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Immunopharmacology and Inflammation

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Preface

Inflammation is a complex physiologic response that protects the body against potentially harmful stimuli, including pathogens, damaged cells and tissue components, malignant elements, irritants and toxins. Both acute and chronic inflammatory responses involve immune cells, blood vessels, proteins, amines and lipid mediators that orchestrate events like vascular permeability, leukocyte migration and pain.

In recent years, the notable advancements in our understanding of the immune system, the huge body of accumulating evidence on the pharmacology of the immune responses and the significant biotechnological advances made available novel targeted therapeutics that challenge current unmet medical needs. These drugs comprise monoclonal antibodies and other biologics, as well as small-molecule enzyme inhibitors that interact with well-defined targets. Along with the wide repertoire of classic and revisited therapeutic interventions, these innovative tools attract high interest and investment from the biopharmaceutical, biotechnological, academic and health care sectors. Thus, immunopharmacology is currently one of the most rapidly expanding and promising areas in biomedicine, offering cutting-edge modalities to treat illnesses ranging from allergies, autoimmune diseases and malignancies to neurological, cardiovascular and metabolic disorders.

This book is supported by members of the Immunopharmacology Section – *ImmuPhar* of the International Union of Basic and Clinical Pharmacology (IUPHAR) – and reflects the future trends and challenges in the area of inflammation and immunopharmacology. By providing a comprehensive, yet non-exhaustive overview of the current advances in these fields, it addresses an audience with basic knowledge in the inflammatory and immune processes and is useful as a source of the most up-to-date information for researchers and clinicians already working in these areas.

The chapters highlight the basic knowledge and introduce new concepts on the mediators of inflammation, including the putative immunomodulatory role of histamine; the molecular mechanisms and the resolution of inflammation; the immunopharmacological perspective of innate and adaptive immunity; and the regulation of immune mechanisms in health and disease, including neural regulation of inflammation and atherosclerosis. The chapters on the actions and side effects of glucocorticoids underscore the continuing

timeliness and relevance of these classic therapeutic agents for the treatment of immune and inflammatory disorders. On the other hand, the chapters on the innovative drugs for allergies and on the immune mechanisms in atherosclerosis emphasize the development and exploitation of targeted therapies in the indented new era of personalized or precision medicine.

Special thanks are due to all authors for contributing to this effort.

Finally, we gratefully acknowledge that completion of this book could not have been accomplished without the skillful efforts and the constant support of Professor Simona Ronchetti, Department of Medicine, University of Perugia, Italy.

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Jerusalem, Israel — Francesca Levi-Schaffer
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Mediators of Inflammation

Izabela Galvão, Michelle A. Sugimoto, Juliana P. Vago, Marina G. Machado, and Lirlândia P. Sousa

Abstract

Inflammation is a physiologic response against noxious stimuli and microbial invaders. The basic elements of inflammation include host cells, blood vessels, proteins and lipid mediators, which work together to eliminate the inflammatory stimulus as well as initiate the resolution and repair. Mediators of inflammation are regulatory molecules that control the generation, maintenance and resolution of this response, which is triggered after recognition of infection or injure. The initial recognition of the inflammatory stimuli leads to the production of pro-inflammatory mediators. These mediators are derived from immune cells (e.g. vasoactive amines, lipid mediators, platelet-activating factor, reactive oxygen species, nitric oxide, cytokines and chemokines) or are acute phase proteins produced by the liver that circulate in the plasma (e.g. the complement, coagulation and kallikrein-kinin systems). Together, the mediators of inflammation orchestrate all the inflammatory events such as blood vessel dilatation, vascular permeability, leukocyte migration to the affected tissue and pain.

Keywords

Inflammation · Vasoactive amines · Lipid mediators · Cytokines · Chemokines · Plasma-driven mediators

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1 Introduction

Inflammation is a protective response, which involves immune host cells, blood vessels, proteins and lipid mediators [1]. During an inflammatory response, molecular patterns are sensed by receptors from innate immunity leading to the production of inflammatory mediators. The aim of inflammation is to eliminate the initial cause of cell injury, such as the necrotic cells/tissues that result from the original damage and, by doing so, paving the way to start the resolution and repair

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processes, restoring tissue homeostasis [2]. Although inflammation helps in the removal of infectious or sterile stimuli and in the initiation of repair, it can cause considerable tissue damage if it is overshooting or if there is failure in the resolution process [3].

Clinically, acute inflammation is classically characterized by symptoms known as the cardinal signs of inflammation: *rubor* (redness), *calor* (increased heat), *tumor* (swelling), *dolor* (pain), and *functio laesa* (loss of function). The physiological basis of these signs is centered in the migration of leukocytes out of blood vessels and into the surrounding tissue, in order to eliminate the inflammatory stimuli and clear the inflammatory site from damaged cells and dead tissues. This is allowed by vascular changes characteristic of an acute inflammatory response, such as vasodilation and plasma leakage. Inflammatory mediators of a vast range of nature, structure and cellular origins are involved in the basis of all the cardinal signs of inflammation and the physiological events associated to the inflammatory response [4].

The immune system deals with infection and tissue damage through a sequence of events involving molecular, cellular and physiological alterations. In order to coordinate these sequences of events, inflammatory mediators are produced by blood or local-derived inflammatory cells in response to a noxious stimulus [5]. The major cell types that produce mediators of acute inflammation are platelets, neutrophils, monocytes /macrophages and mast cells, but cells such as fibroblasts, endothelial cells and smooth muscle cells, can be activated to produce some of these mediators. Plasma mediators such as complement proteins, kinins and fibrinolysis proteins are produced primarily in the liver and are present in the circulation as inactive precursors that need to be activated, usually by a series of proteolytic cleavages, to acquire their biological properties [2]. Once release, these mediators initiate and amplify the inflammatory response by a control system which can be regulated at receptors' and signalling pathways' levels, controlling which tissue respond, and also the length and the extent of the response [4]. On this chapter, we will discuss some of the most important inflammatory mediators, beginning with cell-derived mediators followed by acute-phase proteins found in plasma.

2 Cell-Driven Mediators

2.1 Vasoactive Amines

Changes in the caliber of blood vessels can be made by a variety of substances. Here we will focus on the vasoactive amines. These substances have an important role in the inflammatory response once they have the ability to cause smooth muscle contraction, vasodilation and vascular permeability. The main vasoactive amines with this ability are histamine and serotonin [6].

2.1.1 Histamine

Histamine plays an important role in inflammatory responses, mainly in hypersensitive responses. It is a vasoactive amine released mostly by mast cells that causes vasodilation, edema and smooth muscle contraction. Clinical manifestations vary from anaphylactic reaction and urticaria, to local wheal and flare reactions. Many signs of allergic reaction result from the ability of histamine to affect blood vessels, enhancing blood flow, vascular permeability and inducing vasodilation [7].

2.1.2 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) was first isolated, purified and identified in 1948 by Rapport and colleagues [6]. This molecule was named so because it was found in the serum (*sero*) and was observed that it promotes vascular constriction/tonus (*tonin*) [6]. Although serotonin is best known for its activity in the central nervous system (CNS), it is mostly produced by enterochromaffin cells and predominantly found in the periphery and in the gastrointestinal tract [8]. It is also produced by neuronal cells, T lymphocytes, monocytes and mast cells but all these sources combined are still smaller than enterochromaffin production [9, 10]. Besides the cells that produce serotonin, it can also be present in other cells, like blood platelets, dendritic cells

and B lymphocytes, due to the uptake mechanism [11, 12]. Serotonin functions as a hormone, an immune modulator and a neurotransmitter. Therefore, it contributes to the regulation of many physiological functions, like vasoconstriction, inflammatory responses, intestinal motility and wound healing [8, 13, 14].

Serotonin Synthesis

Serotonin synthesis occurs in two steps and around 5% of all L-tryptophan (Trp) obtained from the diet is consumed in the first step [15]. This step consists of tryptophan hydroxylation by tryptophan hydroxylase (TPH), which uses Fe^{2+} as a co-factor and O_2 and tetrahydrobiopterin as co-substrates, producing 5-hydroxytryptophan (5-HTP). In the second step, 5-HTP is decarboxylated by aromatic amino acid decarboxylase (AADC), resulting in the production of serotonin (5-HT) [16] (Fig. 1).

Serotonin synthesis rate is limited by TPH action, which is a non-heme iron pterin-dependent oxygenase. It has been suggested that TPH action is limited by low concentrations of its co-substrate: Tetrahydrobiopterin (BH_4) [15]. This enzyme has two isoforms, TPH1 and TPH2, distributed in different sites [16]. 5-HT is synthesized by TPH1 in enterochromaffin cells and by TPH2 in neuroendocrine cells, then it is transported by vesicular monoamine transporters ($VMAT_1$ and $VMAT_2$, respectively) to secretory granules, where it is stored [17]. Serotonin is commonly released from vesicles via Ca^{2+}-dependent exocytosis [18]. In addition, it can be released through binding and activation of many receptors, like adrenoceptors, muscarinic receptors or $5\text{-}HRT_3$ autoreceptors, while its release can be inhibited by activation of $GABA_A$, nicotinic or somatostatin 2 (SST_2) receptors or $5\text{-}HRT_4$ autoreceptors. After being released, serotonin can bind and activate its receptors or it can be taken up by other cells through serotonin transporter. The serotonin transporter like all monoamine transporters, is a twelve transmembrane domain spanning sodium-dependent transporter that is expressed in many cells, like platelets, pulmonary cells and dendritic cells [19].

Serotonin Functions and Inflammation

As described previously, serotonin may induce multiple effects in different tissues. These effects are due to the presence of different receptors that will activate distinct signaling pathways. Serotonin receptor family (5-HTR) comprises at least 13 receptors classified in subfamilies from $5\text{-}HTR_1$ until $5\text{-}HTR_7$, they are G-protein coupled receptors (GPCRs) and one is a Cys-loop ligand-gated ion channel receptor [20]. Activation of $5\text{-}HTR_1$ downregulate cyclic AMP. On the other hand, activation $5\text{-}HTR_4$, $5\text{-}HTR_6$ and $5\text{-}HTR_7$ upregulate cAMP [21].

Serotonin is responsible for the regulation of many aspects of cognition and behavior, platelet coagulation, gastrointestinal motility and immunity. 5-HTRs expression in neutrophils, eosinophils, monocytes, macrophages, dendritic cells (DCs), mast cells and natural killer (NK) cells highlight the importance of serotonin in immunological responses (Table 1).

Besides all physiological functions, serotonin can be involved in many pathological processes

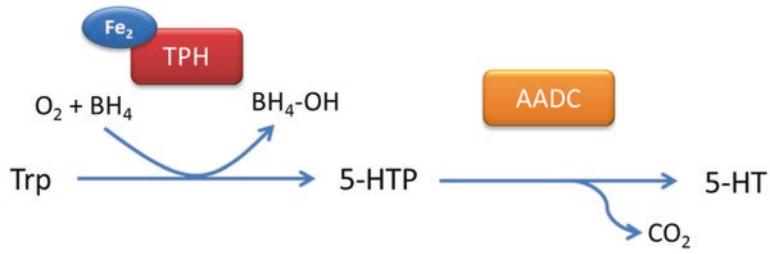

Fig. 1 Serotonin synthesis. Tryptophan is hydroxylated by tryptophan hydroxylase (TPH), which uses Fe_2 as a co-factor, and O_2 and tetrahydrobiopterin (BH_4) as co-substrates, producing 5-hydroxytryptophan (5-HTP). Then, aromatic amino acid decarboxylase (AADC) decarboxylases 5-HTP to produce 5-HT (serotonin) [16]

Table 1 Effects of serotonin in different cell types

Leukocytes	Effects of serotonin
Mast cells	Acts as a chemoattractant [22]
T lymphocytes	Promotes the activation through macrophages [12, 23, 24]
B lymphocytes	Promotes the activation and proliferation [25]
Eosinophils	Acts as a chemoattractant [26]
Basophils	Induces IL-4 production [27]
Macrophages/ monocytes	Increases the production of pro-inflammatory cytokines, such as IL-1 and IL-6 [28] Enhances the phagocytic capacity [29] Acts as a chemoattractant [26] Promotes an anti-inflammatory polarization [30]
Neutrophils	Acts as a chemoattractant [31].
Dendritic cells	Acts as a chemoattractant [26] Alters cytokines' production [32] Modulates the differentiation of DCs from human monocytes [11] Triggers the generation of inflammatory adaptive immune response [33]
Natural killer cells	Stimulates the production of interferon-gamma Enhances cytotoxicity [24] Increases proliferation [11]

due to the deregulation of the serotonin-signaling pathway. These diseases can vary from neurological to inflammatory disorders. It was recently suggested that serotonin uptake by lymphocytes may trigger serotonylation. This process is described as the covalent linkage of serotonin to small intracellular GTPases, such as RhoA and Rab4, by intracellular transglutaminase leading to constitutive activation of G-protein-dependent signaling pathways [34]. As these GTPases are also present in other immune cells, serotonylation may help explain the pathophysiological effects of enhanced intracellular serotonin in various inflammatory diseases. In addition, serotonylation subsequently downregulates efferocytosis, resulting in the progression of some chronic inflammatory diseases, like systemic lupus erythematosus, rheumatoid arthritis, obesity, cardiovascular disease, neurodegenerative disease, asthma and chronic obstructive pulmonary disease [35].

It has been recently identified that platelet, and not mast cell-derived, serotonin contributes to allergic airway inflammation [36]. Patients with Crohn's disease have high levels of serotonin due to enterochromaffin cells production. On the other hand, patients with ulcerative colitis, intestinal inflammation and diverticulitis have reduced expression of serotonin transporter, which also results in increased levels of serotonin [37]. Additionally, in white adipose tissue, it has been shown that reactive oxygen species (ROS) generated by serotonin metabolism in human adipocyte promotes lipid accumulation *in vitro* [38]. This may ultimately lead to adipocyte hypertrophy. Serotonin has been associated with hypertrophy of cardiac cells [39], with release of pro-inflammatory mediators by hypertrophic/hypoxic adipocytes which, according to current literature, are initiators of chronic low-grade systemic inflammation that defines obesity and associated disorders [40].

Serotonin Metabolism

The serotonin released by enterochromaffin cells into the portal blood is rapidly cleared from the plasma by three different processes: Uptake by platelets, metabolization by the liver and metabolization by the lungs. In the liver, serotonin can suffer oxidative deamination, glucuronidation and sulfation. In the lungs, it can suffer oxidative deamination or it can be uptaken. Thus, serotonin plasma levels in homeostatic situations are low [15].

Oxidative deamination is catalyzed by monoamine oxidase (MAO) and produces 5-hydroxyindoleacetaldehyde (5-HIAL). 5-HIAL can either be oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by acetaldehyde dehydrogenase (major metabolic pathway) or reduced to 5-Hydroxytryptophol (5-HTPOL) by the action of aldehyde reductase (ALDR) [41] (Fig. 2).

Other minor metabolic pathways include: Serotonin sulfotransferase that can catalyze the formation of serotonin-O-sulfate, which is not biologically active; Serotonin can be glucuronidated by uridine diphosphate glucuronyltransferase; Serotonin can also be N-acetylated and O-methylated to form melatonin in the pineal gland [42].

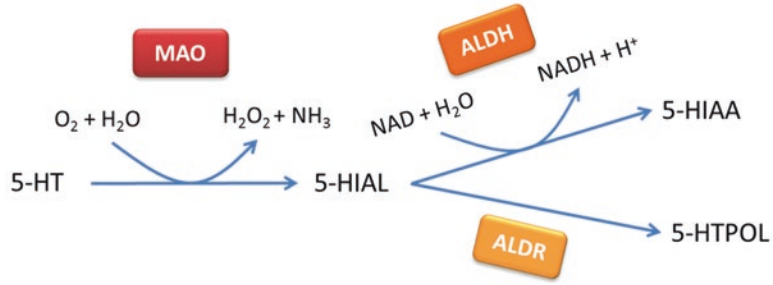

Fig. 2 Metabolism of Serotonin. 5-hydroxytryptophan (5-HT) is metabolized by monoamine oxidase (MAO) producing 5-hydroxyindoleacetaldehyde (5-HIAL). The aldehyde can either be oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by acetaldehyde dehydrogenase (ALDH), which is the major metabolic pathway, or reduced to 5-hydroxytryotiphol (5-HTPOL) by the action of aldehyde reductase (ALDR) [41]

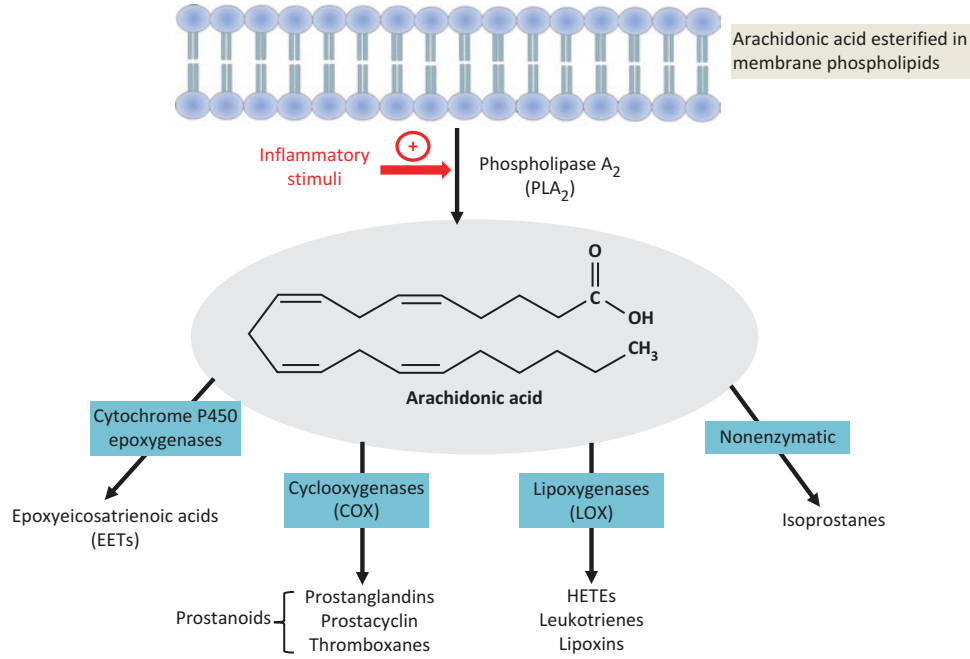

Fig. 3 Overview of arachidonic acid (AA) release and metabolism. Phospholipase A_2 (PLA_2) enzyme catalyses the release of AA from membrane phospholipids. Free unesterified AA is subsequently metabolized into eicosanoids via different pathways

2.2 Arachidonic Acid Metabolites

Eicosanoids comprise a family of locally acting bioactive lipid mediators that contain 20 carbons. The word eicosanoid has a Greek root that means 20. They are biosynthesized from arachidonic acid (AA) and related polyunsaturated fatty acids (PUFAs) by the initial activities of cyclooxygenases (COX), lipoxygenases (LOX), cytochrome P450 (CYP) and by a non-enzymatic pathway (Fig. 3) [43]. The first described eicosanoids were those with pro-inflammatory activities, such as prostaglandins (PG), prostacyclin (PGI_2), thromboxanes (TX) and leukotrienes [44, 45]. This vast collection of lipids trigger *in vivo* eicosanoid signaling networks in cells through binding to their cognate receptors. Eicosanoid signaling is critical for generating, maintaining

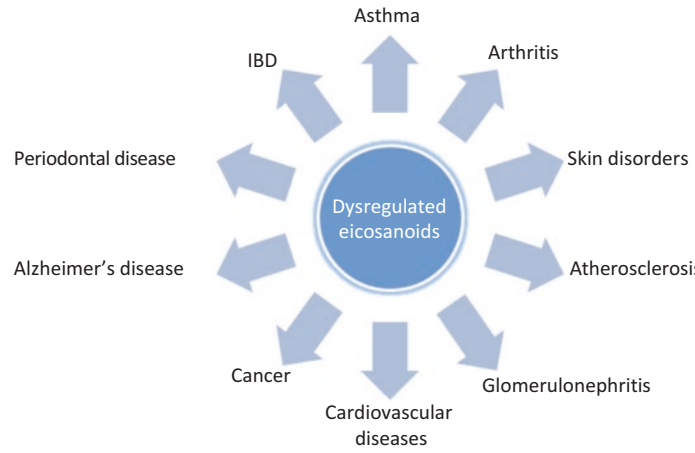

Fig. 4 Important human diseases with dysregulated synthesis of eicosanoids

and mediating inflammatory responses, but also has homeostatic functions and important roles in cardiovascular and reproductive physiology [43, 46].

Recent advances in lipidomics have helped to elucidate unique eicosanoids and related docosanoids with anti-inflammatory and pro-resolution functions, demonstrating that not all eicosanoids are pro-inflammatory [47, 48]. The fine-tuned class "switch" from pro-inflammatory to anti-inflammatory/pro-resolving eicosanoid production provides temporal and spatial regulation of the inflammatory responses and stimulates natural resolution [49]. As eicosanoids are important for the maintenance and amplification of the inflammatory response, and also for inflammation resolution and tissue repair processes, imbalances in eicosanoid synthesis can lead to abnormal immune functions that drive chronic inflammation (Fig. 4) [50].

Due to the pro-inflammatory bioactions of many eicosanoids produced at the initial phase of inflammation, pharmacological interventions in eicosanoid pathways during inflammatory conditions remains a prevailing strategy in the clinical managing of pain, swelling, fever and asthmatic conditions. For this purpose, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), leukotriene receptor antagonists and leukotriene inhibitor are largely employed in current clinical practice [51]. On the other hand, the recent advances in the field of lipid mediators may lead to the development of new therapeutics for the treatment of inflammatory conditions, autoimmune diseases, asthma and a variety of human conditions not previously thought to have an inflammatory component, such as atherosclerosis, cancer and Alzheimer's disease. These new therapeutic strategies may benefit from the anti-inflammatory/pro-resolving features of lipid mediators that act as agonists of the resolving phase of inflammation [52].

Important advances in mass spectrometry-based lipidomic profiling [53] have allowed scientists to properly identify, monitor and quantify hundreds of distinct eicosanoids and related PUFA species [54]. These lipidomic approaches are essential for identifying the lipid mediators involved in infection and in inflammation, as well as in the resolution of inflammation [55]. Indeed, lipidomics is essential for the screening of potential disease biomarkers [56] and to provide a better understanding of eicosanoid biosynthesis and signalling. When combined, the latest advances in genomics (transcriptomics), proteomics and lipidomics allow a better understating on the roles of eicosanoids and related PUFAs in inflammation and in infection, and can provide the basis for the development of innovative therapeutics for inflammatory diseases [55].

2.2.1 Biosynthesis of Eicosanoids

Unlike many inflammatory mediators which are stored in granules after transcription and mRNA translation, eicosanoids are generated from phospholipids precursors during inflammation.

Hundreds of eicosanoids with distinct structures and stereochemistry can be synthesized from AA and other ω-6-derived polyunsaturated fatty acids (PUFAs) [55].

Arachidonic acid (AA), or 5,8,11, 14-eicosatetraenoic acid, the most abundant eicosanoid precursor, is a 20-carbon (C20) fatty acid containing four double bonds (Fig. 3). AA is a normal constituent of membrane phospholipids. Phospholipids found in biological membranes are composed by a glycerol backbone on which two fatty acid chains are esterified in position *sn*-1 and *sn*-2, respectively. The third carbon atom of the glycerol backbone (position *sn*-3) is linked to a phosphate or a phosphorylated alcohol. To enter in the eicosanoid cascade, phospholipase A2 (PLA_2) must first release sn-2 fatty acids from phospholipids at the membrane interface [57].

PLA_2 represents a superfamily of at least 15 groups that have wide-ranging roles in biological processes [58]. There are at least three classes of phospholipases that mediate the release of AA from membrane lipids: cytosolic ($cPLA_2$), secretory ($sPLA_2$), and calcium-independent ($iPLA_2$). Among them, the highly regulated $cPLA_2$ is one of the most important for eicosanoids biosynthesis [59, 60]. Chemical and physical stimuli activate the Ca^{2+}-dependent translocation of $cPLA_2$, which has a high affinity for AA in membranes, where it releases arachidonate (Fig. 3) [61]. On the other hand, $iPLA_2$ is mainly involved in AA release in basal conditions, contributing to membrane remodelling, but not to eicosanoid synthesis. This is because most of the AA liberated by $iPLA_2$ is reincorporated into cell membranes, resulting in insignificant accumulation of free AA substrate for conversion by cyclooxygenases [62]. Most isoforms of $sPLA_2$ generally function extracellularly where it hydrolyses phospholipid substrates [63]. AA can also be released by a combination of phospholipase C or D and diglyceride lipase actions.

Following mobilization, intracellular AA is oxygenated by four separate routes: the enzymatic cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase (cytP450) pathways, and the non-enzymatic isoprostane pathway.

The synthesis of local eicosanoids is dictated, among other factors, by:

- substrate lipid species;
- expression pattern of the different enzymes that belong to the AA cascade;
- pathways engaged;
- types of cells; and
- how the cell is stimulated.

Myeloid cells at different stages display specific agonist and phenotypic eicosanoid profiles. For example, human non apoptotic PMN cells from peripheral blood produce predominantly LTB_4, while apoptotic PMN cells produce PGE_2, LXB_4 and RvE_2, which signal to resolution of inflammation. Indeed, as stated above, the biosynthesis profile of eicosanoids is cell-type specific, as observed by the expression pattern of different enzymes of the AA cascade in specific cells. For example, PGI_2 is the main product of vascular endothelial cells, while platelets produce mainly TXA_2. Furthermore, the quantities produced are altered by the activation state and the physiological conditions of the specific tissues in which they reside. For example, the eicosanoid metabolism differs in profile and in quantity between macrophages of different tissue origins and activation states [64].

2.2.2 Cyclooxygenase Pathways

Cyclooxygenases are glycosylated, homodimeric, membrane-bound, heme-containing enzymes that catalyze the first committed step in the synthesis of prostaglandins, prostacyclin and thromboxanes (collectively called prostanoids) [65]. COX catalyzes two sequential reactions where AA is converted to PGG_2, and subsequently to PGH_2, an unstable transient precursor of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, TXA_2 and PGI_2 [66] (Fig. 5). Two isoforms of cyclooxygenases were initially described in humans: a constitutive (COX-1) and an inducible (COX-2). Later, a splice variant of COX-1 mRNA that retains the intron-1 gene sequence was identified and named COX-3 [67]. COX-1 is constitutively expressed in different tissues (e.g., gastric mucosa) and is believed to have crucial functions in homeostatic

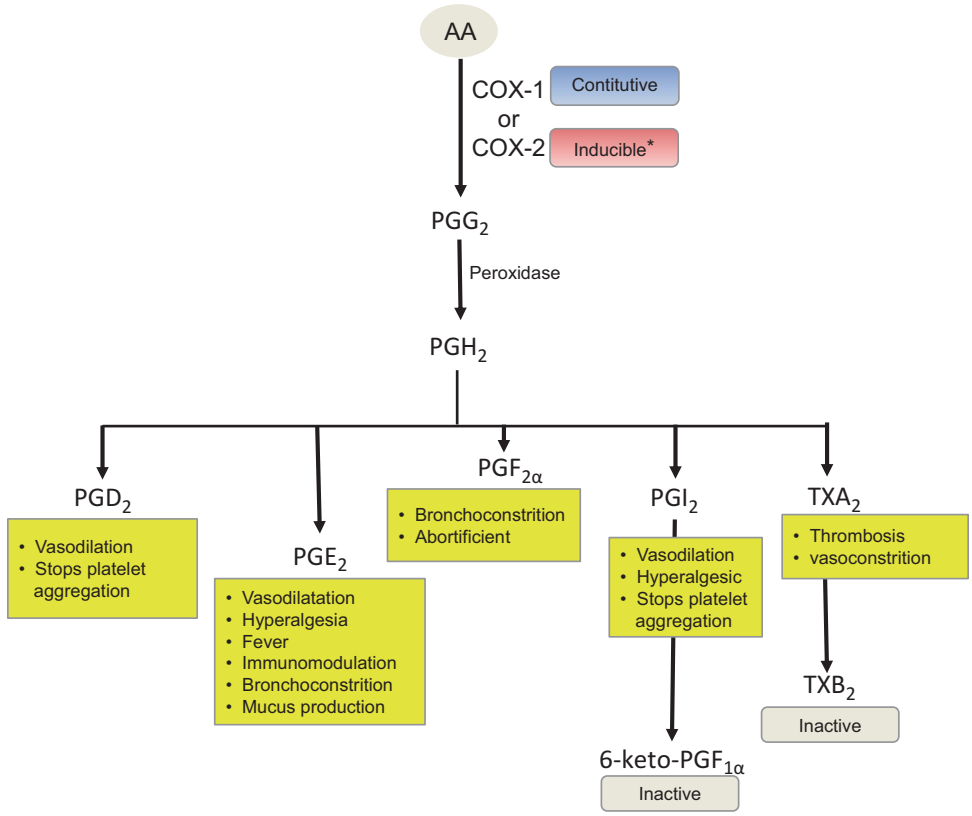

Fig. 5 Overview of cyclooxygenase (COX) pathway from AA to prostaglandins (PGD_2, PGE_2 and PGF_{2a}), prostacyclin (PGI_2) and thromboxanes (TXA_2 and TXB_2). The main bioactions of these eicosanoids are also outlined. Generally, the expression of COX-1 and COX-2 is considered constitutive and inducible, respectively. However, COX-2 is also constitutive in some parts of the nervous and the vascular systems

processes with roles in vascular homeostasis, platelet function and antithrombogenesis, renal and gastrointestinal blood flow, and also in renal function and intestinal mucosal proliferation. COX-2 is inducible and generally expressed when needed during inflammation and different physiological processes such as ovulation, placentation and mitogenesis (especially in the gastrointestinal epithelium). Although they are similar in structure and function, COX-2 utilizes endogenous AA while COX-1 preferentially uses AA derived from exogenous sources, such as dietary intake or released during acute inflammation or cell injury [68]. In humans, COX-3 mRNA is found mainly in the central nervous system, and is also described in several other tissues such as human aorta, and rodent heart, endothelium, kidney and neuronal tissues. However, there has been limited success in isolating the resultant enzyme in humans. COX-3 is known to be less potent and to produce less prostaglandin E_2 than either COX-1 or COX-2 [67, 69, 70].

Prostanoids are released during the inflammatory response by immune cells, local tissues and blood vessels. PGE_2, PGI_2 and PGD_2 in combination with histamine and bradykinin, are potent vasodilators, and thus contribute to redness and increased blood flow in the inflammatory area. These prostanoids also are hyperalgesic, acting as enhancers of responses to painful stimuli by sensitizing afferent C fibres. Besides, they contribute to the increased vascular permeability promoted by histamine and bradykinin. PGE_2 is also pyrogenic (i.e. induce fever) and found in high concentrations in cerebrospinal fluid during

infection. Table 2 summarizes the general bioactions of prostanoids [71].

2.2.3 Lipoxygenases Pathways

In the lipoxygenase (LOX) pathways, AA is converted to several intermediates that culminate in the formation of leukotrienes (Fig. 6) and lipoxins. The biosynthesis of potent bioactive leukotrienes is initiated by the formation of 5-Hydroperoxyeicosatetraenoic acid (5HpETE) from AA by 5-LOX, named for the position that the molecular O_2 is inserted in the fatty acid. This is a calcium dependent process that involves the translocation of both 5-LOX and cPLA to the nuclear envelope. For this purpose, the 5-LOX interacts with a 5-LOX-activating protein (FLAP), enhancing the interaction of 5-LOX to AA. The 5-LOX product 5-HpETE is the direct precursor to LTA_4, which is converted into LTB_4 and LTC_4 by the correspondent LT synthases. LTC_4 is then metabolized to LTD_4 and LTE_4. Together, LTC_4, LTD_4, and LTE_4 represent the cysteinyl-leukotrienes (due to the presence of the amino acid cysteine in their structure) that bind to CysLT1 receptors resulting in important actions on the respiratory and cardiovascular systems. For this reason, cysteinyl-leukotrienes are of particular importance in asthma due to their ability to cause contraction of bronchial muscle. LTB_4 is an important mediator of inflammation acting as a chemotactic agent for neutrophils and macrophages, and stimulating cytokine release by and proliferation of macrophages and lymphocytes [72].

Table 2 Major bioactions of prostanoids in humans

Prostanoid	General bioactions
PGD_2	Induces bronchoconstriction Inhibits platelet aggregation Induces vasodilation Induces relaxation of gastrointestinal and uterine muscles Modifies hypothalamic/pituitary hormones
PGE_2	Induces vasodilatation Induces bronchoconstriction Induces mucus production Promotes fever Induces hyperalgesia
$PGF_{2\alpha}$	Promotes vascular tone Induces bronchoconstriction Induces myometrial contraction Induces abortion
PGI_2	Induces vasodilation Inhibits platelet aggregation
TXA_2	Induces vasoconstriction Promotes platelet aggregation

2.2.4 Cytochrome P450 Pathways

Cytochrome P450 (CYP450) pathways form epoxygenase products and are the major pathways in tissues that do not express COX or LOX, such as, certain cells of the kidneys. CYP450

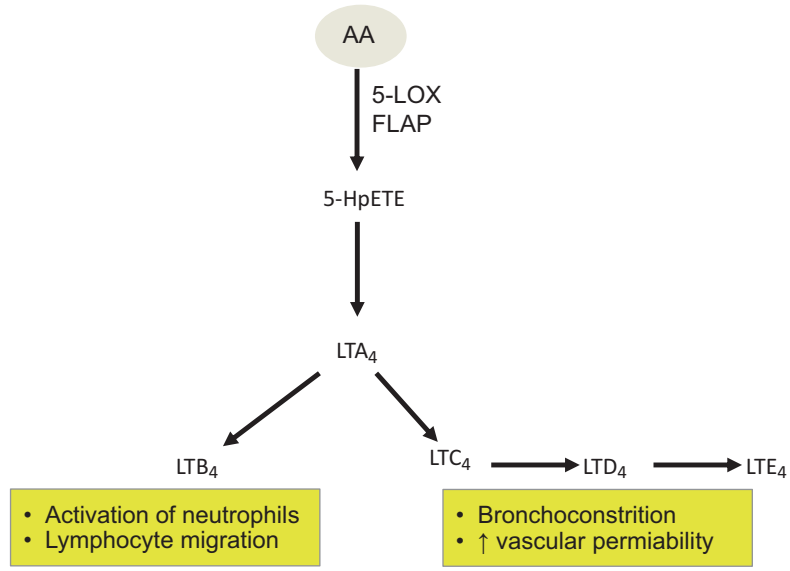

Fig. 6 Leukotrienes biosynthesis from AA and their major bioactions

epoxygenases oxygenate the AA resulting in four epoxyeicosatrienoic acid regioisomers, 5,6-, 8,9-, 11,12-, and 14,15-EET and hydroxyacid derivatives. The EET product depend on which double bond in AA is oxygenated. Dihydroxy derivatives of EETs play physiologic roles such as in renal function, and regulation of smooth muscle cells and vascular tone. Regarding inflammation, specific EETs may play a role in regulating inflammation at certain sites and within distinct tissues [73, 74].

2.2.5 Isoprostane Pathways

The non-enzymatic free radical-initiated peroxidation of AA give rises to a unique series of prostaglandin-like compounds termed isoprostanes (IsoPs) [75]. Although considered products of the AA cascade, isoprostanes are not known to have direct roles in inflammation. Significantly levels of isoprostanes are found in the blood during oxidative stress. Remarkably, isoprostanes can reach much higher levels in the circulation than those of COX products. As isoprostane is a product of cellular oxidation, its levels may be indicative of oxidative stress and it can be used as a marker of oxidative damage that accompanies a wide range of pathologies [76].

2.3 Platelet-Activating Factor (PAF)

Platelet-activating factor - PAF (1-0-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) is a phospholipid classified as autacoid due to its ability to act as a local hormone. PAF was first described by the French immunologist Jacques Benveniste in the 1970s [77]. Benveniste made a great contribution in elucidating the role of PAF and its mechanism of action in diverse pathological and physiological situations [78–80]. This phospholipid was named PAF because it can cause platelet aggregation, which was its first described function [77]. However, after many years of studies, other functions were established for this biological mediator, but its name remained the same. It is well known that PAF is recognized by a specific G-protein receptor (PAFR-platelet-activating factor receptor), which is located in many cell types. PAF is released in the early inflammatory response by a variety of cell types and it works as an intercellular mediator and also as an intracellular messenger [81]. It has been stated that PAF plays a role in the pathogenesis of cancer, asthma, allergy, septic shock and autoimmune conditions [82].

2.3.1 PAF Synthesis

Essentially, all cells can synthesize PAF, but macrophages, monocytes, neutrophils, platelets and endothelial cells are the main producers of PAF [83]. PAF can be synthesized through two different pathways: the lipid remodeling, which is the main one; and the *de novo* synthesis.

2.3.2 Remodeling Pathway

The remodeling pathway begins with the removal of the AA from phosphatidylcholine (PC) by phospholipase A_2 (PLA_2); this reaction forms the intermediate lyso-phosphatidylcholine (lyso-PC). Then, lyso-PC acetyltransferase (LPCAT) transfers an acetyl group from acetyl-CoA to the lyso-PAF to produce PAF (Fig. 7). [83]

As these precursors are bioactive lipids, they are present in many metabolic pathways. Therefore, they are constitutively expressed in the cells. So, due to this availability of precursors, the synthesis of PAF is controlled by the enzyme PAF-AH and by desensitization of PAF receptor. It is already known that there are two groups of PLA_2 involved in the hydrolysis of phosphatidylcholine. None of PLA_2 groups are able to distinguish the length of the alkyl side-chain at sn1 position. Thus the length of sn-1 alkyl group in PAF is different among cell types and species and it reflects the sn-1 alkyl phosphatidylcholine composition of the cell, which is mainly composed by saturated C16 or C18 chains [84]. The Group IVA cytosolic PLA_2, specific for arachidonic acid, depends on calcium to produce AA and lyso-PC quickly and in large amounts. The group VI PLA_{2s}, which is independent of calcium, hydrolyzes unspecific fatty acids slowly and continuously as part of normal membrane remodeling. Therefore, lyso-PC is constantly available for PAF generation, but in small

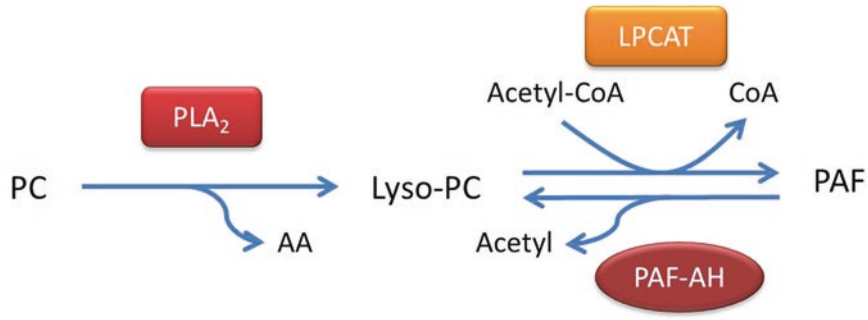

Fig. 7 Synthesis of PAF by lipid remodeling pathway. Phospholipase A2 (PLA2) cleaves arachidonic acid (AA) from phosphatidylcholine (PC) to form lyso-phosphatidylcholine (lyso-PC). Then, lyso-PC receives an acetyl group from acetyl-CoA by lyso-PC acetyltransferase, generating PAF. PAF can be inactivated through acetyl removal by PAF acetyl hydrolase [83]

amounts. Thus, to produce considerable amounts of lyso-PC (and posteriorly PAF) it is necessary to have a trigger, like an inflammatory stimulus. During the production of lyso-PC, AA is also produced. The AA produced can be enzymatically converted to eicosanoids, which are pro-inflammatory lipids with diverse potent actions in inflammation [85]. In the same way, LPCAT also needs a stimulus to change from the basal production of membrane-bound PAF precursor to the production of appreciable amounts of PAF.

2.3.3 De Novo Pathway

The *de novo* pathway has been described as responsible for the maintenance of PAF levels under a condition of physiological cell function. PAF *de novo* synthesis starts with the acetylation of 1-0-alkyl-sn-glycero-3-phosphate (AGP) by acetyl-CoA acetyltransferase (AGP-AT). Then, a phosphohydrolase cleaves the phosphate group of 1-0-alkyl-2-acetyl-sn-glycero-3-phosphate to produce 1-0-alkyl-2-acetyl-sn-glycerol (AAG). AAG has a fatty acid added to sn1 position followed by the addition of phosphocholine in the sn3 position by cholinephosphotransferase (CPT), resulting in the formation of PAF (Fig. 8) [86].

As the *de novo* pathway is constitutively active and in low activity, it does not need a rigid control of PAF synthesis as the remodeling pathway does. However, PAF synthesis is controlled by acetyl-CoA acetyltransferase that has a low activity, and by substrate availability. DTT-I-CPT does not have the same role, since it has high cellular activity [87].

PAF has a variety of homologues (variation in the length of alkyl group – sn1 position) and analogues (modification in sn1, sn2 or sn3 position). PAF homologues have the same biological function and they are the result of different cellular composition of alkyl phosphatidylcholine. PAF analogues have reduced or different biological function and they are the result of PAF metabolism. Modification in the alkyl group (sn1 position) or its removal can decrease or abolish PAF function. Also, modification of the short-chain acyl residue (sn2 position) or demethylation of phosphocholine head group (sn3 position) can reduce PAF biological activity or even abolish it [86].

After PAF is synthesized, it can be secreted, and it is now clear that the percentage secreted varies dramatically in different cells and under different conditions. Endothelial cells secrete little or none of the PAF they synthesize. To facilitate PAF secretion, it binds to albumin, and when it gets into the plasma, PAF associates with low-density and high-density lipoproteins [88].

2.3.4 PAF Signalling Pathway and Inflammation

The secreted PAF is recognized by a receptor from the rhodopsin family, PAFR, which is a G-protein coupled receptor with seven transmembrane domains. This receptor is not specific for PAF, since it also recognizes and binds to oxi-

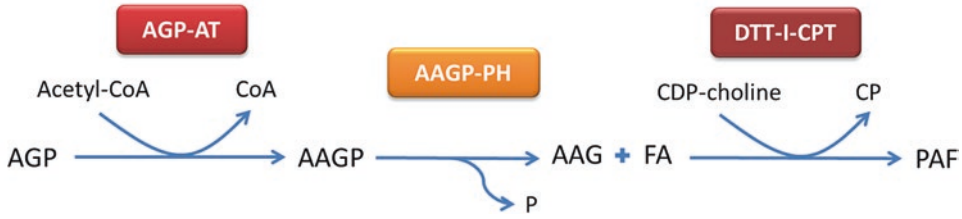

Fig. 8 De novo synthesis of PAF pathway. Acetylation of AGP (1-0-alkyl-sn-glycero-3-phosphate) by AGP-AT (1-0-alkyl-sn-glycero-3-phosphate: acetyl-CoA acetyltransferase). AAGP-PH (phosphohydrolase) cleaves the P (phosphate group) of AAGP (1-0-alkyl-2-acetyl-sn-glycero-3-phosphate) to produce AAG (1-0-alkyl-2-acetyl-sn-glycerol). AAG has a FA (fatty acid) added to sn1 position followed by the addition of phosphocholine from CDP-choline (cytidine diphosphocholine) in the sn3 position by dithiothreitol-insensitive cholinephosphotransferase (DTT-I-CPT), resulting in the formation of PAF [86]

dized phosphatidylcholine (PAF-LL) [89] and to bacterial membrane compounds, such as lipopolysaccharide [90]. PAFR is distributed within many organs, like kidneys, small intestine, lungs, brain and liver, and it has low expression in skeletal muscle, stomach, heart and pancreas [91]. When PAFR is activated by PAF, PLCβ-mediate the hydrolysis of PIP_2 to produce IP_3 and DAG, leading to a transient elevation of cytosolic Ca^{2+} released from intracellular stores and to activation of PKC. The increase of cytoplasmic Ca^{2+} also activates PLA_2 that can drive the synthesis of PAF. Therefore, when PAF binds to PAFR, it activates phospholipase A_2 e phospholipase C. Phospholipase A_2 contributes to the production of more PAF and phospholipase C induces the increase in intracellular Ca^{2+} and activates protein kinase C [92]. This process happens in different types of physiological events, leading to an increase in vascular permeability, leukocyte adhesion and stimulation of uterine contraction [77].

In pathological conditions, PAF triggers the activation of platelets, neutrophils and monocytes, followed by migration, adherence and interaction of platelets and leukocytes with endothelial cells [93]. It also activates endothelial cells and stimulates endothelial cell migration and angiogenesis [94]. In addition, PAF can induce the expression of many genes, including iNOS, MMP-9, COX-2, IL-6, TIMP-2 and MT1-MMP. Taken together, all these effects triggered by PAF activation contribute to the inflammatory response.

PAF has been studied for its action as a mediator of inflammation. It functions as a protective molecule in the innate host defense, but it is also involved in uncontrolled inflammation leading to pathological conditions. It has been described that PAF contributes to sepsis, allergy, stroke, anaphylaxis, ulcerative colitis, cancer and multiple sclerosis [91, 94–98]. PAF signaling is activated in sepsis and its dysregulation is involved in the pathobiology of lethal septic syndromes [93]. PAF-mediated events alter vasoreactivity, increases vascular permeability, and may cause tissue injury and disruption of host defenses that then predispose to bacteremia or endotoxemia leading to systemic manifestations of sepsis.

Due to differences in the length of the alkyl group of PAF, this molecule can trigger different responses. For example, C16-PAF and C18-PAF act through different pathways leading to the same result: death of cerebellar granule neurons [99]. In addition, this variety in the alkyl group can cause different signaling by PAFR, causing or preventing apoptosis, for example. Analogues of PAF may also have biological activities, and they can act in the same way as PAF/precursors or they can exert opposite effects. The edelfosine (PAF analogue) has a potential anticancer effect, while PAF is associated with cancer and metastasis [100]. Phosphatidylethanolamine (a PAF analogue) has an activity similar to that of PAF, but this analogue is less potent. Many studies have demonstrated that some types of cancer overexpress PAF and PAFR in cancer cells. Besides this, PAFR antagonists are able to inhibit cancer cell growth and motility [94]. Thus, PAF can play dual role in immune response and inflammation.

2.4 Reactive Oxygen Species (ROS)

Free radicals are naturally produced by cells and have the oxygen as a principal element for oxidative phosphorylation. Free radicals are characterized by one or more unpaired electrons and they were described for the first time in biological materials in 1954 [101]. Fridovich and co-workers described that the biological systems are able to convert oxygen into a compound with high reactivity, the anion superoxide [102]. In 1973, Babior described the production of anion superoxide by phagocytizing leukocytes, suggesting the involvement of reactive oxygen species (ROS) in host defense [103]. Professional phagocytes such as neutrophils, eosinophils and mononuclear phagocytes produce anion superoxide at the expense of a large amount of oxygen. This abrupt rise of oxygen consumption is called respiratory burst [104].

Reactive oxygen species are reactive molecules generated by an incomplete reduction of oxygen generating oxygen radicals such as superoxide anion (O^{-}_{2}), hydroxyl (OH•), peroxyl ($RO•_2$) and alkoxyl (RO•), and nonradicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1O_2$). The generation of ROS consists in a cascade reaction that starts with the production of superoxide anion (O^{-}_{2}) [105].

1. $O_2 + NADPH + H^+ \rightarrow 2O^{-}_{2} + NADP^+$
2. $2O^{-}_{2} + 2H^+ \rightarrow H_2O_2 + O_2$
3. $O^{-}_{2} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$

ROS drive opposite actions depending on the context. Once formed, ROS can react with a large number of molecules including lipids, proteins, carbohydrates and nucleic acids, promoting irreversible damage or alteration in the function of the target molecule. Due to this stress mediation, ROS is associated with a large number of diseases including cardiovascular disease [106], Alzheimer's disease [107] and pulmonary disease [108]. On the other hand, ROS have a beneficial role due to the involvement in host defense, leading to the death of invading pathogens and serving as inflammatory mediators [109].

2.4.1 Source of ROS

ROS can be produced by enzymatic and non-enzymatic ways. The enzymatic source includes a membrane-associated enzyme, which catalyzes the one-electron reduction of oxygen, consuming one NADPH [104]. Although there are other endogenous sources of ROS such as xanthine oxidase, myeloperoxidase and p450 cytochrome, the Nicotinamide Adenine Dinucleotide Phosphate-NADPH-oxidase (NOX) is associated to several diseases and it is well characterized. Two membrane subunits (gp-91phox/NOX2 and p22phox), three cytosolic subunits (p47phox, p67phox and p40phox) and the G-protein Rac compose the enzyme NOX [110]. There are seven members of the Nox family: Nox1, Nox2, Nox4, Nox5, Duox1 and Duox2. In resting phagocytes, NADPH oxidase is unassembled and inactivated. Once the phagocyte is exposed to an effective agonist, the cytoplasmic subunit p47phox is phosphorylated by GTP. This phosphorylation promotes a conformational change in the complex, which allows the cytoplasmic subunits p47phox, p40phox and p67phox to associate with the membrane subunits gp91phox and p22phox. Once activated, the enzyme complex transports electrons from the cytoplasmic NADPH to the extracellular space or to phagosomal oxygens, generating superoxide anions (Fig. 9) [111].

The non-enzymatic source includes prosthetic groups such as flavins or iron-sulfur clusters, and xenobiotics such as the anticancer agent Adriamycin and the mitochondrial electron transport chain, which contain redox centers that can leak electrons to oxygen [112]. The respiratory chain complexes I and III appear to be responsible for most part of anion superoxide production by the single electron reaction, which favour the monovalent reduction of oxygen [113].

2.4.2 ROS in Host Defense and Inflammation

Cells from the innate immune system use the NADPH oxidase complex to promote the death of pathogens. After an appropriate stimulus, phagocytes increase the uptake of O_2, leading to a state of respiratory burst. The activation of NADPH

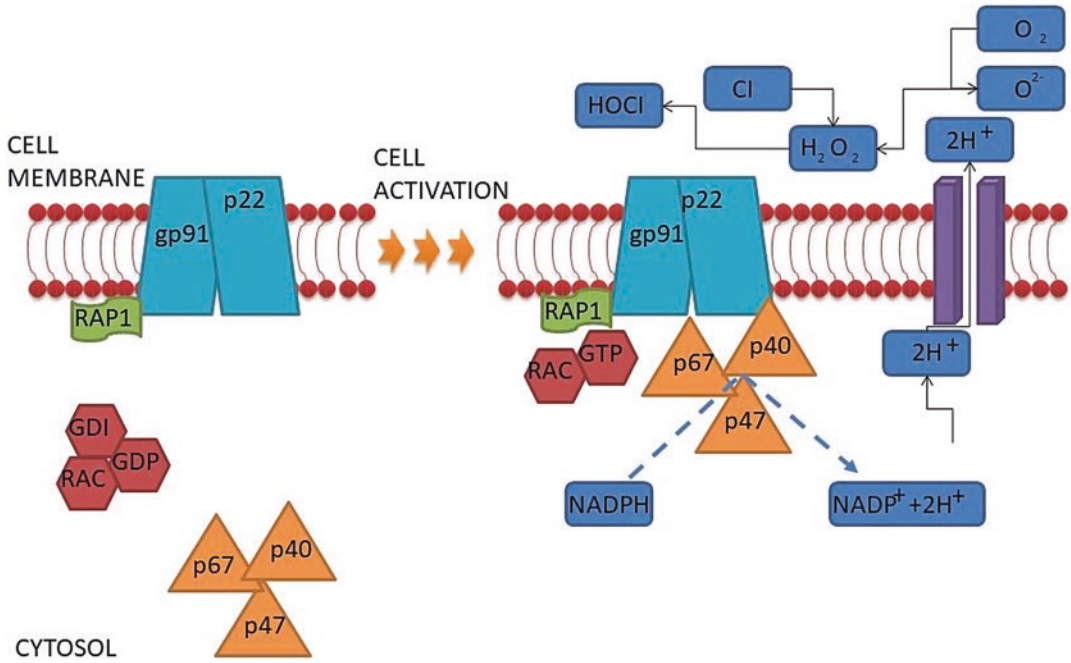

Fig. 9 Activation of NADPH oxidase complex. The NADPH oxidase is unassembled and inactive in physiological conditions. Upon stimulation, phosphorylation of p47phox induce conformational change in the complex, allowing the subunit p47phox, p40phox and p67phox to associate with the membrane complex gp91phox and p22phox. The active complex transfers electrons from the substrate to oxygen. The activation of the complex also requires RAC complex. Upon activation, RAC lose the interaction with the inhibitor GDI and binds to GTP, which translocate to the membrane along with p47phox, p40phox, p67phox

oxidase complex reduces O_2 to superoxide anion, which is required to kill some bacteria [114]. However, superoxide has a low bactericidal action, so H_2O_2 also participates in bacterial killing. H_2O_2 can react with O_2^- to produce hydroxyl radical, which is highly reactive and toxic. H_2O_2 can also react with chloride, being a substrate for MPO to produce *hypochlorous* acid (HOCl), one of the most microbicidal agents [115].

It is becoming evident that ROS can initiate and amplify inflammation by the upregulation of several different genes involved in the inflammatory response, such as those, which produce pro-inflammatory cytokines and adhesion molecules [116]. ROS serves as a second messenger in the inflammatory response. It can modulate the transcription factor nuclear factor-kappa B (NFκB) dependent transcription in early toll-like receptor (TLR)-4 mediated cellular response [117] and in response to antigen-containing immune complexes through FcγR [109].

2.5 Nitric Oxide

Molecular oxygen can form free radicals, which can damage molecules and play a central role in oxidative stress. In contrast, nitrogen gas does not have the same properties. Nitric oxide (NO) is a hybrid between molecular nitrogen and oxygen and is normally less reactive than oxygen, since NO has only one unpaired electron [118]. NO is considered a biological messenger molecule and it is involved in diverse biological processes including vasodilation, bronchodilation, neurotransmission, tumor surveillance, inhibitor of platelet aggregation, antimicrobial defense and regulation of inflammatory-immune response [119].

2.5.1 NO Synthesis

The synthesis of NO occurs by the conversion of the amino acid L-arginine to L-citrulline, mediated by a family of isoforms known as nitric oxide synthases. There are three NOS isoen-

zymes, which are encoded by distinct genes: NOS1, known as neuronal NOS (nNOS); NOS2, known as inducible NOS (iNOS); and NOS3, known as endothelial NOS (eNOS) [120].

eNOS is a constitutively expressed enzyme that is found mainly in the endothelium and in all blood cells [121]. Endothelium-derived NO is a homeostatic controller of physiologic functions of the endothelium, including the control of vascular tone, leukocyte migration and blood clotting [121]. In physiological conditions, eNOS is a beneficial enzyme against diseases. In contrast, the expression of iNOS is marked under inflammatory conditions, following cell stimulation by immunological or microbial stimuli [122].

2.5.2 NO in Host Defense and Inflammation

It has been demonstrated that NO has an effective participation in host response to infection. NO production by iNOS isoform is induced by microbial products and pro-inflammatory cytokines including TNF-α, INF-γ, IL-1 and IL-12 [123]. There is a direct correlation between NO production and the ability of the host to contain microbial proliferation in many animal models [123]. The NO specie is freely permeable to membranes and repeatedly diffuses, allowing the pathogen killing in a widespread way by damaging lipids, proteins and nucleic acids. NO combines with superoxide radical to form peroxynitrite (ONOO-) that has microbicidal effect in several bacterial species. NO also plays a role in the eradication of virus infection by nitrosation of cysteines residues from proteins which are important for viral infectivity, replication and maturation [124].

NO is clearly involved in inflammatory reactions due to the vasodilation role. Under inflammatory stimuli iNOS releases high amount of NO [125]. iNOS is highly expressed upon activation of the transcription factor nuclear factor-kappa B (NFκB) in response to cytokines, bacterial and viral components [126]. NO is generated in high levels during inflammatory response, such as asthma [127] and also in the skin, enhancing vascular permeability, erythema and cellular infiltration. Although, the role of NO in this process is not fully understand, during contact allergen-induced skin inflammation, NO has dual role. At low concentration, NO has pro-inflammatory actions by vasodilation and neutrophil recruitment. On the other hand, at high concentration, NO has anti-inflammatory role by downregulation of adhesion molecules and inducing apoptosis in inflammatory cells [128].

2.6 Cytokines

Cytokines are polypeptides produced by various cell types in response to different stimulus. It depends on the binding to their specific receptors to mediate the communication between leukocytes. They act as mediators of inflammation and immune response. Although there is a variety of cytokines with different functions and actions, they are synthesized in response to external stimuli such as microbes products, pattern recognition or others cytokines [129]. There are different types of cytokines, including interleukins, interferons and tumor necrosis factor TNF, which can act alone, synergistically, or even by modulating the functions of each other, to perform the proper regulation of immune system.

2.6.1 Interleukins

Since the discovery of IL-1 in 1977 [130], interleukins have been studied and grouped in the same family based on the sequence homology, receptor chain similarity or biological properties [131]. IL-1 was the first protein described in the induction of fever and in the stimulation of the acute phase response. Besides the induction of other pro-inflammatory mediators, IL-1 also upregulate cell adhesion molecules that are crucial to an effective defense mechanism. Two different molecules were described: IL-1α that is bound in the membrane and IL-1β that is secreted. IL-1α is produced in a biologically active form, whereas IL-1β is translated into pro-IL-1β, which needs to be cleaved by caspase-1 to produce the biologically active form [132]. Over the past few decades, several other members of the IL family have been identified (IL-1 to IL-37) and their role in innate and adaptive immune responses has

Table 3 Interleukin families

Family	Members	Receptors	Functions
IL-1	IL-1α, IL-1β, IL1Ra, IL-18, IL-33, IL-37	IL-1RI, IL-1RII, IL-18R, ST2	Effects on cell proliferation, differentiation and function of many innate and specific immune competent cells [130]. Pro-inflammatory effects – except for IL-37, with the suppression of cytokines effects [134]
Common γ-chain	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21	IL-2R, IL-4R type I and type II, IL-7R, sIL-7R, IL-9R, IL-15R, IL-21R	Growth and proliferation factors for progenitors and mature cells and also have roles in lineage-specific cell differentiation
IL-10	IL-10, IL-19, 1 L-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, IL-29	IL-10R1/IL-10R2 complex, IL-20R1/ IL-20R2, IL-22R1/ IL-20R2, IL-10R2/ IL20R1, IL-28R1/ IL10R2	Inhibit the expression of many pro-inflammatory cytokines, chemokines and chemokines receptors. Regulate allergen tolerance [135, 136]
IL-12	IL-12, IL-23, IL-27, IL-35	IL-12Rb1, IL-12Rb2, IL-23R, WSX-1, gp130	Depend on cell expression in different cell types and combinations of different receptors chains
Th2 like	IL-4, IL-5, IL-9, IL-13, IL-25, IL-31 and IL-33		Mediate immune response against helminthic infections, IgE production during allergic response and eosinophilia
ILs with chemokine activities	IL-8 and IL-16	CXCR1, CXCR2 and CD4	Neutrophil-specific and T cell chemotactic factors
IL-17	IL-17A,IL-17B, IL17C, IL-17D, IL-17F	IL-17RA, IL-17RB	Pro-inflammatory effects mainly in chronic neutrophilic diseases
Others	IL-3, IL-6, IL-11, IL-14, IL-32, IL-34	CD131, IL-6R/gp130, IL-1R/gp130, (CSF)-1R	Growth factors [137], regulation of immune responses [138], stimulation of hematopoiesis [139], stimulation of B cell proliferation [140], stimulation of pro-inflammatory cytokines [141], stimulation of monocyte proliferation

This table was adapted from Akdis et al. [133]

been elucidated [133]. The families of ILs are described in the following table (Table 3).

2.6.2 Tumor Necrosis Factor (TNF)

TNF was discovered in 1975 as an endotoxin inducible molecule that caused necrosis of tumors *in vitro* [142]. Later, it was described that TNF was also an essential mediator of inflammation [143]. TNF is a transmembrane protein with 26 kDa that is cleaved by metalloproteases [144], releasing a trimeric, soluble 17 kDa-cytokine of TNF (sTNF) that is found in the plasma. Both soluble and membrane forms bind to two transmembrane receptor molecules, TNFR1 and TNFR2. TNFR1 signaling induces apoptosis pathways and conversely, TNFR2 signaling triggers cell survival pathways [145].

The major role of TNF is in the host defense. TNF is important for the response to infections; however, an excessive production can be dangerous [146]. TNF has effects on the vascular endothelium and endothelial leukocyte interaction [146]. TNF can also induce the expression of COX2, which results in vasodilation, causing the *rubor* and *calor* observed during an inflammatory response [147]. TNF mediates the increase in vascular permeability and induces the expression of pro-coagulant proteins that can cause intravascular thrombosis [148]. Small molecules or therapeutic proteins (such as monoclonal antibodies) can be used for anti-TNF therapy and TNF is well established as an effective target to control certain human diseases [149].

2.6.3 Interferons

Interferons (IFNs) are cytokines that have a role in the antiviral immune response. There are three distinct types of IFNs recognized (Type I, Type II

and Type III) based on their receptor usage, structural and biological activity [150]. Type I INF consists in a big family including 13 members such as INF-α and IFN-β, which binds to a cell-surface receptor, the type I IFN receptor [151]. Type II IFN consists in only one member, IFN-γ, which binds to another cell-surface receptor, known as type II IFN receptor [152]. Type III IFN consist in three members such as IFN-λ1, IFN-λ2 and IFN-λ3 that signalling by a distinct receptor complex composed of two subunits, the IFN-λR1 and IL-10R2 [153]. The name interferon came from the ability to restrict viral replication and all types of IFNs signal through Janus-kinases/signal transducers activation of transcription (Jak-STATs) pathway. Type I interferon also enhances the action of dendritic cells and monocytes, promotes CD4+ and CD8+ T cells responses, and enhances NK cell and B cell responses [154]. Type II IFN is an essential regulator of immune responses, since it inhibits viral replication, induces a pro-inflammatory CD4+ T cell response (Th1), regulates macrophage activation and improves antigen recognition [155]. Type III IFN induce antiviral activity in cells, upregulate MHC class I and is usually coexpressed together with type I IFN [156].

2.7 Chemokines

Chemokines are chemotactic cytokines of the immune system that control the migration and residence of all immune cells in tissues. Chemokines are a family of small proteins that are classified into four major groups, according to the arrangement of the cysteine residues (C) conserved in the mature proteins (C-, CC-, CXC and CX3C chemokines). There are approximately 50 endogenous chemokine ligands and 20 G protein–coupled seven-transmembrane signaling receptors that are critical for the generation of immune responses (Table 4) [157].

Chemokines control homeostatic cellular migration during tissue development and maintenance. They also control innate and adaptive responses against pathogens. In the context of inflammation, the main function of chemokines is the guidance of leukocytes during their recruitment into inflammatory sites. They are produced after stimulation by cytokines or microbial products. After a tissue injury, local cells are activated and release inflammatory cytokines and chemokines that promote the entry of additional innate effector cells such as neutrophils and monocytes. These cells follow chemokine gradients to the site of inflammation with the purpose of controlling the damage [158].

3 Plasma Driven Mediators

A range of phenomena in the inflammatory response is mediated by plasma proteins belonging to three interrelated systems: the complement, coagulation and kallikrein-kinins systems. In general, plasma derived mediators are produced in the liver and, once released into the bloodstream, they can contribute to important functions of innate immunity, coordinating various events during inflammation, defending the host against pathogens and bridging innate and adaptive immune responses.

3.1 Complement System

The complement system plays a key role in the innate immune response contributing to the recognition of endogenous danger signals including dying host cells or abnormal molecular structures. In addition, it contributes to the elimination of invading pathogens, acting as a bridge between innate and adaptive immunity [161]. This is a complex system that consists of a series of proteins that are synthesized in the liver and released into the bloodstream [162]. The components of the complement system are found as inactive precursors (zymogens), which are activated by a dependent or an independent IgM/IgG manner, in the plasma and on cell surfaces. The complement mediates early host defense responses through a coordinated sequential enzyme cascade to fight against pathogens through recognition, opsonization and lysis. On the other hand, the complement is also involved in the control of inflammatory

Table 4 Chemokine nomenclature and key immunoregulatory functions

Chemokine	Other names	Receptor	Functions
CXCL1	GROα, MGSA, mouse KC	CXCR2	Promotes neutrophils trafficking
CXCL2	GROβ, MIP-2α, mouse MIP2	CXCR2	Neutrophils trafficking
CXCL3	GROγ, MIP-2β	CXCR2	Neutrophils trafficking
CXCL4	PF4	CXCR3B	Pro-coagulant
CXCL5	ENA-78, mouse LIX	CXCR2	Neutrophils trafficking
CXCL6	GCP-2 (human)	CXCR1, CXCR2	Neutrophils trafficking
CXCL7	NAP-2	CXCR2	Neutrophils trafficking
CXCL8	IL-8 (human)	CXCR1, CXCR2	Neutrophils trafficking
CXCL9	Mig	CXCR3	Th1 response; Th1, CD8, NK cells trafficking
CXCL10	IP-10	CXCR3	Th1 response; Th1, CD8, NK cells trafficking
CXCL11	I-TAC	CXCR3	Th1 response; Th1, CD8, NK cells trafficking
CXCL12	SDF-1	CXCR4	Bone marrow homing
CXCL13	BLC, BCA-1	CXCR5	B cell and T_{FH}-positioning in the lymph nodes
CXCL14	BRAK	Unknown	Macrophages skin homing (humans)
CXCL15	Lungkine (only in mouse)	Unknown	Not determined
CXCL16		CXCR6	NKT cells and ILCs migration and survival
CCL1	I-309, mouse TCA3	CCR8	Th2 and Treg cells trafficking
CCL2	MCP-1, mouse JE	CCR2	Inflammatory monocytes trafficking
CCL3	MIP-1α	CCR1, CCR5	Macrophages and NK cells migration;
CCL4	MIP-1β	CCR5	T cells–DCs interactions
CCL5	RANTES	CCR1, CCR3, CCR5	Macrophages and NK cells migration;
CCL6	C-10, MRP-1 (only in mouse)	Unknown	Not determined
CCL7	MCP-3, mouse Fic or MARC	CCR2, CCR3	Monocytes mobilization
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5 (human); CCR8 (mouse)	Th2 response; skin homing (mouse)
CCL9/10	MIP-1γ, MRP-2 (only in mouse)	Unknown	Not determined
CCL11	Eotaxin-1	CCR3	Eosinophils and basophils migration
CCL12	MCP-5 (only in mouse)	CCR2	Inflammatory monocytes trafficking
CCL13	MCP-4	CCR2, CCR3, CCR5	Th2 responses
CCL14	HCC-1	CCR1	Not determined
CCL15	Leukotactin-1, HCC-2, MIP-5 (human)	CCR1, CCR3	Not determined
CCL16	HCC-4, NCC-4, LEC	CCR1, CCR2, CCR5	DCs maturation factor
CCL17	TARC	CCR4	Th2 responses, Th2 cells migration, Treg cells homing to the lungs and skin
CCL18	PARC, DC-CK1 (human)	CCR8	Th2 response; marker of AAM, skin homing
CCL19	ELC, MIP-3β	CCR7	T cells and DCs homing to lymph nodes
CCL20	MIP-3α, LARC	CCR6	Th17 responses; B cells and DCs homing to the gut-associated lymphoid tissue

(continued)

Table 4 (continued)

Chemokine	Other names	Receptor	Functions
CCL21	SLC, 6CKine	CCR6, CCR7	T cells and DCs homing to lymph nodes
CCL22	MDC	CCR4	Th2 response, Th2 cells migration, Treg cells migration
CCL23	MPIF-1, MIP-3 (human)	Unknown	Not determined
CCL24	Eotaxin-2, MPIF-2	CCR3	Eosinophils and basophils migration
CCL25	TECK	CCR9	T cell homing to the gut; thymocytes migration
CCL26	Eotaxin-3	CCR3, CX3CR1	Eosinophils and basophils migration
CCL27	CTAK	CCR10	T cells homing to the skin
CCL28	MEC	CCR3, CCR10	T cells and IgA plasma cells homing to mucosa
XCL1	Lymphotactin α, SCM-1α	XCR1	Cross-presentation by $CD8^+$DCs
XCL2	Lymphotactin β, SCM-1β	XCR1	Cross-presentation by $CD8^+$DCs
CX3CL1	Fractalkine	CX3CR1	NK cells, monocytes, and T cells migration

This table was adapted from Bachelerie et al. and Sokol and Luster [159, 160]. AAM, alternatively activated macrophage; GALT, gut-associated lymphoid tissue; GCP, granulocyte chemotactic protein; HPC, hematopoietic progenitor cell; ILC, innate lymphoid cell; IP-10, interferon-induced protein of 10 kDa; I-TAC, interferon-inducible T-cell α-chemoattractant; NAP, neutrophil-activating protein; NK, natural killer; NKT, natural killer T; PF, platelet factor; SDF-1, Stromal cell-derived factor-1; TECK, thymus expressed chemokine; TFH, follicular helper T cell; Th1, type 1 helper T; Treg, regulatory T

responses, since it binds to immune complexes and apoptotic cells, and assists in their removal at inflammatory sites [163]. Currently, there are three well-established pathways involved in complement activation: Classical, Alternative and Lectin (mannose-binding lectin) pathways. The mechanisms of activation and the consequent responses to these pathways are discussed below.

3.1.1 Classical Pathway

The classical pathway is initiated by the interaction between IgM or IgG with the antigens, leading to a complex formation. The complex antigen/antibody binds to C1q (one of the three proteins of the C1 complex) leading to the activation of C1r, which in turn activates the pro-enzyme C1s (Fig. 10). Then, the activated C1s acts in the next two components of the classical pathway, cleaving C4 and C2 to generate two fragments, C4b and C2a, which together form the *C3 convertase* of the classical pathway, which in turn cleaves C3 into C3a and C3b (Fig. 10) [164]. While C3a acts as a chemoattractant of leukocytes (anaphylatoxin), C3b binds to the C4b2a complex to form *C5 convertase*. The *C5 convertase* initiates the formation of the Membrane Attack Complex (MAC), which inserts pores into bacterial membrane, leading to its lysis [165]. The classical pathway can be activated independently of antibodies; it can also be activated by other danger signals such as C-reactive protein, viruses, apoptotic cells and others [163, 166].

3.1.2 Alternative Pathway

The alternative pathway is based on observations that the complement system is activated by direct binding to molecules of the microbial surface and is independent of antibody interaction. The alternative pathway is continuously activated at a low level as a result of spontaneous C3 hydrolysis due to the breakdown of the internal thioester bond (highly reactive) which resembles C3b [$C3(H_20)$]. This C3b binds to Factor B, forming *C3 convertase* C3bBb [or C3(H2O)B] and then it is cleaved by Factor D (a process which is stabilized by magnesium ions) (Fig. 10). C3bBb is a proteolytic complex that initiates the amplification process by forming more C3b molecules, constructing further C3bBb convertases (*C3 convertase*), resulting in surface deposition of C3b

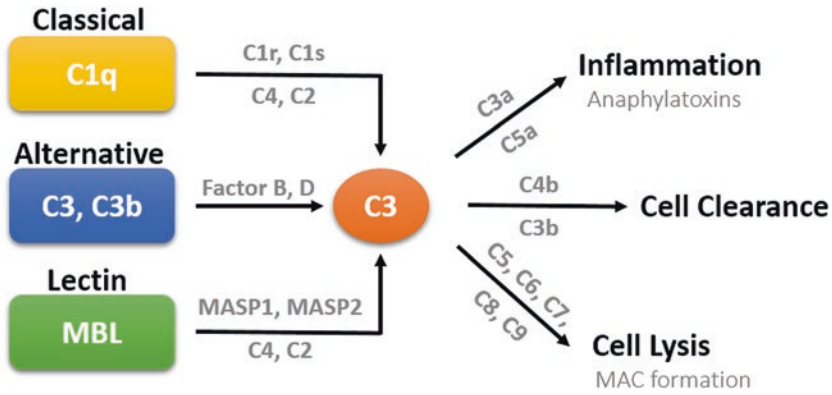

Fig. 10 Pathways of the complement system. The main function of the complement system is the host protection against infection/inflammation. After the activation of the complement system, all the three pathways (Classical, Alternative and Lectin) lead to the generation of *C3 convertase* that cleaves the C3 protein into C3a and C3b. While C3a acts as an anaphylatoxin, C3b covalently binds to the activating surface and participates in the self-activation loop of complement activation via the alternative pathway. C3b also associates with *C3 convertases* (C4b2a or C3bBb) to form the *C5 convertase*, which cleaves C5 complement into C5a and C5b. The interaction of C5b with C6, C7, C8 and C9 leads to the formation of MAC, a multimolecular structure that inserts into the membrane, creating a functional pore leading to cell lysis. The anaphylatoxins (C3a and C5a) are key players in the recruitment of inflammatory cells and release of mediators that amplify the inflammatory response

molecules. C3b is able to create a new *C3 convertase* in the presence of Factors B and D, thus acting as an 'amplification loop' for other pathways, as well as for the alternative pathway. The alternative pathway is independent of the components C1, C2 and C4 (Fig. 10) [163, 164, 167, 168].

3.1.3 The Mannose-Binding Lectin Pathway

Like the alternative pathway, the mannose-binding lectin pathway may be activated in the absence of immune complexes. The MBL-pathway is initiated by molecules called collectins (MBL and ficolin), which are multimeric lectin complexes. In this pathway, MBL binds to specific carbohydrate patterns that are common to pathogens, activating the pathway through the enzymatic activity of MASPs (MBL-associated serine proteases) (Fig. 10). When the carbohydrate-recognizing heads of MBL bind to specifically arranged mannose residues on the surface of a pathogen, MASP-2 is activated to cleave complements C4/C2, generating C4b2a, the classical and MBL-pathway *C3 convertase*, which is capable of enzymatically split hundreds of molecules of C3 into C3a and C3b. While MASP-2 is effective in generating *C3 convertase*, MASP-1 may also cleave C3, but at a very slow rate [169, 170]. Neither MASP-1 nor MASP-3 can cleave C4 and, therefore, they cannot compensate for the absence of MASP-2. Thus, the formation of the MBL pathway *C3* and *C5 convertase* complexes is impossible in the absence of MASP-2 [163]. Once formed, the *C3 convertase* cleaves and activates the remaining complement factors leading to the formation of a pore in the bacterial membrane by Membrane Attack Complex (MAC - subunit composition C5b-C6-C7-C8-C9) that lyses the bacterial cell (Fig. 10).

3.2 Blood Coagulation System

Inflammation and blood coagulation systems have complementary roles in eliminating invading pathogens, limiting tissue damage, and restoring homeostasis. Both systems are intertwined, with each one activating the other. During the initial steps of inflammation, the increase of vascular permeability allows the exudation of plasma proteins, which include a number of pro-mediator systems. Pro-inflammatory cytokines stimulate the production of tissue fac-

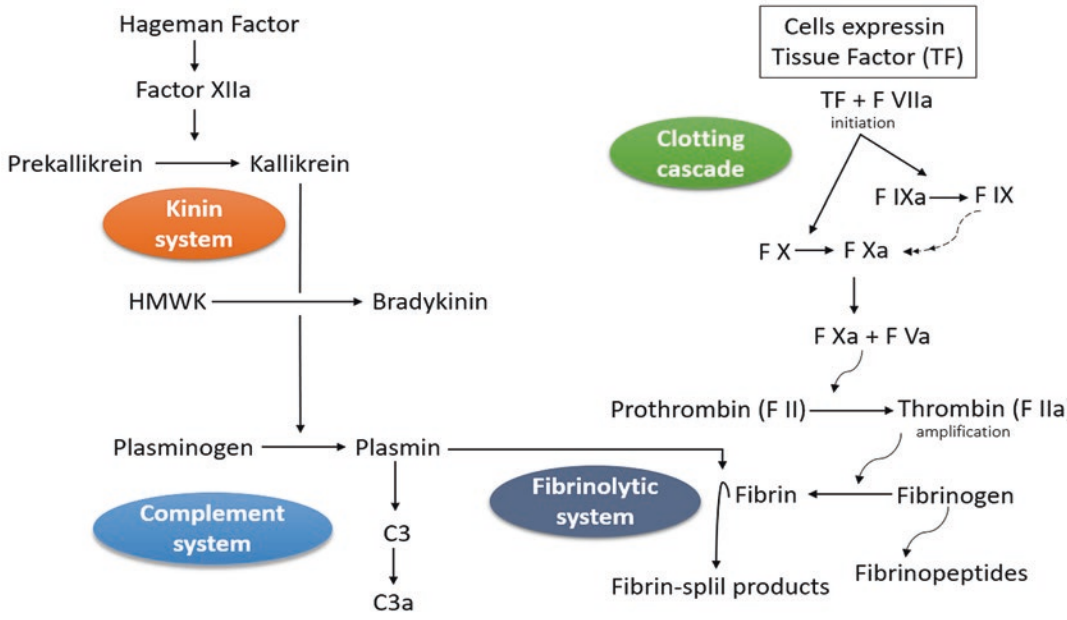

Fig. 11 Cross-talk among coagulation, complement, kallikrein-kinin and fibrinolytic systems

tor (TF). TF is nowadays considered the main initiator of blood coagulation *in vivo* (Fig. 11). It is present in the subendothelial tissue, fibroblasts and in activated monocytes. In physiological conditions, TF is not in contact with the blood. After vascular injury, TF activates the plasma Factor VII, forming a complex (TF-VIIa) that activates Factor X (FX). FXa forms the prothrombinase complex (FVa-FXa) in the presence of FVa. This complex activates prothrombin (FII) to thrombin (FIIa), which plays a central role in the coagulation protease cascade [171]. Additionally, TF activates FIX. FXIa activates more FX the presence of FVIIIa, contributing to the generation of more prothrombinase complex and thrombin. Thrombin is an enzyme able to break fibrinogen into fibrin, which coagulates the blood. Activated coagulation factors affect specific receptors on inflammatory cells and endothelial cells and, thereby, modulate the inflammatory response. Thrombin promotes inflammation by engaging receptors that are called protease-activated receptors (PARs), since they bind to multiple trypsin-like serine proteases in addition to thrombin [172].

Coagulation can limit bacterial pathogenesis. The fibrin network seems to trap bacteria limiting the infection and also protecting from their clearance [173]. However, while the coagulation response is a mechanism involved in bacteria control (mainly by fibrin deposition), the response itself can lead to major tissue injury. Coagulation factors are mostly pro-inflammatory. For example, fibrinogen, fibrin and also fibrin degradation products, can increase inflammatory responses by affecting leukocytes migration and cytokines production [174]. The thrombin and tissue factor are essential for coagulation and have a role to activate TLRs and PARs that mediate pro-inflammatory response [175]. The fibrinolytic system, centered in the enzyme Plasmin, is mainly known by its functions in the degradation of fibrin clot and control of thrombotic events, but is also recognized to trigger the inflammatory response by activating pro-inflammatory pathways in leukocytes [176, 177]. Besides the pro-inflammatory functions, the fibrinolytic system has been recently associated with the termination of the inflammatory response [178] and tissue repair [179–182].

3.2.1 Protease-Activated Receptors (PARs)

Protease-activated receptors (PARs) are important players in the interaction between coagulation system and inflammation [183]. The PARs (PAR-1, -2, -3, and -4) belong to a G-protein coupled receptor family activated by proteases, such as activated coagulation factors, which induce the proteolysis of the N-terminus of the receptor, causing a self-activation by a "tethered ligand". This activation of PARs by enzymatic cleavage is irreversible. The proteolytic activation of PARs can induce a range of cellular reactions that include cytokines release, expression of adhesion molecules, cell migration, or proliferation [172].

PARs are mainly expressed in vascular cells. PAR-1 and PAR-2 are expressed in smooth muscle cells and endothelial cells. Also, PARs are expressed in inflammatory cells including monocytes, mast cells, and eosinophils [44, 184–186]. PARs are activated by the action of serine proteases such as thrombin (acts on PARs 1, 3 and 4) and trypsin (PAR-2) [187]. PAR-1 agonists or thrombin induce IL-8 and IL-6 synthesis in mononuclear leukocytes and endothelial cells. In addition to coagulation factors, other serine proteases, for example matriptase, that are secreted by monocytes stimulate pro-inflammatory cytokines release in endothelial cells via PAR-2 activation [188, 189]. In addition, PAR-1 and PAR-2 agonists induce cytokines release in smooth muscle cells [188].

3.3 Kallikrein-Kinin System

The kallikrein-kinin system is an endogenous metabolic cascade widely involved in blood pressure control, coagulation, inflammation and pain. The activation of this system induces the release of vasoactive kinins (bradykinin-related peptides), potent pro-inflammatory peptides, through the cleavage of kininogen by prekallikrein [190]. The kallikrein-kinin and coagulation systems are also intimately connected [191]. The active form of factor XII, FXIIa, converts the plasma prekallikrein into the active proteolytic form, the enzyme kallikrein, which breaks down the plasma precursor glycoprotein, kininogen, to produce bradykinin [191] (Fig. 11). It is well established that bradykinin is an inflammatory mediator that causes dilation of blood vessels and increased vascular permeability.

Bradykinin and kinins (bradykinin-related peptides) act on two types of G protein-coupled receptors designated as B_1 and B_2 receptors [192]. These bradykinin receptors are involved in the regulation of various physiological and pathological processes. B_2 receptor is constitutively expressed in many types of cells, including vascular cells, and it is rapidly desensitized. On the other hand, the B_1 receptor is resistant to desensitization and is induced by pro-inflammatory cytokines, such as TNF-alpha and IL-1beta [193]. During inflammatory reactions, activated leukocytes and endothelial cells express B_1 receptor, which is sensitive to bradykinin-related peptides. The activation of B_1 receptor is involved in a range of pro-inflammatory effects including edema, pain and promotion of leukocytes trafficking. When kinin B_2 is activated, vascular permeability increases mainly through NO-induced relaxation of perivascular smooth muscle cells. The action of bradykinin is short because it is rapidly inactivated by a number of peptidases (kininases), including angiotensin-converting enzyme (ACE), also termed kininase II [192–194].

3.3.1 Cross-Talk Among Coagulation, Complement, Kallikrein-Kinin and Fibrinolytic Systems

A range of serine proteases that belong to the coagulation system is also associated with the activation of the complement cascade and fibrinolytic system. FXIIa activates the fibrinolytic system. This cascade counteracts clotting by breaking the fibrin, thereby solubilizing the clot. Kallikrein, as well as the plasminogen activator, cleaves plasminogen, a plasma protein that binds to the fibrin clot formed to generate plasmin. The primary function of plasmin is to cleave fibrin clots, but during inflammation it also activates the complement protein C3. Actually, several serine proteases that belong to the coagulation sys-

tem are also capable of activating the complement cascade. It has been already demonstrated that the coagulation factors Xa, Xia and also plasmin can cleave both C5 and C3, generating C5a and C3a (anaphylatoxins), while kallikrein only directly converts C5 to chemoattractant C5a. Both plasmin and kallikrein can activate the Hageman factor, which can trigger multiple cascades, amplifying the response [195, 196].

3.4 Proteases

Proteases are present in all human tissues, playing a role in many biological functions. Among these functions, the inflammatory response is of particular interest [197]. In inflamed tissues, proteases are involved in the first line of defense against invading microorganisms by killing these pathogens, inducing tissue remodeling, inducing immune recognition by resident or inflammatory cells, e.g., via TLRs, thereby contributing to the innate immune response [198]. It was also demonstrated that proteases may be associated with an excessive inflammation response, since treatment with anti-proteases during the onset of inflammation promotes resolution by the release of pro-resolving mediators such as annexin A1 [199].

4 Concluding Remarks

The inflammatory response is regulated by mediators produced by immune cells and those produced by the liver and delivered in plasma, which are important to mediate and control the response from the beginning to the end. These mediators act with some redundancy to ensure that protective response occurs. Although the main purpose of inflammation is to eliminate the stimulus and promotes resolution/repair, in some cases overshooting inflammation with excessive production of inflammatory mediators and infiltrating leukocytes may lead to chronic inflammatory disease.

Many pro-inflammatory molecules have its bioactions primarily related to the inflammatory response. Vasoactive amines are able to cause vasodilation and increase vascular permeability, contributing for the edema, redness and increased heat, which are cardinal signs of inflammation. Serotonin can also act as chemoattractant and can induce production of many cytokines. As vasoactive amines, PAF can be involved in vascular permeability, and like serotonin, it can also induce cell recruitment and activation. Many cytokines and chemokines play a role in the communication between leukocytes, guiding them during their recruitment into inflammatory sites. Prostaglandins in combination with histamine and bradykinin, are potent vasodilators, and thus contribute to redness and increased blood flow in the inflammatory area. Nitric oxide as a vasoactive substance, have a role in the inflammation response increasing vascular permeability, cellular infiltration and defense against infectious agent. Reactive oxygen species, as well as NO, act like second messenger during inflammation, promotes the death of pathogens and cytokines release.

Some molecules currently recognized to mediate inflammation were initially described to have bioactions in other systems. This is the case of components of the hemostatic system. While thrombin and tissue factor are essential for coagulation, they are also able to activate TLRs and PARs, and mediate pro-inflammatory response. Indeed, the fibrinolytic system, centered in the enzyme Plasmin, is mainly known by its functions in the degradation of fibrin clot and control of thrombotic events, but is also recognized to trigger the inflammatory response by activating pro-inflammatory pathways in leukocytes and to be associated to the termination of the inflammation and tissue repair. The dual actions of fibrinolytic proteins, by inducing the production of pro-inflammatory response in leukocytes and by stimulating neutrophil apoptosis and efferocytosis (essential events in inflammation resolution) supports the idea that the beginning, and so its mediators, programs the end of inflammation (as reviewed by Serhan and Savill [200]). All these intriguingly and complexity events have a common final physiological propose that is limit bacterial spread, maintain haemostasis and promote vascular and tissue repair.

While the knowledge of the inflammatory response reveals the complexity of the inflammatory mediator network, production and actions, it also opens new venues for the development of innovative therapeutics for inflammatory diseases.

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Innate Immunity and Inflammation: The Molecular Mechanisms Governing the Cross-Talk Between Innate Immune and Endothelial Cells

Daiane Boff, Caio Tavares Fagundes, Remo Castro Russo, and Flavio Almeida Amaral

Abstract

The innate immune response comprises the initial events that occur during tissue insult, causing cellular activation and triggering inflammation. Innate immune cells, including resident and early migrated cells from the bloodstream, sense a plethora of molecules called molecular patterns, that are derived from microorganisms or host cells. Once activated, pattern recognition receptor (PRR) signalling is triggered intracellularly and promotes the synthesis and release of vasoactive molecules, which target endothelial cells and cause inflammation. In addition, circulating molecules and pathogens also activate PRRs that are expressed on endothelial cells. These events modify endothelial cell metabolism, changing their conformational state and promoting the expression of pro-inflammatory molecules. Importantly, gain-of-function mutations in PRRs are associated with continuous cellular activation, leading to the development of autoinflammatory diseases. Here, we discuss the relationship among the cellular and humoral arms of the innate immune system in inflammatory processes, with special attention given to endothelial cell activation.

Keywords

Innate immunity · Inflammation · Pattern recognition receptors · Cellular metabolism · Autoinflammatory diseases

1 Introduction

Inflammation is a protective response of the body to ensure removal of harmful stimuli and to stimulate a healing process for tissue repair [1]. Inflammation is closely associated with the innate immune response to microbial infection, tissue injury and other sterile stimuli [2]. Resident cells are key elements that orchestrate the release of potent pro-inflammatory mediators by sensing a plethora of stimuli, ranging from pattern molecules to cellular stresses. The produced cytokines

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and other classical vasoactive molecules in the tissue bind to and activate the nearby blood vessels, causing profound changes in endothelial cell (EC) metabolism, conformational structures, and the synthesis of molecules that create a local environment that facilitates the translocation of blood molecules and cells to the affected tissue. These early events are the basis of the clinical signs of acute inflammation, characterized by redness, swelling, heat, pain and loss of tissue function [3].

EC activation is the basis of the inflammatory response. ECs are monolayers of cells that form the inner lining of blood and lymphatic vessels. These cells form the endothelium, a barrier between the vascular space and the interstitium [4]. In adults, the endothelium weighs approximately 1 kg, comprising 1.6×10^{13} cells, and has a surface area of 1–7 m^2 [5]. During homeostasis, ECs control blood fluidity in different ways, and they inhibit coagulation and platelet adhesion throughout the vascular system [6, 7]. ECs also regulate the muscular tonus by releasing vasodilators, such as nitric oxide (NO) and prostacyclins, or vasoconstrictors, such as endothelin [8, 9]. Protein transport and endothelium permeability occur mainly through interendothelial junctions that connect the ECs into a continuous monolayer. In that way, plasma proteins are prevented from moving from the blood to tissues through endothelium in non-inflamed tissues [10]. In addition, the interactions among ECs and leukocytes are minimal during the EC resting phase since they sequester the proteins necessary for these interactions, such as selectins and chemokines, in specialized secretory vesicles [11].

ECs participate and regulate different steps of inflammation during the innate immune response [12, 13]. ECs express pattern recognition receptors (PRRs) and complement protein receptors. Once activated, ECs increase the permeability of the endothelium, facilitating the leakage of serum components and extravasation of leukocytes, and they are also a source of cytokines, chemokines, acute phase proteins, and reactive oxygen species [14–17]. Here we discuss the contributions of key innate immune elements during inflammatory responses, focusing on how ECs sense a plethora of stimuli by innate receptors, change their metabolism, and drive inflammation. In addition, we discuss the involvement of innate receptors in the development of autoinflammatory diseases.

2 Endothelial Cell Heterogeneity and Tissue Specialization During Inflammation

The circulatory system comprises the blood and lymphatic vasculature and plays an essential role in physiology, interconnecting and transporting gases, nutrients, metabolites and cells. The blood vasculature, consisting of arteries, veins and capillaries that exhibit distinct architectures, molecular and functional properties, is essential for normal organ function and disease [18]. The endothelium of blood vessels forms a continuous monolayer, whereas capillary endothelial cells can be classified as continuous, fenestrated or discontinuous, depending on the tissue-specific type in which they reside [18]. Leukocyte migration from the blood flow of post-capillary venules and influx into tissue is a coordinated process involving multistep signals in endothelial and leukocytes via a hierarchy of adhesion molecule activity that mediates the steps of leukocyte tethering, rolling, arrest, activation, firm adhesion and transmigration [19, 20]. Leukocyte tethering and rolling on the endothelial surface is mediated by selectins [19]. Inflammation causes endothelial cell expression of P-selectin and E-selectin, whereas L-selectin is expressed by leukocytes [19–22]. Leukocytes express selectin ligands, including P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 and CD44, which interact with the endothelial selectins [20–22]. L-selectin mediates leukocyte rolling interactions by binding to glycosylated proteins on activated ECs, such as GlyCAM-1 and CD34 [20]. Interestingly, Pentraxin 3 (PTX3), a protein with well-known functions in innate immunity, reduces neutrophil migration and inflammation *in vivo* by binding to P-selectin, impairing the rolling of neutrophils on vessels [23]. Rolling brings the leukocyte into close proximity with the

endothelium, allowing them to respond to chemotactic arrest signals from chemokines present at the endothelial surface [20, 22]. A wide variety of molecules possess this chemoattractant function, including proteins such as chemokines. Leukocytes respond to chemoattractants by rapidly upregulating the affinity of their β2 and α4 integrins [19, 20, 22]. This enables these adhesion molecules to bind to their endothelial-expressed ligands, with β2 integrins binding to molecules, such as ICAM-1 and fibrinogen, and α4 integrins interacting with VCAM-1 and MAdCAM-1 [19, 20, 22]. This interaction causes arrest of the rolling leukocytes, which then firmly attach to the endothelial surface. After arrest, leukocytes migrate across the endothelial surface [19, 20, 22] to identify an optimal location.

