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SECRETION OF CYTOKINES AND CHEMOKINES BY INNATE IMMUNE CELLS

Topic Editor
Paige Lacy

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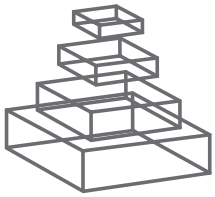

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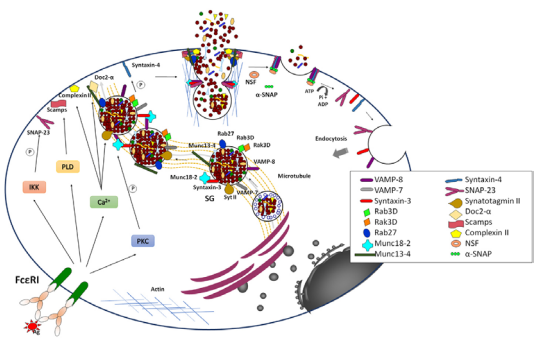
SECRETION OF CYTOKINES AND CHEMOKINES BY INNATE IMMUNE CELLS

Topic Editor:
Paige Lacy, University of Alberta, Canada



Distal signaling events that have been characterized in anaphylactic degranulation from mast cells. From Blank et al. Front. Immunol. 2014, doi: 10.3389/fimmu.2014.00453.

The release of cytokines, chemokines, and other immune-modulating mediators released from innate immune cells, including eosinophils, neutrophils, macrophages, dendritic cells, mast cells, and epithelial cells, is an important event in immunity. Cytokine synthesis and transportation occurs through the canonical protein trafficking pathway associated with endoplasmic reticulum and Golgi. How cytokines are released upon their exit from the trans-Golgi network varies enormously between cell types, and in many cells this has not yet been characterized. This issue delves into the plethora of cytokines released by innate immune cells, and where possible, shines light on specific mechanisms that regulate trafficking and release of Golgi-derived vesicles. Each cell type also shows varying degrees of dependency on microtubule organization and actin cytoskeleton remodeling for cytokine secretion. Understanding the mechanisms of cytokine secretion will reveal the inner workings of individual innate immune cell types, and allow identification of critical regulatory steps in cytokine release.

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EDITORIAL
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Editorial: Secretion of cytokines and chemokines by innate immune cells

Paige Lacy *

Pulmonary Research Group, Departments of Medicine and Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada

Keywords: SNAREs, macrophages, neutrophils, eosinophils, dendritic cells, epithelial cells, exocytosis, degranulation

Cytokines and chemokines are released from a wide range of immune cells, and are essential for the communication of signals in both innate and adaptive immunity. While there is a substantial body of literature regarding the function of cytokines and chemokines in immunity, remarkably little has been done to determine how these are packaged and secreted from a range of immune cells. Our knowledge of how cells package and release cytokines, chemokines, and other immunomodulatory factors is limited and only just emerging. Understanding how innate immune cells release cytokines and chemokines is important, as these factors are indispensable for communication with other immune as well as non-immune cells for the coordination of inflammatory responses. Moreover, cytokine and chemokine release by innate immune cells is a fundamental mechanism for cross-talk with the adaptive immune system.

Protein trafficking and secretion through both constitutive and regulated exocytosis are well described from many cell types. For this reason, many researchers assume that immune cells secrete cytokines and chemokines through the canonical endoplasmic reticulum (ER) and Golgi pathway before being transported to the cell surface via simple, unidirectional secretory vesicles. However, the reality is much more complex than this. Immune cells utilize a myriad assortment of secretory pathways, involving a combination of secretory granules and vesicles, for trafficking cytokines and chemokines. Perhaps counterintuitively, the secretion of cytokines and chemokines is often associated with endocytic pathways that are engaged in retrograde transport of proteins to the cell interior. The trafficking of cytokines and chemokines occurs through distinct vesicular pathways in each cell type, which adds further complexity to our understanding of this process. In this Research Topic, we will review the ability of a group of innate immune cells to synthesize and secrete various cytokines and chemokines. These are macrophages, neutrophils, mast cells, eosinophils, dendritic cells, and epithelial cells.

Submissions in this Research Topic include reviews that delve into some of the mechanisms described for their release. We begin with macrophages, which are best characterized for their ability to transcribe, translate, and process cytokines and chemokines for release (1, 2). Macrophages lack secretory granules and instead rely on a constitutive secretory pathway that engages recycling endosomes for the secretion of cytokines, including tumor necrosis factor (TNF), interleukin-6 (IL-6), and IL-10. The trafficking machinery required for the secretion of cytokines include soluble NSF attachment protein receptors (SNAREs) and accessory proteins that facilitate membrane fusion, such as GTPases, Munc13, and others. Stimulation of macrophages with the bacterial lipopolysaccharide (LPS) results in *de novo* synthesis of the transmembrane TNF precursor, which is transported through ER and Golgi to be trafficked via recycling endosomes to the cell surface by SNARE-dependent membrane fusion. These steps will be reviewed in detail and set the standard for our understanding of cytokine trafficking in innate immune cells.

Neutrophils will also be reviewed for their ability to release a plethora of cytokines and chemokines (3), which are essential for modulation of immune responses in acute and chronic inflammation. In spite of our knowledge of neutrophil cytokine and chemokine synthesis and

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Claudia Kemper, King's College London, UK

***Correspondence:**
Paige Lacy
paige.lacy@ualberta.ca

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secretion, remarkably little is known regarding if or how these are packaged in the neutrophil's secretory granules, which are well characterized. To explore the secretory capacity of neutrophils, we will review the mechanisms of neutrophil granule protein processing and regulated secretion, and how dysregulation of these mechanisms leads to diseases (4).

In mast cells, we will learn the differential release of granule proteins and the secretory pathways involved (5), and review their ability to regulate post-transcriptional control of cytokine production (6). Mast cell secretion of cytokines occurs through a trafficking mechanism that may resemble that of macrophages. Cytokines are dependent on SNARE-mediated secretion in mast cells, although mast cells appear to utilize different set of SNAREs for vesicular transport of cytokines than that of anaphylactic degranulation.

Eosinophils are major effector cells in allergic inflammation and have the ability to secrete numerous cytokines and chemokines (7). They also have the ability to synthesize and store cytokines for subsequent release. We will review the distinct intracellular membrane trafficking pathways that are used to transport pre-formed cytokines from their intracellular site of storage in eosinophil crystalloid granules to the cell membrane (8). Specifically, we will focus on the secretion of IL-4 and the chemokine CCL5/RANTES through a tubulovesicular transport system associated with piecemeal degranulation, which is also dependent on SNARE-mediated membrane fusion.

Relatively little is known regarding how dendritic cells secrete cytokines and chemokines, and in this Research Topic, we will learn the role of the SNARE syntaxin-3 in the secretion of IL-6 following stimulation of toll-like receptors (9). This will also be reviewed to understand more about how SNAREs may be required for the secretion of cytokines from dendritic cells, and this remains an area of investigation (10).

Finally, we will review the role of secreted cytokines and chemokines from airway epithelial cells and their role in shaping the immune response in allergy (11). The mechanisms of cytokine and chemokine secretion from epithelial cells have not yet been investigated, although this review helps us to understand the importance of epithelium-derived factors in altering immunity.

