

Recently, it has been suggested that modulating the function of dendritic cells could reduce GvHD while maximizing GvL [127]. Studies on reconstitution of dendritic cells after HSCT have been contradictory. Maraskovsky et al. [128] have shown that treatment with Flt3-L can expand DC subsets; however, when administered after HSCT, it can worsen GvHD [38]. Gauthier et al. [38] have demonstrated that SDF-1α therapy can expand the DC1 subsets and lower the severity of GvHD. Because of their immunosuppressive properties, mesenchymal stem cells have recently been used for suppressing GvHD [129–131]. Mesenchymal stem cells release cytokines such as IL-7, which improve T-cell survival and promote reconstitution of dendritic cells by secreting SDF-1α [132].

NK-cell immunotherapy is one of the novel strategies underway to reduce GvHD and enhance graft-versus-leukemia effect in a KIR-HLA mismatched haploidentical HSCT [133–135].

6. Future directions

Recently, few studies have identified the association of reconstitution of certain immune subsets with predicting post-HSCT outcomes. However, these studies are often limited by small sample size, lack of detailed immune reconstitution, and secretome profile, which could be used as biomarkers to predict immune reconstitution. Prospective studies involving a large number of patients should be conducted to determine which immune factors and tests to detect the same could have prognostic value and understand the impact of such predictive risk factors on transplant outcomes. This is most beneficial, especially for recipients of
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Conflict of interest

The authors declare no conflict of interest.

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Section 6

Treatment of Leukopenia after Ionizing Irradiation
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Chapter 13
Sorption Detoxification as an Addition to Conventional Therapy of Acute Radiation Sickness and Iatrogenic Leukopenia

Oksana O. Shevchuk, Elisaveta A. Snezhkova, Anatoliy G. Bilous, Veronika V. Sarnatskaya, Kvitoslava I. Badakhivska, Larysa A. Sakhno, Vasyl F. Chekhun and Volodymyr G. Nikolaev

Abstract
Leukopenia is an essential part of the clinical course of acute radiation sickness and is a side effect of anti-cancer treatment. In both situations, the main factors which determine the survival are the degree of bone marrow suppression and gastrointestinal tract damage due to the presence of a large pool of fast-dividing cells. Leuko- and neutropenia are main limiting factors which may contribute to chemotherapy failure. Hematopoietic cytokines the part of conventional therapy in this field, but their effects require boosting. That is why the use of means and methods of adsorption therapy is considered promising. Sorption therapy creates a basis for sorption detoxification, a doctrine of curative measures directed to the removal of toxic endogenous or exogenous compounds from body fluids. The most widely used types are the purification of blood or its components (hemosorption), oral administration of sorption materials (enterosorption) and application-sorption therapy of wounds and burns. In this chapter, the results of early and recent research and prospects for the use of carbon adsorption therapy for the treatment of acute radiation sickness and cytostatic myelosuppression are discussed.

Keywords: leukopenia, ionizing irradiation, anti-cancer chemotherapy, granulocyte colony stimulating factor, hemosorption, enterosorption, application-sorption therapy

1. Introduction
The danger of acute and chronic radiation injuries, which provoke leukopenia, is not just a myth today. The explosion at Unit 4 of Chernobyl Nuclear Power Plant (NPP) in 1986 showed how unprepared people were to such a problem. The collective dose of irradiation for liquidators (clean-up workers) was huge; no one knows the exact numbers (all dosimetric equipment measured only gamma irradiation). And until today, about five million people, who live in areas of Belarus, the Russian...
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1. Introduction

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Federation and Ukraine, which are contaminated with radionuclides, still experience the consequences of pollution [1–3]. An earthquake and tsunami struck Fukushima Dai-ichi NPP in 2011 contaminated the soil and water with radioactive cesium, iodine, etc. It poses significant risks of exposure to the residents [4, 5]. Terroristic threats or military conflicts with the use of radioactive weapons could be considered as a potential risk of injuries also.