The leukocyte recruitment established in post-capillary venules is not exactly the same in all organs. The microvasculature of the lung, liver and kidney are characterized by structural specializations that are required for their functions. Recruitment of leukocytes into the diverse tissues through the specialized capillary network contrasts with the recruitment of leukocytes through post-capillary venules at sites of inflammation (Fig. 1).

2.1 Leukocyte Recruitment in Alveolar Capillaries

The dense capillary network of the lung is a major site of physiological sequestration of leukocytes from the systemic circulation [18, 24, 25]. Compared with blood in the large vessels of most vascular beds, the blood from alveolar capillaries contains circa 50-fold more neutrophils, lymphocytes and monocytes [24], which contribute to the innate immune response and haematopoiesis in the lungs [24, 26, 27]. The alveolar capillaries provide a vascular defensive niche whereby the endothelium and neutrophils cooperate for immediate detection and capture of disseminating pathogens during host defence, mediating vascular protection [24, 27], and crawl throughout the endothelium via a TLR4/CD11b-dependent process [27]. Pulmonary ECs also constitutively express ICAM-1 at much higher levels than in other organs [25]. This process of margination is induced in part by the delay of neutrophils as they undergo the deformation required to pass through the narrow lung microvasculature [24]. Marginated leukocytes are in a dynamic equilibrium with those in the circulation, a situation that is maintained by the ongoing entry and exit of leukocytes from the marginated pool. During inflammation, much of the sequestration and infiltration occurs through vessels so narrow that physical trapping is sufficient to stop the flowing neutrophils [24, 25].

In comparison to post-capillary venules, the tethering mechanisms required to capture neutrophils from flowing blood in larger vessels is not necessary in the alveolar capillary bed. The diameters of neutrophils are larger than the diameters of many capillary segments, and 50% of the capillary segments therefore require changes in the shape of neutrophils for transmigration [24]. The events following the initial sequestration of neutrophils within alveolar capillary beds are apparently influenced by adhesion molecules. Systemic activation of neutrophils by intravenous injection of the chemokine CXCL8 results in rapid neutropenia with massive sequestration of neutrophils within alveolar capillaries. This event is not dependent on L-selectin or β2-integrins, but the retention times within this capillary bed are influenced by these adhesion molecules [24, 28], and this type of adhesion is likely orchestrated by the interaction of leukocyte adhesion molecules and endothelial adhesion molecules, such as VAP-1 [29]. These studies demonstrate that leukocyte recruitment to the pulmonary microvasculature does not necessarily follow the conventional paradigm in that the requirement for archetypal adhesion molecules is variable and recruitment to the lung can occur in the absence of both β2 integrins and the selectin family of adhesion molecules.

2.2 Leukocyte Recruitment in Sinusoid Capillaries

ECs lining hepatic sinusoids are also unique in that they are highly fenestrated and lack a basal lamina [18, 25, 30]. These openings in the endothelial layer allow plasma to flow freely into the

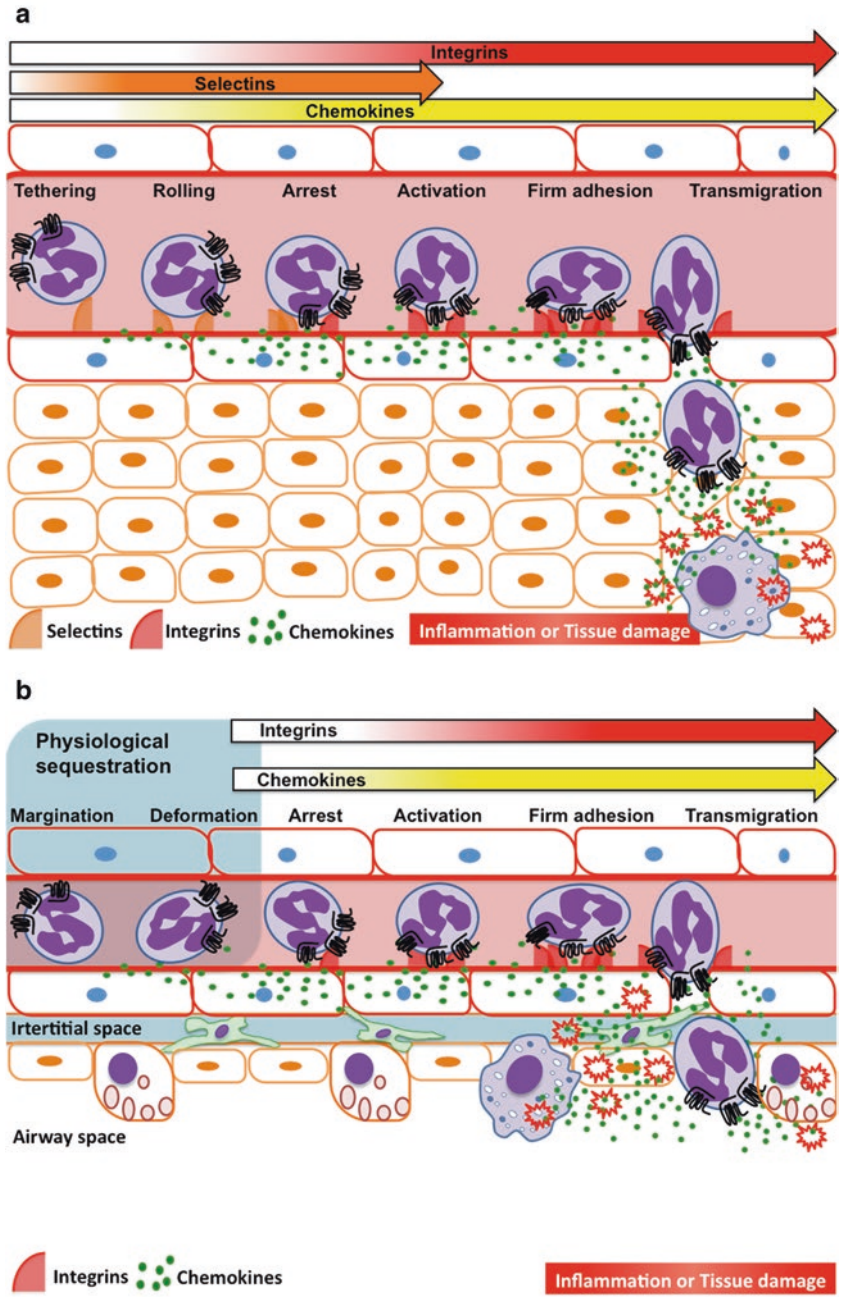

Fig. 1 The multistep cascade of leukocyte recruitment in post-capillary venules (**a**) and lungs (**b**): (**a**) Leukocyte extravasation from the blood into inflamed tissues follows a multistep cascade that involves the sequential action of molecular signals and adhesion molecules. Selectins (such as P-selectin and E-selectin) initiate leukocyte tethering and rolling along the inflamed endothelium. Rolling slows down circulating leukocytes, bringing them into close proximity with endothelial cells and allowing the binding of chemokines that are displayed on the inflamed endothelium to specific G-protein-coupled chemokine receptors on leukocytes. The activation of chemokine receptors triggers intracellular signalling pathways that activate leukocyte integrins. Interactions between integrins expressed on the endothelium mediate the firm adhesion of leukocytes. The interactions between β2-integrins and their ligand intercellular adhesion molecule 1 (ICAM1) and between integrin very late antigen 4 (VLA4) and its ligand vascular cell-adhesion molecule 1 (VCAM1) are of crucial importance for leukocyte adhesion. Leukocytes are directed by

sub-endothelium, where it comes into contact with hepatocytes. Sinusoidal endothelial cells also have a limited capacity to express P-selectin, E-selectin and VCAM-1, while they constitutively express high levels of ICAM-1, as well as less commonly expressed adhesion molecules, such as vascular adhesion protein-1 (VAP-1) [25, 30]. During the mechanisms regulating leukocyte adhesion in the sinusoids in the model of focal necrosis, Mac-1 and ICAM-1 are central to neutrophil recruitment [31]. Moreover, neutrophil recruitment occurs via a sequential process initiated by arrest induced by ATP released from damaged cells. Subsequently, intravascular migration is induced by the CXC family of chemokines, finally resulting in migration into necrotic tissue occurred via formylated peptides [31–33]. In addition, the steps of tethering and rolling of leukocytes in liver sinusoids also occur independently of selectins, but integrins mediate leukocyte arrest and transmigration, as observed in lung tissue.

2.3 Leukocyte Recruitment in Glomerular Capillaries

Glomerular capillaries are lined by specialized ECs that are highly fenestrated [18]. The luminal surface of glomerular ECs is covered by an endothelial surface layer consisting of negatively charged glycoproteins, glycosaminoglycans and proteoglycans, a structure that contributes to the barrier function of the glomerulus [18, 25, 34]. Regarding adhesion molecule expression, evidence suggests that glomerular endothelial cells do not express pre-formed P-selectin but use platelets as a mechanism for expression of P-selectin under inflammatory conditions [35]. In contrast, these cells constitutively express ICAM-1 and can, under inflammatory conditions, express VCAM-1 and E-selectin de novo, as well as increase ICAM-1 expression [36, 37]. In experiments, leukocytes could be observed undergoing adhesion in glomerular capillaries, but no rolling interactions were observed [25, 38]. In accordance with this finding, a selectin inhibitor failed to reduce glomerular leukocyte recruitment [38]. In contrast, inhibition of the β2-integrin Mac-1 prevented inflammation-associated adhesion of leukocytes in glomeruli [25, 36]. Taken together, these findings indicate that while leukocyte adhesion in glomerular capillaries requires an adhesion molecule-mediated interaction, selectins are not essential for this process.

3 Cellular Innate Immune Response: Role of Pattern Recognition Receptors on Endothelial Cells

In 1989, Charles Janeway Jr. proposed that innate immune cells should present germ line-encoded pattern recognition receptors (PRRs) to recognize conserved microbial components named pathogen associated molecular patterns (PAMPs) [39, 40]. It was later demonstrated that PRRs also recognize endogenous molecules released from damaged cells, called damage-associated molecular patterns (DAMPs),

Fig. 1 (continued) immobilized chemokines under flow (chemotaxis) or by chemokine gradients (haptotaxis) to migrate across the endothelium and into the inflamed tissue. (**b**) The initial steps of leukocyte migration, such as tethering and rolling, are absent in tissues during inflammation. Neutrophil migration across the alveolar capillary wall can occur through endothelial-independent selectin activity; however, the pulmonary capillary architecture sequesters leukocytes in small capillaries, and leukocytes marginate and deform to pass through this very small vasculature. Chemokines secreted by alveolar macrophages or endothelial cells activate more endothelial cells and induce leukocyte arrest. Leukocytes that have crossed the endothelium traverse the basement membrane and, through interactions with fibroblasts and neutrophils in the interstitium, adhere to the fibroblast surface. Adhesion is known to involve leukocyte β2-integrin (CD18) and fibroblast intercellular adhesion molecule 1 (ICAM-1). Motility is regulated by CD18 and leukocyte β1-integrins. The unique positioning of the fibroblasts within the interstitium provides directional information, guiding neutrophils toward type II pneumocytes and, thus, leading them to emergence between the margins of two type I pneumocytes and one type II pneumocyte in airways

or alarmins [16, 41]. There are different classes of PRRs that can be subdivided according to their structure, localization, and binding and signalling properties. The transmembrane PRRs include Toll-like receptors (TLR) and C-type lectin receptors (CLR), while the cytoplasmic PRRs are represented by retinoic acid-inducible gene (RIG)-I-like receptors, nucleotide-binding oligomerization domain-like NOD-like receptors (NLR) [42], AIM2-like receptors (ALR) [43], and cyclic GMP-AMP synthase (cGAS) [44].

PRRs are present in virtually all cell types. However, most knowledge about the association of PRRs with tissue inflammation has been obtained from activated innate immune cell recognition of exogenous and endogenous patterns. Once activated, resident and early migrated cells release a plethora of vasoactive molecules, including prostanoids, histamine, cytokines, and chemokines, which cause profound changes in ECs, contributing to inflammation [45]. Here we explore the mechanisms used by ECs to sense pathogens and endogenous molecules and how these interactions result in tissue inflammation and disease (Fig. 2).

3.1 Toll-Like Receptors (TLR)

The TLR family is the major and most extensively studied class of PRRs [46]. TLRs were first described in 1997 and were originally discovered based on homology to the *Drosophila melanogaster* Toll protein, which plays a role in dorso-ventral patterning during embryogenesis as well as in the antifungal response [47]. TLRs are glycoproteins characterized by an extracellular or luminal ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signalling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain [48, 49]. TLRs detect distinct patterns derived from bacteria, viruses, parasites and self-components. In humans, 11 TLRs have been identified, while 13 are described in mice [50]. TLRs are expressed in different cell compartments, favouring the recognition of extra and intracellular patterns. While TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the cell surface and are important for interactions with extracellular patterns, the intracellular compartments contain TLR3, TLR7, TLR8, and TLR9 and are important for binding to nucleic

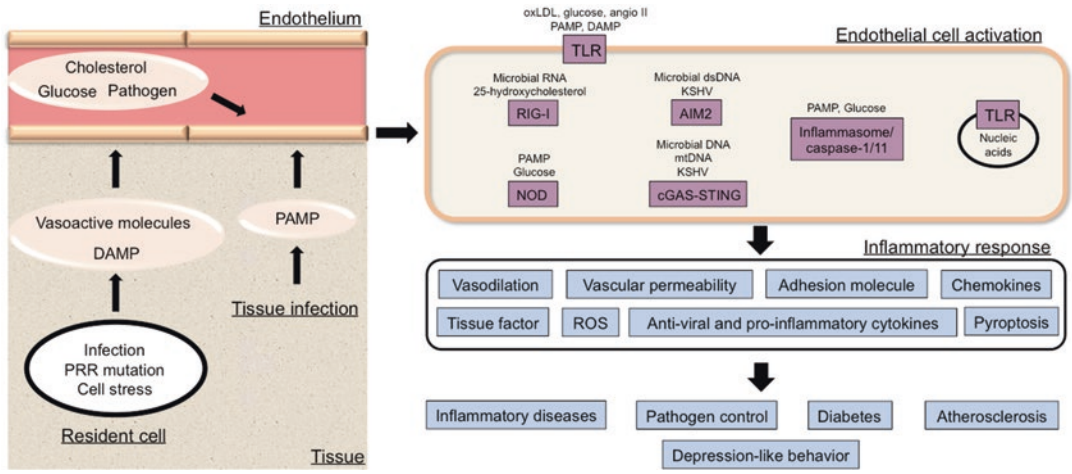

Fig. 2 Endothelial cells sense endogenous and microbial molecules that contribute to tissue inflammation. Different molecules derived from tissue resident cells, microorganisms, and plasma components interact with innate immune-associated receptors expressed on endothelial cells. The intracellular signalling cascades result in endothelial cell activation, promoting adhesion molecule expression and morphologic changes in the endothelium, which facilitate interactions with circulating leukocytes. Furthermore, activated endothelial cells produce and release biologically active mediators that contribute to tissue inflammation and are important for pathogen control and for the development of inflammatory diseases

acids [51]. TLR11 is non-functional in humans but present in murine endosomal compartments together with TLR12 and 13 [52], and it plays a role in the recognition of *T. gondii* profilin [53, 54]. Almost all TLRs signal through the adaptor molecule myeloid differentiating factor 88 (MyD88), mainly by activating the nuclear factor kappa-B (NFκB) and mitogen-activated protein kinase (MAPK) transcription factors [55]. In addition, signalling by TIR domain-containing adapter-inducing interferon-β (TRIF) activates interferon regulatory factors (IRFs) responsible for type I interferon production [56]. Of note, only TLR3 and TLR4 signal via TRIF, although the latter also signals via MyD88 [57, 58]. This ligand-receptor interaction induces receptor oligomerization and triggers a signalling cascade into the cytosol that culminates in the transcription of several genes involved in immune and inflammatory responses.

All TLRs have been detected in ECs [59]. ECs present in the aorta, subclavian, carotid, mesenteric, iliac, and temporal arteries express basal levels of TLR1, TLR2, TLR4, and TLR6. TLR3 is predominantly expressed in human aorta and carotid macrovessels [60] and human brain ECs [61]. However, TLR7 and TLR9 have only been detected in iliac ECs, although at low levels [62, 63]. It has been shown that in humans, ECs from lymph nodes express TLR 1–6 and TLR9 [64, 65]. In addition, human endothelial colony forming cells (ECFCs), comprising a subpopulation of endothelial progenitor cells characterized by their ability to differentiate into mature ECs, express detectable mRNA of all TLRs, albeit higher levels of TLR4 [66, 67].

In general, TLR ligands profoundly alter EC homeostasis, interfering in the coagulation cascade, vascular permeability, and synthesis of pro-inflammatory molecules [68, 69]. TLR activation upregulates the expression of adhesion molecules, such as E- and P-selectins, favouring platelet and leukocyte attachment on the EC surface [70, 71]. Additionally, TLR2 and TLR4 agonists lead to cytokine and chemokine expression by cultured ECs [72, 73]. Intravenous injection of TLR3 agonist long double-stranded RNA in mice impairs EC function, causing vasodilation, increased vascular permeability, and the production of reactive oxygen species [74]. During sepsis, TLR2 and TLR4 activation on ECs increases endothelial permeability and modulates the expression of coagulation factor molecules [66, 75]. In addition, TLR9 activation in human coronary artery endothelial cells by bacterial DNA shifts the balance of tissue factor and tissue factor pathway inhibitor toward a procoagulant phenotype and activates blood coagulation in mice, representing an important mechanism during the coagulation cascade in various pathologies [76].

Although much attention has been given to the participation of TLRs in the context of infectious diseases, non-infectious stimuli also activate TLRs on ECs, contributing to sterile inflammatory diseases. *In vitro*, shear stress modulates TLR expression on human coronary artery ECs [77]. During hypertension, elevated blood pressure can cause tissue damage resulting in DAMP and EC activation [78]. Importantly, angiotensin II infusion in mice cause an upregulation of TLR4 in the aorta, which is associated with EC dysfunction and activation, releasing pro-inflammatory molecules [79]. Oxidized low-density lipoprotein (oxLDL) activates TLR2 and TLR4 in human coronary artery ECs and stimulates the synthesis of bone morphogenetic protein-2 (BMP-2), which plays an important role in atherosclerotic vascular calcification [80]. Vascular dysfunction is an important event in diabetic complications. Hyperglycaemia upregulates TLR2 and TLR4 in human macrovascular aortic ECs and blocks TLR signalling, attenuating hyperglycaemia-induced inflammation, leukocyte adhesion and glycocalyx dysfunction in these cells [81]. Closely related, hyperglycaemia also induces TLR2 and TLR4 expression in human retinal ECs, contributing to the pathogenesis of diabetic retinopathy [82]. Taken together, the control of TLR signalling in ECs must still be considered as a potential strategy to regulate EC activation, facilitating the reduction of infectious and non-infectious inflammatory diseases.

3.2 NOD-Like Receptors (NLR)

The NLR belongs to a class of intracellular receptors/sensors consisting of 23 representatives in humans and 34 in mice [83]. Sequence homology revealed *NOD1* as the first NLR member [84, 85]. *NOD1* encodes an intracellular multi-domain scaffolding protein consisting of a caspase activation and recruitment domain (CARD), a nucleotide-binding oligomerization domain (NOD), and multiple leucine rich repeats (LRRs). NOD2 is a closely related protein with an additional CARD domain [86]. Both NOD1 and NOD2 recognize different pathogen and endogenous patterns, triggering activation of the NF-κB family of transcriptional regulators [87, 88]. Different ECs express NOD1, including HUVECs, HAECs and microvascular ECs. Incubation of ECs with the Gram-negative bacterium *Chlamydophila pneumoniae* contributes to the inflammatory response by increasing the synthesis of CXCL8 via a mechanism that is dependent on NOD1/NF-κB activation [89]. In a similar way, HUVECs incubated with Gram-positive bacteria *Listeria monocytogenes* also produce NOD1-dependent CXCL8. In addition, inhibition of p38 MAPK blocks NOD1-induced CXCL8 production [90].

Although NOD2 is only marginally expressed in ECs, it is quickly upregulated in the presence of microorganisms and pro-inflammatory cytokines, facilitating or potentiating the immune response and tissue inflammation. Under LPS, IL-1β, or TNF-α stimulation, HUVECs show increased NOD2 expression and become responsive to its agonist muramyl dipeptide (MDP), resulting in NF-κB activation [91]. In response to different stimulation, human aortic ECs incubated with Gram-positive bacteria *Streptococcus mutans* promotes the expression of IL-6 and the chemokines CXCL8 and CCL2 via a mechanism that is dependent on NOD2 and TLR2 [92]. Furthermore, IL-6 secretion by ECs under NOD2 activation is associated with $CD4^+$ T helper cell-17 (Th17) polarization while inhibiting $CD4^+$ Th1 and Th2 responses [13, 93].

In an infectious context, *Porphyromonas gingivalis* also stimulates NOD2, NOD1, and TLR2 expression in HUVEC cells. *P. gengivalis* are Gram-negative bacteria that are associated with periodontal disease and atherosclerosis. *P. gengivalis* stimulates NF-κB activation and E-selectin synthesis in HUVECs via a mechanism that is dependent on the three receptors [94]. On the other hand, NOD2 can also contribute to non-infectious inflammatory diseases, such as diabetes. High concentrations of glucose induce NOD2 expression in glomerular endothelial cells (GEnCs). In addition, overexpression of NOD2 is positively associated with the severity of diabetic nephropathy since it is associated with the loss of EC and gain of mesenchymal characteristics, a phenomenon called endothelial-to-mesenchymal transition, resulting in albuminuria and subsequent renal disorder [95].

The other branch of NLRs is represented by the inflammasomes, a group of intracellular multimeric protein complexes that activate pro-inflammatory caspases, leading to pro-IL-1β and pro-IL-18 cleavage and inducing, in particular, a type of cell death called pyroptosis [96]. The best-known inflammasome prototypes comprise NLRP1, NLRP3, NLRP6, NLRC4, and Pyrin. In several cases, the activated inflammasome engages the adaptor molecule ASC, which, in turn, activates caspase-1 [97]. NLRP1, NLRP3, ASC and caspase-1 are expressed in ECs [98]. Lipopolysaccharide (LPS) and ATP induce activation of the NLRP3 inflammasome in human umbilical vein endothelial cells (HUVECs) [99]. In a mouse model of Kawasaki disease, *Lactobacillus casei* cell wall fragments (LCWE) can activate the NLRP3 inflammasome in coronary arteries, resulting in EC dysfunction [100]. Likewise, DAMPs can also activate the NLRP3 inflammasome by lysosomal destabilization in HUVECs, leading to the production of IL-1β, that, in turn, induces IL-6 and CXCL8 in an autocrine manner in HUVECs [101]. High levels of glucose can also activate NLRP3 in ECs, and NLRP3 ablation prevents inflammasome activation and tight junction disassembly in the coronary arterial endothelium of diabetic mice [100]. Similarly, NLRP3 gene silencing prevents high glucose-induced down-regulation of tight junction proteins in cultured mouse vascular endothe-

lial cells (MVECs) [102]. In atherosclerosis, plasma triglycerides and VLDL cholesterol obtained from patients promote *in vitro* NLRP1 inflammasome expression in HAECs [103].

Inflammasome activation also induces cell death via pyroptosis. It has been demonstrated that cadmium, an important and common environmental pollutant that has been linked to cardiovascular diseases, induces pyroptosis in HUVECs through NLRP3 activation in a mechanism that is dependent on mitochondrial ROS generation [104]. Additionally, systemic exposure to LPS causes severe human lung microvascular EC (hMVECs) pyroptosis via a mechanism that is mediated by the activation of human caspase 4/5 or its homolog caspase-11 in mice *in vivo* [105]. Furthermore, hyperhomocysteinaemia (HHcy) is an independent risk factor for cardiovascular disease (CVD). HHcy preferentially induces EC pyroptosis via caspase-1-dependent inflammasome activation, leading to EC dysfunction [106]. Thus, EC dysfunction can occur in response to different types of inflammasome stimulation, revealing inflammatory disease mechanisms and identifying new opportunities for therapies.

3.3 Absent in Melanoma 2-Like Receptors (AIM2)

Absent in melanoma 2 (AIM2) is cytoplasmic dsDNA sensor belonging to PYHIN (IFI20X/IFI16 protein) family. Once binding microbial and host dsDNA, it complexes with ASC for caspase-1 activation, leading IL-1β maturation and release [107]. As part of the innate immune response, AIM2 activation helps host protection against different pathogens [108]. Few studies had investigated the role of AIM2 in EC activation. HUVEC cells stimulated with cell-free DNA increase the expression of AIM2 and also activate NOX4 in an AIM2-dependent manner [109]. dsDNA, IFN-γ, and TNF-α also induce AIM2 synthesis in human aortic ECs, smooth muscle cells, and T/G-human aortic vascular smooth muscle cells. Interestingly, AIM2 is overexpressed in lesions derived from abdominal aortic aneurism and atherosclerotic carotid artery when compared to intact aortic wall, suggesting a possible role of AIM2 in ECs activation and vascular inflammation in these diseases [110]. Another representative of PYHIN group, IFI16, also causes EC activation. Human dermal microvascular endothelial cells infected with Kaposi's sarcoma-associated herpesvirus promote oligimerization of a complex formed by IFI16, ASC, and caspase-1, leading to IL-1β maturation [111].

3.4 RIG-I-Like Receptors (RLR)

The RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) constitute a further PRR family of receptors called RIG-I-like receptors (RLR) [93]. Both proteins are localized in the cell cytosol and consist of a DexD/H box RNA helicase domain as well as two CARDs motifs [112]. These receptors have major function during viral infections, detecting replicating viruses in cytoplasm, particularly at early phases of viral infection [113]. Activation of this innate immune response lead to the induction of type I and III interferons (IFN) and inflammatory cytokines, whose antiviral activity blocks viral replication and facilitate the activation of antigen-presenting cells to activate antigen-specific immunity against viral pathogens [114]. RIG-I and MDA5 are constitutively expressed in ECs and they are upregulated during viral infection and other pro-inflammatory stimuli [112, 115]. However, RIG-I activation cause EC dysfunction. Intravenous injection of a RIG-I agonist in mice impairs EC vasodilatation and increases aortic oxidative stress. Similar events occurred using different EC lineages [116].

Measles virus-infected HUVEC cells synthesize chemokines and IFN-β, which, in turn, upregulates RLR synthesis in these cells [117]. Using glomerular ECs (GEnC), the RIG-I agonist poly I:C RNA stimulated the synthesis of several pro-inflammatory molecules, including IL-6, CCL2, CCL5, and CXCL10, demonstrating a potential involvement of GEnC for the pathogen-

esis of glomerulonephritis caused by viral infections [118]. Dengue virus also stimulates ECs via RIG-I. It is well known that Dengue virus infection leads to structural and functional alterations in the vascular endothelium [119]. In addition, Dengue virus serotype 2 replicated in human brain microvascular ECs. In addition to increasing the synthesis of RIG-I in these cells, Dengue virus 2 induced the production of IFN-β, IL-6, different chemokines, and the adhesion molecule ICAM-1 in a RIG-I-dependent manner [120]. Furthermore, activated ECs during viral infections not only amplify tissue inflammation but also could be associated with sickness behaviour. Type I IFN production from brain endothelial cells during RIG-I stimulation was associated to mouse cognitive impairment and depression-like behaviour [121]. Thus, activated RLR in ECs have crucial contribution to tissue inflammation during viral infections.

Activation of RLR in ECs seems to contribute to inflammation also in bacterial infections and non-infectious diseases [122] suggesting an important function for RIG-I during sepsis, where there is EC dysfunction. LPS and TNF-α induced the synthesis of pro-inflammatory and adhesion molecules by HUVEC cells and promoted leukocyte adhesion in a mechanism controlled by RIG-I and mitochondrial antiviral signalling (MAVS), the RIG-I downstream molecule [122]. 25-hydroxycholesterol increased RIG-I levels in HUVEC cytosol and stimulated CXCL8 expression through RIG-I-dependent mechanisms, which could favour neutrophil and T cell recruitment into the subendothelial space during atherogenesis [116].

3.5 Cyclic GMP-AMP Synthase-Stimulator of Interferon Genes (cGAS- STING)

Cyclic GMP-AMP synthase (cGAS) is a cytosolic protein that senses dsDNA. The downstream signalling in response to this interaction leads to the synthesis of the second messenger cyclic GMP-AMP, which binds to the protein stimulator of IFN genes (STING) present on the endoplasmic reticulum membrane. This cascade culminates in the recruitment of the transcription factor IRF3, which mediates the transcription of IFN-β [123]. STING is expressed in ECs [124]. During viral infections, ECs actively participate in the innate immune response, creating local conditions for tissue inflammation and viral removal. *In vitro* studies have demonstrated that HUVECs infected with the dsDNA of virus Kaposi's sarcoma-associated herpesvirus or cytomegalovirus produce high levels of IFN-β in a mechanism that is dependent on cGAS-STING activation [125, 126].

cGAS-STING has been demonstrated to play different roles in other innate immune response to infectious diseases, regulating EC function and activation. ECs in biopsy samples from patients who present a gain-of-function mutation of the *TMEM173* gene (which encodes STING) express inflammatory EC markers, such as inducible nitric oxide synthase, tissue factor, E selectin, and intercellular adhesion molecule 1. Similarly, HUVECs incubated with the STING-binding molecule cGAMP produce the same inflammatory markers but also engage apoptosis [124]. In human aortic ECs, palmitic acid (PA) leads to cellular stress, releasing mitochondrial DNA (mtDNA) into the cytosol, which activates cGAS-STING, resulting in IRF3 phosphorylation and nuclear translocation. PA is used in studies investigating diabetes since it negatively regulates insulin activity. cGAS-STING has been found to be important for ICAM-1 synthesis and monocyte–endothelial cell adhesion [127]. Using STING-deficient mice, researchers have demonstrated a dependence of the STING pathway on diet-induced obesity, adipose tissue inflammation and insulin resistance [127]. In another study, human aortic ECs incubated with a high concentration of PA showed impaired proliferation, migration, and angiogenesis capacities via a mechanism that was dependent on cGAS-STING activation by mtDNA. These events culminated in the synthesis of MST1 (mammalian Ste20-like kinases 1), a pro-apoptotic protein kinase [128]. Thus, cGAS-STING plays a very important role during tissue inflammation by controlling EC activation during infection and sterile disorders.

4 Role of the Complement System in Endothelial Cell Activation and Inflammation

Pre-formed and newly synthesized soluble molecules during the innate immune response are fundamental to initiate the control of pathogen invaders by activating host cells and creating an inflammatory environment or via the direct lysis of pathogens. However, these molecules also trigger or amplify tissue inflammation in non-infectious disorders, such as autoimmune, auto-inflammatory, and metabolic diseases [129]. Complement system and acute phase proteins are examples of the humoral arm of the innate immune response and strongly influence EC activation. The complement system is an integral part of the innate immune response and acts as a bridge between innate and acquired immunity. It consists of a series of proteins that are mostly synthesized in the liver and exists in plasma and on cell surfaces as inactive precursors (zymogens) [130]. Complement mediates responses to inflammatory triggers through a coordinated sequential enzyme cascade leading to the clearance of foreign cells through pathogen recognition, opsonisation and lysis. There are three known pathways leading to complement activation: classical, alternative and lectin, which vary according to the initial cascade and its components [131].

ECs express complement factors, regulators, and receptors. Complement deposition in ECs leads to cell activation, the expression of adhesion molecules, release of pro-inflammatory cytokines and chemokines, and promotion of membrane attack complex (MAC) formation and cytolysis [132–134]. C3a and C5a are well-known molecules that cause profound inflammatory changes in endothelium and also present chemotactic properties. Several studies have reported that targeting C3a and C5a receptors results in a reduction of tissue inflammation, which leads the development of several compounds for clinical use [129, 135]. Human microvascular and umbilical ECs (hMUECs) have been demonstrated to activate the classical complement pathway when exposed to shear stress by the continuous flow loop [136]. In a complementary manner, activation of this pathway in ECs promotes neutrophil adhesion to the endothelium [137]. ECs can also activate the lectin pathway: MASP-1, the key protease in this pathway, induces IL-6 and CXCL8 production by ECs, which leads to neutrophil chemotaxis [138]. In addition, HUVECs that are activated by MASP-1 can decrease ICAM-2 and increase E-selectin expression, leading to adherence between neutrophils and endothelial cells [139]. Thus, both the cellular and humoral arms of the innate immune system have potential effects on diverse ECs, leading to EC modification and activation and actively contributing to the inflammatory process.

5 Cellular Metabolism Reprogramming and Its Contribution to Inflammation

Traditionally, cell metabolism has been considered as a series of pathways that are responsible for extracting energy from fuel sources, such as glucose, fatty acids, ketones and amino acids. However, in the past few years, cellular metabolism and its by-products have demonstrated much wider implications, including the outcomes of inflammatory responses and of several pathologic processes [140–143]. Hence, metabolic processes, such as glycolysis, the tricarboxylic acid (TCA) cycle, and fatty acid metabolism, have highly specific effects on the function of macrophages and ECs. The manipulation of these pathways can interfere dramatically in the function of these cells in very specific manners, impacting their ability to produce inflammatory mediators and to exert their effector functions rather than simply being involved in energy generation or general biosynthesis [140–143]. In the next section, we will address how shifts in the cellular metabolism of macrophages and ECs, termed metabolism reprogramming, govern the outcome of inflammatory processes by determining cellular activation upon inflammatory stimulation.

5.1 Metabolism in Resting Macrophages

All cell types derive energy from the catabolism of three major biomass sources, glucose, fatty acids, and amino acids, using mainly two processes, glycolysis and oxidative phosphorylation (OXPHOX). In brief, one molecule of glucose can yield up to 38 molecules of ATP, of which 2 are derived from glycolysis, 2 from the TCA cycle, and 34 from OXPHOX. Based on this amount of ATP produced per glucose molecule, OXPHOX would be the most efficient bioenergetic pathway and should be preferred. This is the case for several cell types, including resting macrophages, which, under normoxic conditions, use OXPHOX to generate ATP. The major components of the electron transport chain (ETC) utilize the NADH and FADH generated during reactions in the TCA cycle, which is fuelled by the above-described biomass sources. Glycolysis is low due to expression of the *PFKFB1* gene, resulting in higher levels of the liver isoform of the phosphofructokinase (PFK) 2 enzyme, which yields low levels of the glycolysis activator fructose-2,6-bisphosphate (F-2,6-BP) and slows down the rate of the reactions involved in pyruvate generation from glucose [144]. In fact, rather than glucose, macrophages obtain much of their energy in the resting state from fatty acid oxidation (FAO) and OXPHOX because they express high levels of fatty acid transporters and catabolic enzymes, as well as proteins involved in ETC and those that drive Acetyl-CoA into the TCA cycle [145]. This oxidative metabolism is usually controlled by specific transcription factors, such as PGC-1β, which are present at higher levels in quiescent macrophages [141, 145]. Therefore, macrophages in the resting state obtain much of their energy from FAO and oxidative metabolism, which can efficiently sustain their basal activities for long periods of time.

The metabolic profiles of resting macrophages are usually shifted during cellular activation, in an event called metabolic reprogramming [142, 146]. Metabolic reprogramming can be simply considered as a response of cells to critical changes in the environment. For example, when oxygen tension is low, cells can switch their metabolic profile to enable the proper generation of energy, even in this altered environment. Hence, under hypoxic conditions, cells usually generate ATP through glycolysis independently from OXPHOX, but this pathway is highly dependent on glucose as the sole fuel source [142, 143]. The core metabolic pathways are integrated to interchange carbons between sugars, fatty acids, nucleic acids and proteins, and therefore metabolic flexibility can play an important role due to the changes in prevailing nutrient and oxygen conditions. This metabolic flexibility also seems to be important when cells are faced with distinct functional demands. Hence, recent work has emphasized that changes in key metabolic regulatory events in macrophages (and to a lesser extent in ECs) are initiated not only by shifts in nutrient or oxygen availability but also by downstream activation of PRRs and cytokine receptors [142, 143]. Thus, these cells present the potential to switch their metabolic activities in response to signals from other cells or from changes in the environment, such as those present in the inflammatory milieu. Importantly, metabolic reprogramming has been shown to govern the phenotype of macrophages by controlling transcriptional and post-transcriptional events that are central to their activation status [142]. The mechanisms involved in metabolic reprogramming in these cell types are outlined in the following section.

5.2 Metabolic Reprogramming in Activated Macrophages

Approximately 50 years ago, early work on leukocyte metabolism indicated an increase in oxygen consumption during phagocytosis [147]. In addition, it was shown around this time that monocytes engage glycolytic metabolism during phagocytosis [148]. Thirty years later, Fukuzumi and co-workers showed that LPS-activated macrophages present increased glucose uptake via a mechanism involving the upregulation of the GLUT1 glucose transporter [149]. More recent work has established that, upon activation by

TLR ligands, such as LPS, a shift towards glycolysis and fatty acid synthesis and down-modulation of OXPHOX characterizes proinflammatory macrophages [150, 151]. Inflammatory macrophages also upregulate the pentose phosphate pathway (PPP), which branches from glycolysis and generates NADPH for redox balance [150, 152]. Importantly, this metabolic reprogramming is essential for some effector functions of activated macrophages, such as the production of IL-1β, lipid mediators and reactive oxygen and nitrogen species [142, 146]. Glycolysis also seems to be pivotal for macrophage migration to inflammatory sites [153].

This metabolic shift towards glycolysis in activated macrophages is termed aerobic glycolysis because it occurs even under normoxic conditions. The shift towards aerobic glycolysis seems to be optimally suited to the fast, short-term burst of activation that is required at infectious or sterile inflammatory sites [142, 146]. Recent work has provided extensive evidence that changes in the metabolites associated with macrophage metabolic reprogramming are able to facilitate or promote the specialized activities of these cells. One of the changes in gene expression that increases glycolytic capacity in activated macrophages is the expression of the ubiquitous u-PFK2, the highly active isoform of phosphofructokinase 2, which generates higher quantities of the glycolysis activator F-2,6-BP [144]. In addition, increased expression of the PKM2 isoform of the pyruvate kinase enzyme favours the generation of lactate from pyruvate and diverts it from entry in the TCA cycle [154]. In fact, the TCA cycle is broken at two points in activated macrophages, after citrate and after succinate, leading to an accumulation of these metabolites [150, 151, 155]. Citrate accumulates in activated macrophages as a result of two major causes: increased citrate synthase expression under these conditions [151] and reduced citrate catabolism by isocitrate dehydrogenase due to expression of the *Idh1* gene encoding this enzyme, which is downregulated after inflammatory activation [150]. The concentration of succinate accumulates as a result of other three phenomena. First, glutamine-dependent anaplerosis generates succinate, whereby glutamine is used to generate glutamate and subsequently α-KG in the TCA cycle [156]. Second, succinate is generated through the γ-aminobutyric acid (GABA) shunt, which involves transamination of α-KG by the enzyme GABA α-oxoglutarate transaminase, generating L-glutamic acid (L-GA). Glutamic acid-decarboxylase catalyses the conversion of L-GA to GABA, which is then converted to succinic semialdehyde, a source of succinate [155]. Third, expression of the *immunoresponsive gene 1* (*Irg1*) gene during inflammatory activation of macrophages leads to conversion of cis-aconitate (generated from citrate) to itaconate, a metabolite that inhibits succinate dehydrogenase (SDH), diminishing succinate catabolism and thereby linking citrate and succinate accumulation [157]. The accumulation of these two metabolites is essential for several effector functions of activated macrophages.

Citrate accumulation seems to be a key event for the production of three important classes of inflammatory mediators: prostaglandins, nitric oxide (NO) and reactive oxygen species (ROS). To achieve this goal, citrate must be transported from mitochondria to the cytoplasm, and interestingly, LPS induces expression of the mitochondrial citrate carrier (CIC) [158]. Citrate is used for synthesis of phospholipids, which are a source of the arachidonic acid precursor of prostaglandins and other lipid mediators. Citrate can also lead to NADPH generation via malic enzyme and pyruvate. The generated NADPH is used by the inducible nitric oxide synthase enzyme to catalyse NO generation from arginine. Finally, NADPH is also used by NADPH oxidase to produce ROS. Is important to note that NADPH can also be generated by PPP, which is strongly upregulated in activated macrophages [150]. Nevertheless, inhibition of CIC expression by gene silencing decreases the production of NO, ROS and prostaglandins [158], emphasizing how a single TCA intermediate is involved in the production of key inflammatory mediators. Additionally, the generation of itaconate from citrate improves the antibacterial activities of macrophages because itaconate inhibits microbial metabolism, affecting their viability [159].

Succinate accumulation, on the other hand, is a key event leading to enhanced IL-1β production [155] and greater mitochondrial ROS production by activated macrophages. Succinate controls IL-1β production by enhancing the activity of the transcription factor HIF1α. HIF1α then directly induces expression of the IL-1β gene because its promoter region contains HIF1α-binding sites [155]. Succinate oxidation by SDH and enhanced mitochondrial membrane potential favour ROS production [160]. Enhanced mitochondrial ROS production by SDH amplifies IL-1β production [161]. Finally, succinate may also act as an alarmin because it can be released from inflammatory macrophages and lead to autocrine and paracrine activation of the GPR91 receptor, further increasing HIF1α-induced IL-1β production [162]. GPR91 has also been shown to drive leukocyte migration [163]. In conclusion, metabolic reprogramming of activated macrophages towards glycolysis and consequent accumulation of the two cited TCA cycle intermediates is a key event for the proper effector function of macrophages during inflammation.

5.3 Metabolic Reprogramming in ECs

Much less is known about metabolic reprogramming in ECs, especially in inflammatory contexts. However, there is now sufficient evidence showing that ECs in hypoxic or pro-angiogenic environments also adapt their metabolism to sustain their effector functions in these conditions. This phenomenon has been extensively revised elsewhere [140, 143, 164]. Here we will summarize the metabolic alterations documented in activated ECs that are potentially involved in their responses to mediators or cells present in inflammatory environments.

In contrast to resting macrophages, quiescent ECs (independently of the subtype) are highly glycolytic [165]. Even in healthy quiescent vasculature with plenty of oxygen availability, ECs show high rates of glycolysis, although the rates of other metabolic pathways remain to be well characterized [143]. ECs present a relatively low mitochondrial content [166], and their glycolytic rates are approximately 200-fold higher than their OXPHOX activity [165, 167, 168]. Although the lower ATP yield per molecule, glycolysis might provide more ATP in a shorter period of time than OXPHOX when available glucose is unlimited and has the advantage of shunting to glycolysis side branches (such as the PPP) for macromolecule synthesis [152]. Other advantages of glycolytic metabolism in ECs are thought to be the reduction in OXPHOX-generated ROS, diminishing oxidative stress, the preservation of maximal amounts of oxygen for use by perivascular cells, the adequate adaptation of ECs to hypoxic environments during angiogenesis, and the observed ability of the glycolytic by-product lactate to exert pro-angiogenic activity [140].

Interestingly, EC activation by hypoxic or angiogenic factors (such as VEGF) further enhances glycolytic flux by upregulating PFKFB3. Indeed, when PFKFB3 is silenced, the levels of its product F-2,6-BP, are decreased, leading to a reduction of the glycolytic flux by approximately 35% [165]. Glycolytic intermediates are also shunted to PPP, which provide the ribose units necessary for nucleotide synthesis and generates NADPH, which is used for the redox balance [152]. Decreasing the activity of PPP by inhibiting the expression of its rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD) reduces effector responses in ECs activated by VEGF [169]. In addition to ROS scavenging, NADPH generated by PPP is involved in endothelial NOS (eNOS)-mediated NO production, which is involved in vasodilation [169] .

Another metabolic driver of NO production in endothelial cells is the glycolytic transcription factor HIF1α. HIF1α directly drives eNOS transcription through binding to hypoxia-responsive elements present in the promoter region of the eNOS gene [170] and through tax-responsive elements [171]. Hypoxia or VEGF-activated ECs upregulate HIF1α, further increasing glycolytic metabolism [172, 173] and enhancing NO production, which

leads to vasodilation [174], an important event in the inflammatory milieu. Reduced shear stress present in the vasodilated vasculature also seems to regulate glycolytic metabolism in ECs. Laminar shear stress elevates the expression of Kruppel-like factor 2 (KLF2) in ECs, downregulating glycolytic enzymes, including PFKFB3 [175]. As the vasculature in inflammatory sites is often subjected to a reduction in blood flow, it is conceivable that these changes lead to enhanced PFKFB3 expression and glycolytic flux in ECs. Finally, pro-inflammatory mediators also upregulate glycolysis in ECs [176] and glycolysis further promotes EC pro-inflammatory activity [177]. Therefore, glycolytic metabolism is further induced in activated ECs and seems to be involved in their inflammatory functions in a manner similar to that observed in inflammation-activated macrophages (Fig. 3).

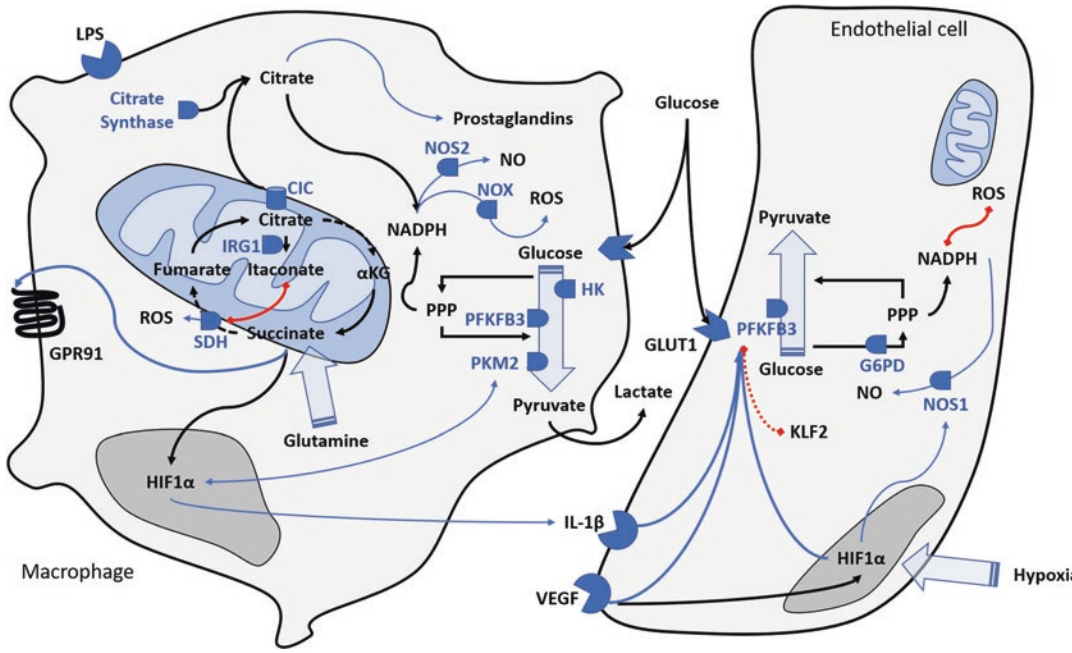

Fig. 3 The metabolism of activated macrophages and endothelial cells control their effector functions: Upon activation by TLR ligands, such as LPS, macrophages reprogramme their metabolism by enhancing glycolytic activity and reducing OXPHOX. The series of glycolytic reactions depends on glucose availability, which is increased by GLUT1-mediated glucose uptake, and by increased transcription of the glycolytic enzymes HK, PFKFB3 and PKM2. Glycolysis also provides energy and substrates for the PPP flux, which is important for the redox balance. This metabolic shift leads to the accumulation of TCA intermediates. Citrate accumulation depends on reduced citrate catabolism in the TCA cycle and increased citrate synthase activity. The citrate carrier transports citrate to the cytoplasm where it is used for lipid synthesis and prostaglandin production. NADPH generation is important for NOS2-mediated NO production and for NOX-mediated ROS generation. Succinate accumulation is induced by reduced SDH activity due to conversion mediated by the IRG1 enzyme of citrate to itaconate, which inhibits SDH activity. Additionally, via a series of reactions, glutamine is converted to succinate. Succinate accumulation leads to mitochondrial ROS production, HIF1α activation and consequent IL-1β production, in addition to chemotaxis mediated by the GPR91 receptor. Endothelial cells are usually glycolytic, but upon activation, this metabolism is further enhanced. HIF1α activation by hypoxia and angiogenic factors, such as VEGF, fosters this glycolytic programme, and the PFKFB3 enzyme is key for glycolysis-mediated endothelial cell effector function. In addition to energy, glycolytic metabolism favours NO production and vasodilation due to PPP-generated NADPH. Inhibition of PPP by targeting the enzyme G6PD inhibits NO production. Pro-inflammatory mediators, such as IL-1β, also foster glycolytic metabolism in endothelial cells, and a reduced blood flow in inflammatory environments might promote glycolytic metabolism by inhibiting KLF2-mediated suppression of PFKFB3 transcription

6 Autoinflammatory Diseases

Dysregulation of the innate immune system is directly associated with the development of autoinflammatory diseases, such as disorders characterized by recurrent episodes of fever, rash, and swelling that affects different tissues, and are dissociated with infectious and autoimmune components (revised by [178]). The causative agents for autoinflammatory diseases are diverse, including those that present mutations of PRR platforms, impaired cytokine signalling, and altered cell metabolism. However, the development of several of these diseases occurs due to gain-of-function mutations in the inflammasome, causing increased production and maturation of IL-1β with consequent neutrophilia, which are potential targets for effective therapies. Nonetheless, other diseases share common features of autoinflammatory diseases despite the absence of point mutations in the inflammasome/IL-1β axis, including atherosclerosis, diabetes, gout, and osteoporosis [178–180]. Here we list examples of autoinflammatory disorders caused by mutated inflammasome platforms.

One of the most common and well-known autoinflammatory diseases is Familial Mediterranean Fever, which is an autosomal recessive syndrome caused by mutations in alleles of the *MEFV* (Mediterranean fever) gene encoding the protein Pyrin [181]. Currently, more than 300 sequence variants have been described in the *MEFV* gene [182]. Pyrin is a crucial molecule that controls caspase-1 activation via the interaction between molecules carrying a Pyrin domain (PYD), such as ASC and some inflammasome prototypes. Nonetheless, Pyrin can also be interpreted as a PRR since it can sense pathogens during the innate immune response [183]. In FMF, mutated Pyrin binds to ASC and causes caspase-1 activation with the consequent release of mature IL-1β [184]. The inflammatory attacks in FMF are self-limited, but recurrent attacks could lead to chronic inflammation. There are different recommendations for FMF management, but colchicine and IL-1β blockers are the most effective options. By disrupting microtubule dynamics, colchicine inhibits Pyrin-ASC aggregation and, consequently, decreases IL-1β maturation and release [185].

Some autoinflammatory diseases are associated with mutations in genes responsible for the synthesis of inflammasome regulatory molecules. For instance, a mutation in the actin regulatory gene *WDR1* is characterized by periodic fever, neutrophilia, and thrombocytopenia, associated with high serum levels of IL-18 but not IL-1β [186]. In this study performed in two girls born to consanguineous parents, their monocyte-derived dendritic cells and neutrophils produced high levels of IL-18 under LPS stimulation, with no alterations in IL-1β. Mechanistically, the authors demonstrated a co-localization of the pyrin inflammasome with mutant WDR1 aggregates, culminating in caspase-1 activation [186]. Previous studies have shown that mice carrying a hypomorphic allele of *Wdr1* present spontaneous autoinflammatory syndrome and thrombocytopenia [187, 188]. The mechanisms responsible for this murine disorder are similar to those identified in humans, although monocytes seemed to be the most active cells in IL-18 release in humans. In the murine model, the depletion of monocytes *in vivo* or the inhibition of actin polymerization prevented the development of autoinflammatory-related symptoms [188].

Distinct autoinflammatory disorders resulting from a gain-of-function mutation in the *NLRP3* gene occur in cryopyrin-associated periodic syndromes (CAPS), comprising familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID) [189]. The increased levels of tissue and serum IL-1β are associated with cutaneous, neurological, ophthalmologic, and rheumatologic manifestations of inflammation. However, the continuous activation of mutated NLPR3 in CAPS could result from poor control of NLRP3 inhibition [190], as demonstrated by the blockade of human NLRP3 activation due to phosphorylation of ser295 in NLRP3 by prostaglandin E_2-induced cAMP-PKA. Interestingly, HEK293A cells transfected with plasmids encoding NLRP3 mutations previously identified in CAPS patients adjacent to ser295 NLRP3

lost the negative regulatory effect of PKA, keeping the cells in an activated state [190]

Recently, a study demonstrated that adult-onset Still disease (AOSD) patients had high levels of the mRNA for NLRP3 in peripheral blood mononuclear cells [191]. AOSD is a rare systemic inflammatory disease that shares common autoinflammatory disease symptoms, such as fever, arthralgia (with or without synovitis), skin rash, and striking leucocytosis with neutrophilia [192]. Although that study did not investigate any mutations in the *NLRP3* gene in AOSD patients, the authors also detected increased levels of caspase-1, IL-1β, and IL-18 in these patients compared with healthy volunteers [191].

Mutations in the NLRP1 inflammasome are associated with autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus [193–195]. However, a recent study identified a new autoinflammatory disease in patients exhibiting a systemic juvenile idiopathic arthritis phenotype. The authors identified a homozygous mutation in the *NLRP1* gene in three patients from two unrelated families that positively correlated with high levels of caspase-1 and IL-18. Clinically, these patients had recurrent fever, arthritis and dyskeratosis. The authors named this disease NAIAD (NLRP1-associated autoinflammation with arthritis and dyskeratosis) [196]. Interestingly, the mechanisms underlying this disease had been identified previously. A gain-of-function mutation in the N-terminal PYD of *NLRP1* is responsible for self-oligomerization and activation of NLRP1, which is highly expressed in skin, promoting an increase in the release of IL-1β by keratinocytes that leads to skin inflammation and epidermal hyperplasia [197].

More recently, gain-of-function mutations in the *NLRC4* gene have also been associated with autoinflammatory disorders. A mutation in the nucleotide-binding domain of the NLRC4 inflammasome is associated to early-onset recurrent fever flares and macrophage activation syndrome (MAS). This phenotype was observed in a single patient who presented spontaneous inflammasome assembly with IL-1β and IL-18 overproduction and increased macrophage pyroptosis [198]. Similarly, a distinct *NLRC4* point mutation was detected in a family with neonatal-onset enterocolitis, periodic fever, and fatal or near-fatal autoinflammatory attack. In this second report, overstimulation of macrophages was also observed, releasing excessive amounts of IL-1β and IL-18 associated with pyroptosis [199]. In addition [200], identified a heterozygous *NLRC4* mutation in a patient diagnosed with NOMID. As mentioned previously, NOMID is an autoinflammatory disorder associated with NLPR3 mutations. However, not all NOMID patients present the *NLPR3* mutation [201]. In that study, exome sequencing of the patient revealed somatic mosaicism (the occurrence of two genetically distinct populations of cells within an individual, derived from a postzygotic mutation [202]) of a novel *NLRC4* mutation. Interestingly, knockout of the *NLRC4* locus in a mutant-induced pluripotent stem cell clone using CRISPR/Cas9 technology abrogated the excessive IL-1β and IL-18 secretion by these cells [200].

The use of next-generation sequencing (NGS) technology has been fundamental for the discovery of different mutations in genes related to autoinflammatory disorders, improving diagnosis and directing the best options for disease management. The most frequently inflammasomopathies can be effectively treated using anti-IL-1 therapies [203–205]. However, in autoinflammatory diseases characterized by increased levels of IL-18, including those with gain-of-function mutations in the *NLRC4* gene, therapy based on recombinant IL-18 binding protein could be the best option [206].

7 Concluding Remarks

Recent knowledge regarding the function of cells and molecules associated with the innate immune response have substantially contributed to unravelling important mechanisms in inflammatory diseases. ECs are very active during the initial events of inflammation by expressing receptors that are classically associated with the innate immune response, enabling them to recognize different exogenous and endogenous molecules.

This recognition modifies their metabolism, promoting the synthesis of pro-inflammatory molecules, and changes their conformational state, establishing tissue inflammation. Nonetheless, altered cellular metabolism and the function of innate immune receptors, mainly on leukocytes, can trigger the development of innate immune-associated autoinflammatory diseases. Thus, attention should be focused on the function of innate immune elements in regard to endothelial cells and leukocytes during the course of inflammatory diseases, which will facilitate the development of novel and proper anti-inflammatory therapies.

Conflicts of Interest The authors declare no conflicts of interest.

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Adaptive Immunity of Airway Inflammation in Asthma

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Abstract

Respiratory immunity is responsible for pathogen elimination and prevention of chronic inflammation through both innate and adaptive mechanisms. Inappropriate activation of these immune systems in the respiratory mucosa results in chronic inflammatory airways disease such as asthma. Adaptive immunity is stimulated for example by allergen exposure that activates T and B lymphocytes leading to IgE production and influx of eosinophilic granulocytes into the airways. Presence of IgE and eosinophilia are diagnostic hallmarks as well as key pathogenic components that have been utilized in the search for improved therapies of allergic asthma for the past several decades. The recent breakthroughs in successful clinical application of biologicals in asthma were driven by improved genetic, biochemical, and immunological screening methods, novel imaging and bioinformatics technology, biomarker discovery and a better understanding of immune regulation of allergic airway inflammation. In this chapter we discuss our current understanding of immune regulation of airway inflammation in asthma, with a special focus on the interactions between the adaptive and innate immune systems and the epithelial mucosal tissue.

Keywords

Asthma · Epithelial cells · Immune homeostasis · Pattern recognition · Host defense

1 Introduction

The history of discovering the importance of the adaptive immune regulation of asthma started with John Bostock's description of hay fever as being an allergic disease affecting the upper airways, in 1819 [1]. It took almost a century however to realize that cellular/humoral immune pathways are involved in development of the allergic condition. In 1906, Clemens von Pirquet first described the 'hypersensitivity' symptoms that some of his diphtheria patients developed when treated with a horse serum antitoxin, and he coined the term 'allergy'(From the Greek allos = "other" + ergon = "work") [2, 3]. Unfortunately, because anaphylaxis was already introduced as a medical term by Besredka [4], Von Pirquet's concepts didn't gain wide acceptance during his lifetime. Nonetheless, between 1911 and 1914

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Leonard Noon and John Freeman were able to establish the foundations for clinical allergy treatment by immunotherapy or "allergy shots" on an empirical basis [4]. It took another fifty years until a new immunoglobulin isotype, (IgX) was discovered by Johansson and Bennich [5] (1967) and identified by Kimishige and Teruko Ishizaka (at the National Jewish Center in Denver) as the principal molecular mediator of the allergic reaction, and was then named IgE. How allergic responses are regulated at the cellular level, however, was still unclear. In 1986 Tim Mosmann & Bob Coffman observed that the "T-cell growth factor 2" (IL-4) producing T cell clones were the same that helped IgE production by B lymphocytes, but different from the IFN-γ producing clones and they published the "Th1-Th2 hypothesis" [6]. In the early 1990s, A Barry Kay's laboratory in London established that atopic asthma is associated with activation of Th2 cells, characterized by IL-4 and IL-5 but not IFN-γ release [7]. IL-4 and IL-5 are cytokines essential for IgE synthesis and tissue eosinophilia, respectively. Discovering the central importance of T cells in asthma pathogenesis explained the phenomenal success achieved in its treatment by the introduction of glucocorticosteroids (by Philip Hench and Edward Kendall, in 1948). Chronic glucocorticosteroid (steroid) treatment of asthmatic patients, however, came with the price of severe metabolic and immune side effects, including global immunosuppression [8]. In addition, while steroids have been effective in general and became the mainstay of treatment, a significant proportion of asthmatics remained unresponsive to treatment (steroid resistant) [9–13]. Alternative approaches, therefore, had to be found. Further advances in selective targeting of the adaptive/allergic immune response were made possible by the ability to synthesize biologicals (monoclonal antibodies, 1975), awarded by the 1984 Nobel prize to Kohler & Milstein. In 2003 for adults and last year also for children above 11 years of age, the Food and Drug Administration (FDA) approved Omalizumab (Xolair; Genentech/Roche and Novartis), a non-anaphylactogenic monoclonal antibody against IgE for the treatment of asthma. This was followed by approval of an anti-IL-5 monoclonal antibody, Mepolizumab (Nucala, GSK), in 2014, and the anti-IL-5R antibody Benralizumab (Fasenra, MedImmune), the anti-IL-4R antibody Dupilumab (Regeneron, Sanofi) just last year, in 2017. The significance of a monoclonal antibody-based treatment lies in the capability to specifically target the major pathogenic mechanisms (IgE production and eosinophilic airway inflammation), while preserving the rest of the functions of the immune system. This long-awaited introduction of selective biologicals to the clinic represents a significant breakthrough in asthma treatment that was (and continues to be) driven by better understanding of how the antigen-driven T cell-dependent immune response (adaptive immunity) is involved in regulation of asthma. T cells normally function to eliminate infections by certain pathogens and to activate B lymphocytes and innate immune cells. Adaptive immunity is based on the unique function of T cells to recognize antigens (through their T-cell receptor) presented by MHC bearing antigen presenting cells (APCs) and respond in an antigen-specific manner. Class II MHC molecules display antigenic peptides derived from extracellular proteins while class I MHC molecules present peptides from cytosolic proteins to CD4 and CD8 expressing T cells, respectively. Allergy develops because of altered T cell responses to otherwise innocuous environmental-derived antigens.

2 How Do Antigens Become Allergens?

Allergens are diverse materials and can be defined according to their chemical structures, by their origins and by the route of exposure (Table 1) [14, 15]. Generally, they have a low molecular weight, are highly soluble, often carry carbohydrate side chains and have various enzymatic activities.

How do antigens become allergens is not well understood [15, 17] but the presence of "danger" signals, a compromised physical barrier [18, 19] and cooperation between structural components

Table 1 Classification of the most common allergens according to origin and biological functions

Internal tissue-derived:	Enzymes (especially proteases), ligand-binding proteins or lipocalins, albumins, tropomyosins and calcium-binding proteins
Foods:	Lipid transfer proteins, profilins, seed storage proteins and tropomyosins The FDA identified the following list as allergenic food products by law (>10 ppm) (https://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAllergens/ucm530854.htm): 1. Milk 2. Eggs 3. Peanuts 4. Tree nuts (almonds, cashews, walnuts) 5. Fish (bass, cod, flounder) 6. Shellfish (crab, lobster, shrimp) 7. Soy 8. Wheat Other common allergenic foods: celery and celeriac, corn or maize; fruit, pumpkin, eggplant, legumes, beans, peas, soybean milk; seafood; sesame, pecan nuts
Animal products	Fur and dander; cockroach calyx; wool
Insect stings	Bee sting venom; wasp sting venom; mosquito stings
Mold spores	*Alternaria alternata; Aspergillus fumigatus*
Plant pollens	The most widespread groups of plant proteins that contain allergens are the cupin and prolamin superfamilies and the protein families of the plant defense system [16]
	Pathogenesis-related proteins, calcium-binding proteins, pectate lyases, β-expansins and trypsin inhibitors; Grass: ryegrass, timothy-grass Weeds: ragweed, plantago, nettle, *Artemisia vulgaris*, *Chenopodium album*; sorrel Trees: birch, alder, hazel, hornbeam, Aesculus, willow, poplar, *Platanus tilia*, Olea, *Ashe juniper*, *Alstonia scholaris*
Drugs	Salicylates (also found naturally in numerous fruits) penicillin, sulfonamides
Other	Latex, metal, wood

Modified from http://www.allergen.org/; https://en.wikipedia.org/wiki/Allergen

of mucosal organs (or skin) and immune cells through released mediators and physical interactions are thought to be important in driving this process [20–33]. Involvement of innate (lymphoid cells/macrophages/dendritic) cells and epithelial cells in allergic (Th2 type) responses are also essential [34–37].

2.1 How Allergens Are Sensed?

Differential recognition of allergen molecular patterns by the adaptive immune system is dependent on a prior sensitization process and a complex antigen presentation between antigen presenting cells and antigen-specific T lymphocytes. Antigen-specific (cognate) activation of T and B cells is a prerequisite of the adaptive immune response.

Allergens can function as adjuvants shaping their own immune response. Innate recognition of allergens is mediated by constitutively active pattern-recognition through a range of receptors (PRRs) in epithelial and immune cells of the barrier surfaces. PRRs can be surface bound, such as the toll-like receptors (TLRs) or C-type lectin receptors (CLRs); cytoplasmic, such as the nucleotide-binding oligomerization domain protein (NOD)-like receptors (NLRs) or the RNA helicases (RIG-like receptors [RLRs]) [38–42]. PRRs can also be soluble such as the pentraxins, mucins, and the initiator molecules of the complement system, C1q, collectins and ficolins [43, 44]. A recently described secreted pathogen sensor is PLUNC (Palate, Lung, Nasal Epithelium Clone), an abundant secretory product of epithelia present throughout the conducting airways of humans and other mammals. PLUNC is evolutionarily related to the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family [45].

PRRs specifically recognize conserved motifs called pathogen-associated molecular patterns (PAMPs) and newly discovered self-derived molecules after cell damage or death, through damage-associated molecular pattern (DAMP) recognition. DAMPs have recently emerged as important proinflammatory mediators in chronic allergic airway inflammation and include S100 proteins, the high mobility group box 1 (HMGB1) protein, actin filaments, extracellular ATP, glucose, monosodium urate (MSU) crystals and calcium pyrophosphate dihydrate (CPPD). Intratracheal administration of house dust mite (HDM) to mice induced the production of uric acid by epithelial cells and promoted Th2 sensitization by amplifying production of IL-25, IL-33, and TSLP. Uricase treatment reduced allergic inflammation and airway hyperresponsiveness [46]. Epithelial injury after fungal airway exposure resulted in the release of ATP, acute extracellular accumulation of which induced IL-33 and subsequent Th2 response [47]. Thus, DAMPs produced by epithelial cells can promote initiation and persistence of allergic inflammation. DAMPs are recognized by NOD-like receptors (NLRs) [48]. NLRs can also recognize alum, cholesterol, environmental irritants, silica and asbestos [49]. A subset of NLRs (NLRP1, NLRP3 and NLRC4) can assemble and oligomerize into a common structure (inflammasome) that activates the caspase-1 cascade leading to production of IL-1β and IL-18, thereby initiating the inflammatory response.

C-type Lectins (CLRs) form a large family of receptors that bind to carbohydrates in a calcium-dependent manner through conserved carbohydrate-recognition domains. CLRs include type I (DEC 205, MMR) and type II (dectin-1, 2, Mincle, DC-SIGN, DNGR-I) membrane molecules [50–53]. Soluble CLRs are the collectins (SP-A, SP-D and MBL) that participate in allergen opsonization and suppression of inflammatory cell activation.

Allergens can also have ***protease activities***. Extracellular serine, aspartic, and metalloproteases (components of many airborne pathogens and allergens [54–56]) and novel membrane-associated proteases, such as yapsins and ADAMs [57, 58], can induce Th2 inflammatory responses

by altering the permeability of epithelial barrier (disrupting the epithelial tight junctions [21]) and allowing allergens to cross and interact with mucosal dendritic cells [59]. Proteolytic enzymes contribute to inflammation through interactions with the kinin system [60] as well as the coagulation and fibrinolytic systems [61] and the complement cascade [62–64]. Proteases can also induce proinflammatory cytokines through protease-activated receptors (PARs) [57]. PARs are identified on immune, inflammatory and structural cells. Specifically, epithelial cells express PAR-1 and PAR-2 receptors, the more prominent of the four identified protease-activated receptor molecules. The mechanisms by which the seven transmembrane G-protein coupled protease receptors interact with proteases involve cleavage of the N-terminal region exposing a cryptic "receptor activating sequence". If the cleaved region includes the receptor activating sequence, the receptor becomes inactivated. Thus, proteases may activate or inactivate PARs depending on the proteolytic cleavage site [65]. Because its major activator is thrombin, PAR-1 links the coagulation and inflammatory cascades. PAR-1 mediates production of protein C, an anti-inflammatory/cytoprotective molecule. The PAR-1/protein C pathway is impaired in asthmatic patients and in mouse models of allergic airway sensitization [61]. PAR-2 is activated by trypsin, mast cell tryptase, leukocyte proteinase-3, bacterial enzymes and a wide variety of cockroach [66], mold and mite [67] allergens that exhibit serine protease activity. A number of studies in animals suggested that PAR-2 activation plays a proinflammatory role in asthma [67–71]. A protective role of PAR-2 activation was also suggested using a PAR-2 activated peptide in allergen-challenged rabbits [72]. Of note, pollen grains with distinct allergenic abilities can also release proteases. These, however, work by attacking the epithelial tight junctions and facilitating allergen delivery across the epithelium (Fig. 1) [54].

TLR Activation is critical not only to detect pathogens in the epithelia but also to regulate Th2 cell responses induced by inhaled allergens as well [29, 73–80]. Activation of TLRs by allergens has been implicated in allergic sensitization and experimental evidence demonstrates an increase in allergen-induced asthma severity after exposure to LPS [81]. Interestingly, Derp2 allergen of the HDM showed structural homology with the LPS-binding protein MD-2 (TLR4 associated adaptor protein). Derp2 acted on structural cells to increase production of Th2-inducing cytokines, such as thymic stromal lymphopoietin (TSLP), IL-33, IL-25 and GM-CSF, all closely associated with the development and pathogenesis of allergic inflammation [77, 82]. Under conditions of low LPS exposure, Derp2 interaction facilitated LPS signaling through TLR4 in the absence of MD-2 and shifted the immune response towards a Th2 type [75]. Allergens may also trigger synergistic effects between PAR-2 and TLR signaling in the epithelial cells [70], mounting Th2 responses and allergic airway inflammation through the release of Th2-inducing cytokines. Lastly, a recent paper indicated that innate immunity of the upper and lower airways was distinctive, because HDM-derived beta-glucans, rather than LPS, activated innate immunity in the nasal mucosa dependent on TLR2, but not on TLR4. In contrast, the LPS/TLR4 signaling axis, rather than beta-glucans/TLR2, was critical to HDM-induced allergic asthma in mice. Thus, differential TLR activation on airway epithelial cells may be responsible in determining the nature of innate immune response of the nose and lungs, leading to allergic inflammation of the upper or lower respiratory tract, respectively [83].

Role of Viruses in allergic airway inflammation: Epithelial cells are the primary target and residence for respiratory viruses such as the human rhinoviruses (HRV), respiratory syncytial virus (RSV) and influenza A virus. In fact, there is considerable evidence that viral respiratory infections and respiratory allergies are the two most significant risk factors for the onset of

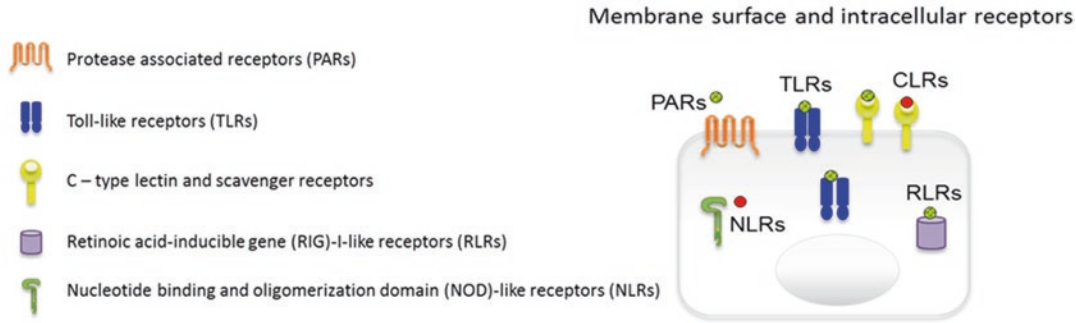

Fig. 1 Cell associated innate immune receptors**:** PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns) are recognized by PRRs (pattern recognition receptors). The receptor families listed here are relevant to allergen-induced inflammation because they bind pathogens that have a modulating role in the inflammatory changes. The Protease associated receptors (PARs), Toll-like receptors (TLRs), C-type lectin receptors (CLRs), can also directly bind allergens. Antibody binding receptors (not listed here) are part of the adaptive immune response

asthma in children and exacerbations of asthma in adults [84, 85]. Respiratory virus infection occurs mainly through receptor-mediated endocytosis. In case of influenza A virus infection, epithelial mediated asthma exacerbations could be due to a damage to epithelial cells and production of inflammatory mediators [86, 87] or activation of innate immune effector and inflammatory cells [88]. Influenza A virus attacks epithelial cells via hemagglutinin that recognizes sialic acid (N-acetyl neuraminic acid) bound to underlying sugars on the tips of the cell glycoproteins [87]. RSV and HRV receptors on the other hand include a number of shared putative molecules such as intercellular adhesion molecule (ICAM)-1 and TLRs. Heparin, annexin II, and fractalkine (CX3CL1) receptor, CX3CR1 additionally were shown to mediate some of the biological functions of RSV, that now also has a newly discovered selective receptor, nucleolin [reviewed by [89]]. HRV infection is the most common cause of asthma exacerbation. The majority of HRV-A and -B strains bind to ICAM-1 (CD54) and only the minority (approximately 10%) of the HRV-A binds to members of the LDL receptor family. HRV upregulates its own receptor, ICAM-1 on epithelial cells in an NF-kB-dependent manner [90, 91] and causes production of inflammatory mediators and loss of glucocorticoid responsiveness [92].

In chronic airway inflammation, there is also evidence for a role of membrane-bound and cytoplasmic RNA sensing molecules, such as TLR3 and TLR7/8 [93], that are localized to endosomal and plasma membranes and bind double-stranded and single-stranded RNA, respectively. Activation of TLR3 leads to induction of the RNA helicases (RIG-like receptors [RLRs] and melanoma-differentiation-associated gene 5 [MDA-5]). All three of these (TLR3, RIG-1 and MDA-5) cooperate in the upregulation of innate interferon (IFN) responses to HRV infection [94]. In asthma the early TLR3 signaling is defective, leading to inadequate IFN-β and IFN-λ responses that account for the impaired viral clearance and consequent epithelial damage in this disease [95]. Viral infections with single- or double-stranded RNA can induce NLRP3 activation and IL-25, IL-33, and TSLP release by epithelial cells *in vitro* and *in vivo*, mediating Th2-type airway inflammation. Indeed, infection of mice with influenza virus induces IL-33 release from alveolar macrophages, resulting in the production of IL-13 by group 2 innate lymphoid cells [88, 96].

Expression of IL-25, IL-33, and TSLP through innate immune signaling is critical to drive the Th2 response. These cytokines are initially produced by epithelium at the mucosal surfaces in response to challenges but they are subsequently

amplified by other tissue-resident and immune cells during inflammatory responses. In recent years, the roles for IL-25, IL-33, and TSLP in Th2 type airway inflammation have been extensively studied in a variety of experimental models and in human systems. These studies consistently find potent activities of these cytokines to induce and amplify Th2 type immune responses [reviewed in [97]]. Transgenic overexpression of these cytokines induces Th2 cytokine expression in tissues, airway eosinophilia, increased release of IgE antibodies and Th2 cytokines into the serum, mucus overproduction, with goblet cell hyperplasia, airway thickening, and airway hyperresponsiveness, all consistent with human asthma [98–100]. Such cytokine-induced pathological airway changes are also apparent in mice deficient in the Rag gene, emphasizing the significance of innate immune cells in initiating the allergic immune responses [101].

Chemokine and Cytokine Release also plays an important role in driving Th2-type immune responses. Following allergen challenge, CCL17 and CCL22 mediate migration of Th2 and dendritic cells to the airways by interacting with their common receptor, CCR4 [78, 102]. CCL20 (the only chemokine that ligates CCR6) is also important in attracting T cells and dendritic cells to the epithelial area of the respiratory tract [77]. Autocrine cytokine stimulation of residential cells in the airways amplifies the initial PRR-induced proinflammatory gene activation. For example, in the early stages during allergen-induced airway inflammation, IL-17 can upregulate release of the CXCR2 ligands, CXCL1, CXCL6 and CXCL8 as well as GM-CSF and G-CSF. It can also induce release of CCL20 and β-defensins, both of which act on CCR6 to attract dendritic cells and memory T cells [103]. IL-17 can also act synergistically with viral infection or other proinflammatory cytokines such as TNF-α and IL-1β to enhance inflammatory responses [104]. Transgenic expression of IL-17 in airway epithelial cells induced airway eosinophil and lymphocyte infiltration and structural changes with mucus metaplasia [105]. Mice deficient for the IL-17 receptor are protected from allergen-induced airway inflammation, and in humans, asthmatic subjects have higher levels of BAL and sputum IL-17 [106–108]. Importantly, after cessation of allergen exposure, the timely resolution of allergic airway responses is also governed by regulation of the Th17 pathway.

Genetic and Epigenetic Influences Genome-wide association studies (GWAS) and meta-analyses of GWAS discovered new asthma susceptibility genes including IL1RL1/IL18R1 [109], IL-33, ORMDL3/GSDMB, SMAD3 [110], and TSLP [111]. Associations with variation in the genes encoding IL1RL1/IL18R1 (the IL-33 receptor ST2), IL-33, SMAD3, and TSLP indicated the importance of both innate immune response promoting Th2 pathways (atopy) and asthma. On the other hand, variation at the 17q21 asthma locus, encoding the ORMDL3 and GSDML genes were specifically associated with risk for epithelial remodeling and childhood-onset asthma but not IgE [112]. Recent technical advances facilitated assessment of the effect of epigenetic modifications on development of allergic airways changes. Epigenetic changes include post-translational modifications of histones and chromatin remodeling, DNA methylation or mRNA regulation through small noncoding RNAs, including microRNAs (miRNAs) that interfere with translation [reviewed in [113]]. Epigenetic regulation can affect the promoter region or the gene itself. It can result in gene silencing or aberrant expression. Epigenetic alterations can be carried over in the genes through meiosis, therefore specific periods in life such as in utero, during childhood or adolescence are highly susceptible to these influences. Environmental factors such as air pollution [114], psychosocial stress [115], cigarette smoke exposure, nutrition/obesity [116], can all alter the epigenome that can in turn influence T cell phenotype and affect the development of asthma [113]. For example, methylation array data in clinic visits of 141 subjects from the Normative Aging Study found that exposures to black

carbon and sulfate were significantly associated with the gene methylation pattern in the asthma pathway [114]. In a study of the gene ADCYAP1R1 in Puerto Rican children 9 years and older, exposure to violence was associated with promoter methylation and increased odds of asthma [115]. DNA methylation in the Neuropeptide S Receptor 1 (NPSR1) promoter was also found to be significantly linked with cigarette smoking and obesity in relation to asthma [116].

3 Cellular Interactions with T-cells Driving Th2-type Inflammatory Response in Asthma

Dendritic Cells were named after their membrane projections resembling the dendrites of neurons. There are two major classes of these cells, *Classical* (or *conventional*) and *Plasmacytoid*. Conventional dendritic cells are originated from myeloid (bone marrow-derived) precursors. Similarly to tissue macrophages, dendritic cells constantly sample the environment in which they reside and capture tissue antigens, migrate to the draining lymph nodes while becoming activated, upregulate costimulatory molecules, produce inflammatory cytokines, and initiate adaptive T cell responses. Classical dendritic cells may be divided into two major subsets: (1) BDCA-1/CD1c (in humans) or CD11b (in mice), is most potent at driving $CD4^+$T cell responses and (2) BDCA-3 (in humans or in mice), CD8 in lymphoid tissues or CD103 in peripheral tissues, is an efficient cross-presenter (described later in this chapter). Some dendritic cells may be derived from monocytes, especially in situations of inflammation (Fig. 2).

Plasmacytoid dendritic cells (looking like plasma cells) are poorly phagocytic and do not sample environmental antigens. They secrete large amounts of type I interferons and in viral infections, present antigens to virus-specific T cells [117]. Activation of selective TLRs on epithelial cells enhances dendritic cell motility and antigen sampling through the production of Th2-promoting chemokines CCL17 and CCL22 [78]. Recruitment and local survival of dendritic cells is mediated by CCL20 (a ligand for CCR6 present on immature dendritic cells) and GM-CSF, respectively [32, 80, 118, 119]. Recruitment of dendritic cells to the airways is essential in the adaptive B, T and NK cell-mediated immune responses important in defense against infection by viruses and bacteria and in allergen-driven inflammation of the airways [120, 121]. The nature of the immune response that occurs after dendritic cell exposure to antigens is determined by the state of dendritic cell activation and the context in which they present antigen to T cells, i.e. the level and type of co-stimulatory molecules and cytokine pattern expressed by the dendritic cells. TSLP can directly activate dendritic cells to prime naïve $CD4^+$ T cells to differentiate into proinflammatory Th2 cells that secrete IL-4, IL-5, IL-13 and TNF, but not IL-10; it can also stimulate the expression of the prostaglandin D2 receptor CRTH2 [122–124]. The process by which this polarization occurs involves the induction of the Th2-cell associated co-stimulatory molecule OX40L (Tumor necrosis factor receptor superfamily, member 4) by dendritic cells [125]. The polarization of Th2 cells induced by TSLP-matured dendritic cells is further enhanced by IL-25 [126].

Macrophages have a central role in the maintenance of immunological homeostasis and host defense in the lung. They can be classified depending on their localization *as* "alveolar" vs. "interstitial"; depending on their origin as "migratory vs. residential" and depending on their function as "M1 vs. M2" (or "classically activated" vs. "alternatively activated") [127, 128]. Dependending on the activating stimulus received, M2 macrophages can be further subdivided into M2a (after exposure to IL-4 or IL-13), M2b (in response to immune complexes in combination with IL-1β or LPS), M2c (IL-10, TGFβ or glucocorticoids), and M2d (induced by TLR agonists through the adenosine receptor) [129, 130]. Depending on their differential

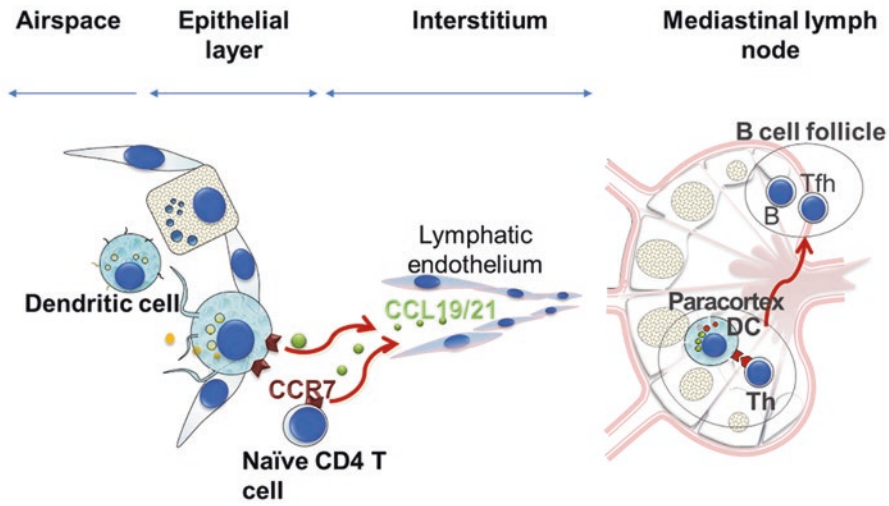

Fig. 2 Dendritic cells and initiation of adaptive immunity of airway inflammation. Classical dendritic cells have the ability to either enter the air spaces or to extend their dendrites out and sample the environment and capture tissue antigens. Upon antigen uptake, dendritic cells mature and express CCR7. This chemokine receptor is ligated by CCL19 or CCL21 that are produced by endothelial cells of the lymphatic vessels in increasing concentrations towards the lymph nodes. While becoming activated, dendritic cells upregulate costimulatory molecules, produce inflammatory cytokines, and initiate adaptive T cell responses in the paracortex. T helper cells then become follicular helper cells (Tfh), migrate to the B cell follicle and help allergen specific B cells in class switch recombination and IgE production

expression of mediators, M2 cell subclasses can support allergic airway inflammation; help resolution of airway changes through a heightened phagocytic capability; facilitate remodeling; and provide immunosuppressive activities. During the allergic airway response, bone marrow-derived monocytes migrate through the circulation and differentiate under the effects of locally released cytokines, chemokines, and other inflammatory mediators. These local factors are important in the priming and skewing of these cells into "alternatively activated" macrophages, a proinflammatory Th2-type subclass of M2 macrophages [131]. The "classically activated" M1 macrophage phenotype promotes Th1-type inflammation [132]. In Th2-type allergic asthma, the M2 macrophage phenotype is stimulated by the absence of IFNγ signaling to the M1/Th1 pathway [132]. Indeed, in three different mouse models of allergic inflammation, the M2 macrophage phenotype was predominant in severe asthma [133]. The M2 phenotype can be induced by IL-33 in alveolar and polarized macrophages [134, 135]. M2 macrophages express CD206 (mannose-binding receptor, MR) and promote the adaptive allergic immune response through a unique mediator profile, including IL-4, IL-5, IL-10, IL-13 and IL-33, CCL17, CCL18, CCL22, CCL24, HO-1, Arginase 1, chitinase-like Ym1, and Fizz1 (found in inflammatory zone-1) [133, 136–138].

Innate Lymphoid Cells In 2010, three independent groups identified a novel innate immune cell type that following alarmin cytokine (IL-33, IL-25, TSLP) activation, was an early producer of IL-5 and IL-13 during allergic inflammation [139]. These cells have since been named group 2 innate lymphoid cells (ILC2s). In mouse models, ILC2s were demonstrated to play an essential role in parasitic diseases and in allergic asthma, producing levels of IL-5 and IL-13 that resembled those derived from Th2 cells [140–145]. Subsequent investigations provided the first direct evidence that in response to IL-25 and IL-33, these cells mediated airway inflammation by secreting Th2 cytokines [146, 147]. A pathogenic role of ILC2s was shown in nasal polyps of

patients with chronic rhinosinusitis and in severe asthmatics with high levels of eosinophils present in the airways [147, 148]. Studies from our laboratory and many others used mouse models to demonstrate that ILC2s are required for Th2-type inflammation [147, 149–151] and allergen-induced airway hyperresponsiveness [152]. These findings indicate that as an innate counterpart to Th2 cells, ILC2s can significantly shape Th2-type immunity.

Eosinophils are the major effector cells in allergic asthma, contributing to airway hyperresponsiveness. As such, targeting and preventing these cells from migrating to the airways has been the focus for asthma therapeutics for some time [153]. Epithelial cells infected with RSV produce eotaxin and RANTES, major chemoattractants for eosinophils [154]. TSLP and IL-33 directly activate eosinophils, increasing their survival and inducing superoxide anion and CXCL8 production [155]. IL-25 induces production of IL-6, CCL2, CCL3, and CXCL8 in human eosinophils [156]. As a positive feedback, eosinophil-derived cationic proteins can activate epithelial cells to produce growth factors, matrix metalloproteinases (MMPs), and other factors involved in airway remodeling processes [157].

Neutrophils are the earliest cells to migrate into damaged or infected tissues, usually recruited by CXCL8 and CXCL1–3, chemokines released from residential cells at the site of inflammation [158, 159]. Growth factors, including G-CSF and GM-CSF, are induced by allergens and IL-17 [160, 161]. GM-CSF expression activates and promotes the survival of many inflammatory cell types and is enhanced in patients with allergic rhinitis and asthma [162]. Granulocytes in turn can also alter host defense functions. Neutrophil elastase can induce expression of CXCL8, mucins, and epithelial anti-microbial substances, such as secretory leukocyte peptidase inhibitor, and human β-defensin 2, providing a self-amplifying defense mechanism [163, 164]. Neutrophil elastase also increased the biological activity of IL-33 [165]. In turn, IL-33 enhanced neutrophil migration in sepsis and psoriasis by upregulating CCR2 expression [166, 167]. While eosinophils are thought to be the main culprit in allergic asthma, more severe forms of disease are associated with chronic neutrophil accumulation in the airways [168]. During acute exacerbations, neutrophils are also found in significant numbers in the airways, suggesting their importance in airflow obstruction [169]. Because of their apparent importance in acute exacerbations and severe, chronic disease, understanding the early and late mechanisms that drive their recruitment to the airways is an important future research direction.

Mast Cell infiltration of the airways mucosal tissue is considered a hallmark of asthma [170]. Mast cells express TSLP receptor and respond to TSLP by producing high levels of cytokines including IL-5, IL-6, IL-13, GM-CSF, CCL-1 and CXCL8 without degranulation [171]. IL-33 also stimulates mast cells to produce Th2 proinflammatory cytokines and chemokines and enhances their maturation and survival [172, 173]. These new findings suggest that mast cells are a non-T-cell-dependent mechanism for initiating and maintaining Th2 inflammation in allergic asthma. Histamine released within minutes from activated mast cells in turn can promote proinflammatory responses and promote airway smooth muscle constriction, impairing breathing [174]. Current research trends seek to understand how T-cell-mast cell crosstalk is skewed towards proinflammatory Th2 responses in allergic asthma, while preventing mast cell activation is an appealing therapeutic target.

Basophils are activated by epithelial-derived TSLP and IL-33, while IL-3 is critical for their development and activation [175]. Upon stimulation with IL-33, basophils produce proinflammatory cytokines including IL-1, IL-4, IL-5, IL-6, IL-8, IL-13, and GM-CSF and chemokines RANTES, CCL2, CCL3, and CCL4 [176]. IL-33 and TSLP can also induce basophil expansion

and their differentiation from the bone marrow [177–179]. Functionally, several reports pointed toward an important role for basophils in providing an early source of polarizing IL-4 to enhance Th2 development [73]. In human patients that died from asthma, basophils were highly enriched in lung tissue [180]. Overall, the significance of basophil-derived inflammatory mediators is poorly understood.

Natural Killer T Cells that express the IL-17RB (receptor for IL-25) and predominantly produce IL-13 and chemokines upon stimulation with IL-25 were implicated in AHR and airway inflammation induced by airway administration of lipid antigens or bacterial glycolipids [181, 182]. Invariant NKT cell responses to ambient antigens promote sensitization to conventional respiratory allergens [183]. NKT cells acted as an adjuvant to enhance Th2-type inflammation and allergic airway inflammation in asthmatic mice [184]. In nonhuman primates, α-galactosylceramide exposure (that directly activated NKT cells) induced AHR in monkeys not sensitized to an allergen, suggesting that NKT cells impact lung function even in naïve animals [185]. Both increased and decreased numbers of NKT cells have been observed in human patients with asthma, so their pathogenic role in allergic asthma is still highly debated [186, 187].