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REVIEW ARTICLE
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Macrophage cytokines: involvement in immunity and infectious diseases

Guillermo Arango Duque*[1,2]* and *Albert Descoteaux*[1,2]

[1] *INRS-Institut Armand-Frappier, Laval, QC, Canada*
[2] *Centre for Host-Parasite Interactions, Laval, QC, Canada*

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Mikhail A. Gavrilin, Ohio State University, USA
Rachael Zoe Murray, Queensland University of Technology, Australia

***Correspondence:**
Guillermo Arango Duque and Albert Descoteaux, Institut National de la Recherche Scientifique–Institut Armand-Frappier, 531 boul. des Prairies, Laval, QC H7V 1B7, Canada
e-mail: guillermo.arango-duque@iaf.inrs.ca, guillo.ad@gmail.com; albert.descoteaux@iaf.inrs.ca

The evolution of macrophages has made them primordial for both development and immunity. Their functions range from the shaping of body plans to the ingestion and elimination of apoptotic cells and pathogens. Cytokines are small soluble proteins that confer instructions and mediate communication among immune and non-immune cells. A portfolio of cytokines is central to the role of macrophages as sentries of the innate immune system that mediate the transition from innate to adaptive immunity. In concert with other mediators, cytokines bias the fate of macrophages into a spectrum of inflammation-promoting "classically activated," to anti-inflammatory or "alternatively activated" macrophages. Deregulated cytokine secretion is implicated in several disease states ranging from chronic inflammation to allergy. Macrophages release cytokines via a series of beautifully orchestrated pathways that are spatiotemporally regulated. At the molecular level, these exocytic cytokine secretion pathways are coordinated by multi-protein complexes that guide cytokines from their point of synthesis to their ports of exit into the extracellular milieu. These trafficking proteins, many of which were discovered in yeast and commemorated in the 2013 Nobel Prize in Physiology or Medicine, coordinate the organelle fusion steps that are responsible for cytokine release. This review discusses the functions of cytokines secreted by macrophages, and summarizes what is known about their release mechanisms. This information will be used to delve into how selected pathogens subvert cytokine release for their own survival.

Keywords: macrophage, cytokine, trafficking, exocytosis, proinflammatory, anti-inflammatory, *Leishmania*, *Mycobacterium ulcerans*

INTRODUCTION: CYTOKINES AND MACROPHAGES

Macrophages are phagocytic cells of the innate immune system that are located in various tissues. The Russian scientist Elie Metchnikoff received the 1908 Nobel Prize in Physiology or Medicine for his work on immunity when he observed that when he punctured starfish larvae, a population of cells migrated to the wound. He also observed cells that were able to uptake particles that had been placed in the digestive tracts of the larvae. Elie Metchnikoff coined these cells as phagocytes and later called them white blood cells for their first-line-of-defense role against infection in living organisms (1). Later, the term macrophage was introduced by Aschoff in 1924 to designate a set of cells of the reticuloendothelial system formed not only by monocytes, macrophages, and histiocytes, but also by fibroblasts, endothelial, and reticular cells. After 1969, the concept of the mononuclear phagocyte system – formed by a variety of macrophages derived from monocytes from the bone marrow – was introduced to replace the concept of the reticuloendothelial system, which is constituted of functionally and immunologically distinct cells. Most macrophages are derived from bone marrow precursor cells that develop into monocytes. These are formed in the bone marrow from stem cells of the granulocytic–monocytic lineage that are exposed to cytokines such as the granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3). Differentiation from stem cells is associated with the expression of specific membrane receptors for cytokines. Monocytes remain in the bone marrow <24 h and they move into the bloodstream and circulate throughout the body. In normal healthy adults, the half-life of a circulating monocyte is estimated at 70 h. Monocytes constitute 1–6% of total leukocytes in healthy peripheral blood. After crossing the walls of capillaries into connective tissue, monocytes turn into macrophages. This differentiation process involves many changes as the cell increases in size from 5 to 10 times, its organelles increase both in number and complexity, phagocytic capacity increases, etc. It is important to note that not all macrophages, such as Langerhans cells and brain microglia, develop from monocytes (2).

The main function of macrophages is to engulf foreign agents that enter the body. These include microbes and other particulate matter. In addition, they eliminate apoptotic cells and recycle nutrients by digesting waste products from tissues. Macrophages are hence essential not only for immunity, but also for development and tissue homeostasis (2). These cells are normally at rest, but can be activated by a variety of stimuli during the immune response (3, 4). Albeit phagocytosis may provide the initial antigen stimulus, the activity of macrophages can be increased by cytokines secreted by helper T cells, with interferon gamma (IFN-γ) being one of the most potent macrophage activators. In addition, these multifaceted cells are also capable of chemotaxis,

namely the process of being attracted and displaced to a particular location by specific molecules. Besides phagocytosis, macrophages play a central role in inflammation. They initiate the immune response against microorganisms, since macrophages are some of the first cells to come in contact with these invaders. This is in part due to their toll-like and scavenger receptors, which have broad ligand specificity for lectins, lipoproteins, proteins, oligonucleotides, polysaccharides, and other molecules. In addition to these functions, macrophages express major histocompatibility complex (MHC) class II molecules on their membranes, and as such, also present antigens to lymphocytes. When macrophages engulf a microbe, its antigens are processed and situated on the outer surface of the plasmalemma, where they will be recognized by T helper cells. Following this recognition, T lymphocytes release cytokines that activate B cells, and activated B lymphocytes then secrete antibodies specific to the antigens presented by the macrophage. These antibodies attach to antigens on microbes, or to cells invaded by microbes; in turn, these antibody-bound complexes are phagocytosed more avidly by macrophages.

Cytokines and chemokines are potent signaling molecules that are as important to life as hormones and neurotransmitters. They are low molecular weight proteins that mediate intercellular communication and are produced by many cell types, primarily those of the immune system. They were discovered in the early 60–70s, and today, over 100 different proteins are known as cytokines (5). These molecules orchestrate a variety of processes ranging from the regulation of local and systemic inflammation to cellular proliferation, metabolism, chemotaxis, and tissue repair. In other organisms, such as fruit flies and lizards (6), cytokine-like molecules are known to regulate host defense and temperature homeostasis. The primary function of cytokines is to regulate inflammation, and as such, play a vital role in regulating the immune response in health and disease. There are proinflammatory and anti-inflammatory cytokines.

Each cytokine binds to a specific cell surface receptor to generate a cell signaling cascade that affects cell function. This includes the positive or negative regulation of several genes and their transcription factors. This may ensue in the production of other cytokines, in an increase in the number of surface receptors for other molecules, or eventually in the suppression of the cytokine's own effect. Each cytokine is produced by a cell population in response to different stimuli; they induce an array of agonist, synergistic, or antagonistic effects that functionally alter target cells. A primary feature of cytokine biology is that of functional redundancy: different cytokines share similar functions. Furthermore, cytokines are pleiotropic since they act on many different cell types, and cells may express more than one receptor for a given cytokine. To generalize the effect of a particular cytokine is virtually impossible. Cytokines are classified as paracrine if the action in the vicinity of the place of release is restricted, autocrine if the cytokine acts on the cell that secretes it, and endocrine if the cytokine reaches remote regions of the body. Most cytokines are short-lived and act locally in an autocrine and paracrine fashion. Only some cytokines present in the blood, such as erythropoietin (EPO), transforming growth factor beta (TGF-β), and monocyte colony stimulating factor (M-CSF), are capable of acting at a distance.

Cytokines are mainly produced by macrophages and lymphocytes, although they can also be produced by polymorphonuclear leukocytes (PMN), endothelial and epithelial cells, adipocytes, and connective tissue. Cytokines are essential to the functions of macrophages. They mediate the unleashing of an effective immune response, link innate and adaptive immunity, and influence the macrophage's microenvironment (4, 7). Multiple subsets of macrophages have been characterized depending on the origin and microenvironment in which the macrophage is found. Contingent on activation status, macrophages have been classified as classically and alternatively activated. In turn, these different macrophage types drastically differ in the cytokines that they secrete, and consequently, their functions (8). The process of cytokine secretion is masterfully regulated by a series of interorganellar exchanges that rely on vesicular trafficking and cytoskeletal remodeling (9). Proteins regulating neurotransmitter release, notably members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family (9, 10), and more recently synaptotagmins (Syt) (11), are pivotal for the spatiotemporal regulation of cytokine secretion. In immune cells, SNAREs and Syts have been found to regulate processes ranging from cytokine trafficking to cell migration and phagocytosis.