One more source of contact with myelosuppressive factors is radiation therapy, which is routinely used in oncology (up to 70% of patients with malignant tumors are treated with) as well as anti-cancer chemotherapy with cytostatics [6–8]. Medical use of radiation accounts for 98% of the population dose contribution from all artificial sources and represents approximately 20% of the total exposure. Annually worldwide, more than 3600 million diagnostic radiology examinations are performed, 37 million nuclear medicine procedures are carried out and 7.5 million radiotherapy treatments are given [9]. In spite of side effects, the concomitant use of radiotherapy and chemotherapy resulted in significantly improved clinical outcomes [10–12]. Different radiomimetics have effects similar to ionizing irradiation. Among them, a lot of anti-cancer drugs and leukopenia is a common side effect of dose-dense and dose-intense tumoricidal chemotherapy.

The organs and tissues with high speed cell proliferation is the most sensitive for radiation- and radiomimetic damage. Leukopenia, because of aggressive direct ionizing irradiation or anti-cancer chemotherapy with cytostatics, is an important prognostic factor for overall survival [13, 14]. The association between chemotherapy-induced leukopenia and clinical outcome has been reported for several types of cancer. The development of such health impairments gains more and more attention, especially after the success of modern techniques such as stem cell transplantation and cytokine treatment to restore hematopoietic functions. But even now, it is not enough for the treatment of acute radiation sickness.

In last decades, we observe combined injury by ionizing radiation and toxic effects of xenobiotic, thermal burns, mechanical trauma, etc. Despite significant achievements in oncology, precise and targeted irradiation of tumors, the development of effective means for enhancement of bone marrow cell and peripheral blood cells proliferation (granulocyte colony stimulating factors (G-CSF), erythropoietin, interleukin-11 and others), the problems of fighting the negative consequences of ionizing radiation and radiomimetics remain very important.

In this chapter, the results of early and recent research and prospects for the use of carbon adsorption therapy for the treatment of myelosuppression caused by acute radiation sickness and cytostatics use are discussed.

2. About radiation injuries

Acute radiation syndrome is a definition to reflect severe damage to specific organs that occurs because of whole-body or significant partial-body irradiation greater than 1 Gy, over a short time period (high dose rate) [15]. The main syndromes are hematopoietic (doses >2–3 Gy), gastrointestinal (doses 5–12 Gy) and cerebrovascular one (doses 10–20 Gy) [16]. Depending on exposed and absorbed doses and its duration, cells exposed to ionizing radiation or radiomimetics present DNA mutations, apoptosis, necrosis, chromosomal aberrations or increased mutation frequency [17, 18]. The most profound injury is to lymphoid organs (lymphatic nodes, spleen and thyroid gland), bone marrow, testicles, ovaries, gastrointestinal mucosa. Parenchymal organs, namely liver, adrenal glands, kidneys, salivary glands and lungs possess quite high radioresistance. According to World Health Organization (WHO), acute radiation sickness (ARS) is composed of the hematopoietic subsyndrome...
(HS), gastrointestinal subsyndrome (GIS), neurovascular subsyndrome (NVS) and cutaneous subsyndrome (CS) [19]. The main factors which determine the survival of victims are the degree of bone marrow suppression and gastrointestinal tract damage due to the presence of a large pool of fast-dividing cells [20–22]. Acute radiation sickness (ARS) could be considered as a sequence of immediate radiation injury and long-lasting bystander cross-effects.

Management of patients with ARS includes early use of hematopoietic cytokines, antimicrobials and transfusion support; in addition, antiemetic agents and analgesics, and even hematopoietic stem cells transplantation [16, 23]. Since 1997, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are used and their doses are driven by the radiation dose and physiologic responses for ARS [24] and by clinical protocols for leukopenia and neutropenia caused by anti-cancer treatment [25, 26]. However, these drugs still are of high cost, and pharmacoeconomic benefits seem to be questionable [27]. Singh et al. concluded that cytokine therapy has significant but modest effects [28]. All these facts force the researches to search new methods and means for additions to the management of post-aggressive iatrogenic leukopenia and related ARS- and radiomimetic-induced damage.

3. Adsorptive hemoperfusion therapy for ARS

Sorption detoxification types, quite widely used today in medicine, are: (1) hemoperfusion (when blood is filtered through the column with activated carbon); (2) enterosorption—enteral use of oral adsorbents of a different type and (3) application-sorption therapy - use of carbon dressing for the healing of the burns and wounds.