B cells B cells have been demonstrated to play a critical role in allergic asthma, particularly when the amount of allergen is low, by their ability to uptake and present antigen on MHC II, similarly to dendritic cells. In the germinal center of the lymph nodes, B cells undergo rapid clonal expansion (proliferation) and express the genome mutator enzyme activation-induced cytidine deaminase (AID). AID is indispensable for two key GC processes, somatic hypermutation (SHM) and class-switch recombination (CSR) [188]. SHM introduces non-templated mutations in the immunoglobulin (Ig) variable region, changing the antibody's affinity for antigen [reviewed in [189]]. CSR alters the antibody effector functions by recombination of the variable region (linked initially to the μ and δ heavy chain exons, encoding IgM and IgD, respectively) with one of the downstream constant region genes of the α, γ or ε isotypes, encoding IgA, IgG and IgE. Each antibody isotype initiates different downstream immune reactions and thus adapts the response to the nature of the challenge faced. Together these processes shape the antibody response, producing germinal center B cells with high-affinity, class-switched antibodies that can differentiate into either antibody-secreting plasma cells or long-lived memory B cells [188, 189]. In addition to their key function in the production of immunoglobulins, B cells can regulate immune responses through their surface molecules and secretion of cytokines (reviewed in [190]). Regulatory B (Breg) cells produce IL-10, IL-35 and TGF-β. Breg cells in mice can be CD5(+) CD1d(hi) B10 cells, CD21(hi)CD23(hi)CD24(hi) transitional stage 2-like B cells, and CD138(+) plasma cells and plasmablasts. Human Breg cells are CD27(+)CD24(high) B10 cells, CD24(hi) CD38(hi) immature transitional B cells, and CD73(−)CD25(+)CD71(+) BR1 cells and a subset of plasma cells [190]. Breg cell numbers increase during the course of allergen-specific immunotherapy. Human BR1 cells selectively upregulate IgG4 antibodies on differentiation to plasma cells that promotes the protective effects of immunotherapy. Thus, Breg cells mediate allergen tolerance.

$CD34^+$ Hematopoietic Stem/Progenitor Cells can also function as inflammatory effector cells during the asthmatic response without having to first undergo differentiation. The hematopoietic progenitors express receptors for TSLP and IL-33 and when stimulated with these molecules produce very high levels of Th2 cytokines including IL-5, IL-6, IL-13, and GM-CSF [191].

Epithelial Cells, Fibroblasts and Smooth Muscle Cells can all significantly shape the tissue response during the adaptive immune effector function. These structural cells respond to

environmental stimulants such as cigarette smoke, lipopolysaccharide, and oxidant and virus infection, by rapid activation of proinflammatory signaling and induction of immune and inflammatory cell migration cascades [192, 193]. Th1 cells are attracted to the respiratory mucosa by the CXCR3 ligands CXCL9, CXCL10 and CXCL11, produced upon exposure to interferons or infection with respiratory viruses [194–196]. On the other hand, structural cells of the airways induce epithelial migration of Th2 cells via production of the CCR8 ligand CCL1 and the CCR4 ligands CCL17 and CCL22 [197, 198]. Epithelial expression of CCL17 and levels of CCL17 in BAL indeed is increased upon allergen challenge of asthmatic subjects [199]. CCL1 production can be induced in airway epithelial cells by Th2 cytokines, IL-4 and IL-13, but also IFN-γ [200] suggesting that epithelial cells may control and amplify both Th1- and Th2-type inflammatory responses locally depending on the predominant cytokine milieu.

4 Transcriptional Regulation of Th2 Cell Differentiation:

After the cognate interaction between dendritic cell MHC-II:CD4$^+$ TCR (T cell receptor) signaling occurs, the prevailing cytokine milieu determines the phenotypic fate of the naïve CD4$^+$ T cell [201]. IL-12 secreted by dendritic cells induces activation of Th1 cells, which then produce TNF-α and IFN-γ. These cells are specialized to help during viral or bacterial clearance, but autoimmunity and inflammation can occur if they become sensitized to self-antigens. High doses of allergen have been also shown to favor Th1 polarization. Th1 cells express the transcription factor Tbet.

IL-4 and IL-10 induce differentiation of Th2 cells which then produce IL-4, IL-5, and IL-13, induce eosinophilia and B cell class-switching to IgE. These Th2 cells are classically associated with eosinophilic allergic asthma, and the IgE bound to the high-affinity IgE receptor of mast cells causes degranulation upon contact with allergen, perpetuating the allergic response. Th2 cells express STAT6 and GATA3 [202].

IL-4, IL-6, and TGF-β in combination induce Th9 cells, that produce IL-9 and are thought to play a role in asthma and autoimmunity by inducing survival and proliferation of Th2-cytokine secreting ILC2s. The transcription factor Foxo1 is essential for IL-9 production [203].

IL-21, IL-23, TGF-β and IL-6 induce Th17 cells, that secrete IL-17A and IL-17F, attracting neutrophils that contribute to neutrophilic airway inflammation and asthma [reviewed in [204]]. Many patients have been found to exhibit a dual Th2/Th17 phenotype and are particularly refractory to current treatments. Th17 cells express STAT3 and RORs.

TNF-α and IL-6 induce Th22 cells, which secrete IL-22 and can be proinflammatory or protective to the epithelium depending on the context [205]. Inflammation is mediated by induction of antimicrobial peptides from the epithelium and TNF-α, IL-17, and IL-1 secretion. Tissue protection is carried out by amphiregulin production, strengthening the epithelial barrier.

IL-21 and IL-27 lead to differentiation of Tfh cells, which play a critical role in the induction and maintenance of B cell class-switching inside lymph node germinal centers. These cells are responsible for the production of high-affinity, antigen-specific IgE by plasma B cells. They express STAT3 and Bcl6 transcription factors.

IL-10 and TGF-β induce Treg cells, whose role is to dampen inflammation once the antigen has been cleared. They also play a role in preventing autoimmunity. The typical Treg transcription factor is FOXP3.

Allergic airway inflammation is driven by production of IL-4, IL-5 and IL-13. A major producer of these cytokines is the differentiated CD4+ T-helper 2 (Th2) cell. IL-4 is important in allergic sensitization, IgE production and Th2 cell differentiation while IL-5 is critical in accumulation of eosinophilic granulocytes and their activation in the lung. IL-13 has pleiotropic effects in tissue remodeling and the development and progression of airway hyperresponsiveness. Th2 cytokines were, therefore, identified as

therapeutic targets with high hopes for the treatment of asthma [206]. Anti-Th2 cytokine therapeutics, however, originally had poor results [207], because these cytokines have numerous overlapping functions. The fact, therefore, that the IL-4, IL-13 and IL-5 genes fall under similar transcriptional regulation [204, 205], especially during allergic airway inflammation [208], is therapeutically intriguing. The genes for IL-4, IL-13 and IL-5 are clustered in a 120 kb region on chromosome 11 in mice and a 160 kb region on chromosome 5 in humans and are activated in a T cell lineage-specific fashion. Their transcription is coordinately regulated by an intricate network of transcription factors and other regulators of the gene promoters that activate and/or repress them [reviewed in [208]]. This process results in characteristic chromatin structure changes [209] in the Th2 cytokine locus that also serves as a general model system of chromatin conformation of immune genes [210]. Transcription activators interact with the core transcription machinery through binding to *enhancers* while repressors work by recruiting *co-repressor complexes* leading to chromatin condensation of enhancer regions. Repressors and activators may also function by competing with each other for occupation of shared DNA-binding sequences [211]. Whether a transcription factor is activated or not is determined by its localization and ability to bind DNA. The initiation, termination and regulation of transcription are influenced by epigenetic and metabolic processes [212, 213] and requires "DNA looping", an important mechanism to bring the promoters, enhancers, transcription factors and RNA polymerases together [214].

The transcription factors that can induce signature cytokines for distinct effector CD4 T cell lineages and are both necessary and sufficient to elicit this process, are called "master regulators" [212]. These bind to a specific region of DNA, critical for transcription factor binding and complex formation that allows for efficient activation of the Th2 cytokine genes [215]. These genes are expressed preferentially from one chromosome rather than at random, suggesting coordinated expression. A locus control region (LCR) is a cis-acting element that confers tissue-specific high-level expression to linked genes [reviewed in [208]]. To identify the Th2 cytokine LCR, investigators deleted a conserved noncoding sequence-1 (CNS-1) between the IL-4 and IL-13 genes and found that this reduced the ability to produce IL-4 by Th2 cells but not mast cells [216] in transgenic mice suggesting that CNS-1 is not an LCR but rather an important T-cell specific enhancer. Another study demonstrated that none of the previously described DNase I hypersensitive sites or conserved sequences in the Th2 cytokine locus had LCR activity, either individually or in combination as a minilocus. However, by evaluating copy number dependency in mice containing transgenes encoding the whole Th2 cytokine locus, other investigators recently found the LCR, located within a 25 kb region containing the 3′ portion of the *RAD50* gene [217, 218]. The *Rad50* gene separates the IL-4 and IL-13 genes from the IL-5 gene [219–221]. There are four sites within the Th2 LCR that are "DNase I hypersensitive", [i.e. exposed chromatin, highly susceptible to binding by proteins like DNase I or transcription factors [219]], implying their importance in transcription. The DNase I hypersensitive sites of the *Rad50* gene are RHS4, RHS5, RHS6, and RHS7 [220, 222]. Indeed, it has been demonstrated that these sites coordinate transcription of the Th2 cytokine genes, although the importance of RHS4 has not been fully established [213, 221, 223]. RHS6 is thought to be the most important of the four sites for Th2 cytokine transcription as it is essential for the expression of each of the IL-4, IL-5 and IL-13 genes [221]. RHS6 contains a number of sites for transcription factor binding, including two direct sites for GATA3 [224, 225]. While several transcription factors are implicated in the regulation of the Th2 cytokine genes, GATA3 is the master regulator of Th2 cell differentiation and is essential for expression of IL-5 and IL-13, but not IL-4 [226]. Large protein complexes formed at the RHS6 DNA subregion suggest that additional transcription factors may also participate in Th2 cytokine gene activation [208, 224, 225].

Such transcription factors are SATB1 and IRF4, also found in all the isolated subregions of RHS6. SATB1 regulates GATA3, coordinating Th2 development [227]. IRF4 is required for Th2 cell differentiation, it binds the IL-4 promoter and acts both upstream and downstream of GATA3 [226]. In the absence of RHS6, GATA3, IRF4, and SATB1 binding was reduced at all sites in the Th2 cytokine locus control region [225]. Thus, RHS6 is a critical regulatory element for effective Th2 cytokine transcription, as suggested by others [221]. The role of RHS6 in mediating Th2 cytokine transcription is conserved among cell types. Because RHS6 is located between the IL-4/IL-13 and IL-5 genes, it is intriguing, that RHS6 can mediate the transcription of up- and downstream targets. This fact brings up the importance of the three-dimensional structure of chromatin and suggests that regulatory elements throughout the genome can have profound effects on genes both up- and downstream.

Thus, GATA3, IRF4, and SATB1 physically complex at RHS6 in the *Rad50* gene of the Th2 cytokine locus, during allergic airway inflammation. A number of genome-wide association studies have revealed that single nucleotide polymorphisms in the *Rad50* gene are associated with asthma and allergy [228–230] but the involvement of the RHS6 region remain unclear in these. Interfering with the physical association of the GATA3, SATB1, and IRF4 complex by targeting RHS6 maybe an attractive, novel therapeutic possibility to reduce Th2 cytokine production during chronic airway inflammation.

5 Targeting T-cell Dependent Inflammation in Asthma

Allergen-specific Immunotherapy (AIT) T cells can be manipulated to induce or suppress immune responses for example, with the use of vaccines (biological preparations of disease-causing agents). When successful, such manipulation can elicit broad and specific immune responses [231, 232]. The development of vaccines has been among the greatest medical breakthroughs [233] aimed at preventing infections, treating allergic and autoimmune diseases, organ transplantation pathologies, chronic infection and cancer [234, 235]. In AIT vaccines (increasing doses of allergens) are administered to restore immune tolerance and reduce allergic symptoms, once the cause(s) of allergy are identified. The allergen can be given intramuscularly, subcutaneously, sublingually, orally, or intra-lymphatically. The doses of allergen are increased as the patient's tolerance grows over time [236]. AIT has been used for IgE-mediated allergic diseases since the early 1900s [234, 237–242], though it remains fraught with problems [234, 237–242]. It is not easy to produce a closely regulated composition for the allergenic compounds and adjuvants, or to establish consensus in regards to the doses, intervals, and length of application. The danger of anaphylaxis occurring during administration of AIT also remains high. When successful however, AIT treats and prevents development of allergic asthma, rhinitis and venom-induced anaphylaxis and was demonstrated to prevent sensitization with new allergens for up to 12 years [234, 243]. Therefore, the quest to develop safer and more effective AIT continues today [244].

Induction of allergen tolerance requires a decrease in IgE and increase in allergen-specific IgG levels [245]. Successful allergen-specific immunotherapy can result in a 10- to 100-fold reduction in the allergen-specific IgE/IgG4 ratio [190]. IgG4 is a non-proinflammatory immunoglobulin that is uniquely capable to form bispecific and functionally monovalent antibodies through Fab arm exchange [246]. IgG4 has low affinity for activating Fcγ receptors and cannot activate *complement*. Allergen-specific IgG4 protects against allergic responses by competitively sequestering the allergen from IgE binding [245, 247, 248]. By changing the ratio of allergen-specific IgE and IgG, effective AIT should desensitize the high affinity IgE-receptor-(FcεRI) bearing mast cells and basophils, decrease the numbers and activity of eosinophils and basophils in the circulation and mast cells in

mucosal allergic tissues, and induce regulatory T and B cell activation [reviewed in [235]].

The heat-labile enterotoxigenic *E. coli* enterotoxin (LT) contains an "A" and a pentameric "B" subunit (AB_5) and it has been successfully used for vaccination purposes. Skin-patch vaccines containing LT have been recently tested in a large clinical trial for travelers' diarrhea. Although it did not prevent diarrhea, the skin patch vaccine induced a strong IgG response in volunteers [249] indicating high immunogenicity. In addition to its proinflammatory and immunogenic properties, when co-administered with different antigens, LT also markedly enhances the antigen-specific immune response (against the co-administered antigens) indicating adjuvant effects [reviewed in [250–252]]. However, neither the proinflammatory properties nor the high immunogenicity of this molecule can explain its remarkable "adjuvanticity". It is speculated that this effect is due to induction of MHC-class II expression on antigen presenting cells, cell clustering and delay/arrest of T cell proliferation [253]. Based on the literature showing that LT-treated dendritic cells had a slower rate of antigen endocytosis, suggesting that prolonged retention of intact antigen on the cell surface may allow B cells to recognize the bound antigen by their immunoglobulin receptors [254] emphasizing the importance of dendritic cells in mediating "adjuvanticity".

Acting as the crucial link, dendritic cell targeting is a major vaccination strategy against many diverse pathogens. In the skin, these cells are the prime initial mediators of protection. As they become activated upon exposure to allergen, they migrate to the draining lymph nodes (Fig. 1). This in turn activates the adaptive immune system, inducing a T cell cytokine profile that favors the production of large amounts of IgG antibodies by plasma cells that are able to block allergy-causing IgE molecules specific for the same allergen [255]. In addition to the use of novel adjuvants, current immunotherapy research is focused on enhancing antigen-presenting cell function in several different ways. For example, in a design targeting dendritic cell subsets, recombinant proteins with the vaccine epitope in question were fused to GM-CSF [256]. To investigate alternate routes of administration, allergen tolerance was recently induced after intra-lymph node injections of a molecular allergen translocation-Fel d 1 vaccine, mediated by increased cellular internalization of the allergen, activation of inflammasome, and generation of allergen-specific peripheral T-cell tolerance [257]. Adoptive transfer of antigen-specific T cells to allogeneic hematopoietic transplant recipients is also being explored in human studies and holds the promise of vaccinating against aspergillosis [233]. Other potential future strategies employ microspheres made of biodegradable polymers containing the epitope of the allergen in question, given to maximize delivery to antigen presenting cells. This method has a specific advantage in that it is designed to induce both B and T cell responses and lasting memory to certain immunodominant epitopes [258]. Epicutaneous patch AIT may be suitable for various allergens in patients. Patch vaccination is an attractive needle-free alternative to allergy shots (offering the possibility of self-administration for patients) and it targets professional antigen-presenting cells (i.e., dendritic cells, Langerhans cells) residing in the skin [259].

The fields of vaccine design and allergen desensitization are constantly evolving. Recent advancements in AIT mechanisms and use in clinical practice were described in the practical allergy (PRACTALL) initiative report that was endorsed by both the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology [234, 243]. AIT, especially in light of the recent evidence of Sublingual Immunotherapy (SLIT) tablet efficacy and safety in mite allergic asthma patients, is considered to be a prototype of Precision Medicine [244]. In spite of all the advances made, however, safer and more effective AIT strategies are still needed, especially for patients with asthma, atopic dermatitis, or food allergy [234, 243].

Glucocorticoids Despite continued advances in the development of small molecule and biological anti-inflammatory therapeutics, monotherapy with corticosteroids remains the most effective first-line treatment for asthma. Glucocorticoid receptors in the cytoplasm bind to corticosteroids and translocate to the nucleus, where they act as transcription factors to induce the synthesis of anti-inflammatory proteins as well as to repress proinflammatory transcription factors such as NF-kB and AP-1. Because of the complex, heterogenic inflammatory pathophysiology that underlies the syndrome of asthma, the broadly distributed mechanism of action of glucocorticoids in downregulating inflammatory signaling underlies their utility in clinical practice. However, the pleiotropic effects of corticosteroid therapy extend beyond general immunosuppression into regulation of central metabolism and development, and these often adverse effects limit their therapeutic potential. Many strategies have been employed to mitigate the off-target effects of corticosteroids; drug development has focused on creating inhaled corticosteroid formulations with reduced systemic availability and characterizing the differences in tissue-specific gene expression induced by steroid analogs. However, a subset of asthma patients is either steroid-dependent or unresponsive to steroid treatment, due to acquired or inherited conditions.

Biologicals Glucocorticoid unresponsiveness bears significant therapeutic consequences and strongly motivated efforts to search for alternative, better means to target T-cell dependent events in allergic airways inflammation. The paradigm of Th2 polarization as being a central driver of allergic inflammation in asthma has led to several attempts to develop targeted monoclonal antibody immunotherapies against Th2 cytokine pathways. In 2003 for adults and in 2016 also for children above 11 years of age, the FDA approved Omalizumab (Xolair; Genentech/Roche and Novartis), a non-anaphylactogenic monoclonal antibody against IgE for the treatment of asthma. Omalizumab is a humanized monoclonal antibody that specifically targets the circulating immunoglobulin E, which in turn impedes and reduces subsequent releases of the proinflammatory mediators [260]. Omalizumab inhibits the binding of IgE to FcεRI on mast cells and basophils by binding to an antigenic epitope on IgE that overlaps with the site to which FcεRI binds. This is important because ordinary anti-IgE antibodies can cross-link cell surface FcεRI-bound IgE and induce mast cell and basophil degranulation. Omalizumab, however, is sterically prevented from crosslinking FcεRI. Interestingly, Omalizumab also inhibits IgE binding to the low-affinity IgE receptor (FcεRII), CD23 on B cells and many other cell types and attenuates IgE-mediated allergic responses. Even more intriguing that Omalizumab has recently been found to be beneficial in non-allergic diseases mediated by mast-cell degranulation [261]. The mechanisms of this effect are not well understood and are under intensive investigation [260–262].

Because of the prominence of eosinophilic granulocytes in the pathogenesis of asthma, monoclonal antibody blockade of IL-5 signaling was believed to have the most potential as a therapeutic due to the central role of IL-5 in the initialization of eosinophilic airway inflammation. However, the early clinical trials of anti-IL-5 and anti-IL-5Ra immunotherapies did not result in significant reductions in clinical asthma exacerbations. More recent clinical trials have met with better outcomes when patients were selected using biomarkers. Mepolizumab was approved in 2015 for the treatment of severe eosinophilic asthma. Results of major clinical trials in severe asthma (MENSA [263] and SIRIUS [264] on the effects of an anti-IL-5 monoclonal antibody, mepolizumab and ZONDA on benralizumab [265–271]) indicated that exacerbation rates as well as oral steroid intake was reduced by 50–75% in severe asthmatic patients. These and other previously published investigations [272, 273] shared important clinical features: significant therapeutic effect was demonstrated only in patients who had persistent symptoms and frequent exacerbations together with peripheral blood and sputum

eosinophilia *despite* high dose inhaled corticosteroid treatment. Further characterization of asthma patient subgroups benefitting from biological treatment, however, is warranted by the high variability of the data obtained so far and additional patient clustering results [274]. Importantly, some of the placebo-treated asthmatics in the trials showed a marked improvement in symptoms. Thus, not all patients need treatment with biologics. Given their high cost, careful selection of patients who will best benefit from this treatment is imperative. Future studies need to provide a way to distinguish patients with true glucocorticoid-resistant asthma as candidates for treatment with biologics from those with adherence/compliance problems or difficulties with correct inhaler technique, in whom conventional glucocorticoid-based therapy would otherwise work effectively.

Other monoclonal antibodies, targeting different cytokines (IL-13, IL-4, IL-17 and TSLP) are still under evaluation, though the preliminary results are encouraging. Tezepelumab for example, was recently developed for blocking the epithelial-cell-derived cytokine thymic stromal lymphopoietin (TSLP) [122, 275], in patients whose asthma remained uncontrolled despite treatment with long-acting beta-agonists and medium-to-high doses of inhaled glucocorticoids. Indeed, among patients treated with long-acting beta-agonists and medium-to-high doses of inhaled glucocorticoids, those who received tezepelumab had lower rates of clinically significant asthma exacerbations than those who received placebo, independent of baseline blood eosinophil counts [35, 276–279]. Thus, targeting inflammation upstream of adaptive immune processes may be more effective in treating both neutrophilic and eosinophilic asthma [276]. Recent clinical trials for anti-IL-33 immunotherapy that have been completed and are awaiting publication, CNTO 7160 and AMG 282. Immunotherapies targeting the alarmin cytokines are currently believed to be some of the most promising drugs in development for asthma treatment [280].

6 Conclusions

Clinical failures in drug treatment of allergic asthma greatly contributed to our understanding of the molecular and cellular players in the pathogenesis of this complex disease and drove the research efforts that lead to recent advancements. Recognition of the significance of airway inflammation was followed by discoveries of the importance of T cells and adaptive immunity with the Th2-polarization hypothesis. The past decade of translational research has, however, largely challenged the Th2-polarization hypothesis and implicated a greater role for airway epithelial and innate immune cells in modulating the adaptive immune response in asthma, than previously thought. We now understand asthma to be a syndrome encompassing diverse etiologies. The continued and rigorous characterization of the molecular determinants and mediators; both intrinsic and extrinsic, that underpin the many endotypes of asthma will lead to the exploration of new druggable targets in the proteome, genome, and beyond. It is possible that therapeutics which target pathways upstream of Th2 cells will have a broad impact in the clinic and thus be more effective than the treatments that directly target IL-4, IL-5, and IL-13. These studies are currently underway. Other therapeutic strategies may seek to impair the transcription factor complexes required to synthesize the molecular products of the immune cells that contribute to asthma or remodel the epigenetic architecture that programs inflammatory signaling pathways. In the era of precision medicine, it seems likely that the most successful novel asthma therapeutics will seek not to treat all asthmatics, but rather all asthmatics of an endotype. The most effective drugs in practice will balance specificity with utility, targeting converging elements of asthma pathophysiology. Without the extensive work of many laboratories around the world to define the basic molecular pathways in asthma, the development of such therapeutics would not be possible. The era of precision medicine holds much promise for more effective asthma therapeutics.

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Role of Histamine in Inflammatory Diseases

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Abstract

Histamine plays a major role in inflammation associated with many different diseases, including allergies, autoimmune diseases and cancer, but it also has important physiological effects. Although mast cells and basophils are the main sources of histamine it can be differentially produced and released by enterochromaffin-like cells, certain nerve cells, and even neutrophils in certain pathological settings. This chapter highlights the recent developments regarding the major contributions of histamine to inflammation by acting not only through H1-receptors (H_1R) but also H_2R and H_4R. These receptors determine both the severity of inflammation and also have immunomodulatory effects in various lymphocytes. Recent evidence also suggests a dual role for histamine in various autoimmune inflammatory disease and certain cancers, where differential receptor expressions for the amine determine the severity of these diseases but also play a role in tumour surveillance.

Keywords

Histamine · Mast cells · Basophils · Allergy · Inflammation · Cancer

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1 Introduction

1.1 What Is Histamine?

Histamine, (chemically also known as 2-(4-imidazolyl)ethylamine), is small molecule derived from the decarboxylation of the amino acid histidine by the enzyme histidine decarboxylase (HDC). It has 2 pathways of degradation, either by diamine oxidase (DAO) or by histamine N-methyltransferase (HNMT) (reviewed in [73]). Histamine was first discovered and synthesized in 1907 and then characterized in 1910 (reviewed in [71]). Not long thereafter the connection between histamine and anaphylaxis was established [71]. This gave rise to considerable interest in histamine and much knowledge has been gained since using pharmacological and mutational studies, cloning of receptors as well as murine knock-out (KO) models.

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1.2 Physiological and Pathological Effects of Histamine

The actions of histamine are mediated by its binding to different types of histamine receptors which are widely distributed in many different cells, including histamine-producing cells themselves, suggesting that histamine is an important regulator of a variety of cellular responses [71]. The major known functions of histamine include allergic and inflammatory reactions, gastric acid secretion, and control of neurotransmission in the central nervous system as wells as effects on the cardiovascular system (reviewed in [28, 107]).

1.3 Sources of Histamine

The most characterized source of histamine is mast cells and basophils which produce and store histamine in granular vesicles that are released following IgE-mediated and non-IgE-mediated stimulation. Other well-characterized sources include enterochromaffin-like cells and neuronal cells, which also store histamine in vesicles [28, 71]. In the gut, enterochromaffin-like cells produce and release histamine to stimulate gastric acid secretion from parietal cells that aid in digestive processes [28, 107]. In neurons histamine is used as a neurotransmitter where it is involved in the circadian (sleep/wake) cycle, appetite, learning and memory responses and also involved in controlling its own release and that of other neurotransmitters [28, 107]. Recently, it was discovered that basophils and mast cells are not the only immune cells that store histamine. Although much lower compared to human basophils and mast cells, which store ~1 and ~1–3 pg histamine/ cell, respectively, human neutrophils were found to contain ~0.3 pg histamine/cell and, like the other immune cells, release the amine following both IgE-mediated and non-IgE-mediated stimuli [8]. Other cells that secrete histamine, have been identified by the presence of HDC expression. These cells, such as T cells, platelets, mouse neutrophils, dendritic cells, monocyte/macrophages, and keratinocytes do not store histamine, but release it directly after *de novo* synthesis [28, 71, 107].

1.4 Histamine Receptors

Histamine causes most of its effects by direct binding to four main types of G-protein coupled receptors (H_1R-H_4R), although with different intracellular mechanisms and affinities for the amine [28, 71]. Furthermore, a fifth intracellular receptor, termed $H_{IC,}$ which interacts with cytochrome P450 (CYP450) has been suggested, although this receptor has not been fully characterized [28, 71]. The differential distribution of receptors, as well as their regulation in healthy and pathophysiological conditions, may explain the pleiotropic effect of histamine in different cells and tissues. Table 1 gives an overview of the distribution of the different histamine receptors. The expression of these receptors is normally identified at mRNA level by RT-PCR and at the protein level using specific antibodies shown by Western blotting, flow cytometry or immunohistochemistry. However, in the case of H_4R protein expression, the data should be considered with caution, since some of the currently available H_4R antibodies do not yield a specific signal when evaluated in transfected or H_4R −/− cells [11]. An additional problem arises in classifying these receptors based on the effects of histamine acting as either an agonist, inverse agonist or antagonist because of dual specificity, thus requiring careful controls [101]. Figure 1 gives an overview of the downstream cellular mechanisms of the 4 major histamine receptors.

Histamine, and analogue agents, can therefore have varying effects depending on the concentrations used and the differential expressions of the four major histamine receptor types [28, 101, 107]. Furthermore, there may also be off-target effects, mediated by non-H-receptor binding [71, 101, 107]. Biased signaling may also affect the outcome of experimental studies due to putative differences in receptor ligand pharmacology [83]. Finally, the effects of histamine are dependent on the mammalian model employed, including strain differences, healthy *vs* disease, and cell developmental stage [107]. Thus extrapolation to humans in clinical settings may be problematical. For example, histamine increases the heart rate in humans and other species, which is abolished by

Table 1 Overview of histamine receptors distrubition and major function

Receptor subtype	Kd	Distribution	Major effects
H1	~10 µmol/L	Widely Airway and vascular smooth muscle cells, epithelial cells, endothelial cells, keratinocytes, fibroblasts, hepatocytes, chondrocytes, dendritic cells, neutrophils, T cells and B cells, mast cells, brain tissue and gastrointestinal tract	Allergic inflammation
H2	~30 µmol/L	Widely Parietal cells, smooth muscle cells, endothelial cells, dendritic cells, neutrophils, T cells and B cells, basophils, mast cells, neutrophils keratinocytes, brain and cardiac tissue	Gastric acid secretion, immune suppressive
H3	~10 nmol/L	Brain tissue Histaminergic neurons at CNS and PNS	Neurotransmitter and modulation of neurotransmitter release
H4	~20–40 nmol/L	Blood/bone marrow, intestine T cells, dendritic cells, mast cells, eosinophils, basophils, epithelial cells, neutrophils, Langerhans cells, keratinocytes	Immune modulation
H_{IC}	~ µmol/L	Hematopoetic	Regulation of histamine and cytokine synthesis

the use of H_2R antagonists but not in in rabbits and dogs (reviewed in [114]). Furthermore, murine basophils have been shown to express the organic cation transporter 3 (OCT3) which is involved in histamine uptake from the microenviroment. High exogenous histamine concentrations could thus increase intracellular histamine which then stimulates H_{IC} (interacting with cytochrome P450 and cytochrome c; although this receptor has yet to be fully characterized) leading to a downregulation of histamine synthesis and Th2 cytokines such as IL-4 [28, 98, 99]. A pilot study stuggested that this is also the case in humans [99], but our own preliminary data suggest that human basophils do not express OCT3 (unpublished observations). Because of these species differences we will focuss primarily on the involvement of histamine in human inflammatory diseases.

2 Histamine and Inflammatory Disease

It has long been recognized that histamine plays a crucial role in allergic inflammation, and the development of H_1R antagonists/inverse agonists have confirmed this by relieving some of the symptoms of allergy. With the discovery of the H_4R, the role of histamine in inflammatory diseases has been re-investigated [107]. Elevated levels of histamine have been found in the bronchoalveolar lavage fluids from patients with allergic asthma, which was associated with a negative impact on airway function [14, 54]. Increases in histamine levels have also been found in the skin and plasma from patients with atopic dermatitis (AD) [7, 22, 52] and in the skin of chronic urticaria (CU) [91, 107]. Other diseases which are not related to atopic diseases have also been associated with increased histamine levels, e.g. multiple sclerosis (MS) [57], rheumatoid arthritis (RA) [60], atherosclerosis [49] and some types of cancers (melanomas, breast cancer, and colorectal carcinoma) [76]. Furthermore, a dysregulated expression of histamine receptors have been described for gastric intestinal (GI) disorders [23], such as irritable bowel syndrome (IBS) [95] and inflammatory bowel disease (IBD), which also exhibit an increase in mucosal histamine levels [103].

2.1 Atopic Disease

Histamine is one of the main contributors to allergic inflammation, where it is mainly released from mast cells and basophils upon degranulation after

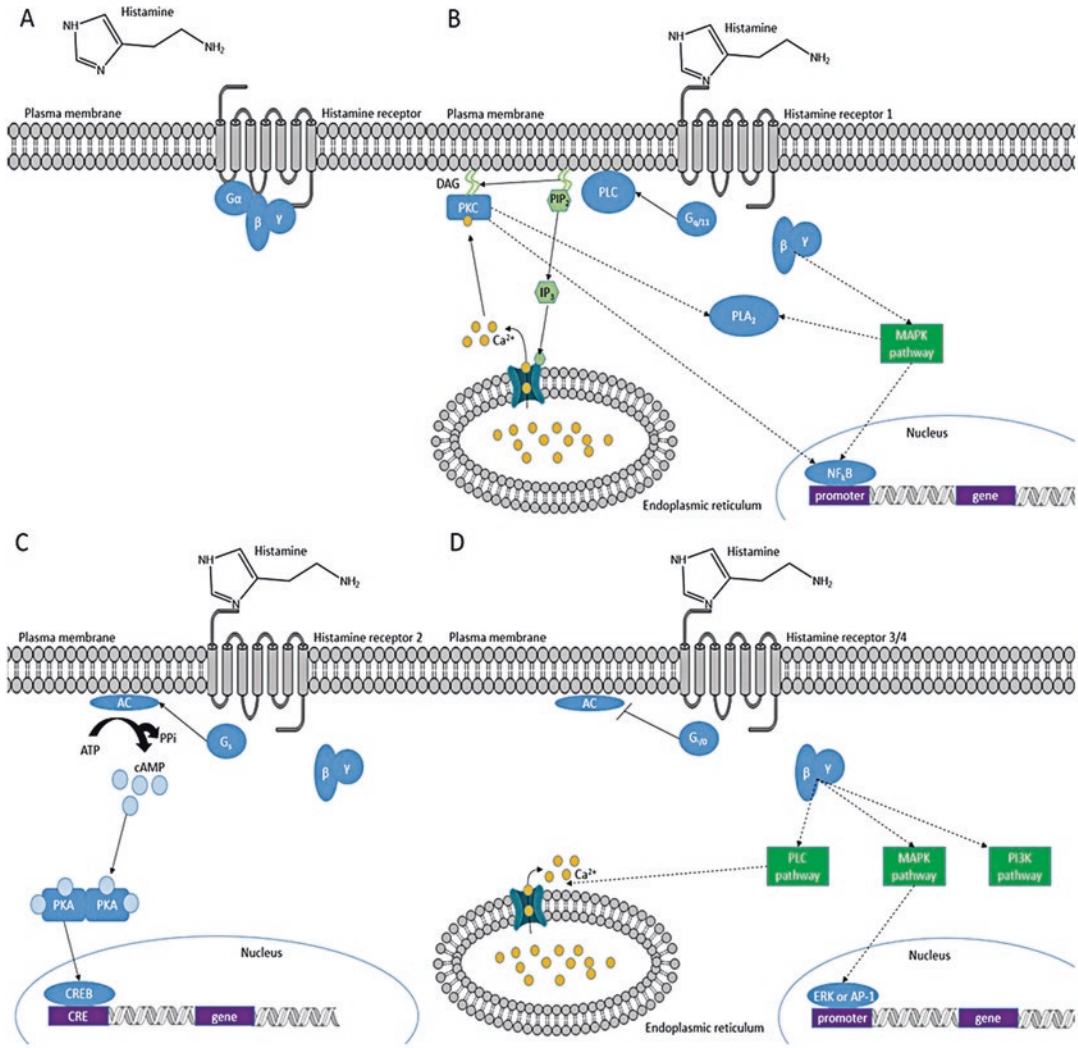

Fig. 1 Histamine receptor signaling (*A*) Depicts an H1 histamine G-protein couple receptor (GPCR) which, upon binding to histamine (*B*) signals *via* $G_{q/11}$, to activate phospholipase C (PLC) leading to an increase in free intracellular calcium. (*C*) Shows H2R signalling, which is couples to G_s, the main characteristic is activation of adenylate cyclase (AC) and an increase in intracellular cAMP. (*D*) Shows signalling downstream of H3 and H4 receptor activation, of which both are coupled to $G_{i/0}$. The main characteristic is inhibition of AC as well as activation of both PLC and the mitogen-activated protein kinase (MAPK) pathway, which leads to decreases in cAMP, an increase in intracellular free calcium and activation of transcription factors

stimulation through the high-affinity IgE receptor (FcεRI). Mast cells and basophils first need to be sensitized with allergen-specific IgE before they can respond, upon re-exposure to the allergen, by cross-linking of FcεRI-bound IgEs by the allergen. This induces a signalling cascade leading to degranulation, with the release of pre-formed mediators such as histamine, and the *do novo* synthesis of eicosanoids (e.g. LTC_4) as well as the production and secretion of cytokines such as IL-4 and IL-13 [40]. Histamine was identified very early on to mimic the signs and symptoms of anaphylaxis and since has found to be involved in the pathogenesis of many atopic diseases such allergic rhinitis, allergic conjunctivitis, allergic asthma, and atopic dermatitis as well as urticaria [28, 71, 107].

2.1.1 Histamine and Anaphylaxis

Anaphylaxis is a serious allergic reaction that is rapid in onset and may be fatal. Its pathophysiology is caused by multiple mediators, such as histamine, PAF, PGD2, TNF-α, etc., which causes a systemic reaction involving multiple organs with symptoms such as urticaria, angioedema, bronchospasm and other respiratory symptoms, hypotension, nausea, cramping and other gastrointestinal symptoms [90]. Of these symptoms, respiratory distress and cardiovascular collapse are potentially life-threatening. Histamine was soon found to have a role in anaphylaxis primarily mediated through both H_1R and H_2R [67]. The heart expresses both H_1R and H_2R, and cardiac myocytes are in close proximity to mast cells, the main sources of histamine in humans. It has been shown that anaphylaxis can be induced directly in the heart, and this is characterized by tachyarrhythmias, decreased coronary flow, and contractile failure [35]. In this case H_1R mediates vascular permeability, coronary artery vasoconstriction and negative chronotropic effects that cause atrioventricular conductance disturbances, whereas H_2R mediates coronary artery vasodilation, increased heart rate (positive chronotropic effect) as well as contractility (inotropic effect) [35, 67]. The first-line of standad therapy for anaphylaxis, however, is epinephrine (adrenaline), since the onset of therapeutic action of antihistamines is often too slow, though they are considered prophylactically. Even though it seems that H_1R and H_2R-mediated effects counteract each other in the heart, a retrospective study found that a combinational therapy of H_1R/H_2R antihistamines was more effective in preventing anaphylaxis than either of them alone [67].

2.1.2 Role of Histamine Acute and Late-phase Allergic Reactions

Histamine affects both early and late phase reactions of allergic inflammation by modulating the responses of various effector cells (e.g. the chemokinetic actions of histamine on eosinophils) and target cells (e.g. sensory nerves, airway smooth muscle) since all of them express at least one of the four main histamine receptor types.

It is well known that activation of H_1R causes many of the symptoms of acute allergic inflammation. The most characterized effect of H_1R activation is vasodilatation, increased vascular permeability, smooth muscle cell contraction (bronchoconstriction) activation of nociceptive nerves, wheal and flare reaction and itch response. However, H_1R antihistamines are not usually able to sufficiently abolish allergic symptoms, especially in asthma and chronic pruritus (reviewed in [85, 107]). Because of its restricted expression in hematopoietic cells, H_4R activation is currently being investigated in various inflammatory conditions and autoimmune diseases, and appears especially to be involved in chemotaxis and pruritus (reviewed in [85, 107, 117]). Other than gastric acid secretion, the H_2R is mainly known as immunomodulatory receptor often with immunosuppressive effects (reviewed in [85, 107]).

In allergic reactions, early phase reactions may progress to the late phase reaction and here histamine can also contribute to this response by modulation of cytokines and chemokines as well as upregulating of adhesion molecules. An *in vitro* study examining the effects of histamine on human airway epithelial cells showed that histamine can exacerbate asthma by potentiating NFκB-dependent transcription of IL-6 and IL-8 [50], where the latter is a very potent chemoattractant for neutrophils. This effect was inhibited by the H_1R antagonist mepyramine, suggesting that H_1R activation is involved in the response [50]. Other studies have found that histamine not only causes vasodilation and increased vasculature permeability of human endothelial cells but also transiently upregulates expression of the cell adhesion molecule P-selectin [25]. P-selectin has been shown to be important for neutrophil adhesion to endothelial cells, and infiltrating neutrophils are more abundant in the nasal epithelium after nasal challenge, suggesting some involvement in allergic rhinitis [25]. Furthermore, histamine-induced IL-6 production was also seen, which was mediated through both H_1R and H_2R activation. IL-6 is an important inflammatory mediator which induces B-cell proliferation and production of acute phase proteins.

2.1.3 Effects of Histamine on Pro-allergic Immunity

To promote isotype switching to IgE, B cells require cytokines such as IL-4 and IL-13 which are produced in primarily by $CD4^+$ helper T cells 2 (Th2) cells and basophils. Histamine has the ability to affect T cells either directly by targeting histamine receptors expressed on Th cells or indirectly through modulation of Th cell polarization by affecting dendritic cells. A study examining histamine receptor expression on human Th cells found that they are differentially expressed [56]. H_1R are predominately expressed on Th1 cells and this expression could be enhanced by IL-3. This suggests that IL-3 is part of a negative feedback mechanism to prevent pro-allergic immunity to predominate by promoting Th1 responses. In contrast to Th1, H_2R was predominantly expressed on Th2 cells [56]. Following the differentiation of naïve T cells, histamine receptor expression was observed to be regulated by the cytokine environment, where IL-12 enhanced H_1R expression whereas IL-4 suppressed it. Histamine was shown to increase intracellular calcium mobilization in Th1 cells whereas with Th2 cells an increase in intracellular cAMP was observed, effects which were attenuated by H_1R antagonists and H_2R antagonists, respectively [56].

Histamine also increased Th1 proliferation, whereas it suppressed Th2 proliferation. The effect of histamine was further investigated in an *in vivo* T cell-dependent antibody production model in mice [56]. H_1R KO mice showed a decrease in IFNγ (a Th1-type cytokine) and an increase in IL-4 production (a Th2-type cytokine) and this was reflected in increased IgG1 and IgE production. H2R KO mice showed an increase in both Th1 and Th2 cytokines, especially IFNγ, resulting in a decreased production of IgG3 and IgE [56]. Taken together, this suggests the H_1R signal in Th cells induces Th1 responses which are involved in attenuating the humoral response by IFNγ production, whereas H_2R stimulation acts as a general negative signal for Th cells. Th cells also express H_4R, and this expression is upregulated by IL-4, thus Th2 cells express higher levels of H_4R than Th1 [48].

Th2 cells are particularly important in the pathogenesis of atopic dermatitis, a chronic inflammatory skin disease, which is characterized by a dysfunctional epithelial barrier resulting in skin lesions and pruritus. In fact, $CD4^+$ T cells show higher expression of H_4R in patients with atopic dermatitis than healthy controls [48]. Signalling through H_4R induces DNA-binding of AP-1, which is required for the production of Th2 cytokines, and its effects are attenuated by H_4R antagonists [48]. Even though AP-1 activation was observed there was no effect on Th2 cytokine production but rather an upregulation of IL-31 mRNA following H_4R stimulation. IL-31 is a cytokine that has been associated with Th2 cells and the induction of pruritus, and it is also especially upregulated in patients with atopic dermatitis (AD) through H_4R signaling [48]. Another cytokine that is upregulated in skin diseases such as psoriasis and AD is IL-17. A study examining human memory Th17 cells, found that histamine induces the release of IL-17 mediated by H_4R signalling in a similar manner as with Th2 cells and IL-31, namely through AP-1 [77].

In addition to its induction of IL-31 and IL-17 in AD, histamine has also been shown to play a role in preventing the terminal differentiation of epidermal keratinocytes, promoting keratinocyte proliferation and causing skin barrier dysfunction (reviewed in [7, 22]). Since human keratinocytes express H_1R, H_2R, and H_4R, the impact on differentiation and skin barrier in *in vitro*-cultured human keratinocytes as well as a 3D organotypic human skin model was studied [45]. Here, it was found that histamine reduced the expression of late differentiations markers, such as keratin, fillagrin, and loricrin, and disrupted tight junctions (TJ) and desmosomal junctions, which play an important role in skin barrier function by reducing key TJ molecules, such as claudins, occludins as well as desmosomal proteins [45]. These defects were mimicked by H_1R agonists and attenuated by H_1R antagonists, suggesting that these are H_1R-mediated. Histamine caused a defect in skin barrier function which readily allowed biotin to penetrate the TJ, whereas this was blocked in untreated controls [45].

Furthermore, it was found that patients with AD express increased H_4R mRNA levels than controls and histamine induces the proliferation of human keratinocytes in an H_4R-dependent manner [43].

H_4R, as well as H_2R, signalling has also been shown to induce IL-16 release from human $CD8^+$ T cells [41]. IL-16 is plays a crucial role in recruiting $CD4^+$ T cell into the lungs of asthma patients. Clobenpropit (an H_4R agonist) induced IL-16 release which resulted in the migration of T helper cells [41]. H_1R antihistamines are a standard treatment for allergic rhinitis. However, they have been less successful for treating allergic asthma and atopic dermatitis. Given the role of histamine in T cells and keratinocytes, H_4R antihistamines might be more beneficial in reducing pruritus symptoms, by reducing IL-31 production and preventing skin barrier dysfunction in AD, and in asthma by reducing Th2 cells in the lungs which produce Th2 cytokines and thus contribute to eosinophilia. Since H_1R-mediated effects play a role in both AD as well as asthma, a combinational approach using H_1R and H_4R antihistamines may prove to be an effective therapeutic strategy (reviewed in [88, 107]).

The polarization of Th cells requires three signals, (1) antigen presentation from antigen-presenting cells (APC) though the interaction of MHC-II and T cell receptors, (2) co-stimulatory signalling and (3) presence of polarizing cytokines. Histamine can modulate this polarization by affecting APC cytokine secretion. Human monocyte-derived dendritic cells express all 4 histamine receptors at mRNA level. IL-12 is important for Th1 polarization and the lack of it is associated with Th2 responses. Since histamine reduces the IL-12p70 in monocytes (reviewed in [46]), it was also investigated in dendritic cells. Using different histamine receptor antagonists it was found that histamine was able to inhibit IL-12p70 production through H_1R, H_2R, and H_4R [46, 47]. H_1R signalling normally leads to Ca^{2+} mobilization but this was not detected in this study [46]. However, histamine did increase cAMP, an effect which was mimicked by H_2R agonists and abrogated by H_2R antagonists, suggesting that the mechanism was H_2R-mediated [46, 47]. H_3R and H_4R are thought to signal through Gi/o and inhibited adenylate cyclase. However, H_3R/H_4R agonists could not inhibit forskolin-induced cAMP. Instead it was shown that H_4R activation could block polyinosinic-polycytidylic acid (polyIC) induced IL-12p70 production, and this was mediated through the activation of AP-1, independent of ERK phosphorylation [47]. Furthermore, it was also shown that histamine could induce chemotaxis of monocyte derived dendritic cells (MoDC) by H_2R and H_4R [47]. This suggests that histamine can recruit MoDCs to sites of allergic inflammation and promote them to induce a Th2 milieu by inhibition of IL-12.

Histamine has been shown to also modulate antibody production from human B cells [38, 61]. An *in vitro* study showed that histamine enhances IL-4 and IL-13-induced IgE and IgG4 production in human B cells and this was related to increases in IL-10 and IL-6 production [61]. Histamine and IL-4-induced antibody production was inhibited by thioperamide (an H_3R/H_4R antagonist), whereas histamine and IL-13-induced antibody production was attenuated by diphenhydramine (an H_1R antagonist) [61]. This was related to the histamine-mediated enhancement of IL-10 production which was attenuated by thioperamide and mimicked by R-α-methylhistamine (an H_3R/H_4R agonist), whereas IL-6 production was inhibited by diphenhydramine [61]. Spontaneous antibody production could also be inhibited by diphenhydramine and thioperamide *in vivo* differentiated $sIgE^+$ and $sIgG^+$ B cells from atopic donors. Furthermore, when atopic patients were given oral H_1R antihistamines the spontaneous production of IgE and IgG4 were reduced [61]. These effects were originally ascribed to H_1R and H_3R, however, since the H_4R was not discovered at that time, and given that B cells are of hematopoietic cell lineage, it is possible that the effect observed was H_4R-mediated. Another study of human B cells found that dimaprit (an H_2R agonist) inhibited IgG and IgM production, since cimetidine (an H_2R antagonist) abrogated the response [38].

This underlines the notion that histamine can modulate antibody production and that H_1R, and maybe also H_4R, antagonists may be beneficial in reducing the IgE antibody production in allergic inflammation.

2.1.4 Effects of Histamine on Allergic Effector Cells

Eosinophils are important effector cells in allergic inflammation, particularly in the pathogenesis of asthma, which is associated by eosinophilia and where they contribute to damage of the airway epithelium. Histamine has been shown to mediate eosinophil chemotaxis [13, 69, 86] and modulate mediator release [32]. Eosinophils can be recruited to sites of allergic inflammation by histamine as well as IL-5 and eotaxin. However, it has been shown that while histamine induces eosinophil chemotaxis at low concentrations high concentrations are inhibitory (reviewed in [86]). Histamine stimulates actin polymerization, shape change and upregulation of adhesion molecule such as CD11b in eosinophils [13, 69]. This was mimicked by clobenpropit and clozapine (H_3R antagonists/H_4R agonists) while thioperamide (H_3R/H_4R antagonist) attenuated these responses. These data, and the fact that pertussis toxin also inhibited this response, suggests H_4R activation through Gi/o coupling [13]. Furthermore, H_4R also appear to be involved in priming of eosinophils for increased chemotaxis towards eotaxin [13, 69], but by itself the amine is only a weak chemoattractant [69].

H_4R activation does not only induce chemotaxis in eosinophils but also in human mast cell precursors [44] and basophils [78]. Human skin mast cells express H_2R and H_4R, though expression of H_4R seems to predominate [70]. In mast cells, H_4R activation causes the release of inflammatory mediators not only associated with allergic diseases but also other inflammatory conditions [55]. Thus, like the observations from T cells, it would seem that H_4R antagonists might be beneficial in atopic disease. Conversely, in basophils while H_4R activation induces chemotaxis, it reduces basophil activation by a reduction of CD63 and CD203c expression (indicating reduced degranulation) and decreases the release of leukotrienes [78]. In fact, specific H_4R stimulation was more effective than H_2R agonists in allergen-specific desensitization of basophils in bee venom allergic patients [78]. Thus this suggest that H_4R (along with H_2R) has differential immune suppressive actions on basophils.

The immunomodulatory and immunosuppressive properties of H_2R have been found in many immune cells. In human basophils, H_2R activation is well known to inhibit degranulation and histamine release (viewed in [32]). The importance of H_2R and H_4R in allergen desensitization has been demonstrated with respect to human basophils [84]. As with H_4R activation on human basophils, H_2R agonists reduced the expression of the activation markers CD63 and CD203c, as well as the release of histamine, leukotrienes and cytokines (IL-4 and IL-8) and this was mimicked by forskolin supporting a receptor coupled to Gs [84]. H_2R signaling is not only suppressive in basophils but also in human eosinophils [32] and neutrophils [12, 36]. A study investigating the effect of histamine on eosinophil degranulation found that histamine inhibited the release of eosinophil peroxidase (an eosinophil degranulation marker) and increased intracellular cAMP [32]. These effects were mimicked by H_2R agonists and attenuated by H_2R antagonists, suggesting the suppression of degranulation is H_2R-mediated [32]. These data, together with data from monocytes, DC and T cells, suggest that H_2R agonists theoretically could be a beneficial treatment for atopic diseases (if targeting approaches to avoid their effects on gastric acid secretion could be developed).

Although neutrophils are not normally considered as major contributors to allergic disease, there is increasing evidence to demonstrate that these cells play a role in allergies, especially in severe asthma where there is a poor response to inhaled corticosteroids [79]. Neutrophils are recruited to sites of allergic reactions by chemoattractants such as mast cell chymase and tryptase [8]. At these tissue locations neutrophils are affected by many mediators and also themselves contribute to inflammatory mediators, such as eosinophil cationic protein (ECP), matrix metalloproteases, reactive oxygen species (ROS),

as well as histamine itself [79]. ROS are important mediators of inflammation and, while oxidative burst is important for the elimination of invading microorganisms, the overproduction of ROS contributes to chronic inflammation (reviewed in [16]) which is characteristic in asthma and some types of cancers. Studies using histamine receptor antagonists and agonists, suggest that neutrophil function, like degranulation and ROS production, is influenced by histamine and neutrophils also express H_1R, H_2R and H_4R receptors (reviewed in [16]). A study comparing atopic and non-atopic subjects found that fMLP-induced ROS production was higher in atopic subjects and the inhibitory effect of histamine was less in atopics, suggesting that neutrophil function in atopic subjects is more difficult to suppress than healthy controls [104].

The H_1R-antagonist, azelastine, was shown to inhibit human neutrophil phagocytosis and generation of ROS, without having any scavenging effect, supporting a role for H_1R in neutrophils [5]. H_2R also have an effect on neutrophils where both stimulatory and inhibitory actions have been reported (reviewed in [16]). The most widely accepted role for histamine in neutrophils is its H_2R-mediated inhibitory actions on oxidative burst, by suppressing NADPH oxidase-dependent formation of oxygen radicals [12]. H_2R activation also inhibits leukotriene (LT) biosynthesis, including LTB_4, which is a potent chemoattractant and activates polymorphonuclear leukocytes (PMN) [36]. It was found that H_2R agonists mimic the inhibitory effect of histamine on agonist-stimulated and thapsigargin-activated PMN and that H_2R antagonists attenuated these effects. Further investigations showed that the molecular mechanism is mediated through an increase in cAMP, which is involved in decreased arachidonic acid (AA) release and prevents translocation of 5-lipoxygenase (5-LO) to the nucleus [36].

These data suggest that H_1R antagonists/inverse agonists and H_2R agonists could reduce inflammation in asthma by minimizing ROS production and the release of LTB_4 and thus the recruitment of neutrophils to affected tissue sites. Furthermore, once recruited neutrophils may themselves contribute to histamine release. Although the level of histamine is low compared to basophils and mast cells, neutrophil numbers are higher in comparison to these other effector cells and therefore their contribution to histamine levels may significantly potentiate allergic reactions [8]. Human neutrophils have been shown to release histamine by IgE-dependent and -independent mechanisms in certain settings and HDC expression in these cells is higher in atopic individuals [8].

Overall, histamine plays a multifacetted role in the development and exacerbation of atopic diseases. Targeting histamine receptors can reduce allergic symptoms but more effective combinational therapies (e.g. H_1R & H_4R) are required.

2.2 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, characterized by demyelination and axonal damage where the inflammatory lesions are characterized by high infiltration of T cells, B cells, macrophages, microglial cells and mast cells (reviewed in [53]). There have been extensive studies in MS and its experimental animal model equivalent, experimental autoimmune/allergic encephalomyelitis (EAE) [18]. However, the exact etiology of MS still remain elusive, though both genetic and environmental factors play a role. The exact pathogenesis of MS is also not fully understood but it has been proposed that histamine is involved [53]. In the brain, neuronal histamine mediates physiological responses by activating H_1R and H_2R which are involved in regulating many functions such as the circadian rhythm, appetite and behavior. H_3R has autorregulatory effects on histamine itself and other biogenic amines in a negative feedback loop. The data regarding H_4R expression in brain tissue is currently controversial, where most cells in the brain expressing this receptor are thought to be immune cells (reviewed in [97]).

Histamine has been associated with neuronal inflammation of the brain seen in MS,

although studies comparing blood or cerebral spinal fluids (CSF) histamine levels from patients with MS and healthy subjects have, overall, not been conclusive [53, 57]. The histamine producing cells of the brain consist of histaminergic neurons and mast cells [53] and it is believed that histamine release from mast cells is involved in MS pathogenesis (reviewed in [118]). The complex interaction of histamine with its four different receptors makes it currently difficult to state what the net effect of histamine in MS is. Histamine may have both detrimental and protective effect in MS and EAE, and the amine can change the permeability of the blood-brain barrier, which could result in increased infiltration of cells in the CNS as well as neuroinflammation. However, in EAE using an HDC KO model, the severity has been reported to be greater, suggesting a protective role of histamine in the pathology of EAE [119]. The role of histamine receptors in EAE have been analyzed, but with relatively unclear results (reviewed in [53, 89, 97]). For instance, It has been shown that dimaprit (an H_2R agonist) significantly reduces clinical signs of EAE [30] but H_2R deficient mice showed improvement [106]. These conflicting results could be because dimaprit has also been reported to act on H_4R [2], and that the beneficial effect of dimaprit is actually mediated through the H_4R. In support of this notion, JNJ-7777120 (an H_4R antagonist) has been shown to exacerbate clinical and pathological signs of EAE [10]. In addition, H_4R deficient mice also showed more severe symptoms, and is related to a lower frequency of Treg and increased numbers of Th17 cells [24]. However, in other mouse models stimulation through the H_4R seem to promote a Th17 response [19]. Taken together, histamine may play a role in the pathogenesis of MS. However, interpreting the role of histamine receptors in EAE may depend on the strain and induction of method of EAE. Furthermore, it is important to determine the downstream signaling of the receptors when using agonists and antagonists, to exclude dual specificity.

2.3 Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is a systemic autoimmune disease that involves chronic inflammation of the synovial membrane that causes joint pain, bone and cartilage erosion. The inflammation is characterized by infiltration of T cells, macrophages, B/plasma cells and activation of synoviocytes and molecular mediators such as TNF-α, IL-1, IL-6, IL-17, RANKL, autoantibodies/immune complexes etc. (reviewed in [58]). The exact role of histamine in the pathogenesis of RA is currently unclear since some reports have shown that histamine levels in blood and synovial fluids (SF) are lower in RA patients than healthy controls [4] but others have found increased levels [60]. However, experimental animal models, have suggested a role mediated through the H_4R [1, 19, 82]. In addition, it has been reported that human synoviocytes express H_1R, H_2R, and H_4R [51, 87]. Since most immune cells that infiltrate the synovium express these receptors, in particular H_4R, this suggests for a role for histamine in modulating the function of these cells. Studies using a mouse collagen antibody-induced arthritis (CAIA) model that included either H_4R-defienct mice or H_4R antagonist treated mice, showed decreased inflammation, cartilage and bone damage and overall lower severity scores [19]. This suggests that H_4R might be involved in the pathogenesis of this arthritis model.

In the mouse collagen-induced arthritis (CIA) model, which has astrong T cell component, it was found that H_4R-defienct mice or H_4R antagonist treated mice had a lower number of Th17 cells and reduced IL-17 production [19]. Both IL-17 secretion and the number of Th17 cells correlate with disease severity of rheumatoid arthritis [9], suggesting that the effects of H_4R could be mediated by targeting Th17 cells. In fact, human memory Th17 cells have been shown to express H_4R and stimulation through this receptor induces IL-17 production [77]. On the other hand, *in vivo* activated human monocytes from RA patients have been shown to promote Th17 responses in a contact-dependent manner [31]. Thus increases in IL-17 could be mediated by an H_4R-independent mechanism, although

human monocytes have been shown to express H_4R and these were increased in monocytes from RA patients compared to healthy controls [60].

There may be a network between histamine, histamine receptor expression and Th17 cytokines (IL-17, IL-21, and IL-22). Histamine, IL-17, IL-21 and IL-22 all increase H_4R expression in human monocytes, and H_4R antagonists attenuate this increase in all case except for IL-21. Furthermore, RANKL is also increased by histamine and IL-22, which was shown to be inhibited by an H_4R antagonist [60]. RANKL is important for osteoclast activity which results in bone erosion. To examine if histamine had an osteoclastogenic role, human monocytes were cultured with M-CSF and histamine with and without H_4R antagonist. Monocytes differentiated into osteoclasts by an H_4R-dependent mechanism [60]. Taken together, there is evidence to suggest that histamine may play a role in RA which is possibly mediated through the actions of the H_4R, although it remains to be seen whether H_4R antagonists have therapeutic applications for this disease, especially in humans.

2.4 Atherosclerosis

Atherosclerosis is an inflammatory disease of the large- and medium-sized arteries, and is characterized by the formation of atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, inflamed smooth muscle cells (SMCs), endothelial cells (ECs), leukocytes, and foam cells (reviewed in [39]). Atherosclerosis is a complex disease where many different mediators are involved. High levels of Low-density lipoprotein (LDL) is one of the most important risk factors for atherosclerosis, however, immune and inflammatory mechanisms also play a role [39]. The main immune cells involved in the initiation and progression of atherosclerosis are macrophages, monocytes and T cells, all of which are able to secrete cytokines (e.g. TNF-α), chemokines (eg. CCL2) as well as other factors like, MMP, ROS, and histamine, contribute to pathogenesis. High blood levels of histamine in combination with low levels of ascorbic acid plays a role in the initiation of atherosclerosis since histamine reduces TJ on endothelial cells, and a lack of ascorbic acid induces endothelial cell gaps (causing initial damage) (reviewed in [17]). H_1R mRNA is expressed in macrophages, vascular SMC [105] and vascular EC in atheromatous plaques, suggesting that histamine could regulate some responses in atherosclerosis [96].

Histamine also enhances the expression of adhesion molecules, such as VCAM and ICAM, in atherosclerosis [62], as well as enhancing the production of cytokines such as IL-6, IL-8 and TNF-α. These adhesion molecules are needed for the recruitment of leukocytes, especially monocytes, into the intima wall [96]. Monocytes express H_1R, H_2R, and H_4R, where H_2R is most predominately expressed [26, 108]. CCL2/MCP-1 is an important chemoattractant for especially monocytes, and it was found that histamine can upregulate the expression of both CCL2 and its receptor CCR2 by an H_2R-mediated mechanism in monocytes [62]. However, histamine also induces IL-10 production while inhibiting IL-12 production and TNF-α through H_2R [29, 110]. In contrast to H_2R, H_4R down-regulates CCL2 production in monocytes [26]. Once in the tissue, monocytes start to differentiate to macrophages, and this process results in a switch of histamine receptor expression to H_1R predominance [108, 112].

Macrophages are crucially important in the pathogenesis of atherosclerosis, and are found in all stages of the disease. Macrophages not only express H_1R but also produce histamine, and it is thought that macrophage-derived histamine plays an important role in atherosclerosis [96]. This is supported by a study investigating HDC expression in relation to atherosclerosis where increased HDC expression in the human aortic intima in relation to the progression of the disease, and this was mainly produced by monocytes/macrophages and to a lesser extent T cells [49]. It has been proposed that histamine and IL-4 synthesis in the arterial intima stimulate both monocytes and macrophages to phagocytes oxidized LDL, that then leads to the formation of foam cells [49]. Some of the target cells for

macrophage-derived histamine are SMC. Reports have shown that histamine could, through H_1R, stimulate the proliferation of SMC contributing to the thickening of the intima as well as inducing the production of MMP-1, which is involved in extracellular matrix degradation in an atherosclerotic lesion [96].

2.5 Gastric Intestinal (GI) Disorders

H_1R, H_2R and H_4R (both mRNA and protein) are expressed in the human gastric intestinal tract [95]. Histamine has a role in multiple physiological processes in the GI tract, including immunological responses, visceral nociception, modulation of intestinal mobility and gastric acid secretion. However, histamine also plays a role in many GI disorders such as systemic mastocytosis, food allergy (FA), malignancies, gastric ulcer, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (reviewed in [23]). In these disorders, increased histamine levels have often been found both in plasma and inflamed lesions [103]. This increase could be due either to an increase in histamine availability or decreased degradation and both circumstances might occur in patients with IBD. An alteration in mast cell numbers as well as an increase in their activation state resulting in an excessive histamine release has been reported [23]. In addition, DAO gene expression was found to be lower in IBD patients [103]. Furthermore, it also appears that the dysregulation of histamine receptor expression also could play a role in some of these diseases, since H_1R and H_2R mRNA have been shown to be upregulated in FA and IBS patients [95]. Inflamed tissue of IBD patients (both Crohn's disease and ulcerative colitis) is associated with an upregulation of H_2R and H_4R mRNA compared to non-inflamed tissue from the same donor [103].

Although the exact etiology of IBD remains unknown, genetic susceptibility, environmental factors, immune dysregulation, and epithelial barrier dysfunction all contribute to disease pathogenesis (reviewed in [66]). Histamine may affect immune regulation and modulation of epithelial barrier function. A recent study, examining the effect of histamine and H_2R on the bacterial-induced inflammatory response in patients with IBD [103], reported an increase in Th1 and Th17 cells. Furthermore, an inverse correlation was found between Th17 cells and monocytes expressing the H_2R as well as a switch in the balance of histamine receptor expressions in monocytes from H_2R to H_4R. The ability of histamine to suppress the PBMC TLR-induced cytokines, such as IFNγ, TNFα, IL-6, IL-12, and IL-17, which are normally increase in IBD was examined. Here, the suppressive effect of histamine was significantly less in patients with IBD than controls for all the above cytokines except for IL-17, an effect which was H_2R-mediated since it was abolished by the H_2R antagonist famotidine [103]. In support of this, a study using the probiotic *Lactobacillus reuteri* (clade II strain which expresses HDC) suppressed intestinal inflammation in a trinitrobenzene sulfonic acid (TNBS)-induced mouse colitis model [42]. The anti-inflammatory effect of *L.reuteri* was diminished by ranitidine, an H_2R antagonist, suggesting colitis is H_2R-mediated. The consequence of H_2R-stimulation was reduced gene expression of pro-inlfammatory IL-6 and IL-1β [42].

Frei et al. showed that histamine plays an important role in the maintenance of mucosal homeostasis [37]. Here, it was shown that human monocyte-derived dendritic cells (MDDC) stimulated with LPS (*via* TLR4) or Pam3Cys (via (TLR2) in the presence of histamine significantly reduced the expression of TNF-α, IL-12p70 and the chemokine CXCL10, while enhancing the expression of the anti-inflammatory cytokine IL-10. [37] Furthermore, a decrease in T cells positive for T-bet (a Th1 transcription factor) decreased after stimulating MDDC with LPS and histamine. The suppressive effect of histamine was also observed for stimulated DCs isolated from human peripheral blood and shown to be H_2R-mediated.

Since histamine does not appear to influence TLR-induced IL-17 secretion directly, it has been suggested that the amine influences Th17 cell

polarization [103]. A mouse study using preclinical models of arthritis, has shown that H_4R antagonism reduces Th17 cell populations and IL-17 secretion [19]. This could be attributed to an H_4R-mediated effect on Th17 cells. In another recent study, polarized human memory Th17 cells, as well as IL-17 positive T cells in skin lesion of psoriasis, expressed H_4R. Upon stimulation with histamine or the H_4R agonist, 4-methylhistamine, increased IL-17 mRNA and secretion of IL-17 were observed, effects which were blocked with selective antagonist JNJ-7777120. These actions could be mediated by activator protein-1 (AP-1) since both histamine and H_4R agonists induce AP-1 DNA binding and H_4R antagonists reduce it [77]. The switch in histamine receptor expression on monocytes from H_2R to H_4R, may play a role in the induction of a Th17 response. This is supported by a report demonstrating that *in vivo* activated human monocytes promote a Th17 response in a cell contact dependent manner [31].

Although it is recognized that histamine in the gut contributes to gastric ulcers in an H_2R-dependent manner, it would seem that, in the case of IBD, H_2R stimulation should have a suppressive effect on the inflammatory mucosa in IBD [23, 66]. However, histamine has a much higher affinity for the H_4R than for H_2R thus, although the expression of H_1R and H_2R receptors are more prominant in the GI tract, histamine could contribute to inflammation *via* H_4R-stimulation and induce Th17 responses. Whether by H_2R or H_4R histamine-dependent effects are mediated by the modulation of cytokine secretion. The cytokines IFNγ, TNFα, IL-1β, IL-6, and IL-17 have all been shown to impair epithelial barrier function, which is also one of the hallmarks of IBD [66]. From the reported studies here, histamine suppresses IFNγ, TNFα, IL-1β and IL-6 *via* H_2R, but enhance IL-17 by H_4R. Taken together, the balance between H_2R and H_4R may be critical in controlling mucosal inflammatory responses and the microbiota (with the presence of certain bacterial strains) might be involved in this control in IBD.

3 Tumorigenesis

Tumorigenesis is a multistep process and arises from various genetic and epigenetic alterations that drive the progressive transformation of normal cells into malignant cells [59, 76]. Chronic inflammatory processes are implicated in the initiation and progression of cancer, such as infiltration of mononuclear cells, upregulation of pro-inflammatory molecules (cytokines, ROS and NFκB), tissue destruction, fibrosis, increased angiogenesis and invasion of tissue, increased in genomic DNA damage and disruption of DNA repair pathways as well as increased proliferation and inhibition of apoptosis. Thus inflammation may help provoke key mutations for cancer development [34].

Chronic inflammation and cancer development are associated in several organs including breast, urinary bladder, ovary, liver, stomach, intestine, skin, and prostate. For instance, the risk of colorectal cancer has been shown to be much higher in patients with IBD such as Crohn's disease and ulcerative colitis [34]. Atopic diseases are known to involve the release of histamine release and other mediators (cytokines, chemokines, and leukotrienes), often resulting in chronic inflammation. However, the association between cancer risk and atopic disease is controversial. Some reports have found that atopic disease is associated with a lower risk of cancer (e.g. leukaemia and glioma), suggesting a protective effect which is most likely due to hyperstimulation of the immune system which could enhance immune surveillance, thus inhibiting abnormal cell growth (reviewed in [21]). In contrast, atopic diseases such as asthma, associated with chronic inflammation and release of inflammatory cytokines and ROS, increase the risk of lung cancer (reviewed in [21, 92]). Atopic diseases therefore increase the risk of certain types of cancers whereas in others they may be protective.

Mast cells and their mediators, such as histamine, may promote a microenvironment for tumor development, since chronic inflammation induces both proliferation of resident mast cells and recruitment of mast cell as well as mast cell progenitors. These have been found to be

associated with angiogenesis, tumor growth and metastases [34]. An increase in histamine levels (or HDC activity) has been shown in many cancer types, such as breast cancer, melanoma, small cell carcinoma, endometrial cancer and colorectal cancer (reviewed in [15]) and this increase is thought to be attributed by overexpression of HDC by tumor cells, mast cell mediator release and a dysfunction in histamine metabolism (von [72]). Furthermore, the expression of histamine receptors has been found in many types of tumors, such as breast cancer, leukemia, melanoma, cervical, ovarian and colorectal cancer (reviewed in [76]). Histamine appears to play a dual role where, on the one hand it has cancer-promoting effects by acting in an autocrine and paracrine manner to support tumor growth but, on the other hand, anti-tumour effects by also inhibiting cell proliferation and enhancing NK cell cytotoxicity. These effects largely depend on the histamine concentration, the tumor type (and even subtype) and the type of histamine receptors expressed [34]. Furthermore, it is also necessary to identify the downstream mechanism, because some cancer types are more complex to investigate due to atypical signaling pathways of the histamine receptors.

3.1 Colorectal Cancer (CRC)

H_1R, H_2R, and H_4R are expressed in the GI tract and elevated HDC levels have been found in colorectal cancer (CRC) tissue [23] where mast cell infiltration is associated with poor prognosis (reviewed in [3, 94]). This suggests that histamine influences the growth of CRC.

H_2R activation has been associated with histamine-mediated tumor growth of several different types of tumors, such as melanoma, colorectal cancer and glioma cells as well as in breast cancer (reviewed in [15, 76]). Adams et al. showed that histamine induced moderate cell proliferation in a CRC cell line in a bell-shaped concentration-dependent manner [3]. The H_2R antagonist cimetidine inhibited this proliferation suggesting that tumor growth is associated with H_2R activation. Xenografted tumor cells in mice were also examined where similar results were obtained supporting the *in vitro* data [3]. This suggests that H_2R antagonists could be a beneficial treatment for CRC. In addition, it has been shown that cimetidine appears to improve diminished immunity after surgical resection of CRC. Here, perioperative administration of cimetidine increased total T cells, Th cells, and NK cells, and increased tumor infiltrating lymphocytes [68]. This study did not investigate the molecular mechanism of action, and thus it is not known if this is directly mediated by inhibiting H_2R or some off-target effect of cimetidine. For instance, cimetidine has been shown to enhance antitumor cell-mediated immunity by improving the antigen presenting capacity of dendritic cells, especially in advanced cancer patients. This effect is thought to be due to some off-target effect, since another H_2R antagonist, famotidine, did not show similar results [65].

H_4R expression levels in CRC are known to differ compared to adjacent normal tissue (ANT). In a recent study it was shown that H_4R expressions, both at mRNA and protein levels, are reduced in CRC compared to ANT and that advance stages of CRC had even lower H_4R mRNA compared to earlier stages, suggesting reduced H_4R could be a marker for tumor progression [33]. To investigate the effect of different H_4R expressions, Fang et al. compared a CRC cell line, which constitutively expressed relatively low levels of H_4R, to the same cell line overexpressing H_4R [33]. Here, histamine and the H_4R agonists clozapine and clobenpropit inhibited proliferation by inducing cell cycle arrest in cells that overexpressed H_4R while not influencing the growth of untransfected cells. This was verified by pretreatment with the H_4R antagonist JNJ7777120, which prevented the cell cycle arrest [33]. The effect on the cell cycle was due to H_4R regulation of cell cycle proteins CDK2, cyclin D1, p21, and p27 and the molecular mechanism for H_4R-mediated cell cycle arrest was due to suppression of the cAMP-dependent pathway. Thus, it appears that CRC tumors downregulate H_4R to increase proliferation by allowing progression through cell cycle arrest. Furthermore, H_4R activation enhanced apoptosis

induced by 5-fluorouracil (a chemotherapeutic drug), suggesting that H_4R-agonists could be play a therapeutic role in CRC in combination with 5-flurouracil [33].

3.2 Histamine and Tumour Surveillance

Histamine can also inhibit or promote tumour growth by affecting tumour surveillance. Combination therapy with IL-2 and histamine have shown to enhance tumor surveillance by enhancing the cytotoxicity of anti-tumor cells such as NK cells and T cells [20, 27, 115]. In other cases, histamine was observed to contributed to immune escape [81, 109].

In metastatic renal cell carcinoma, malignant melanoma, and acute myeloid leukemia combination immunotherapy with IL-2 and histamine have shown to be beneficial [20, 27, 115]. IL-2 is involved in the proliferation and cytotoxic activation (upregulation and maintenance of activation receptors, such as NKGD2) of NK cells and T cells, which promote tumour clearance [20]. However, it cytotoxic activity is diminished in the presence of phagocytes. The combination therapy using IL-2 and histamine is based on the oxidative stress hypothesis, in which histamine inhibits the formation and release of phagocyte-derived (neutrophils, monocytes/macrophages) ROS and thereby protects anti-tumor cells, such as NK cell and T cells, against oxidative damage and apoptosis [12, 27]. The molecular mechanism for the attenuated production of ROS is due to H_2R-mediated inhibition of NADPH oxidase [12].

Another mechanism for the combination effect of IL-2 and histamine is its effect on myeloid cells. A subset of monocytes comprises of myeloid-derived suppressor cells (MDSCs), immature cells with immunosuppressive functions that increase in number during cancer progression. The immune suppressive functions of MDSCs includes inhibition of cytotoxic T cell and Th cell activation and proliferation, induction of regulatory T cells, impaired NK cell activity, and induction of immunosuppressive macrophage phenotypes (reviewed in [111]). Yang et al. demonstrated that HDC deficient mice accumulate more immature myeloid cells and exogenously administrated histamine prevents this accumulation [116].

An *in-vitro* study using cultured human monocytes with growth factors in either the presence or absence of histamine showed that histamine induced differentiation of DCs by increasing the levels of maturation markers such as HLA-DR, C86, CD40 [75]. It was also observed that these DCs were more efficient in promoting T cell proliferation and producton of cytokines such as IFNγ and IL-4. It appears that the differentiation of DCs is NADPH-dependent. In the presence of high ROS, maturation markers were lower than controls. The importance of histamine on NADPH was verified in an NOX2-KO cell line (NOX is a component of NADPH). Here, histamine had little effect on cell differentiation compared to wild type and this was attenuated by ranitidine, an H_2R antagonist, implying that the effect was H_2R-mediated [75]. Thus histamine inhibits the activity of NAPDH though H_2R activation and this prevents the formation and release of ROS, which improve MoDC differentiation.

Histamine also affects another tumor surveillance T cell, the subset γδ T cell, which recognizes antigen bound to CD1 molecules. Like NK cells γδ T cell cytotoxic activity is mediated by the release of perforin and granzyme. A study investigating the effects of histamine on human γδ T cells showed that these cells express H_1R, H_2R and H_4R receptors, suggesting that histamine is able to regulate their function. It was shown that H_4R mediated chemotaxis though pertussis-sensitive G_i signalling led to an increase in intracellular calcium and actin reorganization [109]. However, histamine reduced the cytolytic capacity of γδ T cells towards cancer cell lines. This effect of histamine was mimicked by dimaprit, an H_2R agonist (and partial H_4R agonist) whereas H_2R antagonists abolished this effect, suggesting that the modulatory actions of histamine on T cell-mediated cytotoxicity requires H_2R activation. It was determined that the effect on cytotoxicity was mediated to the cholera toxin sensitive G_s, which increases

intracellular cAMP, again suggesting H_2R-mediated input [109].

Histamine also contributes to immunological escape of monocytic leukemia cells by reducing their susceptibility to NK cells, $CD8^+$ T cells and γδ T cells, by down-regulating NKG2D ligands. The NKG2D receptor is a key activating receptor expressed on these cells, and thus by down-regulating their ligands (MICA/B and UL16BP) leukaemia evade the anti-tumor cell activities. A study examining the histamine effect on NKG2D ligands on THP-1 leukaemia cells showed that IFNγ increased the surface expression of these ligands whereas histamine attenuated this effect [81]. This was most likely mediated through H_1R and H_2R activation since H_1R and H_2R agonists also reduced NKG2D expressions. The molecular mechanism for this reduced expression is due to enhanced poly-ubiquitination which target the ligands for proteasomal degradation, subsequently reducing susceptibility to NK cell-mediated cytotoxicity [81].

Taken together, the role of histamine receptors in cancer is complex. H_2R appear to promote the tumor growth in cancer, most likely by autocrine and paracrine mechanisms. In immune surveillance cells, H_2R also seems to suppress their activity but at the same time H_2R activation on phagocytes prevent the production and release of ROS and thus protect anti-tumor cells from oxidative stress and apoptosis. Thus, in any future therapeutic applications, it would be important to identify the subset of histamine receptors in each cancer type and the molecular mechanisms to unveil the most suitable therapeutic strategy in terms of modulating the actions of histamine.

4 Histamine Intolerance

Histamine intolerance (HIT) is a relatively controversial topic which has been poorly researched and is characterized as an accumulation of histamine due to increase availability (e.g. ingestion of histidine/histamine-rich food) or reduced capacity of histamine degradation (e.g. alcohol or drugs that can inhibit catabolic enzymes). The enzyme DAO is the main enzyme for catabolizing ingested histamine and it has been proposed that its reduced activity (e.g. decrease expression, drugs or metabolites which inhibit DAO activity) can result in an excess of histamine, causing pseudo-allergic symptoms, such as diarrhea, headache, rhino conjunctival symptoms, asthma, hypotension, arrhythmia, urticaria, pruritus, and flushing [73, 93, 102].

The scientific evidence to support an association of ingested histamine and adverse reactions is limited [93]. Furthermore, studies on HIT is difficult to initiate because it is difficult to distinguish and diagnose in potential patients. Patients first have to be excluded for allergies or other diseases that might increase histamine levels, such as mastocytosis. In addition, that lack of reliable laboratory tests for objective diagnosis is also a hinderance [93].

The gold standard in diagnosis for HIT is a double-blind, placebo-controlled histamine provocation following a histamine-free diet for 4 weeks [80]. However, the interpretation of provocation results is often prombelmatical due to a lack of symptom reproducibility [64]. Assuming that intestinal DAO levels reflect that of serum, low serum DAO concentrations could be a biomarker for histamine intolerance [80, 113]. However, measurement of serum DAO is somewhat controversial. A study using a commercially available kit to measure serum DAO reported that DAO activity in histamine intolerant patients was lower than in healthy controls, and patients with highly reduced DAO activity displayed symptoms of HIT after the intake of histamine-rich food [80]. This is supported by a retrospective study which examined serum DAO levels and the clinical response to DAO supplementation where serum DAO levels were also reduced in patients suspected of HIT compared to controls [74]. In contrast, other studies found no correlation between DAO serum levels and clinical status due to high interassay variation [63] [100]. This suggests that it is not currently possible to conclude whether the accumulation of histamine causes intolerance and whether reduced histamine catabolism is the cause. A more recent concept which could in part be related to HIT-phenomena is mast cell activation

syndrome, which is currently being investigated as a possible cause for a variety of idiopathic mast cell or histamine-related disorders [6].

5 Conclusions

It is increasingly clear that histamine plays an important, yet highly complex, role in many different inflammatory diseases. It does so not only by driving the classical hallmarks of inflammation but the studies highlighted in this chapeter also show that it modulates the functions of a variety of adaptive immune cells (antigen-presenting cells; T cells, B cells, NK cells) which govern the underlying immunology of inflammatory diseases. The net effect of histamine on these cells is crucially dependent on the expression of the various histamine receptor subtypes. However, this is not so easily addressed due to heterogeneous expressions between different cell lines and species, as well as biased signaling and differential off-target effects of drugs used to study histamine receptor actions. Despite these issues, new developments in the H_4R field have shown that this receptor is crucially involved in pruritus (itch) and clinical trials underline its potential therapeutic use, especially in combination with H_1R antagonists/inverse agonists and anti-IL-31approaches. In contrast, the therapeutic use of H_4R antagonists in RA have yet to be proven in humans despite promising results in mouse models. However, in cancer, recent evidence demonstrating that histamine plays a major role in tumour surveillance (H_2R-mediated) may have therapeutic implications and considerably enhance our understanding of inflammatory processes in an oncological context.

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Neural Regulation of Inflammation: Pharmacological Mechanisms and Therapeutic Perspectives

Marco Cosentino and Franca Marino

Abstract

During the past three decades, our knowledge about the close relationship and functional integration between the immune system and the nervous system hugely increased, and the relevance of the neuroimmune network in health and disease is now established, providing novel and unanticipated opportunities for the modulation of the immune response by means of conventional neural targets. Primary and secondary lymphoyd organs are extensively innervated by the autonomic nervous system, and cells of adaptive as well as innate immunity express receptors for neurotransmitters and neurohormones, including noradrenaline, adrenaline, acetylcholine and many others, which control critical immune functions. In addition, immune cells themselves may produce and utilize classical neurotransmitters, providing additional complexity to the network but also additional opportunities to develop novel immunomodulating strategies.

Neuroimmune pharmacology is a young but rapidly growing discipline, encompassing interdisciplinary research in pharmacology, immunology and neuroscience, offering original therapeutic approaches to investigate the neuroimmune network. The present chapter provides an overview of the main neurotransmitter-operated pathways affecting the immune system, as well as of their clinical and translational potential with regard to major diseases such as multiple sclerosis, cancer and rheumatoid arthritis.

Keywords

Neuroimmunology · Neuroimmune · Pharmacology · Dopamine · Noradrenaline · Adrenaline · Acetylcholine · Glutamic acid · Multiple sclerosis · Cancer · Rheumatoid arthritis · Drug repurposing

1 Immune System-nervous System Communication Pathways

The main and most finely tuned communication between the nervous system and the immune system occurs primarily through the autonomic nervous system (ANS) that innervates lymphoid organs with both sympathetic and parasympa-

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thetic fibers, including primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs [1]. In the immune system, the presence of different neurotransmitters and neuropeptides (and receptors on immune cells for these mediators) is in line with the close functional relationship between the nervous and the immune system.

In this neuro-immune network, a key role is played by the sympathetic nervous system (SNS) that, together with the hypothalamic–pituitary–adrenal (HPA) axis, represents the major pathway involved in the cross-talk between the brain and the immune system [2].

Nerve terminals reach immune cells in lymphoid tissue, as well as in the vascular wall and in other tissues and organs, and release catecholamines (CA), acetylcholine (ACh) and other neurotransmitters and neuromodulators (e.g., neuropeptide Y, glutamic acid, etc.) [3]. Additional complexity to the network derives from observations showing that human immune cells express on their surface receptors for neurotransmitters such as noradrenaline (NA), dopamine (DA), acetylcholine (ACh), serotonin and other neurotransmitters, together with the machinery for their synthesis and storage [4–12]. Communication between the central nervous system (CNS) and the immune system has many physiopathologic and clinical implications, as shown e.g. by evidences about worsening of inflammatory disease such as rheumatoid arthritis after the disruption of sympathetic nerve fibres [13] or the documented dysregulation of cholinergic pathways in different types of cancer [14].

2 Inflammation and Neurotransmitters

Inflammation is an essential immune response triggered by infection and injury, and represents a nonspecific reaction involving first of all the innate immune system (monocytes/macrophages, neutrophils, dendritic cells, natural killer, and the other components of the innate branch of immunity). Adaptive immunity (primarily T and B lymphocytes) thereafter contribute through several mechanisms, including production of cytokines, which are considered the "immune hormones" [15], with the aim to maintain/ reestablish tissue homeostasis. Both pro- and anti-inflammatory cytokines are involved in this process, and acute-phase reactants, such as C reactive protein (CRP), are produced during inflammation. Although inflammation is a critical response to acute infection or injury, chronic or excessive inflammation may be detrimental for health.

In specific inflammatory conditions, such as stress-related inflammation, CA and cortisol are major modulators of the immune response and their effects may well explain the consequences of stress on immunity. In fact, not only glucocorticoids, but also NA is known to interfere with most of the immune functions, including the innate response with the release of proinflammatory cytokines [16–18], such as interleukin (IL)-6 [16, 19, 20]. Indeed, noradrenergic dysfunction, including overactivity of the SNS, is recognized as a characteristic of emerging diseases such as obesity-related metabolic syndrome, or contributing to the pathophysiology and negative clinical prognosis in cardiovascular diseases [21]. Adrenergic nerve endings in blood vessels play a role in metabolic dysregulation [22, 23], and SNS overactivation is involved in increasing of body weight [21]. Similarly, the parasympathetic branch of the ANS plays a key role regulating inflammation, mainly with an antiinflammatory role. Stimulation of vagus nerve reduces the production of proinflammatory cytokines by means of ACh possibly acting on nicotinic receptors. On this basis, the existence of a "nicotinic antinflammatory pathway" has been proposed [24].

Besides CA and ACh, other neurotransmitters such as glutamic acid play a key role in inflammation. Glutamic acid seems to be involved in several diseases affecting the CNS, such as multiple sclerosis [10], amyotrophic lateral sclerosis [25], as well as the periphery (e.g. cancer) [26]. Recently, glutamic acid has been proposed to play a role also in the inflammatory component of neuropathic pain [27].

2.1 Catecholamines

The term "catecholamine" (CA) includes a wide group of neurotransmitters called "monoamines" that are chemically organized with a single amine (-NH2) group, a cathecol nucleus (i.e. a benzene ring with two adjacent hydroxyl groups), and an ethyl-amine side chain [28]. The most important CA are dopamine (DA), noradrenaline (NA) and adrenaline (A), which are synthesized from the amino acid tyrosine. The first step is the transformation of tyrosine in levodopa by the enzyme tyrosine hydroxylase (the rate-limiting enzyme in the biosynthetic pathway of CA). In adrenergic and dopaminergic neural and neuroendocrine cells, levodopa is then decaborxylated to produce DA which in dopaminergic cells is just stored in vescicles, while in adrenergic cells is transformed in NA by the enzyme dopamine β-hydroxylase (DHBA), and is eventually finally converted to A by phenylethanolamine N-methyltransferase (PNMT) [29, 30]. This latter conversion occurs mainly in periphery (in particular in the adrenal gland).

2.1.1 Dopamine

DA is usually considered first of all a neurotransmitter of the CNS, involved in the control of several key functions, such as cognition, motivation, movement and reward [28], while in the periphery DA is known to regulate blood pressure, sodium balance, adrenal and renal function, glucose homeostasis and body weight. Recently however an important role of DA as key transmitter in the interconnection of the CNS and the immune system has been extensively characterized, and now DA is known to be produced also by immune cells themselves [31–33]. Almost all human immune cells are affected by DA, and some cells (e.g. lymphocytes and dendritic cells) are also able to produce and utilize DA as an autocrine/paracrine mediator [34, 35].

DA exerts its effects interacting with five different dopaminergic receptors (DR), which are 7-transmembrane, G protein-coupled receptors [36], usually classified in two main families: D1-like (D1 and D5, that activate Gαs/olf proteins to stimulate cyclic adenosine monophosphate (cAMP) production by adenyl cyclase (AC)), located both pre- and post-synaptically, and D2-like (D2, D3 and D4, that activate Gαi/o proteins, inhibiting AC and resulting in reduced cAMP levels), which are mainly post-synaptic.

In the periphery, the gastrointestinal tract represents the major source of DA, but DA can be produced at least by three different districts: the neuroendocrine cells, the adrenal glands and the neuronal fibers. Recently, it was hypothesized that an increase in the dopaminergic tone of the striatum may represent a risk factor for obesity and, in line with this hypothesis, it was shown that, consumption of carbohydrates stimulates the production of DA and is linked with food reward and release of DA in the brain.

Dopamine and the Immune System

The presence of DA in immune compartments is suggestive of a role of this mediator in influencing immune cells. For example, treatment of lymphocytes with IFN-β (a cornerstone in the treatment of multiple sclerosis) leads to increased production and release of DA [37], while profound changes in DR expression on CD4+ T lymphocytes (in comparison to healthy subjects) occurs in Parkinson's disease [38] and in multiple sclerosis [39]. Immune dopaminergic pathways are also extensively involved in other diseases such as rheumatoid arthritis [40] and obesity [41].

DA profoundly affects both the innate and the adaptive branch of immunity. Both innate immune cells and B and T lymphocytes express all the five DR and possess the enzymatic machinery for the synthesis, storage and release of DA (as well as of the other two CA, NA and A) [6, 42], and dopaminergic dysregulation occurs in immune cells of patients with reumathoid arthiritis [40, 43] or with Parkinson's disease [38]. Tables 1 and 2 summarize the main evidences about the presence of DR on cells of innate and adaptive immunity.

Evidence also exists suggesting that dopaminergic drugs used in clinics may affect immunity. Dopaminergic agonists like cabergoline, pramipexole and ropinirole are used as antiparkinson agents, while antagonists like chlorpromazine, halperidol or metoclopramide are used as antipsychotics and antiemetics. Interestingly e.g.

Table 1 Examples of effects induced by dopamine and drugs acting on dopaminergic receptors on cells of innate immunity

Model	Function	Cells	Effects	Ref.
Neutrophils				
Human	fMLP-induced superoxide anion production	Isolated neutrophils	Inhibition/reduction	44
	CD11b/CD18 expression, adherence to endothelium, phagocytic activity	Isolated neutrophils	Inhibition/reduction	45
	Apoptosis	Isolated neutrophils	Inhibition/reduction	46, 47
	IL-8 induced migration	Isolated neutrophils	Inhibition/reduction	48
	Neutrophil count	Whole blood (anaphylactic shock)	Reduction induced by chlorpromazine and pimozide, increase induced by apomorphine	49
	fMLP-induced cell migration, fMLP-induced ROS generation	Isolated neutrophils	Inhibition/reduction	50
	Cell count	Whole blood	L-DOPA-induced neutropenia in patients with Parkinson's disease	51
Eosinophils				
Human	Eosinophils miocarditis	Explanted heart/blood count	Reduction of myocarditis/ peripheral eosinophilia	53
Rat	Cell count	Whole blood	Inhibition with high dose of L-DOPA/ increases with low dose of L-DOPA	52
Mast cells				
Mouse	Cell degranulation	RBL-2H3 cell line	Inhibition	54
Monocytes-macrophages				
Human	Chemokinesis	CD14+CD16+	Increased by SKF-38393	55
	Monocyte-induced HIV entry into the BBB	CD14+CD16+	Increase	56
	HIV entry and replication	Primary macrophages	Increase of entry and inhibition of replication	57, 58
	Reverse transcriptase activity		Increased activity	59
Mouse	LPS-Induced Systemic Inflammation In Vivo NLRP3 inflammasome activation	Intraperitoneal injection of LPS or bone derived cells	Inhibition	60
Dendritic cells (DC)				
Mouse	Th17-mediated immunity, cytokine production		Inhibition	61
	DC-mediated Th17 differentiation		Inhibition	62
Human	IL-10, IL-6, IL-8, TNF-α, IP-10 and IL-12 production	Monocyte-derived DC	Risperidone increased IL-10, IL-6, IL-8, TNF-α, and decreased IP-10 and IL-12	47
Natural Killer (NK)				
Mouse	Cytotoxic activity	Spleen-derived NK	Increased by SKF-38393 and decreased by quinpirole	63
Rat	Killing activity		Increased in hyperactive and decreased in hypoactive dopaminergic system	64
Human	Fc-receptor-mediated human NK activation	Peripheral NK	Inhibition by antagonists	65

(continued)

Table 1 (continued)

Model	Function	Cells	Effects	Ref.
Astrocytes				
Rat	NLRP3 inflammasome activation		DA increases Ca^{++} levels	60
	Calcium intracellular signaling		DA increases Ca^{++} levels	66
Complement and antibacterial peptides				
Human	Complement component C3a and levels of IL-6, IL-8	Blood of patients with coronary artery bypass grafting		67

chlorpromazine may reduce neutrophil count and affects other innate immune functions (Table 1), while DA or dopaminergic agonist dobutamine (used in the clinical setting as inotropic drugs) may counteract eosinophilic myocarditis (Table 1). DA and DR agonists may have an anti-inflammatory profile, and may also act as antiangiogenic agents (Table 2).