This review will present the functions of macrophage cytokines and, where known, summarize findings on how these cytokines are released. The types of macrophages that secrete these cytokines will also be depicted. To illustrate the importance of macrophage cytokines in health and disease, we will describe selected examples of how pathogens use cytokines to their advantage.

THE MACROPHAGE CYTOKINE PORTFOLIO

PROINFLAMMATORY CYTOKINES

When macrophages are exposed to inflammatory stimuli, they secrete cytokines such as tumor necrosis factor (TNF), IL-1, IL-6, IL-8, and IL-12. Although monocytes and macrophages are the main sources of these cytokines, they are also produced by activated lymphocytes, endothelial cells, and fibroblasts. Additionally, macrophages release chemokines, leukotrienes, prostaglandins, and complement. All of these molecules, in concert, may induce increased vascular permeability and recruitment of inflammatory cells. Aside from local effects, these mediators also produce systemic effects such as fever and the production of acute inflammatory response proteins. The inflammatory response is beneficial for the host when the aforementioned cytokines are produced in appropriate amounts, but toxic when produced in a deregulated fashion. For example, excessive production of IL-1β and TNF triggers an acute generalized inflammatory response characteristic of septic shock and multi-organ failure (12).

TNF

Tumor necrosis factor (formerly known as TNF-α) is a 185-aminoacid glycoprotein that was initially described for its ability to induce necrosis in certain tumors (13). It stimulates the acute phase of the immune response. This potent pyrogenic cytokine is one of the first to be released in response to a pathogen, and is able to exert its effects in many organs (12). As such, TNF is one of the main cytokines responsible for septic shock. In the hypothalamus, TNF stimulates the release of corticotropic releasing hormone,

suppresses appetite, and induces fever. In liver, it stimulates the acute inflammatory response by elevating the synthesis of C-reactive protein and other mediators. TNF induces vasodilation and loss of vascular permeability, which is propitious for lymphocyte, neutrophil, and monocyte infiltration. It helps recruit these cells to the inflammation site by regulating chemokine release. TNF, in concert with IL-17, triggers the expression of neutrophil-attracting chemokines CXCL1, CXCL2, and CXCL5 (14) and can also augment the expression of cell adhesion molecules (15) that facilitate diapedisis. This in turn increases CXCR2-dependent neutrophil migration to the inflammation site. Being an inducer of the inflammatory response, excess amounts of TNF have been found to play pathological roles in ailments such as inflammatory bowel disease, psoriasis, rheumatoid arthritis, asthma, cancer, infectious diseases, and other auto-immune pathologies. Some of these conditions are currently co-treated with monoclonal antibodies that neutralize this cytokine (16).

In macrophages, TNF is released to the extracellular milieu via the constitutive secretion pathway, and its trafficking is the best understood of all cytokines (9, 17, 18). Details on TNF trafficking will be discussed in another article of this issue. After synthesis in the ER, the SNARE proteins Stx6, Stx7, Vtib mediate the fusion of TNF-containing vesicles from the Golgi complex with $VAMP3^{+}$-recycling endosomes (17, 19). Thence, the Stx4/SNAP23/VAMP3 complex facilitates the passage of TNF from recycling endosomes to the cell membrane (17, 18). Rho1 and Cdc42, two proteins that govern cell shape via actin remodeling, also regulate the post-recycling endosome trafficking of TNF to the plasmalemma (20). Moreover, LPS was found to increase the expression of vesicle trafficking proteins that regulate TNF trafficking (17, 18). Finally, release of mature TNF from the plasmalemma requires cleavage of the membrane-bound precursor by the TNF-α-converting enzyme (TACE) (21). The process of phagocytosis requires extensive membrane exocytosis from several organelles that also partake in TNF secretion (7). Interestingly, it was found that TNF is not only secreted to the extracellular milieu at the plasma membrane, but also in a polarized manner at the phagocytic cup (17). This highlights an efficient and elegant strategy where macrophages can promptly release cytokines at the same time that they phagocytose microbial invaders. The importance of regulating TNF secretion implies that there exist negative regulators for its secretion. One such regulator is the recently characterized protein Syt XI, which associates to recycling endosomes and lysosomes in macrophages (11, 22). Syts constitute a group of membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis (11, 23) and phagocytosis (11, 24, 25). Syts control vesicle fusion by virtue of their Ca^{2+}-binding C2 domains (26). However, Syt XI cannot bind calcium and inhibits vesicle fusion (27). Upon LPS stimulation of macrophages treated with siRNA to Syt XI, more TNF and IL-6 are released. The inverse is true when Syt XI is overexpressed (11). Though the mechanism for this finding is not yet known, Syt XI likely regulates cytokine release by interacting with members of the SNARE complex. Indeed, Syt XI was found to interact with the Golgi SNARE Vti1a (28), raising the question of whether Syt XI regulates SNARE complex formation at the Golgi.

IL-1

Three forms of IL-1 are known: IL-1α, IL-1β and IL-1Ra. Although both IL-1α and IL-1β are strongly proinflammatory, perform many of the same functions and bind to the IL-1 receptor (IL-1R), there is only 25% aminoacid homology between them. Similarly to TNF, IL-1β is also an endogenous pyrogen that is produced and released at the early stages of the immune response to infections, lesions, and stress. Although monocytes and macrophages are the main sources of IL-1β, it is also released by NK cells, B cells, dendritic cells, fibroblasts, and epithelial cells. During inflammation, IL-1β stimulates the production of acute phase proteins from the liver and acts on the central nervous system to induce fever and prostaglandin secretion. In mast cells, IL-1β induces the release of histamine, which in turn elicits vasodilation and localized inflammation. It is also a chemoattractant for granulocytes, enhances the expansion and differentiation of CD4 T cells (29), and increases the expression of cell adhesion molecules on leukocytes and endothelial cells. Additionally, IL-1β augments the expression of genes that produce it (30). To quell the proinflammatory action of IL-1α and IL-1β, IL-1Ra competes for the same receptor. IL-1Ra is secreted via the classical secretory, though the exact mechanism is not well known. Its binding to the IL-1R does not induce the proinflammatory signaling program induced by IL-1α and IL-1β.

In stimulated macrophages, IL-1α is synthesized *de novo* and can be actively secreted (31) or passively released from apoptotic cells (32). It can also exert its effects in an intracrine fashion and act as a transcription factor (29, 30). IL-1β is synthesized as a leaderless precursor that must be cleaved by inflammasome-activated caspase-1. After activation, autophagy plays a major role in the release of this cytokine. Autophagy is a highly conserved process in eukaryotes in which the cytoplasm, aberrant, or damaged organelles are sequestered in double-membrane vesicles and released into the lysosome for breakdown and eventual recycling of resulting macromolecules (33). This process plays a crucial role in adaptation to changing environmental conditions, starvation, cellular remodeling during development, and senescence. Autophagy is characterized by the formation of double-membrane vesicles, called autophagosomes, which capture and transport cytoplasmic material to acidic compartments where material is degraded by hydrolytic enzymes (33). Autophagy has also been recognized to mediate the secretion of proteins (34) – such as IL-1β and IL-18 (35, 36) – that would otherwise not enter the classical secretory pathway due to lack of a leader peptide. In the case of IL-1β, the autophagic protein Atg5, the Golgi protein GRASP55, and Rab8a are essential for translocating IL-1β-containing cargo to the outside of the cell. In peritoneal macrophages, it has been shown that IL-1β is transported to the extracellular milieu via membrane transporters (37); knockdown of ABC transporters inhibits IL-1β secretion (38). Additionally, exocytosis of P2X7R-positive multivesicular bodies containing exosomes has also been reported to play an important role in the release of this cytokine (39). The various modes of IL-1 secretion highlight the exquisite machinery that macrophages have evolved as a means for rapidly responding to inflammatory stimuli.