The ground for use of direct perfusion of the blood through an adsorbent column for its purification (hemoperfusion) was the Kuzin A.M. Structural-Metabolic Theory in Radiobiology (1970) [29]. Organs and tissues exposed to ionizing radiation and radiomimetic influences are damaged by radiotoxins, which affect radiosensitive structures, and direct radiation-dependent changes in the macromolecules of the genome. Further investigations demonstrated that “radiotoxins” are reactive oxygen species (ROS) formed by water radiolysis. Oxidative stress causes DNA, protein and lipid oxidation and is responsible for the whole range of signs and syndromes of ARS [29]. Because of excessive lipid peroxidation, a lot of damaged cells appear that deepens the primary radiation injury repeatedly. In summary, ARS is a sum of primary damage due to oxidative stress plus so-called bystander effects [18], when cells exposed to ionizing radiation or radiomimetics can release signals that induce very similar effects on non-targeted neighboring cells.

Our first research of adsorptive therapy effects for acute radiation sickness (ARS) started in 1976 [30]. In this study, 69 inbred dogs were irradiated by external X-ray at the dose of 525 Rad (5.25 Gy). They were randomly assigned to three groups: first control group (n = 31), which received standard antibiotics therapy; second group (n = 19) got antibiotics + hemoperfusion 2 hours after irradiation and third group (n = 19) underwent saline infusion 4–5 hours after irradiation plus furosemide, and hemoperfusion 24 hours later. The results are presented in Table 1.

The highest survival rate was in the second group—68.4%, while in the control group, it was only 3.2%. Late hemoperfusion also resulted in a high survival rate—62.4%. Only 16% of an animal with hemoperfusion treatment (three dogs in each group) had critical leukopenia. In the control group, it was 93.5% of animals.

It is noteworthy, that mitotic index (a marker of the rate of cells division) (Figure 1) was significantly higher in the second group compared to the control one.
possessing intrinsic antioxidant properties) would be more effective against ROS and simulate the effects of free oxygen radical scavenging enzymes.

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A team of researchers who carried out the experiment on dogs by irradiating them at the dose of 5.25 Gy, witnesses that perfusions of the blood through the column with a carbon adsorbent were quite short. Slugging of columns was the main reason for incomplete procedures (only 0.3–0.5 of circulating blood volume was purified) [35]. Despite these factors, the survival rate and other studied parameters were quite successful. We suppose that it could be explained by washout of dust particles from the surface of the adsorbent in the moment of primary contact with the blood, and viscosity changes inside the column after the replacement of rinsing solution to the blood also contributed to it. We think that positive secondary effects could be provided by nano- and microparticles (1–2 μ) of activated carbon, which contact with the blood. Their content is not controlled according to the standards of British (BP) and American (USP) Pharmacopeia.

Today, we have a lot of evidence that positive curative effects of carbon nanoparticles, alone or as a part of a composite, are obliged to their ability to scavenge the ROS and simulate the effects of free oxygen radical scavenging enzymes. Sandhir R. et al. [36] believe that nanoantioxidants (inorganic nanoparticles possessing intrinsic antioxidant properties) would be more effective against (6 hours after irradiation) and even to the initial level (14th day after exposure to ionizing irradiation) [31].

Hemoperfusion with activated carbon also provided survival of 50% of dogs exposed to ionizing irradiation at the doses of 3.46 and 3.65 Gy [32]. These results were re-tested and developed within a special closed program of Research institutions of the Ministry of Health and the Ministry of Defense of USSR. Hemoperfusion methods were implemented into clinics [33, 34].

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<table>
<thead>
<tr>
<th>Group</th>
<th>Survival rate, %</th>
<th>Bone marrow cellularity &lt;1.0 × 10^9/L</th>
<th>Leukopenia &lt;1.0 × 10^9/L</th>
<th>Thrombocytopenia, &lt;50.0 × 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 31)</td>
<td>3.2</td>
<td>13.4 ± 1.1</td>
<td>93.5</td>
<td>70</td>
</tr>
<tr>
<td>2 (n = 19)</td>
<td>68.4</td>
<td>17.0 ± 1.6</td>
<td>16.0</td>
<td>52.6</td>
</tr>
<tr>
<td>3 (n = 19)</td>
<td>62.4</td>
<td>16.6 ± 1.9</td>
<td>16.0</td>
<td>38.9</td>
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Table 1. Hemoperfusion for ARS treatment [30].