2.1.2 Noradrenaline and Adrenaline

The names "adrenaline" (A) and "noradrenaline" (NA) derive from the early identification of these mediators: "adrenaline" means "near the kidney" (Latin roots: "ad renes"), like the US name epinephrine (Greek roots: "epi" and "nephros", i.e. "on the kidney"). Noradrenaline/norepinephrine present the prefix "nor" standing for "normal", which indicates the demethylated form of a compound (although according to others it is the acronym for nitrogen "öhne radikal" (without radical)). NA was first identified in 1946 by von Euler, and a few years later in 1950 the same substance was found in the brain by Holtz. Previously, its presence was strictly associated with autonomic fibers innervating the smooth muscles of cerebral blood vessels (and NA was called sympathin). Finally, in 1954, Vogt found that the presence of NA in the brain was not only correlated with vessel, and its role as central neurotransmitter was established. The major source of A into the bloodstream are the chromaffin cells in the adrenal glands, stimulated by the SNS through its preganglionic fibers. Chromaffin cells release A (~80% in humans) and in minor part NA (~20%). In the CNS, noradrenergic neurons are located mainly in the *locus coeruleus* (LC), and their axons project to hippocampus, septum, hypothalamus and thalamus, cortex and amygdala, to cerebellum, as well as to spinal cord. Adrenergic control includes attention, arousal and vigilance, and regulation of hunger and feeding behavior. In some areas of the CNS (e.g. in the medullary reticular formation) also A acts as neurotransmitter, affecting eating behavior and blood pressure [28]. In the ANS, NA is the main transmitter of sympathetic postganglionic fibers, which affect smooth muscle contraction in blood vessels and exocrine glands, heart rate and force of contraction, glycogenolysis in liver and muscle, lipolysis in adipose tissue, thermogenesis in brown adipose tissue, and secretion of insulin and renin. On the contrary, adrenergic control of smooth muscle in the gut wall, bronchi, and blood vessels supplying skeletal muscles is mainly inhibitory [85].

Adrenergic receptors (AR) were first described by Ahlquist in 1948 and classified in two different classes named α- and β-AR [86]. They are both G-protein coupled receptors and are divided in: $\alpha 1_1$ and 2, and β1, 2 and 3, functionally linked to different intracellular second messengers according to the different Gα subunit. This initial classification was later upgraded by Bylund, and now we classify α-AR subtypes in α1A, α1B, α1D and α2-AR subtypes in α2A, α2B and α2C [87]. α1-AR are linked to a Gq and their stimulation induces the activation of phospholipase C (PLC) that promotes hydrolysis of phosphatidylinositol bisphosphate producing inositol trisphosphate (IP3) and diacylglycerol (DAG), acting respectively on non-mitochondrial pools or protein kinase C and mediating intracellular Ca^{++} release. α2-AR are linked to a Gi and their stimulation induces AC inhibition with consequent reduction of cAMP levels. β-AR are coupled to the stimulatory G- protein Gs that leads to

Table 2 Examples of effects induced by dopamine and drugs acting on dopaminergic receptors on cells of adaptive immunity

Model	Function	Cells	Effects	Ref.
Mononuclear cells				
Human	Cell apoptosis; ROS generation	Circulating PBMC	Increased by high and decreased by low DA	75
	Cytokine production		DA suppressed IL-17 production	76
Mouse	Cell proliferation	Splenic mononuclear cells	Increased by high and decreased by low DA, increased by SKF38393 or LY171555 i.v.	74
B Lymphocytes				
Human	Mitochondrial potential	Epstein-Barr virus (EBV)-transformed human B-lymphocytes	Inhibited by DA	73
Mouse	Inflammatory infiltration in the airways	B cells	Inhibited by SCH23390 and increased by SKF83959	70
	LPS-activated cells; cAMP response; Ig synthesis	B lymphocytes from spleen, mesenteric lymph nodes and Peyer's patches	Decreased by DA	71
	Chemotaxis, calcium influx	Pre B cell line	Induced by DA	72
T Lymphocytes				
Human	Con-A activated T cells	Naïve T cells	Inhibited by DA	4
	Cell adhesion	Naïve isolated T cells	Induced by DA	77
	Cell migration	CD8+	Induced by DA	72
	Cell adhesion, cytokine production	CD8+	Induced by DA	78
	Cytokine production	Naïve T cells	Induced by DA	79
	Cell proliferation and cytokine production	Regulatory T cells	Inhibited by DA	34
	mitogens and cell proliferation and cytokine production	Peripheral T lymphocytes	DA enhanced in vitro T cell proliferation and Th17-related cytokines in MS-derived cell cultures	80
	Cell proliferation, IL-2 production	Activated T cells	Reduced by DR agonists	81
Mouse	Expression of Th1/Th2- and Treg-specific transcription factors and cytokines in Con A-activated lymphocytes	Mesenteric lymph nodes lymphocytes	Quinpirole upregulated the expression of Th2- and Treg-specific transcription factors and cytokines in Con A-activated lymphocytes, and downregulated the expression of Th1- and Th17-specific transcription factors and cytokines	82
	Cell proliferation	Lymph nodes lymphocytes	Carbidopa inhibited T cell activation in vitro and in vivo	83
	cAMP and ERK1/2-phosphorylation	Splenic T lymphocytes	Inhibited by DA	84

activation of AC and accumulation of the second messenger cAMP. Under certain circumstances, β-AR, and particularly β3-AR, can be coupled also to Gi [88]. β1 and β2-AR are the most well characterized and mediate several important physiological functions. β1-AR in particular are the most important receptors mediating cardiovascular responses to NA released from sympathetic nerve terminals and to circulating A. They are stimulated or blocked by many drugs currently used as therapeutic agents for cardiovascular diseases, such as hypertension, cardiac arrhythmias, and ischemic heart disease. β2-AR are primarily localized on airway smooth muscle cells and are involved in bronchial relaxation. Drugs acting as agonists of β2-AR are employed

as first line-treatment of asthma and chronic obstructive pulmonary disease. β3-AR were previously described as mediating lipolysis in rat adipocytes [89], and thereafter they were also found in blood vessels inducing vasodilatation [90]. Pharmacological evidences and molecular studies suggest the existence of a fourth β-AR subtype [91].

Noradrenaline and Adrenaline and the Immune System

The discovery of sympathetic fibers innervating spleen and lymphoid organs [92, 93] was rapidly followed by several studies showing a link between the immune system and the ANS [94]. Together with the hypothalamic-pituitary-adrenal axis, the SNS represents, the major pathway involved in the cross-talk between the brain and the immune system. Sympathoadrenergic fibers innervate both primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid organs, where NA and A are released from nerve varicosities and derive from the bloodstream to act on AR expressed on immune cells. AR on immune cells represent an important example of extra junctional receptors and of intercellular (nerve-immune) communication [95]. Recently, data about the pathophysiological relevance of such interaction were provided in several pathological conditions [68, 95–97].

Over the last two decades, several reports showed the profound influence exerted by sympathetic innervation in the regulation of the immune responses and vice versa the ability of cytokines released by immune cells to cross the blood brain barrier (BBB) and influence CNS functions [94, 98, 99]. NA may be proinflammatory as well as anti-inflammatory (Table 3) depending on the function or the cell type involved. For example, NA may increase the expression of inflammatory cytokines and chemokines in resting and activated memory CD8+ T cells, while it may reduce cell migration or increase adhesion of activated cells. In the innate branch of immunity, NA and the β- AR agonist isoprenaline are usually anti-inflammatory, reducing respiratory burst, reactive oxygen species production and migration of humn neutrophils and inhibiting histamine release from mast cells. Table 3 summarizes several examples of the influence of adrenergic mechanisms affecting human immune functions. On these basis, drugs acting on AR warrant careful consideration for their potential antinflammatory effects. Repurposing adrenergic (and dopaminergic) agents as antinflammatory and immunomodulating drugs could benefit patients and the health systems, providing drugs with a usually favourable therapeutic index and expectedly at low price.

2.2 Acetylcholine

Acetylcholine (ACh) was firstly described in 1914 by Dale, who showed that stimulation with ACh induced responses superimposable to those elicited by parasympathetic nerve stimulation [135]. In other studies, Dale showed that the effects of ACh were mimicked by two alkaloids: nicotine and muscarine, and proposed the existence of two main classes of receptors, the nicotinic and the muscarinic receptors, a classification which is still in use at present. The biosynthetic pathway of ACh in nerve terminals starts from choline (actively transported through the BBB) and acetylcoenzyme A, which ae bound to each other by cholinacetyltransferase (ChAT). ACh is then stored in vesicles, and when released acts on nicotinic (nAChR) and muscarinic (mAChR) receptors. The nAChR are composed by four glycoproteic subunits (α, β, γ and δ). The nAChR are divided in neuronal and muscular (each of them including different subtypes) on the basis of the presence of the presence of the ε glycoprotein (homologous of the γ subunit) in the muscular subtypes [136]. Both nAChR types are coupled to a Na+ channel, which opens upon receptor interaction with two molecules of ACh [137]. The mAChR presently identified are five, and all are G-protein coupled receptors, named M1, M2, M3, M4 and M5. The M1, M3 and M5 are coupled to a Gq protein which induces the activation of PLC and intracellular Ca++ mobilization. On the

Table 3 Examples of effects induced by noradrenaline, adrenaline and drugs acting on adrenergic receptors on human immune cells

Functions	Cells	Effects	Ref.
Innate immunity			
Neutrophils			
Respiratory burst	Isolated neutrophils	Inhibited by Isoprenaline	100
cAMP formation; respiratory burst		Inhibited by β2-agonists	101
ROS generation, cell migration, CD11b expression	Isolated neutrophils, whole blood	Inhibited by A and reverted by propanolol	102
CD15, CD44, CD54 adhesion molecule expression		Inhibited by A at high concentrations	103
IL-8 induced migration; Cell migration, superoxide anion generation	Isolated neutrophils	Inhibition/reduction	48, 104
Cell count	Whole blood	Reduced by A and increased by β-blockers	105
Leukotriene C4 production, EPO production	Isolated neutrophils	Reduced by β-AR agonists	106
Mast cells			
Histamine release	Lung mast cells	Inhibited by isoprenaline and clenbuterol	107
Histamine release	Mast cells cultured from venous blood	Inhibited by isoprenaline and β-AR agonists	108
Monocytes/macrophages			
Oxygen radical production	Isolated monocytes	Inhibited by β-AR activation	109
TNF-α receptor expression and production		Induced by CA	110
Phagocytosis induced by *C. albicans*		Inhibited by β-AR activation	111
Cytomegalovirus receptor promoter	Whole blood	Induced by CA	112
Cell adhesion to laminin Phagocytosis induced by oxidized-lipoprotein	Isolated monocytes	Increased by A	113
IgE-induced production of IL-6, IgE, superoxide anion generation, nitric oxide, TxB2	Isolated monocytes	Induced by β-AR activation	114–116
LPS or IL-1 stimulated production of IL-8		Induced by β2-AR activation	117
Surface expression of L-selectin		Increased by A	118
MMP-1 production	Circulating and isolated macrophages	Increased by NA and A	119
Complement components C2-C5, factor B, properdin, beta 1H, and levels of IL-6, IL-8	Isolated monocytes	Synthesis increased by A and NA	129
LPS-induced IL-1β production		Induced by A	120
Tumor recruitment of macrophages		Increased by CA	121

(continued)

Table 3 (continued)

Functions	Cells	Effects	Ref.
Dendritic cells (DC)			
CD-40 stimulated cAMP and IL-12 production	DC	Inhibition of IL-12 and Th1 differentiation, increased cAMP production and Th2 differentiation induced by β2-AR activation	122
LPS-induced IL-23, IL-12 p40, TNF-α, IL-6	DC from cord blood	Inhibited by NA	123
Cell migration	DC	Inhibited by A	124
Proinflammatory cytokine production	DC	Inhibited by CA	125
Natural killer (NK)			
Cytotoxic activity	Peripheral NK	Decreased by NA and A	126
Cell adhesion to endothelium		Increased by β2-AR activation	127
Cell migration		Increased by NA and A	128
Fc-mediated NK activation		Increased by β2-AR activation	65
Other cells			
Infection by *Mycoplasma penumoniae*	Asthma airways epithelial cells	Albuterol and formoterol protect	130
Neutrophil peptide expression	Synovial tissue culture	Inhibited by NA	131
Adaptive Immunity			
Apoptosis	PHA-stimulated PBMC	Increased by CA	132
Superoxide production	Peripheral PBMC	Increased by NA through α2-AR	133
Cell-to-cell interaction, cytokine production		NA, A and isoproterenol inhibited AGE-2- and AGE-3-induced adhesion expression and cytokine production	134
Cytokine production in resting and stimulated cells	Isolated CD8+ T cells	NA induced an elevated expression of inflammatory cytokines and chemokines in resting and activated memory CD8 T cells in addition to a reduced expression of growth-related cytokines	133
Migration of activated CD8+ T lymphocytes; cell adhesion		NA reduced cell migration while increased adhesion of activated cells	134

contrary the M2 and M4 are coupled to a Gi and result in cAMP reduction. ACh is inactivated by the enzyme acetylcholinesterase (AChE, existing in different isoforms) and this is the only way to remove ACh from the synaptic space. In the brain, cholinergic transmission plays important roles such as control of motor neuron and regulation of arousal, attention, memory and motivation. In addition, ACh is an important neurotransmitter in the ANS, in both ortho- and parasympathetic preganglionic fibers (acting on nAChR) and in parasympathetic postganglionic fibers (acting on mAChR). Finally, ACh is released by somatic nerve terminals impinging the motor end plate on skeletal muscles (nAChR).

2.2.1 Effect of Acetylcholine on Immune Cell Functions

The presence of receptors, synthetisizing and storing systems for ACh on cells of the immune system was described during the last century, at the end of the seventies, when Gordon and described the ability of ACh to modulate lymphocyte functions [8]. Later on, different lymphocyte subsets were found to express mAChR [138]. In 1999, Sato and coworkers, using RT-PCR on human leukemic cell lines and peripheral blood mononuclear cells demonstrated the presence of both mAChR and nAChR [139]. AChR occur also on microglia and astrocytes [140] where the activation of nAChR reduces neuroinflammation and production of proinflammatory cytokines,

such as TNF-α [141–143]. Different nAChR were described on B and T lymphocytes, on neutrophils, macrophages, dendritic cells and microglial cells [144–146], and may be involved in the pathogenesis of several autoimmune diseases [145]. The contribution of nAChR in the regulation of immune cell functions has been extensively documented by the group of Kawashima [147–149]. Fo example, in a mouse model of multiple sclerosis treatment of T cells with nicotine significantly attenuates inflammatory responses to myelin antigens [150]. In the periphery, nicotine exposure inhibits the proliferation of autoreactive T cells and alters the cytokine profile of T helper cells [151].

Stimulation of the vagus nerve, through the activation of nAChR, results in anti-inflammatory effects, such as inhibition of proinflammatory cytokines production, and suggests the existence of a "nicotinic antinflammatory pathway" [24], possibly contributing to counteract severe pathological responses such as sepsis [145, 152]. Main evidence comes from studies on the effects of nicotine on the immune response. Such studies are abundant, even if also often conflictual: for example, nicotine may increase production of proinflammatory cytokines in dendritic cells [153], chemotactic activity of neutrophils [154], and antigen receptor-mediated signal transduction in lymphocytes [155], however it may also reduce CD14 and TLR4 expression in human monocytes [156], and inhibit the production of proinflammatory cytokines in monocytic cell lines [157]. Nicotine acting on nAChR affects immunity [158, 159] and is possibly neuroprotective, for example in Parkinson's disease [160].

Dysregulation of cholinergic pathways may occur in disease conditions. T leukemic cell lines derived from relapsed patients produce significantly higher levels of ACh in comparison to T cells derived from healthy subjects, and mAChR expression is altered [14]. Decreased ACh levels in plasma and increased levels of butyrylcholinesterase (BuChE, the enzyme devoted to ACh catabolism in plsma and tissues) may occur in multiple sclerosis patients, suggesting a possible role in the inflammatory events underlying autoimmunity [161]. Promising results cholinergic pathways as therapeutic targets comes from a recent studies in HIV-infected patients showing that pyridostigmine (an inhibitor of AChE), used as add-on therapy, may increase CD4+ T cells [162].

2.3 Glutamic Acid

In the earliest 1950s, a possible physiological role, in the brain, of glutamic acid (or glutamate) and aspartic acid (or aspartate) (the two-main nonessential amino acids present in the brain) was described [163, 164], but only in the late 1970s, glutamate was recognized as the main and most abundant excitatory neurotransmitter in the CNS of vertebrates. At present, glutamate is known to be involved in different functions in the normal brain, including cognition, memory, learning, and it is also a key regulator of neuronal development and synapses formation. In periphery, glutamatergic transmission affects heart, kidney, intestine, muscles, liver, bone, pancreas and glands such as adrenal, pituitary and pineal. Glutamate exerts its effects through two main distinct classes of receptors (GluR): the ionotropic (iGluR) and the metabotropic (mGluR). Both are found in neuronal and glial cells. The iGluR are ion channels opened/gated by glutamate, while the mGluR are G- protein coupled receptors. The iGluR are divided into three main subgroups and are recognized on the basis of the different ligands which bind their recognition sites: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4 propionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopenylpyrrolidine (Kainate, KA). All the three subtypes occur in the CNS and in periphery. The NMDA iGluR are widely expressed on neurons and are present in three main forms: type 1 (NR1), type 2 (NR2) and type 3 (NR3A/B), composed by a tetramer or a pentamer, with NR3 that seems to have a prevalent regulatory role for the activation of the others [165]. The combination of the NR3 and NR1 generates a glycine receptor, that is not responsive to glutamate [166]. A unique property of the NMDA iGluR is their voltage-dependent

activation and the blocked of the ion channel by extracellular Mg++. From a pharmacological point of view, several antagonists of these receptors such as amantadine, ketamine, phencyclidine and others, are used as therpeutics, for example as anesthetics, on s secnd line drugs for Parkinson's disease, or even as recreational drugs due to hallucinogen properties [167]. The AMPA iGluR are the most common iGluR present in CNS and are widely distributed also outside the brain. These receptors are homo and hetero- oligomers composed by four subunits (GluR1-GluR4) organized in a tetramer [168], and possess at least four different binding sites for glutamate as well as for other ligands [169]. In the CNS, AMPA iGluR are necessary for the regulation of neuronal plasticity and synaptic transmission. The KA iGluR are not chracterized in detail, and their distribution in the brain is limited in comparison to the other two iGluRs. Their physiological role is not clearly established. KA receptors are involved in both pre and post-sinaptic transmission and it is generally accepted that pre-sinaptic KA receptor activation is involved in inhibitory transmission (e.g. by modulating of GABA release), while post-sinaptic activation is generally involved in excitatory responses [170]. The mGluR are widely distributed both in the peripheral nervous system and in the CNS, where they affect anxiety, learning, memory, and are involved in pain perception. mGluR are typical G-protein coupled include changes in excitability of synapses [171]. At present, three different subtypes are identified (named I, II and III), mGluRI including 1 and 5 and being associated with a Gq (coupled to PLC), mGluRII including 2 and 3, and mGluRIII including 4, 6, 7, and 8 (coupled to a Gi and G0).

2.3.1 Effect of Glutamic Acid on Immune Cell Functions

Earliest evidence about presence and physiological relevance of glutamate in the immune system was published in 1972, concerning the pesence of glutamate in thymus cells [172] and in guinea pig immune cells, where functional effects of glutamate were also reported [173]. Susequently, both iGluR and mGluR were described on different immune cell subsets, and their possible relevance in health and disease was proposed [10, 26, 174–176]. The direct effects of activation/inhibition of GluR on immune cells include for example the ability of isoflurane (an anesthetic drug known to act as antagonist on NMDA iGluR) to reduce NO production from zymosan activated rodent neutrophils. In the CNS, increased levels of glutamate induce microglia activation (with a shift towards the M1 proinflammatory phenotype). In addition, in microglia glutamate may also increase inflammation and apoptosis [177]. Glutamate is also a modulator of T cell responses,. for example resulting in inhibition of cell proliferation [178], adhesion molecule expression [179], increasing the adhesion of lymphocytes to laminin and fibronectin [26], and modulating the release of several pro and anti-inflammatory cytokines, such as IFN-γ, IL-10 [180], IL-6, TNF-α, IL-2, and many others [181]. Glutamate seem to be involved in several immune-mediated diseases of the CNS such as multiple sclerosis [10], amyotrophic lateral sclerosis [25], as well as in peripheral diseases such as cancer [26]. Gluatamate has also key role in glia, in the development of neuropathic pain with a rich inflammatory component, and different drugs such as MK801 (antagonist of the rNMDA iGluR) are proposed as putative therapeutics [27]. Glutamate plays also a key role in neuroinflammation during HIV infection, with peripheral macrophages affecting glutamate-induced excitotoxicity [182].

2.4 Other Neurotransmitters

Many other neurotransmitters are present in immune cells and are able, through interaction with their receptors, to affect immune functions, and fibers positive for these neurotransmitters are recognized in lymphoid tissues. For example, 5-hydroxytriptamine (5-HT), also called serotonin, and isolated by Ersparmer in 1935, at present is known to occur in several immune cells and is able to interfere with different functions. Seven families of receptors, named 5-HT1–7 (and in turn classified in different subtypes) are described, and are able to affect different key functions in

both CNS and in periphery [9]. 5-HT has important immunomodulatory roles [183]. In the nervous system, 5-HT interferes with adrenergic and cholinergic transmission and affects peripheral transmission [184]. Several line of evidences suggest that 5-HT is present in cells of immune system and that these cells express the receptors for this neurotransmitter; in addition, 5-HT is able to affect the functions of both cells of innate and adaptive immunity acting on their different receptors presents on cell membrane. For example, in monocyte/macrophages 5-HT induces phagocytosis and reduces the TNF-α release; similarly, an inhibitor role was described in human alveolar macrophages activated by LPS, where 5-HT reduces the TNF-α and IL-12 release and increases the IL-10, nitric oxide, and prostangladin-E2 production. By contrast in monocytes, 5-HT is able to enhances the LPS-induced secretion of IL-12p40, or the LPS induced secretion of IL-6, IL-1β, IL-8/CXCL8. In T cells, 5-HT seems to play a key role in the ability of these cells to produce proinflammatory cytokines, such as IL-2 and IFN-γ or to affect cell migration and is involved in antibody responses in autoimmune diseases. Dysregulation of 5-HT pathways seem involved in different diseases such as fibromyalgia or in the ability of the immune system to counteract infection (both viral or bacterial) or in typical inflammatory diseases such as asthma and rheumatoid arthritis.

Histamine (a biogenic amine) is another key neurotransmitter in the CNS which is also present in peripheral fibers and is able to affect immunity, interacting with its four receptors [185] Cells of both the innate and adaptive immune system can be regulated by histamine (H) and these cells are found to express all the four receptors (H1-4R). The H1R is expressed on the most part of the cells of innate and adaptive immunity and is involved in the typical hypersensitivity responses and involved in disease such as allergic rhinitis, atopic dermatitis, urticaria, asthma and anaphylaxis. The H2R seems to play a role in inflammatory bowel disease while the activation of the H3R is involved in the increased severity of neuroinflammation. Finally, a key role of the H4R is described and the evidences come from data from animal models showing that the treatment with H4R antagonists can be used to counteract immunological diseases such as colitis, arthritis and asthma. Vasoactive intestinal peptide (VIP) is a peptide composed of 28 amino acids that was firstly detected in 1970 in the porcine small intestine and subsequently found in other peripheral tissues (such as heart, lung, kidney, etc), including the immune system and the CNS. VIP is involved in several functions such as vasorelaxation or stimulation of different tissues and is proposed as neuromodulator. The presence of VIP receptors (G-protein coupled receptors) on immune cells has been reported together with their immunoregulatory role in autoimmune diseases such as multiple sclerosis or in other conditions such as sepsis [11].

Somatostatin (SST), initially extracted from bovine hypothalamus, attracted interest for its ability to exert a lot of different effects not only in the CNS, where it interacts with five different G-protein coupled receptors (SSTR1–5), but also in the peripheral nervous system and in different organs and tissues. SST occurs in sympathetic fibers innervating lymphoid organs, and plays an active role in different immune-mediated diseases. Together with neuropeptide Y (NPY), VIP and corticotrophin- releasing factor (CRF) are also involved in the gut-brain axis [186], and contribute to different inflammatory diseases such as rheumatoid arthritis [187].

NPY is a peptide of 36 amino acids and represent one of the most abundant peptides in the nervous system. It was originally isolated from pig brain and it is known to exert its effects through five different G-protein co upled receptors (named Y1–5). In periphery, NPY was described not only as modulator of sympathetic fibers but also affecting immune responses, and its receptors ae expressed on immune cells [188]. NPY modulates immune cell trafficking, it is involved in T helper cell differentiation and affects cytokine secretion. It also regulates cell phagocytosis and production of reactive oxygen species, suggesting a possible involvement in immune-mediated diseases [188]. GABA, discovered in 1950, is the main inhibitory neurotransmitter in the mammalian brain and is known to act through

two classes of receptors: GABA-A (a pentameric chloride ion channel) and GABA-B (a metabotropic receptor coupled to a Gi protein), although a third class, the GABA-C has been also proposed. The synthesis of GABA starts from glutamate, and GABA is an important regulator of glutamate excitotoxicity. The peripheral role of GABA is still debated, in particular in the gastrointestinal tract, where it is involved in the circuitry of the enteric nervous system. Recent evidences suggest a possible role of GABA in enteric carcinogenesis and in inflammatory diseases [189]. GABA receptors mediate the effects of a lot of drugs, such as barbiturates and benzodiazepines, commonly used in different diseases, as well as of other substances such as ethanol. Many other drugs able to interfere with GABA synthesis, release and reuptake are used as therapeutics, for example as antiepileptic drugs. At the end of the past century, a peripheral receptor for benzodiazepines (PBR) was described and this receptor was identified prevalently on immune cells where the PBR was described both on cell membrane and intracellularly on mitochondria [190]. Agents acting on GABA-R affect immune responses both in animal models and in human cells, for example by reducing the production of proinflammatory cytokines in macrophages or decreasing inflammatory responses in animal models [191], suggesting a possible application as antinociceptive drug. GABA also modulates T cell proliferation [192], impairs chemotaxis and phagocytosis [193], and reduces the number of infiltrating leukocytes in the brain [194], suggesting a protective role of GABA pathways in neuroinflammation during e.g. multiple sclerosis, Parkinson's disease, Alzheimer's disease [195].

Opioids derive their name from "opium", produced by the seed capsules of the flower *Papaver somniferum*, and are well known for their analgesic and addictive properties. Opium contains different alkaloids such as morphine, papaverine, codeine and others, endowed with analgesic, antidiarrhoic and antitussive properties. Despite this old use, the discovery of the mechanism of action of these drugs, the receptors involved in their action and the identification of endogenous ligands (the endorphins) represent a recent achievement of pharmacology. Endogenous opioids are small peptides named endorphins and at present more that 20 different molecules divided in three main classes (enkephalines, endorphins and dynorphins) have been identified. Endorphins are produced in the CNS and are released from nerve terminals as a result of painful stimuli. All endorphins derive from the cleavage of the bigger precursors pro-enkephalin, pro-opiomelanocortin and pro-dynorphin. These peptides were discovered in the CNS, but at present the synthesis of some of them is known to occur also in lymphocytes, granulocytes, and monocytes/macrophages, and endorphin-positve fibers are present outside the CNS [196, 197]. The endorphins as well as the exogenous opiods act through interaction with G-protein coupled receptors that are classicaly named by the greek letters μ, κ and δ. Analgesia is predominantly evoked by stimulation of the μ subtype, but this receptor is also the main responsible of the main adverse effects such as addiction and respiratory depression. Analgesia is also induced by stimulation of the other two subtypes. In the CNS, these substances induce a lot of side effects that are well known and widely described (hypnosis, drowsiness, mood changes, mental clouding). Many of the effects of opioids derive from their ability to modulate the release of different neurotransmitter such as NA, DA, ACh and 5-HT. Opioids peptides are present in enteric neurons and in the cells of the immune system [198]. For long time the possible immunosuppressive effects of opiod treatment were a matter of active discussion [199]. Interestingly, leukocyte-containing opioids are found close to nerve terminals in periphery, and during inflammation increased opioid receptor expression may occur [200]. In response to opioid stimulation, CD4+ T cells and other immune cells increase their ability to migrate and reach inflamed tissues [201]. Opioids modulate the response of different immune cells [197], but the actual contribution of such modulation in immune-mediated diseases awaits clarification. Opioid peptides are involved in the dysregulation of enteric transmission and in the regulation of the gut-brain axis contribut-

ing to inflammatory processes such as inflammatory bowel disease [198]. A direct effect of opioid peptides was also described specifically in the immune system. Apart from epidemiological studies showing negative effects on disease progression in HIV-infected patients that are also opioid abusers [197], evidences in other diseases are limited. Large studies conducted in cancer patients, where opioid represent the first line of treatment for cancer-associated pain, suggest that the different types of opioids (morphine, phentanyl, tramadol, etc.) can exert opposite effects on immune response. For example, morphine may reduce and tramadol may increase NK cytotoxicity [202].

3 Therapeutic Perspectives

The ability of the different neurotransmitters to affect immunity potentially provide new therapeutic approaches in several immune-mediated diseases. For instance, catecholaminergic modulation of the immune response plays a role in autoimmune diseases, like multiple sclerosis [30, 39], rheumatoid arthritis [40, 203] or cancer [32, 204]. Similarly, a role in the modulation of different immune-mediated diseases was proposed for ACh [152], glutamate [10], serotonin [183] and for many other neurotransmitters [188, 195, 202]. Hereafter, the main findings showing a possible contribute of these neurotransmitter in multiple sclerosis, rheumatoid arthritis and cancer will be briefly summarized.

3.1 Multiple Sclerosis

Multiple sclerosis (MS) is the most frequent autoimmune demyelinating disease of the CNS and affect about 2.4 million individuals worldwide; MS is characterized by a progressive loss of neurological functions due to the destruction of the axonal myelin in different areas of the brain and spinal cord [205]. Disease progression is affected by dysregulated immune-component, in particular various T-cell subsets [205]. The role of the ANS, in particular the sympathetic branch, has been shown not only in the pathogenesis of MS but also in comorbodities such as depression, fatigue, and osteoporosis [39, 206, 207]. The contribution of DA is also well characterized in MS. A dysregulation of DR on immune cells has also been proposed as a peripehral marker of the disease [208]. Cholinergic pathways may also play a role in the development of the inflammatory pattern of MS. In MS patients, decreased plasma levels of ACh and increased levels of butyrylcholinesterase (BuChE, the tissue enzyme devoted to ACh catabolism) has been described [161]. Evidences from animal models of experimental autoimmune encephalomielitis suggest a possible contribution of nAChR activation [209]. Evidences about a role of GABA transmission in MS are more recent but promising for new possible therapeutic approaches [210].

3.2 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a common and frequent autoimmune disease with increased frequency in women and occuring tipically between 40 and 60 years of age [211]. It is characterized by irreversible joint destruction associated with pain and progressive disability [212]. The key role of innate immunity in the pathogenesis of RA is well established, with macrophages infiltrating the synovial tissues as primary actors. In *loco*, these cells produce proinflammatory cytokines, particularly TNF-α and IL-1 [213] and display a pro-inflammatory phenotype [213]. Together with increased presence of macrophages, in synovial fluid of patients DC, NK and neutrophils can be found. All these cells actively participate in the disruption of joints [214]. Similarly, the contribution of SNS in the onset and progression of the disease is well established and a double opposite role of NA (released both from sympathetic fibers and by the immune cells themselves) in disease progression is described [215]. DA and dopaminergic pathways are co-actors of the inflammatory events leading to disease progression, as shown by dysregulation of DR on synovial fibroblasts of patients with RA

[69], and by DR agents which may reduce the disease at least in animal models [216]. Other neurotransmitters or neuromodulators, such as substance P, glutamate, opioid peptides and ACh, may be involved in RA [217]. GABA administration in animals reduces the inflammatory responses and ameliorates disease-related symptoms [218]. Similarly, cholinergic antinflammatory pathways may have a role, as the activation of nAChR seems to improve clinical signs and symptoms in arthritis and to reduce the cytokines-induced joint destruction [219].

3.3 Cancer

DA improves the efficacy of cyclophosphamide in animal models of cancer, reducing its hematotoxicity (suggesting the possibility to use DA or DR agonists as addon-drugs) [220], while the lack of sympathetic innervation and reduced levels of endogenous DA favour cancer progression [221]. In line with such evidence, the ablation of peripheral dopaminergic nerves stimulates malignant tumor growth [222]. The administration of exogenous DA acting on pericytes and endothelial cells induces a normalization of vessel morphology in cancer [221]. Similarly, a contribution of SNS dysregulation has been proposed in different tumour types, which overexpress β-AR, and propranolol or other β-AR blockers ae increasingly considered as addon anticancer drugs [223, 224]. In the last decade, also glutamate was proposed as a mediator involved in the stimulation of non-neuronal tumor cell proliferation, such as osteosarcama or lung, breast, thyroid and gastric cancer cell, possibly through NMDA iGLUR [225]. Non only the sympathetic branch of the peripheric nervous system was shown to play a role in cancer progression, but also the parasympathetic component. A dysregulation of cholinergic pathways is suggested by the occurrence of higher levels of ACh in T leukemic cell lines in comparison to healthy T cells [14]. The contribute of endogenous opioids in cancer is still controversial, primarily dur to conflicting evidences related to the use of exogenous opioids in the management of cancer-related pain. Different types of opioids (morphine, phentanyl, tramadol, etc.) may exert opposite effects on immune function, with a reduction of immune responses after morphine and, by contrast, increased activity with tramadol [202]. Methionine enkephalin may also promote tumoricidal activity [226].

4 Conclusions

The close relationship and functional integration between the immune system and the nervous system and the relevance of the neuroimmune network in health and disease are increasingly providing novel and unanticipated opportunities for the modulation of the immune response by means of conventional neural targets. Knowledge about how the nervous system affects immunity and inflammation fostered the development of highly novel and inter/transdisciplinary fields of basic and clinical research, such as neuroimmunology and neuroimmune pharmacology. Results from these fields are increasingly supporting the opportunity to repurpose e.g. classical adrenergic or dopaminergic drugs, used for example in clinical neurology, neuropsychiatry or cardiology, as novel immunomodulating agents. Beta-blockers in cancers, beta-AR agonists in peripheral and central inflammatory diseases, dopaminergic agents in multiple sclerosis or rheumatoid arthritis are just a few examples. Similarly drugs acting on glutamatergic or cholinergic pathways can be proposed in a new perspective in diseases that are not classically considered as affected by these transmitters. Hopefully, in the near future more drugs classically acting on the peripheral and central nervous system will be translated to the clinics as novel agents acting on the immune response.

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Part II

Resolution of Inflammation

Pro-resolving Mediators

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Abstract

Acute inflammation is a self-limiting process of the immune system, which resolves through the initiation of a program referred to as the resolution of inflammation. It has been argued that uncontrolled inflammation may be the basis of a variety of chronic inflammatory and autoimmune diseases. The resolution of inflammation is an active process coordinated by the production of proresolving mediators. The release of proresolving mediators prevents further migration of granulocytes, and increases leukocyte apoptosis. Moreover, some proresolving molecules are able to promote the infiltration of nonphlogistic macrophages, which are fundamental cells to efferocyte of apoptotic granulocytes. This event, in turn, triggers macrophage reprogramming towards more restorative and resolutive roles, thereby promoting resolution and reestablishment of tissue homeostasis. Here, we summarize the most prominent pro-resolving mediators relevant to the resolution of inflammation.

Keywords

Inflammation · Pro-resolving mediators · Resolution

1 Resolution of Inflammation

In the previous chapter, we discussed the molecular mechanisms of inflammation and the importance of molecules and cells during the initiation and maintenance of inflammation. In this chapter, we will focus on those mediators that are relevant to the final phase of inflammation, denoting the resolution of inflammation.

Acute inflammation consists of an immediate protective response that occurs following tissue damage or infection. The ability to trigger an inflammatory response is critical to the survival of the host. However, a lack of control during this acute response can lead to exacerbation of tissue injury, organ failure, and progression of several diseases mediated by chronic inflammation or autoimmunity, including metabolic syndrome, cardiovascular diseases, neurodegenerative

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disorders, asthma, arthritis, and periodontal disease [1–4]. In this way, after elimination of the stimulus that induced inflammation, the process known as *resolution of inflammation* must dampen this first inflammatory phase and promotes some key activities to restore the homeostasis of the inflamed tissue or organ. For a long time, the resolution of inflammation has been considered a passive process, conducted by the simple removal/buffering of pro-inflammatory mediators. However, the current understanding regards this endogenous phenomenon as an ***active process*** [5], guided by the action of pro-resolving mediators to equilibrate and provide a physiological response [6]. To better understand which pro-resolving mediators are involved and how they act during the resolution of inflammation, it is important to emphasize the difference between anti-inflammatory and pro-resolving mediators. Anti-inflammatory mediators contribute to blocking leucocyte recruitment, as well as reducing endothelial activation and vascular permeability (Fig. 1) [2, 5, 7]. On the other hand, a pro-resolving action involves altering the progression of an established inflammatory process in a clinically relevant manner by releasing endogenous mediators, affecting either signalling cascades or cellular interactions, and acting as inflammatory switches to promote resolution [8].

According to the consensus among renowned researchers studying the resolution of inflammation, certain criteria have been established to define a mediator as a pro-resolving factor. In brief, pro-resolving mediators can induce and activate important events during resolution, including: (1) the cessation of effector leukocyte infiltration, (2) regulation of chemokine and cytokine levels, (3) switch-off of signalling pathways associated with leukocyte survival and the induction of apoptosis, (4) induction of apoptotic neutrophil effero-

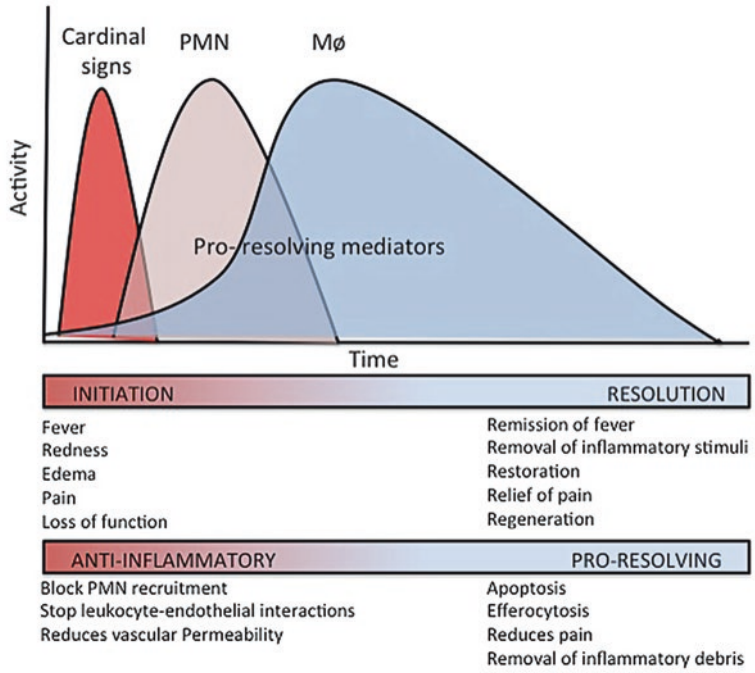

Fig. 1 The inflammatory process. At the onset of acute inflammation, cardinal signs occur (fever, redness, oedema, pain, and loss of function). Polymorphonuclear cells (PMNs) are among the first responders during an acute inflammatory response, followed by macrophages. The first cellular hallmark of tissue resolution is an anti-inflammatory response that blocks additional PMN recruitment. The resolution phase is an active process involving the removal of inflammatory stimuli and improvement of cardinal signs, and the return to homeostasis. This occurs due to pro-resolving actions such as apoptosis, efferocytosis, and the removal of inflammatory debris. Inadequate or insufficient resolution results in chronic inflammation, excessive tissue damage, and dysregulation of tissue healing, which can lead to fibrosis and loss of function

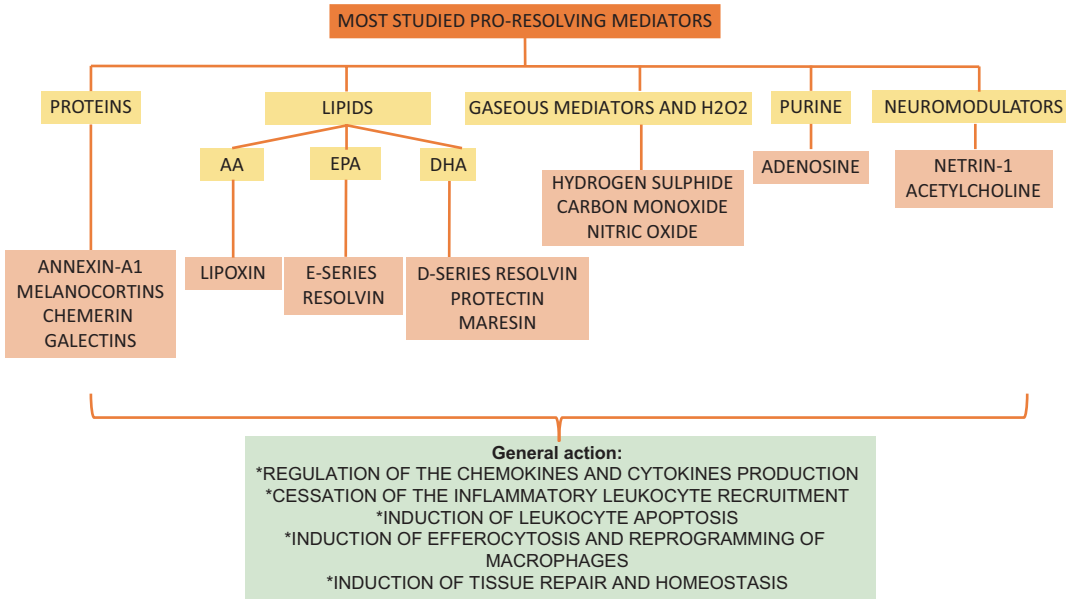

Fig. 2 The pro-resolving mediators: The pro-resolving molecules activate intracellular pathways that terminate inflammation and promote the restoration of homeostasis in damaged tissues by regulation of the chemokines and cytokines production, cessation of the inflammatory leukocyte recruitment, induction of leukocyte apoptosis, induction of efferocytosis and reprogramming of macrophages. Among the diversity of molecules that comprise this endogenous system, this figure will introduce bioactive lipids, proteins and peptides, gaseous mediators, purines, neuromodulators, and reactive oxygen species (ROS)

cytosis by macrophages, (5) reprogramming of macrophages from classically activated to alternatively activated, and (6) stimulating the production of anti-inflammatory mediators for tissue repair and regeneration [2, 9–14] (Fig. 2).

This ***active process*** guided by pro-resolving mediators has two important implications, according to the perspective of Perretti and colleagues in the review "*Immune resolution mechanisms in inflammatory arthritis*" published in 2017 [15]. First, chronic inflammation could result from inadequate resolution rather than an exacerbated response during the pro-inflammatory phase. Second, the emerging pathways and molecules that govern the resolution processes create interesting therapeutic possibilities to manage chronic inflammatory diseases. The latter implication subverts the current management of inflammatory diseases that mainly focus on the pharmacological inhibition of key pro-inflammatory mediators [16], and provides new insight to pharmacological-based modulation of resolution activity through endogenous resolving mediators [10].

As such, emerging classes of pro-resolving mediators have been shown to be associated with the initiation and orchestration of the resolution of inflammation. These molecules activate intracellular pathways that terminate inflammation and subsequently promote the restoration of homeostasis in damaged tissues [17]. Among the diversity of molecules that comprise this endogenous system, this chapter will introduce bioactive lipids, proteins and peptides, gaseous mediators, purines, neuromodulators, and reactive oxygen species (ROS) [2, 10, 18] (Fig. 2). Next, the roles of these major pro-resolving molecules in the context of resolution of inflammation will be discussed.

2 Pro-resolving Mediators

2.1 Bioactive Lipids

During adverse conditions, such as tissue injury or pathogen invasion, essential polyunsaturated fatty acids (PUFAs) are released from the phos-

pholipids of cell membranes and used to synthesize inflammatory mediators that trigger vascular and cellular events to the inciting stimulus [19, 20]. However, during inflammation the synthesis of PUFA-derived pro-inflammatory mediators switches to the synthesis of PUFA-derived pro-resolving mediators, namely specialized resolving mediators (SPMs), resulting in the activation of the resolution program [3, 22, 23, 273, 405]. These SPMs are formed by biosynthetic pathways that convert the essential PUFA arachidonic acid (AA) to lipoxins (LXs) [24, 25], omega-3-derived eicosapentaenoic acid (EPA) to E-series resolvins, as well as docosahexaenoic acid (DHA) to D-series resolvins, protectins [52, 405] and maresins [21].

Next, we will discuss the synthesis, mainly cellular sources and receptors of pro-resolving mediators, as well as some examples of how these molecules push on resolution process.

2.1.1 Lipid Mediators Derived from Arachidonic Acid (AA): Lipoxins

At the onset of inflammation, the enzymatic conversion of AA is initiated to synthesize several lipid mediators, such as leukotrienes, prostaglandins, and thromboxanes [2]. These eicosanoids elicit pro-inflammatory activities, resulting from an increase in vascular permeability and leucocyte recruitment [2].

Lipoxygenase-***in***teracting products, or lipoxins, are unique compared to pro-inflammatory eicosanoids, and are the first bioactive lipids proven to have anti-inflammatory and pro-resolving activities [404]. Their production by lipoxygenase (LO)-dependent biosynthetic pathways occurs in different cellular types and requires a sequence of steps (for more details, see Serhan [404]). In macrophages, dendritic cells (DCs), epithelial cells, eosinophils and monocytes; [28–30, 404], AA is converted (by the oxygenation of AA in the C15 position) to 15-HpETE through 15-lipoxygenase (15-LO) action. In leukocytes, 5-LO transforms this compound to DIH(p)ETE, and subsequently to 5(6)-epoxytetraene [24, 30, 31, 404]. Lastly, specific hydrolases catalyse the step to form lipoxin A_4 (LXA_4) or its positional isomer lipoxin B_4 (LXB_4) [31, 404].

The interaction between neutrophils and platelets provide another mechanism of lipoxin synthesis. In summary, leukotriene A_4 (LTA_4) derived from 5-LO-AA-dependent conversion in neutrophils serves as the substrate to the 12-LO to produce LXA_4 and B_4 in platelets [32–34, 402, 404]. Cyclooxygenase 2 (COX-2) acetylation by aspirin, which leads to the inhibition of PG and TX synthesis, also promotes the conversion of AA to 15R-HETE in endothelial cells. Likewise, this intermediate can also be synthesized by cytochrome P450 enzymes [36, 37]. Subsequently, 5-LO acts on 15R-HETE to form 15-epi-lipoxins (A_4 or B_4; aspirin-triggered LX (ATL) [30, 31, 35, 37, 404].

The reported pro-resolving effect of LXs and ATLs (as well as annexin A1 (AnxA1) is mediated through the activation of the formyl peptide receptor 2 (FPR2; also known as the lipoxin A_4 receptor (ALX) [38]. The FPR2/ALX receptor, a member of the formyl peptide receptor family (FPR), is a promiscuous G protein-coupled receptor that is activated by lipids and peptides, triggering distinct ligand-dependent signalling pathways [31, 39, 40]. FPRL2/ALX detection has been reported in several cell types, such as neutrophils, monocytes, macrophages, eosinophils, T cells, epithelial cells, and fibroblasts [38, 41–47].

The FPR2/ALX as an emergent master receptor of inflammation resolution [403]. This concept could raise new promising perspectives in the development of FPR2/ALX- mediated resolution agonists for pharmacological modulation of several inflammatory conditions [48, 403].

For example, in mice LX-activated FPR2/ALX were found to regulate inflammatory mediators and stimulate IL-10 production [48]. A summary of important pro-resolving responses in generated by LX-activated FPR2/ALX during experimental models as well in human cells are provided in Table 1.

Table 1 Main pro-resolving actions of Lipoxins related to resolution of inflammation

Bioactive lipids	Action	Disease or experimental model	References
Lipoxins (Lxs)	Reduce inflammation-induced pain	Carrageenan-induced hyperalgesia	[233]
	Decrease the number of neutrophils or their infiltration	Monocyte/neutrophil culture; Dermal inflammation; Human skin blister; Peritonitis induced by Zymosan; Skin ulcers	[234] [384] [235] [236] [237]
	Decrease eosinophil infiltration	Human eosinophils culture	[238]
	Stimulate the expression of anti-inflammatory and pro-resolution genes	Human neutrophils culture	[239]
	Decrease inflammatory mediators	Synovial fibroblasts culture; Skin ulcers; Murine air pouches; Ischemia and reperfusion; Leukocytes culture	[240] [385] [47] [48] [237]
	Decrease leucocytes, degranulation from mast cells and eosinophils, and promote a protective effect in the different strati of the airway	Mice allergic inflammation	[241]
	Stimulate IL-10 production	Ischemia and reperfusion	[48]
	Increase noninflammatory monocyte infiltration	Human monocytes culture	[386]
	Stimulate efferocytosis	Human monocytes and neutrophils culture	[242] [243] [244]
	Demonstrate a protective role	Human bronchial epithelial cell culture; Suplementation of transgenic rabbits	[245] [387]
Lipoxin A4 analogue (BML-111)	The lipoxin A4 analogue decreases pro-inflammatory cytokines, increases microglial/macrophage cell populations and protects against neuroinflammation	Neuroinflammation after ischemic stroke	[246]

2.1.2 Lipid Mediators Derived from Omega-3 Fatty Acids

Lipid Mediators Derived from Eicosapentaenoic Acid (EPA): E-Series Resolvins

E-series **r**esol**v**ins (resolution phase interaction products, Rv) are bioactive lipids derived from **E**PA [405]. Both EPA and DHA are PUFAs obtained from the diet (eg. from fish oils), as the human body cannot produce them naturally [22, 49, 50]. EPA-derived SPMs were the first mediators identified in inflammatory exudate, and their resolving action includes the decrease of leukocyte recruitment and decrease in the magnitude of inflammatory responses (refer to [51, 405]). The E-series resolvin family consists of RvE1, RvE2 and RvE3 [50]. The synthesis of these mediators involves EPA conversion by acetylated COX-2 to 18(R) hydroxyEPA (18(R)HEPE) in endothelial cells from the vasculature, and subsequent conversion to RvE3 due to the action of 15-LO in leukocytes [30, 52, 53, 55, 56, 58] or to RvE1 and RvE2 by 5-LO in leukocytes [26, 30, 50, 51, 53, 57, 58]. Cytochrome P450 also converts EPA to 18(R)HEPE [50, 53, 407]. Several authors have demonstrated that the expression of biosynthetic enzymes responsible for RvE generation lead to low RvE levels in normal tissue, and increased levels during acute inflammation [50, 59–62]. These described pathways have been observed in neutrophils to produce RvE1 and RvE2 [63], and in eosinophils to produce RvE3 [55, 56].

The pro-resolving and anti-inflammatory effects of RvE1 and RvE2 are mediated by calcitonin gene-related peptide (CGRP) chemerin receptor 23 (Chem23), also called chemokine-like

receptor 1 (CMKLR1) and leukotriene B_4 (LTB_4) receptor (BLT1) [3, 30, 54, 64, 65]. However, RvE1 is a partial agonist to BLT1, and RvE2, a partial agonist to Chem23 [3]. Isobe et al. [406] suggest that there might be a high-affinity receptor to RvE3 in neutrophils because, although RvE3 presents an inhibitory effect in neutrophil chemotaxis, its interaction with LBT1 does not explain this effect [56]. Thus, it is necessary to identify the receptor mediating the effects of RvE3.

Both receptors, Chem23 and BLT1, have been detected in several tissues and cell types. Chem23 is found in the testis, prostate, heart, aorta, liver, kidney, liver, lung, brain, and gastrointestinal organs [51]. Moreover, this receptor has been detected in monocytes, macrophages, microglia, neurons of the dorsal root ganglia (DRG), DCs, $CD4^+$ lymphocytes, natural killer cells, platelets, intestinal epithelial cells, as well as endothelial and vascular smooth muscle cells [50, 64, 66–69, 406]. The BLT1 receptor is expressed in eosinophils, neutrophils, monocytes, and T cells [50, 54, 70].

Important pro-resolving actions that occur following signal transduction induced by RvE receptor activation during experimental models as well in human cells, for example, the neutrophil resolvin E1 promotes phagocytosis or efferocytosis in type 2 diabetes in mice, and elevates anti-inflammatory mediators [71]. Others pro-resolving actions are described in Table 2.

Lipid Mediators Derived from Docosahexaenoic Acid (DHA): D-Series Resolvins, Protectins and Maresins

DHA is a precursor in the synthesis of D-series resolvins, protectins, and maresins [72, 405]. The anti-inflammatory, pro-resolving, and protective roles of these emerging mediators has increased interest for the development of new pharmacological strategies to treat many inflammatory

Table 2 Main pro-resolving actions of Resolvin E related to resolution of inflammation

Bioactive lipids	Action	Disease or experimental model	References
4Resolvin E (RvE)	Correlates with reduced pain and neuronal sensitivity	Arthritis; Dorsal root ganglia neuron culture	[247] [248]
	Reduces neutrophil infiltration or its accumulation	Human neutrophil culture; Murine peritonitis; Murine dorsal air pouch; Corneal HSV (Herpes simplex virus) infection	[249] [53] [251] [63] [392]
	Diminishes the expression of pro-inflammatory mediators or their gene expression	Human neutrophils culture; Murine peritonitis; Murine dorsal air pouch; Corneal HSV infection; Allergen-initiated respiratory inflammation; Microglial cells culture; Suture-induced inflammatory corneal angiogenesis; Periodontitis	[63] [251] [393] [252] [394] [428]
	Elevates anti-inflammatory mediators	Corneal HSV infection	[251]
	Promotes phagocytosis or efferocytosis	Human neutrophils culture; Murine peritonitis; Murine dorsal pouch; Murine acute lung injury; Dorsal air pouch	[7] [396] [63] [253] [71]
	Plays a protective role	Ligature-induced periodontitis; P. gingivalis- induced periodontitis; Bronchial asthma; Experimental diet-induced atherosclerosis	[254, 255] [256] [257] [258]
	Prevents platelet aggregation	Human fresh venous blood	[259]

conditions [403]. Below, a brief review regarding the main features of these important DHA-derived SPMs will be explored.

D-Series Resolvins (RvDs)

Sequential lipoxygenase-dependent reactions are required to synthesize DHA- derived resolvins (D-series). This bioactive lipid group is composed of RvD1, RvD2, RvD3, RvD4, RvD5 and RvD6. For their endogenous synthesis, the first reaction is catalysed by 15-LO to form 17-(S) HpDHA from DHA. This product undergoes subsequent reactions catalysed by either 15-LO or 5-LO. First, the 17-(S) HpDHA is oxygenated in the C7 position, producing RvD5 or a 7S-8S-epoxide precursor. The 7S-8S-epoxide is then converted to RvD1 and RvD2 by enzymatic hydrolysis. Second, 17-(S) HpDHA is oxygenated in the C4 position, producing RvD6 or a 4,5-epoxi precursor. Moreover, in the presence of acetylated COX-2, the DHA conversion generates 17-(R) HpDHA, followed by the formation of RvD positional isomers (AT-RvD1-6) via 5-LO (for more details, refer to [30, 51, 73]). The biosynthesis of some RvDs has been shown to occur in inflammatory exudates and in a murine model of ischemic stroke [52, 75, 76], as well as in human glial cells, neutrophils, and whole blood samples [75, 97]. More recently, RvD1, RvD3, and RvD5 were found to be present in the synovial fluid from arthritic patients [77, 78], similar to other SPMs (for more details, refer to [15]). In addition, Chatterjee et al. [79] demonstrated that in human intact arteries incubated with DHA, saphenous vein endothelial cells and vascular smooth muscle cells can also produce RvDs.

Evidence from that literature has supported that the RvD1 receptor (DRV1/GPR32), DRV2/GPR18 and FPR2/ALX CGRP receptors can be activated by RvDs. DRV1/GPR32 has been detected in monocytes and neutrophils [80, 81] and is activated by RvD1 [3, 15, 51, 57, 80, 81, 82], RvD3, and RvD5 [3, 50, 83, 84]. DRV2/GPR18 expressed in neutrophils, monocytes, and macrophages [4, 85], is activated by RvD2 [15, 51, 81]. The interaction between DRV2/GPR18 and RvD2 has been confirmed in several transgenic mouse studies [3, 85–87]. Activation of the FPR2/ALX-mediated pro-resolving effect is initiated by RvD1 [3, 15, 50, 80]. The specific receptor involved in RvD4 and RvD6 signal transduction that triggers the pro-resolving effect remains to be discovered [50]. In Table 3, important pro-resolving effects of RvDs during experimental models as well in human cells have been described.

Protectins (PDs)

As suggested by the name, protectins (docosatrienes derived from DHA) are SPMs that present with not only pro-resolving features but also protective properties [76, 88, 89, 405]. Frequently identified in the neuronal system, this DHA-derived lipid exerts beneficial protection at this location [76, 88–90, 405]. Moreover, as expected, PDs play an important role in controlling the duration and magnitude of inflammatory responses [51, 91].

The first enzymatic reaction of protectin synthesis is the 15-LO-dependent conversion of DHA into 17-(S) HpDHA. Next, this intermediate undergoes enzymatic epoxidation, forming 16,(17)-epoxy-docosatriene, which is then hydrolysed to produce PDs [51, 73, 88]. From this biosynthetic pathway, two bioactive lipids, PD1 and PD2, can be formed [92].

Originally identified in murine brain cells and human microglial cells [52], PD1 (10,17(S) docosatriene) is termed *neuro*protectin when detected in neuronal tissue [73, 88, 405]. However, PD1 is reported to be synthesized in murine exudate, brain and, human airways, microglial cells and retinal pigment epithelium, as well as in mononocyte and $CD4^+$ T cells, neutrophils, and eosinophils [52, 55, 74, 76, 88, 89, 93, 94, 95]. Furthermore, omega-3 fatty acid supplementation has been shown to increase protectin levels in rat placenta [96].

PD1 is related to the promotion of several pro-resolving responses by affecting various cell types [51, 73]. For example, PD1 stimulates the phagocytosis of apoptotic cells by macrophages [7], and reduces cytokine production by glial cells [97], in addition to helping protect retinal pigment epithelium [89] and triggering a response

Table 3 Main pro-resolving actions of Resolvin D related to resolution of inflammation

Bioactive lipids	Action	Disease or experimental model	References
Resolvin D (RvD)	Presents antinociceptive action or decrease in allodynia	Complete Freund's adjuvant (CFA) induced hind paw inflammation; CFA-induced inflammatory pain and arthritis; Chronic pancreatitis-induced chronic pain	[260] [261] [262] [263]
	Limits or blocks neutrophil migration	Peritonitis; Murine zymosan-initiated peritonitis; Neutrophils and monocytes culture; Lung infection	[395] [85] [429]
	Decreases pro-inflammatory mediators or their gene expression	Mpp+- Induced Parkinson Disease in vitro; Dextran sulfate sodium (DSS)-induced colitis; Chronic pancreatitis-induced chronic pain; Human monocytes and macrophages culture; Streptozotocin (STZ)- induced diabetic retinopathy; Microglial cells culture; Abdominal aortic aneurysm	[264] [261] [265] [266] [267] [252] [397]
	Increases the anti-inflammatory mediators	Human monocytes and macrophages culture	[265]
	Increases M2 macrophages	Human monocytes and macrophages culture; Abdominal aortic aneurysm	[265] [397]
	Stimulates macrophage phagocytosis or efferocytosis	Microbial- initiated peritonitis; Murine dorsal skin pouches; Neutrophils and monocytes culture; Peritonitis; Ischemia reperfusion injury; Cecal ligation and puncture;	[84] [80] [83, 395] [4] [85]
	Presents a protective role	Murine Zymosan- initiated peritonitis; Neutrophils and monocytes culture; Cecal ligation and puncture; STZ-induced diabetic retinopathy	[85] [4] [267]

in eosinophils [95] and T cells [98]. Moreover, Marcheselli et al. [74] have demonstrated that PD1 binds with a high affinity at the neutrophil surface. However, a specific PD1 receptor is not well known [99], and a detailed characterization needs to be elucidated [81]. The protective, anti-inflammatory, and pro-resolving actions of protectin during experimental models as well in human cells have been shown in Table 4.

Maresins (MaRs)

Maresins (**ma**crophage mediators in **re**solving **in**flammation; MaRs) are macrophage-derived resolution mediators with pro-resolving, anti-inflammatory, and regenerative roles [81, 83, 100, 101, 409]. The main cellular source of these particular DHA-derived SPMs are macrophages [3, 15, 57, 99, 101, 409]. Moreover, Abdulnour et al. have demonstrated that MaR1, a MaR family member, can originate from platelet/neutrophil interactions [102].

The biosynthetic pathway to MaR generation incorporates 12-LOX-dependent lipoxygenation of DHA, yielding the 14-(S) HpDHA precursor [57, 26]. This product undergoes subsequent oxygenation at the omega-1 position to produce MaR3, or is converted to 13S,14S-epoxy-maresin following soluble epoxy hydrolase action to generate MaR1 or MaR2 [83, 101, 410]. MaR1 synthesis can also occur via platelet/neutrophil interactions in the vasculature [102]. This biosynthetic pathway begins in the platelets, with 12-LOX-dependent production of 13S,14S-epoxy-maresin from DHA, and finishes with MaR1 production in neutrophils [102]. Hong et al. also identified the production of maresin-like (L)1 and maresin-L2 via platelets and/or leukocytes [103]. Of note, the 13S,14S-epoxy-maresin intermediate also contributes to potent pro-resolving mechanisms—for example, blocking the hydrolase that synthesizes LTB_4 [410].

Table 4 Main pro-resolving actions of protectins related to resolution of inflammation

Bioactive lipid	Action	Disease or experimental model	References
Protectins (PDs)	Improve pain	Acute and persistent inflammatory pain induced by CFA or formalin	[268]
	Inhibit neutrophil infiltrate	Middle cerebral artery occlusion and reperfusion; Human neural progenitor cells in primary culture; Murine peritonitis; Kidney ischemia/ reperfusion model	[76] [269] [270] [408]
	Prevent eosinophil recruitment	Allergen-initiated respiratory inflammation	[95]
	Decrease pro-inflammatory mediators or their gene expression	Middle cerebral artery occlusion and reperfusion; Human neural progenitor cells in primary culture; Murine peritonitis; Allergen-initiated respiratory inflammation; Concanavalin A- induced hepatitis; Corneal HSV infection; Human T cells culture; Murine peritonitis	[52] [98] [269] [95] [76] [250] [271]
	Increase anti-inflammatory IL-10	Corneal HSV infection; diabetes mouse model	[430] [103]
	Increase macrophage efferocytosis	Leukocytes cell culture	[408]
	Stimulate tissue protection, specifically neuroprotection	Middle cerebral artery occlusion and reperfusion; Human neural progenitor cells in primary culture; Ischemia-reperfusion model; Alzheimer disease; Human ARPE-19 cell culture; Kidney ischemia/reperfusion model; Allergen-initiated respiratory inflammation; Transient middle cerebral artery model (experimental stroke); Human retinal pigment epithelial	[76] [89] [90] [270] [95, 398], [272], [399, 400]

All three MaR family members can stimulate anti-inflammatory and pro-resolving responses at low-range levels [104], including several effects that benefit tissue regeneration, healing, and pain [22, 57, 100]. Nevertheless, there is no mention concerning which specific receptor is activated by MaRs [99]. The pro-resolving effects of MaRs during experimental models as well in human cells can be seen in Table 5.

2.2 Proteins and Peptides

2.2.1 Annexin A1

In the 1980s, many researchers studied the mechanisms of inflammatory response reduction mediated by glucocorticoids (GCs). They found that the inhibition of AA release following GC treatment was dependent on the production of an inhibitory protein that was first named lipocortin-1 [105]. This protein, today known as annexin A1 (AnxA1), is a 37-kDa calcium-dependent phospholipid-binding protein that belongs to the annexin protein superfamily [1, 105].

AnxA1 is expressed in various cell types, including epithelial cells (gut, lung, and skin) [106, 107], endothelial cells [108], synoviocytes [109], fibroblasts [27, 110, 111], and skeletal muscle [112], as well as neutrophils, monocytes, mast cells, and macrophages [107, 113, 114]. AnxA1 is also present in biological fluids such as seminal fluid and plasma [115]. In resting cells that can produce AnxA1, there are elevated cytoplasmic levels of AnxA1 that can be quickly externalized and/or secreted following activation. Cellular activation causes the relocation of AnxA1 to the outside of the plasma membrane in a calcium-dependent manner. The increase in extracellular Ca^{2+} can also change the conformation of AnxA1, resulting in its activation [114].

Table 5 Main pro-resolving actions of maresins related to resolution of inflammation

Bioactive lipids	Action	Disease or experimental model	References
Maresins (MaRs)	Diminish inflammatory pain	Capsaicin- induced spontaneous pain	[100]
	Limit neutrophil chemotaxis, adhesion or infiltration	Isquemia-reperfusion; Peritonitis	[100] [101] [411]
	Stimulate efferocytosis or phagocytosis. Enhance Kupffer cell phagocytic capacity*.	Zymosan-induced peritonitis; *Escherichia coli*- induced peritonitis; Ischemia- reperfusion; Obesity-induced nonalcoholic fatty liver disease	[401] [100] [411] [101] [274, 275]
	Increase regulatory T cells and decrease the production of IL-5 and IL-13	Allergic inflammation	[276]
	Promote regeneration or healing	Planaria fluorescent lectin-conjugated stainin; Ischemia-reperfusion	[100] [411]
	Induce macrophage polarization towards an M2 phenotype	Genetic (*ob/ob*) obese mice	[277]
	Revert pro-inflammatory cells and pro-fibrotic effects induced by high glucose in mouse glomerular mesangial cells	Mouse glomerular mesangial cell culture	[278]
	Enhance platelet aggregation and spreading, and suppress the release of pro-inflammatory and prothrombotic mediators. These are important events for the resolution of inflammation in cardiovascular diseases	Human platelets spreading	[279]
	Prevent atheroprogression, suggesting that MaR1 represents an innovative strategy to resolve arterial inflammation	High-fat diet	[280]

After transport to the extracellular space, AnxA1 interacts with its receptor, FPR2/ALX (already been detailed in the section "Bioactive lipids"), and can exert autocrine, paracrine, or juxtacrine effects [92, 114, 116].

The AnxA1 pathway is modulated by GCs that have overlapping activities in the regulation of inflammatory responses. GCs induce ANXA1 gene expression and stimulate the release of protein to the extracellular medium [117]. An increase in FPR2/ALX expression occurs 12–24 h after human monocyte incubation with dexamethasone or other synthetic glucocorticoids [118].

In this context, AnxA1 has emerged as a potent pro-resolving mediator, regulating the inflammatory response through a variety of methods, such as the modulation of pro-inflammatory mediator production, as well as stimulating the release of immunosuppressive and pro-resolving molecules [2, 412]. In Table 6, the pro-resolving effects of annexin-A1 during experimental models as well in human cells are described in detail.

2.2.2 Melanocortins

Melanocortins, which include adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), β-MSH, and γ-MSH, are peptides derived from the cleavage of pro-opiomelanocortin (POMC) [119–121]. POMC is mainly found in the central nervous system, but is also present in smaller amounts at other sites, including the skin, spleen, lungs, gastrointestinal tract, genitourinary tract, adrenal gland, thyroid gland, and immune cells (such as lymphocytes and monocytes) [119, 120, 122].

There are five transmembrane G protein-coupled receptors related to the activation of the melanocortin pathway (abbreviated MC1R to MC5R). All melanocortin peptides can bind to MC1R, MC3R, MC4R and MC5R, but with varying affinities. On the other hand, the MC2

Table 6 Main pro-resolving actions of Annexin-A1 related to resolution of inflammation

Protein	Action	Disease or experimental model	References
Annexin-A1	Inhibits neutrophil recruitment	Zymosan-induced neutrophil rolling, adhesion, and emigration model; Human neutrophils cell culture; Peripheral blood PMN cell culture; Murine air-pouch; Murine peritonitis; Murine ischemia reperfusion; Human umbilical vein endothelial cells culture; Skin edema induced by fMLF; Paw edema induced by carrageenan	[281] [282] [283] [284] [92] [285]
	Induces apoptosis of inflammatory cells	Rat pheochromocytoma (PC12) cells culture; Rat thymocytes culture; Rat pheochromocytoma cell culture; U937 cell culture; Mammary glands of adult rats; BZR cell culture; Human PMN cell culture; LPS-induced pleurisy model	[286] [287] [288] [289] [290] [117] [219, 388]
	Stimulates efferocytosis of apoptotic neutrophils by macrophages	Human monocyte-derived macrophages (Mphi) cell culture; Mice Bone Marrow derivated macrophage cell culture; LPS-induced pleurisy model	[291] [292] [388]
	Elevates cAMP, which in turn stimulates the pro-apoptotic program in neutrophils, leading to the resolution of inflammation	Human neutrophils cell culture; pleurisy model	[232]
	Induces macrophage reprogramming	Jurkat and THP-1 cells culture; Human Primary Neutrophils and Monocytes cell culture; HEK 293 cell culture; Nonalcoholic steatohepatitis in mice	[293] [116] [302] [413]
	Inhibits the inducible nitric oxide synthase (iNOS) enzyme	Nonalcoholic steatohepatitis in mice; Microglial secondary culture	[294] [295]
	Stimulates IL-10 release[1,2] and inhibits nitric oxide synthesis[1]	Macrophage (J774) cell line culture; Murine intestinal ischemia and reperfusion	[1][296] [2][48]
	Reduces cerebral microvascular dysfunction and tissue injury associated with middle cerebral artery occlusion and reperfusion by the reduction of pro-inflammatory cytokine levels, rolling and cell adhesion and attenuation of the infarct volume	Middle cerebral artery occlusion and reperfusion	[297]
	Contributes to the process of the healing of gastric mucosal damage	Gastric mucosal injury	[298]
	Regulates bleomycin-induced lung fibrosis and inflammation	Bleomycin-induced lung fibrosis model	[299]

Table 6 (continued)

Protein	Action	Disease or experimental model	References
Annexin-A1	Contributes to skeletal muscle tissue regeneration	Mouse myoblast cell culture and In vitro Wound-healing Assay	[391]
	Contributes to the resolution of cerebral inflammation in sepsis, reducing rolling and adhesion leucocytes in cerebral venules	Murine model of endotoxin-induced cerebral inflammation by intraperitoneal injection of LPS	[300]
	Binding to a novel intestinal epithelial FPR promotes mucosal wound repair by the activation of focal adhesion kinase and paxillin	Human intestinal epithelial cells culture, mechanical colonic mucosal wounds in mice by colonoscopy and culture of human intestinal cell of patients with ulcerative colitis	[301]
	Release of AnxA1 by hepatic macrophages modulates hepatic inflammation and fibrogenesis during nonalcoholic steatohepatitis progression.	1. Murine model of nonalcoholic steatohepatitis	[302]
	Reduces atherosclerotic plaque formation.	Western Type Diet in LDLR-/- mice and intraperitoneal injection of Human recombinant annexin A1 (hr-anxA1)	[303]
	Promotes timely resolution of inflammation in murine gout by reducing neutrophil influx, IL-1β and CXCL1 production in the periarticular joint, hypernociception and improving articular injury.	2. Murine model of acute gout	[304]
	Contributes to the resolution of inflammatory responses during *Leishmania braziliensis* infection.	*L. braziliensis*-infected BALB/c mice	[228]

receptor interacts only with ACTH [119, 120, 122, 123]. The melanocortin 1 receptor (MC1R) participates in skin and hair pigmentation, and contributes to immune cell regulation [124]. This receptor is considered the classical melanocyte α-MSH receptor and is expressed by fibroblasts, cutaneous melanocytes, keratinocytes, endothelial cells, and antigen-presenting cells. Monocytes, macrophages, neutrophils, mast cells, fibroblasts, DCs, astrocytes, and microglia also express MC1R, contributing to the immune system regulation realized by melanocortins [122, 414]. MC2R is expressed in the zona reticularis and zona fasiculata of the adrenal gland, and interacts only with ACTH in this organ, controlling the synthesis and release of GCs [122, 415]. This receptor was also found in adipose tissue, likely mediating stress-induced lipolysis in response to ACTH [125]. MC3R is expressed in the central nervous system (CNSIt) and in several peripheral tissues such as the placenta, gastrointestinal tract, and heart, as well as in human monocytes and murine peritoneal macrophages [119, 122, 126, 127]. This receptor also participates in immune system regulation [128]. MC4R is expressed mainly in the CNS. MC4R contributes to hunger control, pain, and sexual health [129, 416–418]. MC5R is expressed in many locations, such as the adrenal gland, adipocytes, and leucocytes (including B and T- lymphocytes), and can regulate lipid metabolism, exocrine function, and inflammatory activity [122, 123, 126].

The exact role of melanocortins in the resolution of inflammation remains unclear, but many achievements have demonstrated significant anti-inflammatory and immunomodulatory properties of melanocortins during experimental models as well in human cells as seen in Table 7.

2.2.3 Chemerin Peptides

Chemerin is a protein that was discovered and described in 2003 as a natural ligand for chemokine-like receptor 1 (CMKLR1 or chemR23), an orphan G protein-coupled recep-

Table 7 Main pro-resolving actions of Melanocortins related to resolution of inflammation

Protein	Action	Disease or experimental model	References
Melanocortins	Reduce pro-inflammatory cytokines, including IL-1, IL-8, IL-6, TNF-α, IFN-γ, and IL-17	Lymph Node T cell culture; Myocardial infarction; Rheumatic disorders; Endotoxemia; HIV infection; TNF-alpha activated human C-20/A4 chondrocytes culture; Urate crystal-induced monocyte activation and neutrophil responses in vitro; Peripheral blood mononuclear cells human culture; Psoriasis-like skin inflammation	[305] [306] [307] [308] [309] [310] [311]
	Increase the levels of anti-inflammatory cytokines such as IL-10 and TGF-β	Human monocytes culture; T cell culture; Macrophage-like RAW 264.7 cells culture; TNF-alpha activated human C-20/A4 chondrocytes culture	[389] [313] [314] [310]
	Inhibit the CD86[1,2] and CD40[2] co-stimulatory receptors	Human monocytes culture; Human peripheral blood-derived monocytes and dendritic cells culture	[1][312] [2][315]
	Inhibit NF-kappaB activation, which reduces pro-inflammatory mediators	Macrophage-like RAW 264.7 cells culture; U937 cell culture; Human melanocytes and melanoma cells	[316] [317] [314]
	Inhibit iNOS[1] and reduce NO production[1,2]	Macrophage-like RAW 2647 cell culture; Microglial cells culture; U937 cell culture	[1][318] [1][314] [2][308]
	Reduce the expression of ICAM-1,VCAM-1, and E-selectin	Human keratinocytes culture; Lipopolysaccharide-induced vasculitis	[319] [320]
	An analogue of α-MSH, AP214, inhibits cell infiltration and cytokine release and stimulates efferocytosis	Gouty inflammation	[321]
	Induce tolerogenic dendritic cells[2] and immunosuppressive Tregs[1,2]; suppress the activation and proliferation of effector T cells[1,2]	T cell culture; Psoriasis-like skin inflammation	[1][313] [2][311]
	Reduce chemoattractant activity for neutrophils and expression of CD11b in these cells; reduce reactive oxygen intermediate production	Urate crystal-induced monocyte activation and neutrophil responses in vitro	[309]

tor, already detailed in the section "Bioactive lipids" [65, 130, 131]. This protein was first named tazarotene-induced gene 2 protein (TIG2) or retinoid acid receptor responder 2 (RARRES2), since the anti-psoriatic synthetic retinoid tazarotene used to treat psoriatic skin injuries was shown to up regulate the chemerin gene [132, 133]. There are more than two receptors for chemerin binding—chemokine CC motif receptor-like 2 (CCRL2) and G protein-coupled receptor 1 (GPR1)—of which chemR23 is the main chemerin receptor, and is associated with chemerin chemotactic action [133]. Chemerin was initially isolated from inflamed biologic fluids, such as rheumatoid arthritis synovial fluid and ovarian cancer ascites [134, 135, 136]. This protein is also released by several tissues and organs, including the liver, spleen, lymph nodes, epithelia, pancreas, lung, skin, platelets, adipose tissue, and immune cells (as reviewed by [133]).

Chemerin is synthesized and secreted as a precursor named prochemerin [137]. Human prochemerin is synthesized by the removal of a 20-aa hydrophobic signal peptide from chemerin by an unknown protease, generating a prochemerin that contains 143 aa ($chem^{21–163}$) [137]. This prochemerin has little activity, but can be converted into a fully active form by proteolytic removal of the last 6 amino acids by different proteases, such as elastase and cathepsin G (neutrophil-derived proteases), tryptase (derived from mast cells), and proteases of the

coagulation cascade [134–137, 139]. Based on the cleavage in the C-terminal domain, different chemerin peptide isoforms can be produced, presenting either high activity when the peptide isoforms are chemerin-^{157} and chemerin-^{156}, or low activity/inactivity, when the peptide isoforms are chemerin-^{158}, chemerin-^{155}, chemerin-^{154} and chemerin-^{152} [137].

Traditionally, the production of bioactive chemerin is mediated mainly by the serine proteases cathepsin G (CG) and elastase (HLE), which are released following neutrophil activation and are responsible for producing chemerin-^{157} and chemerin-^{156}, respectively. These chemerins stimulate chemokine production and recruitment of macrophages and DCs [134, 135]. However, some studies have now demonstrated the participation of other proteases in producing chemerin peptides that regulate inflammation. Guillabert and colleagues [138] showed the ability of the neutrophil-derived serine protease proteinase 3 (PR3) to convert prochemerin into chemerin-^{155}. Moreover, these researchers found that chymase, an enzyme present in mast cells, can convert active chemerin-^{157} and chemerin-^{156} into chemerin-^{154}. Neither chemerin-^{155} produced by PR3 of neutrophils nor chemerin-^{154} produced by chymase of mast cells induced DC chemotaxis, and thereby regulated the action of chemerin during immune responses. In 2008, Cash and colleagues [139] also demonstrated that classically activated macrophages release cysteine proteases, mainly calpains and cathepsin S, which cleave prochemerin into peptides with potent anti-inflammatory properties, including inhibition of pro-inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-12 and RANTES (regulated on activation, normal T cell expressed and secreted) and induced the mRNA expression of the anti-inflammatory cytokines transforming growth factor (TGF)-β and IL-10. Peptide chemerin15 (C15) is another peptide derived from chemerin cleavage, which has been considered an anti-inflammatory and pro-resolving peptide, since it inhibits pro-inflammatory mediators produced by macrophages, promotes phagocytosis of apoptotic cells, inhibits integrin activation and clustering, reduces neutrophil adhesion and chemotaxis, and contributes to the reprogramming of macrophages by reducing inducible nitric oxide synthase (iNOS) and TNF-α and increasing arginase-1 expression [139–142]. Thus, chemerin is a protein that can contribute both to the onset and termination of acute inflammation [133].

Confirming the role of chemerin as an anti-inflammatory and pro-resolving mediator, Zhao and colleagues [143] have demonstrated that chemerin administration is positively related to reduced $CD4^+$ T-cell accumulation, lower mRNA expression of CCL17 and CCL22 (Th2-attracting chemokines) and decreased recruitment of $CD11c^+$ $CD11b^+$ inflammatory DCs in a murine model of asthma. Chemerin treatment could also reduce neutrophil infiltration and inflammatory cytokine release in a mouse model of acute lung inflammation induced by LPS [144]. Despite the studies presented here, participation of chemerin in the resolution of inflammation remains poorly understood and needs to be studied further.

2.2.4 Galectins

In recent years, several studies have focused on understanding the role of proteins and glycans in the modulation of the immune response [145]. Galectins are conserved glycan-binding proteins presented in mammals, birds, fish, sponges, nematodes, and microorganisms during evolution [146, 148]. To date, 15 galectin members have been identified, and all of them share a conserved carbohydrate-recognition domain (CRD) of approximately 130 amino acids, which promote carbohydrate binding [148, 149].

Based on their structure and number of CRDs, galectins can be classified into three families: (1) prototypic galectins that have one CRD, and exist as monomers or dimers, represented by galectin-1, 2, 5, 7, 10, 11, 13, 14 and 15; (2) tandem-repeat galectins that are composed of two different CRDs separated by a set of more than 70 amino acids, represented by galectin-4, 6, 8, 9 and 12; and (3) chimaera-type galectin-3 group that has a single CRD and a large amino-terminal domain, represented only by galectin-3 [148–152].

Galectins are involved at all stages of immunity and inflammation, from the initiation

through to the resolution [153, 154]. There are no specific receptors for galectin binding, and these proteins act both extracellularly and intracellularly [148, 149]. Intracellularly, galectins bind to cytoplasmic and nuclear proteins regardless of their interaction with carbohydrates and regulate various biological responses [147, 419]. Through an unknown mechanism, galectins can be exported extracellularly and act via the recognition of glycoproteins on the cell surface (such as adhesion receptors and cytokine receptors), controlling the responses and properties of these receptors [148, 152].

According to the galectin type, concentration, location, and glycan-binding preferences of each galectin member, these proteins can perform diverse biological response, regulating positively or negatively distinct cellular events, including cell activation, cytokine secretion, migration, proliferation, differentiation, polarization, and viability of immune cell populations [148, 149, 155]. Thus, some members of this family, such as galectin-3, contribute to the pro-inflammatory response, and others (for instance galectin-1 (Gal-1) and Gal-9) participate mainly in the resolution of inflammation [153, 156]. Here, we focus on the main characteristics of Gal-1 and Gal-9, and their contribution to the resolution of inflammation.

Gal-1 is expressed by epithelial, endothelial, and stromal cells, as well as macrophages, polymorphonuclear cells, eosinophils, DCs, and activated T cells (as reviewed by [157]). Galectin can be found in the nucleus, cytoplasm, inner or outer surface of the cell membrane, and extracellular matrix [167]. The participation of endogenous or exogenous Gal-1 to limit or resolve inflammation has been demonstrated in multiple inflammatory disease models, including nephritis, arthritis, encephalomyelitis, and hepatitis [158].

Gal-9 is expressed in various tissues and cell types, such as lung and immune cells, including T cells, B cells, DCs, monocytes, and eosinophils (as reviewed by Rao [167]). Some researchers have demonstrated anti-inflammatory and pro-resolutive properties related to Gal-9, such as the induction of T cell apoptosis and stimulation of phagocytic clearance [159–162].

The participation of Gal-1 and -9 in the resolution of inflammation remains controversial since some studies have also demonstrated pro-inflammatory properties of these molecules [163, 164]. Thus, Table 8 summarizes certain studies in which Gal-1 or Gal-9 has pro-resolution activity during experimental models as well in human cells.

2.3 Gaseous Mediators: Hydrogen Sulphide, Carbon Monoxide, and Nitric Oxide

Gaseous mediators are gases with very low molecular weights, which diffuse freely through cell membranes. The half-lives of these molecules are very short, and they do not have specific receptors [165]. The main representatives of gaseous resolving mediators are hydrogen sulphide (H_2S), carbon monoxide (CO), and nitric oxide (NO), whose functions are mediated through the interaction with many genes and proteins [165].

Hydrogen sulphide used to be strictly considered a toxic gas. However, in 1996, Abe and Kimura demonstrated the physiological role of this gas in the nervous system [166]. In 2001, Wang and colleagues also showed that H_2S is an important endogenous vasorelaxant. Subsequently, several studies have explored the role of this gaseous mediator in various tissues [165]. H_2S is derived from cysteine through the enzymatic action of cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CSE), [166–169] and 3-mercaptopyruvate sulphur transferase (3MST) [170]. CBS and CSE can be found in the liver, kidney, brain, ileum, uterus, placenta, and pancreatic islets. The CSE enzyme is also found in the portal vein and thoracic aorta [423]. 3MST is localized to the liver, kidney, heart, lung, thymus, testis, thoracic aorta, and brain [423].

CO is produced by the action of the enzyme heme oxygenase (HO) on heme. There are two isoforms of this enzyme: HO-2 (constitutive expression) and HO-1 (inducible expression). The detection of HO-1 can be used to identify cellular stress and produces CO to limit tissue damage. Moreover, the products of HO-1 activity are involved in various defence mechanism [171].

Table 8 Main pro-resolving actions of Galectins related to resolution of inflammation

Protein	Action	Disease or experimental model	References
Galectin-1	[1]Inhibits pro-inflammatory cytokine production, [1,3]reduces cell adhesion and lymphocyte trafficking and [2]deletes T helper (Th)1 and Th-17 cells selectively	Human T cells culture; Murine T cells culture; Experimental autoimmune encephalomyelitis; Human umbilical vein endothelial cells culture; Delayed-type hypersensitivity response induced in mice by met-BSA	[1][146] [2][322] [3][323]
	[1]Inhibits neutrophil extravasation and mast cell degranulation and [2,3]reduces neutrophil adhesion and transmigration across the inflamed endothelium	Rat hind paw edema; Human recombinant Gal-1 and human PMNs assay; Neutrophil culture	[1][324] [2][390] [3][420]
	Inhibits nitric oxide production by reducing inducible nitric oxide synthase (iNOS) expression	Peritoneal rats macrophages culture	[325]
	Suppresses antigen presentation by regulating constitutive and inducible FcγRI expression and FcγRI-dependent phagocytosis; inhibits IFN-γ-induced MHC class II (MHC-II) expression and MHC-II-dependent Ag presentation in a dose-dependent manner.	Human monocytes culture and murine macrophages culture	[326]
	Induces the differentiation of tolerogenic dendritic cells and regulatory T cells	Experimental autoimmune encephalomyelitis; Murine and human T cells culture; Human DCs and monocytes culture	[322] [327]
	Enhances IL-10 production	J558L, HL-60, Wehi-3, and PC-3 cells culture; Mouse Th cells culture; Hapten-dependent contact hypersensitivity; Human skin-explant T cell culture; Human knee synovial fluid culture of patients with rheumatoid arthritis; Human PBMC culture; MOLT-4 T cells culture; Human neutrophils and T cell culture	[328] [329] [330]
	Induces 12 and 15-lipoxygenase expression in macrophages and favours their conversion towards a pro-resolving phenotype	Mouse peritonitis; Mouse peritoneal macrophages and neutrophils culture	[331]
Galectin-9	[1]Exogenous Gal-9 inhibits airway inflammation by binding to CD44 and preventing CD44-hyaluronic acid interaction, reducing, in turn, leucocyte adhesion and migration to the lung; [2]Exogenous Gal-9 also suppresses airway resistance and eosinophil recruitment; [3]Reduces the inflammatory response related to asthma by inducing IL-10 production	Murine model of allergic asthma; RBL-2H3 cells culture; Mouse mast cell line MC/9 culture; Asthmatic reaction in guinea pigs and passive-cutaneous anaphylaxis in mice; Induced sputum samples of asthma patients	[1][161] [2][333] [3][153]
	Binds to IgE and prevents IgE-antigen complex formation and mast cell degranulation	RBL-2H3 cells culture; Mouse mast cell line MC/9 culture; Asthmatic reaction in guinea pigs and passive-cutaneous anaphylaxis in mice	[333]
	Induces apoptosis of activated eosinophils but not non-activated eosinophils	Bronchoalveolar lavage fluid of patients with acute and chronic eosinophilic pneumonia	[332]
	Exogenous Gal-9 induces T-cell apoptosis, limits leucocyte recruitment and oedema formation	Carrageenan-induced paw edema model	[162]
	Exogenous Gal-9 suppresses Th17 cell development and expands Foxp3$^+$ Tregs from naïve CD4 T cells	MOG-induced experimental allergic encephalomyelitis model	[334]

NO was first described in the 80s as a molecule that was able to regulate vascular tone [172]. It is now known that this inorganic free radical is related to many diverse functions in physiological and pathological situations [165, 173]. NO production results from the catalysis of L-arginine by a family of enzymes termed nitric oxide synthases (NOS). There are three known isoforms of NOS: two constitutive isoforms nNOS (or NOS1, present in nervous system tissue and skeletal muscle) and eNOS (also called NOS3, present in the endothelium), and an inducible isoform (iNOS or NOS2, present in immune cells and the cardiovascular system). nNOS and eNOS are present as preformed proteins and produce loss of NO levels after increased intracellular calcium, which is generally associated with physiological processes. iNOS produces large quantities of NO upon stimulation, by pro-inflammatory cytokines for example, and is therefore usually related to pathological processes [174].

Production of these gases is related to both physiological and pathological processes. In the context of resolving inflammation, they present similar actions that contribute to the termination of the inflammatory response [165]. Table 9 details the specific activities mediated by each of these gases that participate in the resolution of inflammation during experimental models as well in human cells.

2.3.1 Purine: Adenosine

Adenosine is a purine that can be detected in the extracellular space at nanomolar levels under normal conditions [175], and is found in every cell of the body [176]. Following cellular damage or stress, increased levels of adenosine can be produced by various cell types through its intracellular formation and export via nucleoside transporters, or extracellular degradation of adenine nucleotides (ATP and/or ADP) [175, 177, 424].

There are four specific G-protein-coupled receptors associated with adenosine binding: A1, A2A (high-affinity), A2B (low-affinity), and A3. The A1 and A3 receptors are known to inhibit adenylyl cyclase (the enzyme responsible for catalysing the conversion of adenosine ATP to 3′,5′-cyclic AMP (cAMP), whereas the A2 receptor stimulates this enzyme [99, 175, 178, 424]. Thus, activation of a certain adenosine receptor may result in contrasting physiological effects, based on the location and level of expression of these receptors [99, 175].