IL-6

IL-6 is a pleiotropic cytokine that has both proinflammatory and anti-inflammatory functions that affect processes ranging from immunity to tissue repair and metabolism. It promotes differentiation of B cells into plasma cells, activates cytotoxic T cells, and regulates bone homeostasis. As with other proinflammatory cytokines, IL-6 is has been implicated in Crohn's disease and rheumatoid arthritis (40). Similar to TNF and IL-1β, IL-6 is an endogenous pyrogen that promotes fever and the production of acute phase proteins from liver. Proinflammatory properties are elicited when IL-6 signals in trans via soluble IL-6 receptors binding to gp130, which is ubiquitous in all cells. Inhibition of trans signaling via gp130 blockade in murine sepsis models rescues mice from widespread inflammation and death (41). IL-6 trans signaling also leads to recruitment of monocytes to the inflammation site (42), promotes the maintenance of Th17 cells, and inhibits T cell apoptosis and development of Tregs (43). In contrast, anti-inflammatory properties are elicited when IL-6 signals through the classical pathway, which occurs via the IL-6 receptor that only few cells express. The anti-inflammatory properties of IL-6 are illustrated by IL-$6^{-/-}$ mice, which exhibit hepatosteatosis, insulin resistance, and liver inflammation (44). IL-6 classic signaling also mediates apoptosis inhibition and the regeneration of intestinal epithelial cells (43).

IL-6 is a soluble cytokine that is synthesized in the ER and, unlike TNF, is not processed as a membrane-bound precursor. Upon stimulation of macrophages with LPS, IL-6 starts accumulating in the Golgi after 4 h of stimulation (45). From the Golgi, IL-6 exits in tubulovesicular carriers that may also contain TNF. Golgi-derived vesicles then fuse with VAMP3-positive recycling endosomes. Three-dimensional reconstruction of fluorescence images showed that recycling endosomes can harbor both TNF and IL-6, albeit both occupy different subcompartments (45). The post-Golgi trafficking of IL-6 follows a route that is also dependent on Stx6 and Vti1b, which form a complex with cognate SNARE VAMP3 at recycling endosomes (17, 18). Knockdown and overexpression of these SNAREs decreases and augments IL-6 release, respectively (45). Syt XI may be negatively modulating the secretion of this cytokine by regulating the formation of these SNARE complexes (11, 28). Unlike TNF, IL-6 is not secreted at the phagocytic cup (45).

IL-12

IL-12 is produced primarily by monocytes, macrophages, and other antigen-presenting cells; it is essential for fighting infectious diseases and cancer. IL-12 is a heterodimeric cytokine comprised of the p35 and p40 subunits, which come together after their synthesis. Deletions within the p40 gene have been observed in patients suffering from concurrent multiple bacterial infections (46, 47). IL-12 promotes cell-mediated immunity via stimulation of Th1 cells. It synergizes with TNF and other proinflammatory cytokines in stimulating IFN-γ production, as well as the cytotoxicity of NK and CD8 T cells (48). IL-12 can also inhibit angiogenesis through IFN-γ-mediated upregulation of the anti-angiogenic chemokine CXCL10. The involvement of this cytokine in these processes has made it a target in both auto-immune pathologies and cancer (46, 47). After protein synthesis, both p40 and p35 subunits associate at the ER, where they undergo subsequent glycosylation steps prior to being released at the cell membrane (49). Although the precise post-Golgi trafficking mechanisms in macrophages are not known, the release route is likely to resemble that of TNF and IL-6 (9). Data from neutrophils localized the SNAREs VAMP2, VAMP7, Stx2, Stx6, and SNAP23 in the granules that contain and secrete IL-12 (50, 51). Although macrophages do not possess secretory granules, IL-12 release from these cells may involve some of the same SNARE complexes. Furthermore, IL-12 is secreted in a polarized manner from lymphocytes; this process is dependent on Cdc42 (52), which also regulates release of TNF to the plasma membrane. This raises the interesting prospect that IL-12 may be released in a polarized fashion, along with TNF (17), at nascent macrophage phagosomes.

IL-18

IL-18 is a member of the IL-1 family and also an inducer of IFN-γ production. It synergizes with IL-12 to activate T cells and NK cells. Albeit the fact that IL-18 signals similarly to IL-1β, IL-18 is not a pyrogen, and can even attenuate IL-1β-induced fever (53). Lack of fever induction may be explained by the fact that IL-18 signals through the MAPK p38 pathway instead of the NF-κB pathway, which is used by IL-1β (54). IL-18 trafficking is similar to that of IL-1β, with secretory autophagy also playing a major role in its release (35, 36).

IL-23

IL-23 is also an IFN-γ inducer and T cell activator that is involved in a variety of diseases ranging from psoriasis to schizophrenia (47). It is similar to IL-12 in that both induce inflammation. Moreover, both IL-12 and IL-23 share the IL-12p40 subunit and thus have similar signaling pathways. In contrast to IL-12, IL-23 augments IL-10 release and induces IL-17 synthesis by activated naïve T cells (55).

IL-27

IL-27 is a member of the IL-12 family, and is composed of subunits p28 and Epstein–Barr virus-induced gene 3. Similar to TNF, it is produced early in monocytes and macrophages stimulated with LPS and IFN-γ. Knockout of its receptor ensues in increased susceptibility of mice to bacterial and parasitic infections due to impaired IFN-γ production (56). In addition to favoring the differentiation of naïve T cells to Th1 cells via IFN-γ induction, IL-27 can also inhibit the differentiation of Th17 cells (57). IL-27 also has anti-inflammatory properties, which are exemplified by the fact that IL-27 receptor-deficient mice are more susceptible to auto-immune encephalomyelitis, which correlates with increased levels of Th17 cells (55). The fact that this cytokine has selective inflammatory and anti-inflammatory properties supports the concept that the inflammatory response is prompt, but also carefully calibrated to avoid damage to the host.

ANTI-INFLAMMATORY CYTOKINES

IL-10

Inflammation is tightly regulated by multiple inhibitors and antagonists. IL-10 is a 35 kD cytokine identified in 1989, and is produced by activated macrophages, B cells, and T cells (58). Its main

activities concern the suppression of macrophage activation and production of TNF, IL-1β, IL-6, IL-8, IL-12, and GM-CSF (59). IL-10 suppresses MHC-II expression in activated macrophages and is thus a potent inhibitor of antigen presentation (60). Of particular interest is that IL-10 inhibits the production of IFN-γ by Th1 and NK cells, and induces the growth, differentiation, and secretion of IgGs by B cells (61, 62). Macrophages themselves are affected by IL-10 in that exposure to this cytokine lowers their microbicidal activity and diminishes their capacity to respond to IFN-γ (63, 64). Experiments in murine models have shown that blocking or neutralizing IL-10 leads to increased levels of TNF and IL-6; on the contrary, exogenous IL-10 improves survival and reduces the levels of inflammatory cytokines (65). It has been observed that reduced levels of IL-10 favor the development of gastrointestinal pathologies such as inflammatory bowel disease (65). Recombinant IL-10 has indeed been effective in the treatment of some of these diseases.

The mechanism of IL-10 trafficking and release resembles that of TNF and IL-6 (66). IL-10 traffics from Golgi tubular carriers associated with p230/golgin-245 along with TNF-containing vesicles, or in golgin-97-associated tubules. The Golgi-associated p110δ isoform of PI3K was also found to be a positive regulator of IL-10 release. From the Golgi, IL-10-containing vesicles move to recycling endosomes, where VAMP3 and Rab11 then modulate the transit of this cytokine – and of TNF and IL-6 – to the cell surface. Independent of recycling endosomes, IL-10 was also observed to exit directly from the Golgi to the cell surface in apoE-labeled vesicles (66).