Figure 1. Mitotic index (%) in the bone marrow of the dogs, exposed to external ionizing and hemoperfusion. Notes: * p ≤ 0.05 compared to the initial level; ** p ≤ 0.05 compared to the control group.
ROS-induced damage because they cross the blood-brain barrier. It is a potential application in treating and preventing neurodegenerative conditions [36]. Arifa R.D. et al. research demonstrated that nanocomposite with fullerol decreases the intensity of irinotecan-induced leukopenia and gastrointestinal damage in mice and do not diminish the tumoricidal effects of the drug [37]. The aftertreatment with the same nanocomposite ameliorates the graft-versus-host disease reactions in mice and reduces intestinal lesions and bacterial translocation; prevents mortality and morbidity [38]. Nano-fullerenes promote osteogenesis of human adipose-derived stem cells and possess a great antioxidant capacity [39].

Encouraging results have been found concerning the amelioration of side effects of one more radiomimetic—anthracycline antibiotic doxorubicin (DOX), which also is known by its ability to cause oxidative stress and leukopenia. Fullerenol C60(OH)24 nanoparticles improved the myocardial morphology of DOX-treated animals, but cause a certain degree of parenchymal degeneration by itself [40]. Such and similar cases [41] evidence the need for designing and searching for the nanocomposites with specific features, which will possess antioxidant capacity without notable cytotoxicity. One of the solutions could be the conjugation of carbon nanomaterials with albumin [42]. It was found that C60(OH)24 decreases the consequences of DOX-induced excessive oxidation in the tissues of kidneys, testis and lungs in mice [43]. An aqueous solution of fullerenol was quite effective to fight experimental arthritis in rats [44]. Andrievsky G.V. et al. demonstrated significant (but only by 15%) radioprotective properties of hydrate C60 fullerene in X-ray irradiation of the mice at the lethal dose of 7 Gy [45]. Water-soluble polyvinilpyrroli done-wrapped fulleren derivative showed to significantly inhibit UVA-promoted melanogenesis in normal human epidermis melanocytes and human melanoma HMV-II cells within a non-cytotoxicity dose range [46]. Huq R. et al. showed that nontoxic poly(ethylene glycol)-functionalized hydrophilic carbon clusters, known scavengers of the ROS superoxide and hydroxyl radical, are preferentially internalized by T lymphocytes over other splenic immune cells [47]. It was successfully used to reduce T-lymphocyte-mediated inflammation in experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis) [47].

Another type of carbon material—carboxylated nanodiamonds, diminish the biochemical and histological signs of damage of γ-irradiated human erythrocytes [48]. On the other hand, hydrogenated nanodiamonds dramatically increase the sensitivity to radiation effects of human radioresistant cancer cell lines [49]. The same effect was seen considering the radiomimetic neocarcinostatin. Single-walled carbon nanotubes were found to be the efficient nanocarriers for drug delivery in the murine model of breast cancer [50, 51]. The team of researchers [52] synthesized the magnetic particles Fe3O4 in the shell from partially graphitized carbon and demonstrated their high intrinsic peroxidase-like catalytic activity, which promotes oxidative stress in human prostate cancer PC-3 cells in the presence of ascorbic acid. One more interesting study with a composite system of reduced graphene oxide—iron oxide nanoparticles showed that such a combination can synergistically induce physical and chemical damage to methicillin-resistant Staphylococcus aureus (MRSA) [53].

We must notice, that carbon nanoparticles possess great antioxidant properties and could be perspective for designing the nanopharmaceutical means and drugs to treat the disorders, when oxidative stress is an intrinsic part of pathogenesis, for leukopenia also. It means that further studies of carbon micro- and nanoparticles effects at parenteral routes of administration could finalize the discovery of quite a new method of mass treatment of acute radiation sickness.

Recently, several detailed reviews have been published on the pharmacological potential and prospects for the therapeutic use of cerium nanoparticles as traps of highly reactive oxygen (ROS) and nitrogen species (RNS) [54–56]. These reviews...
are based on a variety of experimental studies both in vitro and in vivo. Not less interesting results for use of nanocrystal cerium dioxide (CeO₂) on the model of DOX-induced cardiomyopathy in rats we got [57]. Cardiomyocytes mostly are damaged because of the radiomimetic impact of the drug, and the violation of blood components was quite similar to the effects of ionizing irradiation. It is known that oxidative stress is an intrinsic part of the cytotoxic effects of DOX, and heart tissues are vulnerable because of a lack of intracellular antioxidant defense factors compared to other organs and systems [58].