Adenosine represents an impressive pro-resolving mediator that can impact the initiation, duration, and resolution of the inflammatory response [175]. Production of this purine has been demonstrated to be associated with the modulation of several cell types, including neutrophils, macrophages, endothelial cells, DCs, and lymphocytes (see [175, 177] and references therein for more details). In summary, the following points regarding adenosine receptor interactions and their roles in the resolution of inflammation in various cells types can be highlighted:

- *Neutrophils:* This cell type can release adenosine after activation. Adenosine binding to A2A and A3 receptors is related to the inhibition of neutrophil activation [179]. Adenosine inhibits the phagocytic activity of neutrophils, diminishes neutrophil production of oxygen radicals and other potentially deleterious mediators, as well as reduces neutrophil adhesion to the vascular endothelium [180, 181].
- *Macrophages:* The differentiation of monocytes into macrophages is related to increased expression of A1, A2A and A3 receptors. Activation of A2A, A2B and A3 receptors promotes the polarization of the anti-inflammatory alternatively activated macrophages (or M2), which are associated with decreased levels of TNF, IL-6, IL-12 and macrophage inflammatory protein (MIP)-1α, as well as increased levels of the anti-inflammatory cytokine IL-10. A2B receptor activation also increases the expression of arginase-1 and Mgl-1 expression, and increases the production of tissue inhibitor of metalloproteinases-1 (TIMP-1) in alternatively activated macrophages. These changes also favourite tissue repair [182–186].
- *Endothelial cells:* Adenosine binding of the A2 receptor increases vascular endothelial

Table 9 Main pro-resolving actions of gaseous mediators related to resolution of inflammation

Gas	Action	Disease or experimental model	References
Hydrogen sulphide	Acts as a scavenger of cytotoxic substances, including peroxynitrite[1], hypochlorous acid[2] hydrogen peroxide[3], and superoxide anion[4]	Human neuroblastoma SH-SY5Y cells culture; Murine RAW 264.7 macrophage culture; Human vascular smooth muscle cells culture	[1][354] [2][355] [3][356] [4][357]
	Exerts antinociceptive effects in the gastrointestinal tract by activating K_{ATP} channels	Colorectal distension in rats	[339]
	Suppresses leucocyte infiltration and oedema formation: inhibits the expression of cell adhesion molecules in both the endothelium (ICAM-1 and P-selectin) and on leucocytes (LFA-1)	Mesenteric microcirculation evaluation by intravital; Carrageenan air pouch model in rats	[340]
	Helps to restore tissue function by upregulating enzymes that drive tissue repair and preserve mitochondrial function	Human colon adenocarcinoma cell lines culture; Mitochondria isolated from mouse kidneys, liver, and heart; Human colonic epithelial HT-29 Glc - /+ cells culture	[341, 342] [343]
	Reduces the inflammation-associated-upregulation of COX-2 expression and reduces the range of pro-inflammatory cytokines such as IL-1, TNF, IFN, IL-12, and IL-23	LPS-induced endotoxic shock in rats	[344]
	Enhances ulcer healing	Experimental gastric ulcer in rats	[345]
	Inhibits leucocyte adherence to blood vessels walls and induces vasodilatation in the cardiovascular system	Haemorrhagic shock model in rats	[346]
	Induces neutrophil apoptosis	Human PMN cells culture	[347]
	Stimulates angiogenesis	Human umbilical vein endothelial cells culture; chick chorioallantoic membrane model	[348]
	Stimulates the translocation of annexin-A1 from the cytosol to the plasma membrane	Murine bone marrow–derived macrophages culture; Intravital microscopy in mouse mesenteric microcirculation; Human PMN cells culture	[421]
	Protects blood-brain barrier integrity[1] and promotes angiogenesis after cerebral ischemia[2]	Middle cerebral artery occlusion in mice; Middle cerebral artery occlusion in rats	[1][422] [2][349]
	Promotes gastrointestinal mucosal integrity and repair[1,2] Promotes stronger barrier function of the GI mucosa, limiting exposure to luminal bacteria[2]	Gastroenterophaty; Hapten-induced colitis in rodents	[1][350] [2][351]
	Inhibits the activity of the human myeloperoxidase (MPO)	Circulating and endothelium-bound human MPO	[352]

(continued)

Table 9 (continued)

Gas	Action	Disease or experimental model	References
Carbon monoxide	At lower concentrations, produced by eNOS and nNOS: usually is cytoprotective At supra-physiological concentrations, produced by iNOS: triggers cell death	Culture of endothelial cells, hepatocytes, thymocytes, neurons, leukocytes and several tumor cell lines; Brain ischemia; Alzheimer's and Parkinson's Disease; Murine macrophage culture; Human peripheral blood mononuclear cells culture; Human neutrophils culture; Eosinophils culture; Rheumatoid arthritis, acute pancreatitis, bacterial pneumonia, inflammatory bowel disease, asthma, and following surgery	[353] [354] [355] [173]
	Suppresses leucocyte adherence to the vascular endothelium and reduces pro-inflammatory cytokine expression by inhibiting NF-kB	Human osteoarthritic chondrocytes and cartilage cell culture; Acute peritonitis induced by Zymosan in mice; Model of adaptive immune response in mice	[356] [357] [358]
	Reduces neutrophil infiltration and stimulates the activity of HO-1 and phagocytosis by resolution macrophages	Murine peritonitis and human macrophage and PMN cell culture	[359]
	Exhibits cell and tissue protection through anti-apoptotic, anti-inflammatory, and anti-proliferative effects	CO gas inhalation by rats, mice and pigs; Murine model of chronic colitis; Pulmonary arterial hypertension and right ventricular hypertrophy in mice; Experimental model of cerebral malaria in mice	[360]
Nitric oxide	Inhibits platelet and inflammatory cell activation	Vascular smooth muscle cells culture; Human and mice neutrophils culture	[361] [362]
	Promotes ulcer healing	Gastric ulcer in rats	[363]
	Induces apoptosis in smooth muscle cells	Vascular smooth muscle cells culture	[364]
	Stimulates angiogenesis, proliferation and metastasis in cancer at lower levels (<100 nM), but promotes cytotoxicity and cell apoptosis in cancer at higher levels (>400–500 nM):	Breast cancer; Gastric cancer; Melanoma cells; HL-60 human leukemia; JEG-3 choriocarcinoma cells; Ovarian carcinoma cells; Medullablastoma cells; Human prostatic epithelial cells; Human bladder carcinoma cells; Murine melanoma cells; Human adenocarcinoma cells; Fibrosarcoma cell; Renal cell carcinoma; B-cell chronic lymphocytic leukemia cells; Colon cancer cells; Skin tumors (murine)	[365] [366]

growth factor (VEGF) expression by macrophages, stimulates the proliferation and migration of endothelial cells, and promotes angiogenesis, tissue repair, and wound healing [187–189].

– *Dendritic cells:* A1 and A3 receptors are expressed on immature human DCs and contribute to the recruitment of this cell type to inflamed tissues. Mature DCs mainly express the A2A receptor, which is associated with the reduction of interferon (IFN)-α, IL-6, TNF, and IL-12, as well as the inhibition of antigen presentation and costimulation [177, 190, 425].
– *Lymphocytes:* The binding of adenosine to A2A, A2B, and A3 receptors is related to reduction of IL-2 and IFN-γ levels, and inhibition of effector T-cell proliferation. CD39 and CD73, molecules present on the surface of Tregs and Th17 cells, play important roles in the production of adenosine that binds the A2 receptor of effector T cells, suppressing both $CD4^+$ and $CD8^+$ T cell effector functions [191, 192, 193].

2.4 Neuromodulators

Interaction between the nervous system and immune system have been well documented in many studies. Following activation, immune cells can stimulate neuronal circuits that participate in innate and adaptive immune response regulation [194–198]. This is possible since there are cholinergic, catecholaminergic, peptidergic, and other types of neurons distributed throughout different organs, including the liver, spleen, lymph nodes, and thymus. These neurons transmit information to and from the nervous system during infection or sterile inflammation [198]. Moreover, some structures, such as Toll-like receptors (TLRs) and cytokine receptors, are present in both leukocytes and neurons, facilitating neuro-immune communication [197].

In the context of neuro-immune regulation, vagus nerve-mediated cholinergic signalling plays an important role in the resolution of inflammation via the inflammatory reflex arc [194–198]. This inflammatory reflex arc is characterized by the recognition of signs of injury, infection, or inflammation by an afferent sensory neural arc, and the transmission of responses by an efferent motor neural arc that regulates the immune system [196]. The neurotransmitters involved in this process can bind to acetylcholine receptors and adrenergic receptors expressed in immune cells such as macrophages, T lymphocytes, and DCs, enabling the neural regulation of immune responses (as reviewed by [199]). Furthermore, acetylcholine, dopamine, and other neuromodulators can be produced and released by immune cells, contributing to local immune regulation [197, 199–201].

Netrin-1 and acetylcholine are two relevant neuromodulators associated with the resolution of inflammation that act via the vagus nerve-mediated reflex [197]. Mirakaj and colleagues [6] have shown that vagotomy after zymosan-initiated peritoneal injury in mice reduced acetylcholine and netrin-1 levels and delayed the resolution of inflammation, by increasing pro-inflammatory cytokines and leukocyte recruitment.

The cholinergic anti-inflammatory activity induced by the vagus nerve occurs via acetylcholine binding to the alpha 7 nicotinic acetylcholine receptor (α7nAChR). This receptor is broadly expressed by immune cells, such as B cells, T cells, DCs, monocytes, and macrophages [196, 197, 202, 426]. α7nAChR activation results mainly in the suppression of pro-inflammatory cytokines via three pathways: (1) cAMP response element binding protein (CREB) phosphorylation, followed by increased c-Fos expression, which inhibits nuclear factor (NF)-κB; (2) through the interaction of α7nAChR with Janus kinase 2 (JAK2), causing the phosphorylation and subsequent nuclear translocation of STAT3 (signal transducer and activator of transcription 3) or (3) via rapid acetylcholine influx into the cytoplasm following ATP entry, which attenuates the release of mitochondrial DNA and inflammasome activation [197].

Netrin-1, the other neuromodulator regulated by the vagus nerve, is a laminin-related protein expressed by the vascular endothelium. Its expression is regulated by inflammatory cytokines and infectious agents [427]. Unc5 netrin receptor B (UNC5B) is highly expressed in leukocytes, and can interact with netrin-1 to regulate the immune response [427]. The binding of netrin-1 to the A2B receptor is also associated with a reduced inflammatory response [6, 203, 204]. This receptor is expressed in several tissues and organs, including the vasculature, large intestine, and brain. Moreover, different cell types express high levels of A2B, such as neurons, astrocytes, endothelial cells, mast cells, neutrophils, DCs, macrophages, and lymphocytes (as reviewed by [205]). The participation of netrin-1 in the resolution of inflammation is related to the overexpression of resolvins, decreased recruitment of neutrophils, and overactivation of efferocytosis [6, 203, 204, 383].

The effects of acetylcholine and netrin-1 that contribute to the resolution of inflammation during experimental models as well in human cells are highlighted in Table 10.

Table 10 Main pro-resolving actions of neuromodulators related to resolution of inflammation

Protein	Action	Disease or experimental model	References
Acetylcholine/ cholinergic anti-inflammatory pathway stimulation	Attenuates the release of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18 and TNF	Lethal endotoxaemia in rats and mice; Human macrophage culture; Acute Hypovolemic hemorrhagic shock in rats; Septic peritonitis induced in mice; Murine model of chronic relapsing colitis; Spleen cell culture; Microdialysis in mice; Kidney ischemia- reperfusion injury in mice	[367] [368] [369] [431] [370] [374] [371] [200, 372] [373]
	Upregulates the protective immunoresolvent PCTR biosynthetic pathway in human group 3 innate lymphoid cells (ILC3), promoting the resolution of bacterial infections	*Escherichia coli* peritonitis in mice	[222]
	Decreases circulating HMGB1, attenuating sepsis	Endotoxemia and polymicrobial sepsis	[374]
	Impairs the migration of B cells, neutrophils, monocytes and dendritic cells and reduces antibody production	Immunization of mice with *Streptococcus pneumoniae*	[375]
	Improves inflammation, pannus formation, cartilage destruction and bone erosion in collagen-induced arthritis	Rat collagen-induced arthritis	[376]
	Reduces myocardium inflammation, decreasing IL-6 and TNF-α; monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, RANTES, CCR1, CCR2, and CCR5. Reduces matrix metalloproteinase-14, natriuretic peptide precursor B, tissue inhibitor of metalloproteinase-1 and osteopontin	Murine autoimmune myocarditis	[377]
Netrin-1	Increases the expression of alternatively activated macrophages and promotes proliferator-activated receptor γ (PPARγ) expression; decreases cardiac serum Troponin T (TnT) expression and reduces allograft infiltration of neutrophils and monocytes/macrophages	Cardiac ischemia reperfusion injury	[378]
	Inhibits leucocyte migration	Peripheral blood lymphocytes culture; Human umbilical vein endothelial cells culture; Mouse sepsis model; Mouse peritonitis model	[379]
	Shortens the resolution interval, decreases neutrophil recruitment, reduces pro-inflammatory mediators and stimulates the production of resolvins, protectins and lipoxins	Murine peritonitis; Human neutrophils and monocytes culture; Murine LPS inhalation model; Murine ventilator-induced lung injury model; Peritonitis model; Caco-2, T84 and HMEC-1 cells culture; DSS-colitis; Murine model of hepatic ischemia reperfusion injury	[6, 203, 383] [204, 380] [381]

2.5 Hydrogen Peroxide – H2O2

The reactive oxygen species (ROS) family is comprised of a group of molecules, such as hydrogen peroxide (H_2O_2), are essential for host defence, and are produced through the mitochondrial electron transport chain or the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) complexes in the plasma membrane and cytosol [206]. ROS are normally produced during cellular energy production in aerobic cells, and are removed by antioxidant enzymes. ROS can also be produced by phagocytes in response to microbial and inflammatory stimuli, within the mitochondria, or through a process known as "oxidative burst" mediated by NOX, and are generally considered to be pro-inflammatory mediators [207].

A reduction in ROS production is associated with several human pathological conditions, such as chronic granulomatous disease (CGD). In this disease, ROS-deficiency is associated with severe infections and is characterized by excessive inflammation [208]. Patients with CGD have a decreased ability to resolve inflammation [209]. In addition, H_2O_2 is described as an inducer of apoptosis in epithelial cells, endothelial cells, human hepatocytes, myocytes, neutrophils, and eosinophils [210–215].

It has also been demonstrated that H_2O_2 derived from NOX is directly linked to the induction of eosinophil and neutrophil apoptosis. Moreover, exogenous administration of H_2O_2 at the peak of the inflammatory response decreases the number of viable eosinophils and increases the number of apoptotic eosinophils, which consequently shortens the duration of allergic inflammation [210, 211]. In addition, in an antigen-induced arthritis model, Lopes et al. [211] showed that H_2O_2 increased neutrophil apoptosis, anticipating the natural resolution of inflammation. Indeed, at controlled low concentrations, H_2O_2 is a key second messenger of physiological processes such as cell proliferation [216] and migration [217]. Thus, in addition to the canonical pro-inflammatory roles, H_2O_2 has also been shown to act as an important pro-resolutive mediator.

3 Pharmacological Strategies for Resolution: Clinical Trials

Several studies have focused on the discovery of new compounds with a therapeutic potential to treat inflammatory diseases [6, 71, 210, 211, 218–222]. The development of pharmacological pro-resolution strategies to treat chronic inflammatory pathology intrinsically affords a greater scope than conventional anti-inflammatory approaches. Current treatments that focus on the inhibition of the productive phase of inflammation (anti-inflammatory treatments), act mainly to reduce both leukocyte recruitment and the release of pro-inflammatory mediators [3, 8]. By contrast, pro-resolving treatment aims to increase the production or use of mediators to augment important aspects during resolution, such as apoptosis, efferocytosis, recruitment of resolving macrophages, and tissue repair. In addition, these treatments seek to simulate the mechanisms and endogenous biochemical pathways related to the resolution of inflammation [8, 57, 210, 220].

Several authors have investigated the anti-inflammatory and pro-resolving properties of endogenous and synthetic lipids derived from PUFAs, which has demonstrated that these specialized lipid mediators limit leukocyte accumulation and enhance the influx of pro-resolving macrophages and efferocytosis in animal models. Many of these studies have translational potential, and some have already been tested in clinical trials [26, 72, 100].

Most clinical trials aiming to resolve inflammatory exudates use omega-3 fatty acids to produce structurally distinct families of signalling molecules, including SPMs. A resolvin E1 analogue improved the signs and symptoms in a Phase II clinical trial in patients with dry eye syndrome [223], and this study has progressed to a Phase III clinical trial (Safety and Efficacy Study of RX-10045 on the Signs and Symptoms of Dry Eye, identifier NCT00799552; www.clinicaltrials.gov). This was the first demonstration of clinical efficacy using mediators associated with the resolution phase of the inflammatory response. SPMs have demonstrated several pro-operative effects, are associated with a low cost

Table 11 Pro-resolving strategies in clinical trials

Pro-resolution drug/ compounds	Action	Disease	References
Protectin D1	Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness	Asthma	[95]
Resolving macrophages	Efferocytosis of neutrophils	Periodontal disease	www.clinicalTrials.gov Ekstein, J
Fish oil supplementation	Production of Omega-3 Fatty Acid-Derived Mediators	Peripheral Artery Disease	[226]
Neuroprotectin D1	This report provides a demonstration of the role of neuroprotectin D1 in cell survival and its potential deficiency in Alzheimer's disease	Alzheimer	[90, 225]
Docosahexaenoic acid	The ratio of pro-resolution/pro-inflammatory lipid markers was increased in the plasma of the intervention group over the entire study	Obesity inflammation	[224]
Specialized pro-resolving lipid mediators	SPMs promote macrophage phagocytosis of blood clots	Coronary artery disease	[382]
RvE1 analogue	Reduces inflammation	Dry eye syndrome	www.clinicalTrials.gov Phase 3
Specialized pro-resolving lipid mediators	Increase pro-resolution signals	Allergic inflammation	www.clincalTrials.gov Barning C, completed study
E-series resolvin (RvE) 1	RvE1 rescues the dysregulation in the neutrophil receptor profile and, following a therapeutic dosage, activates phagocytosis and resolution signals in type 2 diabetes	Type II Diabetes	[71]
Monoglyceride of DHA	Monoglyceride of DHA (DHA-MAG) is a lipid compound for which intestinal absorption would increase the DHA/arachidonic acid (AA) ratio and promote the synthesis of specific metabolites involved in the resolution of inflammation. Reduces lung inflammation and improves pulmonary function	Cystic fibrosis	www.clinicalTrials.gov Phase 2

and can be found in the diet, and have also been widely used as supplementation in pregnant, obese, and diabetic patients [240]. In addition, several studies have demonstrated the importance of these mediators in diseases such as allergic asthma, Alzheimer's disease, coronary artery disease, and peripheral artery disease [71, 90, 95, 225–227].

However, these mediators carry a high price tag for large-scale use in the clinic. Thus, certain animal studies seem promising, [2, 10, 12, 17, 77, 92, 210, 211, 220, 221, 228, 229, 230, 231, 232] especially those related to the use of alternative therapies that use other mechanisms associated with the resolution phase of an inflammatory response, and those associated with the activation of endogenous mechanisms that trigger an acceleration of the resolving phase. Table 11 is a summary of pro-resolving strategies in clinical trials.

In summary, this chapter highlighted the main pro-resolving mediators and their roles in the resolution of inflammation. Moreover, some clinical trials were presented to confirm the importance of these mediators in regulating inflammatory responses in different diseases. Research into the resolution of inflammation and its mediators continues to be a promising field of study, still requiring constant progress.

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Part III

Immunopharmacology

Innate Immunity in Inflammation

Andrea Cignarella and Chiara Bolego

Abstract

A fine balance between prompt response to pathogens and avoidance of unregulated inflammation, as well as that between protection and self-damage drives the complexity of the immune system, at the same time pointing out the challenge for effective and safe immunopharmacological intervention. A wide variety of clinically relevant drugs are currently used in the treatment of human inflammatory and immune-system associated disorders. Classical therapeutic approaches are now integrated with emerging strategies that largely derive from advances in signalling and regulatory networks and the pathological consequences of their dysregulation in the field of innate immunity. This chapter provides an account of: (i) the interplay between innate immunity and inflammation; (ii) main immune signalling molecules in inflammation including cytokines, prostanoids and cancer-related immune response, and the main aspects of pharmacological control thereof; and (iii) emerging options for therapeutic interventions on cells of innate immunity.

Keywords

Innate immunity · Inflammation · Anti-cytokine agents · Macrophage polarization · Immunometabolism · Histamine · Immune and inflammatory pathologies

1 Introduction: Innate Immune System and Inflammation

The innate immune system is fundamental to protect us from pathogens. Although the core components were first elucidated in model organisms such as Drosophila and mice, exactly how they are regulated in human health and contribute to disease remains to be fully defined. Over the past several years, technological advances have begun to reveal new components of the innate immune system and have led to improved understanding of its function. Importantly, these new insights have allowed to better understand the pathophysiological basis of certain human diseases and to identify new therapeutic targets to alleviate them. Thus, the field of innate immunity has seen advances in signalling and regulatory networks and in understanding how their dysregulation contributes to immunopathologies and auto-inflammatory conditions.

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The inflammatory response involves a complex interaction of vasculature and cellular processes which is controlled by a host of biological mediators. Because inflammation plays an important role in health and disease, the pharmacology of the mediators and cells driving inflammation has attracted considerable interest since ancient times. Hence, much effort has been devoted to developing drugs targeted at modulating immune, inflammatory and/or infectious components of disease. The IUPHAR Guide to Immunopharmacology website provides a searchable database with quantitative information on drug targets and 179 medications which are currently, or have been in the past, approved for human clinical use by a regulatory agency, as well as experimental drugs that act on them [1]. These clinically relevant agents including non-steroidal anti-inflammatory drugs, H_1 receptor antagonists, antileukotrienes, mast cell membrane stabilizers, glucocorticoids, immunosuppressants, cytotoxic agents, protein kinase inhibitors, cytokines/chemokines, monoclonal antibodies and vaccines are used in the treatment of human inflammatory, allergic and other immune system-associated disorders [2]. Isolated antibodies represent effective drugs when developed as biopharmaceuticals, as demonstrated by the ever-expanding list of approved molecules as well as by the large number of antibody products in development in a wide range of therapeutic areas [3]. The antibody landscape is changing as the field has moved from empirical to knowledge-based, designer approaches for which understanding of mechanisms of action at the molecular level is critical.

2 The Innate Immune System at Work

The innate immune system responds to common structures shared by a vast majority of threats. The common structures are called pathogen-associated molecular patterns, or PAMPs, and are recognized by the Toll-like receptors (TLRs). These transmembrane proteins activate signaling pathways that launch immune and inflammatory responses to destroy invaders. This triggers the release of cytokines, particularly interleukin (IL)-1 and tumour necrosis factor (TNF)-α, as well as various chemokines. The innate immune system works as the first line of defence in protection from pathogenic microbes and host-derived signals of cellular distress. One way in which these "danger" signals trigger inflammation is through activation of inflammasomes [4]. Inflammasome multiprotein signalling platforms typically consist of a cytosolic pattern recognition receptor (PRR), an adaptor protein, and pro-caspase-1. A number of distinct inflammasome complexes have been identified, each with a unique PRR and activation triggers [5]. Understanding of the signalling mechanisms by which PRRs sense microbial infections and cellular stress engage innate immune responses is moving at an unprecedented rate. A wealth of clinical information supporting the key roles of PRRs not only in host-microbe interactions, but also chronic autoinflammatory and autoimmune diseases in patients has emerged. This has spurred important translational medicine efforts to bring new therapies and diagnostics to the clinic.

TLRs are primarily found on macrophages, mast cells and dendritic cells, the three sentinel cells of the innate immunity. Dysregulation of TLR signalling has severe consequences, and causes chronic pathological inflammation and many autoimmune diseases [6, 7]. TLR signalling has been shown to be involved in not just infection, but also diseases such as insulin resistance. Analysis of this innate immune pathway may identify novel TLR roles in other diseases. TLRs are involved in the development of atherosclerosis, an inflammation-driven condition that is the leading cause of cardiovascular disease [8]. Accordingly, mice genetically deficient in TLR5 develop hallmark features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity, indicating that malfunction of the innate immune system promotes the development of metabolic syndrome [9]. Interestingly, TLR9 expression in tumour-associated macrophages has been exploited for TLR9-targeted delivery and intracellular

processing of CpG oligonucleotides as inhibitors of STAT3, a transcription factor with established tumorigenic properties in human cancer [10]. One of the few examples of TLRs as pharmacological targets of approved drugs is TLR7, which is expressed on monocytes/macrophages, mast cells and B-lymphocytes. Among other viral ligands, it also recognises some synthetic imidazoquinoline antiviral drugs such as imiquimod [11]. The ability of these drugs to provoke TLR activation likely underlies their clinical effectiveness. By contrast, although hydroxychloroquine has direct effects as an antimalarial agent, this drug also appears to be an antagonist of two of the human TLRs, namely TLR7 and TLR9 [12]. Antagonism of these receptors on dendritic cells is likely related to the anti-inflammatory action of this drug in some auto-immune diseases including moderate active rheumatoid arthritis and juvenile arthritis.

3 Pharmacological Control of Immune Signalling Molecules in Inflammation

Our knowledge of the complex innate immune response is rapidly increasing. An organism's survival depends on a prompt response to pathogens, but it is equally important to avoid unregulated inflammation that can lead to dangerous pathologies such as sepsis and autoimmune disease [13]. It is this fine balance between protection and self-damage that drives the complexity of the innate immune response. The main pathways related to immune cell activation and inflammation that are targeted by clinically relevant drugs are depicted in Table 1. Drugs should be used in dangerous and not self-resolving sepsis and in autoimmune diseases and in allergy in which both innate and adaptive responses are involved.

Table 1 Main drug classes affecting immune cell function in inflammatory disease

Drug class	Comments	Section in this chapter
Nonsteroidal anti-inflammatory drugs	Little or no effect on immune cells at clinically relevant concentrations Immune cell activation appears to be protective in paracetamol overdose	3.2
Antileukotriene drugs	Antagonists of mast cell and macrophage CysLT receptors used in asthma	3.2
Anti-histamines	Selective H_1 antagonists widely used in the prevention of allergic inflammation investigational H_4 antagonist under clinical development for atopic dermatitis	3.3
Glucocorticoids	Established first-line anti-inflammatory agents – Skew monocyte subsets and macrophage activation towards anti-inflammatory phenotypes	3.2, 4.1, 4.2
Estrogen	*In-vitro* and *ex-vivo* evidence of macrophage anti-inflammatory phenotype rescue that is impaired in post-menopausal women	4.3
Immunosuppressants	Rapamycin skews macrophage immunophenotypes and induces phenotype-specific apoptosis	4.3
Glitazones	PPARγ promotes lipid metabolism and limits inflammation in metabolically activated adipose tissue macrophages	4.3
Anti-cytokine agents – monoclonal antibodies	Established efficacy in inflammatory disease The anti-IL-1β mAb canakimumab reduces CV events	1, 3.1
Cytotoxic agents	Immune checkpoint inhibitors are the current wave of cancer treatment	3.4

3.1 Cytokines

Inflammation is a pervasive phenomenon that operates during severe perturbations of homeostasis, such as infection, injury, and exposure to contaminants, and is triggered by innate immune receptors that recognize pathogens and damaged cells. Among vertebrates, the inflammatory cascade is a complex network of immunological, physiological, and behavioural events that are coordinated by cytokines, immune signalling molecules. In auto-immune disease (e.g. rheumatoid arthritis, where the adaptive immune system is activated), tumour necrosis factor (TNF) appears to be the predominant influence and blocking its action is therapeutically effective [14–16]. In auto-inflammatory diseases (e.g. gout, where only the innate system is involved), IL-1 seems to be the key mediator [17] because, by regulating cell migration to sites of infection, it produces fever and pain. Both TNF-α and IL-1 are important targets for anti-inflammatory biopharmaceuticals [18, 19]. The biology of transforming growth factor beta (TGF-β) also impacts on multiple immune cell types and immune settings, including innate-lymphoid cells, hematopoietic stem cells and innate immunity [20] through antiproliferation signalling that may affect cancer progression.

Inflammation plays a major role in heart attack and stroke, and patients with elevated inflammatory biomarkers have increased vascular risk. IL-1β plays several roles in heart disease and increases in cells where cholesterol crystals deposit, leading to clinical events. Of note, although many cell types can produce IL-1 family members, monocytes and macrophages, key cells in atherosclerotic plaque biology, produce the bulk of IL-1β [21, 22]. The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS; [23]) tested whether reducing inflammation in patients who have had a prior heart attack could reduce the risk of future cardiovascular events. The study was conducted in more than 40 countries and specifically tested whether blocking IL-1β with canakinumab, as compared to placebo, reduced rates of cardiovascular outcomes among patients at high risk due to a persistent elevation of the inflammatory biomarker high sensitivity C-reactive protein (hsCRP; ≥2 mg/L) despite best medical care. Canakinumab is a human monoclonal antibody that neutralizes IL-1β, and is approved in the United States and Europe as a treatment for several rare inflammatory diseases such as juvenile arthritis. Canakinumab significantly reduced hsCRP levels from baseline, as compared with placebo, without reducing the LDL cholesterol level, and the 150-mg dose resulted in a significantly lower incidence of recurrent cardiovascular events over a median follow-up of 3.7 years. Importantly, while a significantly higher incidence of fatal infection and sepsis was not unexpectedly observed with canakinumab with respect to placebo, the trial also found fewer cases of lung cancer in those on treatment [24], suggesting that the same inflammatory pathway may initiate or spur the growth of such tumours. It should be pointed out though that the trial was not sufficiently powered to study that disease. These studies also raise a potential area for further research aimed at investigating shared, as yet poorly defined pathogenic pathways as well as beneficial effects of medications in the fields of cardiology and oncology [25].

3.2 Prostanoids

In areas of acute inflammation, prostaglandin (PG)E_2 and PGI_2 are generated by the local tissues and blood vessels, while mast cells release mainly PGD_2. In chronic inflammation, cells of the monocyte/macrophage series also release PGE_2 and thromboxane (TX)A_2. Together, the prostanoids exert diverse effect in inflammation, stimulating some responses and decreasing others. Nonsteroidal anti-inflammatory drugs (NSAIDs) induce anti-inflammatory and analgesic effects by blocking vasodilating and pain-sensitising effects of prostanoids, and exert antipyretic action by inhibiting PGE_2 synthesis in the hypothalamus. Although NSAIDs have additional anti-inflammatory effects independent of COX inhibition that may affect immune cell

function [26], they only occur *in vitro* and their clinical relevance remains to be determined. As a class, NSAIDs generally have little effect on aspects of inflammation such as cytokine/chemokine release, leukocyte migration, lysosomal enzyme release and toxic oxygen radical production, which contribute to tissue damage in chronic inflammatory conditions such as rheumatoid arthritis. Furthermore, little is known on NSAID-mediated downstream inhibition of anti-inflammatory effects of prostainoids (e.g. PGE_2) which are relevant during the resolution phase of inflammation.

Of note, the innate immune system is involved in specific NSAIDs adverse effects such as paracetamol hepatotoxicity. In particular, while the underlying mechanism involves formation of a reactive metabolite, glutathione depletion and protein adduct formation, a substantial sterile inflammatory response follows the initial cell death. Cytokines produced mainly by activated Kupffer cells modulate intracellular mechanisms of cell death whereas monocyte-derived macrophages and potentially neutrophils support tissue repair and resolution of the inflammatory response. Thus, the innate immune response after paracetamol-induced liver cell injury fosters the repair of the tissue damage [27]. In addition, NSAIDs (as well as steroids) have broad effects all over the body, and NSAIDs under certain conditions can spur inflammation along with blunting it. The widespread use of NSAIDs drugs worldwide is associated with emerging safety concerns including potential cardiovascular hazard upon prolonged exposure [28]. The main mechanism contributing to NSAIDs-induced cardiovascular effects is COX-2 inhibition in the vasculature, the heart, and the kidney. This public health issue should be tackled with focused research.

Cysteinyl-containing leukotrienes LTC_4, LTD_4, LTE_4 and LTF_4 (also referred to as the sulfidopeptide leukotrienes) are produced mainly by eosinophils, mast cells, basophils and macrophages. Their biological actions including bronchoconstriction, chemotaxis and stimulation of cytokine release are mediated by CysLT (two subtypes) receptors, and are important on the respiratory and cardiovascular systems [29]. The CysLT-receptor antagonists zafirlukast and montelukast are now in use in the treatment of asthma, often with a corticosteroid [30].

3.3 Histamine

Histamine is released from mast cells by exocytosis during inflammatory or allergic reactions. Four types of histamine receptor have been identified: $H_{1\text{-}4}$ [31, 32]. All of them are G protein-coupled receptors implicated in diverse aspects of the inflammatory response. While selective H_1 and H_2 antagonists have been used for decades in the prevention of allergic inflammation and acid secretion, respectively, the selective inverse agonist pitolisant acting on H_3 receptors in the brain was approved for the treatment of narcolepsy in 2015 [33]. The H_4 receptor has been discovered recently and is primarily expressed in cells of the immune system such as mast cells and eosinophils. While this receptor offers new perspectives as a new drug target in a variety of conditions, an investigational selective H_4 antagonist has been tested in a phase 2 trial for treatment of atopic dermatitis that has just been completed [34]. This illustrates the still expanding set of biological functions of histamine, a biogenic amine discovered over 100 years ago, and the potential for novel pharmacological intervention on its receptors.

3.4 Cancer-Related Inflammation and Immune Cell Signalling

The functional interplay between cancer cells and immune cells is extremely complex. An immune cell infiltrate is a characteristic feature of many tumours, and it is increasingly appreciated that immunity and inflammation are key determinants of tumour development and progression. Immune cells initially inhibit tumour growth; however, tumour cells often suppress not only the tumour-inhibitory function of immune cells, but they can also hijack immune cells to promote tumour growth. The immunological mechanisms involved in cancer growth provide

numerous potential points for intervention with small molecules, including extracellular enzymes, receptors and intracellular signal transduction pathways [35]. Therapeutic intervention in this system aims either to promote antitumor immune responses [36] or to block the immunosuppressant tumour microenvironment. In recent years, drugs aiming to restore the tumour suppression function of the immune system have shown remarkable clinical results, although not all cancer patients benefit from these. While immunotherapy is emerging as a major cancer therapeutic modality, the interplay between cancer, inflammation, and immunity remains an area of active investigation.

Immune checkpoint molecules, such as indoleamine 2,3-dioxygenase (IDO), tryptophan 2,3-dioxygenase (TDO), programmed cell death (PD)-1 and cytotoxic T-lymphocyte-associated protein (CTLA)-4, are co-stimulatory or inhibitory proteins that contribute to the ability of tumours to evade the immune system. Immune checkpoint inhibitors are the current wave of cancer treatment [37, 38]. In particular, ipilimumab is an immunomodulatory monoclonal antibody directed against the cell surface antigen CTLA-4, thereby acting as an immune checkpoint inhibitor. This drug initiated the rise of checkpoint inhibitors as cancer therapy [39]. Nivolumab is a genetically engineered, fully human immunoglobulin (Ig) G4 monoclonal antibody directed against the negative immunoregulatory human cell surface receptor PD-1 (PCD-1) with immune checkpoint inhibitory and antineoplastic activities. The FDA approved nivolumab to treat patients with advanced (metastatic) non-small cell lung cancer whose disease progressed during or after platinum-based chemotherapy [40, 41]. The FDA approved pembrolizumab for the treatment of patients with metastatic non-small cell lung cancer whose tumours express programmed death ligand (PD-L) 1 as determined by an FDA-approved test [42, 43]. Atezolizumab, avelumab, and durvalumab block PD-L1 on tumour cells and/or tumour-infiltrating immune cells. Atezolizumab is a fully humanized, IgG1 monoclonal antibody that blocks the interaction of PD-L1 with both PD-1 and B7.1, but not the interaction of PD-L2 with PD-1 [44–46]. This antibody is approved to treat the most common type of bladder cancer, urothelial carcinoma. Dendritic cell-derived exosomes (Dex), with surface expression of MHC-peptide complexes, are also able to interact with immune cells. These nanometer-sized Dexs are able to mediate immune response in patients as a feasible and safe immunotherapy for cancer [47]. Outside the cancer field, it is worth recalling that the presence and regulatory capacity of immune checkpoint proteins is established in the circulation and atherosclerotic lesions of cardiovascular patients as well. Hence, pharmacological modulation of these proteins offers additional opportunities to control pro-inflammatory immune responses in atherosclerosis [48].

The autophagy pathway and proteins balance the beneficial and detrimental effects of immunity and inflammation, and thereby protect against infectious, autoimmune and inflammatory diseases [49]. These pathways of immune cell activation and death are involved in the pharmacodynamics of anticancer drugs. In particular, TLR-2 and TLR-9-MyD88 signalling pathways have a central role in initiating the acute inflammatory response to the immunogenic form of apoptosis induced by doxorubicin. By contrast, the inflammasome does not have a major role in doxorubicin-induced acute inflammation, providing important new insights into how the innate immune system senses immunogenic apoptotic cells [50].

4 Emerging Areas for Pharmacological Intervention in Innate Immune Cells

Active research in the fields of immunology, pharmacology and drug development is devoted to the identification of new targets as well as the repositioning of existing therapeutics. Selected areas for potential pharmacological intervention are discussed in the following and summarized in Table 2.

Table 2 Emerging areas for identification of novel immunopharmacological targets

Research field	Comments	Section in this chapter
Distribution of monocyte subsets	Dynamic changes occur in inflammatory disease and may be regarded as risk biomarkers	4.1
Immuno-metabolism	Metabolic pathways are major determinants of the behaviour of immune cells, which in turn are major determinants of metabolic homeostasis. The intricate links between immunology and metabolism may offer a new perspective for therapeutic options in a variety of disease conditions	4.2
Pharmacological macrophage targeting	A number of therapeutic strategies may target macrophage polarized activation occurring *in vivo* in health and disease	4.3
Inflammatory pathways in hypertension	ACE, target of established vasoactive agents, is involved in macrophage expression of cytokines and shapes the immune response to hypertension	4.4

4.1 Monocyte Subsets

Based on recent evidence [51], the discrete nature of human monocyte subsets may be best represented as continuous changes rather than incremental differences as described in the literature [52, 53]. Dynamic changes of monocytes subsets may occur during the course of disease such as major systemic inflammation [51]. Importantly, the frequency and phenotype of human monocyte subsets may be regarded as a useful biomarker for clinical outcomes in inflammatory and cardiovascular diseases [54, 55]. In fact, a large prospective study in a general population (n = 659) demonstrates that subjects who had the highest tertile of the pro-inflammatory classical $CD14^{++}CD16^{-}$ monocytes had the lowest event-free survival during a 17-year follow-up period predicting incident cardiovascular events independently of other risk factors [56]. Hence, monocyte phenotypes are likely to change following pharmacological treatment. For example, an extensive *in vitro* study highlights that overnight exposure to glucorticoids (fluticasone propionate) induces a specific anti-inflammatory phenotype of monocytes with characteristic functions [57]. Accordingly, distribution of monocyte subsets is likely to be skewed over a course of glucocorticoid therapy in patients. Vallelian et al. [58] found that glucocorticoid treatment of monocytes *in vitro* and in patients on glucocorticoid-pulse therapy polarizes monocytes into a M2/alternatively activated phenotype with high hemoglobin-scavenger receptor (CD163) expression and enhanced hemoglobin clearance and detoxification.

4.2 Immunometabolism

Metabolic and immune systems are among the most fundamental requirements for survival [59]. The field of immunometabolism aims to explore metabolic pathways in immune cell function and has thrived over the last decade, revealing the major roles of immune cells in metabolic homeostasis as well as the impact of specific metabolic pathways on immune cell development, fate, and behaviour. Local and systemic metabolism is integrated at the cellular level to regulate immune cell function. Cell types, mediators and pathways orchestrate the immunological-metabolic cross-talk, which highlights emerging immunological targets for treatment of metabolic diseases. Therefore, therapeutic opportunities emerge for a number of diseases, including metabolic and cardiovascular diseases, autoimmunity, and cancer [60, 61] For instance, the immune responses of dendritic cells has just been reported to be fuelled by an intracellular storage of glycogen as opposed to external glucose, which might be exploited to suppress immune reactions in autoimmune disease or hyper-inflammatory conditions [62]. Current research is leading to potential new

players in immunometabolism such as dopaminergic signalling in peripheral blood mononuclear cells. In fact, dopamine receptor expression in these cells correlates with improved metabolic and inflammatory pattern, also showing a protective role in the development of obesity [63].

4.3 Macrophage Targeting

Macrophages are phagocytes that engulf and digest cellular debris and pathogens. They play a critical role in both innate and adaptive immunity, and hence are associated with many diseases related to the immune system. Currently there is substantial focus on macrophage activation as well as the relationship of macrophages to e.g. infection and cancer. In macrophages, which orchestrate inflammatory response, transcriptional control of inflammation enables the autonomous modulation of different functional programs, such as cell migration, antimicrobial defense, tissue repair and phagocytosis [64].

Specific macrophage-targeted therapies including the possibility to reshape deranged macrophage polarization are currently under development [65, 66]. Evidence suggests that polarized phenotypes are reversible *in vitro* and *in vivo* [67]. Macrophages derived from spontaneously differentiating monocytes display all the features of plasticity of tissue or *in vitro* induced M1 and M2 macrophages. Dexamethasone, an immunosuppressive glucocorticoid capable of potent suppression of inflammatory pathways, has been shown to polarize human blood-derived monocytes toward the anti-inflammatory (M2) phenotype [68, 69], consistent with the immunosuppressive effect of glucocorticoids on modulating macrophage activation. It is also worth noting that dexamethasone treatment in macrophages up-regulates the expression of B7-H4, a negative costimulatory molecule, in an inverse correlation with miR-125b-5p, suggesting a further suppressive mechanism of glucocorticoids resulting from interference with macrophage-to-T cell interactions [70]. These findings have been recently validated *in vivo* following local delivery from siloxane-based three-dimensional scaffold platforms [71]. Estrogen is also involved in macrophage activation and polarization in isolated cells challenged with specific immune stimuli [72]. 17β-estradiol exerts anti-inflammatory actions in human monocytes-derived macrophages. We demonstrated that 17β-estradiol-dependent estrogen receptor (ER)α activation usually induces M2-macrophage polarization, thus attenuating inflammatory responses. While challenge of LPS with interferon gamma decreases ERα levels, *in vitro* treatment with estrogen rescues the M2 phenotype after M1 polarization, but does not increase it over baseline expression [73]. We also found a role of systemic estrogen in controlling the macrophage-polarizing signaling by showing that M2-associated stimuli and activation is markedly impaired in macrophages from postmenopausal compared with premenopausal women *ex vivo* [73]. In contrast, treatment with the immunosuppressant rapamycin induces apoptosis in M2 but not in M1 macrophages, and enhances M1 surface markers and pro-inflammatory cytokines production together with a reduction of typical M2 markers [74].

Modulation of macrophage function is an off-target effect for a number of diverse therapeutic agents. For instance, PPARγ agonists used in the treatment of diabetes have been linked to M2 polarization and hence to the metabolic role of adipose tissue macrophages. PPARγ is expressed in both adipocytes and macrophages. Notably, recent studies suggest that metabolic activation in human adipose tissue produces a complex macrophage phenotype that is mechanistically distinct from classical activation, suggesting that metabolic-disease-specific pathways drive macrophage inflammation via mechanisms that are different from those operative during infection. In this context, PPARγ is a key factor that promotes lipid metabolism and limits inflammation in metabolically activated adipose tissue macrophages [75]. Rosiglitazone treatment in mice promotes macrophage infiltration and increases adipose abundance of alternatively

activated macrophages, thereby suppressing production of inflammatory mediators [76].

In a study of 63 patients, statin therapy for at least 6 weeks was associated with a reduction in the number of aortic wall $CD68^+/iNOS^+$ (M1) and an increase in the number of $CD68^+/CD163^+$ (M2) macrophages [77], thereby resulting in a shift towards atheroprotective phenotypes [78]. This study supports the notion that drug treatment affects macrophage phenotypes *in vivo*.

4.4 Immune-Mediated Hypertension

Cellular inflammation plays a critical role in the development of hypertension. Whether challenged by the developing hypertension or by other initiating agents, it has been demonstrated that innate immunity is involved in the early reaction steps [79]. Here, monocytes, macrophages and other cells detect an initial insult and react by elaborating pro-inflammatory cytokines (TNF-α, IL-12 etc) that amplify the initial response. The angiotensin converting enzyme (ACE) is involved in macrophage expression of cytokines during the initial (innate) immune response to hypertension [80]. Furthermore, ACE plays a direct role in influencing several different aspects of the immune response. This on one hand may lay the ground to a new approach for improving the immune response to a variety of stimuli, including infections and tumours. On the other hand, the beneficial effects of ACE inhibitors in treating hypertension and heart failure are well established [81, 82]. Thus, expanding knowledge on ACE and the renin-angiotensin-aldosterone system in inflammation and hypertension will provide new opportunities for pharmacological intervention [83].

5 Conclusions

The cost and lack of oral bioavailability of biotechnological drugs, the evidence that only a limited number of patients do respond adequately to them, and the cardiovascular hazard linked to prolonged NSAID use are among the main limitations of currently approved drugs to treat disease conditions linked to inflammation and immune dysfunction. By contrast, the discovery of new properties of old medications and the increasing appreciation of the links between immune cell function, inflammation and metabolism in health and disease have spurred further studies to consider novel or previously overlooked pathways as viable targets for pharmacological intervention. This research areas show much promise for the development of drugs with incremental benefit over existing treatment in inflammatory disease.

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Regulatory Mechanisms in Neutrophil Degranulation

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Abstract

Bone marrow-derived circulating neutrophils of the innate immune system extravasate through blood vessel walls to sites of infection and injury where they orchestrate a myriad of protective and destructive host responses during acute inflammation. Although neutrophils comprise the first line of defense against exogenous and endogenous insults, these abundantly produced white blood cells can damage tissues and consequently increase the severity of inflammatory diseases. Neutrophils undergo receptor-mediated respiratory burst and release inflammatory mediators by degranulation of membrane-bound secretory granules after migrating from the bloodstream in response to chemotactic signals generated at inflammatory foci. Many studies point to degranulation as the chief causative process involved in inflammatory disorders, but the underlying mechanisms remain poorly understood. We discuss the complex interplay of distal, intracellular pathways involving numerous signaling proteins that are implicated in the exocytosis of granular contents. This review summarizes current knowledge of neutrophil biology and highlights mechanisms that regulate degranulation.

Keywords

Actin cytoskeleton · Exocytosis · Granules · Granulocytes · Guanosine triphosphatases · Kinases · Myeloperoxidase · Phosphoinositides · Reactive oxygen species · SNAREs

Abbreviations

a2V	V-ATPase subunit
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BoNT	Botulinum neurotoxin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate

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GTPase	Guanosine triphosphatase
GTPγS	Guanosine 5′-O-(γ-thio) triphosphate
IL-8	Interleukin-8
LTF	Lactoferrin
MAP	Mitogen-activated protein
MARCKS	Myristoylated alanine-rich C kinase substrate
MMP	Matrix metalloprotease
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
Nexinhibs	Neutrophil exocytosis inhibitors
NSF	*N*-ethylmaleimide-sensitive factor
PI3K	Phosphatidylinositol 3-kinase
PIKfyve	FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PMA	Phorbol 12-myristate 13-acetate
PTP-MEG2	Protein tyrosine phosphatase MEG2
Q-SNARE	SNARE expressing a key glutamine residue in the SNARE-binding domain; also known as t-SNARE in exocytosis; members include syntaxins, SNAP-23, and SNAP-25
Rac	*ras*-related C3 botulinum toxin substrate
ROS	Reactive oxygen species
R-SNARE	SNARE expressing a key arginine residue in the SNARE-binding domain; also known as v-SNARE in exocytosis; members include VAMPs
SFKs	*src* family of non-receptor tyrosine kinases
SNAP	*N*-ethylmaleimide-sensitive factor attachment protein or synaptosomal-associated protein
SNARE	*N*-ethylmaleimide-sensitive factor attachment protein receptor
SV	Secretory vesicle *N*-ethylmaleimide-sensitive factor attachment protein receptor
TeNT	Tetanus neurotoxin
TLR	Toll-like receptor
VAMP	Vesicle-associated membrane protein

1 Receptor-Mediated Exocytosis of Granule-Derived Mediators from Neutrophils

Neutrophil granulocytes are highly responsive, short-lived white blood cells characterized by multilobed nuclei and tightly packed cytoplasmic secretory granules. These cells are key effector cells of the innate immune system and form the first line of defense against invasive pathogens. Derived from the bone marrow, neutrophils are phagocytic white blood cells that respond within minutes by chemotaxis to injured or infected tissue signals, and are usually the first to arrive at inflammatory sites. As sentinel cells, neutrophils migrate along and bind to adhesion molecules on endothelial cell surfaces that line blood vessel walls, then squeeze through them or their tight junctions to subsequently infiltrate the inflamed or infected tissue. Peripheral blood neutrophils are the most abundant leukocyte, accounting for 40–80% of the total population of circulating white blood cells in a healthy human. The largest proportion of the tissue-marginated pool of neutrophils exists in the lungs, where they are fundamentally important in maintaining alveolar homeostasis with lung-resident microorganisms. There is a large body of evidence indicating that neutrophils perform their role in immunity as a double-edged sword, by playing dual contrasting functions in tissue inflammation and its resolution (e.g., pathogen clearance and wound healing) [1]. This is evident in infants born with neutropenia, characterized by neutrophil deficiency, who are susceptible to life-threatening bacterial and fungal infection and require bone marrow transplantation from a suitable HLA-matched donor to survive, while conversely, accelerated accumulation and overactivation of neutrophils may be fatal

to those experiencing sepsis or those diagnosed with acute respiratory distress syndrome [1].

Neutrophil-mediated tissue damage is entirely attributed to the cellular process of degranulation and mediator release. Degranulation is defined as the receptor-mediated release of cytoplasmic mediators and occurs either intracellularly via docking (i.e., anchored) and fusion (i.e., joined) with microbe-laden phagosomes or extracellularly through the exocytotic fusion of granules with the plasma membrane. A diverse array of secreted molecules including antimicrobial proteins, proteolytic enzymes, and other pro-inflammatory substances are stored within granules or synthesized de novo in response to receptor stimulation [2]. These granule-derived mediators contribute primarily to pathogen killing following phagocytosis, and may damage host tissue following extracellular release by exocytosis (refer to Sect. 2). Concomitantly, neutrophils produce and release reactive oxygen species (ROS) by respiratory burst, and these inflammatory mediators promote pathogen clearance as well as facilitate leukocyte recruitment to the inflamed or infected area. The production of ROS serves many functions in neutrophils, and may interact with mechanisms that control degranulation. For example, using the mitochondria-targeted antioxidant SkQ1, Vorobjeva et al. [3] demonstrated the involvement of mitochondrial ROS in oxidative burst promotion of granule exocytosis.

Excessive neutrophil degranulation is a shared characteristic of several inflammatory disorders including acute lung injury, ischemia/reperfusion injury, rheumatoid arthritis, septic shock, and severe asphyxic episodes of asthma [4]. Although inhibition of neutrophil degranulation is a desirable outcome, existing therapies have not been effective in targeting this specific mechanism in neutrophils. Altogether, targeted therapies aimed at inhibiting specific signaling pathways, including calcium (Ca^{2+}) and phospholipid signaling [5, 6], involved in granular exocytosis in neutrophils, that would ultimately result in downregulation of the degranulation response, may prove effective in attenuating undesirable inflammatory sequelae.

1.1 Four Distinct Types of Neutrophil Granule Populations

Neutrophil granules are categorized as primary, azurophilic or peroxidase-positive granules (the latter nomenclatures are based on their affinity for the dye azure A or the presence of the marker, myeloperoxidase [MPO], respectively), secondary or specific granules, gelatinase or tertiary granules, and secretory vesicles (SVs). Granules are released in an hierarchical order from neutrophils based on their developmental stage (reviewed in Cowland and Borregaard [7] and Scapini et al. [2]). Primary granules, as their name indicates, are the first to originate during granulopoiesis at the promyelocyte maturation stage, and contain the oxidant enzyme MPO (stored in azurophilic granules), the serine proteases cathepsin and elastase, as well as other neutrophil-derived bactericidal and cytotoxic mediators [2]. Both MPO and elastase are associated with tissue damage and their presence in blood and tissues is considered a hallmark of systemic inflammation [8]. Secondary and tertiary granules encapsulate an arsenal of antimicrobial substances such as lysozymes, express common adhesion molecules (e.g., CD11b/CD18) on their surfaces, and share classes of functional proteins including vesicle-associated membrane protein 2 (VAMP-2) that play a role in membrane docking and fusion [2]. Despite having similar histological and morphological traits, secondary and tertiary granules are often differentiated using gradient density centrifugation techniques that apply the concept of buoyancy to separate molecules [9]. Finally, secretory vesicle membranes are rich in fMLP receptors and the internal presence of the major human blood plasma protein, serum albumin implies that they form by endocytosis of extracellular fluid [10]. All the above-mentioned granule subtypes remain immobilized in the cytoplasm until phagosomal or plasma membrane receptors transmit signals through a cascade of molecular switches to cytosolic signaling pathways, resulting in actin cytoskeleton-mediated movement of the granules to their site of secretion (Fig. 1).

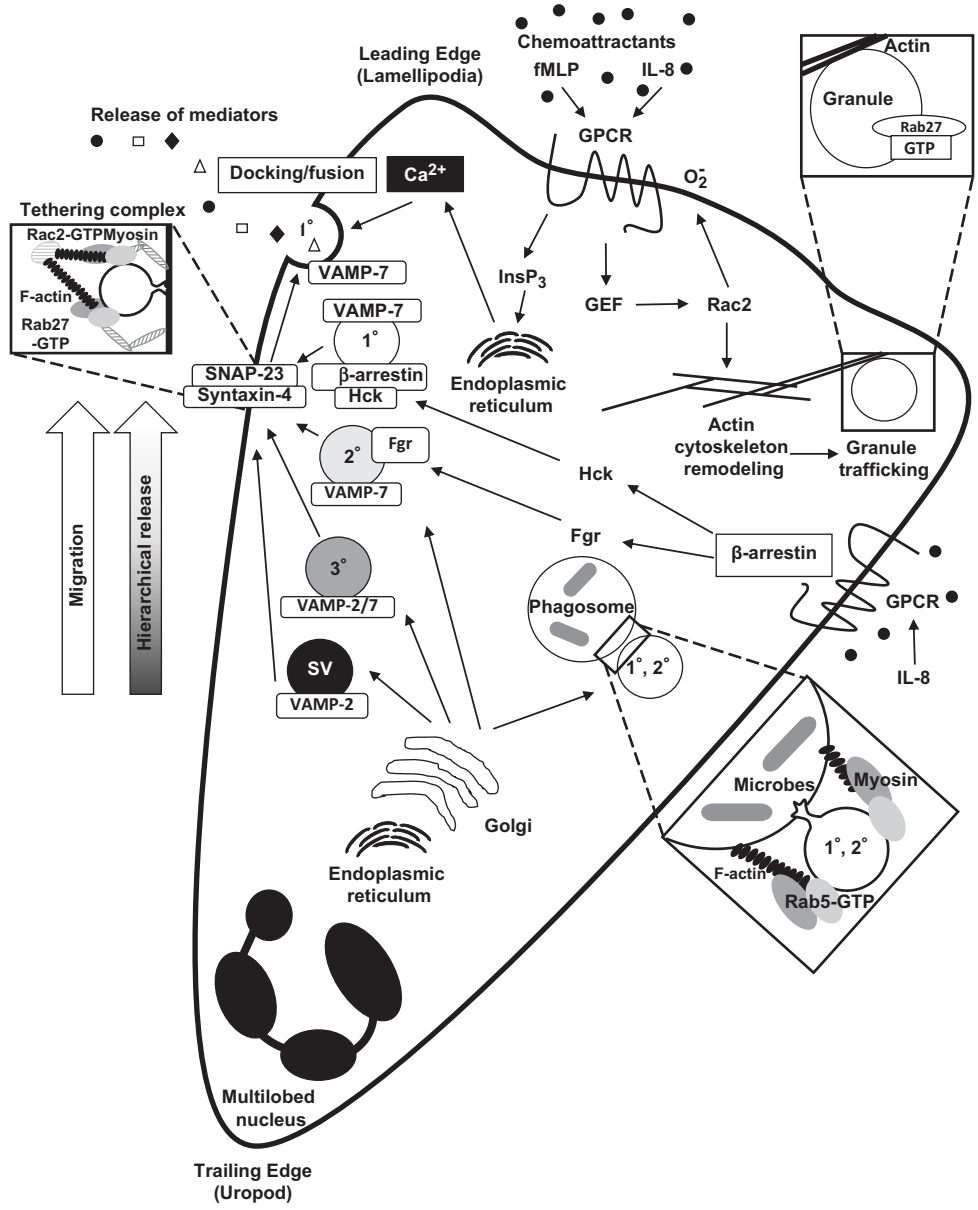

Fig. 1 Schematic representation of postulated regulatory mechanisms and signaling pathways involved in polarized neutrophil degranulation. Activation of GTPase and SNARE signaling pathways play a role in Ca^{2+}-dependent neutrophil degranulation, beginning with binding of a chemoattractant (e.g., fMLP, IL-8, etc.) to a GPCR on the plasma membrane. Chemoattractant exposure causes polarization of the neutrophil to form a leading edge with lamellipodia, in which active actin reorganization takes place, and a trailing edge with uropod formation. Rac is proposed to regulate actin remodeling at the leading edge, as well as granule movement and superoxide release. This receptor-ligand interaction initiates a signal transduction cascade through multiple overlapping and non-redundant signaling pathways that selectively regulate the exocytosis of granule subsets (i.e., primary, secondary, and tertiary granules, as well as secretory vesicles) and the release of their mediator contents. The selective mobilization of primary granules via Rac2 activation by the G protein-mediated GEF is an example of a non-redundant pathway. During hypothesized Rho and Rab GTPase activation, F-actin directs the transport of granules and facilitates their docking and fusion with phagosomes or the plasma membrane for mediator release. Membrane transport facilitated by Rab5 and Rab27 GTPase may differentiate between target membranes, and attach vesicles to

1.2 Four Discrete Stages Involved in Neutrophil Exocytosis

Degranulation from neutrophils may occur through exocytosis or necrosis, leading to release of granule contents or entire intact granules, respectively. Exocytosis, which is defined as the final step of granule fusion with the phagosomal or plasma membrane, occurs through either regulated or constitutive pathways [11]. Regulated exocytosis involves receptor stimulation of granule release, while constitutive exocytosis does not depend on receptor-mediated signaling mechanisms. All cells exhibit constitutive exocytosis, while only specialized secretory cells undergo regulated exocytosis.

In regulated exocytosis, binding of secretagogues to specific neutrophil receptors prompts the transmigration to and subsequent docking and fusion of granules with either phagosomal or plasma membranes where they release their payload (i.e., mediators). This process is generally considered to occur through a series of four discrete stages [12]. The first stage involves the actin cytoskeleton remodeling and microtubule assembly-dependent trafficking of cytoplasmic granules to the target membrane [13]. The outer surface of the granule must be near the inner surface of the desired membrane to initiate Rab and *soluble N*-ethylmaleimide-sensitive factor- (NSF) attachment protein (SNAP) receptor (SNARE) protein-mediated tethering and docking [14] in the second stage prior to contact and ultimate fusion of the two lipid bilayers. The docked granules undergo a series of preparatory reactions (i.e., they become "primed") both to acquire fusion competence and to ensure rapid fusion in the third stage. During the fourth and final stage, fusion of the granule with its target membrane forms a continuous lipid bilayer and promotes the development of a reversible (i.e., able to open and close) fusion pore that facilitates cargo release into the extracellular environment [15]. Completion of these steps not only increases the total surface area of the cell, but also exposes the granule's interior membrane to the exteriorof the cell.

The presence of the energy-rich molecule guanosine triphosphate (GTP), hydrolysis of adenosine triphosphate (ATP) and elevated levels of the second messenger Ca^{2+} represent the minimum functional requirements for intracellular trafficking and exocytosis of neutrophil granules [16, 17]. Target molecules including Ca^{2+}- (e.g., annexin and calmodulin) and GTP- (e.g., heterotrimeric and small monomeric proteins G proteins) binding proteins that accompany the abovementioned effectors are numerous. The high-energy molecule ATP is employed by protein kinases and adenosine triphosphatases (ATPases) to phosphorylate and thus activate downstream effector molecules. Moreover, Ca^{2+}-mediated actin cytoskeleton reorganization produces a mesh that prevents aberrant targeting, docking, priming and fusion of granules with the cell periphery, and must be disassembled for granule exocytosis to occur (refer to Sects. 2.1 and 2.2). Altogether, exocytosis is a selective and energy-dependent process that serves to expel granule-derived mediators out of the cell into the extracellular milieu.

2 Protective Host Defense Mechanisms Exist to Prevent Tissue Damage

Although receptor-mediated degranulation is essential to control the vigor and duration of an inflammatory response while avoiding tissue damage (in the case of phagosome-directed degranulation), it is not the only regulatory mechanism that plays a pivotal role in host defense. Over a decade has passed since Brinkmann et al. [18] first described a novel

Fig. 1 (continued) myosin-type motors required to propel granules along "actin tracks." Finally, chemotactic mediator-bound GPCRs directly bind β-arrestin, which signals through Hck and Fgr, and these phosphoproteins also translocate to primary and secondary granules along with Hck and Fgr to induce granule movement. G protein-coupled receptor (GPCR); guanine nucleotide exchange factor (GEF); guanosine triphosphate (GTP); interleukin-8 (IL-8); inositol trisphosphate ($InsP_3$); *N*-formyl-methionyl-leucyl-phenylalanine (fMLP); secretory vesicle (SV); synaptosomal-associated protein of 23 kDa (SNAP-23); vesicle-associated membrane protein (VAMP)

mechanism that enables the efficient capture and killing of microbial pathogens and coined the term "neutrophil extracellular traps (NETs)" (reviewed in detail elsewhere: [19–21]. Neutrophil-generated NETs composed primarily of histones and deoxyribonucleic acid with primary, secondary and tertiary granule proteins (e.g., elastase and MPO) attached to its backbone are formed via ligand (e.g., interferon-α coupled with complement 5a, interleukin-8 [IL-8], lipopolysaccharide [LPS], and phorbol 12-myristate 13-acetate [PMA]) activation [18, 22–24]. These web-like nuclear structures were deemed highly effective at trapping and killing foreign bodies in vitro by concentrating them into a fibrous mesh, which consequently reduces host tissue exposure [18]. The discovery of NETs was indeed a landmark in neutrophil biology, particularly in relation to mediator release and microbicidal activity explorations, and their formation is now documented as a fundamental cornerstone of the molecular mechanisms. NETs have been shown to be central in a variety of human diseases including atherosclerosis, atherothrombosis [25], lupus-like disease [26], sepsis [27, 28], non-infectious diseases [29], and recently, rhinovirus-induced asthma exacerbations [30].

2.1 Actin Cytoskeleton Dynamics During Exocytosis

Numerous cellular activities including chemotaxis, exocytosis, and phagocytosis depend on remodeling of the actin cytoskeleton. Activated effector molecules target downstream remodeling of the actin cytoskeleton around the periphery of various secretory cell types (e.g., endocrine cells, neurons, neutrophils and mast cells) during receptor-mediated exocytosis. This dynamic, mesh-like cytoskeletal structure acts as a protective barrier against docking and fusion of abnormally accumulated granules beneath the plasma membrane and must be disassembled during exocytosis [31–33]. Studies suggest that stimulation with the bacterial tripeptide fMLP induces cortical ring assembly of typically diffuse F-actin in neutrophils, though this finding may have been attributed to their methodology [34, 35]. Authors of the latter studies used fMLP to activate live neutrophils already adhered to glass coverslips; therefore, cortical F-actin formation may have been induced by adhesion itself rather than by fMLP stimulation. Our group developed a "reverse method" of stimulating suspended neutrophils with fMLP to initiate exocytosis first, and then fixing them in suspension immediately prior to placement on glass coverslips for cell adherence and subsequent staining [36]. This technique allowed us to visualize actin cortical ring assembly and disassembly in resting and cytochalasin B/fMLP-stimulated neutrophils in suspension prior to adherence. Staining with rhodamine-phalloidin revealed an elaborate interconnecting meshwork of cortical F-actin that prevented granule docking and fusion in resting, unstimulated cells in suspension, that was disassembled during receptor activation by fMLP, leading to exocytosis.

Actin filament remodeling has also been implicated in directional neutrophil migration (reviewed in Affolter and Weijer [37]). Elevated levels of polymerized actin are apparent on the leading edge of a neutrophil crawling along a chemotactic gradient toward specific inflammatory foci. Remarkably, uniform concentrations of chemoattractants produce an F-actin-dependent polarized response (i.e., competing signaling pathways promote a 'frontness' and a 'backness' at opposite poles of the cell [38]) in a mobile neutrophil, and its 'frontness' is maintained by the continuous production of activated effector molecules (e.g., 3′-phosphoinositol lipids) in the plasma membrane [39, 40]. A comprehensive immunoblot analysis by Jog et al. [41], demonstrated that the actin cytoskeleton regulates exocytosis of all four neutrophil granule subtypes (i.e., azurophil, gelatinase, secretory, and specific) described above by controlling their access to the plasma membrane and this work partly supports our findings. In agreement with the concept that cytoplasmic F-actin formation is a requirement for primary granule exocytosis, our colocalization analyses revealed an association between fluorescein isothiocyanate-conjugated anti-CD63-labeled granules and polarized F-actin

formed by chemoattractant stimulation [36]. Therefore, the formation of the so-called "actin tracks" that mediate cytoskeleton remodeling in a chemotaxis-dependent manner may also mediate directed granule movement to the plasma membrane for polarized exocytosis.

2.2 Calcium Signaling-Mediated Exocytosis

Second messenger signaling is essential for exocytosis and sufficient neutrophil granule release and can be achieved by increasing intracellular Ca^{2+} concentrations using Ca^{2+} ionophores (e.g., A23187 or ionomycin). The existence of a functional hierarchy of granule subtypes allows for their sequential release (i.e., SVs > tertiary granules > secondary granules > primary granules) in response to increasing Ca^{2+} levels [42–44], which appears to be differentially regulated by specific intracellular signaling pathways. Activation of neutrophil receptors including seven transmembrane-spanning G protein-coupled receptors (GPCRs; e.g., fMLP ligand coupled to the formyl peptide receptor) and chemokine receptors (e.g., the IL-8 receptor, CXCR1) is known increase intracellular Ca^{2+} levels [45, 46]. Although specific target molecules for Ca^{2+} in the context of neutrophil degranulation are likely to be numerous and have not yet been fully identified, several Ca^{2+}-binding proteins including annexins, calmodulin and protein kinase C have been proposed to play a prominent role in granule translocation and exocytosis in neutrophils [47–51]. Taken together, Ca^{2+} is an essential signaling compound for regulated endocytosis processes in neutrophils and a variety of other excitable cells.

2.3 Role of Phospholipids in Regulating Neutrophil Degranulation

The regulatory role of phospholipids, especially polyphosphoinositides, in neutrophil degranulation has been studied extensively [52–54]. The mammalian phosphatidylinositol transfer protein mediates phosphatidylinositol transport between membrane compartments [55]. Phosphatidylinositol 4,5-bisphosphate (PIP_2) not only regulates the actin cytoskeleton but also acts as a substrate for both phospholipase C and phosphatidylinositol 3-kinase (PI3K) [55]. Regions likely rich in PIP_2 (e.g., plasma and granule membranes) provide important binding sites for pleckstrin homology-containing intracellular signaling molecules (e.g., the guanine nucleotide exchange factor [GEF], Vav, which signals its downstream effector, Rho family guanosine triphosphatases (GTPases) [56–58], which regulate many cellular functions by catalyzing hydrolysis of GTP to guanosine diphosphate (GDP). Using permeabilized HL-60 cells, Fensome et al. [59] demonstrated that PI3K catalytic subunit γ (PI3Kγ)-induced production of PIP_2 is necessary for and restores granule exocytosis. Moreover, phospholipase D and its product, phosphatidic acid, are of central importance for neutrophil degranulation and have been implicated in the release of primary and secondary granules [60]. Membrane curvature generation and membrane lipid remodeling are key for intracellular trafficking and other degranulation-related processes such as membrane fusion and scission [61]. Phosphatidic acid formed from lysophosphatidic acid regulates intracellular membrane fission and although the exact molecular mechanisms remain unknown, it has been postulated that phosphatidic acid plays a role in the bending, fission and local curvature of the membrane [62]. The lipid kinase FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) generates phosphatidylinositol-3,5-bisphosphate and phosphatidylinositol-5-phosphate [63]. While lysosomal-associated membrane protein 1-positive lysosomes were shown to become engorged, primary, secondary and tertiary granule secretion was not affected by PIKfyve inactivation [63]. Although PIKfyve has little direct control of granules, its deficiency may impair their biogenesis and function in neutrophils [63]. Altogether, phospholipids play a central role in degranulation, and are fundamental regulatory molecules in signaling to protein kinases for phosphorylation of downstream targets.

2.4 Phosphorylation Signaling and Protein Kinases in Granule Exocytosis

Receptor-mediated phosphorylation events by kinases, a highly conserved mechanism for orchestrating neutrophil degranulation, commence at the level of receptor activation and lead to granule exocytosis. Kinases play a major role in cellular activation processes and these key regulatory enzymes themselves are often phosphorylated upon activation by upstream molecules. In addition, different amino acid resides in effector molecules determine the kinase-substrate interaction affinity. Kinase phosphorylation specificity involves the transfer of a high-energy phosphate group from a donor molecule (i.e., intracellular ATP) to an amino acid site causing conformational changes and ensuing activation of the effector molecule. Formyl peptide receptor stimulation by the fMLP ligand triggers phosphorylation of various kinases and subsequent downstream activation of their respective effector pathways. Two distinct families of kinases, serine/threonine and tyrosine, play a role in cell receptor signaling by transferring a phosphate group to specific amino acid residues (Ser/Thr or Tyr) in target proteins. The latter family is further subdivided into receptor-associated and nonreceptor or cytoplasmic (e.g., Src) tyrosine kinases based on the presence of a transmembrane domain and intracellular location, respectively.

The Src family of non-receptor tyrosine kinases (SFKs) regulate receptor-mediated exocytosis of granule-derived mediators from neutrophils. Three neutrophil-expressed, fMLP-activated SFK members, Hck, Fgr, and Lyn, have been implicated in the release of proinflammatory mediators from activated neutrophils in vitro [64]. Cytosolic Hck and Fgr kinases translocate to and associate with MPO-positive primary and lactoferrin (LTF)-containing secondary granules [65, 66], while leading edge recruitment of Lyn controls neutrophil adhesion [67]. In addition to their actin polymerization and superoxide anion releasing capabilities, Hck and Fgr also regulate activation of Vav1, an upstream GEF activator of Rac GTPases (see Sect. 2.3) [58]. Selective recruitment of SFKs implies that diverse signaling pathways have evolved to facilitate exocytosis of each granule subset from neutrophils. The SFK inhibitor PP1 was shown to prevent the release of all granule subtypes, except tertiary granules, from fMLP-stimulated neutrophils [68]. Using $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ triple knockout mice-derived neutrophils, Mocsai et al. [68] also demonstrated a p38 mitogen-activated protein (MAP) kinase activity-correlated deficiency in the release of LTF from secondary granules, and suggested that SFKs carry out their functions upstream from p38 MAP kinases. While the p38 MAP kinase inhibitor, SB203580, decreased both primary and secondary granule release, treatment with the extracellular signal-regulated kinases (ERKs) 1 and 2 activity-blocking MAP kinase inhibitor PD98059 did not affect primary and secondary granule nor secretory vesicle exocytosis from fMLP-stimulated neutrophils [68]. The latter finding suggests that ERK1/2 are not involved in granule release from fMLP-stimulated neutrophils. Overall, secretory vesicle and specific granule exocytosis depend on p38 MAP kinases, and Hck, Fgr, Lyn, as well as p38 MAP kinases likely execute their functions near the formyl peptide receptor to regulate an early step of the exocytosis process.

In contrast, incubation of neutrophils with the periodontal pathogen *Filifactor alocis* (live and heat-killed) was recently shown to induce granule exocytosis via interaction with Toll-like receptor 2 (TLR-2) and through ERK1/2 and p38 MAP kinase activation [69], suggesting that more complex secretagogues than fMLP may rely on ERK1/2 for degranulation. Moreover, Potera et al. [70] found that tumor necrosis factor-α (TNF-α) stimulation resulted in ROS generation and gelatinase granule mobilization, and this priming likely involved both ERK1/2 and p38 MAP kinases [69]. Low-level ROS production by priming was also reported by Volk et al. [71], who found that low concentrations of TNF-α increased expression of CD11b located at the cell surface through degranulation. Although TNF-α was not solely sufficient to mobilize primary granules, secondary stimulation with fMLP in addition to TNF-α increased the release of granule-derived elastase

[70]. Elastase release was even more pronounced when ROS from (NADPH) oxidase was absent, which suggests that the latter enzyme plays a role in decreasing elastase-related inflammation [70]. Overall, kinases are essential signaling molecules that play a modulatory role in neutrophil degranulation.

2.5 β-Arrestins Activate Granule Exocytosis Signaling Pathways

Cytosolic β-arrestin scaffold proteins play an important role in chemokine receptor-mediated signaling [72] and promote primary and secondary granule exocytosis from neutrophils [73] by acting at plasma and granule membranes. These phosphoproteins initially characterized for their role in endocytosis of the high-affinity IL-8 chemokine receptor, CXCR1, uncouple GPCR from heterotrimeric G proteins and bind to the CXCR1 receptor's cytoplasmic tail [73, 74]. Indeed, treatment with high concentrations of IL-8 can promote granule release via CXCR1 through Hck and Fgr-mediated processes [72]. Interaction of both β-arrestins 1 and 2 with cofilin and chronophin were deemed essential in actin reorganization, pseudopodia polarization and subsequent directional migration of neutrophils [75]. Increased neutrophil infiltration to the inflammatory site was observed in mice lacking β-arrestin 2 [76]. Altogether, β-arrestins play key roles in chemokine-mediated migration and degranulation of neutrophils.

2.6 Requirement of Guanosine Triphosphatases in Granule Exocytosis

The binding of GTP to intracellular effector molecules is also needed for exocytosis of granules. Granule-derived mediator secretion has been reported in non-hydrolyzable or slowly hydrolyzable G protein-activating analog guanosine 5′-O-[γ-thio]triphosphate (GTPγS)-treated permeabilized or patch-clamped neutrophils [77] suggesting that GTP-binding proteins (e.g., GTPases) are important in granule trafficking within and release from neutrophils. Heterotrimeric G proteins and *ras*-related monomeric GTPases are among the most extensively studied and best understood families of regulatory GTPases identified in eukaryotic cells [78]. Heterotrimeric G proteins are typically associated with the cytosolic face of the plasma membrane and transmit receptor signals to the cytoplasm, whereas the *ras*-related GTPases likely found near the actin cytoskeleton, in the cytoplasm, or on membranes play diverse regulatory roles in neutrophil activation. *Ras*-related GTPases behave as on–off molecular switches that control intracellular signaling events, and when turned on via GTP-binding, these small monomeric molecules trigger the association of other GTPases with their respective sites. Hydrolysis of GTP to GDP on the *ras*-related GTPase activates the next effector molecule in the signaling pathway. Noted effects of the poorly-hydrolyzable yet potent exocytosis stimulator, GTPγS, indicate that GTP-bound forms of GTPases induce downstream signaling events leading to exocytosis, rather than formation of GDP by GTP cleavage, in neutrophils and other myeloid cells [16, 79]. Interestingly, GTPγS strongly inhibits Rab GTPases [80], suggesting that distally positioned *ras*-related GTPases (such as Rho-related GTPases, which are strongly activated by GTPγS) are required rather than Rab GTPases during the final steps of membrane fusion.

2.6.1 Rho GTPases in Cytoskeletal Arrangement and Reactive Species Generation

Three prototypical members of the Rho GTPase subfamily, Rho, Rac, and Cdc42 [81–83], fill major regulatory roles in chemotaxis, actin cytoskeleton reorganization, and ROS release. Rho GTPases may be inhibited upon glucosylation by several bacterial toxins including *Clostridium difficile* toxin B and *Clostridium sordellii* lethal toxin, which have been useful tools in deciphering the role of Rho GTPases in cellular functions [84, 85]. Three ras-related C3 botulinum toxin substrate (Rac) isoforms, Rac1, Rac2, and Rac3,

exist in neutrophils, and Rac1 and Rac2 share 92% amino acid sequence homology (the last 10 amino acids in their carboxyl termini differ). This high sequence homology contributes to the interchangeably regulatory roles of Rac1 and Rac2 in pathogen-induced superoxide anion production through NADPH oxidase activation [86–89]. Although Rac binding is undoubtedly important for activation of NADPH oxidase, it is unclear whether GTP binding by Rac is essential for the assembly of this enzyme complex. The commonly used inducer of superoxide anion production, PMA, for example, was found to have both positive [90] and negative [91] effects on Rac-GTP formation in human neutrophils. In addition, the Rac inhibitor NSC23766 [92] failed to block PMA- or fMLP-induced superoxide anion release, while it was shown to significantly decrease fMLP-induced Rac1-GTP and Rac2-GTP formation in neutrophils [36], suggesting that activation of the NADPH oxidase complex may occur without the need for GTP-bound forms of Rac protein. Moreover, Rac2 has been shown to induce F-actin formation [93] and is the preferred NADPH oxidase activator in neutrophils due to its distinct carboxyl-terminal sequence from Rac1 [94]. Rac2 also serves important selective functions in neutrophil degranulation, and homozygous gene deletion of *rac2* in murine models disrupts primary granule exocytosis from bone marrow-derived neutrophils [95]. Compensatory overexpression of Rac1 occurs in $Rac2^{-/-}$ neutrophils, suggesting that Rac1 is unable to rescue the Rac2-deficient phenotype, and emphasizes the selective and distinct (from Rac1) role of Rac2 in regulating translocation and exocytosis of primary granule from neutrophils [93, 94, 96]. Defective primary granule exocytosis from $Rac2^{-/-}$ neutrophils was attributed to actin-dependent translocation machinery that requires cytoskeletal remodeling to direct the movement of granules to the plasma membrane. Effector molecules that regulate actin rearrangement downstream of Rac2 must be identified to fully describe and enhance our understanding of the complex signaling pathways involved in Rac2-mediated release of primary granules from neutrophils.

2.6.2 Rab GTPases Catalyze Downstream Exocytotic Events

Signaling events downstream of receptors and kinases must act proximally to activate certain factors (i.e., Rab GTPases) involved in early granule transmigration to and fusion at the plasma membrane. Unlike Rho GTPases that may promote the distal steps of granule movement via actin remodeling, the large Rab family (>60 isoforms [97]) of small monomeric GTPases (and SNAREs described in Sect. 2.7) catalyze exocytotic events through SNARE molecules. The membrane-associated transport-facilitating Rab GTPases attach vesicles to myosin V-type motors, and engage "velcro" effector complexes involved in vesicle tethering (distinguished from SNARE-mediated docking) to exocytosis sites [98, 99]. Rab proteins and their effector complexes are found in granule membranes where they direct specific subsets to and select docking sites for exocytosis [98, 100]. The Rab GTPase isoforms Rab3 and Rab27 have been reported to facilitate vesicle docking at the plasma membrane of neuronal and endocrine cells [101, 102], as well as cytotoxic T lymphocytes [103], respectively. Neutrophils also express these Rab isoforms (reference). While the function of Rab3 in neutrophil degranulation is not clear, Rab27 has been extensively investigated for its role in neutrophil exocytosis.

Human neutrophil granules copurify with three Rab GTPases, Rab3a, Rab4, and Rab5a [104]. Upregulated expression of Rab3D is associated with myeloid cell differentiation into granulocytes [105]. Furthermore, the Rab3D isoform was shown to interact with the Rab3-associated kinase, which is capable of phosphorylating and consequently inactivating the Q-SNARE syntaxin-4 predominantly found at the plasma membrane of mast cells [106]. This interaction implies a negative regulatory step between Rabs and SNAREs, and offers an explanation as to why Rabs must go through a ubiquitous biochemical cycle of GTP binding and hydrolysis to serve as molecular switches for catalyzing the assembly of the protein complexes that activate fusion machinery [107]. However, defects in granule maturation but not exocytosis were noted in

Rab3D knockout mice-derived mast cells and endocrine tissues, suggesting that Rab3D may not be important in regulation of exocytosis, but rather for homeostatic maintenance of granules [108]. In contrast, Rab5a regulates intracellular fusion between granules and pathogen-containing phagosomes in neutrophils [109] and other phagocytic cells, suggesting an endosomal function [110–112].

Recently, Johnson et al. [8] identified small-molecules (i.e., nitroaromatic group-containing structures) named neutrophil exocytosis inhibitors (Nexinhibs) that prevent the interaction between Rab27a and its effector, JFC1. These small molecule inhibitors of neutrophil exocytosis and inflammation did not affect other critical immune responses including phagocytosis and NET production [8]. Certain reversible Nexinhibs (e.g., Nexinhib20) have been shown to decrease neutrophil infiltration into the liver and kidney of mice, as well as reduce levels of plasma secretory proteins released by neutrophils [8]. Cell membrane-associated adhesion molecules that mediate neutrophil adhesion are upregulated through the exocytotic process [8]. The inhibitory effect on tissue infiltration was likely due to inhibition of cell membrane adhesion molecule upregulation in endothelial cells, as well as diminished exocytosis of primary granules from neutrophils [8]. A similar decrease in plasma secretory protein levels from neutrophils was observed in Rab27a knockout mice, though the number of infiltrating neutrophils remained unaffected. Although inhibition of Rab27a-JFC1 interactions resulted in decreased neutrophil exocytosis and in vivo inflammatory activity, the innate immune functions of neutrophils were not compromised [8]. Proinflammatory components were not selectively released during degranulation; therefore, numerous components are secreted from the cell in Rab27-dependent secretion [8]. Rab27a is also known to regulate ROS production, and Nexinhibs reduced extracellular ROS production by decreasing upregulation of the NADPH oxidase subunit, cytochrome *b*558, at the cell membrane [8].

Singh et al. [113] exposed Rab27b knockout and Rab27a/b double knockout mice to chemokines (macrophage inflammatory protein 2 and leukotriene B_4) and noted impaired neutrophil migration into tissues. The Rab27a/b double knockout mice showed impaired neutrophil recruitment in the lungs, but this effect was not evident after LPS-stimulation [113]. These results indicate that Rab27b regulates chemotaxis-induced migration of neutrophils. Given that neutrophil movement from both mouse models were equally impaired, Rab27a likely functions upstream to transport granules to the cell periphery, and then Rab27b regulates their fusion with the membrane [113].

It is known that Rac and Rho GTPase cross-talk directs exploration of cellular shape space and morphological heterogeneity [114]; therefore, it would be interesting to determine whether Rho and Rab GTPases cross-talk during degranulation. Cross-talk mediated by the regulatory protein Rho GDP dissociation inhibitor α has been shown to occur between closely related Rho GTPases [115] and these signaling pathway communication mechanisms drive migration toward chemotactic stimuli in neutrophils [40, 82] and in other cell types [116, 117]. Mechanisms driving cross-talk between multiple GTPases would allow granules to spatially and temporally synchronize Rho GTPase activation, to induce "actin track" formation and subsequent Rab GTPase association, and to self-regulate their exocytosis by recruitment of actin-based motors.

2.7 SNARE Molecule-Mediated Granule Docking and Fusion in Exocytosis

The SNARE paradigm is based on the notion that intracellular receptors recognize secretory granules and guide their docking/fusion to target membranes during the final stage of exocytosis. This model states that a vesicle-associated SNARE (R-SNARE, named for its expression of a key arginine residue in the SNARE-binding domain, and formerly known as v-SNARE) protein on a SV binds (in trans) to a Q-SNARE protein (named for expression of a glutamine residue in SNARE domain) on the target membrane to

form a SNARE complex that mediates interactions between the vesicle and the plasma membrane [118, 119]. Mechanistic insights into highly conserved, membrane-bound SNARE complexes that play key roles in vesicle docking and fusion by all cell types were initially gained from in vitro studies using neuronal and yeast cell models [119, 120]. The exocytotic SNARE complex comprised of both v-SNAREs (e.g., VAMP-2/synaptobrevin) and t-SNAREs (e.g., synaptosomal-associated protein 25 kDa [SNAP-25] and syntaxin-1A) can stimulate vesicle fusion [118]. Formation of the SNARE complex requires four coiled-coil structure α helices, two from syntaxin-1A and VAMP-2 and the other two helices are derived from the SNAP-25 molecule [118]. The SNARE motif is a term used to describe the binding region of the three SNARE molecule associated with the four α helices. Membrane fusion depends on the cytosolic AAA ATPase family protein, NSF, that functions to disassemble the SNARE complex through interactions with α, β, or γ-SNAPs allowing reuse of SNARE component proteins after exocytosis [118, 119].

Remarkably, the bonds within a SNARE complex are highly stable and resistant to treatment with the detergent sodium dodecyl sulphate [121]. However, SNARE molecules including VAMP/synaptobrevin are targeted and susceptible to cleavage by clostridial neurotoxins containing zinc endopeptidase activity including botulinum neurotoxins (BoNTs) and tetanus neurotoxin (TeNT) [122]. The deleterious effects of BoNTs and TeNT holotoxins on intracellular SNARE molecules likely form the molecular basis of flaccid and spastic paralyses, respectively. Both BoNTs and TeNT specifically act on neuronal cells that possess membrane ganglioside-binding sites for the heavy chain components of these neurotoxins [123]. Neuronal and non-neuronal tissues widely express VAMP-2 [124] and other SNARE isoforms (e.g., syntaxin-4 and SNAP-23) were identified in cells not associated with the nervous system [125].

Neutrophils also express several identified SNARE isoforms including VAMP-2 and syntaxin-4 [44]. The latter study demonstrated that VAMP-2 was predominantly localized in $CD35^+$ SVs and tertiary granules, and Ca^{2+} ionophore stimulation caused movement of these $VAMP\text{-}2^+$ vesicles to the plasma membrane. Using reverse transcriptase-polymerase chain reaction, Martin-Martin et al. [126] detected several human messenger ribonucleic acid encoding syntaxin genes (i.e., 1A, 3, 4, 5, 6, 7, 9, 11, and 16) in both neutrophils and in the neutrophil-like human promyelocytic leukemia (HL-60) cell line. Antibodies and permeabilizing agents are powerful tools for exploring the exocytotic functions of SNARE molecules in neutrophils. Antibodies against SNAP-23 and syntaxin-6 applied to Ca^{2+}- and GTPγS-stimulated electropermeabilized neutrophils revealed that these SNARE isoforms are important in regulating secondary granule exocytosis [127]. Moreover, Ca^{2+}- and GTPγS-induced exocytosis from electro-permeabilized neutrophils were effectively blocked by syntaxin-4 and VAMP-2 antibodies [128]. The latter two exocytosis-focused studies employed flow cytometry analyses to determine whether surface expression of the primary granule marker, CD63, and the secondary granule marker, CD66b, was upregulated in Ca^{2+}- and GTPγS-stimulated cells. Anti-VAMP-2 did not affect $CD63^+$ primary granule exocytosis but prevented CD66b upregulation on the surfaces of stimulated cells indicating the involvement of VAMP-2 in secondary granule exocytosis. Syntaxin 4- and VAMP-1/7-mediated release of primary granules has also been reported [127–129].

The Munc13-4 tethering factor functions via a Ca^{2+}-favoured syntaxin 7 and VAMP-8 interaction [130]. Neutrophils lacking Munc13-4 display exocytotic defects and improper movement of late endosomal proteins to the phagosome [130]. Furthermore, Munc13-4 directly regulates TLR-9-dependent signaling that increases neutrophil adhesion molecule/receptor CD11b at the cell surface [130]. Altogether, these findings indicate that Munc13-4 regulates TLR-9 activation and mediates endosomal release [130]. In a separate study, Johnson et al. [131] demonstrated that Munc13-4 is a Rab11-binding protein that regulates $Rab11^+$ vesicle trafficking and docking at the plasma membrane during exocytosis. Therefore, the interaction between Rab11 and

Munc13-4 is a potential target for controlling inflammation [131]. It has been demonstrated that Munc13-14 binds to Rab27a [131]. Using Rab27a or Munc13-4 deficient neutrophils, Ramadass et al. [132] showed that GM-CSF cytokine-dependent priming did not induce exocytosis in the absence of Rab27 and interestingly, exocytosis was present in Munc13-4 deficient cells. Rab27a and its effector Munc13-4 were not required for Cd11b mobilization in GM-CSF-primed neutrophils unless the cells were stimulated with nucleic acid-sensing TLR ligand, which suggests that both Rab27a and Munc13-4 play a role in endocytic TLR maturation [132]. Furthermore, Rab27a is required for proper matrix metalloprotease 9 (MMP-9; stored in specific and tertiary granules) and MPO exocytosis [132]. Although the secretory factors Rab27 and Munc13-4 mediate MMP-9 and MPO release, their involvement in exocytosis after GM-CSF priming is less consistent [132].

In addition, characteristics of VAMPs 4 [133], 5 [134], 7 [135–138], and 8 [139–141] have been described in non-neuronal tissues. We and others have reported high levels of VAMP-7 expression in most granule subsets and have proposed that the exocytotic release of primary, secondary and tertiary granules is mediated by this SNARE isoform [79, 129]. Treatment of streptolysin O-*permeabilized* human neutrophils with low anti-VAMP-7 antibody concentrations blocked the release of pre-stored MPO, LTF, and MMP-9 mediators from neutrophil granules, similar to eosinophils [142]. The above findings suggest that the SNARE VAMP-7 broadly controls exocytotic trafficking of many granule populations in neutrophils. Similarly, cytokine release is dependent on the Q-SNARE STX3 in differentiated HL-60 cells that resemble neutrophils [143]. STX3 was required for the release of IL-1α, IL-1α, IL-12β, and CCL4, as well as MMP-9 exocytosis in gelatinase granules of differentiated HL-60 cells [143]. Furthermore, STX3 has been shown to partly colocalize with and regulate exocytosis of gelatinase granules and secretory vesicles [143]. Altogether, SNARE isoforms bind several cognate partners and fill key binding roles in membrane fusion.