TGF-β

Together with IL-10, TGF-β is another powerful anti-inflammatory cytokine that acts on many target cells and tones down the inflammatory effects of TNF, IL-1β, IL-2, and IL-12, etc. (61, 67, 68). TGF-β is a potent suppressor of both Th1 and Th2 cells, but foments the maintenance and function of Tregs (67, 69). The importance of TGF-β in the immune system is highlighted by the fact that mice lacking the TGF-β1 isoform, which is predominant in cells of the immune system, develop severe multi-organ inflammation and die by week 4 (70). TGF-β is also implicated in hematopoiesis and has a crucial role in embryogenesis, tissue regeneration, and cell proliferation and differentiation.

Transforming growth factor beta is synthesized as a precursor and is directed to the ER by virtue of its signal peptide. Cleavage by the endoprotease furin, which can happen at the ER or in the extracellular environment, is required for activation of this cytokine (70). Although the secretory mechanism of this cytokine has not been explored, it is possible that it follows a post-Golgi pathway similar to that of TNF, IL-6, or IL-10.

CHEMOKINES

Chemokines are a special family of heparin-binding cytokines that are able to guide cellular migration in a process known as chemotaxis. Cells that are attracted by chemokines migrate toward the source of that chemokine. During immune surveillance, chemokines play a crucial role in guiding cells of the immune system to where they are needed (71). Some chemokines also play a role during development by promoting angiogenesis, or by guiding cells to tissues that provide critical signals for the cell's differentiation. In the inflammatory response, chemokines are released by a wide variety of cells involved in both innate and adaptive immunity (71). As already mentioned, chemokine release is often induced by proinflammatory cytokines such as TNF, IL-6, and IL-1β. Below is a description of the main chemokines released by macrophages.

CXCL1 and CXCL2 (MIP-2α)

CXCL1 and CXCL2 (also known as macrophage inflammatory protein 2-α, MIP) share 90% amino acid similarity and are secreted by monocytes and macrophages to recruit neutrophils and hematopoietic stem cells (72, 73). Both chemokines are angiogenic and may promote the development of tumors such as melanomas (74).

CCL5 (RANTES)

CCL5, or the regulated upon activation normal T cell expressed and secreted (RANTES), is an inflammatory chemoattractant for T cells, basophils, eosinophils, and dendritic cells to the site of inflammation (75). Aside from this role, it can also mediate the activation of NK cells into chemokine-activated killers (CHAK) (76). Similar to CXCL1 and 2, it promotes tumorigenesis and metastasis (74). CCL5 is synthesized in the ER and traffics to the Golgi complex before being exported outside of the cell. The secretory carrier membrane protein (SCAMP)5, a recycling endosome-associated protein, governs post-Golgi trafficking of CCL5 to the plasmalemma. Stimulation of macrophages with ionomycin induces SCAMP5 translocation to the plasma membrane, where it colocalizes and interacts with Syt I and II, which in turn mediate interactions with various SNAREs (77).

CXCL8 (IL-8)

CXCL8 is a potent chemoattractant for neutrophils, in which it also induces degranulation and morphological changes (78, 79). Since macrophages are some of the first cells to respond to an antigen, they are likely the first cells to release CXCL8. Other cells such as keratinocytes, endothelial cells, eosinophils, and basophils also respond to this chemokine. The importance of IL-8 has made this chemokine important in inflammatory diseases such as psoriasis, Crohn's disease, and cancer (80, 81).

CXCL9 (MIG)

CXCL9, also known as monokine induced by gamma interferon (MIG), is a strong T cell chemoattractant to the site of inflammation (71, 82). It mediates cell recruitment necessary for inflammation and repair of tissue damage. CXCL9 also inhibits neovascularization (83) and has anti-tumor and anti-metastatic effects (74).

CXCL10 (IP-10)

CXCL10, or interferon gamma-induced protein 10, is secreted not only by monocytes and macrophages, but also by fibroblasts and endothelial cells (83). It serves to attract T cells, NK cells, dendritic cells (84), and also has potent anti-cancer activity.

CXCL11 (IP-9)

Similar to CXCL9 and CXCL10, CXCL11 is interferon-inducible and also mediates T cell recruitment, although more potently than

CXCL9 and CXCL10 (85). It also inhibits angiogenesis and tumor formation (74).

ALTERNATIVELY ACTIVATED MACROPHAGES AND THEIR CYTOKINES

The microenvironment in which a macrophage is found provides it with diverse signals that divergently bias the macrophage's phenotype toward "classically activated" (M1) or "alternatively activated" (M2a, M2b, or M2c) (**Figure 1**) (55). Polarization signals may be apoptotic cells, hormones, immune complexes, or cytokines provided by lymphocytes or other cells. Exposure of naïve monocytes or recruited macrophages to the Th1 cytokine IFN-γ, TNF, or LPS, promotes M1 development. Those macrophages in turn secrete proinflammatory cytokines TNF, IL-1β, IL-6, IL-12, IL-23, and promote the development of Th1 lymphocytes. In addition, M1 macrophages secrete high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), produce and secrete iNOS, and promote the metabolism of arginine into nitric oxide and citrulline. As a result, M1 macrophages foster a highly microbicidal environment, and have a role in mediating the destruction of pathogens and tumor cells. M1-derived chemokines help recruit NK and Th1 cells. In stark contrast, exposure or treatment of monocytes with IL-4 and IL-13 polarizes these cells toward an M2a phenotype (8, 86). Those macrophages express a series of chemokines that promote the accrual of Th2 cells, eosinophils, and basophils. M2b macrophages are induced by a combination of LPS, immune complexes, apoptotic cells, and IL-1Ra. They secrete high levels of IL-10, but also proinflammatory cytokines TNF and IL-6 and express iNOS. Through chemokine production, M2b macrophages also promote recruitment of eosinophils and Tregs that foster a Th2 response. M2c macrophages are induced by a combination of IL-10, TGF-β, and glucocorticoids. In turn, those macrophages secrete IL-10 and TGF-β, both of which are immunosuppressive cytokines that promote the development of Th2 lymphocytes and Tregs. They also express high levels of arginase and promote tissue regeneration and angiogenesis (8, 87). The capacity of M2c macrophages to induce Tregs makes them more effective than M2a macrophages at protecting organs from injury caused by inflammatory infiltrates (88). Macrophage bias is reversible. For example, if an M1 macrophage is given apoptotic cells, it may transform into an M2 macrophage.

The characteristics of M1 and M2 macrophages have implicated them in the development of infectious disease and cancer. For example, helminth-derived molecules can strongly bias