In this study, we used 21 female white mongrel rats, which were randomly assigned to the next groups (n = 7): first control groups got weekly intraperitoneal (IP) injection of saline; rats of second (DOX) and third groups got three times a week IP injections of doxorubicin at a dose of 2.5 mg/kg (n = 7); rats of third (DOX + CeO₂) group got twice weekly IP injections of nanodisperse CeO₂ (0.2 mg/kg) next day after doxorubicin injections additionally. Treatments lasted for 2 weeks (Figure 2).

Injections of nanodisperse CeO₂ caused positive changes in myocardium structure. We observed improvement of a structure, decreased vacuolization of sarcoplasm, a number of cells with nuclei pathology was much lower (Figure 4) compared to the second group (Figure 3). A part of myocardium cells still had pyknotic nuclei with karyolysis signs. But mostly, the intensity of dystrophy and necrosis reduced and nuclei acquired oval shape again.

Figure 2.
Myocardium tissue of rat of the control group. H&E. ×600.

Figure 3.
Myocardium tissue of rat of the DOX group. H&E. ×600.
Cells of the Immune System are based on a variety of experimental studies both in vitro and in vivo. Not less interesting results for use of nanocrystal cerium dioxide (CeO2) on the model of DOX-induced cardiomyopathy in rats we got [57]. Cardiomyocytes mostly are damaged because of the radiomimetic impact of the drug, and the violation of blood components was quite similar to the effects of ionizing irradiation. It is known that oxidative stress is an intrinsic part of the cytotoxic effects of DOX, and heart tissues are vulnerable because of a lack of intracellular antioxidant defense factors compared to other organs and systems [58].

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Figure 4. 
Myocardium tissue of rat of the DOX + CeO2 group. H&E. ×600.

Also, we observed an increased number of lymphoid follicles in the spleen, which restored a circle-like shape (Figures 5–7).

There were no significant positive changes in the structure of liver parenchyma. We may just note restoring nuclei sizes and shape and a little bit lighter pale pink
color of cytoplasm. It witnesses that the synthetic function of the liver was partly restored. Concerning the kidneys, no positive changes had been found.

Biochemical indices of lipid and protein peroxidation, antioxidant defense system showed that CeO₂ increased the activity of catalase by 24.6%, raised the level of reduced glutathione by 10.9% and decreased the level of oxidative modification of protein and lipids by 28.1 and 23.6%, respectively (compared to the group with untreated DOX-induced cardiomyopathy).

Bakht M.K. et al. proposed to reduce the actual radiation burden in patients exposed to radioisotope studies by arranging radiolabels for cerium oxide [59], and Colon J. et al. could achieve a good prophylactic result for radiation pneumonitis in mice that received nanocrystalline dioxide Ce [60]. One more fact should be mentioned here: because of bone marrow suppression and leukopenia development, lungs are fragile to injury by ionizing irradiation. They have their own host defense system, based on alveolar macrophages. Because of leukocytes toxic damage (by ionizing injury or radiation therapy or as the side effects of anti-cancer chemotherapy), resting macrophages can no longer be transformed which lead to radiation pneumonitis [24]. Heslet L. et al. showed that systemic administration of myelo stimulative cytokines was not helpful to prevent it because they do not penetrate the alveoli. That is why we suggest that oral adsorbents and/or parenteral use of CeO₂ (it penetrated the alveoli and prevents radiation pneumonitis on mice model) will enhance the prophylaxis and treatment of ARS and decrease the intensity of side effects of radiation therapy and cytostatic drugs.

4. Local signs of whole-body irradiation and efficacy of application-sorption therapy

External exposure to ionizing irradiation frequently results in radiation burns of the skin. Leukopenia just deepens the injury because of oppressing the regeneration processes. A retrospective report on injuries caused by the atomic bombing of Hiroshima showed that up to 65% of all type of injuries were “radiation-combined injury;” when ionizing irradiation was coupled with burns, wounds and infections [61]. Regarding these facts and negative contribution of leukopenia also, we want to demonstrate the efficiency of activated carbon. The remarkable result was observed on the model of the thermal non-full depth burn in Albino rats [62]. The early application (within first
Cells of the Immune System

Figure 7. Spleen structure of rat of the DOX + CeO2 group. H&E. ×600.

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Histological analysis demonstrated that adsorptive carbon dressings’ application promoted the restoration of skin structure on the 7th day after injury in rats with the non-full burn (Figures 9–11).