2.8 Potential Role of Other Regulatory Molecules in Neutrophil Exocytosis

Other regulatory molecules involved in neutrophil degranulation include the a2 isoform of V-ATPase (a2V), the protein tyrosine phosphatase MEG2 (PTP-MEG2), and myristoylated alanine-rich C kinase substrate (MARCKS). The putative endosomal pH sensor a2V is located chiefly on primary granules and to a lesser extent on the surface of other three granule subtypes (i.e., secondary and tertiary granules, as well as SVs) [144]. Indeed, a2V was not present on the surface of resting neutrophils, suggesting that a2V may be a valuable biomarker for activated neutrophils [144]. The presence of a2V on the plasma membrane when cells were incubated with a weak base indicates that the granule-associated a2V isoform is important for granule fusion and for maintaining proper intraluminal pH gradients [144]. The nonreceptor transport vesicle (e.g., primary, secondary, and tertiary granules)-associated PTP-MEG2 facilitates membrane trafficking throughout the cell via NSF dephosphorylation [145]. NSF is a cytosolic ATPase that allows repeated membrane fusion events by cycling SNARE proteins between their bound and unbound states [146]. The latter study was the first to show that NSF possesses a tyrosine-phosphorylated residue, and that dephosphorylation of this key amino acid residue by PTP-MEG2 induces binding of a different cytosolic protein (i.e., α-SNAP), also required for SNARE cycling between confirmation structures [146]. Both NSF and α-SNAP proteins are needed to promote vesicular fusion with either phagosomal or plasma membranes. Enlarged granules were observed in mutant NSF-expressing cells, indicating that a dephosphorylated form of NSF bound to α-SNAP is required for repeat homotypic fusion of granule with target membranes [146]. Additionally, PTP-MEG2 activation by the polyphosphoinositide PIP_2 further implicates the involvement of this intracellular signaling molecule in granule membrane fusion events [146]. The protein kinase C- and Ca^{2+}-calmodulin-regulated protein MARCKS was originally known for its actin filament cross-

linking abilities [147] and is now considered a requirement for the release of primary granules from neutrophils [148]. This finding may provide new insights into a novel mechanism that integrates Ca^{2+}-induced exocytosis with actin polymerization in neutrophils.

3 Future Prospects

Our understanding of the regulatory mechanisms underlying neutrophil degranulation has progressed considerably over the past decade, and most of what we know about the signaling pathways activated during this process is presented in Fig. 1. Neutrophils and their granule-derived products are key components involved in the regulation of several autoinflammatory diseases and infectious disorders [149]. Recently, several reports in the clinical literature have described the characteristics and functions of a variety of intracellular signaling molecules present in neutrophil granulocytes. These regulatory molecules mediate the transmigration of membrane-bound SVs and facilitate their docking and fusion with either a microbe-laden phagosome or with the plasma membrane for cargo exocytosis. Bacterial toxins target and inhibit the function of many of these molecules; therefore, exotoxins and endotoxins may be employed to modulate neutrophil degranulation. Despite these new contributions to the field of clinical immunology, many challenges in identifying exact molecular mechanisms by which neutrophils traffic granules and release inflammatory mediators remain. We have highlighted several gaps in current knowledge including the roles of Rab3 and Rab27, the docking capabilities of Rab5, and the specific target molecules for Ca^{2+} during neutrophil degranulation.

Further analysis of the activated signaling pathways involved granule exocytosis from neutrophils is required for the development of new drugs that inhibit neutrophil degranulation in both airway diseases and inflammatory disorders. Although multiple inhibitors have been developed for proximal signaling in neutrophils (e.g., the p38 MAP kinase inhibitor JNJ 28610244 [150] and the broadly specific phosphodiesterase inhibitor pentoxifylline [151]), these are not specific for degranulation and can block other neutrophil activation mechanisms such as cell migration, nuclear transcription, respiratory burst, and shape change. To date, at least two inhibitors of the distal signaling pathway involved in neutrophil exocytosis have been reported: the cell-permeable fusion protein, TAT-SNAP-23 [152], and the exocytosis-specific Nexinhibs, described above, that interfere with Rab27a-dependent secretory functions in neutrophils [8]. Intravenous injection of TAT-SNAP-23 was shown to ameliorate neutrophil degranulation-induced acute lung injury in rats, as indicated by reductions in both CD18 surface expression and in bronchoalveolar lavage fluid-derived granule proteins [152]. These molecules have considerable potential for advancing the development of adjuvant therapeutic strategies targeted at acute inflammation, arthritis, sepsis, and other conditions characterized by the release of secretory proteins [8, 151].

Academics continue to collaborate with clinician scientists and patients in search of clinical biomarkers and therapeutic agents for several autoimmune, inflammatory and infectious diseases. However, more concerted and coordinated efforts are required to close the gap between mechanistic understanding and clinical practice. Researchers and clinicians interested in the study of neutrophil degranulation have the potential to build on the great progress already made and we are excited to see the impact that future discoveries will have on human health over the next decade.

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Immune Mechanisms in Atherosclerosis and Potential for Immunomodulatory Therapies

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Abstract

A considerable body of pre-clinical and clinical research data support a pivotal role played by immune-inflammatory responses in atherosclerosis formation and development. Recent clinical trial results confirm the feasibility of targeting immune pathways for the therapeutic control of the pathology. In this chapter, we discuss the key immune-inflammatory mechanisms involved in atherosclerosis development and progression and plaque destabilization. We discuss the anti-inflammatory pleiotropic effects of lipid-lowering drugs and potential therapeutic strategies for the direct control of vascular inflammation. Finally, we discuss vaccination approaches in atherosclerosis and critical questions that should be addressed in future investigations.

Keywords

Atherosclerosis · Canakinumab · Inflammation · Interleukin-1 · Methotrexate · Statins · PCSK9 inhibitors · Vaccination

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1 Atherosclerosis

Atherosclerosis and related cardiovascular diseases (CVDs) are the principal cause of death worldwide [1]. The atherosclerotic lesion, known as atheroma, consists of the accumulation of cells, debris, lipids and extracellular matrix (ECM) components in the inner layer of the artery, inducing a thickening of the intima in small and medium sized arteries. Symptomatic pathologies occur when the atheroma rupture occludes the blood flow through the arteries and, depending on the location of the obstruction, the blood cessation can lead to more severe complications such as myocardial infarction and death [2].

At first, atherosclerosis had been considered exclusively as an arterial occlusive disease in which the accumulation of cells, mainly smooth muscle cells (SMCs) and macrophages, in conjunction

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with lipids represents the central mechanism in the formation of the stenosis. For decades, the degree of the stenosis, combined with the manifestation of symptoms represented the only tools available for the assessment of atherosclerosis [2].

The cholesterol hypothesis is the most accredited pathophysiological theory for the development of atherosclerosis. It postulates that hypercholesterolemia is the causal factor in disease development. Indeed, elevated blood cholesterol levels, in particular low-density lipoprotein (LDL) are directly correlated to adverse cardiovascular events (ACEs) and denote an incontrovertible risk factor. Most importantly, normalising circulating cholesterol levels significantly reduces the burden of disease and its clinical consequences [3]. In addition to actively lowering modifiable risk factors, the identification of multiple molecular pathways that regulate cholesterol metabolism has led to the development of effective drug therapies. Statins are the main class of lipid-lowering drugs developed to reduce circulating levels of LDL and are effective in treating CVDs. More recently, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved monoclonal antibodies that inactivate proprotein convertase subtilisin/kexin type 9 (PCSK9) as new drugs to reduce circulating LDL. Despite these substantial advances in controlling circulating LDL levels, the recurrent rate of ACEs is approximately 20% within 3 years [4], with many individuals who experience myocardial infarction having cholesterol concentrations at currently mandated targets.

1.1 The Immune-Inflammatory Nature of Atherosclerosis

There is currently a wide acceptance that atherosclerosis consists not only of accumulation of lipids in the arterial wall but that a local chronic inflammatory response, consisting of both innate and adaptive immunity, represents a critical factor in the development and progression of the pathology (Fig. 1).

The generation of radical oxygen species (ROS) is a key event in the vessel wall disease. ROS are produced as part of the physiological respiration or pathological processes and participate in the formation of a microenvironment suitable to oxidation. Inflammation is closely interconnected with the production of ROS. In fact, the local production of ROS is able *per se* to induce an inflammatory state [5, 6].

The increase in circulating cholesterol facilitates the deposition of LDL in the subendothelial layer of the artery wall. The local accumulation of lipids is an inflammatory trigger, dictating the recruitment of monocytes/macrophages, the cells deputed to phagocytose and remove arterial oxidative lipids. This process amplifies the production of local ROS formation and the subsequent oxidation of LDL (ox-LDL). In particular, the oxidation of LDL results in the modification of apolipoproteins (apo), such as apoB-100. Interestingly, it has been previously demonstrated that the modification of apoB-100 activates *in vitro* the scavenger receptor mediated uptake of lipid particles by macrophages [7–9].

The ox-LDL particles are a strong activator of endothelial cells (ECs), inducing the expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), which facilitates the subsequent recruitment of monocytes in concert with enhanced local chemokine release. Following differentiation, macrophages display elevated expression of scavenger receptors on their surface (i.e. SR-A, LOX-1, CXCL16 and CD36) [10]. The continuous cycle of LDL deposition, oxidation and endothelial dysfunction mediated by the local inflammatory process leads to the recruitment of a large number of macrophages. The sustained phagocytosis of LDL by-products creates cholesterol ester-laden foam cells. The accumulation of foam cells and lipids within the intima (termed ‘fatty-streak lesion’) is the first step in the development of a more complex atherosclerotic lesion. In parallel, macrophages can proliferate and secrete several inflammatory mediators, most notably interleukin (IL)-1β, which function to sustain and amplify the inflammatory response. Depletion of monocytes from the circulation in hypercholesterolemic

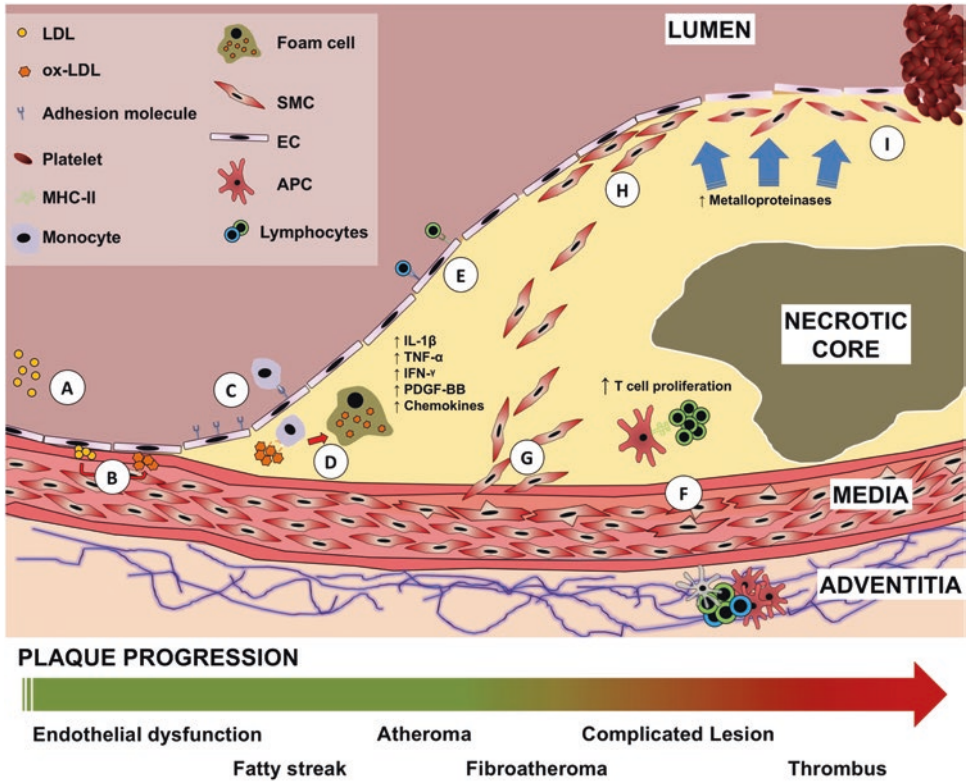

Fig. 1 Inflammation and immune cells are crucially involved in atherosclerosis progression and plaque destabilization. (**A**, **B**) Circulating LDL diffuse and accumulate in the sub endothelial layer of medium and large sized arteries, undergoing a process of oxidation. (**C**) OxLDL are an inflammatory trigger dictating the recruitment of monocytes/macrophages into the vessel wall. (**D**) The activated monocytes differentiate intro macrophages, which are deputed to phagocyte and remove arterial debris. Sustained phagocytosis of LDL by macrophages creates cholesterol ester-laden foam cells. (**E**) The local production of inflammatory mediators induces the intensification of endothelial cell activation. The subsequent expression of other adhesion molecules facilitates the recruitment of multiple subtypes of leukocytes. (**F**) In advanced atheroma, the reactivation and local proliferation of detrimental T cells subtypes is well documented, as is the accumulation of immune cells in the adventitial space and the formation of tertiary lymphoid organs, known as ATLOs. (**G**, **H**) On the other hand, the inflammatory mediators are strong activators of smooth muscle cells. Once activated, they are able to migrate from the media and proliferate in the intimal layer of the vessel producing a protective coating known as fibrous cap. This is an essential feature of the stable plaque phenotype. (**I**) During acute local immune-inflammatory activation, the production of digesting enzymes, e.g. metalloproteinases, critically degrades the protective layer with the subsequent formation of thrombi

rabbits significantly reduced plaque formation, implying a major role for monocytes in atherosclerosis [11].

In the 1980s, the first clear documentation of T lymphocytes in human atherosclerotic plaque was published, which consisted of histological identification of adaptive immune cells located within a human atherosclerotic plaque [12]. Subsequently, the identification of major histocompatibility complex (MHC) class II expression in multiple cell types within the plaque supported the evidence of an active local adaptive immune response [13].

Amongst $CD4^+$ T cell subtypes, Th1 T cells are mainly responsible for driving atherogenesis [14]. Indeed, in advanced human lesions, Th1 markers correlate with ACEs [15] and the plaque microenvironment contains several Th1 pro-inflammatory cytokines, such as IFN-γ and TNF-α [2]. Dendritic cells (DCs) are the most effective antigen presenting cells (APCs) and are located in the intimal and adventitial space of

healthy arteries. The number of DCs increases in the aorta of atherosclerotic mice and in human atherosclerotic lesions. During atherosclerosis progression, other APCs display a parallel increase in number, including B cells and macrophages [16].

T cells recruited to the atherosclerotic lesion can locally recognise antigens presented as peptides on MHC-II by APCs and undergo reactivation. In fact, human lesional $CD4^+$ T cells can be reactivated *in vitro* by plaque antigens [17].

The detrimental role of Th1 T cells is counterbalanced by regulatory T cells (Treg) producing anti-atherogenic TGF-β and IL-10 [17]. We have recently provided strong evidence for a functional local adaptive immune response in the advanced stages of atherosclerosis in hyperlipidemic apolipoprotein-E $(apoE)^{-/-}$ mice in which the formation of artery tertiary lymphoid organs (ATLOs) occurs in the adventitia, adjacent to underlying plaques. These lymphoid structures are organized into well-defined immune cell compartments, including T cell areas, activated B cell follicles and zones with plasma cells. They are characterised by a high content in Tregs and are capable of controlling vascular T cell responses leading to a reduction in plaque size [18].

Of note, a broad range of innate and adaptive immune cells are also present in healthy vessels [18, 19].

1.2 Plaque Progression and Destabilization

In response to multiple inflammatory stimuli secreted by macrophages and ECs, SMCs migrate from the tunica media to the intimal space where they start to proliferate, creating a protective layer, known as the fibrous cap. SMCs are the primary cells responsible for the production of the ECM that further stabilises the fibrous cap. These processes cause the lesion to evolve into a fibrotic plaque that encapsulates the lipid and necrotic core. The rupture of the plaque cap is the most important mechanism underlying the sudden thrombotic occlusion of the vessel lumen, responsible for clinical events.

In the generation of the fibrous protective layer, the role of growth factors such as platelet derived growth factor (PDGF)-BB, cytokines such as tumour necrosis factor (TNF)-α and chemokines such as chemokine (C-C motif) ligands (CCL)-2 are required for the induction, migration and proliferation of the SMCs [20–22].

On the other hand, pro-inflammatory cytokines and chemokines modulate a number of detrimental steps in the control of plaque stability and rupture. A balance between the expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs, tissue inhibitor of metalloproteinases) regulates the degradation and synthesis of the ECM. The expression and activity of MMPs and TIMPs are directly regulated by cytokines and chemokines [23]. Moreover, cytokines such as IFN-γ, TNF-α and IL-1β may promote apoptosis of macrophages and SMCs leading to the enlargement of the necrotic core and the thinning of the fibrous cap [24].

The direct involvement of immune cells in modulating plaque stability has also been evaluated. Adoptive transfer of $CD4^+$ T cells from human plaques into immunodeficient mice engrafted with human atherosclerotic vessels resulted in apoptosis of SMCs in a TNF-related apoptosis-inducing ligand (TRAIL)-dependent manner, inducing fibrous cap thinning and plaque destabilization [25]. Interestingly, Klingenberg et al. [26] showed the considerable increase of Th1 cells in vulnerable plaques and the detrimental effect of Treg depletion on plaque stability in mouse models. This effect could, at least in part, be justified by the reduction in the local level of TGF-β, a cytokine produced by Treg with direct fibrogenic action on SMCs and fibroblasts.

2 A Lesson from Anti-atherosclerotic Drugs

2.1 Statins

Since their introduction, more than 25 years ago, as the first line therapeutic for lipid lowering, statins are a proven class of drugs for effective prevention of CVDs but with benefits beyond those predicted solely by their actions in reducing plasma LDL cholesterol [27].

Indeed, statins have an undefined pleiotropic anti-inflammatory action and are able to reduce plaque formation in hypercholesterolemic mice, without affecting lipid levels [28]. Recently, by injecting simvastatin-loaded reconstituted high-density lipoprotein nanoparticles in apoE$^{-/-}$ mice, Duivenvoorden et al. [29] revealed a selective uptake into the vessel wall with associated local anti-inflammatory activity, sufficient to inhibit plaque progression without any systemic effect on lipid concentrations.

Statins are inhibitors of 3-hydroxy 3-methyl glutaryl coenzyme A (HMG-CoA) reductase and therefore of the synthesis of cholesterol, resulting in reduction of circulating LDL levels. Interestingly, as part of their mechanism of action, statins inhibit biosynthetic intermediates of cholesterol synthesis and their precursors such as geranyl-geranylpyrophosphate (GGPP) [30]. GGPP is a lipidic attachment for Rho-GTPase and its inhibition reduces Rho-GTPase related activity. This is one of the mechanisms that can explain the pleiotropic effect of statins [27, 30]. In fact, the production of nitric oxide (NO) by the endothelium *via* nitric oxide synthase (eNOS) regulates the homeostasis of the vessel. Indeed, atherosclerotic lesions develop faster and grow larger in hypertensive eNOS/apoE double knockout mice when compared to control apoE$^{-/-}$ mice [31]. Statins stimulate NO production by ECs via eNOS. Indeed, pravastatin and simvastatin induce vasorelaxation in mouse aortic rings and NO production by cultured bovine aortic ECs [32]. The direct inhibition of RhoA or GGPP leads to increased expression of eNOS, suggesting that the effect of statins on eNOS is cholesterol-lowering independent [27]. Laufs et al. [33] reported that statins increased eNOS mRNA half-life in ECs. Finally, Scalia et al. [34] demonstrated that an acute treatment with simvastatin in apoE$^{-/-}$ mice is able to increase NO production in the aorta *via* eNOS, without the reduction of circulating cholesterol levels.

Statins also exert canonical anti-inflammatory effects. Simvastatin showed anti-inflammatory activity, similar to that of indomethacin, in a model of acute inflammation in normocholesterolemic mice, the carrageenan paw oedema [28]. The potent anti-inflammatory activity of statins is only partially explained by their action on NO production by the endothelial layer. Reduction in the transendothelial migration of leukocytes to inflammatory sites is one of the most coherent effects of statins. Atorvastatin, simvastatin and cerivastatin inhibit the expression of adhesion molecules on human ECs and peripheral blood mononuclear cells, reducing binding to ECs *in vitro* [35]. Statins reduce the production of chemokine CCL-2 *in vitro* in human ECs exposed to IL-1 and *in vivo* in a mouse model of air-pouch [36]. Statins also reduce *in vitro* CCL-2-induced migration of a human continuous monocyte/macrophages cell line (THP-1) and the secretion of MMP-9 [37], a metalloprotease important for the migration/invasion of leukocytes. Taken together, these data suggest that statins reduce the transendothelial migration by reducing adhesion molecule expression, recruitment signals, migration to and entry into the subendothelial space.

In addition to suppressing cell infiltration into sites of injury, statins also exert direct effects on immune cells. Activation of T cells is mediated by the interaction with MHC-II and costimulatory molecules. Kwak et al. [38] demonstrated that statins inhibit MHC-II-mediated T cell activation. Statins are able to inhibit the expression of CD40 in human ECs, SMCs, macrophages and fibroblasts affecting NOS and peroxisome proliferator-activated receptor (PPAR) signalling pathways [39]. Moreover, treatment of LPS-stimulated human monocyte-derived DCs from healthy patients with simvastatin and atorvastatin reduced the expression of CD83, CD86 and human leukocyte

antigen-DR. Statins also reduced the production of IL-6, IL-8, IL-12, and TNF-α by DCs and suppressed their ability to induce T cell proliferation, activation and Th1 differentiation [40]. Statins have pronounced effects on T cell activation and proliferation through modulation of the Rho-GTPase pathway [41], and atorvastatin in particular has been shown to promote differentiation of T cells into a Th2 subtype with concomitant suppression of the secretion of Th1 cytokines [42]. Finally, lovastatin increases Treg cell recruitment into inflamed sites in a chemokine (C-C motif) ligand 1 (CCL1)-dependent manner. This effect, is in fact, abrogated in CCL1-deficient mice [43].

The atheroprotective effect of statins is exerted not only by reducing plaque development but also stabilizing vulnerable plaques. Atorvastatin inhibits the development of an unstable plaque phenotype in hypercholesterolemic mice, lowering the level of chemokines and chemokine receptors [44]. Intriguingly, rosuvastatin reduces nuclear factor (NF)-κB activation induced by CD40L in human aortic SMCs thus regulating plaque ECM production [45]. Rosuvastatin also reduces the expression of MMP-9 by macrophages [37], a metalloproteinase able to induce acute plaque disruption in apoE$^{-/-}$ mice [46].

Main pleiotropic targets of statins are listed in Table 1.

2.2 PCSK9 Inhibitors

PCSK9 is an enzyme ubiquitously expressed by many cell types. PCSK9 binds the LDL receptor (LDLr), which is primarily expressed in the liver and is a signal for inducing the degradation of the receptor such that is no longer recycled back to the cell membrane surface [47]. Consequently, increased levels of PCSK9 can inhibit LDLr expression on hepatocyte cell membranes and therefore increase LDL levels in the bloodstream. Chang et al. [48] reported for the first time the possibility to neutralize the PCSK9 using a monoclonal antibody, termed mAb1, in mouse and non-human primates (Fig. 2). Interestingly, they demonstrated that a single subcutaneous injection in *cynomolgus monkeys* of the neutralizing antibody led to rapid and significant LDL lowering, 8 h after injection, reaching a maximum of 80% below pre-dose levels by day 10.

In 2015, two PCSK9 inhibitors: the monoclonal antibodies alirocumab and evolocumab, have been approved by FDA and subsequently by EMA for lowering circulating LDL levels. They are used as a second line treatment for atherosclerotic patients who are resistant to statins or affected by familial hypercholesterolemia. Across all clinical trials performed, the use of alirocumab and evolocumab in association with the prescribed statin regimen, reduced the level of LDL (<70 mg/dL) in 90% and 82% of patients respectively [47].

Table 1 Main pleiotropic targets of statins in atherosclerosis

Pathological process	Statin effect	Target cells	References
Endothelial dysfunction	↓ Adhesion molecules	ECs	[35]
	↑ Vascular relaxation	ECs	[32–34]
Inflammatory cell infiltration	↓ Adhesion molecules	ECs; PBMCs	[35]
	↓ Chemokines production	ECs	[36]
	↓ Migration/invasion	Monocyte/macrophages	[37]
Immune cells activation	↓ T-cells activation and proliferation	T-cells	[38, 41]
	↓ CD40 expression	ECs; SMCs; Marophages	[39]
	↓ CD83 and CD86	Monocytes-derived DCs	[40]
	↓ Cytokine production	Monocytes-derived DCs	[40]
T-cells phenotype	↑ Th2 and ↓Th1	T-cells	[42]
	↑ Treg	T-cells	[43]
Plaque rupture	↓ Metalloproteinases expression	SMCs and macrophages	[37]
	↓ chemokine and chemokine receptors	Multiple vessel cells	[44]

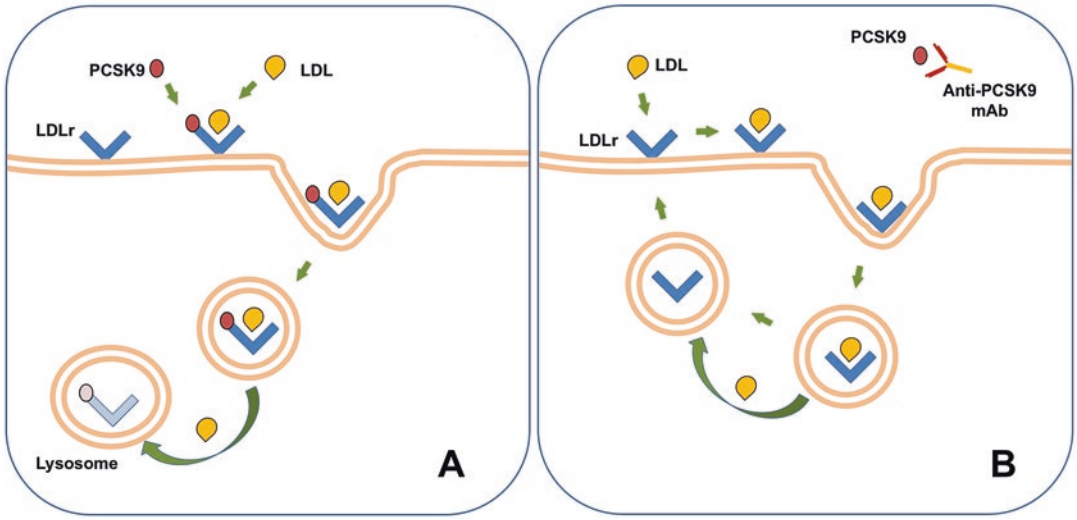

Fig. 2 Proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9) regulates LDL receptor degradation. (**A**) During hypercholesterolemia, the circulating levels of PCSK9 increase and are free to bind LDLr on the hepatocyte surface. The PCSK9-LDLr complex is a signal for degradation via lysosome. (**B**) Binding of circulating PCSK9 by specific monoclonal antibodies results in recirculation of the LDLr and accelerated clearance of circulating LDL

In early 2017, results from the *Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk* (FOURIER) study have been revealed. The study analysed 27,564 patients with CVD and who were receiving a moderate- to high-intensity statin regimen with a LDL cholesterol level between 70 and 100 mg/dL. Researchers randomly assigned patients (1:1) to receive, in association with the canonical statin treatment, a subcutaneous injection of evolocumab or placebo. Relative to placebo, the circulating LDL level reduced by 59%, from a median baseline value of 92 mg/dl to 30 mg/dl. After 2 years of observation, the group treated with the anti-PCSK9 therapy showed a statistically significant 27% and 21% reduction in heart attack and stroke respectively when compared with placebo [49].

The efficacy of these inhibitors is now clearly corroborated by clinical trial data, demonstrating the potency of these new agents in greatly reducing circulating cholesterol levels yet the reduction in cardiovascular risk is only partially identified. Interestingly, experimental studies have suggested also a pro-inflammatory role for the PCSK9 protein, promoting atherosclerosis with a LDL-independent mechanism. PCSK9 reduces the expression of LDLr in human macrophages [50] while modulating expression of stress response and inflammatory genes in liver cells, independent of its effects on cholesterol uptake [51], suggesting a direct role for PCSK9 in foam cell formation and inflammation. Cheng et al. [52], correlated PCSK9 serum levels with the size of necrotic core tissue in coronary atherosclerosis evaluated by IVUS-VH imaging on 581 patients, showing that serum PCSK9 linearly correlates with coronary plaque inflammation, independently of serum LDL cholesterol levels and statin usage. To investigate the direct effects of PCSK9 on vascular inflammation, Tang et al. [53] silenced PCSK9 with the lentivirus-mediated PCSK9 shRNA (LV-PCSK9 shRNA) vector in apoE$^{-/-}$ mice. PCSK9 gene interference decreased atherosclerosis formation by directly reducing vascular inflammation and inhibiting the TLR4/NF-κB signalling. Furthermore, the neutralization of PCSK9 in hypercholesterolemic mice using alirocumab decreased monocyte recruitment, leading to a more stable plaque phenotype [54]. Finally, silencing of PCSK9 inhibited *in vitro* ox-LDL induced human APC maturation and T cell activation [55].

3 Old and New Agents to Target Immune Mechanisms in Atherosclerosis

The establishment that atherosclerosis is a chronic inflammatory disease identifies multiple targets in the molecules sustaining inflammation in the plaque microenvironment. Therefore, known multi-target anti-inflammatory and immunomodulating drugs may find a novel application in the control of atherosclerosis. In parallel, the development of biologics targeting specific components of traditional and emerging immune-inflammatory pathways may greatly enhance selective treatment opportunities.

3.1 Methotrexate

Methotrexate, developed as an antifolate drug for inhibiting cell division in the treatment of cancer, is actually used in the therapy of rheumatoid arthritis although its anti-inflammatory effects are not well characterized. Some of the proposed mechanism of actions are independent from the antifolate activity and include alterations of the cellular redox state, the inhibition of polyamine formation and the increase of extracellular release of the anti-inflammatory adenosine [56]. Treatment with methotrexate downregulated *in vitro* the expression of pro-inflammatory genes including TNF-α, IL-1β, chemokine (C-X-C motif) ligand 2 (CXCL2) and TLR2 while upregulating the anti-inflammatory TGF-β1 gene in TNF-α-stimulated human ECs [57]. Moreover, methotrexate promotes reverse cholesterol transport, reducing foam cell formation in lipid-loaded THP-1 macrophages [58]. Methotrexate reduces levels of TNF-α and increases the expression of IL-10 in thioglycollate-induced peritoneal exudates in mice [59]. This effect was further confirmed in high-fat diet induced obesity in mice. Methotrexate administration reduced TNF-α, IL-6 and leptin production from the adipose tissue of these mice leading to increased production of anti-inflammatory molecules such as adiponectin and IL-10 [60]. In addition, methotrexate reduces circulating levels of the pro-inflammatory cytokine IL-6 in psoriatic patients [61] and adhesion molecule expression in biopsies of human inflammatory tissue from oral bullous pemphigoid [62]. Finally, methotrexate inhibits antigen-induced T cell proliferation in mice [63].

All these actions support the possibility of using methotrexate in atherosclerosis treatment. Indeed, methotrexate reduces atherosclerotic lesion areas in cholesterol fed rabbits leading to a reduction in plaque macrophage content and the presence of apoptotic cells [57] in addition to reductions of MMP-9 and pro-inflammatory cytokines [64, 65].

A randomised clinical trial: the Cardiovascular Inflammation Reduction Trial (CIRT; https://clinicaltrials.gov/ct2/show/NCT01594333), is actually ongoing. The rationale is the use of low-dose methotrexate to reduce heart attacks, stroke, or death in people with type 2 diabetes, metabolic syndrome and heart attack or multiple coronary occlusions. The primary completion date for CIRT will be the end of 2018.

3.2 The IL-1 Pathway

IL-1 is a major mediator of inflammation and immune diseases [66]. IL-1α and IL-1β bind to the same receptor (IL-1R) and share the same downstream pathway. An endogenous inhibitor of the IL-1 signal is the receptor antagonist (IL-1RA) that serves as decoy receptor. Both IL-1α and IL-1β precursors are activated following enzymatic cleavage [67]. Their different properties are primarily related to the cellular location and to the method of activation. Despite the IL-1β precursor being inactive, the IL-1α precursor is active and functions as alarmin, inducing the expression of other cytokines and chemokines [66]. IL-1α mediates the early phases of sterile inflammation [68]. Alternatively, IL-1β is produced as an inactive precursor and is activated by caspase-1, a process controlled upstream by the NLRP3 inflammasome [66, 67].

The IL-1 pathway is unequivocally involved in atherosclerosis. IL-1 induces adhesion molecule expression in human umbilical vein endothelial cells [69], increasing leukocytes adhesion [70]. Mice deficient in IL-1RA developed foam cell lesions once fed a high fat diet supplemented with cholate. While mice genetically deficient in LDLr overexpressing a murine sIL-1RA showed reduced atherosclerosis formation compared to LDLr$^{-/-}$ control mice [71]. Finally, treatment of apoE$^{-/-}$ mice with human recombinant IL-1RA reduced lesion area [72].

Cholesterol crystals and ox-LDL accumulated in the lesions activate the NLRP3 inflammasome, inducing the secretion of the active form of IL-1β by macrophages in the plaque [73, 74]. On the other side, it has been demonstrated that IL-1α-driven vascular inflammation induced by fatty-acids through mitochondrial uncoupling is independent from IL-1β [75].

The blocking of IL-1β represents an interesting target to treat atherosclerosis inflammation. IL-1β is a potent tissutal pro-inflammatory mediator, inducing local vasodilatation and recruitment of leukocytes [76]. Moreover, during inflammation, IL-1β has been linked to the activation of several T cells subtypes, which suggest IL-1β as a link between the innate and the adaptive immunity [76]. To confirm its role in atherosclerosis, IL-1β deficiency [77] or IL-1β neutralization [78] inhibited atherosclerosis development in apoE$^{-/-}$ mice.

Different strategies have been followed to develop biologics able to inhibit the IL-1 pathway. The anakinra is a recombinant version of the human IL1-RA and is approved for the treatment of rheumatoid arthritis. In 2009, the FDA approved canakinumab, a human monoclonal antibody targeted at interleukin-1β, for the treatment of cryopyrin-associated periodic syndrome, a group of rare and heterogeneous autoinflammatory diseases mediated by IL-1. Currently, canakinumab is used clinically as a second line treatment in rheumatoid diseases and is also approved for TNF receptor associated periodic syndrome and familial Mediterranean fever.

3.3 Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS)

The CANTOS is a randomised, double-blinded, placebo-controlled trial on secondary prevention in more than 10,000 patients previously affected by myocardial infarction. The enrolled patients, with elevated levels of the inflammatory marker C-reactive protein (CRP), were randomised and treated with canakinumab at three different doses or placebo, as control. About 90% of the enrolled patients had cholesterol levels under control by statins [79]. Canakinumab significantly reduced rate of recurrent cardiovascular events without reducing all-cause mortality.

Despite the modest cardiovascular benefit CANTOS results finally convey to basic and clinical researchers the demonstration of a theory: that immune-inflammatory responses not only represent a pathophysiological mechanism but also a valid target for the development of new and selective therapies in atherosclerosis [80].

4 Vaccination Strategies

Systemic treatment with immunosuppressant drugs may lead to several side effects and cannot be suggested for primary prevention. In this regard, modulation of antigen-specific adaptive immune responses via vaccination strategies may be a preferable approach.

Several potential antigens have been found in the atherosclerotic plaque. They include endogenous LDL, in particular apoB-100, ox-LDL and heat shock protein 65 (HSP65) or exogenous antigens like *Chlamydia pneumoniae* and periodontal pathogens.

During the 1990s, several publications reported the efficacy of an active immunization against ox-LDL in reducing atherosclerosis severity in rabbits [81, 82]. The better identification of the peptides comprising the protein components of LDL and their modified forms in ox-LDL makes more definitive immunological studies possible.

The immunisation with specific apoB-100 sequences reduced plaque formation by 60% in apoE$^{-/-}$ mice [83]. Scanning the apoB-100 protein aminoacid sequence, Tse et al. [84], identified the portions predicted to bind to the mouse MHC-II molecule I-Ab. Two fragments: $ApoB_{3501–3516}$ and $ApoB_{978–993}$ were used to immunise the mice and resulted in reduced atherosclerosis development in apoE$^{-/-}$ mice *via* increased IL-10 production. Generating T cell hybridomas from human apoB-100 transgenic (huB100(tg)) mice immunised with human apoB-100, Hermansson et al. [85] identified an MHC-II-restricted apoB-100-responding CD4$^+$ T cells hybridoma expressing a T cell receptor variable (V) beta chain, TRBV31. The immunization of hypercholesterolemic mice with a TRBV31-derived peptide reduced atherosclerosis by 65%. Finally, the apoB-100 peptide sequence, named p210, also represents an interesting antigen in vaccine formulations due to its consistent atheroprotective effect [86]. The immunization of mice with p210 is effective in atherosclerosis reduction by the systemic induction of the CD4$^+$IL-10$^+$ [87] and CD4$^+$CD25$^+$FoxP3$^+$ cells [88], suppressing the immune response, with minor effects on the other T cell subtypes [86].

PCSK9 also represents an interesting target for immunisation. As previously described, the injection of a human monoclonal antibody against this enzyme, effectively reduced LDL circulating levels and cardiovascular risk in humans [49]. Active immunisation against this target could achieve similar results with long-term biological effects. Recently the effect of an anti-PCSK9 vaccine, named AT04A, has been evaluated in hypercholesterolemic mice. AT04A was able to induce persistently high antibody levels against PCSK9 with a reduction of LDL. Interestingly, AT04A reduced atherosclerosis formation by 64%, data correlated to the systemic and local reduction in inflammatory markers [89].

5 Conclusions and Perspectives

This chapter highlights the importance of immune-inflammatory mechanisms in the pathogenesis of atherosclerosis and related clinical manifestations. Conventional lipid-lowering treatments exert pleiotropic anti-inflammatory effects and recent results from the CANTOS trial have shown that targeting inflammation may be beneficial in secondary prevention. However, several key questions still need to be addressed before considering widespread use of anti-inflammatory drugs in the prevention and treatment of CVDs.

Atherosclerosis is a life-long pathology, identified mainly as a vascular rather than a systemic immune disorder. As such, it is unlikely that the current generation of biologics will ever be used for primary prevention in atherosclerosis given the high risks associated with chronic systemic immunosuppression. Different immune pathways play may different roles at different stages of the disease development and progression. We may therefore hypothesize that different immunomodulatory options may be required to selectively affect disease onset, progression, and/or plaque stabilization. Finally, the development of better diagnostic tools for the direct evaluation of vascular inflammation is required for better patient stratification and the design of future clinical trials. In summary, treatment of vascular inflammation is still in its infancy, but exciting developments lie ahead this rapidly expanding research field.

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Regulation of Immunity and Disease by the IL-1 Receptor Family Members IL-1R2 and IL-1R8

Martina Molgora, Domenico Supino, and Cecilia Garlanda

Abstract

Interleukin-1 and other IL-1 family members are key players in immunity and inflammation.

The activation of the IL-1 system is tightly regulated, through ligands with antagonistic or anti-inflammatory activity, or decoy and negative regulatory receptors. IL-1R2 and IL-1R8 (also known as SIGIRR) are members of the ILR family acting as negative regulators of the IL-1 system. IL-1R2 binds IL-1 and the accessory protein IL-1RAcP without activating signaling, thus modulating IL-1 availability for the signaling receptor. IL-1R8 dampens IL-1 receptor- and Toll Like Receptor-mediated cell activation and is a component of the receptor complex recognizing the anti-inflammatory cytokine IL-37.

The deregulated activation of the IL-1 system is the potential cause of detrimental local or systemic inflammatory reactions. Here, we summarize our current understanding of the function of IL-1R2 and IL-1R8, focusing on their role in pathological conditions, ranging from infectious and sterile inflammation, to cancer-related inflammation.

Keywords

Interleukin-1 · Inflammation · Infection · Inflammation-associated cancer

1 Introduction

Innate and adaptive immunity cells are tightly regulated by a plethora of cytokines and receptors. The Interleukin-1 system plays a crucial role in controlling immune responses and inflammatory processes [1, 2]. IL-1 family ligands include 7 molecules with agonist activity (IL-1α, IL-1β, IL-18, IL-33, IL-36α, β, and γ), three receptor antagonists (IL-1Ra, IL-36Ra and IL-38), and an anti-inflammatory cytokine (IL-37). The IL-1R family members include 11 molecules [IL-1R1, IL-1R2, IL-1R3 (IL-1RAcP), IL-1R4 (ST2), IL-1R5 (IL-18Rα), IL-1R6 (IL-1Rrp2, IL-36R), IL-1R7 (IL-18Rβ), IL-1R8 (TIR8, also known as SIGIRR), IL-1R9 (TIGIRR-2), IL-1R10 (TIGIRR-1)] (Fig. 1) [2].

ILRs are characterized by an evolutionarily conserved structure which consists of Ig-like

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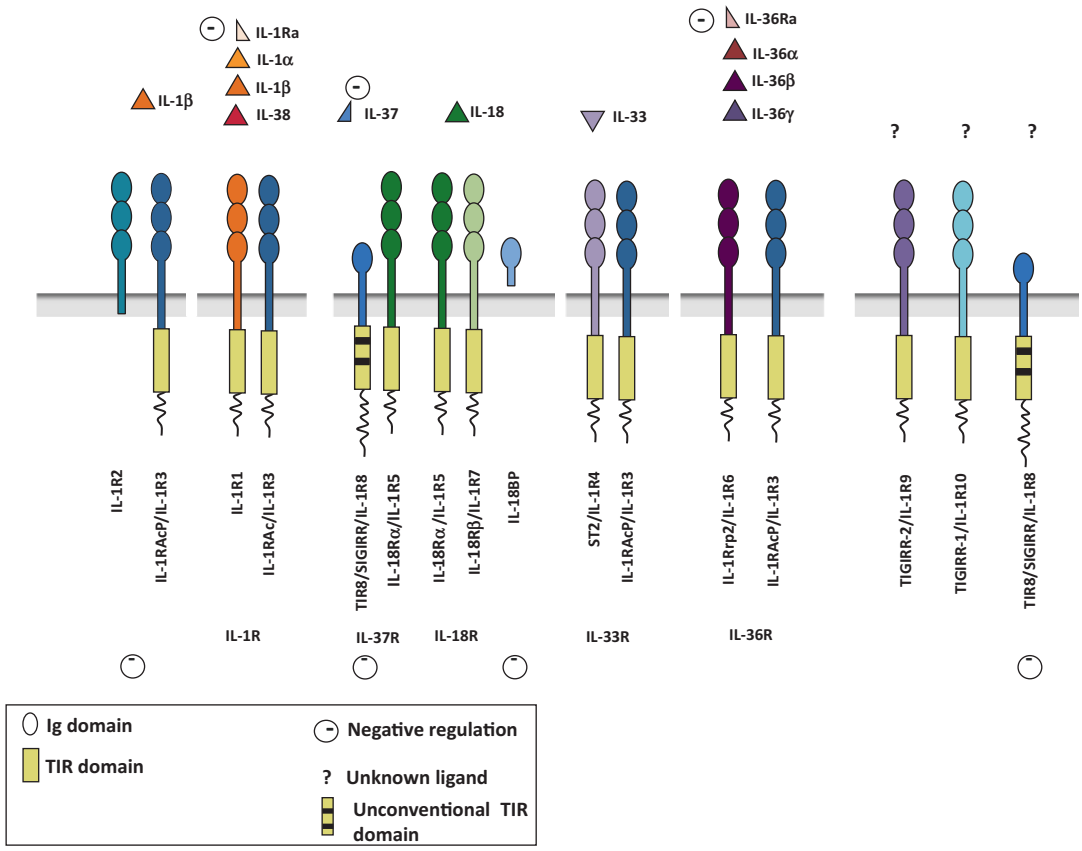

Fig. 1 The IL-1 system Ligands of the IL-1 receptor (ILR) family are shown (IL-1α, IL-1β, IL-38, IL-33, IL-36α, IL-36β, IL-36γ and IL-18). IL-1R, IL-33R, IL-36R and IL-18R complexes activate signal transduction. IL-R2, sIL-1R2, IL-1Ra, IL-36Ra IL-18BP and IL-1R8 are negative regulators acting with different mechanisms. IL-37 is an anti-inflammatory cytokine, signaling upon the formation of a tripartite complex (IL-37/IL-1R5/IL-1R8). IL-1R3 is an accessory protein for IL-R1, IL-1R2, IL-1R4 and IL-1R6. Ligands for IL-1R8, IL-1R9 and IL-1R10 are still partially defined

extracellular domains and an intracellular Toll-IL-1 resistance (TIR) domain, that is shared with Toll-like receptors (TLRs) [3]. Ligand binding induces the dimerization through the TIR domain of the specific receptor with a second receptor molecule, acting as an accessory protein and establishing an intracellular signaling platform, which recruits one of the adaptor proteins MyD88, MAL, TRIF, TRAM or SARM. In turn, these molecular complexes unleash protein kinases activation (e.g. Tumor necrosis factor receptor-associated factor 6 (TRAF6) and IL-1R associated kinases (IRAKs),) and trigger a cohort of downstream targets such as nuclear factor-κB (NFκB), activator protein-1 (AP-1), c-Jun N-terminal kinase (JNK), p38 mitogen-associated protein kinase, extracellular signal-regulated kinases (ERKs), mitogen-activated protein kinases (MAPKs), and interferon (IFN)-regulatory factors (IRF) [4–6]. The modulation of multiple Transcriptional Factors (TFs) orchestrates a robust pro-inflammatory reaction, enforcing both the innate and adaptive immunity [7–9].

The fundamental role of ILR family in inflammation is underlined by a broad spectrum of inflammatory, autoimmune and neoplastic diseases correlated to deregulation of the IL-1 system. For instance, several lines of evidence indicate that IL-1 and its regulation play a pivotal

role in cancer-related inflammation and progressive tissue damages in chronic inflammatory conditions. This link emphasizes the implications of ILR and cytokine targeting as therapeutic strategy in several pathological conditions associated with acute and chronic inflammation, ranging from cardiovascular and autoimmune diseases to cancer [10–16].

The IL-1 system includes several extracellular and intracellular endogenous regulators, which tune ILR signaling and are necessary to restore homeostatic conditions. These "caretakers" are anti-inflammatory cytokines (IL-37, IL-38), receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), scavengers and/or decoy or negative regulatory receptors (e.g. IL-1R2, IL-1R8 and IL-18BP), and miRNAs [17] that tune ILR signaling at transcriptional and post-transcriptional level.

Here, we summarize our current understanding of the structure and function of IL-1R2 and IL-1R8, two negative regulators of inflammation and immune responses, describing their relevance in physiology and pathology.

2 The Decoy Receptor IL-1R2

2.1 IL-1R2 Protein and Function

Human *IL-1R2* is a highly conserved gene localized in chromosome 2, in a large cluster which includes several ILR members such as the receptors for IL-33, IL-18 and IL-36 [18, 19].

IL-1R2 gene encodes an extensively glycosylated 68 kDa protein composed of 386 amino acids. The IL-1R2 extracellular domain has the canonical ILR Ig-like-structure, and shares 28% amino acid homology with IL-1R1. In contrast, the IL-1R2 intracellular domain is peculiar for the absence of a functional TIR domain, which is substituted by a short 29 amino acid-long cytoplasmic tail [20, 21]. Several enzymes, in particular the metalloproteinase ADAM17, cleave the full-length receptor to generate an IL-1R2 soluble form with decoy activity [22–24]. Pro-inflammatory molecules (LPS, TNFα, leukotriene B4, or fMLF) trigger the enzymatic cleavage and the release of soluble IL-1R2 [25–28], which can be also generated by an alternative splicing isoform of the IL-1R2 transcript [29].

IL-1R2 exerts its decoy activity through different mechanisms. First, IL-1R2 sequesters IL-1R3 to generate a dominant negative receptor complex [30], which competes with IL-1R1 for the formation of a signaling receptor complex [31–33]. Second, the IL-1R2/IL-1R3 complex binds IL-1α and IL-1β, without activating the pro-inflammatory signaling cascade [20, 34]. In addition, the soluble form participates in reducing IL-1 availability for the signaling receptor, since soluble IL-1R2 and IL-1R3 are found at high concentration (in the order of ng/ml) in the blood, and their physical interaction increases the affinity for IL-1α and IL-1β [34, 35]. Finally, IL-1R2 is present in the cytoplasm and interacts with pro-IL-1α preventing cleavage and activation by different enzymes (calpain, granzyme B, chymase, and elastase) during necrosis [34–37].

2.2 IL-1R2 Expression and Regulation

IL-1R2 is the predominant IL-1 receptor in the myeloid compartment, in particular monocytes, macrophages and neutrophils, and it is overexpressed in M2 macrophages [20, 34, 38, 39]. In the lymphoid compartment, IL-1R2 shows a high expression level in B cells and in T regulatory cells (Treg) and it is up-regulated upon TCR stimulation [20, 34, 38, 39]. In colorectal and non-small-cell lung cancers, Treg express higher levels of IL-1R2 compared to Th1 and Th17 tumor infiltrating lymphocytes [40]. Similarly, breast cancer infiltrating Tregs express higher IL-1R2 levels compared to healthy breast resident Tregs and circulating Tregs [41]. The functional activity of IL-1R2 in tumor infiltrating Tregs and the molecular mechanisms regulating its expression are still unknown. Interestingly, IL-1β inhibition significantly reduced the risk of incident lung cancer and lung cancer mortality in a large cohort of atherosclerosis patients, suggesting the relevance of IL-1 regulation in cancer [16].

In the mouse, *Il1r2* is widely expressed in innate and adaptive immune cells of myeloid and lymphoid origin [25, 42–50], it is up-regulated by several anti-inflammatory stimuli (e.g. IL-4, IL-13, IL-27, IL-10, glucocorticoid hormones and prostaglandins) [20, 38, 51–56], and down-regulated by pro-inflammatory and chemotactic molecules (e.g. LPS, IFNγ, TNFα, reactive oxygen intermediates and phorbol myristate acetate) [22, 27, 28, 54, 57].

The regulation of IL-1R2 expression on the myeloid compartment has been associated with the pathogenesis of several inflammatory diseases. Atherosclerosis is associated with vessel wall inflammation and IL-1 has long been known to drive atherosclerosis and its complications. Interestingly, reduced expression of IL-1R2 was observed in atherosclerosis vascular lesions, which suggests defective tuning of IL-1 activity [48]. Based on the role of IL-1 in the pathogenesis of cardiovascular diseases, a large prospective study was conducted using anti-IL-1β (Canakinumab) in high-risk atherosclerosis patients, which showed that treatment led to a significantly lower rate of recurrent cardiovascular events [15].

Up-regulation of IL-1R2 in microglia represents a protective mechanism of the central nervous system suppressing IL-1β-mediated brain inflammation and neurotoxicity [45, 46, 58]. IL-1R2 down-regulation has been associated with type II osteoarthritis [59] and correlated to bone resorption upon IL-1 stimulation [47].

The relevance of decoy receptors as fundamental brakes of the immune response is demonstrated by their exploitation by viruses and bacteria as pathogen evasion strategies. For instance, double strand DNA viruses (Poxviruses and Herpesviruses) have acquired decoy receptor genes through genetic recombination with the host genome [60]. In lethal *Listeria monocytogenes* infection, IL-1R2 expression is up-regulated in monocytes [42], and protein A of *Staphylococcus aureus* was shown to induce soluble IL-1R2 by stimulating ADAM17-mediated cleavage, resulting in IL-1β sequestration and decreased bacterial eradication [61].

2.3 IL-1R2 Functional Role In Vivo

Several studies have demonstrated the anti-inflammatory role of IL-1R2 *in vivo*. IL-1R2 deficiency exacerbates endometriosis [62], autoimmune myocarditis [63] and skin inflammation [64], through the inhibition of IL-1 signaling and therefore Th17 cell activation [65].

IL-1R2 deficient mice were also more susceptible to arthritis. In collagen-induced arthritis, IL-1R2-deficient macrophages increased their responsiveness to IL-1 and governed the pro-inflammatory response [59, 66, 67]. In contrast, in the K/BxN serum transfer-induced arthritis, increased joint degeneration has been attributed to neutrophils, through a not cell autonomous mechanism [68]. IL-1R2-deficiency on neutrophils increased the IL-1-induced response of fibroblasts, suggesting that IL-1R2 acts in trans, as soluble form shed upon IL-1β treatment. However, IL-1R2-deficiency did not affect the acute inflammation induced by systemic administration of IL-1β or LPS [64, 68], in contrast with pleiotropic effects of IL-1Ra-deficiency [69, 70].

Recently, it was shown that IL-1R2 was expressed together with the IL-1 receptor antagonist IL-1Ra, by follicular regulatory T (Tfr) cells, which are responsible for the modulation of follicular helper T (Tfh) cell effector functions and therefore B cell activation in the germinal center. IL-1 treatment induced IL-21 and IL-4 production by Tfh cells and this effect was inhibited in the presence of Tfr cells, possibly because of IL-1 capture by IL-1R2 [71].

2.4 IL-1R2 Like Prognostic and Diagnostic Marker

IL-1R2 shedding in pathological conditions has encouraged the studies of IL-1R2 soluble form as diagnostic and prognostic marker. IL-1R2 is released in plasma in physiological conditions (5–10 ng/ml), but its levels are proportionally increased upon infections (acute meningococcal infection, experimental endotoxemia, trauma, necrotizing enterocolitis, acute respiratory

distress syndrome, sepsis) [72]. IL-1R2 soluble form was suggested as biomarker in multiple sclerosis [73] and in Alzheimer's disease [74], whereas in the synovial fluid and plasma of rheumatoid arthritis patients IL-1R2 was correlated with symptom amelioration [75, 76]. Soluble IL-1R2 has been suggested as good prognostic biomarker in pancreas islet transplantation [77] and in inflammatory bowel disease [78] and as biomarker to monitor the clinical outcome of TNFα blockade with Etanercept [79] and in steroid treatment response [80].

Finally, IL-1R2 over-expression has been also observed in psoriatic patients [81] and in several solid tumors such as prostatic cancer, ductal adenocarcinoma [82], benign prostatic hyperplasia [83] and ovarian cancer [84], but the functional implication of IL-1R2 in neoplastic transformation is unknown. In ulcerative colitis, IL-1R2 expression was correlated to remission [78, 85] and to steroid response [80].

3 IL-1R8 (TIR8/SIGIRR)

3.1 IL-1R8 Gene and Protein

IL-1R8 is an antisense gene on human chromosome 11 [86], with three main isoforms that share a common coding DNA sequence. The murine locus is on chromosome 7. The gene is well conserved among vertebrates, and the human IL-1R8 protein has a primary sequence of 414 amino-acid with a high identity score (82%) between human and mouse species [87]. Despite the partial identity with IL-1R1 protein (23%), IL-1R8 has relevant structural differences: the extracellular region of IL-1R8 has only a single Ig domain and the intracellular TIR domain has a long tail of 95 residues. Compared to "canonical" TIR domains, IL-1R8 has two aminoacid substitutions in Ser447 and Tyr536 (switched to Cys222 and Leu305) and the lack of phosphorylation on these two residues influences IL-1R8 signaling activity.

Similarly to IL-1R2, IL-1R8 is N- and O-glycosylated on the extracellular domain, and these post-transcriptional modifications have been described as functionally relevant in a study performed in colon cancer patients (see below) [88].

IL-1R8 is expressed in the majority of epithelial tissues and it is particularly enriched in liver, in kidney and in lymphoid organs. The expression in leukocytes is ubiquitous, showing a higher expression level in NK cells and T lymphocytes, and it is also expressed in platelets [86, 89–91] (Fig. 2).

IL-1R8 was shown to be downregulated upon bacterial infections by *Pseudomonas aeruginosa* [92], or *Toxoplasma gondii* [93], in pyelonephritis induced by *E. coli* [94] and in necrotizing enterocolitis [95]. A reduced expression of IL-1R8 was also observed in acute inflammation, in psoriatic arthritis, in asymptomatic bacteriuria [96, 97], in colitis, and after stimulation with flagellin and LPS *in vivo* and *in vitro* [91, 98–100]. Treatment with LPS was shown to downregulate IL-1R8 in monocytes and neutrophils [98] through the inhibition of SP1 binding on IL-1R8 promoter [98, 101]. However, an increased expression of IL-1R8 was observed in monocytes in sepsis and sterile inflammation and this correlated with a tolerogenic phenotype after LPS and Pam_3CysSK_4 stimulation [102]. Moreover, amyloid β treatment has been proposed to downregulate IL-1R8 in microglia and hippocampal tissue through the transcription factor peroxisome proliferator-activated receptor (PPAR)γ [103]. Other stimuli involved in tuning IL-1R8 are the neuropeptide vasoactive intestinal peptide (VIP), *Lactobacillus jensenii* [104], and bacterial immunogenic molecules [105], which mainly affect myeloid derived cells (macrophages, DC, Langerhans cells).

The deregulation of IL-1R8 has been associated with malignant transformation. In chronic lymphocytic leukemia (CLL), neoplastic B cells showed lower expression of IL-1R8 compared to B cells from healthy donors [106]. Several genes are downregulated through DNA hypermethylation in CLL, but no difference was observed in IL-1R8 methylation. However, treatment with the hypomethylating drug 5-Azacytidine led to IL-1R8 overexpression, suggesting an indirect regulation of IL-1R8 mediated by 5-Azacytidine [106]. IL-1R8 loss of

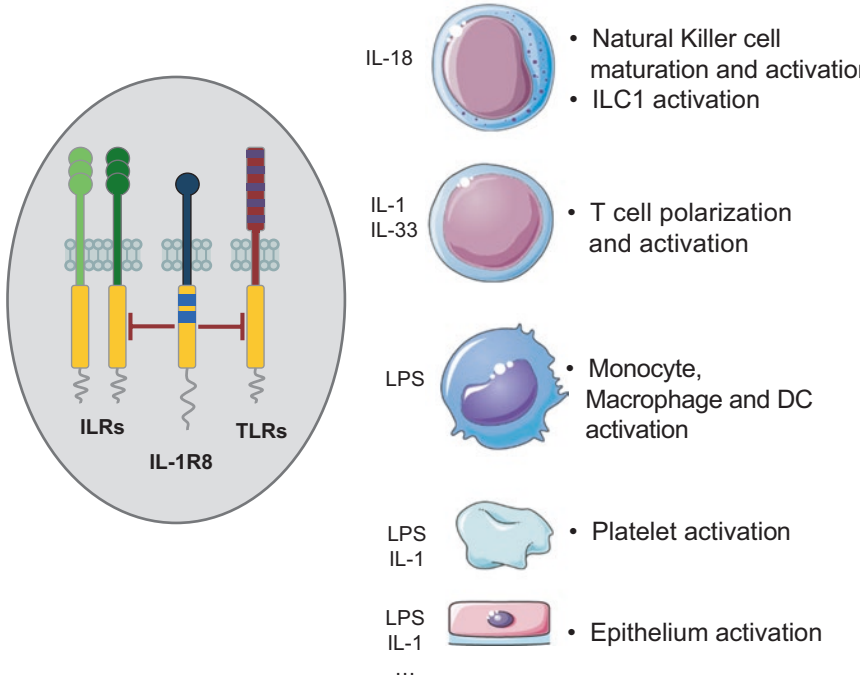

Fig. 2 IL-1R8 functions in different cell types IL-1R8 is widely express in both the hematopoietic and non-hematopoietic compartment and governs cell differentiation and activation. In particular, IL-1R8 modulates NK cell maturation and effector functions; ILC1 activation; T cell activation and polarization; monocyte, macrophage, DC, platelet and epithelium activation, through the negative regulation of IL-1 family members or microbial moieties acting on ILRs and TLRs, respectively

function has been described in colon tumorigenesis, which has been explained with the existence of a dominant negative isoform of IL-1R8, a truncated protein that was shown to trap the main IL-1R8 protein isoform in the endoplasmic reticulum [88]. Finally, RNAseq data and experiments on tumor cell lines showed that IL-1R8 was upregulated in breast cancer [107].

Other IL-1R8 isoforms are emerging, but their function is unknown. Recently a longer isoform called IL-1R8L1 was characterized in tumor epithelial cell lines (e.g. Hela, HT-29 and PC3), in a neuroblastoma cell line (SK-N-HS), in leukemic cell lines (e.g. Jurkat, MEC1, Ramos, Daudi, and THP1), and in human healthy tissues (e.g. hearth, small intestine, kidney, liver, lung, stomach, spleen, ovary, and testis) [108]. LPS stimulation was shown to downregulate IL-1R8L1 in THP1 cell lines, indicating a common regulatory mechanism shared by IL-1R8 isoforms [108].

3.2 Functional Roles of IL-1R8

IL-1R8-deficient mice have demonstrated the role of IL-1R8 in reducing NFκB and JNK activation, inhibiting ILRs and TLRs (e.g. IL-1R1, IL-1R5/IL-18Rα, IL-1R4/ST2, TLR1, TLR2, TLR4, TLR7, TLR9, TLR3) downstream signaling pathways [90, 91, 109–114].

IL-1R8 is recruited to the ligand-receptor complex, and the BB-loop structure of IL-1R8 TIR domain inhibits the dimerization of MyD88 [86, 89, 109, 111, 115, 116]. *In silico* studies of protein modeling have suggested a regulatory mechanism similar to IRAK-M, in which the Myddosome complex is retained on receptors and cannot drive the pro-inflammatory cascade [117]. Furthermore, IL-1R8 extracellular domain inhibits the reciprocal interaction between IL-1R1 and IL-1R3 [111]. The steric competition exerted by IL-1R8 has been also proposed to explain the IL-1R8-mediated regulation of TLR3

signaling, in which IL-1R8 blocks TRAM homodimerization and TLR4-TRAM and TRIF-TRAM interactions [117–119].

The decoy activity of IL-1R8 is also involved in the regulation of JNK and mTOR pathways in lymphoid and not lymphoid cells (e.g. Th17, NK cells and intestinal epithelium) [90, 120, 121].

The deregulation of the IL-1 system is part of pathogen evasion strategies, as mentioned in case of IL-1R2 [122]. Indeed, several bacteria (e.g. *Brucella melitensis*, *E. coli*, *Salmonella enterica*, *Pseudomonas denitrificans* and *P. aeruginosa*) [122–125] have evolved TIR-containing proteins (Tcps) that dampen TIR-related pathway, suggesting that Tcps might be evolutionary linked to IL-1R8.

3.3 IL-1R8 as a Coreceptor of IL-1R5/IL-18Rα for IL-37

In the last decade new anti-inflammatory interleukins involved in controlling TLR pro-inflammatory pathways were identified. In this regard, IL-37 has emerged as a bioactive molecule in leukocytes, in particular in macrophages, and epithelial cells, and IL-37-transgenic mice (IL-37tg mice) were reported to be refractory to inflammation [126]. Intriguingly, the formation of a tripartite complex composed by IL-37, IL-1R8 and IL-1R5 was demonstrated to be required for IL-37 signaling in human PBMCs and murine bone marrow-derived macrophages. This interaction induced an immunosuppressive pathway, inhibiting MAPK, NFκB, mTOR, TAK1 and Fyn, and activating STAT3, Mer, PTEN and p62(dok) signaling [127, 128]. IL-37 mediated protection was abolished by IL-1R8-deficiency in LPS-induced endotoxemia, *A. fumigatus* pulmonary infection [127, 129], and OVA-induced asthma [130].

IL-37 was also implicated in tuning metabolism and in AMPK activation in adipocytes and macrophages [131], with a significant effects on obesity, insulin response and glucose tolerance. In this context, IL-37 and IL-1R5/IL-1R8 receptor complex led to the inhibition of mTOR signaling and activation of STAT6 and Foxo TF family [127], which triggered a pseudo-starvational state in macrophages and DCs. Furthermore, IL-37 was described as a regulatory molecule in muscle cells, orchestrating AMPK pathway and improving exercise performance [132]. At the cellular level, IL-37 potentiated oxidative phosphorylation in mitochondria modulating redox state in the organelles [132]. Finally, recombinant human IL-37 increased muscular resistance in healthy mice and in models of systemic inflammation (upon LPS administration), and IL-1R8-deficiency abrogated IL-37 effects on fatigue tolerance [132]. These lines of experimental evidence support the potential targeting of IL-37/IL-1R8 axis in patients, in which chronic inflammation leaded muscle degeneration and impaired physical mobility [133].

3.4 IL-1R8 in Infections and Inflammation

IL-1R8-deficient mice exhibited an overwhelming local and systemic inflammation and tissue damage after infection with several pathogens (Fig. 3). In fungal infection models such as *Candida albicans* or *Aspergillus fumigatus*, the absence of IL-1R8 led to enhanced susceptibility to mucosal and disseminated or lung infection, respectively, with increased mortality and fungal burden, increased activation of IL-1 signaling and Th17 cell response and reduced Treg activation [134]. In *Mycobacterium tuberculosis* infection, IL-1R8-deficiency was associated with exacerbated inflammation, in terms of macrophage and neutrophil lung infiltration and increased systemic levels of inflammatory cytokines. The higher mortality observed in IL-1R8-deficient mice was prevented by IL-1 and TNFα inhibition and was not dependent on the increased mycobacteria load [135]. In acute lung infections with *P. aeruginosa*, IL-1R8-deficient mice showed deregulation of IL-1 signaling, leading to higher mortality and bacterial load, and increased production of pro-inflammatory cytokines [92]. Moreover, in a model of keratitis induced by *P. aeruginosa*, IL-1R8 was involved in preventing tissue damage, through the negative

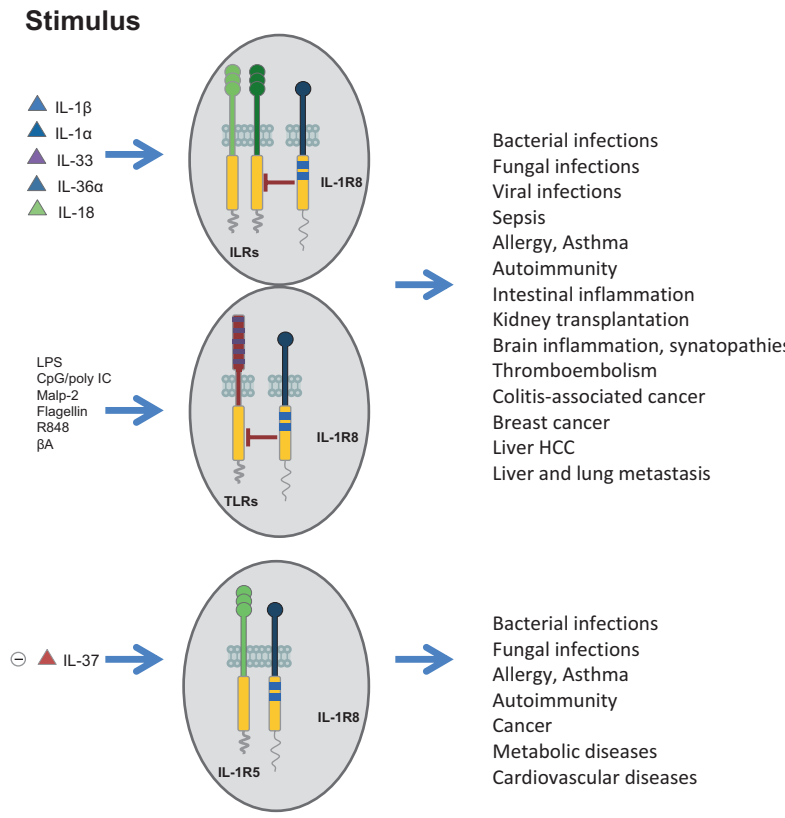

Fig. 3 Roles of IL-1R8 in pathology IL-1R8 emerged as a crucial modulator of inflammation, and innate and adaptive immune responses in several pathological contexts and it is also part of the tripartite complex necessary for IL-37 signaling. IL-1R8 plays a fundamental role in models of infections, autoimmunity, allergy, renal inflammation, platelet activation, brain inflammation and neuronal plasticity, intestinal inflammation and cancer (colorectal cancer, CLL and breast cancer). IL-1R8 acts as a checkpoint molecule regulating NK cell antitumor and antiviral activity

regulation of IL-1R1 and TLR4 signaling in Th1 cells [136]. In humans, 3 SNPs (rs10902158, rs7105848, rs7111432) were identified in the IL-1R8 gene, which correlated with the development of both pulmonary tuberculosis and tuberculous meningitis [137]. Increased susceptibility to LPS-induced mortality was described in IL-1R8-deficient mice on a BALB/c background [109], but not in a mixed C57BL/6 × 129/Sv background [110].

In contrast, IL-1R8-deficiency was protective in a model of experimental urinary tract infection (UTI) induced by uropathogenic *E. coli*, causing reduced renal bacteria outgrowth and renal dysfunction. The initial recruitment of leukocytes in the kidney was increased, in line with increased production of TNFα and chemokines (CXCL1, CCL2 and CCL3) by tubular epithelial cells after stimulation with *E. coli* [94]. In line with this, in a human bladder epithelial cell line (BECs), IL-1R8 silencing was associated with increased JNK, p38 and ERK1/2 activation and IL-6 and IL-8 production, after stimulation with LPS [100]. Similarly, in *Streptococcus pneumoniae* pneumonia and sepsis, IL-1R8-deficiency caused reduced mortality, bacterial outgrowth and dissemination [138].

In *Citrobacter rodentium* infection in mice, that mimics intestinal infections by enteric bacterial pathogens in humans, IL-1R8-deficiency was associated with microbiota depletion, due to enhanced IL-1R1 and MyD88-driven inflammatory and anti-microbial response, and therefore causing exacerbated pathogen colonization [139].

Thus, depending on the effect of inflammatory responses in specific infections, IL-1R8 may play a protective or detrimental role in the innate resistance to pathogens, emerging as a key player in the regulation of the complex and delicate balance between protective immune responses and inflammation and host tissue damage.

3.5 IL-1R8 in Autoimmunity and Allergy

IL-1 family and TLR signaling are involved in the pathogenesis of autoimmune diseases and allergy (Fig. 3). In two different models of arthritis, IL-1R8-deficient mice displayed an higher susceptibility, associated with increased cellular infiltration into the affected joints [140]. In line with this study, IL-1R8 expression was reduced in the peripheral blood of patients with psoriatic arthritis, compared with healthy donors [96]. Moreover, IL-1R8-deficient mice showed enhanced susceptibility to psoriasis, increased infiltration and activation of γδ T cells, and IL-1-driven IL-17A expression by γδ T cells [141]. In experimental autoimmune encephalomyelitis (EAE) IL-1R8-deficient mice developed a more severe disease, due to an increased Th17 infiltrate in the central nervous system (CNS) and enhanced Th17 polarization and pathogenic functions. IL-1R8 was shown to regulate IL-1-dependent Th17 cell differentiation, expansion and effector functions, by controlling IL-1-induced mTOR pathway [120].

In a model of hydrocarbon oil-induced lupus, IL-1R8-deficiency was associated with enhanced TLR7-mediated activation of DCs and expansion of autoreactive lymphocyte clones [142]. In SLE patients, in particular those with nephritis, reduced frequency of IL-1R8^{+} CD4^{+} T cells was reported in [143]. The analysis of IL-1R8 gene allelic variants of a single missense SNP (rs3210908) in a large European population showed no correlation between IL-1R8 polymorphisms and SLE [144], whereas the genetic variants of SNP rs7396562 correlated with the susceptibility to SLE in a Chinese population [145]. In C57BL/6$^{lpr/lpr}$ mice, which develop delayed autoimmunity due to impaired Fas-induced apoptosis of autoreactive B and T cells, the absence of IL-1R8 determined a massive lymphoproliferative disorder, increased autoimmune lung disease, lupus nephritis and hypergammaglobulinemia. The phenotype was associated with increased activation of DCs and B cells and production of proinflammatory cytokines (CCL2, IL-6, and IL-12p40) and B cell antiapoptotic mediators (Baff/BlyS and Bcl-2) in response to RNA and DNA immune complexes or other TLR agonists [146].

In the context of IL-33-dependent allergic responses, IL-1R8-deficient mice showed increased lung inflammation, splenomegaly and serum levels of IL-5 and IL-13 and enhanced production of type 2 cytokines *in vitro* [113]. In contrast, IL-1R8 alleles or haplotypes were not associated with asthma susceptibility or asthma-related conditions in a cohort of Japanese asthma patients [147].

3.6 IL-1R8 in Sterile Inflammation

IL-1R8 is expressed at high levels in the kidney, in particular in tubular epithelial cells, DCs and macrophages [112]. In a postischemic renal failure model, IL-1R8-deficient mice exhibit increased renal injury, caused by a massive activation of myeloid cells, increased intrarenal cytokine and chemokine production and increased leukocyte recruitment [148]. In lupus nephritis, postischemic acute renal failure or kidney transplantation, IL-1R8 expressed by hematopoietic cells was demonstrated to negatively modulate TLR activation by nucleosomes and DAMPs, released during cell necrosis associated with these conditions [142, 146, 148, 149]. In a model of fully mismatched kidney allotransplantation, IL-1R8-deficient grafts were less tolerated compared with control grafts. This phenotype was associated with enhanced allostimulatory activity of DCs and consequently allogeneic adaptive immune responses and increased post transplant kidney inflammatory response, driven by ILR and TLR signaling [149].

3.7 IL-1R8 in the Brain

IL-1R8 is expressed in the brain by neurons, microglia and astrocytes [89, 150, 151] and it regulates LPS- or IL-1-induced neuroinflammation (Fig. 3). IL-1R8-deficient mice exhibited a massive brain inflammation, in terms of CD40, ICAM, IL-6 and TNFα mRNA expres-

sion in microglia and inflammatory cytokine production in hippocampal tissue, upon treatment with LPS [152]. Even in the absence of external stimuli, cognitive and synaptic functions, such as novel object recognition, spatial reference memory, and long-term potentiation (LTP) were impaired. The phenotype was dependent on increased expression of IL-1α and high mobility group box 1 (HMGB1) and enhanced activation of IL-1R1 and TLR4 downstream signaling molecules (IRAK1, c-Jun, JNK and NFκB) [153]. Moreover, IL1R8 negatively regulated the anti-inflammatory activity of IL-36Ra in glial cells [150]. In addition, it was demonstrated that IL-1R8 regulated β-amyloid (Aβ) peptide-induced TLR2 signaling and inflammation in the brain, suggesting a potential role of IL-1R8 in Alzheimer's disease (AD) and AD-associated neuroinflammation [103].

A recent study elucidated the molecular mechanisms underlying cognitive and synaptic function impairment in absence of IL-1R8 [114]. It was shown that IL-1R8-deficiency and the consequent hyperactivation of the IL-1R pathway affected neuron synapse morphology, plasticity and function. Indeed, IL-1R8-deficient hippocampal neurons displayed an increased number of immature, thin spines and a decreased number of mature, mushroom spines along with a significant reduction of spine width, and reduced amplitude of miniature excitatory postsynaptic currents. Spine morphogenesis and plasticity impairment was caused by the IL-1R1-driven hyperactivation of the PI3K/AKT/mTOR pathway in IL-1R8-deficient neurons, leading to and increased expression of methyl-CpG-binding protein 2 (MeCP2), a synaptopathy protein involved in neurological diseases, such as Rett syndrome and MeCP2 duplication syndrome [154]. Pharmacological inhibition of IL-1R1 with IL-1Ra (Anakinra) or IL-1R1 genetic inactivation normalized MeCP2 expression and cognitive deficits in IL-1R8-deficient mice, revealing the key role of IL-1R8 in the fine tuning of IL-1R1 pathway, which is required for correct long-term potentiation [114]. Importantly, in cryopyrin-associated periodic syndrome (CAPS) patients, pharmacological inhibition of IL-1, reversed mental defects of the patients and reduced signs and symptoms of IL-1-dependent inflammation [155]. These results thus identify IL-1R8 as a key molecule involved in synaptopathies through the modulation of IL-1 activity in neurons.

3.8 IL-1R8 in Intestinal Inflammation and Intestinal Cancer

IL-1R8-deficiency is associated with uncontrolled inflammation in the intestine, leading to a reduced survival, weight loss, intestinal bleeding and local tissue injury in the model of dextran sodium sulfate (DSS)-induced colitis [110, 156] (Fig. 3). The phenotype was associated with increased local leukocyte infiltration and higher level of proinflammatory cytokines (TNFα, IL-6, IL-1β, IL-12p40, IL-17), chemokines (CXCL1, CCL2) and prostaglandins [110, 156], and demonstrated the regulatory function of IL-1R8 in epithelial cells.

IL-1R8 was also shown to inhibit the proliferation and survival signals for intestinal epithelial cells in colon crypts, through the regulation of microflora-induced ILR and TLR activation. Indeed, IL-1R8-deficiency was associated with constitutive NFκB and JNK activation and increased expression of Cyclin D1 and Bcl-xL [156]. This phenotype in healthy mice was not confirmed by other studies [110, 157], probably because of animal house-dependent variation of the microflora.

In agreement with the contribution of inflammation in increasing the risk of cancer, IL-1R8 was shown to act as a negative regulator of cancer-related inflammation and therefore cancer development and progression in different murine models of colon cancer. In a model induced by the procarcinogen Azoxymethane (AOM) followed by DSS, IL-1R8-deficiency was associated with increased susceptibility to cancer development, driven by exacerbated intestinal inflammation, as demonstrated by deregulated intestinal permeability, increased *in situ* production of proinflammatory cytokines, chemokines and prostaglandin

E_2 and expression of NFκB-induced genes involved in cell survival and proliferation (Bcl-xL and Cyclin D1) [156, 157]. IL-1R8 overexpression in gut epithelial cells rescued the susceptibility of IL-1R8-deficient mice to colitis-associated cancer development, suggesting that the regulatory activity of IL-1R8 in intestinal epithelial cells plays a central role in this model [156].

In the genetic $Apc^{min/+}$ model, which mimics the Familial Adenomatous Polyposis syndrome [158], IL-1R8 deficiency caused increased susceptibility to cancer development, due to a more sustained activation of the Akt/mTOR pathway, which is involved in cell cycle progression and consequent genetic instability [121].

Interestingly, in human colorectal cancer specimens, IL-1R8 expression was shown to be impaired compared with healthy tissues [88]. Zhao et al. identified a dominant negative isoform of IL-1R8 ($IL\text{-}1R8^{\Delta E8}$), derived from an alternative splicing causing the skipping of the exon 8. $IL\text{-}1R8^{\Delta E8}$ was retained in the cytoplasm, showed reduced N-linked glycosylation, and interacted with full-length IL-1R8, acting as an antagonist and suppressing its function. In agreement, gut epithelium-specific IL-1R8 transgenic mice expressing a mutant form of IL-1R8 ($IL\text{-}1R8^{N85/101S}$) that resembles $IL\text{-}1R8^{\Delta E8}$ isoform showed increased susceptibility to colon cancer. This indicates that IL-1R8 full functionality *in vivo* requires proper post-transcriptional modifications and cell membrane localization [88].

3.9 IL-1R8 in Chronic Lymphocytic Leukemia

TLR and ILR signaling are involved CLL development and progression, together with genetic defects and other microenvironmental contributions [159, 160]. IL-1R8-deficient mice exhibited an earlier and more severe appearance of monoclonal B cell expansion and an increased mortality, in the mouse model of CLL (TCL1), mimicking the aggressive variant of human CLL [161]. In line with these results, human malignant B cells expressed lower levels of IL-1R8 mRNA than normal B cells [160, 162, 163].

3.10 IL-1R8 in Platelets

In a recent study it was shown that both human and murine platelets and megakaryocytes expressed high levels of IL-1R8, which emerged as a key player in the regulation of platelet activation in inflammation and thromboembolism [91] (Fig. 3). Platelets express functional TLRs and IL-1 family receptors (e.g. IL-1R1 and IL-18Rα) [91, 164, 165] and interestingly, IL-1R8-deficiency caused increased platelet/neutrophil aggregate formation, induced by LPS, IL-1β or IL-18 *in vitro* and upon systemic treatment with LPS *in vivo* [91]. IL-1R8-deficient platelets displayed higher active α2bβ3 and P-selectin surface expression in basal conditions, suggesting a hyperactivated phenotype. After *in vitro* stimulation with pro-thrombotic stimuli, $Il1r8^{-/-}$ platelets showed enhanced aggregation amplitude and higher expression of α2bβ3 [91]. Moreover, IL-1R8-deficient mice were more susceptible to ADP-induced pulmonary thromboembolism, as shown by enhanced occlusion of vessels by fibrin clots and systemic levels of soluble P-selectin. IL-1R8-mediated regulation of IL-1 signaling was shown to be responsible for the hyperactivity of platelets in the absence of IL-1R8, since the phenotype was abrogated in $Il1r8^{-/-}/Il1r1^{-/-}$ mice, in line with the reported role of IL-1β in platelet activation [164]. In addition, commensal flora-derived TLR agonists were shown to be also involved in the phenotype, since microflora depletion abrogated the enhanced platelet activation in IL-1R8-deficient mice [91]. In agreement with these results in the mouse, in SIRS/sepsis patients, which exhibit platelet dysfunction [166], IL-1R8 surface expression was significantly downregulated compared to healthy controls and the downregulation correlated with the severity of the disease. Moreover, IL-1R8 expression was shown to be higher in microparticles released from LPS-stimulated platelets or collected from the serum of septic patients compared to controls, suggesting the shedding of the receptor in inflammatory conditions through microparticle release [91]. These results indicate that IL-1R8 contribute to the modulation of platelet activation, aggregation and hetero-aggregation,

both in physiological and pathological conditions *in vitro* and *in vivo*, and unveil a novel function of IL-1R8 in the regulation of thrombocyte function.

3.11 IL-1R8 in Breast Tumors

Tumor recognition and eradication mediated by the immune system can be escaped through various strategies developed by tumors [167]. Recently, we characterize IL-1R8 in breast cancer as a crucial immunomodulatory molecule. Transformed breast epithelial cells upregulated IL-1R8 expression, which was associated with impaired innate immune and T cell response [107] (Fig. 3). IL-1R8 upregulation in breast tumor cell lines led to the inhibition of IL-1-dependent NFκB activation and expression of pro-inflammatory molecules. In agreement, in a genetic model of breast cancer (MMTV-neu), IL-1R8-deficiency was associated with protection from the development of breast lesions and the number of lung metastasis was reduced. *In vitro* and *in vivo* evidences demonstrated that IL-1R8 in tumor cells was responsible for shaping the tumor microenvironment and IL-1R8-deficiency was associated with higher frequency of DCs, NK cells and $CD8^+$ T cells and lower frequency of TAMs [107]. Importantly, RNA sequencing in 1102 clinical samples of breast cancer patients showed that high IL-1R8 expression was associated with a non-T cell inflamed molecular signature, lower expression level of pro-inflammatory cytokines and chemokines, DC and NK cell metagenes, components of the peptide-presenting machinery, cytolytic enzymes and type I IFN-induced genes. Collectively, these data indicate that IL-1R8 emerges as a novel immunomodulatory molecule in breast tumors, affecting the mobilization and activation of immune cells and therefore tumor growth and metastatization [107]. These findings have important therapeutic implications, since the inhibition of IL-1R8 in this context may represent a way to restore the innate immune response and T cell trafficking and activation in the tumor microenvironment.

3.12 IL-1R8 as a Novel Checkpoint in NK Cells

Our group has recently described that IL-1R8 is expressed at high levels in murine and human NK cells and that IL-1R8 expression level increased during NK cell maturation, both in terms of mRNA and protein [90]. IL-1R8-deficient mice displayed a higher frequency and absolute number of NK cells in peripheral blood, higher frequency of mature NK cells ($CD11b^+CD27^-$ and $KLRG1^+$) in blood, spleen, bone marrow and liver. Moreover, IL-1R8-deficient NK cells showed a more active phenotype, in terms of activating receptor expression (NKG2D, DNAM-1, Ly49H), interferon-γ (IFNγ) and granzyme B production, Fas ligand expression and degranulation [90]. Bone marrow chimeric mice and IL-18 depletion experiments demonstrated that IL-1R8 directly acts on NK cells regulating IL-18, which is a key cytokine involved in NK cell activation [168, 169]. RNASeq and protein phosphorylation analysis showed that IL-18 responsiveness was dramatically different in IL-1R8-deficient NK cells, affecting pathways involved in NK cell activation, degranulation, cytokine production and anti-viral response. Moreover, IL-18-dependent activation of mTOR and JNK pathways was enhanced in IL-1R8-deficient NK cells. In contrast, other candidate pathways (i.e. IL-1 and microflora-driven TLR activation) potentially regulated by IL-1R8 in NK cells were not involved in the IL-1R8-deficient NK cell phenotype. In models of DEN-induced hepatocellular carcinoma, MCA-induced lung metastasis and colon cancer-derived liver metastasis, the disease severity and the number and dimension of metastasis were significantly reduced in $Il1r8^{-/-}$ mice. The protection was dependent on IL-1R8-mediated regulation of IL-18 in NK cells, since depletion of NK cells or IL-18-deficiency totally abrogated the phenotype. Finally, in a model of MCMV infection, $Il1r8^{-/-}$ mice controlled the virus more efficiently and the protection was dependent on enhanced NK cell degranulation and IFNγ production. Importantly, the adoptive transfer of $Il1r8^{-/-}$ NK cells was protective in the metastasis and viral infection models, compared to the treatment with $Il1r8^{+/+}$ NK cells. Partial silencing of the molecule

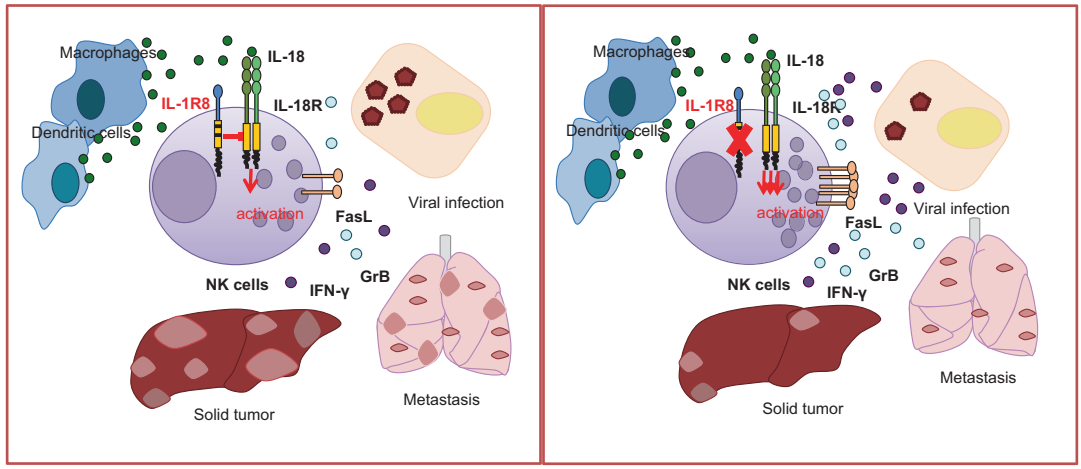

Fig. 4 IL-1R8 as a novel checkpoint of NK cell anti-tumor and anti-viral activity IL-1R8 plays a key role in the regulation of NK cell maturation and effector functions, through the modulation of IL-18-induced signaling pathway. IL-1R8 genetic blockade leads to enhanced NK cell anti-tumor, anti-metastatic and anti-viral activity

demonstrated that also in human IL-1R8 regulates NK cell activation, in terms of IFNγ production and CD69 expression [90].

NK cells are generally not credited to play a major role in the control of solid tumors, whereas evidences suggest that they are involved in the control of metastasis [170–172]. These results indicate that in addition to metastasis, NK cells have the potential to restrain solid tumors upon checkpoint blockade and in NK cell-enriched sites, such as the liver. Thus, IL-1R8 plays a non-redundant role in the regulation of NK cell development and effector functions, by tuning IL-18 signaling and emerges as a novel checkpoint molecule of NK cell antitumoral and antiviral potential [90] (Fig. 4).

4 Concluding Remarks

IL-1 family members are central mediators of the inflammatory process and play a key role in both homeostatic differentiation and activation of immune cells. ILR and TLR pathway activation is crucial for the immune surveillance against infectious agents and sterile damages, but given its broad inflammatory potential it needs to be tightly regulated at different levels. Indeed, the balance of positive and negative regulators, accelerators and brakes is a fundamental concept that governs the delicate equilibrium between host defense and detrimental inflammation leading to tissue damage.

IL-1R8 and IL-1R2 emerge as important regulators in various physiological and pathological conditions and the impairment of their function is an escape mechanism developed by pathogens and tumors. Dissecting their cell-specific and context-specific role is essential for the development and improvement of therapeutic strategies.

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Conflicts of Interest The authors declare no conflicts of interest.

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Part IV

Classical Drugs to Treat Inflammatory and Autoimmune Diseases

Glucocorticoids: Molecular Mechanisms of Action

Diana Cruz-Topete and John A. Cidlowski

Abstract

Since their discovery in the 1940s, glucocorticoids remain one of the most widely prescribed drugs in the world to treat inflammatory and autoimmune disorders, including arthritis, multiple sclerosis, asthma, ulcerative colitis, eczema, and psoriasis. They are also used as potent immunosuppressants after an organ transplant, and in the treatment of hematological cancers. Although the clinical value of glucocorticoids has been known for almost 80 years, the molecular mechanisms underlying their systemic and tissue-specific actions are still a subject of intense investigation. In this chapter, we provide an overview of the mechanisms regulating glucocorticoid synthesis, secretion, bioavailability, physiological effects, and their signaling via the glucocorticoid receptor (GR). We briefly review the gene and protein structure of the glucocorticoid receptor and its isoforms, as well as the glucocorticoid receptor genomic and non-genomic mechanisms of action. Finally, we discuss in detail the current knowledge of glucocorticoid effects on the regulation of the inflammatory response from a molecular perspective.

Keywords

Glucocorticoids · Glucocorticoid receptors · Genomic and non-genomic mechanisms · Inflammation

1 Regulation of Glucocorticoid Secretion

Glucocorticoids are steroid hormones produced by the zona fasciculata of the adrenal cortex in a circadian manner and in response to stress (Fig. 1). Exposure to environmental or biological stressors stimulates the neurosecretory cells (parvocellular neurons) within the paraventricular nucleus of the hypothalamus to release corticotropin releasing hormone (CRH). CRH is released at the median eminence then carried to the anterior pituitary gland via the hypothalamic-hypophyseal portal system. In the anterior pituitary gland, CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) by the corticotroph cells. Once in the general circulation, ACTH binds ACTH receptors located on adrenal cortical cells of the adrenal gland, leading

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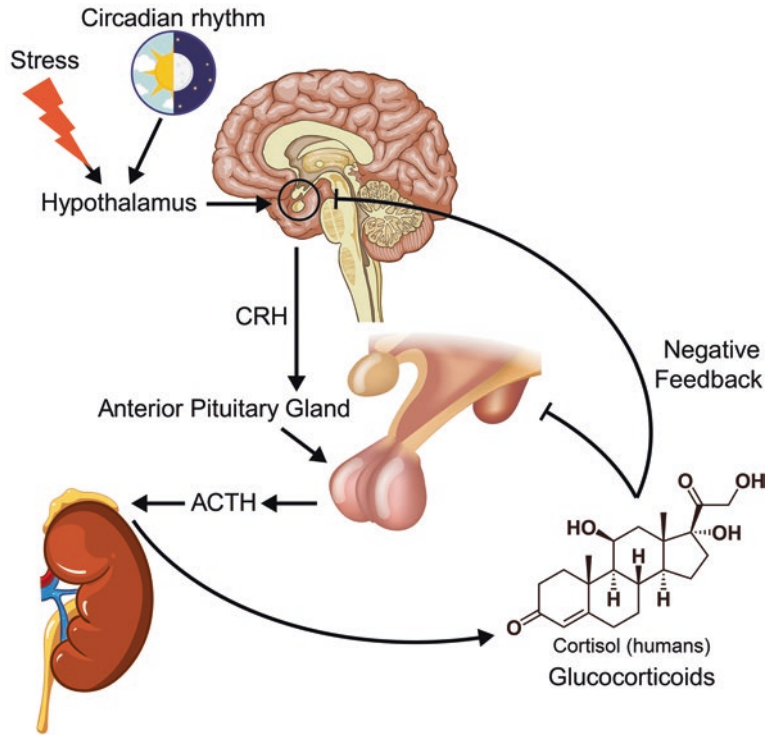

Fig. 1 Regulation of glucocorticoid secretion by the hypothalamic-pituitary-adrenal (HPA) axis in response to circadian variation and stress. The hypothalamus responds to diurnal variations (day/night) and stress stimuli by releasing corticotropin-releasing hormone (CRH). Following secretion, CRH targets the anterior pituitary gland to induce the release of adrenocorticotropin hormone (ACTH). Once in circulation, ACTH binds its receptors in the cortex on the adrenal gland to stimulate the synthesis and releases of the primary stress hormones glucocorticoids (cortisol in humans). Glucocorticoids synthesis and secretion is tightly regulated by a negative feedback to the anterior pituitary gland and the hypothalamus

to glucocorticoid synthesis (steroidogenesis) from cholesterol by the action of mitochondrial and smooth endoplasmic reticulum enzymes [1]. Following release from the adrenal glands, glucocorticoids exert their systemic and tissue-specific actions by binding glucocorticoid receptors (GR, *NR3C1*), which are ubiquitously expressed in all nucleated cells throughout the body. Acute increases in glucocorticoid levels are beneficial and help the body restore homeostasis by regulating the immune system, metabolism and cardiovascular function. However, sustained increases in glucocorticoid levels due to chronic stress, pharmacological treatment or endocrine disorders (*e.g.,* Cushing syndrome) result in an array of pathologies, including autoimmune disorders, cardiovascular disease, metabolic syndrome, infertility, growth suppression and psychiatric disorders [2–13]. Because of their profound physiological effects, glucocorticoid secretion is tightly regulated by a negative feedback loop at the level of the hypothalamus and pituitary gland (Fig. 1). Glucocorticoids negatively feedback to the hypothalamus by both genomic and non-genomic mechanisms, which inhibit the parvocellular neuron release of CRH, and subsequent secretion of ACTH by the pituitary gland [14–16]. This feedback is critical for the maintenance of systemic homeostasis. In addition to this control mechanism for glucocorticoid synthesis and secretion, glucocorticoid tissue-specific effects are further regulated by glucocorticoids binding to the plasma protein corticosteroid-binding globulin (CBG) and by the action of 11β-hydroxysteroid dehydrogenase enzymes (11βHSD1 and 11βHSD2).