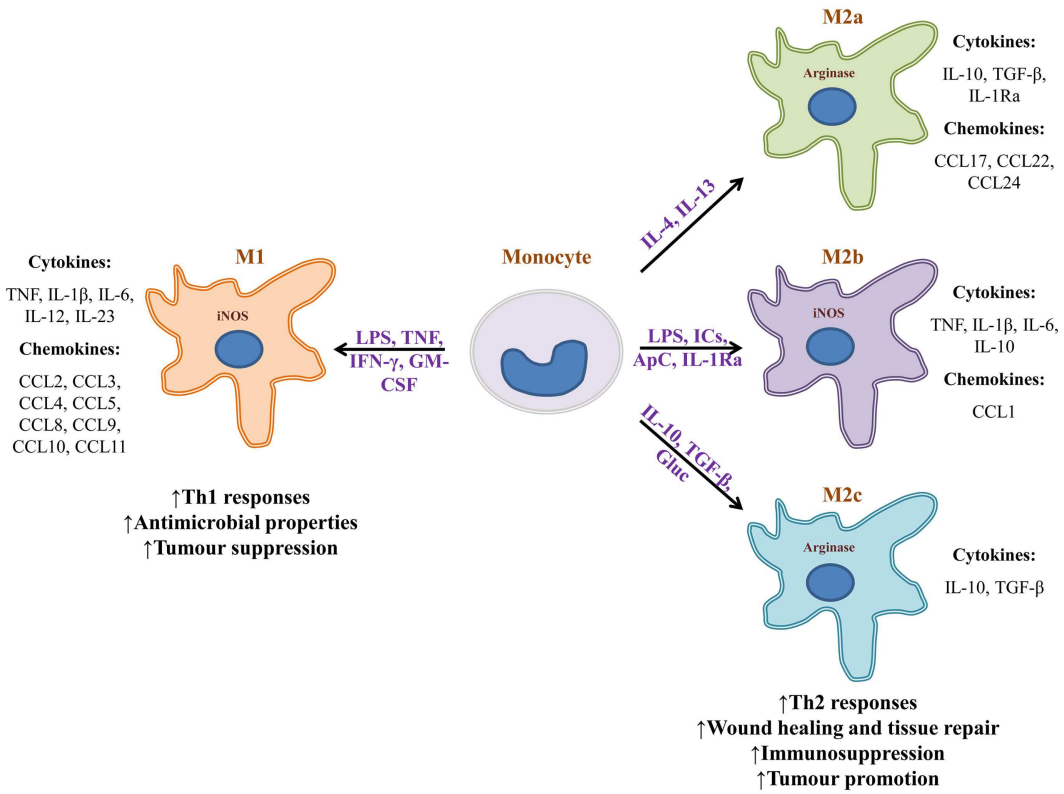

FIGURE 1 | Monocytes can become phenotypically distinct macrophages. Upon encountering different stimuli, monocytes turn into highly microbicidal (M1), or into immunosuppressive macrophages (M2). Stimuli can range from microbial substances to biochemical signals provided by the microenvironment of a given tissue. Many of the cytokines that bias macrophage phenotype are provided by surrounding lymphocytes or other non-immune cells. Macrophage subtypes release a vastly different array of cytokines and chemokines that can either promote inflammation and sometimes tissue destruction, or wound healing and tissue repair. M1 macrophages are known to be tumor suppressive whereas M2 macrophages generally promote tumorigenesis. It is important to note that macrophage bias is a spectrum and is reversible. IC, immune complexes; ApC, apoptotic cells; Gluc, glucocorticoids.

macrophages toward an M2 phenotype. The cytokines and associated Th2 response that ensues promote immunosuppression and parasite survival (89). In cancer, tumor-associated macrophages (TAMs) have been known to either promote or hinder neoplasia (8, 90). In colorectal cancer, TAMs are inflammatory and promote the development of a Th1 response (91). In contrast, many other neoplasms are associated with M2-like TAMs that secrete immunosuppressive cytokines that promote tumor growth and metastasis (8, 90). TAMs may aid tumor growth by facilitating the chemotaxis of Th2 and Treg cells, and by promoting angiogenesis and lymphoangiogenesis via production of VEGF, VEGF-C and -D, PDGF, and TGF-β (92). Additionally, TAMs secrete MMP9, a matrix metalloprotease that promotes tumor growth and spread. Importantly, TAMs induce immunosuppression via release of IL-10 and TGF-β, both of which inhibit the development of cytotoxic T cells and NK cells, and may fuel the appearance of more M2-like TAMs at the tumor site (8, 67, 90). The contribution of alternatively activated macrophages and their cytokines to disease has made them a target for immunotherapies that seek to alter the phenotypic bias of macrophage populations. For instance, helminth-derived molecules could be used to alter the proinflammatory cytokine profile of colitis-associated macrophages (89).

HOW DO PATHOGENS DISRUPT CYTOKINE SECRETION FROM MACROPHAGES?

The evolutionary race that has taken place over millions of years among pathogens and their hosts has given rise to a multitude of adaptations that have allowed these pathogens to resist the defenses mounted by their hosts. Several of these adaptations endow pathogens to evade the immune system in order to survive destruction and thrive. Both intracellular and extracellular parasites have evolved mechanisms to not only avoid or survive the immune response, but also to use it for their own benefit (93, 94). Upregulating or downregulating the production and release of macrophage cytokines can have profound effects on the immune response. A variety of pathogenicity factors target these important molecules of the immune system. The following examples describe how certain pathogens, depending on their needs, deregulate cytokine secretion to aid in their survival and dissemination.

MYCOBACTERIUM ULCERANS USES MYCOLACTONE TO INHIBIT CYTOKINE PRODUCTION

Mycobacteria are intracellular pathogens that cause a variety of human diseases that are difficult to treat. Due to their particular cell wall, these bacteria are very resistant to antibiotics and innate host defenses. *M. ulcerans*, the causative agent of the Buruli ulcer, induces deep necrotizing ulcers that are often ironically painless (95). Lesions can cause incapacitation, disfigurement, and severe deformities (95). The disease is the third-most common mycobacterial infection and affects areas of the world with hot and humid climates. *M. ulcerans* produces a macrolide toxin called mycolactone that is highly cytotoxic and immunosuppressive (96, 97). It causes broad tissue damage in the absence of an acute inflammatory response. Injection of mycolactone alone can induce lesions similar to those caused by infection (96). In contrast to other mycobacterial infections, *M. ulcerans* is found mostly extracellularly. This may be explained by the fact that mycolactone inhibits phagocytosis and hampers phagolysosomal maturation in macrophages (98, 99). In addition, mycolactone contributes to immunosuppression by hampering the production of several cytokines and chemokines from macrophages (**Figure 2A**) (99–101); mycolactone is effective at dampening the production of LPS-induced mediators. Although the mechanism for these findings was unknown, data from multiple studies suggested that inhibition was at the post-transcriptional level. Indeed, Hall et al. found that mycolactone does not cause gross changes in translation, with proinflammatory mRNAs being actively translated (102). That finding prompted the investigators to check whether TNF was being translocated to the ER for processing. Interestingly, inhibiting the 29S proteasome showed that non-glycosylated TNF accumulates in the cytoplasm of mycolactone-treated macrophages, indicating that this causes the failure in TNF secretion. To show that TNF was not being translocated into the ER lumen, Hall et al. performed *in vitro* translation assays with ER-containing membranes to study whether TNF was being protected from proteinase K degradation (102). In the presence of mycolactone, TNF is not protected from proteinase K digestion, indicating that this cytokine does not translocate into the ER under these conditions. These effects were found not to be due to mycolactone disrupting ER membrane integrity or due to induction of ER-associated degradation pathways. It would be interesting to investigate whether mycolactone can physically block the channel activity of the Sec61, or that of other ER translocons. These findings were made more general by showing that – in many cell types – mycolactone was inhibiting the translocation of several secreted and membrane proteins into the ER. Importantly, mycolactone blocked the release of several cytokines, chemokines, and other inflammatory mediators from LPS-activated macrophages (102). Quenching cytokine production in this way can thus severely obstruct the development of the immune response and promote the survival of *M. ulcerans*.