Similar results were observed on the burns caused by external irradiation at the dose of 8 Gy. Epithelialization of burn wounds has been completed on 21.1 ± 4.1 versus 27.3 ± 5.7 days after trauma for carbon and gauze dressings use, respectively. One
more fact relates to the treatment ultraviolet radiation-induced burns. Application of adsorptive carbon dressings significantly (by 1.5–1.7 times) accelerated the burn-healing time. All these data will be published soon.

These results presented the undoubted perspective for use of high capacity carbon fabrics for the treatment of superficial skin lesions, especially complicated by concomitant leukopenia.

5. Enterosorption for leukopenia management

Hemoperfusion as a procedure requires well-trained staff, specific equipment and sterility. It means that such method of sorption detoxification is not adapted to emergency exposure situations, during war-time and large human contingent injury. That is why the use of enteral sorption therapy (ingestion of activated carbon) is a more prospective method for such situations. Among the early studies, the great results were observed in the patients with lymphogranulomatosis undergoing radiotherapy [63], who were treated with fibrous carbon oral adsorbent. Enterosorption treatment allowed to continue planned schemes of radiation therapy and was more efficient than conventional methods for leukopenia healing. In the next study [64], cyclophosphane was given to Guerin tumor-grafted rats at the dose of 100 mg/kg of body weight on 10th and 13th days after tumor transplantation; enterosorption with synthetic SCN carbons (bulk density 0.3–0.4 g/cm$^3$) was administered next day after cyclophosphane injection. These expressed myeloprotective effects we approved and confirmed in the clinic. One more radiomimetic anti-cancer agent cisplatin was used in an experiment on Guerin tumor-grafted rats [65] and highly activated fibrous carbon material Carboline (Ukraine) successfully ameliorated a wide range of its side effects. Carboline is used in clinical practice also and demonstrates promising results [66].

Our latest experiments on rats exposed to X-ray irradiation in a total dose of 6 Gy (63 Rad per min, t = 11 min) demonstrated great results of novel oral carbon adsorbents administration to ameliorate radiation-caused leukopenia. We used two granulated activated carbons (AC) with a diameter of granules (0.25–0.5 mm) and bulk density 0.1 and 0.2 g/cm$^3$ (ES1 and ES2, respectively). Enterosorbents were administrated as radioprotectors, radiomitigators and therapeutic agents (at the dose of 10 ml/kg, admixed to the food, three days before and nine days after ionizing irradiation exposure). Irradiation caused a 10-fold decrease in the white blood cell count. ES1 administration raised the index twice on the 9th day after X-ray exposure, while ES2 produced fewer results (Figure 12).

The same effect was observed concerning the lymphocytes count (Figure 13). These results will be published soon in detail.

So, as we observed, specific oral adsorbents with specified porosity and pores distribution are quite successful to fight the iatrogenic leukopenia because of the influence of ARS or anti-cancer treatment.

Oral carbon materials have a high capability to decrease the emesis caused by anti-cancer treatment [66, 67]. Also, it is a unique mean with anti-diarrhea action, which could be implemented in the clinics for the treatment of ARS-induced gastrointestinal subsyndrome as well as for dyspepsia syndrome caused by tumoricidal therapy.

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Thus, enterosorption for the results on the animal study and use in clinics do prevent hematotoxicity of anti-cancer treatment and significantly ameliorated leukopenia and its consequences.
6. Oral carbon adsorbents as an addition to classical treatment of leukopenia with G-CSF

Abovementioned positive results led us to design of a new improved version of carbon oral adsorbent, which was used in early experiments [64, 68]. An old prototype C1 and his new version C2 were approved on the model of melphalan-induced bone marrow suppression [69–71]. We demonstrated that myeloprotective action of carbon granulated enterosorbent C1 (bulk density of 0.28 g/cm³, specific surface of 1719 m²/g and mesopore area of 239 m²/g) is significantly less compared to effects of adsorbent C2 with bulk density of 0.18 g/cm³, total specific surface of 2162 m²/g and mesopore area of 565 m²/g (Figure 14).

**Figure 14.** White blood cells and neutrophils counts (10⁹/L) on the 8th day after melphalan injection at the dose of 3 mg/kg and administration of oral carbon adsorbents C1 and C2 in a study on rats. Notes. *—p < 0.05 compared to the control group; **—p < 0.05 compared to the melphalan group; #—p < 0.05 and compared to the Melphalan + C1 group.