Glucocorticoid binding to CGB controls the amount of free cortisol that can diffuse into cells; therefore, CGB plays a major role in controlling the activity of glucocorticoids [17]. CBG essential for the systemic distribution of glucocorticoids to target cells. For example, during infectious processes, specific proteases, including neutrophil elastase, target CGB and disrupt its ability to bind cortisol, thereby facilitating the release of active glucocorticoids at sites of tissue injury or inflammation [18, 19].

Once glucocorticoids are distributed to peripheral tissues, the concentration of bioactive corticosteroids is further regulated through 11β-HSD isozymes. Within cells, cortisol can be converted to the inactive glucocorticoid, cortisone, by the action of 11β-HSD-2, and vice versa, inactive cortisone can be converted to cortisol by the action of 11β-HSD-1. By interconverting cortisol and corticosterone, these enzymes play a pivotal role in glucocorticoid metabolism, as they can control the amount of cortisol that will bind the glucocorticoid receptor within a cell. Therefore, changes in 11β-HSD expression have been associated with changes in sensitivity to endogenous glucocorticoids [20].

2 The Glucocorticoid Receptor

The physiological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), which is a member of the nuclear receptor family of ligand-activated transcription factors. The human glucocorticoid receptor (hGR) gene, *NR3C1*, was cloned for the first time in 1985 [21–24]. The hGR gene is composed of 9 exons and is located on chromosome 5. In terms of structure, the GR protein is composed of three domains (Fig. 2) [24–26]: (1) the amino-terminal domain, which interacts with co-regulators and the transcriptional machinery; (2) the DNA binding domain (DBD), which contains two zinc-finger motifs involved in genomic interactions; and (3) the ligand-binding domain (LBD), which contains a hydrophobic pocket for glucocorticoid binding also contains an activation function (AF2) that interacts with coregulators in a ligand-dependent manner. The DBD and LBD are separated by a flexible region of the protein termed the hinge region that is involved in receptor dimerization. Two nuclear localization signals, NL1 and NL2, are located at the DBD/hinge

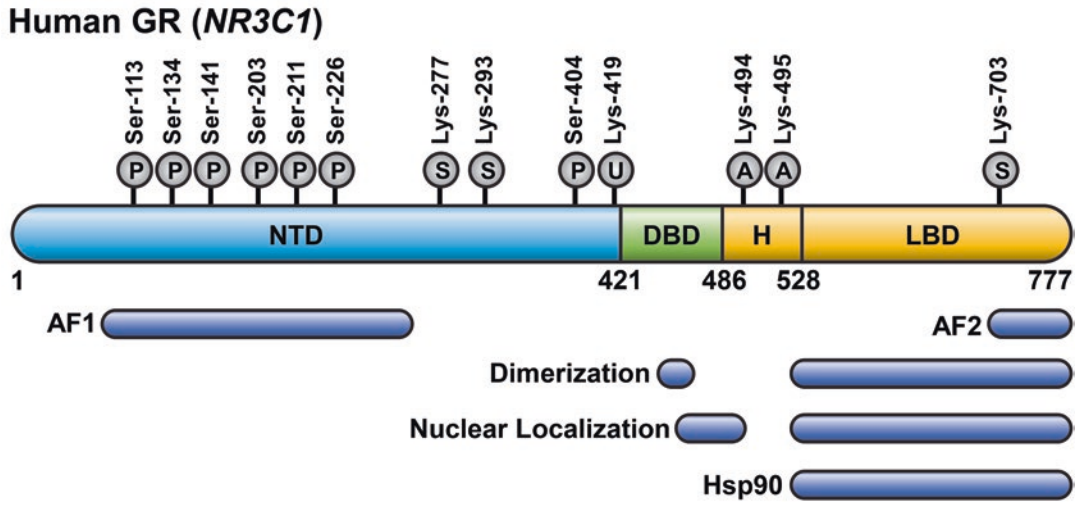

Fig. 2 Schematic representation of the human glucocorticoid receptor (hGR) protein. The hGR protein is composed of an N-terminal domain (NTD) (blue), a DNA-binding domain (DBD) (green), a hinge region (H) (red), and the C-terminal ligand domain (LBD) (yellow). The protein regions involved in transactivation (AF1 and AF2), dimerization, nuclear localization, and hsp90 binding are displayed in light blue. The sites (amino acid number) for the most common known posttranslational modifications, such as phosphorylation (P), sumoylation (S), ubiquitination (U), and acetylation (A), are indicated in the diagram

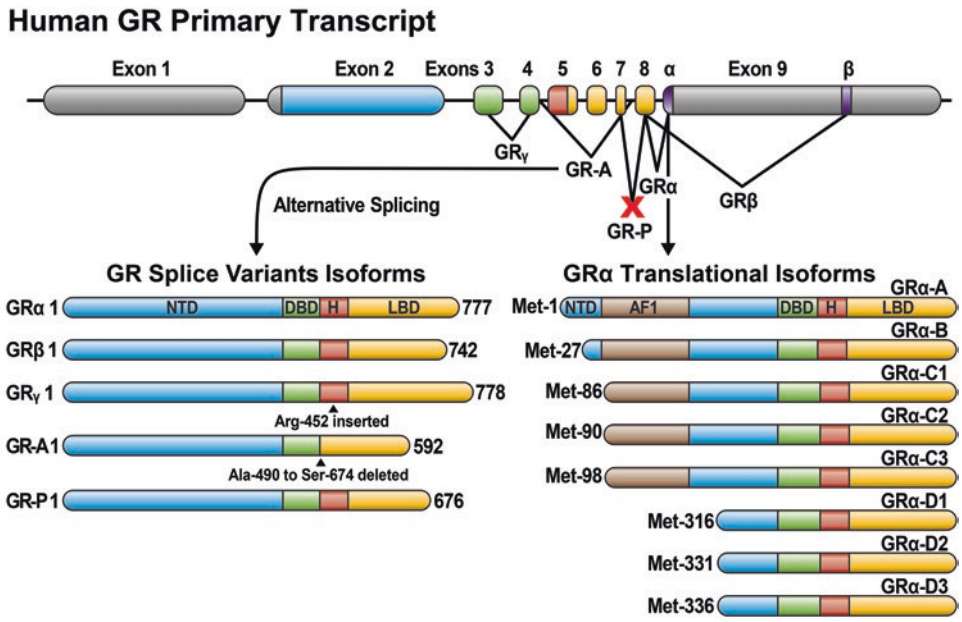

Fig. 3 Human GR (hGR) isoforms are generated by alternative splicing and alternative translational initiation. The primary transcript for hGR is composed of 9 exons (1–9). Exon 2 encodes the N-terminal domain (NTD), exons 3 and 4 the DNA-binding domain (DBD), and exons 5–9 encode the hinge region (H) and the C-terminal ligand binding domain (LBD). Alternative splicing gives rise to 5 distinct isoforms (right panel): GRα results from splicing of exon 8 to the beginning of exon 9 (see arrows). In contrast, GRβ is produced from an alternative splicing between the end of exon 8 to downstream sequences in exon 9 (see arrows), which gives rise to a GR variant encoding 15 unique amino acids at positions 728–742. GRγ is generated from alternative splicing between exons 3 and 4 (arrows), resulting in an extra- amino acid located on the DBD at position 452 [Arginine (Arg)-452]. GR-A arise from splicing that joins exon 4 to exon 8. This form of alternative splicing deletes 185 amino acids of the LBD (Ala-490-Ser-674) encoded by exons 5–7. Finally, GR-P is produced by the retention of the intronic sequence between exon 7 and 8. This event inserts a stop codon, resulting in a truncated receptor mutant missing the distal half of the LBD. The left panel displays GRα translational isoforms that result from the presence of eight different AUG start codons in the GR-mRNA, which generates eight GR isoforms with progressively shorter NTDs. GRα translational isoforms GRα-A, B, C1, C2, C3, D1, D2, and D3. Similar variants are generated from GRβ

region junction and within the LBD, respectively (Fig. 2).

2.1 Glucocorticoid Receptor Variants: Alternative Splicing and Translational Isoforms, and Post-Translational Modifications of GR

The hGR is derived from a single gene; however, alternative splicing of hGR exon 9 leads to the generation of two isoforms, hGRα and hGRβ (Fig. 3) [27–29]. These splice variants are identical through amino acid 727, but diverge thereafter. The hGRα is the classic GR protein that mediates glucocorticoid effects, and contains 50 additional amino acids (Fig. 3). In contrast, hGRβ contains only 15 nonhomologous additional amino acids (Fig. 3). The differences in the carboxyl-terminal sequence of these isoforms confers several distinct properties. For example, while hGRα resides primarily in the cytoplasm of cells and is a ligand-dependent transcription factor, hGRβ does not bind glucocorticoid agonists and resides constitutively in the nucleus of cells. Another important difference is that hGRβ by itself is inactive on glucocorticoid-responsive reporter genes. In terms of physiology, elevated levels of GRβ have been associated with glucocorticoid resistance in a variety of inflammatory diseases including asthma and rheumatoid

arthritis [24, 30]. The mechanisms by which GRβ expression leads to glucocorticoid resistance are not clear, however, one of the proposed mechanisms involves GRβ inhibition of GRα transcriptional activity through competition for GRE binding sites and/or transcriptional coregulators, and via the formation of inactive GRα/GRβ heterodimers [30–32]. In addition, studies employing genetically engineered cells expressing GRβ have also suggested that GRβ directly induces/represses the expression of a vast array of inflammatory genes, independently of its effects on GRα transcriptional activity, by a mechanism involving the recruitment of histone deacetylase complexes [33]. Supporting these data, recent studies in mice expressing hGRβ in the liver have shown that the expression of numerous genes involved in inflammatory processes are directly regulated by GRβ independently of its effects on GRα transcriptional activity [34]. Together, these studies suggest that GRβ has inherent transcriptional activity and plays a direct role as a transcriptional regulator of glucocorticoid actions. The GR gene can also give rise to three additional alternative splice isoforms: GRγ, GR-A and GR-P [26] (Fig. 3). Their roles are not well-characterized, but studies have suggested that they may play a role in the development of glucocorticoid resistant hematological malignancies [35, 36].

In addition to these splicing GR variants, GRα and GRβ can also undergo alternative translation initiation in exon two, which gives rise to several GR isoforms. Eight additional isoforms of GR with truncated N-terminal domains are generated from GRα: GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3 (Fig. 3). Alternative translational isoforms can also be generated from GRβ. Although the precise physiological role of individual GR isoforms is still not clear, studies have shown each isoform exhibits distinct cellular localization, transcriptional activity and tissue-specific patterns of gene expression, which would explain in part the cell- and tissue-specific effects of glucocorticoids [13]. The signaling properties of splicing and translational GR isoforms are discussed elsewhere and will not be elaborated here [26].

Posttranslational modifications (PTMs) play an important role in modulating the metabolic and transcriptional activity of GR. One of the best characterized PTM is phosphorylation. The GR protein can be phosphorylated in at least 9 serine residues (Ser-45, Ser-113, Ser-134, Ser-141, Ser-203, Ser-211, Ser-226, Ser 267 and Ser-404) [7, 37] (Fig. 2). GR can be phosphorylated by different kinases, including MAPKs, cyclin-dependent kinases, casein kinase II, and glycogen synthase kinase 3β [7]. Under basal conditions, GR is phosphorylated in the absence of ligand at several residues and is hyperphosphorylated at other residues upon hormone binding. The hyperphosphorylated GR protein then translocates to the nucleus, bind DNA [38] and interact with additional cofactors [39, 40]. GR phosphorylation has been shown to modulate receptor transcriptional activity, nuclear localization, and half-life [37, 41], which leads to variations in glucocorticoid mediated signaling. For example, phosphorylation of Ser-211, Ser-226 and Ser-404 have been reported to impact GR transcriptional regulation of pro-inflammatory genes, including activator protein-1 (AP-1) and nuclear factor (NF)-kB NFκB [37, 42, 43]. Also, phosphorylation at Ser-226 or Ser-404 has been shown to dampen GR downstream signaling by inhibiting GR nuclear translocation and enhancing GR protein degradation [44, 45].

In addition to phosphorylation, the GR protein is also subject to other PTMs, including ubiquitination at Lys-419, which tags the receptor for protein degradation [46, 47], and sumoylation (Lys-277, Lys-293 and Lys-703) that also promotes GR degradation and modulates the transcriptional activity of GR in genes containing multiple GR-binding sites [48]. Additional PTMs include acetylation, which can also alter glucocorticoid repression of NFκB gene expression [49].

In summary, the existence of GR isoforms and PTMs of the receptor are critical determinants of GR function, signaling and the overall glucocorticoids physiological effects, in a cell and tissue-specific fashion.

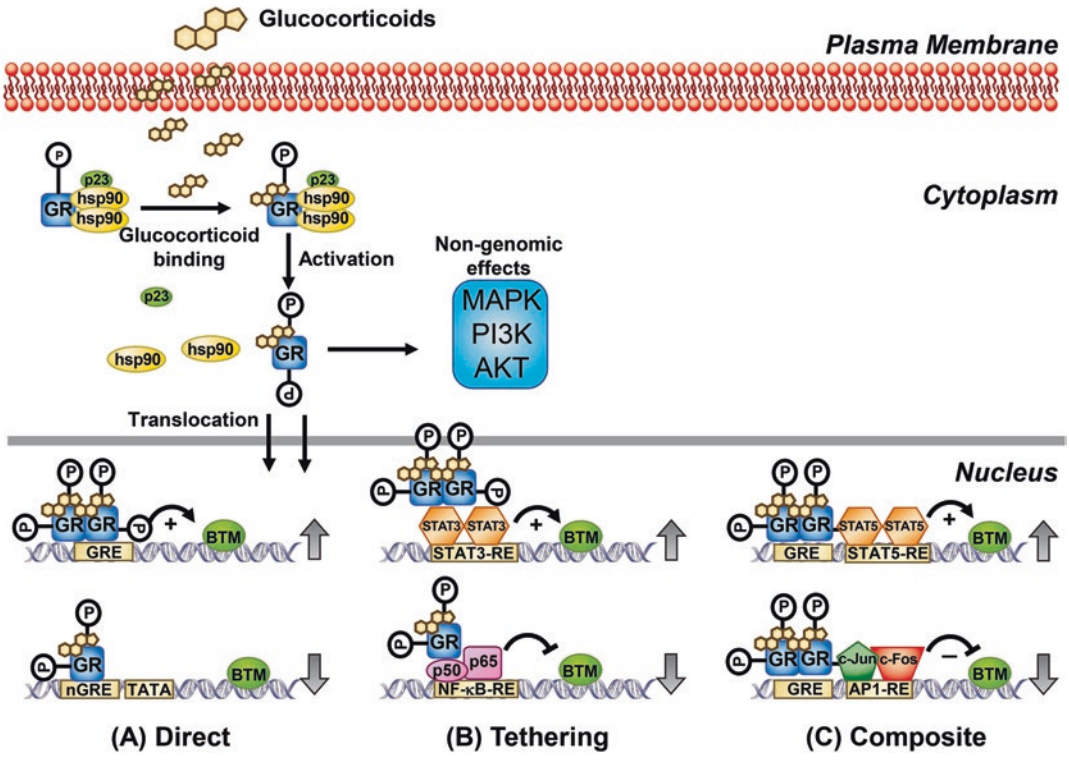

Fig. 4 Glucocorticoid receptor signaling mechanisms. Glucocorticoids are lipid soluble and readily diffuse through cell membranes. Once in the cytoplasm, glucocorticoids bind to the glucocorticoid receptor (GR). GR then undergoes activation (conformational change), becomes hyperphosphorylated (P) and dissociates from the multiprotein complex composed of the chaperone proteins hsp90, hsp70, immunophilins FKBP51 and FKBP52, and p23. Activated GR then exerts its actions by two distinct pathways, genomic and non-genomic. Non-genomic mechanisms involved protein-protein interactions with several kinases, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and AKT. GR can signal in a genomic manner by 3 mechanisms: (**a**) Direct, activated GR binds to GREs or nGREs on the promoter or sequence of target genes; (**b**) Tethering: GR tethers itself to other DNA-bound transcription factors; (**c**) Composite: GR binds directly to a GRE and interacts with neighboring DNA-bound transcription factors. BTM, Basal transcription machinery; NFκB, Nuclear factor-κB; STAT, Signal transducer and activator of transcription; AP-1, Activator protein-1

3 GR Signaling Mechanisms

Non-ligand bound GR resides predominantly in the cytoplasm of the cell as part of a multiprotein complex that maintains the receptor in a conformation that favors ligand-binding, prevents its degradation, and promotes GR nuclear translocation by facilitating GR mobility/trafficking along the cytoskeleton [50]. This multiprotein complex is composed of chaperone proteins hsp90, hsp70, immonophilins FKBP51 and FKBP52, p23, and dynamitin and dynein [50–54]. Upon ligand withdrawal, GR is shuttled again to the cytoplasm by the calcium-binding protein calreticulin, and the chaperone complex reforms to prevent GR degradation and prepare the receptor for another round of ligand-binding and nuclear mobilization [55] (Fig. 4). In the next sections, glucocorticoid signaling through genomic and non-genomic mechanisms will be reviewed.

3.1 GR Genomic Effects

Glucocorticoid-mediated physiological effects are mainly attributed to GR alterations to gene expression through three mechanisms (Fig. 4). The first mechanism involves GR direct binding

to DNA sequences, known as glucocorticoid responsive elements (GREs, consensus sequence GGAACAnnnTGTTCT, where 'n' represents any base), to induce gene expression, or GR direct binding to negative GREs (nGREs, consensus sequence CTCC$(n)_{0\text{-}2}$GGAGA) to repress gene expression. GREs and nGREs are found in gene promoters, intragenic areas and in regions distant from transcription start sites [56]. A second mechanism involves GR forming "tethers" with another transcription factor without contacting DNA. A third and final mechanism includes GR binding DNA sequences that contain both a GRE and a response element for another transcription factor. This final mechanism is known as composite binding.

Most of the anti-inflammatory effects of glucocorticoids are believed to result from GR protein-protein interactions with DNA-bound transcription factors (tethering), by interacting with the classical pro-inflammatory transcriptional regulators NF-kB and AP1, the signal transducer and activator of transcription (STAT), CCAAT/enhancerbinding protein (C/EBP) and the nuclear factor of activated T cells (NFAT) families [57, 58]. GR can repress AP1 and NF-kB activity by binding the Jun subunit of AP1 and the p65 subunit of NF-kB (Fig. 4). Also, GR can also regulate AP1 and NF-kB activity by binding directly to a GRE and physically associating with these pro-inflammatory mediators (Fig. 4).

3.2 GR Non-genomic Effects

Classically, biological effects of glucocorticoids are attributed to GR modulation of gene transcription (genomic effects); however, GR also exerts rapid physiological actions by non-genomic mechanisms involving physicochemical interactions with cytosolic GR or membrane-bound GR that lead to changes in ion transport through plasma membranes (Fig. 4). For example, in lymphocytes, activated GR translocates to mitochondria and regulates apoptosis by promoting alterations in membrane cation-exchange, which has a major impact on mitochondrial coupling efficiency and production of reactive oxygen species [59, 60]. In addition, GR can alter cytoplasmic signaling through its interactions with various kinases, including phosphoinositide 3-kinase (AKT), and mitogen-activated protein kinases [61]. GR has also been shown to localize in caveolae, and through interactions with caveolin-1, glucocorticoids can inhibit cell growth and proliferation independently of GR effects in gene expression [62]. Therefore, GR non-genomic actions orchestrate important control over glucocorticoid intracellular signaling and add complexity to the mechanisms governing glucocorticoid-dependent physiological effects.

4 Glucocorticoid Anti-inflammatory Effects

Glucocorticoids are among the most potent regulators of the inflammatory response [63]. Inflammation is commonly divided into two types: acute inflammation, which occurs within seconds of injury and is mainly mediated by the innate immune system, or chronic inflammation, which takes place over a prolonged period and in which the adaptive immune response plays a major role. Glucocorticoids alter events involved in both acute and chronic inflammation. For example, glucocorticoids block the initial events in the inflammatory response by inhibiting vasodilation and increased vascular permeability, resulting in a decrease in leukocyte migration/trafficking to the inflamed tissues [64]. At the same time, glucocorticoids modulate events in chronic inflammation by altering/suppressing T cell activation [65]. During acute inflammation, glucocorticoids diminish the activation of pro-inflammatory signaling pathways by suppressing the expression of inflammatory mediators [66]**.** For example, glucocorticoids downregulate the classical Toll-Like-Receptor (TLR) signaling pathway by inhibiting, through genomic mechanisms, the expression of TLR downstream transcription factors, including NF-κB, AP-1, and interferon-regulatory factor 3 (IRF3) [67]. Glucocorticoids also upregulate the expression of genes that inhibit TLR signaling activation. Among these glucocorticoid-regulate genes are

the dual-specificity protein phosphatase 1 (DUSP1), which dephosphorylates mitogen-activated protein kinase 1 (MAPK1) and therefore inhibits its activity, and the IL-1 receptor-associated kinase 3 (IRAK3), which represses the TLR and IL-1 receptor signaling [66]. Also, glucocorticoids have been reported to modulate the expression of NF-κB inhibitors, including IκBα and the glucocorticoid-induced leucine zipper protein (TSC22D3, also known as GILZ) [63, 68]. The repression of the NF-κB pathway by glucocorticoids blunts the expression of many pro-inflammatory cytokines, such as IL-1α and β, IL-2, IL-3, IL-4, IL-5, TNF, etc., and genes involved in T-cell development, maturation, and proliferation [68–70]. Therefore, by inhibiting "key" pro-inflammatory modulators of signal transduction, glucocorticoids control/suppress the magnitude of the inflammatory response.

In the next subsection, the anti-inflammatory and immunosuppressing mechanisms of glucocorticoids-via GR- during acute inflammation are reviewed in more detail.

4.1 Glucocorticoid Mechanisms of Action in Acute Inflammation

Upon tissue injury, an inflammatory response is initiated by the expression of pattern recognition receptors (PRRs), complement receptors, and Fc receptors by tissue-resident cells (Fig. 5). Once activated, PRRs bind molecular motifs from microbial organisms known as pathogen-associated molecular patterns (PAMPs) and endogenous cellular molecules produced by injured cells called damage-associated molecular patterns (DAMPs). These cellular events will initiate a cascade of immune cell activation and the production of inflammatory mediators, including cytokines, histamine, bradykinin, prostaglandins, leukotrienes, and cell adhesion-promoting molecules [9].

Glucocorticoids are potent immune regulators that interfere with the inflammatory response at the cellular level [71]. For example, glucocorticoids have been shown to inhibit macrophage cytokine production by the selective inhibition of p38 MAP kinase, which leads to repression of its downstream signaling, in particular, cytokine production [72]. Glucocorticoids also downregulate the expression of chemokines and chemoattractants in leukocytes, including IL-8, IL-16, CC-chemokine ligand 2 (CCL2), CCL3, CCL5, CCL11, CCL24 and CCL26, adhesion molecules, and integrins, which block immune cell migration and adhesion to the sites of inflammation [4, 73, 74]. In addition, glucocorticoids induce apoptosis of T-cells, myeloid cells, basophils, and eosinophils by repressing the activity of pro-survival and proliferation transcription factors, such as AP-1, c-myc, NFkB [75].

Classically, the ability of glucocorticoids to inhibit the production of cytokines, chemokines, and their receptors has been attributed to glucocorticoid inhibition of the TLR signaling pathway. TLRs are pattern-recognition receptors (PRRs) that recognize PAMPs from microorganisms or DAMPs from damaged tissue [76]. Activation of TLR signaling is pivotal for the initiation of the innate immune response and the subsequent development of antigen-specific acquired immunity. In the context of inflammation, glucocorticoids repress TLR signaling by directly regulating the activity of pro-inflammatory transcription factors, such as NF-kB, AP-1 and interferon-regulatory factor 3 (IRF3), or indirectly by inducing proteins that antagonize these inflammatory signaling pathways (Fig. 6).

The NF-κB family consists of five members: p65 (RelA), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (P50/p100) [77]. The mechanisms by which GR represses the activity of NF-kB are unclear. However, several possibilities have been proposed (Fig. 6). Studies have shown that GR physically interacts with RelA, which inhibits its transcriptional activity [78]. Also, GR can block the formation of the p65/IRF3 complex [79]. In addition, GR has been reported to inhibit the phosphorylation of the C-terminal domain (CTD) of Pol II by competing with the CTD kinase pTEFb [80]. Similarly, GR can recruit histone deacetylases to NF-κB-dependent pro-

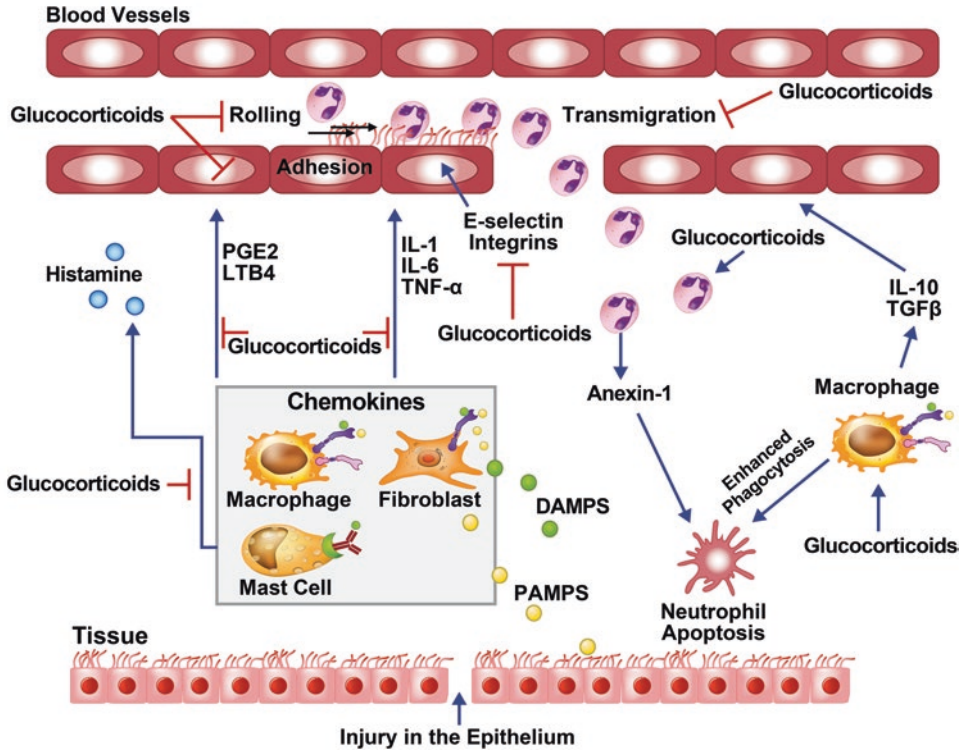

Fig. 5 Anti-inflammatory effects of glucocorticoids. Exposure to pathogens leads to a fast activation of the inflammatory response characterized by the expression of "danger sensors," including pattern recognition receptors (PRRs, in purple), complement receptors (pink), and Fc receptors (green). PRRs bind pathogen-associated molecular patterns (PAMPs, green circles) and damage-associated molecular patterns (DAMPs, yellow circles). Upon activation of PRRs by PAMPs and DAMPs, resident tissue macrophages, mast cells and fibroblasts start producing and secreting inflammatory molecules such as histamine, prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and pro-inflammatory cytokines (IL-1, IL-6, and TNF-α). Together these inflammatory mediators induce the expression of adhesion molecules and chemoattractants that stimulate the recruitment of leukocytes into the injured area. Endothelial cells expressed E-selectins and integrins, which facilitate neutrophils and monocytes rolling, adhesion and transmigration to the inflammatory foci. Extravasated leukocytes then migrate through the tissue following chemokine gradients to inflammatory sites. Glucocorticoids modulate the magnitude of the inflammatory response at several checkpoints. Glucocorticoids act on macrophage and mast cells blocking the production of histamine and lipid mediators (such as PGE2 and LTB4). This effect inhibits the increase in vascular permeability. In addition, glucocorticoids repress the expression of pro-inflammatory cytokines by resident immune cells and extravasated immune cells. On the endothelium, glucocorticoids down-regulate the expression of E-selectin and other adhesion molecules curving rolling, adhesion, and extravasation on neutrophils to the site of inflammation. Glucocorticoids also induce the expression of annexin-1 by neutrophils. Annexin-1 expression promotes neutrophil detachment and apoptosis, and phagocytosis of apoptotic neutrophils by macrophages. Moreover, glucocorticoids induce a switch in resident macrophages gene expression profile from pro-inflammatory to anti-inflammatory and increases macrophages phagocytic activity and the expression of the antiinflammatory cytokines transforming growth factorβ (TGFβ) and IL10, which contributes to the resolution of the inflammatory process

moters [81, 82], or compete with NF-κB for binding to CREB-binding protein and p300 [83]. Finally, GR can also repress NF-kB by inducing the gene expression of the NF-kB inhibitor IkBα [84, 85], although this mechanism is still controversial as it appears to be cell-type specific.

AP-1 is a group of dimeric factors comprising the basic leucine-zipper transcription factors Fos (cFos, Fos B, Fra-1, and Fra-2), Jun (c-Jun, v-Jun,

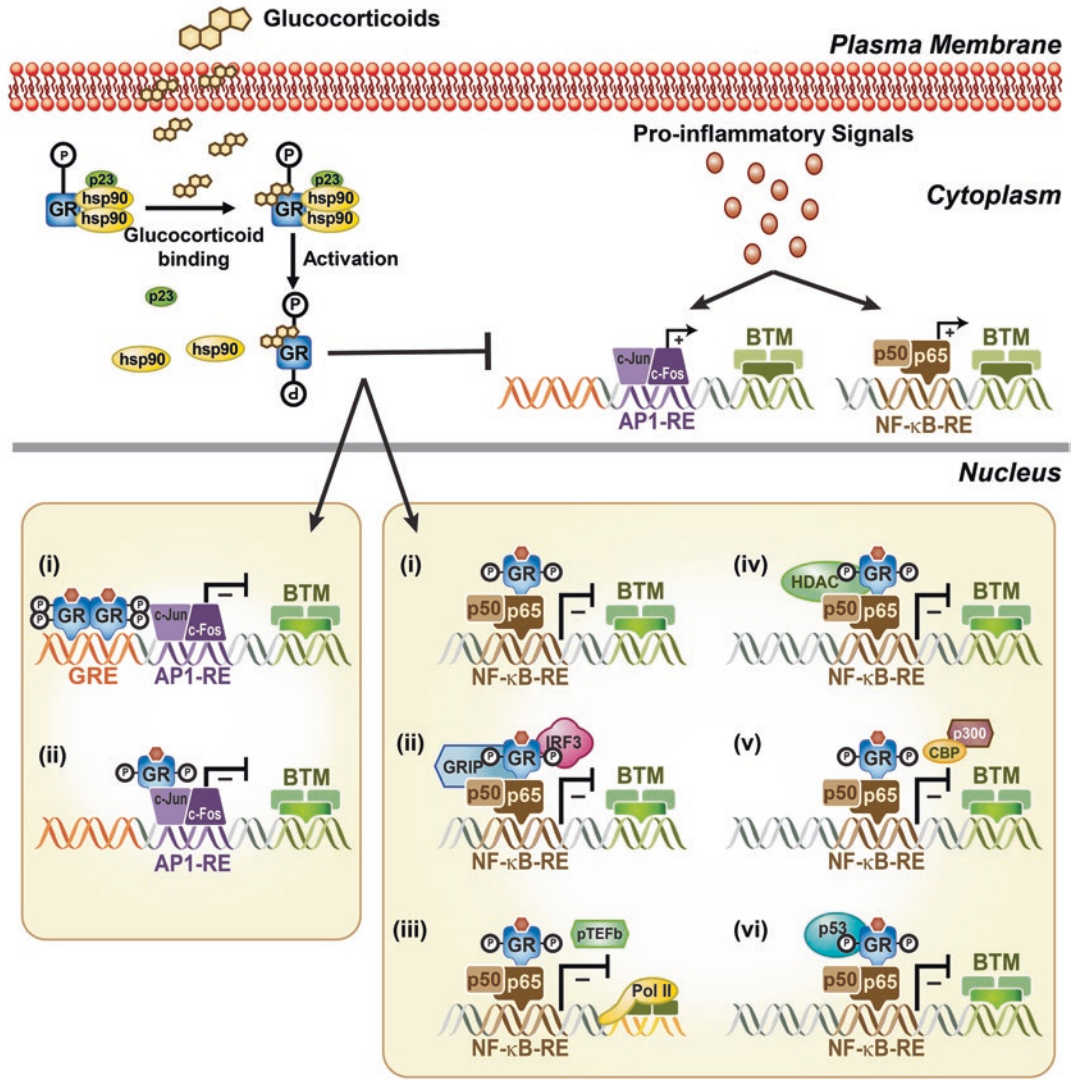

Fig. 6 GR represses pro-inflammatory gene expression by inhibiting AP-1 and NFκB transcriptional activity. Activated GR translocates to the nucleus and blocks AP-1 and NFκB transcriptional regulation of pro-inflammatory genes by different mechanisms. GR represses AP-1 by the following mechanisms: (i) GR binding to a GRE and simultaneously interacts with c-Jun to repress transcription; (ii) GR protein-protein interactions (tethering) with c-Jun. There are six well-characterized mechanisms by which GR represses NFκB activity: (i) GR direct interaction with the p65 subunit; (ii) GR recruitment of GRIP (GR interacting protein) which blocks the formation of NFκB/IRF3 (interferon regulatory factor 3) heterodimer; (iii) GR can inhibit the activation and phosphorylation of RNA polymerase II (Pol II) by blocking the recruitment of pTEFb (positive transcription elongation factor); (iv) GR also can recruit HDAC (histone deacetylases) blocking NFκB transcriptional activity. (v) GR can also prevent NFκB from interaction with p300 and CPB (CREB1-binding protein); (vi) finally, GR can interact with p53, altering NFκB pro-inflammatory transcriptional activity

Jun B, and Jun D), activating transcription factor (ATF2, ATF3, B-ATF, JDP-1, and JDP-2), or MAF (MAFA, MAFB, c-MAF, NRL, MAFF, MAFG, and MAFK) [86, 87]. AP-1 is activated by mitogens, oncoproteins, and pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), and interleukin-1 [88]. AP-1 is one of the most important regulators of the inflammatory

response. Once activated AP-1 regulates the expression of pro-inflammatory genes by binding to the promoters of target genes. The classical model for GR-inhibition of AP-1 transcriptional activity involves GR direct interaction with the c-Jun subunit of AP-1, leading to the inhibition of c-Jun NH2-terminal domain phosphorylation [88–90] (Fig. 6).

Although GR inhibition of NF-kB and AP-1 activities has been suggested to be the primary mechanism by which glucocorticoids induce immunosuppression, studies have also shown GR affects additional modulators of the immune system that contribute to glucocorticoid anti-inflammatory effects (Fig. 6). Interferon regulatory factor (IRF)3 is one of these factors [91]. IRF3 is a mediator of the inflammatory response downstream of Toll-like receptors (TLR) 3 and 4, which are activated by viral double-stranded RNA (dsRNA) [92] and bacterial lipopolysaccharide (LPS) [76], respectively. IRF3 is a transcription factor required for the activation of type I interferons (IFNα/β), chemokines IP10, RANTES and several other pro-inflammatory genes [93]. GR has been shown to regulate IRF3-dependent gene expression by sequestering glucocorticoid receptor interacting protein-1 (GRIP1), a member of the steroid receptor coactivator (SRC) family of transcriptional regulators. Interaction of GRIP1 with IRF-3 is critical for activation [91, 94]. In addition, glucocorticoids can induce the expression of proteins that antagonize pro-inflammatory processes at the post-transcriptional level. For example, glucocorticoids induce an increase in MAPK phosphatase-1 at the mRNA and protein level. Increased MAPK phosphatase-1 then blocks the phosphorylation and activation JNK, leading to decreases in c-Jun transcriptional activity [95]. These events, in turn, prevent the induction of multiple inflammatory genes. Glucocorticoids also inhibit NF-κB transcriptional activity in T lymphocytes and in macrophages by inducing the expression of the anti-inflammatory protein glucocorticoid-induced leucine zipper protein (GILZ) [96, 97]. GILZ specifically associates with the p65/p52 subunits of NF-κB inhibiting its transcriptional activity [98]. Finally, glucocorticoids can also block the actions of pro-inflammatory cytokines by influencing the mRNA stability of proteins that stimulate their degradation. Among these proteins is tristetraprolin (TTP), which stimulates the degradation of transcripts with AU-rich elements such as TNF-α [99].

One hallmark of inflammation is the increase in vascular permeability and tissue edema. Glucocorticoids control these processes by acting on macrophages in inflamed tissues and inhibiting the production of nitric oxide and eicosanoids, such as prostaglandins and leukotrienes, that promote vascular permeability and vasodilation. There are two mechanisms by which glucocorticoids curb the production of prostaglandins and leukotrienes. One mechanism involves GR-dependent induction of annexin A1 expression, which inhibits phospholipase A2 (PLA2) activity [100]. PLA2 catalyzes the release of arachidonic acid (a 20-carbon polyunsaturated fatty acid), which is further modified by cyclooxygenases into prostaglandins and leukotrienes [101]. A second mechanism, by which GR further blocks the production of eicosanoids is inhibition of cyclooxygenase 2 (PTGS2) through the NFκBsuppressing properties of GILZ [70]. Also, glucocorticoids can induce the production of vasoconstrictors (*e.g.,* angiotensin-converting enzyme and endothelin) and inhibit the activity of vasodilators (*e.g.,* bradykinin). The effects lead to a reduction in the flow of blood to the sites of inflammation, which decreases vascular leakage and leukocyte infiltration across post-capillary venules [102].

Glucocorticoids also interfere with leukocyte adhesion by preventing the upregulation of adhesion molecules. Dexamethasone (a synthetic glucocorticoid) and cortisol administration can block E-selectin (CD62E) and ICAM-1 (CD54) expression on human endothelial cells following stimulation with lipopolysaccharide [73]. In vivo studies have shown that glucocorticoids can significantly reduce the expression of ICAM-1 in the rat mesenteric microvascular bed [103]. Moreover, treatment of mice with the synthetic glucocorticoid dexamethasone reduces leukocyte extravasation into the peritoneal cavity and

blocks the upregulation of ICAM-1 by the intestinal vascular bed in endotoxemia [104]. Glucocorticoids also curb the expression of other endothelial adhesion molecules, including P-selectin, E-selectin, VCAM1, and several chemokines and chemoattractants, including IL-8, IL-16, CC-chemokine ligand 2 (CCL2), CCL3, CCL5, CCL11, CCL24 and CCL26, thereby decreasing leucocyte migration and adhesion during inflammation [73].

Studies have demonstrated that glucocorticoids also reduce the expression of adhesion molecules CD44 and the integrin lymphocyte function-associated antigen 1 (LFA1) and very late antigen 4 (VLA4) [105].

Glucocorticoid induction of annexin A1 and the subsequent activation of ALXR in neutrophils and macrophages leads to an increase in neutrophil apoptosis and phagocytosis of apoptotic neutrophils. This action reduces further tissue damage and limits the inflammatory response by resulting in the transduction of anti-inflammatory signals, such as by transforming growth factor-beta [106]. Moreover, glucocorticoid-dependent induction of annexin A1 also blocks T cell recruitment to the sites of inflammation [106].

As the inflammatory process subsides, glucocorticoids upregulate the expression of anti-inflammatory and reparative cytokines such as transforming growth factor-beta and IL-10 by monocytes and macrophages that further promote the phagocytosis of apoptotic cells and debris [107]. In addition, glucocorticoids induce the expression of pro-resolving factors by enhancing the expression of the lipoxin A4 receptors, which halt PMN diapedesis [108]. Therefore, glucocorticoids mediate the control and resolution of inflammation through the direct effects on gene expression and by many effector molecules.

4.2 Glucocorticoid Regulation of Cellular and Humoral Immunity

Glucocorticoids exert important effects in the regulation of T cell development, viability, and activation. Glucocorticoid insufficiency leads to thymocytes increased proliferation, suggesting that glucocorticoids are negative regulators of thymopoiesis [63]. In vitro and in vivo data show that thymocytes are extremely sensitive to glucocorticoid-induced cell death [9, 63]. Mice with conditional deletion of GR in T cells present thymic hyperplasia and impaired T cell response to glucocorticoids, supporting the role of endogenous glucocorticoids in the regulation of thymocytes expansion and T cell activation and survival [109]. The effects of glucocorticoids on T cells are particularly important for the treatment of hematological cancers. Therefore, understanding their mechanisms of action is critical to develop better pharmacological therapies.

Glucocorticoids decrease T cell activation by inhibiting the maturation of antigen-presenting cells (APCs) and by downregulating the expression of MHC class II molecules, the lipid presentation molecule CD1a, co-stimulatory molecules, and pro-inflammatory cytokines [63, 110]. Also, glucocorticoids diminish T cell activation by repressing the activation of the T cell receptor (TCR) signaling through the downregulation of transcription factors FOS, AP-1, NF-κB, and NFAT [111], and by regulating the expression of ITK, TXK, and LCK, kinases involved in TCR signaling [111]. Finally, via non-genomic effects, glucocorticoids dampen the activation of kinases pathways (*e.g.*, LCK and FYN) that play a role in TCR signaling activation [112]. Although glucocorticoids main effects on T cells are associated with decreased activity, some studies suggest that endogenous glucocorticoids also have positive effects on T cells [113]. For example, glucocorticoids can promote the differentiation and activity of T_H2 cell and regulatory T cells [114]. On the other hand, glucocorticoids target T_H17 to control the immune response in animal models of sclerosis and rheumatoid arthritis [115]. One possible explanation for these contradictory data is that glucocorticoids exert differential effects on T cells depending on their activation status and stage of differentiation. Further studies in animal models and humans are needed to clarify the role of these hormones in thymopoiesis and T cell biology.

Regarding the effects of glucocorticoids on humoral immunity, recent studies have shown that glucocorticoids have effects on B cell selection, survival, and homeostasis. However, no mechanistic studies exist on glucocorticoids molecular effects in B cell signaling and antibody production [116]. Additional studies are needed to fully dissect the effects of glucocorticoids in T and B cell function.

5 Conclusion and Future Perspectives

For almost 80 years, glucocorticoids have been and continue to be the first line of treatment for inflammatory and autoimmune disorders, yet the molecular basis for their actions in the regulation of the immune system are still unclear and controversial. State-of-the-art research employing in vitro and animal models have revealed that glucocorticoids exert dual (positive and negative) effects on the immune system depending on the physiological context. During acute inflammation, glucocorticoids restrain the magnitude of the immune response by diminishing the amplification of "danger signals" (expression of PRRs, PAMPs, and DAMPs), blocking vascular dilation and permeability, controlling the migration of inflammatory cells to the site of injury, and by repressing the expression of pro-inflammatory cytokines. Therefore, glucocorticoids "brake" the immune response allowing the body to restore homeostasis by fine-tuning inflammation resolution and promoting the transition to wound healing. Despite the positive effects of glucocorticoids in the attenuation of inflammatory processes, there is considerable evidence that glucocorticoids can also exacerbate inflammation. This pro-inflammatory response has been observed to depend on several factors including dose, type of exposure (acute or chronic), and time of administration (*e.g.,* low-glucocorticoid treatment before inflammatory stimuli). Therefore, alterations in the levels of glucocorticoids, as observed in chronic stress, may impact the sensitivity and overall effects of pharmacological treatment with synthetic glucocorticoids in autoimmune and inflammatory disorders. Although until this day glucocorticoids remain the mainstays in the treatment of inflammatory pathologies, their use is hampered by severe side effects associated with chronic glucocorticoid use. Adverse effects range from ulcers, hypertension, glucose intolerance and insulin resistance, to osteoporosis and obesity. Rheumatoid arthritis and asthma are among the most frequent diseases for which long-term glucocorticoid treatment is prescribed. Therefore, efforts are now focused on designing novel synthetic glucocorticoids for more efficient and safer treatment for patients who required long-term use. To this end, new glucocorticoid receptor agonists are currently being developed in an attend to reduce the adverse side effects while maintaining the anti-inflammatory effects. More research and a deeper understanding of the molecular actions of endogenous glucocorticoids in the regulation of the immune response will provide valuable information on how basal levels of glucocorticoids positively or negatively influence the effects of exogenous glucocorticoid administration. This approach will help to optimize the efficiency of glucocorticoid therapies and minimize the adverse side effects.

Finally, there are significant gaps in our knowledge about the sex-specific effect of glucocorticoids on the immune response, especially regarding the heterogeneity in GR signaling responses between males and females in normal physiology and pathological conditions. Men and women react differently to stress and exogenous glucocorticoid administration. The understanding of the impact of these differences on immune regulation will contribute substantially to the development of effective and safe glucocorticoid therapies for immune disorders that work efficiently and with minimal side effects in both men and women.

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Glucocorticoids: Immunity and Inflammation

Simona Ronchetti and Carlo Riccardi

Abstract

As very powerful anti-inflammatory and immunosuppressive drugs, glucocorticoids have gained attention over the past decades as a first line treatment for chronic inflammatory and autoimmune diseases, in addition to its wide use in the field of oncology. An extensive body of research has accumulated with regards to the molecular anti-inflammatory mechanisms by which glucocorticoids exert their effects on the cells of the immune system. Moreover, some pro-inflammatory properties have recently emerged, based on the analysis of glucocorticoid-regulated genes. Understanding the physiology and the pharmacology of these hormones and drugs in the context of inflammation and the immune system will allow for the comprehension of their still incomplete function in homeostasis and of their practical clinical applications.

Keywords

Glucocorticoid · Inflammation · Immunosuppression · Stress · HPA axis · Lymphocytes

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1 Introduction

When discussing the most potent anti-inflammatory drugs, glucocorticoids (GCs) represent the first class of drugs that is typically considered. Since the discovery of their importance for the life of a human being in the half of the nineteenth century, GCs have been studied for decades; however, the molecular mechanisms of GCs have been incompletely understood to date. Thomas Addison was the first to describe adrenal insufficiency syndrome in 1849, and Harvey Cushing reported hypercortisolism syndrome later in 1932; however, it was not until 1949 when the use of corticosteroids started being introduced into the clinics for patients affected by rheumatoid arthritis, by Hench and Kendall. The discovery of the extraordinary anti-inflammatory properties of GCs was new and unexpected at that time, and it paved the way for the subsequent extensive clinical use of GCs for a multitude of inflammatory diseases that were incurable. The impact of treatment with GCs was so revolutionary that Philip Hench and Edward Kendall received the Nobel prize in 1950 together with Tadeus Reichstein, who first isolated and identified the steroid hormones of the adrenal cortex. Subsequently, approximately 30 synthetic GCs have been synthetized by the pharmaceutical industry and are employed to treat a variety of inflammatory and autoimmune diseases. Despite their therapeutic potency, considerable adverse effects occur during long-term treatment with GCs. The continuous and probing research

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into the molecular mechanisms of action of these compounds is helping to determine which synthetic GCs will exert only beneficial effects.

2 GCs in the Stress-Related Immunomodulation

Stress is defined as an actual or anticipated threat of the homeostasis of an organism, by which external or internal stimuli (e.g. social threats, maternal deprivation, fear, physiological challenge and surgery) termed stressors, activate the so-called "stress system" [1]. The stress system consists of: (1) the *locus caeruleus*/norepinephrine autonomic nervous system that acts within seconds; and (2) the hypothalamic–pituitary–adrenal (HPA) axis, comprising the hypothalamus, pituitary and adrenal glands, whose response starts more slowly, from hours to days. GCs are the final products of the HPA axis, and are released by the *zona fasciculata* of the adrenal cortex as a response to psychogenic (e.g., fear) and physical (e.g., cellular lesion or pathogen invasion) stressors. They act through the GC receptor (GR) via genomic and non-genomic pathways in virtually any tissues, thereby controlling the basic and physiological functions of the cardiovascular and immune systems, the metabolism of carbohydrates, lipids, and proteins, bone and muscles, as well as behavioral processes (e.g. emotion) (Table 1).

During the acute phase of stress, GCs at low concentrations can bind to both the membrane-bound GR and the mineral corticoid receptor (MR). This, in turn, activates multiple signal transduction pathways that ultimately lead to a series of physical and behavioral changes that aim to increase the survival of the organism [2, 3]. While the cytoplasmic GR receptor is ubiquitously expressed in the brain, with high levels of expression in the neurons and microglia, MR is expressed in a few regions (e.g. the hippocampus, amygdala and neurons within the paraventricular nucleus of the hypothalamus) [4]. The cytoplasmic GR is responsible for the genomic effects of GCs, the membrane GR and MR mediate the rapid non-genomic effects of GCs in the brain in response to a stress stimulus, with important consequences to the adaptive changes in the organism. However, how these receptors function still requires further investigation. Following a stress stimulus, GCs restore their own hypersecretion via a negative feedback mechanism on the HPA axis, but if the stressor persists, GC hypersecretion may not be properly controlled and may become a potential threat for the organism. There is evidence that the prolonged exposure to stressful conditions can induce the structural remodeling of neurons and alterations in glial functions, which are frequently maladaptive, thus contributing to the development of acute or chronic diseases [5].

Both acute and chronic stress affects the immune response by activating both the sympathetic nervous system and the HPA axis, from which the release of cortisol is required to re-establish homeostasis. The interplay between these systems are complex and remain only partially understood, occurring in both the peripheral tissues and the central nervous system (CNS). As an example, GCs are known to control the expression of α1B and β adrenergic receptors, with important consequences in the clinical use of GCs [6, 7]. Immune responses are affected by disturbances in HPA axis activity. The hypersecretion of cortisol (e.g. during stress) can increase susceptibility to infections and neoplastic diseases and, simultaneously, enhance resistance to autoimmune or aseptic inflammatory diseases. Conversely, a deficiency in cortisol production reduces the susceptibility to infectious agents while also diminishing the susceptibility to autoimmune or aseptic inflammatory diseases. Therefore, the level of circulating cortisol influences immune cell activity due to the potent effects of GCs on cells of the innate and adaptive immune system (as described later). As an example, surgery-induced stress reduces the number of T cells and shifts the Th1/Th2 balance to a Th2 response in humans, in conjunction with an increase of T regulatory (Treg) cells that contribute to Th1 suppression [8]. Similarly, other studies have demonstrated that corticosteroids, both alone or combined with catecholamines, can increase the expression of Th2 cytokines in the peripheral blood cells of human subjects and shift the Th1/Th2 balance towards a Th2 phenotype during long-term GC exposure. Simultaneously, this shift was accompanied by an altered ratio of IFNγR/IL-4R, with a dominant expression of IL-4R in Th2 cells [9, 10]. A Th1/Th2 unbalance predominantly impacts diseases such as asthma, Th2 cytokine-mediated allergic airway inflammation of the

Table 1 A list of the physiological and adverse effects of glucocorticoids. The adverse effects, as well as the physiological effects, occur after long-term pharmacological treatments and represent an exacerbation of their physiological effects

Tissue/system	Physiological effect	Adverse effect
Carbohydrate/lipid metabolism	Gluconeogenesis increase	Diabetes mellitus
	Peripheral insulin resistance	
	Hepatic glicogen deposition increase	
Adipose tissue	Increase in fatty acids	Fat redistribution, visceral obesity
Bone metabolism	Bone formation decrease	Osteoporosis, osteonecrosis
	Bone mass loss	
Cardiovascular	Stimulation of renal tubular secretion and of glomerular filtration rate	Hypertension, alterations of serum lipoproteins, premature atherosclerotic disease, arrhythmias with pulse infusions
Muscle, connective tissue	Normal muscle function, protein catabolism	Myopathy, muscolar atrophy
Genitourinary and reproductive	Inhibition of release of GnRH from the hypothalamus, of gonadotropin from pituitary, inhibition of testosterone synthesis and release from gonads	Amenhorrhea/infertility, intrauterine growth retardation
Brain	Modulation of physiological homeostasis, coordination of adaptive responses to stressors	Euphoria, dysphoria/depression, insomnia, psychosis, pseudo tumor cerebri
Endocrine system	Decrease of LH, FSH, TSH release	Hypothalamic-pituitary-adrenal insufficiency
Immune system	Immunosuppression	Heightened risk of typical infections, opportunistic infections, herpes zoster
	Anti-inflammatory effects	
Gastrointestinal tract	Inhibition of the intestinal absorption of calcium	Gastritis, peptic ulcer disease, pancreatitis, steatohepatitis, gastrointestinal bleeding, hypocalcemia
Renal	Salt and water retention	Hypokalemia, fluid volume shifts
Skin		Skin thinning and purpura, alopecia, acne, hirsutism, striae, petechia, hypertrichosis, delayed wound healing
Eye		Posterior sub capsular cataract, glaucoma, exophtalmos

lungs. For example, psychological stress exacerbates allergic symptoms due to endogenously released GCs and, simultaneously, induces a reduction in GR expression, causing an insensitivity to the anti-inflammatory effects of exogenously administered GCs for treatment of asthma [11, 12]. Such reduced GR expression is not the only mechanism responsible for the failure to elicit a response to GCs in this pathology. GC resistance also involves other mechanisms, including defects in caspase-induced apoptosis in eosinophils, the major pro-inflammatory effector cells in asthma. Furthermore, GCs inhibit IL-12 release by monocytes and macrophages, thus contributing to the shift towards a Th2 phenotype [13]. This effect is further exacerbated by the reduction of T regulatory (Treg) cells caused by GCs; stress-derived GCs suppress the proportion of FoxP3+ Treg cells in subjects experiencing mental stress, thus worsening the symptoms of asthma. In addition, a decrease in Treg numbers and activity was found in different rodent asthma models, likely due to the reduction of T cell production in the thymus [14, 15].

In the CNS, the primary cell types exhibiting immune functionality is microglia cells. Microglia cells are associated with the HPA axis, respond to infections by secreting pro-inflammatory cytokines, and help mobilize other immune cells to restore homeostasis. They express high levels of GR and represent the first immune target of GCs in the brain [16]. When a stressor activates the HPA axis, the released GCs

stimulate the production of anti-inflammatory cytokines (e.g. IL-4 and IL-10), which function to counteract the inflammatory signals generated by the microglia. Moreover, stress-induced GCs stimulate the proliferation of microglia cells [17]. This cross-talk between GCs and microglia cells is included in the signaling network between the immune, endocrine and nervous systems. Such cross-talk is necessary to restore homeostasis following an inflammatory insult to prevent an inappropriate reaction that can result in detrimental effects to neurons; however, GCs both promote anti-inflammatory effects but also induce pro-inflammatory effects in the CNS following a stress stimulus, depending on the timing of exposure followed by an immunogenic challenge. For example, GCs can potentiate or suppress the same pro-inflammatory cytokines depending on whether the cells have been treated with GCs either before or after LPS administration, respectively [18, 19]. Thus, stress-released GCs via the HPA axis can prime neuroinflammatory processes to subsequent immune events, shifting the surveillance state of microglia cells to a primed one [20]. Since exaggerated immune and inflammatory responses in the brain are currently considered to be pathogenic in the context of a number of psychiatric disorders (e.g. depression or bipolar disorder), stressors that lead to neuroinflammation by means of released GCs are thought to contribute to the development of psychiatric disorders [21].

The immune system is also a target of the stress-related effects in the periphery. Cathecolamines released by the sympathetic nervous system (SNS) are directly released into the lymphoid organs. Increased norepinephrine in the bone marrow causes a release in the circulation of myeloid cells, (e.g. monocytes and granulocytes). These cells are immature and therefore, naturally "inflammatory", producing pro-inflammatory cytokines in the tissues which they easily infiltrate [22]. Furthermore, myeloid cells are insensitive to GCs, thus accounting for the GC-resistance that develops during chronic stress. This functional insensitivity is primarily associated with the reduction of the transcriptional activity mediated by GR. In addition, inflammatory peripherally derived macrophages traffic to the brain, thus contributing to the neuroinflammation of the brain with consequences in neurobiology and behavior [23].

In several stress-related long-term diseases, (e.g. acute coronary syndrome or major depression), the percentage of Tregs is decreased, which may serve as a means to facilitate an immune response [15]. When stress becomes chronic, it can lead an increased percentage of Tregs to counteract the inflammatory status and the increased proliferation of effector T cells [24]. However, heightened levels of Tregs have also been found in acutely-stressed healthy individuals, which may represent an altered response to acute stress that may predispose individuals to future disease development [25].

Immunomodulation by stress can influence tumor growth, progression, and metastasis. Cytokines (e.g. IL-6) can be produced at high levels by immature, inflammatory myeloid derived-cells that are GC insensitive and contribute to enhanced inflammatory responses to infectious agents [26, 27]. Therefore, chronic inflammation can lead to tumor development via the mediation of cells of the immune system [28]. As an example, Tregs can favor tumor progression by locally suppressing the anti-tumor immunity [29–31]. In addition, stress-released GCs induce immunosuppression, thereby decreasing immune defense against cancer and facilitating tumor growth. Furthermore, epidemiologic studies have linked stress to tumor progression through the activation of the SNS [32]. Molecular studies have also revealed a role for the Glucocorticoid-Induced leucine Zipper (GILZ) protein and its isoform long-GILZ as novel anti-proliferative GC-mediators. GILZ can bind to Ras, thereby inhibiting Ras- and Raf-dependent cell proliferation, whereas L-GILZ can bind to p53, thereby exerting an anti-proliferative effect [33, 34]. However, the mechanism by which GCs influence tumor cell growth, either under conditions of stress or during a pharmacological treatment, requires further study.

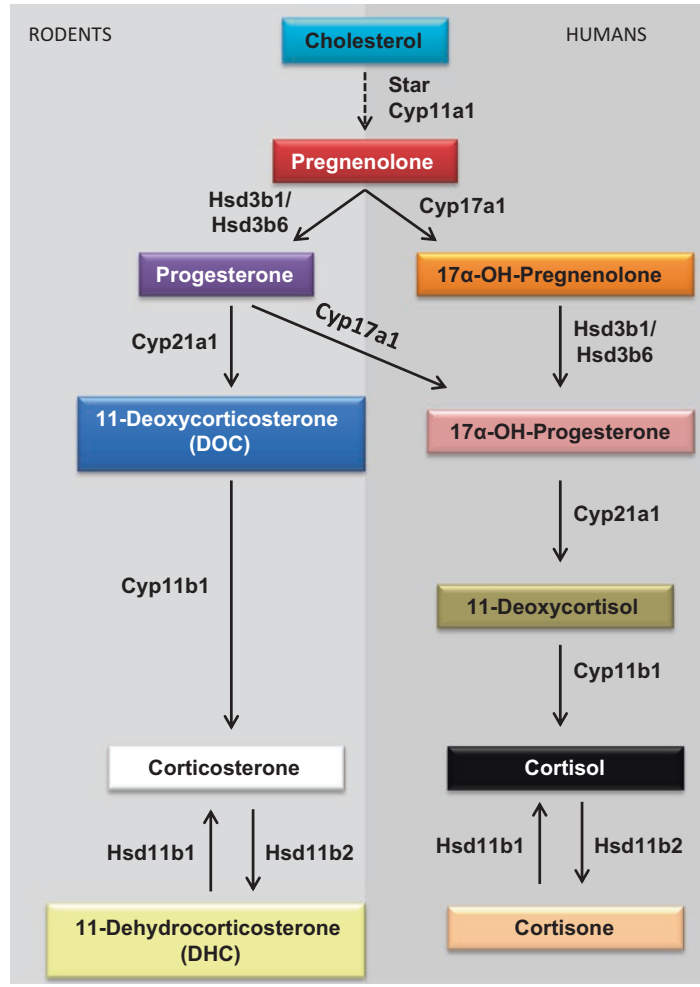

Fig. 1 Glucocorticoid synthesis in different species. A series of enzymatic reactions lead to corticosterone and cortisol production in rodents and humans, respectively

3 Local Production of Glucocorticoids

Circulating GCs are released by the adrenal cortex of the adrenal glands following a circadian local rhythm and this mechanism is independent of systemic influences (e.g. stress or peripheral constant CRH infusion). This regulation protects the organism from threats (e.g. infections) by avoiding possible serious consequences derived from the long-term activation of the immune system, which communicates with the HPA axis via the secretion of cytokines, including TNF-α, IL-1 and IL-6 [35]. GCs are derived from cholesterol via a series of enzymatic conversions (Fig. 1) thus leading to corticosterone production in species, such as reptiles, birds, or mice, and to cortisol release in species such as fish, primates or humans. Both corticosterone and cortisol bind to the GC and mineralocorticoid receptors, thereby regulating the activity of several organs.

In addition to the systemic production of GCs, extra-adrenal synthesis can be found in organs such as primary lymphoid organs, skin, intestine, brain, heart, and vasculature. This local production does not affect serum levels of GCs, since the removal of adrenals does not lead to a detectable amount of circulating GCs; however, it rather seems to exert control over eventual inflammatory conditions [36]. The first organ in which the local

production of GCs was demonstrated was the thymus, approximately 20 years ago [37]. Although the level of enzymes required for the de novo synthesis of GCs is up to 10,000-fold lower than that in the adrenals, the level of GCs produced by thymic epithelial cells (TECs) is sufficient to activate GC-responsive genes in the surrounding cells. TECs are the main cells that produce GCs in the thymus, which is high at birth but decreases with aging. In contrast, corticosterone produced by double positive (DP) $CD4^+$ $CD8^+$ thymocytes increases with age. Once thymocytes have passed positive selection, they upregulate the expression of CD69 and cease producing GCs. Immature thymocytes are the primary target of the effects of both adrenal-released and locally produced GCs. In fact, GCs produced by immature thymocytes induce apoptosis in these cells in an autocrine manner [38]. In contrast, ACTH inhibits GC synthesis in thymocytes while inducing their synthesis in adrenal and TECs. This opposing effect has not been fully characterized, but it is believed to function as a limiting factor for the control of excessive apoptosis in the thymus, thus protecting this organ from a strong activation of the HPA axis that can result in detrimental effects [39, 40]. However GCs can also prevent apoptosis in thymocytes when the TCR is triggered, in so called "mutual antagonism", in which the pathways activated by the TCR and the GR interact. This mechanism helps protect the thymocytes that would be negatively selected if they had received only one signal (from the GR or TCR). In particular, local GCs protect thymocytes with an intermediate affinity for the TCR from pro-apoptotic signals, thus undergoing to positive selection and survival, while low and strong affinity for TCR results in thymocyte apoptosis. Overall, positive selection can occur as a result of GC-mediated TCR antagonism [41]. At the molecular level, GCs induce the upregulation of Bcl2 expression and GR interactions with AP1, NFAT, and NF-κB transcription factors, thus contributing to prevention of apoptosis [40]. The dual production of GCs, one by the adrenal glands and the other by both the local thymocytes and TECs, appears to serve two purposes: (1) peripheral production is required to respond to and regulate strong systemic immune responses; and (2) local production controls homeostasis and the development of thymocytes and possibly other cell types, including dendritic cells, fibroblasts and macrophages.

The thymus is not the only lymphoid organ that produces GCs. The bone marrow and spleen are also lymphoid organs particularly devoted to the synthesis of GCs in early life when the adrenal contribution is low. This production increases with age, suggesting that lymphoid GC synthesis is required for lymphocyte development throughout life. In this context, in addition to T lymphocytes extensively studied in the thymus, B cells may also be the target of locally released GCs because of their high GR expression, and their maturation can be influenced by the effect of local GCs. However, additional studies are necessary to assess the role of lymphoid GCs in extra-thymic lymphoid organs and their effects on poorly studied cells other than T lymphocytes. One interesting feature of locally produced GCs is their synthesis via GC regeneration, as demonstrated by the high expression of the enzyme, Hsd11b1, that converts the inactive 11-keto metabolite DHC and cortisone into active GCs (Fig. 1) [42, 43]. While GC synthesis from cholesterol is independent of serum (adrenal) steroids, GC regeneration is dependent on the availability of circulating synthesized GC metabolites (DHC in mice or cortisone in humans), which can vary acutely in response to stressors. Therefore, stressors, chronic stress, or diseases that affect the availability of circulating GC metabolites can control T cell development via regenerated GCs in the peripheral lymphoid organs.

Another important organ with the ability to synthesize GCs is the intestine. The proliferating cells of the intestinal crypts are devoted to the production of GCs, which have a controlling task in the maintenance of local immune homeostasis. Any stimulus that activates the immune local system triggers the production of GCs that control and limit the immune response to avoid an exaggerated response and consequent tissue damage. Such function of locally produced GCs occurs in the mucosal tissues, in which the strict contact between immune cells and microorganism does exist. In addition to this sentinel role, GCs are also

responsible for maintaining the integrity and permeability of the epithelial barrier by antagonizing the destruction of tight junctions caused by TNFα during inflammation (e.g. Crohn's disease) [44]. TNFα is also the most important cytokine able to induce local GC synthesis: in mice lacking TNFα or its receptor, GC synthesis in the gut is either reduced or absent [45]. In line with this fact, GCs produced in the intestine were found to reduce the damage in inflammatory bowel disease, both in experimental colitis in rodents and in human disease, confirmed by the decreased expression of GC synthetic-enzymes in Crohn's patients [46].

Tumors derived from transformed epithelial cells of the adrenal cortex produce GCs; similarly, transformed cells from intestinal crypts (i.e., colorectal tumor cells) constitutively synthetise GCs. Although the reason these tumor cells release GCs has not yet been elucidated, it is believed that local GC synthesis may exert immune suppression on immune cells infiltrating the tumor microenvironment, as a mechanism of immune escape [47].

Lungs, like the intestine, are lined with a single epithelial layer that allows for the exchange of gases with the external environment; however, this permits access to potential pathogen agents. For this reason, the mucosa is rich in resident immune cells involved in the surveillance against invading microorganisms. Similar to the intestine, the lungs are the site of extra-adrenal GC synthesis that serve to prevent tissue damage caused by an exaggerated immune response. However, the metabolic pathway of GC synthesis in the lungs differs from that in the intestine. The lungs appear to reactivate circulating non-active dehydrocortisone to corticosterone, whereas in the intestine, GCs are synthesized from cholesterol or cholesterol metabolites. Unlike the intestine, steroidogenesis is not triggered by TNFα or other pro-inflammatory cytokines, but rather is dependent on the serum dehydrocorticosterone released by the adrenal glands, as the surgical removal of these glands abolishes local GC synthesis in the lungs [48, 49]. This differential regulation of local steroidogenesis could be due to different regulation of local inflammation in the intestine and lungs. This may be related to the need to restrict the inflammatory response to the lamina propria to prevent bacteria spreading while maintaining hormonal regulation by the adrenal glands in the lungs, since local inflammation could easily turn into systemic inflammation due to the high vascularization of lungs.

Another barrier between the organism and the external environment is the skin. Steroidogenic enzymes have been found in melanocytes, fibroblasts and keratinocytes. Moreover, cortisol is the major steroid produced in the skin. Local production of GCs is induced by inflammation (i.e. TNFα and IL-1β cytokines), UV radiation, and tissue damage, which trigger a reproducible HPA axis response with local ACTH production. GCs produced in the skin play an immunosuppressive and anti-inflammatory role, in contrast to serum GCs, which promote cell migration from the blood to the skin in response to infections or tissue damage in the short-term. Therefore, their function is to control an excessive immune response, similar to other extra-adrenal organs [39].

Rat and human brains express enzymes for glucocorticoid and mineralocorticoid synthesis and the mouse brain can synthesize corticosterone in both hippocampal pyramidal neurons and granule neurons. In developing neurons, it is believed that GC levels are maintained at low levels due to their potential toxic effects, together with low levels of serum GCs; however this hypothesis requires validation [39]. As described earlier in this chapter, GCs exerts extremely important functions in the brain, but dissecting the effects caused by circulating GCs from those triggered by locally synthesized GCs is extremely difficult.

Overall, the local production of GCs appears to control an excessive immune reaction, since it takes place in organs in which the immune surveillance is physiologically relevant (e.g. thymus, intestine and lungs). The concentration of locally synthesized GCs is much higher than the serum levels. Furthermore, circulating GCs are poorly available when needed due to their ability to bind to the serum corticosteroid-binding globulin. This local high concentration allows for a higher number of GC receptors to be activated and initiate the genomic and non-genomic responses with

typical anti-inflammatory and immunosuppressive outcomes at specific sites. In addition, the extra-adrenal and adrenal GC syntheses are occasionally in opposition, such as in the lymphoid organs, where local GC production is high during development and decreases over time, compared to the systemic GC synthesis that is low during development and increases over time. Since local GC production has been found in animal species other than rodents and humans, it is believed that this mechanism represents an evolutionary adaptation that is useful to obtain a focused control of the immune reactions thereby avoiding harmful effects in all other tissues eventually exposed to high levels of circulating GCs. Finally, this local production raises the question whether therapeutic use of synthetic GCs should be adapted to reconstitute local concentration levels of endogenous GCs that have been eventually decreased in diseases.

4 GC Effects on Cells of the Immune System

Among the most sensitive GR expressing cells in an organism, immune cells have been largely studied for being exquisitely responsive to the effects of GC. The anti-inflammatory and immunosuppressive effects of GCs occur in all cells of the immune system, with either overlapping or different mechanisms linked to various cell types.

4.1 T Cells

T lymphocytes are the most studied immune cells under the effects of GCs. Starting from their maturation and development in the thymus, T cells are sensitive to GC effects throughout their lifespan. T cells are either positively or negatively selected at the stage of $CD4^+$ $CD8^+$ cells, in which a strong TCR signal leads to apoptosis as well as an absent TCR signal. At this stage, T lymphocytes are very sensitive to GC-induced apoptosis, which is antagonized by TCR engagement when both stimuli are present, as described earlier in this chapter [50]. The intensity of TCR signaling determines the fate of the cell: only TCRs with an intermediate signal intensity and avidity for self-antigens will allow the survival of the thymocyte due to the "mutual exclusion" effect of GCs, through which the TCR and GR signals oppose each other, allowing the cell to escape apoptosis. Thymic selection of mature T cells has also been explored in studies that have used transgenic mice with a mutated GR. Mice expressing reduced GR levels due to the presence of an antisense transgene exhibit an impaired transition from $CD4^-$ $CD8^-$ precursors to $CD4^+$ $CD8^+$ and increased apoptosis, supporting a role for endogenous GC in balancing TCR-mediated signals during thymic selection and agreeing with in vitro obtained results [51, 52]. Conversely, other studies that have used mice with GR-deficient thymocytes demonstrated that GR signaling is not essential for T cell development or selection in the thymus [53, 54]. To dissect the specific roles of GCs in the thymus, further studies using in vivo mouse models and targeted mutations in the GR will be able to assess the actual role of GC in thymic cell development.

GCs also exert their effects on mature T cells. T-cell-specific inactivation of the GR, as well as mice with a selective functional mutation in the GR are useful for demonstrating that GCs can suppress activation-induced cell death by inhibiting the expression of FasL, which induces cell death by binding to its cognate receptor, Fas, on T lymphocytes, thereby limiting excessive activation [55]. In addition to this genomic pathway, other non-genomic pathways mediate the effect of GCs on T lymphocytes. Soon after GC administration, kinases (i.e. LCK and Fyn) are downregulated with a consequent dissociation from the TCR complex [56]. Although less sensitive to GC-induced apoptosis due to a strong CD28 signal, mature lymphocytes are therefore influenced by GCs with regards to their activation, survival, and cell death through multiple mechanisms.

An important GC function is the ability to drive T cell subtype differentiation. Upon an antigenic or inflammatory stimulus, T lymphocytes can differentiate into distinct subtypes such as Th1, Th2, Th17, Tregs and, more recently, Th9 cells. Each subtype has a specific role, either physiological or even pathogenic, expresses specific transcription

factors, and secretes a distinct pattern of cytokines. Each subset is differently sensitive to GC-induced apoptosis; for example, Th1 cells undergo apoptosis when subjected to GC effects while Th2 and Th17 cells are resistant. Moreover, Tregs have been demonstrated to be both sensitive and resistant in distinct experimental settings. Furthermore, GCs can induce cytokine suppression distinctly in different subtypes, one of the mechanisms of their anti-inflammatory properties. Cytokines from Th1 and Th2 cells, including IFN-γ, IL-4, IL-5, and IL-13, can be suppressed by GCs. In contrast, IL-17A and IL-17F from Th17 cells are resistant to GC suppression in primary Th17 cells, despite being sensitive in multiple sclerosis or severe asthma [57]. Moreover, Th17 cells and their released cytokine IL-17 appear to be partially responsible for the resistance to GC treatment in one third of patients with inflammatory disorders that do not respond to steroid therapy [58].

GCs can cause a shift from Th1 to Th2 immunity at physiological doses by suppressing Th1 cytokines. More specifically, GCs can suppress the production of interleukin (IL)-12, interferon IFN-γ, IFN-α, and TNF-α by antigen-presenting cells (APCs) and Th1 cells, but can upregulate the production of IL-4, IL-10, and IL-13 by Th2 cells, thereby promoting their differentiation [59]. One consequence of this unbalance is the exacerbation of pathologies (e.g. asthma) as mentioned above, in which the Th2 phenotype contributes to disease pathogenesis.

The role of GCs in Tregs remains still not completely defined: data obtained *in vitro* in human PBMC revealed a rapid decrease of FoxP3 expression within 24 h of exposure to Dexamethasone (Dex), not due to apoptosis. In support of these *in vitro* data, in transplanted patients with a normal Treg number, Tregs can be suppressed by GC treatment. However, an increased number of Treg cells was reported in patients with allergic rhinitis, asthma or other autoimmune disease, treated with high doses of corticosteroids, in which the number of Tregs could be modified by the disease. Additionally, in an experimental model of EAE, short-term simultaneous administration of Dex and IL-2 markedly expanded functional suppressive $Foxp3^+$ $CD4^+$ $CD25^+$ T cells in murine peripheral lymphoid tissues [14, 60, 61]. These opposing observations may vary according to the diseases in which Tregs are studied or differential experimental settings. Recently, the mechanism through which GCs increase the number of Treg cells in mice has been discovered; GILZ, an anti-inflammatory protein rapidly induced by GCs, increases the number of Treg cells by virtue of its ability to cooperate with TGF-β in the induction of FoxP3 [62]. The Treg field still remains an incompletely explored field and additional studies are needed to unravel the relationship between GCs and Tregs.

4.2 B Cells

If considered with respect to T lymphocytes, less is known about GC function in B lymphocytes. Only recent work has partially clarified the role of GCs in B cells. GCs are used to treat B cell malignancies due to their ability to suppress B-cell checkpoint genes across multiple developmental stages. A very recent study reported that supraphysiological levels of GCs can either push immature cells to the next stage of development, with consequent apoptosis, or they may arrest cells by removing a positive growth signal [63]. An in-depth analysis in murine B cells derived from the spleen and bone marrow demonstrated that Dex stimulated apoptosis in all B-cell developmental subsets, suggesting that GC signaling plays a pivotal role in B-cell life-or-death decisions [64]. To further underline this important role in the life of a B cell, a GC-induced protein, GILZ, was found to be the mediator of the effects of GCs on B cell lifespan. In mice with a GILZ deletion, an accumulation of B lymphocytes in the bone marrow, blood, and lymphoid tissues was found, as well as decreased B-cell apoptosis. This supports an important role of GCs, through GC-induced genes, in the regulation of B-cell survival [65, 66]. Immunoglobulin synthesis is also regulated by GCs; low levels of GCs do not exhibit any effects on immunoglobulin synthesis, whereas high doses decrease immunoglobulin levels in the blood due to an increase of their catabolism at the beginning, followed by a reduction in synthesis [67, 68].

4.3 Macrophages

Monocytes and macrophages are among the cells of the first line of defense in the immune system and thus targets of the actions of GCs. Suppressing the intracellular signaling cascade of MAP kinases is one of the mechanisms through which GCs exert their anti-inflammatory effects, by inhibiting the transcription of pro-inflammatory cytokines like IFNγ, IL-1α and IL-1β. Furthermore, the suppression of these pro-inflammatory cytokines can be achieved via the direct interaction of ligand-activated GR with transcription factors like AP-1 and NF-κB at the promoter of target genes [68]. GCs can even promote the survival of anti-inflammatory monocytes by exerting anti-apoptotic effects. It is important to protect anti-inflammatory monocytes from apoptosis and let them differentiate so that they can be efficient in the down-regulation of inflammation. The mechanism by which GCs exert this specific effect is via the regulation of the A3 adenosine receptor [69].

Physiologically, low concentrations of corticosterone exert stimulatory effects on naïve macrophage chemotactic and phagocytic activities, in the absence of immune stimuli, and GR is at least partially responsible for these effects. Conversely, supraphysiological GC concentrations do not have any effects on macrophage functionality. Thus, during the early phase of stress, corticosterone may prime innate cells and contribute to defense against an infectious agent [70]. Overall GCs exert distinct functions on monocytes/macrophages, depending on the dose and the presence or absence of an immune stimulus.

4.4 Neutrophils and Other Granulocytes

Different from other immune cell types, neutrophils are protected from apoptosis when treated with GCs, exhibiting a doubling of their half-lives compared to untreated cells. This is due to the expression of members of the Bcl-2 family of survival proteins and the suppression of pro-apoptotic genes. In this manner, GCs can contribute with neutrophils to help the organism fight against infections with a primary defense cell, when all other cells of the immune system succumb to their apoptotic action. In contrast, the persistence of neutrophils in inflamed tissues further increases inflammation and contributes to the resistance to any pharmacological treatments with GCs (e.g. severe neutrophilic asthma, inflammatory bowel disease, and rheumatoid arthritis). It remains to be elucidated whether GCs differentially influence the distinct circulating phenotypes of neutrophils under inflammatory conditions [71]. Another anti-inflammatory effect GCs exert on neutrophils is the prevention of granulocyte trans-migration into inflamed tissues. GCs can arrest the extravasation of neutrophils from blood circulation by multiple mechanisms, including the reduction of selectin expression and integrin receptors on neutrophils and endothelial cells, respectively. Furthermore, GCs have been recently found to reduce neutrophil migration by the upregulation of GILZ protein and consequently Annexin A1, an anti-inflammatory and anti-migration protein [72]. GCs are also able to increase bone-marrow derived neutrophils in the blood stream, so that they are therapeutically useful for the treatment of neutropenia in combination with G-CSF [73]. Although studies describing the effects of GCs on granulocytes began many years ago, we are still far from fully understanding the influence of GCs on neutrophils, under both the physiological and inflammatory conditions.

Eosinophils and basophils are sensitive to GC-induced apoptosis, and this mechanism is mediated by the Fas/FasL system and the increased generation of cell-damaging molecules (e.g. reactive oxygen species [ROS]) by eosinophils [74, 75]. In addition, GCs promote eosinophil clearance by inhibiting pro-survival signals induced by the cytokines IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) [76]. These effects explain why GCs have been used for many years to treat eosinophilic

disorders, although new pharmacological treatments have replaced them as a result of their undesired adverse effects. GCs do not only reduce the number of basophils but can even inhibit their migration in a concentration-dependent manner and prevent the release of histamine. Recently, GCs have also been found to inhibit basophil activation via membrane-bound GR interferences with the formation of lipid raft nanoclusters [77, 78].

4.5 Mast Cells

Mast cells are effector cells characteristic of allergic and inflammatory reactions. When allergen-mediated aggregation of the FcεRI takes place, a signaling cascade is initiated that leads to the production of cytokines, chemokines, arachidonic and eicosanoid production, and cellular degranulation. Long-term treatment with GCs can inhibit mast cell activation by downregulating Erk1/2 and inhibiting of the PI3K signaling cascade, with the subsequent prevention of degranulation and mast cell activation. Other signaling pathways are also reduced by GC treatment, including the phosphorylation of p38 and JNK1/2 [79]. One of the major factors involved in the molecular pathways of the anti-inflammatory actions of GCs on mast cells is the activation of protein tyrosine phosphatases (PTPs) by GCs. Within this family, DUSP1 and DUSP2 have been functionally characterized in mast cells and found to be upregulated by GCs, thus are available for the dephosphorylation of Erk1/2 and the subsequent inhibition of cellular activation. Another important feature of mast cells is their accumulation at the site of inflammation. GCs are known to reduce mast cell accumulation by downregulating the stem cell factor released by fibroblasts with kinetics that remains to be established [80, 81]. While all of these actions mediated by GCs are genomic-derived, other effects occur rapidly after GC administration (e.g. in the treatment of allergic reactions, degranulation is rapidly decreased). Despite the potential role of membrane bound based on studies in other cell types, no studies has still been conducted in mast cells.

4.6 Dendritic Cells

Dendritic cells have been largely studied as a target of GC action. Throughout their life cycle, dendritic cells are influenced by GCs, differently from the monocytes from which they are derived. Dendritic cells mature after encountering an antigen and they are sensitive to GC-induced apoptosis only before this stage. Furthermore, GCs stimulate antigen uptake before maturing, thus helping the organism fight against invading pathogens by keeping these cells in an immature state. More importantly, dendritic cells become tolerogenic once they are exposed to GCs, exhibiting low levels of expression of MHCII molecules, costimulatory molecules, and cytokines (e.g. IL-1, IL-6, and IL-12) [68]. Under this state they can neither prime nor induce the proliferation or activation of T cells; however they can promote the formation of Treg cells [82]. The migration towards the lymph nodes is also inhibited by GCs.

It has been recently shown that endogenous GCs suppress the dendritic cell response to LPS exposure by reducing IL-12 production during sepsis, thus explaining the role of GCs in the treatment of sepsis [83].

5 Anti-inflammatory Versus Pro-inflammatory Effects of GCs

As evidenced throughout this chapter, due to the specific effects of GCs on the cells of the immune system, GCs have historically gained attention as the most important anti-inflammatory and immunosuppressive drugs. Indeed GCs are currently used to treat pathologies such as asthma, rheumatoid arthritis, inflammatory bowel disease or even tumor pathologies such as acute lymphoblastic leukemia or to prevent the graft-vs-host disease in organ transplantations. The ability of GCs to suppress pro-inflammatory cytokines or other inflammatory mediators (listed in Table 2) in a variety of cells, guarantees its success in the treatment of inflammatory diseases. Nonetheless, GCs are not as anti-inflammatory as might be expected. It is generally accepted that in addition to the detrimental side

Table 2 Pro-inflammatory mediators suppressed by glucocorticoids

Cytokines	IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-12, IL-13, IL-16, IL-17,-IL-18, TNFα, GM-CSF, SCF, TSLP
Chemokines	IL-8, RANTES, MCP-1, MCP-3, MCP-4, MIP-1α, CCL1, CCL5, CCL11, CXCL8
Adhesion molecules	ICAM-1, VCAM-1, *E*-selectin
Inflammatory enzymes	iNOS, COX-2, PLA-2
Inflammatory peptides	Endothelin-1
Mediator receptors	Neurokinin (NK1) receptor, Bradykinin (B2)-receptor

effects described in Table 1 and gathered under the Cushingoid syndrome, the long term treatment with GCs may enhance inflammation and immunity depending on the dose, chronicity of treatment, and target organ [84, 85]. Genome-wide expression studies of GC-treated cells have revealed that GCs upregulate genes of innate immune cells that are involved in the recognition of pathogens (e.g. pattern recognition receptors –PRRs-), but inhibit the expression of pro-inflammatory cytokines in cells involved in the adaptive immune response [86, 87]. In other studies, the increased expression of cytokine receptors has been reported (e.g. TNFR, IL-1R, Il-6R, and receptors for IFNγ), as well as increased IL-1β production in response to LPS [88, 89]. The expression of pro-inflammatory genes, including iNOS and TNFα, together with a decreased expression of anti-inflammatory genes, including IL-1ra and IL-10, have also been shown in the frontal cortex of rats; these effects were shown to be GR-mediated and region-dependent [88]. Therefore, in some conditions, while the effects of GCs may be opposing, their functions respond to the specific needs of the organism and the mechanisms through which they occur remain incompletely understood. A model proposed by Cain and Cidlowski suggests that low levels of GCs in the absence of inflammation make cells sensitive to any harmful stimulus by promoting the expression of PRRs and other pro-inflammatory mediators. In contrast, during inflammatory conditions, high levels of GCs induced by stress shorten the duration of the inflammatory response by acting as anti-inflammatory agents [86].

6 Conclusions

There is no doubt regarding the clinical efficacy of GCs for the treatment of pathologies that cannot be treated with targeted drugs, even despite the occasional observance of resistance to GC treatment. However, basic knowledge regarding the functions of GCs on cells, not only of the immune system, but of the whole organism remains incomplete. Thus, gaining insights into the mechanism of GC action is required to both unravel their physiological role and to develop alternative drugs with the same anti-inflammatory properties as GCs, without the harmful adverse effects.

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Strategies and Compounds to Circumvent Glucocorticoid-Induced Side Effects

Astrid Luypaert, Wim Vanden Berghe, Jan Tavernier, Claude Libert, and Karolien De Bosscher

Abstract

Glucocorticoids (GCs) are used in the clinic for the treatment of inflammatory diseases and particular cancers. Although they are highly efficient in combating inflammation, their use is limited. This is caused by quality-of-life-threatening side effects, e.g. osteoporosis and metabolic imbalances. Contemporary efforts still focus on the development and characterization of novel (non-)steroidal ligands that can separate the beneficial anti-inflammatory effects from the plethora of adverse effects. However, early-day views of how GCs mechanistically work appeared too simplistic to cover the complexity and pleiotropicity of glucocorticoid receptor (GR)-mediated therapeutic actions. In this chapter, we will zoom in on the rationale of selective GR agonist design and recent advances in the field. We will discuss in detail how exemplary novel GR-agonists are able or expected to circumvent specific GC-induced side effects. Today, although novel selective GR-drugs have demonstrated therapeutic benefit in pre-clinical and clinical trials, reaching the pharmacy market remains an insurmountable hurdle. This calls for stepping up knowledge-gathering efforts alongside a serious rethinking of strategies within the field.

Keywords

Glucocorticoid receptor · Selective GR agonists and modulators · Dissociating ligands · Glucocorticoid-induced side effects

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1 Introduction

1.1 Glucocorticoids and Their Receptors

Recently, the prevalence of adults using systemic GCs in a representative European country, here exemplified by Denmark, was estimated at 3%, with a notably high prevalence in elderly of up to 10% [1]. The clinical use of glucocorticoids (GC) is narrowed down by the many quality-of-life-threatening side effects and the onset or inherent occurrence of GC-resistance, depending on the

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disease. The greater majority of both beneficial and unwanted GC-effects depend on the activation of the Glucocorticoid Receptor (GR). To improve the therapeutic range and applicability, a much better understanding of the GR-signaling pathways remains crucial. Insights into the mechanism of action via *in vitro* and *in vivo* work stimulated the search for selective GR modulators and subsequent studies on ligand structure-activity relationships. By specifically targeting only one mechanistic arm of how GR works, these so-called *selective* GR modulators are promising new candidates for the treatment of several inflammatory diseases and some kinds of cancers, aimed at separating the unwanted effects from the beneficial therapeutic effect [2]. GR-mediated mechanisms of action are recently reviewed in [2] and a summary is depicted in Fig. 1. Because GR is ubiquitously expressed, it provides a dramatic example of perhaps the most critical property of eukaryotic transcription factors, i.e. their context specificity. Indeed, GR regulates networks of genes that are precisely determined in a given setting, yet differ substantially as a function of cell type and physiological state. Thus, GR structure, together with its determinants, is key to understand both its regulatory precision and plasticity [3].

1.2 Importance of GCs as Anti-inflammatory Drugs

Glucocorticoids (cortisol in humans and corticosterone in rodents) are steroid hormones secreted by the zona fasciculata of the adrenal glands [4–6] involved in a plethora of physiological functions besides controlling inflammation. From the introduction of GC therapy in rheumatic diseases by Dr. Philip Hench in the 1950s and onwards, GCs are considered the most effective anti-inflammatory drugs. Although their use precipitates side-effects, GCs are still one of the most widely prescribed drug classes worldwide, with a long-term GC use estimated at between 1% and 3% of adults [7]. Despite their effective anti-inflammatory actions, their adverse effects when used long-term and/or at high doses, limits their use and therapeutic compliance [2, 8]. Therefore, con-

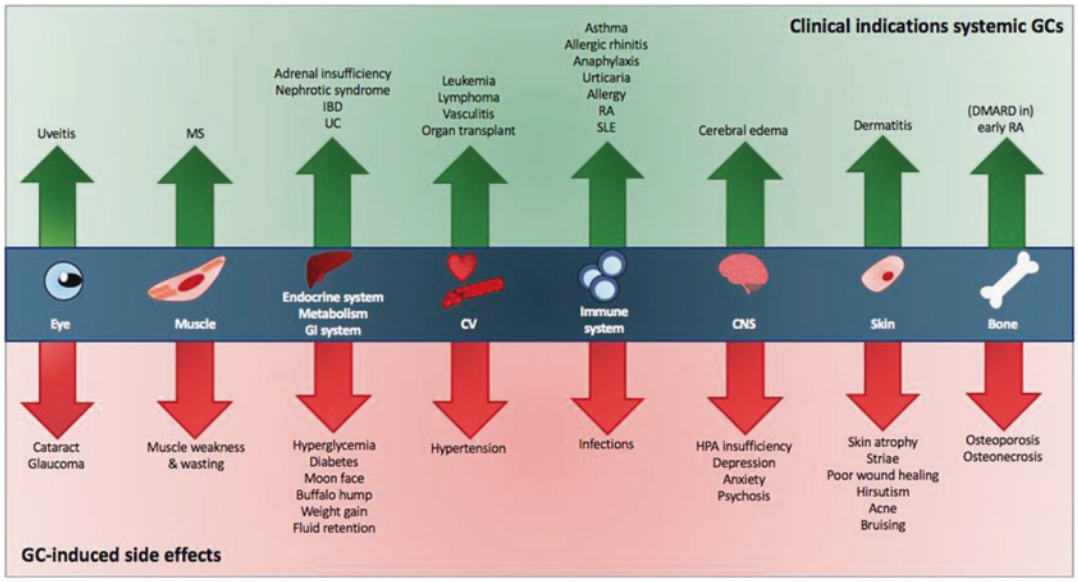


Fig. 1 Representation of the double-edged sword of GC-therapy. MS, multiple sclerosis; IBD, inflammatory bowel disease; UC, ulcerative colitis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; DMARD, DMARD = disease-modifying anti-rheumatic drug; GI, gastro-intestinal; CV, cardio-vascular; CNS, central nervous system; HPA, hypothalamic-pituitary-adrenal. For simplicity, organ transplant is put is part of the CV system. Obviously, GC-therapy is described is immunosuppressive drug for any kind of organ transplant to avoid transplant rejection

tinuous efforts are being made to implement recommendations for optimal dosing of GCs, monitoring for potential adverse events, adverse event prevention and management. Apart from this, more work is underway to develop innovative glucocorticoids or selective glucocorticoid receptor ligands in order to improve the therapeutic balance [9]. In this book chapter we will primarily focus on selective (non-) steroidal GR modulators (SEGRMs) for which side effect parameters have been scored. These novel selective GR-targeting drugs are expected to present the same or better efficacy compared to classical steroids, but, in as much as possible, are able to separate side effects from desired therapeutic effects [10–12].