LEISHMANIA PROMASTIGOTES EMPLOY GP63 TO AUGMENT TNF AND IL-6 RELEASE

Protozoa of the *Leishmania* genus are parasites of phagocytic cells, especially macrophages. Depending on the species, *Leishmania* can cause self-healing cutaneous lesions (e.g., *L. tropica*, *L. major*, *L. mexicana*, and *L. pifanoi*), disfiguring mucocutaneous disease (e.g., *L. braziliensis* and *L. guyanensis*), or severe visceral illness (e.g., *L. donovani* and *L. Infantum chagasi*). Mucocutaneous and visceral disease can be lethal if untreated, but most deaths are attributable to visceral leishmaniasis (103). *Leishmania* has a digenetic lifecycle. Promastigotes are elongated and have a flagellum that allows them to move in extracellular environments. Dividing procyclic promastigotes develop in the gut of infected sandflies, where they transform into infectious non-dividing metacyclic promastigotes that can be ejected upon the sandfly's next blood meal (104). Once inside the host, metacyclic promastigotes are phagocytosed by neutrophils or by macrophages. *Leishmania* promastigotes are able to cripple the microbicidal power of the phagosome, rendering it a propitious parasitophorous vacuole (PV) for the parasite (105, 106). Within PVs, promastigotes differentiate into amastigotes, which are the non-flagellated intracellular

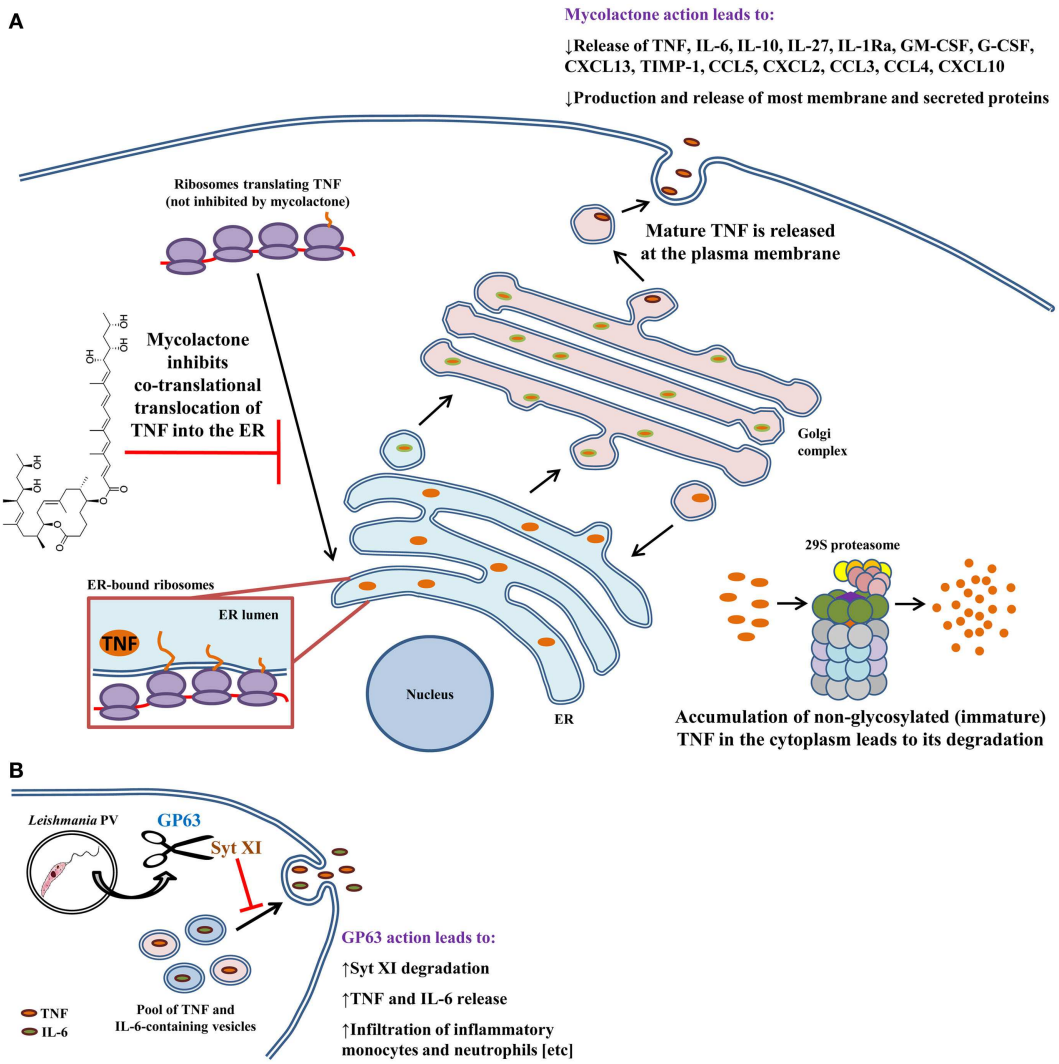

FIGURE 2 | Modulation of macrophage cytokine secretion by *Mycobacterium ulcerans* bacteria and *Leishmania* promastigotes. Disruption of cytokine release has evolved as an effective means by which several pathogens contravene the immune response. **(A)** *M. ulcerans* employs mycolactone to sabotage the immune response by inhibiting the secretion of more than 17 cytokines, chemokines, and inflammatory mediators. TNF, as well as other cytokines and chemokines, undergo post-translational modifications in the ER and Golgi prior to being shepherded outside of the macrophage. Mycolactone hampers delivery of TNF into the ER. As a consequence, immature protein that accumulates in the cytoplasm is eventually degraded by the proteasome. **(B)** Unlike *M. ulcerans*, *Leishmania* promastigotes trigger the release of TNF and IL-6 from infected macrophages via GP63-mediated degradation of Syt XI (a negative regulator of cytokine release). *In vivo*, GP63 also facilitates the infiltration of inflammatory monocytes and neutrophils to the infection site. Both of these phagocytes are infection targets for *Leishmania* and aid in establishing infection. These findings can be explained by the fact that TNF and IL-6 mediate phagocyte infiltration by upregulating the expression of adhesion molecules and chemokines. Arrows indicate multiple steps and drawings are not to scale.

form of the parasite. Amastigotes replicate inside macrophages, and when these apoptose, surrounding macrophages uptake the amastigote cargo (107), eventually propagating the infection. The *Leishmania* lifecycle is perpetuated when free amastigotes and amastigote-containing phagocytes are taken up by sand-flies that bite infected hosts. The GP63 zinc metalloprotease is a multifaceted *Leishmania* pathogenicity factor and is also one of the most abundant molecules on the surface of promastigotes (105, 108, 109). In infected macrophages, GP63 impairs antigen cross-presentation (110), stalls transcription and translation, and deactivates several microbicidal pathways (111–114). Additionally, GP63 hampers lipid metabolism in liver, and helps the parasite evade complement-mediated lysis and avoid killing by NK cells (108, 115, 116). Of particular note is the capacity of GP63 to cleave members of the SNARE complex (105), which raises the possibility that GP63 may cleave other membrane fusion regulators. Earlier studies found that *Leishmania* promastigotes of certain species were able to induce the release of TNF and IL-6 (117–121) following their engulfment by macrophages. However, the mechanisms for this induction were not known. Hence, Arango

Duque et al. hypothesized that Syt XI, a negative regulator of cytokine secretion (11), was targeted by *Leishmania* (**Figure 2B**) (22). Infection of macrophages with $GP63^{+/+}$ or $GP63^{-/-}$ parasites revealed that Syt XI is degraded by GP63, leading to the release of TNF and IL-6. Moreover, cytokine release by infected macrophages positively correlated with the GP63 content of different *Leishmania* species. To highlight the relevance of these findings in an *in vivo* setting, it was demonstrated that intraperitoneal injection of GP63-expressing promastigotes induces TNF and IL-6 release 4 h after inoculation. As already described, these cytokines induce adhesion factor expression and chemokine release (14, 15, 42, 122, 123). Interestingly, it was observed that GP63 also promotes the infiltration of neutrophils and inflammatory monocytes early during infection. Future research will reveal whether phagocyte recruitment is dependent on GP63-mediated cleavage of Syt XI *in vivo*. It will also be interesting to research whether Syt XI is targeted by other pathogens. The involvement of GP63 in cytokine secretion and phagocyte recruitment can aid in the establishment of infection. Infection of recruited inflammatory monocytes and resident macrophages can induce IL-10 secretion, which fosters the immunosuppressive environment observed in chronic infection (124, 125). Infection of inflammatory monocytes (126) may also turn them into arginase-expressing alternatively activated macrophages that trigger the differentiation of naïve CD4 T cells into $FoxP3^{+}$ cells (86), which are immunosuppressive in leishmaniasis (127, 128). Overall, those findings underline the importance of proinflammatory cytokines and phagocytes at the early stages of *Leishmania* infection (22).