C2 enterosorbent administration normalized the prooxidant/antioxidant system too [71]. Oxidative stress is an intrinsic part of ionizing radiation and radiomimetic injury, and enteral sorption therapy possesses notable antioxidant effects. Adding the carbon oral adsorbent to classical scheme for the treatment of leukopenia with G-CSF (caused by single intravenous melphalan injection at the dose of 4 mg/kg) demonstrated significant myeloprotective effect and synergy compared to single-use effects of each agent alone [70] (Figures 15 and 16). C2 and filgrastim combination caused increase of white blood cells count by 138.3% compared to the melphalan group; by 65.8% compared to melphalan + C2 group, and by 51.7% compared to use of filgrastim alone.

We must note that in this study, we got an unexpected significant increase of platelets level from (254.60 ± 45.59) to (505.40 ± 70.68) × 10⁹/L in a group of rats that received the combined treatment with oral adsorbent and G-CSF. Isolated administration of enterosorbent C2 tended to raise the level of thrombocytes, we suppose because of general detoxification action.

Use of enterosorbent or combined use of both preparations provided significantly better effects toward the prooxidant/antioxidant balance in rats. The important issue is that combination of G-CSF and carbon adsorbents [69] as well as enteral sorption therapy use alone [65] does not affect the efficacy of anticancer treatment; we proved it by our experiments on Guerin tumor-grafted rats.

7. Conclusions

Leukopenia is an essential part of the damage caused by ionizing irradiation and/or radiomimetic influences (as tumoricidal chemotherapy). Leukocytes play an important role in immune defense, tissue regeneration, the functioning of the main organs and systems; and the degree of bone marrow suppression determines the survival of victims in ionizing radiation exposure as well as the efficacy of anticancer chemotherapy. All three methods of sorption detoxification with activated carbons such as hemoperfusion (when blood is filtered through the column with activated carbon); enterosorption—peroral use of oral adsorbents and application—sorption therapy (use of carbon dressing for the healing of the burns and wounds), can be successfully used for the leukopenia prophylaxis and treatment in ionizing irradiation exposure, side effects of anticancer chemotherapy, as well as for the boosting of healing of associated skin damage.

**Figure 15.** White blood cells count (10⁹/L) on the 8th day after melphalan injection at the dose of 4 mg/kg and administration of oral carbon adsorbent and filgrastim in the study on rats. Notes. p < 0.05 compared to: *—Control group; **—Melphalan group; ***—Melphalan + C2 group; ****—Melphalan + filgrastim group.
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Enterosorption demonstrates significant synergy with hemopoietic cytokines in the treatment of bone marrow suppression caused by such an aggressive agent as melphalan (a derivative of mustard nitrogen). Nanocrystal cerium dioxide could be useful for oxidative stress modulation caused by such radiomimetic as anti-cancer anthracycline antibiotic doxorubicin. Modification of pathological biochemical processes which provoke bone marrow suppression and leukopenia is a basis of the efficacy of sorption detoxification in acute radiation syndrome as well as to decrease the side effects of anti-cancer chemotherapy. Our findings may contribute to the refinement of current risk stratification algorithms for acute radiation sickness treatment.

Conflict of interest

The authors declare the absence of the conflict of interest.

Abbreviations

- NPP: nuclear power plants
- ARS: acute radiation sickness
- G-CSF: granulocyte colony-stimulating factor
- ROS: reactive oxygen species
- Gy: gray, unit of ionizing radiation dose

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Cells of the Immune System


The cells of the immune system are lymphocytes (T-cells, B-cells and NK (natural killer) cells), neutrophils, eosinophils, and monocytes/macrophages. This book is an overview of some types of these cells and their role in recognizing and/or reacting against foreign material. The immune system is characterized by collaboration between cells and proteins. The development of all cells of the immune system begins in the bone marrow with a hematopoietic stem cell. Two chapters deal with neutrophils, three chapters with T-cells, four chapters with eosinophils, and other chapters review the immunomodulation of macrophages, the role of transcription factor KLF4 in regulating plasticity of myeloid-derived suppressor cells, immune reconstitution after allogeneic hematopoietic stem cell transplantation, and role of sorption detoxification in the therapy of acute radiation sickness.