2 Rationale for SEGRAM

2.1 Transrepression Hypothesis

The development of **selective GR agonists and modulators** (**SEGRAM**) or **selective GR modulators** (**SEGRM**) to specify compounds with a possible atypical GR binding mode is based on the so-called *transrepression hypothesis* [13–15]. Figure 2 summarizes a scheme of what transrepression entails, as opposed to transactivation. Transrepression is the gene regulatory action mechanism whereby GR controls gene expression without a direct contact to DNA. Transactivation, by direct contact of GR to DNA, of gluconeogenic and lipogenic genes typically accounts for GC-induced side effects, whereas transrepression causes anti-inflammatory and immunosuppressive actions. Thus, a drug able to dissociate between these two GC-mediated mechanisms of action should theoretically separate the GC-induced disadvantages from beneficial therapeutic effects. However, strictly following this hypothesis underestimated and oversimplified the problem and appeared not applicable for all GC-treated diseases [16]. For example, acute inflammatory diseases such as sepsis, rather require a dimer-promoting approach of GR, favoring transactivation above transrepression resulting to reach therapeutic benefit [17].

Although helpful in the beginning of SEGRAM discovery, separating molecular mechanisms of GR into transactivation versus transrepression falls somewhat short as an overarching therapeutic model since GR deploys many more transcriptional (and translational) mechanisms. Notwithstanding, selective GR-drugs have been identified that shift balances from transactivation to transrepression, with a therapeutic index considered superior to classical GC. The need for transactivation, depending on the (inflammatory) study model, can be an explanation for enhanced anti-inflammatory effects, but does not allow to fully explain a concomitant reduced side effect profile [18–21]. In other words, side effects cannot be solely linked to transactivation and beneficial effects cannot be exclusively coupled to transrepression. In support, GR-mediated enhanced repression of RUNX2, a crucial regulator of bone health, appeared osteo-destructive. So at least for osteoporosis, a fine balance between GR-mediated beneficial and detrimental mechanisms is crucial to circumvent this particular GC-induced side effect. Summarized, examples exist of enhanced anti-inflammatory effects when stimulating transactivation and of an increased side effect profile when supporting GR-mediated transrepression.

It turned out difficult to pharmacologically dissociate transactivation from transrepression to obtain an effective anti-inflammatory drug with a reduced side-effect profile. Besides this hurdle, little is known about f.e. the non-genomic and microRNA-mediated effects of SEGRAMs [22, 23]. Understanding how GR recruits cofactors to assist in its gene regulatory mechanisms, esp. following SEGRAM treatment would also contribute to an optimized selective GR-drug design [24].

An effective anti-inflammatory therapy would be one that inhibits inflammation but does not interfere with normal homeostasis. However, progress with novel, selective GR agonists is not evident. Extensive knowledge of the structure of the full-length GR protein, decorated with specific cellular cofactor proteins and in complex or not with specific ligands is at this moment

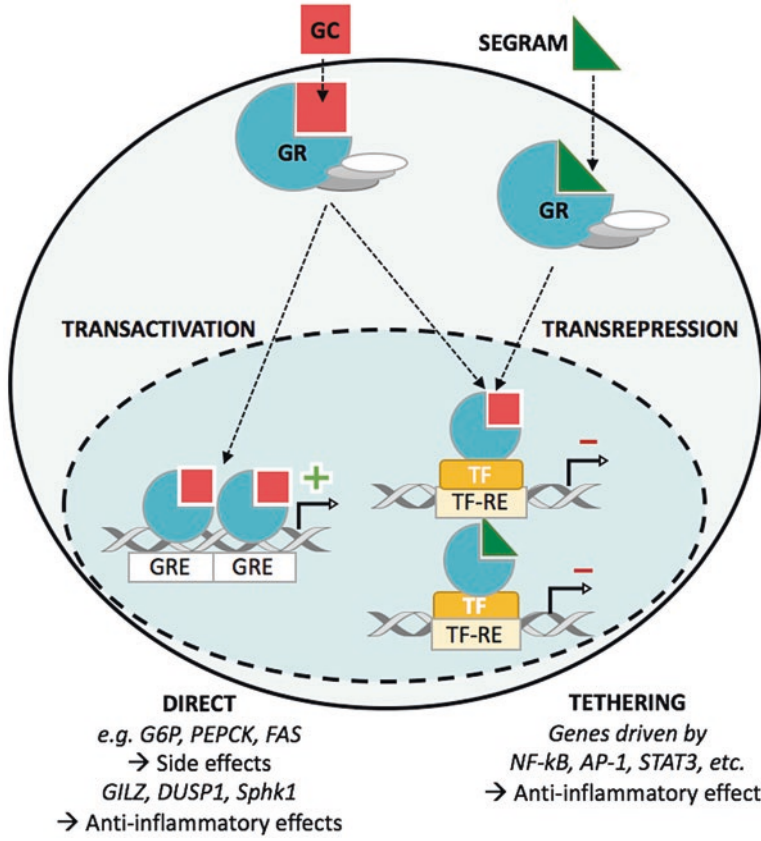
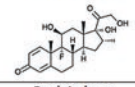
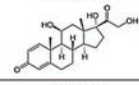
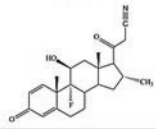
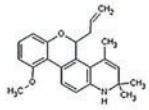
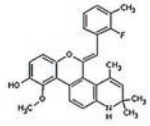
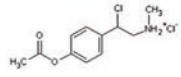
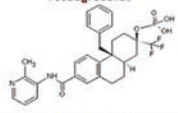
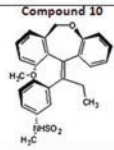
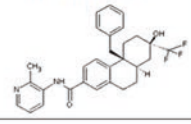
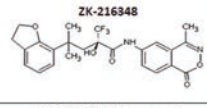
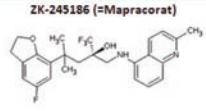


Fig. 2 Scheme of the transrepression hypothesis. Classical GCs trigger both transactivation and transrepression mechanisms, whereas SEGRAM have the potential to separate the unwanted side effects from the advantageous anti-inflammatory actions. See text for more details and critical notes. GR, GRα; GRE, glucocorticoid-response element; TF, transcription factor; TF-RE, transcription factor-response element; SEGRAM, selective glucocorticoid receptor agonist and modulator; G6P, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; FAS, Fas cell surface death receptor; GILZ, glucocorticoid-induced leucine-zipper; DUSP1, dual specificity protein phosphatase 1; Sphk1, sphingosine kinase 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1, activator protein 1; STAT3, signal transducer and activator of transcription 3

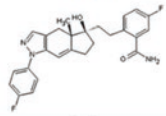
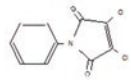
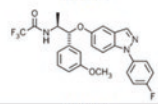
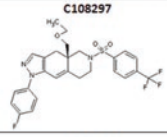
missing. A better understanding of the conformational changes of GR upon DNA and ligand binding is expected to step up the pace in discovering novel side-effect lowering GR-ligands. We believe disease-tailored SEGRAMs can be developed by targeting specific GR conformations. Two decades ago, the first promising SEGRA, i.e. the steroidal compound RU24858, showed AP-1 inhibition but decreased transactivation in an experimental set-up predominantly relying on recombinant reporter gene assays [25]. Disappointingly, the results were not corroborated in a subsequent *in vivo* study [16]. Alongside problems of selectivity towards other steroid receptors, this led researchers away from a steroidal structure as a viable way-to-go to develop innovative, novel, dissociative GR-targeting drugs. As a result, non-steroidal GR targeting drugs were sought after using screening with compound libraries while some were found by serendipity. A selection of some of those (selective) GR-agonists is summarized in Table 1. For many of those, the precise molecular mechanisms remain incompletely understood. Other challenges are a paucity of experimental models (go/no-go decisions are primarily based on vitro

Table 1 Overview of selected GR-targeting drugs

	Small molecule innovator drug	Circumvented GC-induced side effect	Research type	Therapeutic field	Indication	Highest development stage	References
Steroidal, non-dissociating classical GC	Dexamethasone 						
	Prednisolone 						
Steroidal, dissociating compound	RU24858 						
Non-steroidal, Dissociating compounds	AL-438 	Diabetes, hyperglycemia Osteoporosis	In vitro In vivo	Immunology	Inflammation Autoimmune disorders	Preclinical	[45-48]
	LGD-5552 	Hypertension Central obesity Osteoporosis	In vitro In vivo	Immunology Oncology	Inflammation Cancer	Discontinued	[49, 83]
	CpdA 	Diabetes, hyperglycemia Osteoporosis Skin atrophy, poor wound healing Muscle atrophy	In vitro In vivo	Hormonal disorders Immunology Oncology	Autoimmune disorders Inflammation	Preclinical	[41, 53, 72, 92, 97]
	Fosdagrocorat 	Osteoporosis	In vitro In vivo	Immunology	Inflammation	Discontinued	[55, 56]
	Compound 10 	Osteoporosis	In vivo	Immunology	Inflammation	Preclinical	[58]
	PF-802 (=Dagracorat) 	Diabetes, hyperglycemia Osteoporosis	In vitro In vivo	Immunology	Rheumatoid arthritis	Discontinued	[30]
	ZK-216348 	Glaucoma Diabetes, hyperglycemia Central obesity Skin atrophy, poor wound healing	In vitro In vivo	Gastrointestinal Immunology	Colitis Inflammation	Inactive	[15, 47, 87]
	ZK-245186 (=Mapracorat) 	Diabetes, hyperglycemia Skin atrophy, poor wound healing	In vitro In vivo	CNS Ophtalmology	Ocular pain Allergic conjunctivitis Keratoconjunctivitis sicca Ocular Inflammation	Inactive	[88, 110, 111, 124]

(continued)

Table 1 (continued)

	JTP-117968	Diabetes, hyperglycemia	In vitro In vivo	Immunology	Inflammation Autoimmune disorders	Preclinical	[75]
	MK-5932 	Diabetes, hyperglycemia	In vitro In vivo	Immunology	Inflammation Autoimmune disorders Rheumatoid arthritis Dermatitis	Inactive	[76, 77]
	Q40 	Diabetes, hyperglycemia	In vitro In vivo	Immunology	Inflammation	Unknown	[78]
	AZD5423 	Less thymic involution	In vitro In vivo	Respiratory	Asthma COPD	Discontinued	[125]
	C108297 	Depression, mood changes, Diabetes Central obesity	In vitro In vivo	CNS Immunology Oncology	Inflammation Prostate cancer	Preclinical	[81, 82]
	C113176	Depression, mood changes, Diabetes Central obesity	In vitro In vivo	CNS	Hypercortisolism	Preclinical	[82]

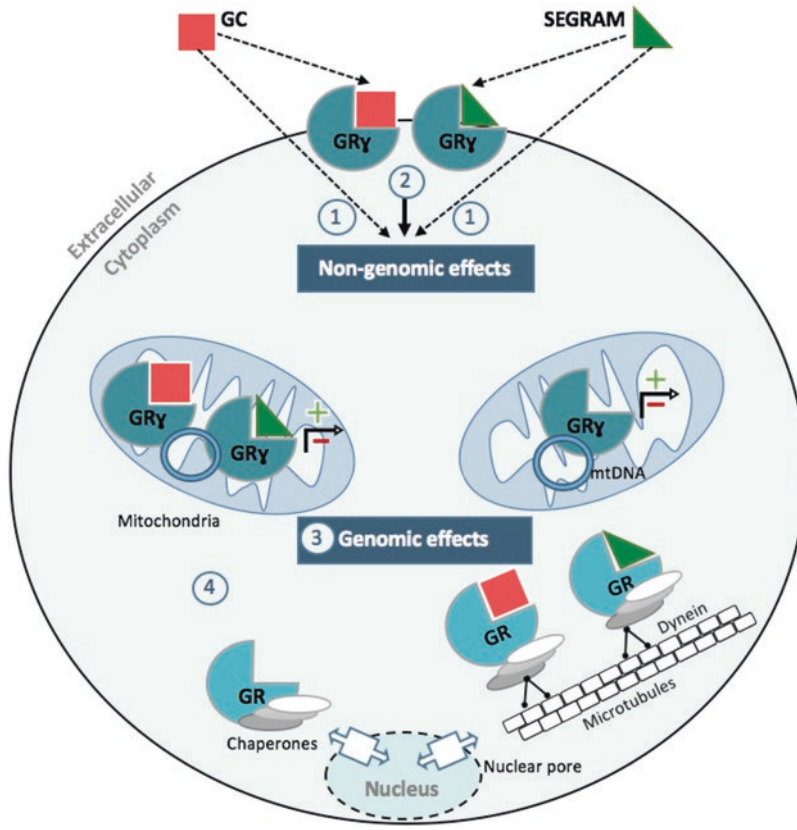


Fig. 3 GR-mechanisms of action. (**a**) Cytoplasmic non-genomic and genomic effects. (1) GCs and SEGRAM, but particularly GCs in high doses, can evoke GR-independent non-genomic effects. (2) Selective and non-selective GR-ligands can induce fast non-genomic effects through the membrane-bound GRy isoform. (3) Genomic mitochondrial effects through GRy. Both ligand-bound and unliganded GRy bound to mitochondrial DNA (mtDNA) can result in genomic effects regulating mitochondrial functions and energy metabolism

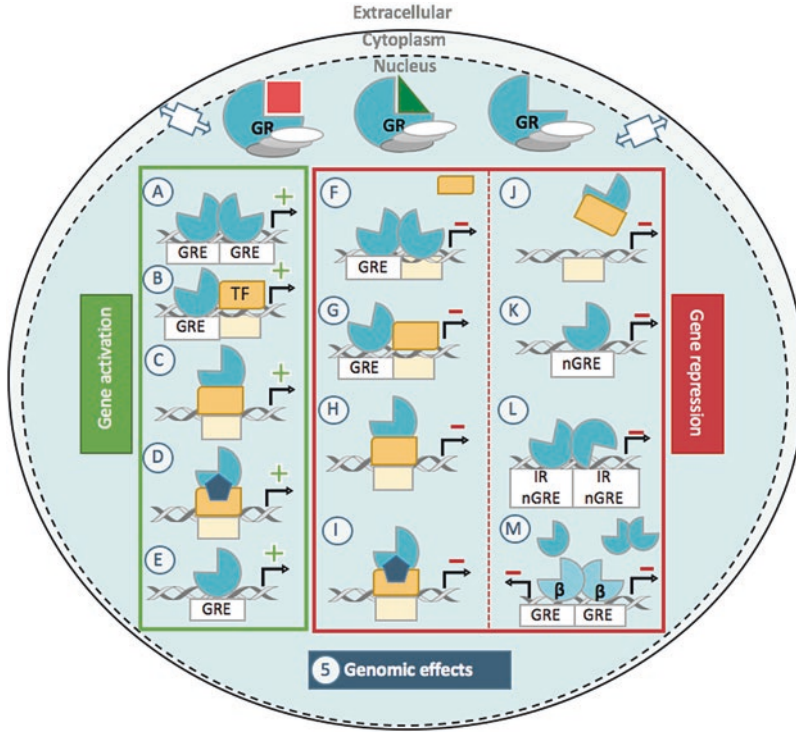


Fig. 3 (continued) (4) The unliganded GR mostly resides in the cytoplasm complexed with accessory proteins. This unliganded shows high affinity for both GCs and SEGRAM. Once ligand-bound, the GR binds to dynein, making transportation through microtubules to the nuclear pore possible. Once translocated in the nucleus, GR dissociates from its chaperones and cause a plethora of genomic effects (see Fig. 3b). Unliganded GR nuclear translocation is not yet completely understood. (**b**) Nuclear genomic effects. (5) Nuclear GR-mediated gene activation (A-E) and repression (F-M) is mediated through different mechanisms of action. (A) Simple GRE: GRa homodimers bound to GREs. (B) Composite GRE: monomeric GRa DNA binding in a concerted manner with another TF. (C) Direct tethering and (D) Indirect tethering (co-factor assisted). (E) Half site-binding of monomeric GRa. (F) Competitive GRE: Direct GR homodimer competition for an overlapping binding site. (G) Composite GRE: gene repression by monomeric GR DNA-binding crosstalk with another TF. (H) Direct tethering and (I) Indirect tethering (co-factor assisted). (J) DNA-bound TF sequestration. (K) Simple nGRE: direct binding of monomeric GR onto a negative GRE (nGRE). (L) Inverted repeat (IR) nGRE: Two monomeric GR bind with inverted polarities to IR nGREs. (M) GRb competition: GRb competition for an overlapping GRE, impairing GR binding. Besides the before mentioned cytoplasmic and nuclear effects, post-translational modifications(PTMs) also contribute to increased or decreased GR-controlled effects in all stages. It is important to note that the acronym GR used in this manuscript refers to the GRα isoform

models) and read-outs and the enormous complexity of GR-based molecular mechanisms (Fig. 2). All these complexities and experimental hurdles have led to a delay in achieving a sufficient knowledge of SEGRAMs to permit their clinical use [26]. An improved therapeutic balance *in vivo* remains hard to achieve and is reflected by the absence of a successful SEGRAM on the pharmaceutical market.

In vitro SEGRAM activity is commonly evaluated by analyzing repression or activation of characteristic GC-regulated genes. Suppression of the inflammatory markers IL6 and IL8 is typically addressed using reporter gene assays, mRNA analysis and ELISA. However, many more inflammatory mediators exist and one realizes these may be essential to analyze as well to get a correct global picture. On the other hand, charting tens to hundreds of markers in a concentration-responsive manner is impossible and unaffordable in a screening program. Enhanced PEPCK mRNA expression is often used as a marker of enhanced transactivation, since enhanced levels of its gene product support hyperglycemia as a side effect. But also here, there are many more markers to measure (e.g. TAT, G6Pase, FAS, etc.). In more

recent work, also target genes with a beneficial outcome of an anti-inflammatory effect are being scored (e.g. GILZ, DUSP1) [24]. Moreover, some of these GC-induced targets are being explored as primary anti-inflammatory drug targets themselves, e.g. GILZ [27]. The team of Riccardi found that GILZ transactivates Anxa1 expression at the promoter level via binding with the transcription factor, PU.1 [28].

The most promising drugs in *in vitro* preclinical trials subsequently proceed to a testing *in vivo*. Even if this stage goes well, ample examples exist of candidates failing to reach the next phase, i.e. clinical trials [29]. Unfortunately, progress toward the goal of selective GR modulation seems mostly based on scientific doubt rather than certainty. SEGRAMs ability to transactivate anti-inflammatory genes, such as DUSP1, MKP-1 and GILZ, and subsequent inflammation resolving effect has not been sufficiently investigated [18, 20, 21].

2.2 Recent Views on GR Mechanisms

Cytoplasmic and nuclear effects of GR-mediated actions are depicted in Fig. 3 (based on [2]). Hu et al. were the pioneers of the 'antagonist hypothesis' [30]. As with the transrepression hypothesis, the idea of a synthetic GR-ligand that can successfully dissociate the therapeutic efficacy from the GC-induced side effects stays central in the search for better anti-inflammatory drugs based on the 'antagonist hypothesis'. It was hypothesized that a novel GR ligand that shifts helix 12 in a differential way from an agonist and antagonist would result in an altered GR conformation, getting closer to the ultimate goal of a superior therapeutic index. Hu et al. investigated non-steroidal, tricyclic scaffolds that showed a dual function in cellular assays, partial but robust agonist activity for inflammatory gene inhibition and full antagonist activity for reporter gene activation. PF-802, an angular benzyl, is an example of such a helix 12 shifting compound with full antagonist activity. Oppositely, analogues that are not able to interfere with helix 12 still have partial agonist activity in reporter gene assays. Hu et al. stated for the first time that full antagonist activity is required for significant unwanted effect dissociation. Although the studied compounds did not exhibit substantial reporter gene activity, a limited number of co-activators (e.g. PGC-1a) were weakly recruited [30]. This once more emphasizes the importance of a full understanding of co-activator recruitment [24]. Recently, Oh et al. monitored in detail the epigenomic landscape of macrophages and found that the gene-inducing activity of GR is crucial for boosting inhibitors of inflammatory factors. They carefully investigated the effect of LPS-treatment pre-and post GC administration [31]. These findings emphasize that GR-binding characteristics depend on the inflammatory and cell-context. For more discussions on recent molecular mechanisms of how GR can regulate gene expression or which proteins are recruited to GR, via genome-wide genomics and proteomics approaches respectively, we gladly refer to other recent reviews and research articles [3, 32–34]. To give a visual comprehensive overview, we have included a summarizing Figure (Fig. 3) on the transcriptional mechanisms, making a distinction between genomic and nongenomic mechanisms [35]. To illustrate the complexity does not end here; we also highlighted how other GR isoforms, here GRγ, may additionally impact GR-mediated transcriptional programs and cofactor recruitment profiles [36, 37].

3 GC-Induced Side Effects and Novel Therapeutics to Circumvent Them

The incidence and severity of the GC-induced side effects depends on the time, amount, dosing regimen, specific GC used and administration mode. Overall, prolonged use and dose are high-risk factors. The term 'side effect' is actually misleading, since many if not all GC-linked side effects are a physiological reflection of endogenous glucocorticoids. Still, when considering therapy these effects are inappropriately augmented, initiated, extended and/or maintained at

an improper time point of the circadian cycle [4, 29]. In Table 1 we presented an overview of studied SEGRM alongside the GC-induced side effects they are able to, or try to, circumvent or tone down.

Hyperglycemia and diabetes, osteoporosis, hypertension and skin and muscle atrophy are clinically well-known worrying adverse effects of GC-based therapies [38]. However, recently an online cross-sectional survey in the UK studied the perceptions of patients suffering from GC-induced adverse effects [39]. Although weight gain was long considered only an esthetical burden of GC-therapy, this specific side effect was ranked as most psychologically burdening to the responders. Insomnia and moon face are completing the top 3 of most quality-of-life threatening side effects. Three side effects from a clinical point of view, i.e. CVD, diabetes and infections were not scored of highest importance by the patient, although research of those events, together with osteoporosis, obviously needs to keep focusing also on these other serious GC-induced complications [39]. Further research on selective GR-targeting drugs, given the high prevalence of patients gaining weight under current GC-therapy, must also consider an impact on adipocyte biology. A patient- and disease-orientated approach for novel drugs design should be promoted, to ensure an optimal quality of life during a balanced treatment at both a clinical and psychological level.

3.1 Bone Health

3.1.1 Osteoporosis

GC-therapy is the leading iatrogenic cause of secondary osteoporosis. Thirty percent to fifty percent of GC-users suffer from this GC-induced co-morbidity. Loss of bone mineral density (BMD) occurs already within the first 6 months in the course of GC therapy and slows down after 1 year. In the first 3 months of treatment, there is a 75% increased risk of fractures before a significant decrease in BMD is observed [40]. Osteoporosis results from an unbalance in reduced osteoblast proliferation and activity and increased bone-resorbing osteoclast activity. GC-induced osteoclast activity partially results from a GRE-mediated transactivation of osteoprotegerin-ligand (OPG-L, also known as RANKL). OPG-L activity stimulates osteoclast differentiation and activity and inhibits osteoclast apoptosis. Skewing the balance even more to osteoclast activity, GCs also induce osteoprotegerin (OPG) transrepression, which results in less binding of OPG-L and subsequent higher osteoclast activity and bone resorption.

The drop in osteoblast function is multifactorial including a GC-induced decrease of adrenal steroid hormones, osteoblast and osteocyte apoptosis, and suppression of bone homeostasis markers (e.g. growth hormone, insulin-like growth factor-1 and transforming growth factor-β). In addition, IL-11, a cytokine targeted by GC use was shown to play a crucial role in osteoblast differentiation, providing another level at which GC-induced bone loss can operate [41]. Next to unbalanced bone-forming and bone-resorbing cells, reduced synthesis of bone-forming extracellular matrix proteins, e.g. collagen type II and osteocalcin, contributes to GC-induced osteoporosis in the long-term GC-user [4, 38].

Contradictory, GCs at low-doses are used in the clinic as disease-modifying anti-rheumatic drugs (DMARDs). It was shown that low-doses of GCs have the ability to slow down the bot erosion process, particularly in the early stages of RA [42–44]. Below, we discuss selective GR-agonists that are able to maintain bone homeostasis, while upholding anti-inflammatory actions.

AL-438 is a prototypical non-steroidal Abbott-Ligand. In vitro experiments showed successful transrepression of the pro-inflammatory genes TNF, IL1a, IL6 and E-selectin. Since it can separate transrepression from transactivation, AL-438 is less potent in GR-controlled aromatase transcription, resulting in a lowered osteoporosis-favoring profile [45]. By not altering osteocalcin mRNA levels in the murine chondrogenic cell line ATDC5, AL-438 lacked osteoporosis-inducing properties, in contrast to classical GC-therapy [46]. In addition, Humphrey et al. described AL-438 as a weak stimulator of the RANKL:OPG

ratio, suggesting that this dissociative GR-ligand could have bone-sparing effects [47] AL-438 has also chondrocyte sparing effects on the growth plate in vitro and in vivo, which is also interesting for the treatment of children suffering from inflammatory diseases [46]. Although AL-438's anti-inflammatory properties are similar to prednisolone, it does retain minor unwanted effects on glycemia [45, 48].

The non-steroidal compound **LGD-5552** has high GR-binding affinity, but antagonizes the mineralocorticoid receptor. Oral administration of LGD-5552 to collagen-induced arthritis (CIA) mice results in a classical GC-transrepression potency, but with less unwanted effects such as decreased bone formation as seen under prednisolone treatment [49].

Compound A (CpdA) was, unlike the other non-steroidal SEGRAM discussed in this review, found by serendipity. It originates from the Namibian desert shrub *Salsola tuberculatiformis* Botschantzev [50]. It is known as the most dissociative selective GR-agonist, because its (1) unable to induce GR-dimerization at GRE in the nucleus and (2) selectively transrepresses NF-kB-mediated signaling, without interfering with the AP-1 cascade [51]. CpdA shows a reduced side effect profile compared to classical GCs with regard to bone health. Although CpdA has lower anti-inflammatory potential in arthritis mice models than classical GR-drugs, it maintains the BMD in these animals [52, 53]. CpdA does not alter RANKL/OPG ratio and IL11 expression and subsequent STAT3 phosphorylation in fibroblast-like synovial cells from patients. These mediators are crucial for proper bone metabolism, and most likely a result from CpdA's ability to distinguish between AP-1 and NF-kB suppression, leaving AP-1's activity intact [51, 54]. Additionally, it was shown that CpdA does not affect osteoblast differentiation *in vitro* and *in vivo*, making it a valuable tool compound to further research in how its biological properties circumvent one of GC most feared side-effects, i.e. GC-induced osteoporosis [41].

Decreased osteocalcin (OC) levels are a typical phenomenon with classical GC treatment. **PF-802** showed unaltered osteocalcin expression and secretion *in vivo* and *in vitro* [30]. Similar effects were observed with CpdA and AL-438 treatment [41, 48]. Encouragingly, pre-clinical bone-sparing effects of PF-802's chemical derivative **fosdagrocorat** were translated to a clinical trial with healthy volunteers. This compound had less impact on osteocalcin [55]. Recently, it was shown in a randomized placebo-controlled phase II clinical trial that this SEGRAM demonstrated efficacy in improving signs and symptoms in rheumatoid arthritis patients, with manageable side-effects [56]. However, follow-up studies are needed to assess the longer-term safety and efficacy of fosdagrocorat. Shoji et al. developed a quantitative tool to find optimal doses of the selective GR-modulator with a reduced unwanted effect on bone formation [57]. Additionally, a 12-week, phase II, randomized double-blind study (NCT01393639) by Pfizer evaluated the efficacy and safety of fosdagrocorat versus prednisone or placebo in patients with RA and publication of the findings is awaited with interest.

Compound 10 is the archetype of a new family of dibenzoxepane and dibenzosuberane sulfonamides. It has full transrepression activity, but still holds partial transactivation ability, making it a valuable fine-tuned alternative to treat autoimmune diseases and inflammation. Its action is mediated by a new binding mechanism, clearly differentiating from the classic GC binding model [58]. Compound 10 has comparable anti-inflammatory actions in the CIA mouse model, but showed improved protection of joint and bone degradation as evaluated by histology [58].

ZK-216348 is a GC alternative without bone-sparing effects. *In vivo* experiments are lacking for ZK-216348-mediated effects on bone metabolism, since *in vitro* experiments already showed a clear inhibition of OPG [47].

3.1.2 Osteoarthritis

The pathogenesis of the metabolic disorder osteoarthritis is partly attributed to adipokines, such as leptin. The exact effect of classic GCs on the progression of this disease is still under study. However, it is known that GCs increase leptin levels and levels of its receptor (Ob-R). Malaise et al. showed previously that glucocorticoids

induce leptin secretion in OA synovial fibroblasts. In contrast, CpdA does not induce increased levels of leptin and its receptor, contributing to a potential improved risk:benefit ratio [59]. This effect may be explained by the transrepression-favoring capacity of CpdA, since it was recently shown that transactivation of GILZ can contribute to enhanced levels of leptin and its receptor in human synovial fibroblasts [60].

3.2 Metabolic Health

3.2.1 Diabetes and Weight Gain

The metabolic effects of long-term GC-use likely results from a combination of increased hepatic glucose output, altered fatty acid metabolism and increased insulin resistance in muscle and fat [48]. Long-term GC use is linked to the development of hyperglycemia. This unwanted effect is predominantly mediated through GR-transactivation of the gluconeogenesis pathway. A glucose flux is followed by elevated glycogen storage in the liver through GC-stimulated transactivation of glycogen synthase. The concomitant risk of diabetes type II and cardiovascular complications is linked to GC-induced insulin resistance and decreased insulin production of pancreatic β-cells [38, 61]. Insulin resistance development is multifactorial, since (1) GCs directly inhibit insulin signaling and insulin-stimulated glucose uptake in myotubes and adipocytes, (2) decreased osteocalcin secretion under GC-therapy reduces insulin secretion and sensitivity and (3) GCs promote lipolysis in white adipose tissue (WAT). All three aforementioned mechanisms account for the insulin resistance-promoting phenotype under chronic GC therapy. Recently, Chen and co-workers demonstrated that Angptl4 is a key player in GC-induced whole-body insulin resistance, through an augmentation in hepatic ceramide production [61].

GC-induced weight gain is considered a huge quality-of-life threatening side effect from the patient's perspective. Patients using higher GC doses for longer periods suffer from a characteristic central fat accumulation, a typical 'buffalo hump' and general increased obesity [39, 62–64]. Adipose tissue can be divided in a white and brown counterpart, WAT and BAT respectively. Both AT depots regulate distinct physiological functions. Whereas WAT stores excess energy, BAT regulates energy expenditure. Globally, increasing BAT activity in WAT reduces body fat accumulation and improves insulin sensitivity [65]. GCs are known regulators of white adipocyte differentiation, but have a role in BAT as well [62, 66]. GCs negatively regulate BAT-specific genes, i.e. uncoupling protein-1 (UCP-1), Cidea, Cox7a1 and Cox8b in BAT in rodents [66, 67]. UCP-1 is an important mitochondrial regulator of thermogenesis and energy expenditure [66, 68]. GCs further inhibit nonshivering thermogenesis and stimulate lipid storage in BAT of rats [69]. Until recently, the role of GCs in the browning of WAT was unclear. Kong et al. demonstrated that micro-RNAs, in particular miR-27b, are key players in the pathogenesis of GC-induced central fat accumulation [62]. GCs regulate miR-27b transcription through direct GR-mediated DNA-binding, resulting in suppressed browning of WAT by targeting the 3′ UTR of the transcriptional coregulator Prdm16 [62].

Below, the impact of SEGRM is discussed on both diabetes and weight gain.

ZK-216348, characterized by Bayer Schering Pharma AG, showed dissociative characteristics *in vitro* and *in vivo*. Mice with croton-oil induced ear inflammation showed reduced blood glucose levels when treated for 19 days with this non-steroidal selective GR-compound compared to mice treated with prednisolone [15]. Topical treatment of this SEGRAM did not increase body weight gain, as expected from a topical use. Taken together, ZK-216348 use results in a superior metabolic health of mice suffering from croton-oil induced skin inflammation [15].

Mapracorat (ZK-24518) successfully reached phase II clinical trials for the treatment of atopic dermatitis. Extensive preclinical studying *in vitro* and *in vivo* actions demonstrated Mapracorat did not induce hepatic tyrosine aminotransferase (TAT) and hyperglycemia, 2 markers of diabetes mellitus II induction [70].

CpdA does not induce GRE-regulated gene expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphate [50], two diabetogenic markers. In mouse models of multiple sclerosis, arthritis and experimental autoimmune neuritis, CpdA did not negatively affect blood glucose homeostasis while being therapeutically efficient [51, 71–73]. Although GR research is mainly focused on type II diabetes, CpdA showed beneficial effects also in a pharmacological model of type I diabetes in mice [74]. Although CpdA has proven beneficial outcomes in reducing metabolic GC-induced side effects while maintaining anti-inflammatory actions, its chemical lability is a major drawback preventing it from proceeding to clinical trials let alone the pharmaceutical market. Nevertheless, as stated above, CpdA serves as an excellent tool compound for research purposes. Chemical optimization, together with a detailed study of its GR-dependent and -independent actions, should be on top of the list when continuing with this research line.

Org 214007-0 is a selective GR modulator (SEGRM) with an improved therapeutic index, as studied by *in vitro* (both cell lines and primary human samples) and *in vivo* experiments. Mice models for both acute (LPS-induced TNF model) and chronic inflammation (CIA mouse model) receiving this compound were not suffering from glucose unbalance as observed under an equivalent dose prednisolone treatment [20].

PF-802, compared to the classical GC prednisolone, reduces phosphoenolpyruvate carboxykinase and tyrosine aminotransferase (TAT) gene expression in primary hepatocytes. Reduced expression of these typically GC-transactivated genes implied a reduced side-effect profile regarding glucose metabolism [30]. Pfizer next conducted an open label study to evaluate the effect of single doses PF-802 compared to prednisone on carbohydrate metabolism in healthy adult males, using an oral glucose tolerance test to evaluate glucose tolerance and insulin resistance (NCT01199900). The results of this phase I clinical trial are not yet made public. Recently, **JTP-117968** was described as a yet another structural analogue of PF-802. *In vitro* and *in vivo* data show an improved transactivation/transrepression ratio of this novel compound [75]. Future experiments with PF-802 should consider inclusion or substitution with JTP-117968, to evaluate the (diabetogenic) side-effect reducing potential of this selective GR-modulator as well.

Fasted, male Sprague Dawley rats treated with **AL-438** and prednisolone only showed a time and dose dependent hyperglycemic response after classical GC-treatment. Pre-treatment of the rats with AL-438 antagonized the plasma glucose flux induced by prednisolone. So, although AL-438 is bound to GR in vivo, it does not induce hyperglycemia at equivalent anti-inflammatory doses [48]. Overall, these results suggested that AL-438 could be a potent alternative to classic GCs to treat inflammatory diseases, since it does not support diabetogenic effects.

Orally administered **MK-5932** displayed anti-inflammatory efficacy in a rat oxazolone-induced chronic dermatitis model, while sparing plasma insulin [76]. In a follow-up *in vitro* and *in vivo* study, MK-5932 showed anti-inflammatory effects and a favorable dissociative dose-dependent effect on glucose metabolism and neutrophil counts. Conclusions were reached using rat and human whole blood, different rat models (i.e. contact dermatitis model, CIA and adjuvant-induced arthritis models) and healthy dogs [77].

Q40 was picked up in a compound library screen for its dissociative potential on GR-transactivation and transrepression. Based on in vitro data in 293 T cells, Q40 has the characteristics of a selective GR-ligand that can be used in chronic inflammatory diseases, without inducing dysregulation of glucose metabolism [78].

As already mentioned above, **LGD-5552** has side-effect reducing properties compared to classical GCs in a CIA mice model. Mice treated for 4 weeks with an oral dose of LGD-5552 failed to accumulate body fat [49]. Despite its metabolic advantageous profile, LGD-5552's MR-antagonistic activity may disturb sodium and potassium homeostasis and may lower blood pressure [49, 79].

Both **C108297** and **C113176** are a peculiar class of SEGRM since they are in fact selective *GR-antagonists*. The team of Meijer showed earlier the dissociative potential of C108297 with regard to brain stress circuits, supporting potential beneficial effects in terms of a GC-induced depression [80]. More recently they proved in 10 week old high-fat-diet mice that C108297 attenuates obesity, while still having anti-inflammatory potential. This is mediated by a combination of reduced caloric intake and increased lipolysis and fat oxidation [81].

Additionally, the Riddell team has developed an established rodent model of rapid-onset diabetes (ROD) [82]. This model combines increased levels of GCs via a slow-releasing corticosterone pellet with a HFD in young male Sprague-Dawley rats. In the ROD model, C113176 is superior to C108297, with regard to body composition, fasting glucose concentrations and insulin metabolism [82]. These findings emphasize the importance of the experimental model used. Including an extensive preclinical study of the hits and leads in primary human cells of different origin, may increase chances of a successful clinical trial course.

3.3 Cardiovascular Complications

3.3.1 Hypertension

Na^+ retention is the predominant cause of GC-induced hypertension. Endogenous GCs that have binding affinity for both the GR and MR can stimulate both the activity and gene expression of epithelial Na^+ channels via a transactivation mechanism. GC-induced SGK transcription further activates epithelial Na^+ channels through enhanced phosphoregulation mechanisms [38].

The only SEGRAM studied for its effect on blood pressure is **LGD-5552**. This compound (1-3 mg/kg) did not evoke hypertension in rats compared to prednisolone with the latter inducing a higher blood pressure already at low doses [83].

3.4 Atrophy

3.4.1 Skin Atrophy and Poor Wound Healing

A combination of decreased keratinocyte and dermal fibroblast proliferation and dermal fibroblast protein synthesis results in GC-associated skin atrophy. GC-induced direct protein-protein interaction between GR and the TGFβ-signalling component and transcription factor Smad3 results in decreased collagen type I transcription and translation. Furthermore, GC-induced skin atrophy is also mediated through the regulation of other extracellular matrix proteins, such as tenascin C [38]. The team of Perez researched skin wound healing in transgenic mice with a keratinocyte-restricted expression of either wild type GR or a mutant GR that is transactivation-defective but maintains transrepression (K5-GR and K5-GR-TR mice, respectively). Their data strongly suggested that the transrepression function of GR negatively regulates early stages of wound closure, while the transactivation function by GR delays later stages of healing [84]. Interestingly, another study revealed that a blockade of GC receptors with RU486 was able to partially improve stress-impaired wound healing [85].

The skin disease settings also impact GR signaling. De novo GC synthesis, and GC receptor expression was found dysfunctional in both non-lesional and lesional psoriatic skin. To distinguish between local (keratinocyte) and systemic GC activity a mice model was used of GR epidermal knockout mice with adrenalectomy. Keratinocyte-derived GC synthesis protected skin from a topical phorbol 12-myristate 13-acetate (PMA)-induced inflammatory assault. Thus, localized de novo GC synthesis in skin is essential for controlling inflammation, and loss of the GC pathway in psoriatic skin represents an additional pathological process in this complex inflammatory skin disease [86].

Treatment of mice suffering from skin inflammation with **ZK-216348** was advantageous over classical GC-treatment with regard to skin atrophy

[15]. However, the exact mechanisms involved were not documented. Reuter et al. next showed potent anti-inflammatory actions of ZK-216348 in an *in vitro* wound healing model. Unlike dexamethasone, this selective GR-agonist has no dose-dependent inhibitory effect on cell restitution. This discrepancy is explained by an unaltered signaling of EGF/ERK1/2/MAPK-pathways following ZK-216348 treatment. The aforementioned DEX-induced pathway hampers intestinal epithelial wound healing by stimulation of MKP-1 and Annexin-1. Both targets were unaffected by ZK-216348 [87].

In 2009, Schäcke et al., already showed a better safety profile in vitro and in vivo after treatment with **Mapracorat (ZK-245186)**. Its anti-inflammatory action was assessed both in vitro and in vivo. In vitro assays were based on inhibition of cytokine secretion and T cell proliferation and in vivo models included irritant contact dermatitis and T-cell mediated contact allergy models in mice and rats. This compound showed a better safety profile with regard to skin atrophy after prolonged topical application [70]. The molecular signaling pathways involved in skin were not elucidated. Effects are nevertheless species independent since similar effects were observed following topical treatment of dogs with inflamed skin responses (i.e. wheal and flare reaction) with mapracorat [88]. Two phase II clinical trials with mapracorat were started in 2009. In the first double blind dose finding study by Intendis, 64 patients were tested for 4 weeks with topical application of mapracorat against atopic dermatitis (NCT00944632).

From the same class of *in vivo* validated non-steroidal SEGRAs as mapracorat is **GSK866**, in which bulky, bicyclic aromatic substituents account for the structural similarity to corticosteroids [89, 90]. A novel class of SEGRA **GSK866 analogues with electrophilic warheads** was synthesized in analogy to covalent-binding kinase inhibitors to improve the clinical safety profile for long lasting topical skin disease applications [91]. Interestingly, adding covalent binding cysteine reactive warheads to GSK866 reduced its GR transactivation properties, while maintaining anti-inflammatory efficacy, in comparison to the non-covalent drug. Such covalently binding drugs should have a prolonged therapeutic effect *in vivo* and smaller doses of the drug can be applied to obtain similar therapeutic efficacy with reduced systemic side effects. In addition, electrophilic properties of covalent binding GSK866 analogues might locally trap anti-inflammatory effects in the epidermis upon topical administration and limit transdermal systemic circulation, which may further reduce systemic side effects upon chronic treatment. However, despite promising preclinical results *in vitro,* further *in vivo* validation and clinical proof of concept for improved safety of topical skin applications with covalent binding GSK866 analogues, remains to be demonstrated [91].

Skin atrophy was assessed by analysis of epidermal thickness, keratinocyte proliferation, subcutaneous adipose hypoplasia, and dermal changes after chronic treatment of mice with a classical GC and CpdA [92]. CpdA was able to inhibit TPA-induced skin inflammation and hyperplasia. Unlike steroidal GR-agonist treatment, CpdA treatment did not cause skin atrophy. This effect is mostly linked to unaltered REDD1 levels, which is considered causative for skin and muscle steroid-induced atrophy [92]. Reuter et al. showed that CpdA has the same beneficial effect on wound healing as already mentioned above for ZK-216348 [87].

Muscle Atrophy

Prolonged use of GCs is linked to muscle atrophy, via a combination of inhibition of protein synthesis and stimulation of protein degradation. GR-controlled gene activation of components of the ubiquitin-proteasome signaling pathway accounts for enhanced protein degradation. Key players in this process are MuRF1, atrogin-1 and members of the forkhead box superfamily of transcription, e.g. FOXO3, which is indirectly activated by GCs via the PI3K/AKT pathway [93, 94]. Other mechanisms involved in GC-induced muscle wasting are upregulation of FOXO1 and C/EBPβ and downregulation of MyoD and myogenin. In addition, GC-stimulated hyperacetylation via increased expression of p300 and its HAT-activity and decreased expression and activity of HDACs may

contribute in explaining GC-linked muscle proteolysis and wasting. Other mechanisms may also be involved in glucocorticoid-induced muscle wasting, including induction of myostatin expression levels, insulin resistance and store-operated calcium entry [93, 95]. Finally, in the specific case of ischemic myocardial injury, macrophage GR was found to regulate myofibroblast differentiation in the infarct microenvironment during the early phase of wound healing [96].

Protein degradation and stimulation of muscle dystrophy markers was no longer observed under CpdA treatment compared to classical GCs in a mouse model for X-chromosome linked muscular dystrophy [97].

3.5 Ocular Health

3.5.1 Ocular Eye Pressure

GCs are often used topically and/or intravitreally to treat ocular inflammation or edema associated with macular degeneration and diabetic retinopathy. Unfortunately, GCs themselves may cause irreversible eye damage through GC-induced ocular hypertension, resulting in secondary open-angle glaucoma [98]. The World Health Organization estimates that 80 million people worldwide will suffer from glaucoma by 2020 [98]. GC-induced intraocular pressure (IOP) has both GR-dependent and independent components. GR-independent factors, which mostly cause short-term effects, include formulation (e.g. acetatic (lipophilic) vs phosphatic), intravitreal injection volume, particulate size and appearance [98]. The GC-induced damage in the trabecular meshwork is mediated via an increase in aqueous humor outflow resistance. This is partially caused by enhanced deposition of extracellular matrix proteins, via a combination of metalloproteases (MMPs) inhibition and stimulation of its natural inhibitor, i.e. tissue inhibitors of metalloprotease (TIMP) [99]. Classical GCs, such as dexamethasone, both stimulate and inhibit a complex network of factors that contribute to IOP. For example, morphological and immunohistochemical examination of trabecular tissues revealed that steroids induce accumulation of fine fibrillar-like material and deposits of type IV collagen, heparin sulphate proteoglycan and fibronectin [100, 101]. DEX further increases unbreakable glycosaminoglycans [102], β3 integrin via the calcineurin/NFAT pathway [103], several matrix proteins [104], and cell stiffness via CLANS [105, 106] as important modulator of the cytoskeleton, e.g. the Rho GTPase Cdc42 [107]. DEX-induced IOP is also linked to inhibition of phagocytosis [108]. Overall, these data demonstrate that GC-induced glaucoma is mediated through a complex combined action of various proteins.

ZK209614 was extensively studied by the Nakamura team. Both *in vitro* and *in vivo* tests in the carrageenan-induced conjunctivitis model and allergic conjunctivitis model in rats were conducted to show its dissociative potential. *In vivo* assessment of the unwanted ocular hypertensive effects was evaluated in a feline model, by topical administration of the compound. In the feline intraocular pressure-elevation experiment, ZK209614 showed to be superior compared to betamethasone phosphate. These results position ZK209614 as an ophthalmic drug with an improved therapeutic index compared to the currently used GCs [109].

Mapracorat showed an anti-inflammatory efficacy comparable to DEX in experimental models of dry eye and post-operative inflammation while demonstrating reduced effects in intraocular pressure [110]. In another study, mapracorat was administered into the conjunctival sac of ovalbumin (OVA)-sensitized guinea pigs following the induction of allergic conjunctivitis [111], which enabled control of the late phase of ocular allergic conjunctivitis promising. A double blind trial conducted by Bausch & Lomb, evaluated in 2010 an ophthalmic suspension of mapracorat in about 550 patients for the treatment of inflammation following cataract surgery (NCT00905450). A successor phase III clinical trial started in the same year and was completed in 2011 (NCT01230125). Although the clinical trials are completed, no detailed study results have yet been published.

4 Selective GR-Therapy and Cancer

Novel GR-ligands that have an improved therapeutic index compared to the classical GCs may become of great value for cancer patients. GCs are for instance widely used for the treatment of hematological malignancies. As for chronic inflammatory diseases, their prolonged use is linked to several quality-of-life threatening side-effects (see above).

CpdA-like properties of a SEGRM could be an advantage in the cancer field providing the anti-cancer potential can be uncoupled from side-effects as observed under the current GC-based therapy [112]. In this respect, CpdA inhibited growth and viability of human T-, B-lymphoma and multiple myeloma cells in a GR-dependent manner [113]. The efficacy of GCs to induce apoptosis of lymphoid malignancies directly relies on the amount of functional GR in the cell. GR protein stability is under the control of the 26S proteasome [114]. The proteasome causes cell desensitization to GCs via an accelerated hormone-induced GR degradation. As a consequence, an interesting pharmacological approach is to make use of proteasome inhibitors to increase GR cell levels [113]. CpdA cooperates with the proteasome-inhibitor Bortezomib, resulting in an enhanced transrepression activity and suppressing the growth and survival of *in vitro* lymphoma and myeloma cell models [113].

GCs inhibit bladder cancer cell invasion in a GR-dependent manner, whereas androgen-induced AR-signaling is responsible for bladder tumor progression. CpdA was tested *in vitro* in 4 human GR-positive bladder cancer cell lines and *in vivo* in mouse xenograft models for bladder cancer [115]. Overall, CpdA inhibits bladder cancer growth predominantly via GR-transrepression and partially through AR-inhibition, making it a favorable compound compared to classical GCs in the treatment of bladder malignancies [115].

Many patients with solid tumors have GCs as a coadjuvant in their therapeutic scheme. GCs are prescribed in these patients to suppress nausea and vomiting, inflammation and cytotoxic side effects [116–118]. Unfortunately, this chemotherapeutic supplementation, resulting in GR-activation, is associated with pharmacotherapeutic failure and tumor progression of different types of solid tumors [119, 120]. The exact mechanisms underlying these effects are not yet elucidated. Chen and co-workers did show that dexamethasone regulated pathways are linked to drug resistance in triple-negative breast cancer (TNBC) cells. The DEX-affected genes are aberrantly expressed in TNBC patients and associated with an undesirable clinical outcome. Interestingly, CpdA regulates only a small subset of genes, not involved in carcinogenesis and therapy resistance. A detailed mechanistic ChIP-exo approach learned that only stimulation with DEX, and not with CpdA, results in binding to a single GRE, causing pro-tumorigenic gene expression (117). For this reason, a stable analogue exhibiting CpdA-like properties would be an attractive alternative co-adjuvant in chemotherapeutic regimens treating TNBC patients.

Not only CpdA is considered as an alternative for currently GC-treated cancers. For example, castration-resistant prostate cancer (CRPC) progression is mediated by increased GR expression and activity following androgen blockade. The two highly selective GR modulators **C118335 and C108297** inhibit GR transcriptional activity in prostate cancer and attenuate in a GR-dependent way CRPC progression as shown in a xenograft model. In contrast to mifepristone (a FDA-approved GR-antagonist), the novel selective GR-modulators are capable of maintaining androgen receptor signaling, improving their therapeutic potential. After inhibiting AR signaling, these SEGRMs were able to decrease GR-mediated tumor cell viability as observed after transcriptome analysis. Genome-wide analysis revealed that these SEGRMs were able to block GR-mediated proliferative gene expression pathways. In vivo treatment with C108297 inhibits proliferation-associated genes (AKAP12, FKBP5, SGK1, CEBPD and ZBTB16). Overall, these results are in support of SEGRM-mediated prostate cancer growth inhibition and their therapeutic potential in GR-expressing CRPC [121].

5 Outlook/Critical Perspective

As for most therapies, future GR-targeting drugs should focus on a personalized medicine approach, taking into account the exact disease condition (e.g. acute versus chronic diseases) and a more holistic view of the patient's needs. Besides the nature of the SEGRAM, the dose and duration of treatment, a more personalized approach may ask for precise timing of medicine intake (e.g. morning versus evening), metabolic state of the patient, disease dynamics and interplay with other medication [122]. A more intense and detailed monitoring of the patient by the clinician allows a better judgement of when a switch to a drug exhibiting selective GR-modulating properties is required to reach an optimal treatment regimen.

Molecularly, as already suggested by Yamamoto and his team, data on priming, (de) sensitization, and (in)activation of GR, together with synergies and cross-talk with other signaling pathways, should be mapped for each patient individually, to reach the proposed precision medicine approach in inflammatory diseases [123]. This concept can only be successfully reached when a multi-disciplinary approach of biological, physical, engineering, computer and health sciences is implemented [122].

In this book chapter we predominantly focused on the commonly studied GC-induced side effects, i.e. adverse effects on bone, metabolic, cardiovascular, skin, muscle and ocular health. Additionally, we provided a short overview of recent advances in SEGRAM application in the cancer field. Although the molecular mechanisms behind SEGRAMs in these research topics are far from fully elucidated, the impact on other GC-induced comorbidities, such as depression, obesity and fertility lag even further behind. Effects are unknown and still need intensive research [81]. Another major pitfall of classical GC-therapy is GR-resistance. This topic is however beyond the scope of this book chapter.

Although CpdA, i.e. the most intensively studied SEGRAM, and the other discussed selective GR-targeting drugs exhibit promising features enabling them to circumvent at least some of the GR-related side effects, other unwanted side-effects may remain or emerge, in case of different scaffolds. If the transrepression hypothesis (Sect. 2.1) would have been as black and white as was hoped for, alternatives for the current GR-targeting drugs would have been found already. Meanwhile, medicinal chemists are faced with the burden that novel compound development is based on still fairly limited structural information about GR. Also, novel drug design remains difficult if not impossible when the (favoured) mode of action of GR itself is not understood in full detail, which is currently still the case. A major step forward would be a full comprehension of GR-conformation and mode of action with the by pharma-synthesized GR-ligands made available to fundamental researchers and a facilitation of more research platforms between pharma and acadamia.

Not only the combined agonistic and antagonistic action and activation of one specific GR-pathway is important for finding new GR-therapies. Another important research field concerns the posttranslational modifications that affect GR and consequently affect its function [2]. Recently, alternative cofactor recruitment profiles were identified that skew and fine-tune GR effects towards an improved therapeutic advantage [24]. Additionally, the GR ligand-independent activation of GR adds another layer of complexity in GR signaling pathways. Notably, PF-802 is the only SEGRM that cannot bind to GR and only CpdA and ZK-216346 proved to elicit a partial or full nuclear translocation of GR. This observation makes it tempting to speculate that the observed anti-inflammatory effects of particular SEGRM may be (at least partially) GR-independent [4].

Unraveling the biological relevance of alternative GR signaling pathways will assist in the development of novel strategies to improve selective GR-targeting drug efficacy causing minimal therapy-induced harm to the patient [2].

A fast and better predictive screening will accelerate the search for novel dissociative compounds. For example, a successful approach for testing mapracorat using a psoriasis plaque test has recently been described [124]. Researchers

should focus not only on drug development but also on robust assay development and the subsequent read-outs onto which go/no decisions are based, already early in fundamental and clinical development. For phase I studies a combination of (1) a small number of subjects, (2) a short study duration time, (3) a highly discriminating power between compounds and concentrations and (4) a parallel measurement of treatment response can significantly increase the success rate of follow-up clinical trials. Researchers in the drug development field should aim for a low attribution rate, especially when the novel drugs have entered the clinical trial track. This can only be reached when the pre-clinical stage is based on solid fundamental results, rather than scientific doubt. Overall, each novel selective GR-drug has its own improved therapeutic index as shown in pre-clinical and clinical trials, but none of them invaded the pharmacological market so far. By adding extra layers of complexity, using different scientific methods, the search for selective GR-drugs will be more based on scientific fundamental insights. By closing these research gaps, researchers must eventually succeed in developing more safe and effective glucocorticoid receptor therapies.

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Part V

Innovative Drugs to Treat Inflammatory and Autoimmune Diseases

Innovative Drugs for Allergies

Ekaterini Tiligada, Kyriaki Gerasimidou, and Francesca Levi-Schaffer

Abstract

Allergic disorders are caused by immune responses to normally harmless environmental antigens. These allergens typically induce the production of type 2 T helper cell (T_H2) cytokines, such as interleukin (IL)-4, IL-5 and IL-13 that promote class switch recombination to immunoglobulin E (IgE) in antigen-activated B cells and the functional activation of mast cells, basophils and eosinophils. The classical therapeutic interventions for the management of allergic diseases include corticosteroids, antihistamines targeting the histamine H_1 receptor and other symptomatic medications, with variable clinical success. In recent years, a variety of pathobiological mechanisms implicated in the heterogeneous allergic phenotypes and endotypes have been exposed, driving the development of optimized small molecule drug candidates and novel targeted biologics. In the indented new era of personalized or precision medicine, numerous monoclonal antibody (mAb) products that are targeted at a specific determinant – usually a cytokine or a cytokine receptor – are at various stages of preclinical and clinical evaluation for their efficacy in managing allergic inflammation. Despite the plethora of emerging targeted options, only a few medications have been approved for human use to date. The anti-IgE mAb omalizumab is the first biologic that has been approved since 2003 for the treatment of patients with moderate to severe persistent asthma, and more recently for the management of chronic spontaneous urticaria. Subsequently, the anti-IL-5 humanised mAbs mepolizumab and reslizumab received market authorization as add-on maintenance therapies for patients with severe eosinophilic asthma that is not adequately controlled with inhaled corticosteroids. The latest addition to the armamentarium of approved medications for allergic disorders is dupilumab, a human mAb that inhibits IL-4 and IL-13 signaling by targeting the IL-4Rα subunit of the IL-4 receptor. Dupilumab received its first global approval in 2017 for the treatment of moderate to severe atopic dermatitis. Certainly, the complexity of asthma, atopic dermatitis and other related pathologies comprises heterogeneous, yet elusive, phenotypes and endotypes. In spite of the frequently disappointing outcomes of trans-

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lational research, the emerging scientific evidence on the cellular and molecular mechanisms underlying the inflammatory responses, and the constantly expanding fields of immunopharmacology and bioengineering are promising indications for the expected successful development of better therapeutic approaches for allergic diseases in the future.

Keywords
Allergy · Asthma · Atopic dermatitis · IgE · Interleukins · Monoclonal antibodies · Urticaria

1 Introduction

Allergic diseases are among the commonest chronic disorders, affecting approximately 15% of the global population and impairing the patient's quality of life [1, 2]. They comprise anaphylaxis, eczema or atopic dermatitis (AD), urticaria (hives), asthma, allergic rhinitis (hay fever) and (rhino)conjunctivitis, as well as food and venom allergy [3]. Their causes are complex local and/or systemic hypersensitivity reactions, which are typically attributed to a wide range of environmental stimuli referred to as allergens, including non-infectious components of some infectious organisms [4–6]. Common allergen sources include pollens, fungi, house dust mite, Hymenoptera venoms, animal dander, latex, a number of medicines and foods such as peanuts, nuts, fish, shellfish, eggs and milk [7].

A first step of a typical hypersensitivity reaction is the induction of allergen-specific immunoglobulin (Ig) E during the sensitization phase of the adaptive immune response (Fig. 1), although in some cases IgE does not seem to be a key component [4, 9]. Subsequent re-exposure of a sensitized subject to the allergen induces an immediate early-phase response followed by a late-phase reaction, whereas persistent or repetitive exposure results in chronic allergic inflammation (Fig. 1) [4, 8–11]. Localized allergic reactions are usually manifested as skin rashes, bronchospasm, rhinitis, conjunctivitis and/or gastrointestinal dysfunction. On the other hand, systemic anaphylaxis involves multiple organ systems and requires immediate intervention [12]. This acute, potentially life-threatening reaction is characterized by a combination of signs and symptoms, including flushing, pruritus, hives, angioedema, shortness of breath, wheezing, nausea, vomiting, diarrhea, hypotension and cardiovascular collapse [13].

Besides allergen avoidance [14], the management of both IgE- and non-IgE-mediated inflammation relies on a repertoire of classic, revisited and novel therapeutic interventions directed against either or both the putative aetiological components or/and the symptoms of the disease (Fig. 2). These include topical and/or systemic therapies with H_1-antihistamines, corticosteroids, mast cell stabilizers, immunosuppressive agents, leukotriene antagonists, anticholinergics, selective β_2-agonists, biologics and the potentially disease-modifying allergen-specific immunotherapy [18–20].

1.1 Novel Therapeutic Options for the Management of Allergic Disorders

The complexity of allergic inflammation is demonstrated by the wealth of related literature and the numerous emerging therapeutic strategies exploiting both small molecules and biologics that interfere with immune cell components and are currently at various stages of development and clinical testing [16, 21–25]. The poor understanding of the pathobiology underlying distinct allergic phenotypes and/or endotypes is a central limiting factor that complicates targeted treatment approaches [6, 20]. This may explain the high frequency of refractory patients and the continuing efforts to stratify potentially responsive patient populations in clinical trials [26]. In addition, the emergence of target-related and off-target adverse effects often exceed the capacity of the biomedical and clinical community to identify exploitable options for the management of many allergic disorders [27].

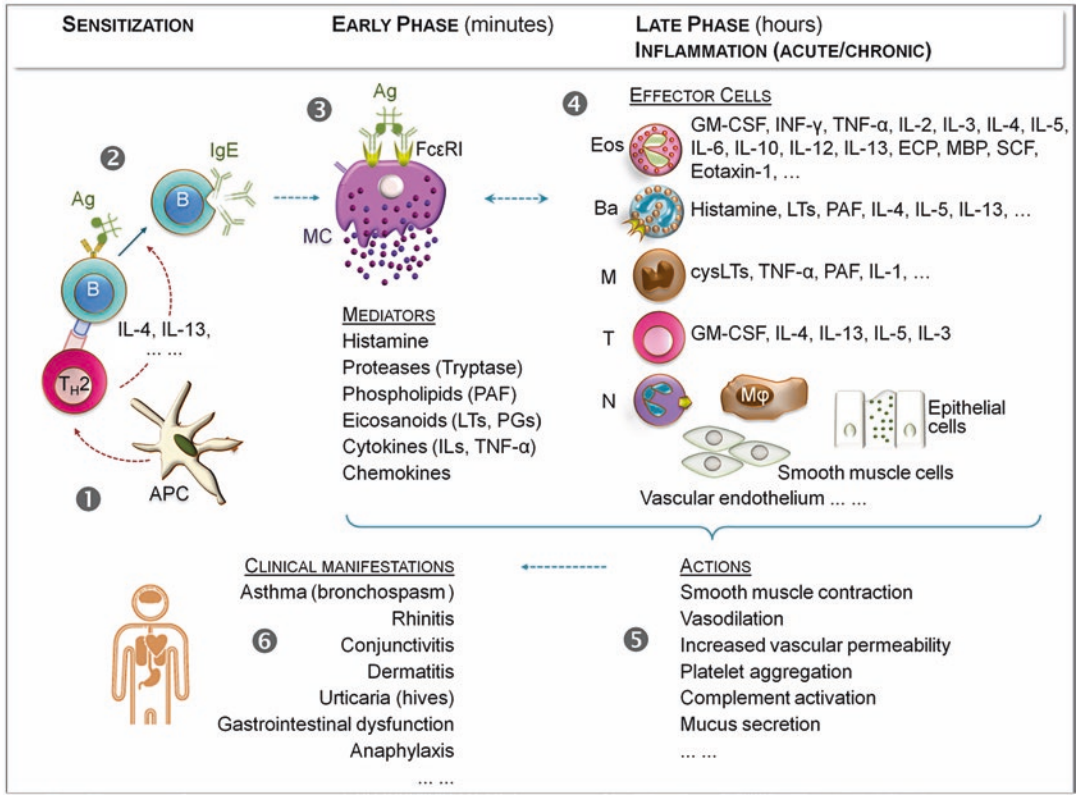

Fig. 1 Overview of the basic pathophysiological processes in allergic diseases. ❶ Allergens (Ag) enter the body predominantly through the skin or the lungs and are phagocytosed by antigen-presenting cells (APC). ❷ During the sensitization phase, the interaction of type 2 helper T cells (T_H2) and B cells (B) results in the production of Ag-specific immunoglobulin (Ig) E that binds primarily to its high-affinity receptor FcεRI on tissue mast cells (MC). ❸ Within minutes of re-exposure, the cross-linking of two bound IgE molecules triggers the release of preformed and newly synthesized mediators, including histamine, proteases, phospholipids such as platelet-activating factor (PAF), eicosanoids like leukotrienes (LTs) and prostaglandins (PGs), diverse chemokines and cytokines such as interleukins (ILs) and tumor necrosis factor (TNF)-α. ❹ These mediators modulate the recruitment and the activation of effector cells (*Ba* basophils, *Eos* eosinophils, *M* monocytes, *T* T cell subsets, *N* neutrophils, *Mφ* macrophages), thus promoting the development of the late-phase response 2–6 h after Ag exposure and/or of chronic inflammation upon persistent or repetitive exposure to the Ag. ❺ The activation of immune cells and the functional alterations in the affected tissues result in ❻ diverse clinical phenotypes that characterize the allergic diseases [4, 8–11]. ECP eosinophil cationic protein, GM-CSF granulocyte-macrophage colony-stimulating factor, MBP major basic protein, SCF stem cell factor

2 Drugs Targeting Histamine Receptors

Histamine is a biogenic amine that exerts its vital effects through binding to four subtypes of rhodopsin-like G protein-coupled receptors (GPCRs), designated as H_1, H_2, H_3 and H_4 [17, 28]. Importantly, histamine plays a key role in the immune responses by driving allergic and chronic inflammation [24, 29–31]. For more than 70 years, allergies are essentially managed with antihistamine drugs targeting the H_1 receptor (Fig. 2) that aim to counteract the effects of histamine released mainly from mast cells and basophils [15, 17, 19, 32].

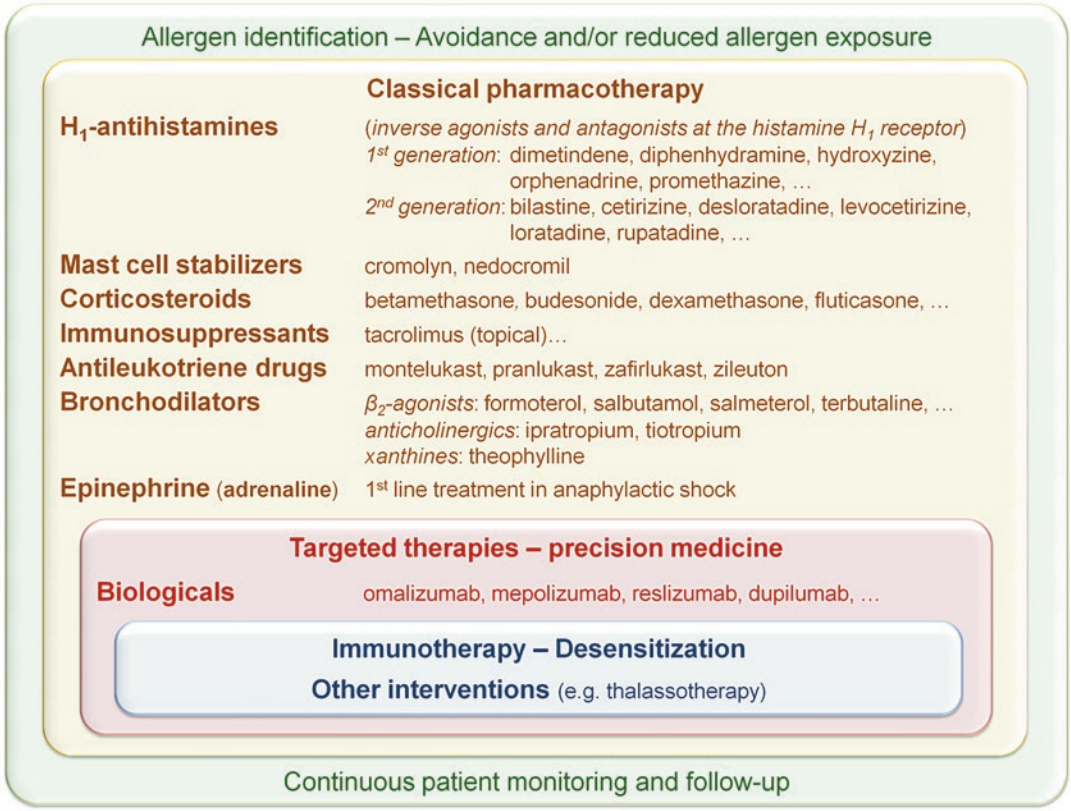

Fig. 2 Therapeutic approaches for the management of allergic disorders and indicative medications [13–17]

2.1 Classical H_1-Antihistamines and Beyond

The established contribution of the H_1 receptor to the allergic phenotype stems from its expression in smooth muscle cells of the vascular, respiratory and gastrointestinal systems, in endothelial and epithelial cells, in neurons of the central nervous system, and in numerous immune cells including eosinophils, mast cells, T and B lymphocytes, neutrophils, monocytes, macrophages and dendritic cells [17, 33]. By acting largely as inverse agonists at the low affinity H_1-receptor, H_1-antihistamines directly interfere with the actions of histamine and decrease, for instance, vascular permeability and smooth muscle contraction during allergic inflammation [17, 19]. Thus, H_1-antihistamines have achieved blockbuster status and their value in the treatment of a range of allergic conditions is universally accepted [34, 35].

Currently, more than 45 H_1-antihistamines are available worldwide including newer molecules for oral administration and topical application with improved benefit-to-risk profile [17, 19]. Nevertheless, the recent advances in GPCR structure, function and regulation exposed novel, yet elusive properties of histamine receptors and their ligands. Illustrative examples are the emerging concepts of ligand residence time and ligand-biased signaling that provide H_1-antihistamine development with novel clinically relevant opportunities, despite the complexity added to their pharmacological properties [33, 36, 37].

2.2 The Challenge of H_4-Antihistamines

In the post-genomic era, the revival of the global interest in histamine research was fueled by the discovery of the high affinity H_4 receptor at the turn of the millennium [38]. This newest addition to the family of histamine receptors introduced novel concepts on the immunomodulatory role of histamine in allergy and inflammation. The exploitation of the prototype H_4 receptor antagonist JNJ7777120 in preclinical pharmacology revealed that both histamine and H_4 receptor ligands recruit β-arrestin-2 in a G-protein-independent manner, besides G protein-dependent signaling [33, 39].

Numerous studies identified a key role of the H_4 receptor in mediating immune cell chemotaxis during the inflammatory response [38, 40]. Experimental evidence has associated the H_4 receptor with diseases that have extensively been managed with H_1-antihistamines with various degrees of success, such as allergic rhinitis, conjunctivitis and pruritus [25, 41, 42]. Consequently, various H_4-targeting compounds with favorable safety profile, including ZPL389 and toreforant are currently under proof-of-concept clinical testing for pruritus, atopic dermatitis, asthma, allergic rhinitis and/or psoriasis [41].

3 Monoclonal Antibodies

In recent years, our understanding of the immune system, the expansion of immunopharmacology and the significant biotechnological advances made available novel cutting-edge targeted therapeutics that challenge current unmet medical needs, largely by shifting drug development from small chemical molecules to biologics [6, 43]. Molecularly targeted therapies comprise therapeutic monoclonal antibodies (mAbs) that are tailored to interact with well-defined targets playing pivotal roles in complex human pathologies [16, 21, 43]. These leading tools of precision medicine (also referred to as personalized, stratified, or P4 medicine) revolutionized treatment options for malignancies and chronic inflammatory diseases and offer significant clinical benefit via monitoring immunological signals and restoring immune balance [16, 22, 23, 44].

In the intended new era of personalized therapy, ~50 mAb products including Fc fusion proteins, ab fragments and ab-drug conjugates, and biosimilars have been approved globally since the licensing of muromonab-CD3 (Orthoclone OKT3®) 30 years ago, whereas >500 products are estimated to be across various stages of development and/or evaluation [21, 44, 45]. Being one of the most rapidly expanding and promising areas in biomedicine with predicted combined world-wide sales of ~€145 billion by 2020, the development of mAb products, including new formats such as bispecific antibodies, nanobodies and peptibodies raise major technological challenges and attract high interest and investment from the biopharmaceutical, biotechnological, academic and health care sectors [46]. The increasingly demanding bioengineering innovations in antibody therapeutics is actually reflected by the ambiguities in the International Nonproprietary Name (INN) of mAb-based drugs as voiced by academic and industry scientists and evidenced by the announcement of the World Health Organization (WHO) in June 2017 [47].

Compared to conventional pharmacotherapy, mAbs are designed to interact with specific targets and to minimize or even eliminate adverse effects [48]. However, their growing use has revealed a number of both foreseeable and unanticipated target-related and/or off-target side effects that frequently hamper their clinical success. The main drawbacks comprise pharmacokinetic restrictions, limited clinical benefit to a subset of patients, (fatal) infusion reactions, antibody-dependent (ADCC) and complement-dependent (CDC) cytotoxicity, immune-complex-mediated pathologies, immunogenicity, autoimmunity, infections, cardiovascular complications and hepatotoxicity [48]. Unfortunately, nonclinical studies cannot always predict the nature, severity, frequency, dose dependency and reversibility of human responses, whereas disease complexity makes most model-based approaches difficult to rely on [49]. Treatment

failure is predominantly attributed to the disease pathobiological heterogeneity and/or occasionally to debatable evidence on the immunogenic and pharmacokinetic clinical assessment of biologics that is typically conducted by individual institutions and often relies on in-house assays for measuring of anti-drug antibodies (ADA) and drug concentrations [27].

Despite these shortcomings, the intense exploration of the yet elusive complex pathogenetic mechanisms underlying the highly heterogeneous disease phenotypes and/or endotypes in allergic patients is leading the rapid development of numerous innovative interventions. These largely target the type 2 immune response involving type 2 helper T cells (T_H2), type 2 innate lymphoid cells (ILC), other T_H subsets, including regulatory T cells (Treg) and T follicular helper cells (T_{HF}), natural killer T cells, mast cells, basophils and epithelial cells [6, 18, 21–23, 26, 50, 51]. The promising biologics-based options that are currently approved for the management of allergic disorders (Table 1) comprise omalizumab targeting IgE [52–55], mepolizumab and reslizumab targeting interleukin (IL)-5 [56, 57], and more recently dupilumab directed against the IL-4 receptor α (IL-4Rα) subunit [58, 59]. In addition, numerous other biologics and small molecules are in the pipeline and at various stages of ongoing clinical development [16, 21, 23, 26, 51].

3.1 Therapeutic Monoclonal Antibodies Against IgE

The IgE-mediated mast cell activation is considered to be a pivotal step during the onset of allergic inflammation, triggering mediator release and subsequently leading to downstream events that mark the late phase response and chronic inflammation (Fig. 1) [9, 11]. Discovered almost 50 years ago, IgE is the least abundant Ig isotype in human blood serum [60]. Accumulated evidence has documented its critical role in human allergic diseases and in host protection against helminth infections [10, 61, 62].

IgE is an evolutionary conserved, heavily glycosylated antibody consisting of two κ or λ light chains and two ε heavy chains that binds predominantly, but not exclusively to the human high affinity receptor FcεRI primarily expressed on tissue mast cells and blood basophils (Fig. 3) [52, 62, 65]. IgE binding to FcεRI provides a long-term and stable IgE loading on effector cells with a half-life of approximately 10 days [62]. Different strategies have been pursued to reduce the effects of IgE in allergic patients, including the development of therapeutic anti-IgE antibodies primarily aiming at decreasing the levels of circulating IgE [52, 62].

3.1.1 Omalizumab and Other Anti-IgE Agents

Omalizumab (Xolair®) is a humanized IgE-neutralizing mAb (Fig. 3) with a good safety and efficacy profile that has been approved since 2003 and 2005 by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), respectively as the first-in-class biologic for the treatment of IgE-mediated asthma in adults, adolescents and children 6–12 years of age [53] (Table 1). In 2014, omalizumab was the first biologic medicine approved for the treatment of chronic urticaria in adult and adolescent patients with inadequate response to H_1-antihistamine treatment [54, 55]. Considering the patent expiry of the innovator biologic in 2017, both in the U.S. and in Europe, FDA approved the initiation of a first-in-human study of GBR 310, a proposed biosimilar of omalizumab, for the treatment of allergic asthma and chronic idiopathic urticaria.

Omalizumab exerts its therapeutic actions predominantly by binding to the circulating IgE and preventing its interaction with FcεRI on effector cells, such as mast cells and basophils (Fig. 3) [52, 62]. In addition, it induces the rapid dissociation of IgE from the FcεRI following binding to a small population of FcεRI-bound IgE molecules and destabilizing the IgE-FcεRI interaction [52]. Moreover, omalizumab appears to block IgE binding to the low affinity cluster of differentiation (CD) 23/FcεRII receptor expressed on B and antigen-presenting cells, as well as on airway and gut epithelial cells [52, 65, 66].

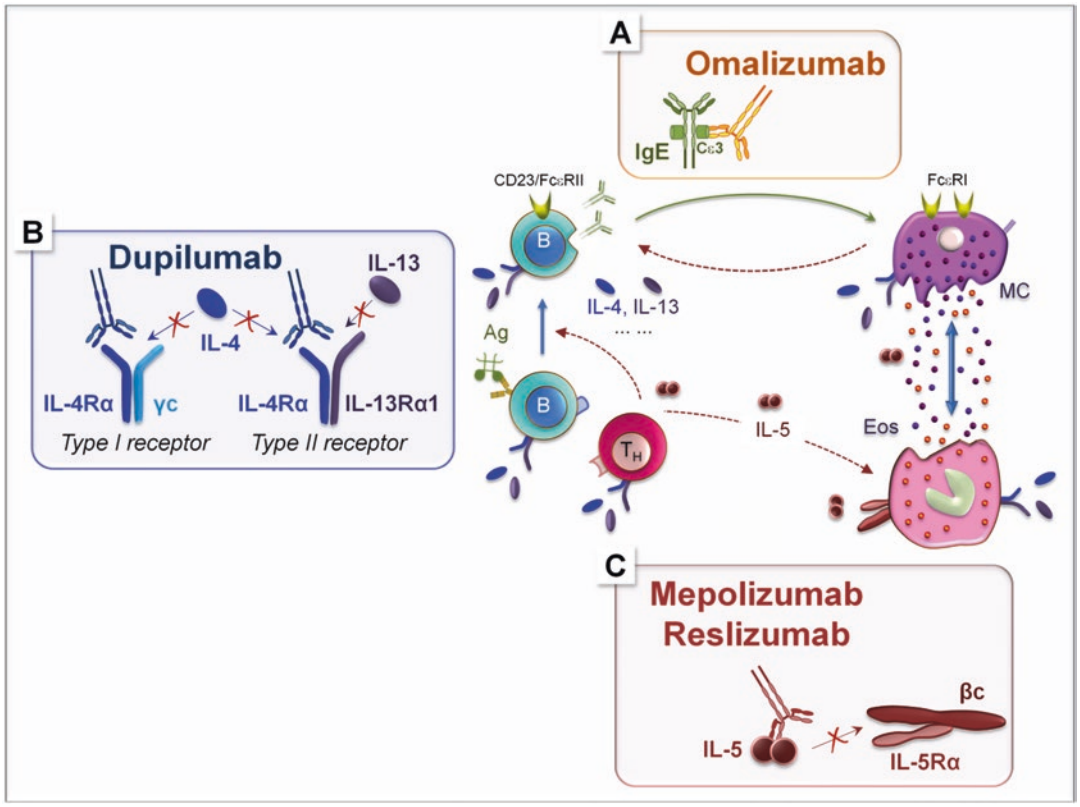

Fig. 3 Simplified schematic illustration of the mechanisms of action of approved biologics for the management of allergic diseases. (**A**) Omalizumab is a humanized immunoglobulin (Ig) G1 monoclonal antibody directed against the Cε3 domain of the constant region of the ε heavy chain of IgE that is essential for binding to the FcεRI receptor, thus preventing the interaction of serum IgE with FcεRI on effector cells, such as mast cells (MC). Additional interactions with IgE molecules bound to the high- and low-affinity receptors FcεRI and CD23/FcεRII, respectively may contribute to the actions of omalizumab in atopic individuals. Omalizumab has been approved for the management of moderate to severe asthma and chronic idiopathic urticaria [52, 62]. (**B**) The heterodimeric type I and/or II interleukin (IL)-4 receptor (R) complexes are composed of the IL-4Rα subunit and the gamma chain (γc), and of IL-4Rα and IL-13Rα1 subunits, respectively. Activation by IL-13 and IL-4 triggers, among others, type 2 helper T cell (T_H) and B cell differentiation, Ig isotype switch and eosinophil (Eos) recruitment. Dupilumab is a human IgG4 monoclonal antibody that inhibits IL-4 and IL-13 signaling through binding to the IL-4Rα subunit of type I and/or II receptor heterodimers. In 2017, dupilumab received its first global approval for the treatment of moderate-to-severe atopic dermatitis [59, 63, 64]. (**C**) The IL-5 receptor is composed of the IL-5Rα subunit and the beta chain (βc). Mepolizumab and reslizumab are humanized IgG1 and IgG4 monoclonal antibodies, respectively that bind to IL-5 and prevent its binding to IL-5Rα resulting in reduced eosinophils survival and activity. Both biologics were approved in 2016 for the management of severe eosinophilic asthma [56, 57]. *Ag* antigen, *x* inhibition

Based on the central role of IgE in allergies, the efficacy of omalizumab in treating patients with allergic rhinitis, atopic dermatitis or food allergy is under proof-of-concept clinical evaluation, yet without success so far [23, 26, 67].

Interestingly, many indications of biologics have been based on historical discovery and licensing rather than on a validated biological basis of disease and a rational use of biomarkers of response. In principle, the target specificity of a mAb does not always reflect the aetiology underlying a disease, but merely the involvement of a component of immunological signaling –typically a cytokine or a CD antigen– that contributes to a variety of unrelated conditions sharing some common inflammatory pathways. A characteristic

Table 1 Approved monoclonal antibodies for the management of allergic disorders

INN ATC code	Trade name Company	Type	Target	Year of approval	Indications	Contraindications Adverse reactions
Omalizumab R03DX05	Xolair® *Genentech (Roche) & Novartis*	Humanised IgG1κ[a] (~149 kD)	IgE	FDA 2003 EMA 2005 PMDA 2011 EMA 2014 FDA 2014 PMDA 2017	*Moderate to severe persistent asthma* (>6 years of age) *Chronic idiopathic urticaria* (>12 years of age remaining symptomatic despite H_1-antihistamine treatment)	Not to be used for acute bronchospasm or status asthmaticus Severe hypersensitivity reaction Anaphylaxis
Mepolizumab R03DX09 (Previously L04AC06)	Nucala® *GlaxoSmith Kline*	Humanised IgG1κ[a] (~149 kD)	IL-5	FDA 2015 EMA 2015 PMDA 2016	*Add-on treatment for severe refractory eosinophilic asthma* (>18 years of age)	Not to be used for acute bronchospasm or status asthmaticus Injection site reactions Hypersensitivity Headache, back pain, fatigue Herpes zoster infections
Reslizumab R03DX08	Cinqair® Cinqaero® *Teva*	Humanised IgG4[b] (~147 kD)	IL-5	FDA 2016 EMA 2016	*Add-on maintenance therapy for severe eosinophilic asthma inadequately controlled with high-dose inhaled corticosteroids plus another medicinal product* (>18 years of age)	Not to be used for acute bronchospasm or status asthmaticus Anaphylaxis Malignancies Oropharyngeal pain Increased creatine phosphokinase levels
Dupilumab D11AH05	Dupixent® *Regeneron & Sanofi-Aventis*	Human IgG4[a] (~147 kD)	IL-4Rα	FDA 2017 EMA 2017	*Moderate to severe atopic dermatitis* (Adult candidates for systemic therapy – used with or without topical corticosteroids)	Injection site reactions Hypersensitivity Conjunctivitis, blepharitis, keratitis, eye pruritus, dry eye Herpes simplex virus infections

ATC Anatomical Therapeutic Chemical classification system used by the World Health Organization for the classification of active drugs ingredients, *EMA* European Medicines Agency, *FDA* U.S. Food and Drug Administration, *Ig* immunoglobulin, *IL* interleukin, *INN* International Non-proprietary Name, *kD* kilodalton, *PMDA* Pharmaceuticals and Medical Devices Agency of Japan, *R* receptor

[a]Manufactured by recombinant DNA technology in Chinese hamster ovary (CHO) cells

[b]Produced by recombinant DNA technology in mouse myeloma cells (NS0)

example is rituximab (MabThera®/Rituxan®) that targets CD20 on B cells and is approved to treat B cell lymphomas since 1998 [68]. Based on their B cell-depleting properties, rituximab, a first biosimilar and other anti-CD20 mAbs are indicated or undergo clinical evaluation for the treatment of a variety of inflammatory conditions characterized by excessive numbers, overactive or dysfunctional B cells [68]. Although, the idea of pan-modulators seems incorrect, a limited number of studies with omalizumab in combination with other therapies, including rituximab suggest that a subgroup of patients might benefit from this strategy [26].

Besides omalizumab, a number of optimized anti-IgE agents have been developed with yet undetermined clinical exploitation potential [23, 66]. For instance, ligelizumab (QGE031) is an investigational anti-IgE mAb that is under phase II clinical evaluation for the treatment of allergic asthma and chronic spontaneous urticaria [69, 70]. Moreover, a phase I clinical trial in atopic subjects receiving MEDI4212, which blocks IgE binding to FcεRI and CD23/FcεRII, essentially failed to show appreciable benefit over omalizumab [71].

3.2 Therapeutic Monoclonal Antibodies Against T_H2 Cytokines

3.2.1 IL-4 and IL-13

Several cell types and inflammatory mediators have been reported to be implicated in allergic reactions, yet their exact role and interactions in driving the heterogeneous local or systemic clinical phenotypes remain unclear [72]. Since the identification of IL-4 as B cell stimulating factor in the late 1980s [73], classical research indicated that class switch recombination to IgG1, IgG4 and IgE in human antigen-activated B cells is modulated by IL-4 and IL-13 secreted during the T_H2 response that characterizes many forms of allergic inflammation [26, 74].

The structurally and functionally related cytokines IL-4 and IL-13 are short four α-helix bundle glycoproteins encoded by adjacent genes and sharing about 25% sequence similarity, receptor subunits and signaling molecules [63]. Being secreted by T_H2-polarised cells, type 2 ILCs, mast cells, basophils, eosinophils and monocytes/macrophages, IL-4 and IL-13 have been recognized as key players in the pathobiology of asthma, atopic dermatitis and allergic rhinoconjunctivitis [26, 58, 59, 63]. The classical T_H2 cytokine IL-4 signals via binding to type I receptors composed of the IL-4 receptor α (IL-4Rα) subunit and the gamma chain (γc) primarily expressed on hematopoietic immune cells, and through type II receptors composed of the IL-4Rα and the IL-13 receptor α1 (IL-13Rα1) subunits that are widely expressed on many cell types, including eosinophils, B cells, monocytes, macrophages, smooth muscle cells and endothelial cells. IL-13 binds to the IL-13Rα1 subunit of the type II receptors with lower affinity than IL-4 for IL-4Rα, as well as to the IL-13Rα2 that reportedly plays a pivotal role in some cancers [63]. Consequently, depending on IL-4 and IL-13 extracellular levels and on the availability of their receptor subunits on the cell surface of the responding cell, IL-4 and IL-13 elicit both overlapping and unique actions, including among others, T_H2 cell differentiation of native T_H0 lymphocytes, M2 macrophage polarization, B cell differentiation and antibody isotype switch, growth and development of mast cells and recruitment of eosinophils [63].

Among the various anti-IL-4 and anti-IL-13 mAbs that have been reported to improve lung and cutaneous functions in asthma and eczema, respectively [16, 23, 51], dupilumab (Dupixent®) was granted the first global approval in 2017 for the treatment of adults with moderate to severe atopic dermatitis that is not controlled adequately by topical therapies, or of patients for whom topical therapies are not advisable (Table 1) [64]. Furthermore, dupilumab has an acceptable safety profile and it is being evaluated for the treatment of atopic dermatitis in paediatric patients, as well as for the management of asthma, nasal polyposis and eosinophilic oesophagitis [58, 64, 75].

Dupilumab is a human IgG4 mAb that inhibits IL-4 and IL-13 signaling, including the release of pro-inflammatory cytokines, chemokines and IgE, by specifically binding to the IL-4Rα subunit shared by the heterodimeric type I and type II receptors for IL-4 and IL-13 (Fig. 3). Interestingly, before this innovative treatment option, the only medications licensed for atopic dermatitis over 15 years ago were the topical calcineurin inhibitors tacrolimus and pimecrolimus, whereas corticosteroids, antihistamines and, for more severe cases, systemically administered immunosuppressant drugs, such as cyclosporine have been used for decades [26].

3.2.2 IL-5

Since the initial characterization of eosinophils by Paul Erlich in the late nineteenth century – based on their staining properties with the acidic aniline dye eosin– these multifunctional leukocytes are widely accepted to be pivotal mediators of host defense in helminth infections and harmful causative components in allergic pathologies [76]. The abundant literature supports the critical role of IL-5 in regulating differentiation, activation, migration and survival of eosinophils, in addition to its modulatory role in the development and function of basophils and mast cells [57, 77]. Moreover, it provides the rationale for the implication of IL-5 in eosinophilic inflammation that commonly underlies asthma, atopic dermatitis and other pathologies associated with IgE and T_H2 cytokines [16, 57].

IL-5 is a homodimeric glycoprotein that elicits its actions through binding to its specific receptor subunit IL-5Rα (Fig. 3), while a separate motif binds to the signaling βc subunit that also binds IL-3 [57]. Eosinophils and basophils both produce IL-5 (Fig. 1) and express IL-5Rα, contrary to other IL-5 sources, such as T_H2 cells, mast cells and natural killer cells that do not seem to express the receptor [57]. The importance of eosinophils in the pathogenesis and severity of asthma and in other eosinophil-associated disorders led to the development of various IL-5/IL-5R targeted therapeutics, yet with limited clinical success [16, 23, 57]. Among them, the humanized anti-IL-5 mAbs mepolizumab (Nucala®) and reslizumab (Cinqair®, Cinqaero®) have been approved as add-on maintenance therapies for eosinophilic asthma (Table 1) [56, 78].

4 Concluding Remarks

Considering the increased prevalence of allergic diseases and the relative modest advancements in allergy treatment for decades, basic and translational research is currently directed towards the dissection of the causative mechanisms implicated in allergic inflammation, hoping to pave the way to next-generation therapies.

In recent years, diverse pathobiological mechanisms have been reported to underlie the heterogeneous allergic phenotypes and endotypes [6, 20, 50, 51]. Consequently, an impressive list of emerging investigative biologics and small molecule options for the management of allergic disorders is in the pipeline or at various stages of ongoing clinical assessment [16, 21, 23, 26, 51]. However, only a few innovative medications have been approved for human use to date. These comprise mAbs directed against IgE [52–55, 78], and the T_H2 cytokines IL-5 [56, 57], IL-4 and IL-13 [58, 59].

Further to the biotechnological challenges, the complexity of asthma, atopic dermatitis and other related pathologies comprises, yet elusive, multifactorial phenotypes and endotypes. In spite of the frequently disappointing outcomes of translational research, the dissection of the cellular and molecular mechanisms underlying allergic inflammation and the constantly expanding field of immunopharmacology are expected to provide better therapeutic approaches for allergic diseases in the future.

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