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Cytokine secretion in macrophages: SNAREs, Rabs, and membrane trafficking

Rachael Zoe Murray [1]*** and Jennifer Lea Stow*** [2]***

[1] Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, QLD, Australia
[2] Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD, Australia

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Robert Adam Harris, Karolinska Institutet, Sweden
Martin Herrmann, Universitätsklinikum Erlangen, Germany

***Correspondence:**
Rachael Zoe Murray, Institute of Health and Biomedical Innovation, School of Biomedical Sciences, Queensland University of Technology, 60 Musk Avenue, Brisbane, QLD 4059, Australia
e-mail: rachael.murray@qut.edu.au;
Jennifer Lea Stow, Institute for Molecular Bioscience, University of Queensland, Building 80, 306 Carmody Road, Brisbane, QLD 4072, Australia
e-mail: j.stow@imb.uq.edu.au

Macrophages have the capacity to rapidly secrete a wide range of inflammatory mediators that influence the development and extent of an inflammatory response. Newly synthesized and/or preformed stored cytokines and other inflammatory mediators are released upon stimulation, the timing, and volume of which is highly regulated. To finely tune this process, secretion is regulated at many levels; at the level of transcription and translation and post-translationally at the endoplasmic reticulum (ER), Golgi, and at or near the cell surface. Here, we discuss recent advances in deciphering these cytokine pathways in macrophages, focusing on recent discoveries regarding the cellular machinery and mechanisms implicated in the synthesis, trafficking, and secretion of cytokines. The specific roles of trafficking machinery including chaperones, GTPases, cytoskeletal proteins, and SNARE membrane fusion proteins will be discussed.

Keywords: cytokine secretion, macrophages, SNAREs, Rabs, TNF, IL-6, IL-10

INTRODUCTION

Macrophages activated by contact with pathogens or danger signals release cytokines and chemokines as a major component of the innate immune response (1). Inflammatory cytokines recruit other immune cells and orchestrate the actions and fates of the cells secreting them and those in the surrounding milieu. Macrophages are one of the most abundant sources of inflammatory cytokines, which normally act in a protective manner; however, these same cytokines underlie many acute and chronic inflammatory diseases. Increasingly, it emerges that macrophages secrete cytokines inappropriately in an ever broadening array of chronic conditions, from diabetes and obesity to vascular and neurodegenerative diseases (2–4). Many of the newer therapeutics target cytokines directly, their modes of release, or receptor engagement.

Macrophage cytokines are synthesized and released in response to activation of pattern recognition receptors or inflammasomes, and there is an abundant literature documenting the many factors, signaling cascades, and transcriptional machinery that lead to cytokine synthesis (5–7). The subsequent organelles, secretory pathways, and cellular machinery that transport cytokines through the cell are also critical for ensuring timing and site of release for new cytokines. These pathways and their regulators will be the subject of this review. In particular we focus on two large families of trafficking regulators that guide cytokine secretion through multiple intracellular steps, the Rab small GTPases, and the SNAREs (Soluble NSF Attachment Protein Receptors).

CYTOKINE TRAFFICKING PATHWAYS

Cytokine secretion pathways are often adapted to suit specific cytokines, their function, and cell type. Many immune cells stockpile cytokines in distinct compartments – namely, secretory granules or lysosome-related organelles (LROs) – that enable rapid release of the cytokines upon cell activation (8, 9). Macrophages, however, lack these granules, and instead cytokines must be synthesized after cell activation and secreted via the constitutive (or continuous) secretory pathway or via non-conventional secretion. The majority of cytokines in macrophages are processed and transported through the constitutive pathway (10–13) (**Figure 1**). To accommodate the need to transport and release large volumes of cytokines in the first hours after cell activation, the cellular machinery and carriers involved in the constitutive pathway are upregulated (11, 12, 14–16) (**Table 1**). Much of our knowledge of this pathway comes from work in other cell types, such as epithelial cells, in addition to studies in macrophages themselves. Multiple cell types have in common routing of newly synthesized proteins from the endoplasmic reticulum (ER) to the Golgi complex, sorting in the trans-Golgi network TGN and then for some cargo, transport directly to the cell surface or transit via recycling endosomes (17, 18). Much less is known about the non-classical secretory pathways for cytokine secretion. IL-β and IL-18 are cytokines secreted from the cytoplasm by non-classical pathways, although the exact route of release is contentious and might, in some cases, be cell type specific.

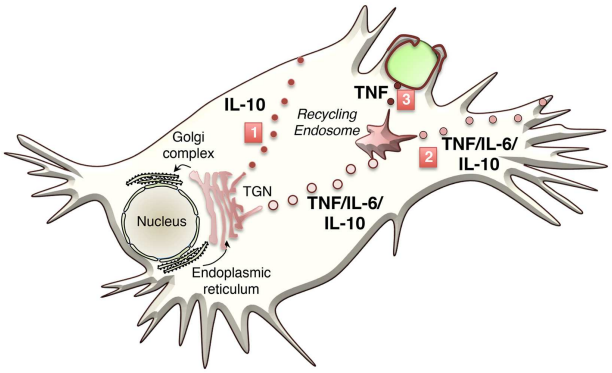

FIGURE 1 | Classical transport pathways used to secrete macrophage cytokines. Three major transport pathways for cytokine secretion have been identified to date. The first involves direct transport to the cell surface (IL-10) from the TGN, the second pathways routes cytokines via the recycling endosome and the out to the cell surface (TNF, IL-6, and IL-10), and the 3rd pathway occurs during phagocytosis where cytokine (TNF) is routed from the recycling endosome to the phagocytic cup.

Table 1 | Proteins and lipids altered by LPS stimulation.

Organelle	Protein/lipid	Proposed function	Reference
ER	iRhom2	Promotes TACE exit from the ER	(19)
TGN	PtdChol	Lipid raft/vesicle biogenesis	(20)
TGN	CCTα	Catalyzing PtdChol biosynthesis	(20)
TGN	p230	Sorting at the TGN	(14)
TGN	Rab6	Recruitment/stabilization of p230 on TGN membranes	(15)
TGN	Stx6	Fusion of TGN-derived vesicles with recycling endosome	(12)
TGN	Stx7	Fusion of TGN-derived vesicles with recycling endosome	(12)
TGN	Vti1b	Fusion of TGN-derived vesicles with recycling endosome	(12)
TGN	SCAMP5	Forms a complex with Stx6 and potentially regulates fusion with RE	(21)
RE	VAMP3	Fusion of TGN-derived vesicle with RE and the RE with PM	(11)
RE	Rab11	Fusion of RE with PM	(11)
RE	Rab37	Fusion of RE with PM	(16)
PM	Stx4	Fusion of RE with PM	(22)
PM	SNAP23	Fusion of RE with PM	(22)
PM	SCAMP5	Forms a complex with Stx4/SNAP23 to regulate exocytosis	(21)

ER, endoplasmic reticulum; TGN, trans-Golgi network; RE, recycling endosome; PM, plasma membrane.

CONSTITUTIVE SECRETION OF CYTOKINES

Cytokines such as IL-2, 3, 6, 10, 12 and TNF contain a signal peptide targeting them to the ER, where once correctly folded, they are packaged into coat protein complex II (COPII) coated vesicles and delivered to the ER–Golgi intermediate compartment (ERGIC) and the *cis* side of the Golgi complex. From here, they are transported through the medial-Golgi to the TGN, a tubular network that emanates from the stacked reticulated ribbon-like structured Golgi cisternae. In addition to the final modifications, proteins might receive in the TGN, this compartment acts as one of the major organizing centers to sort cargo to different post-Golgi carriers and routes (17). This includes the sorting of transmembrane cytokines, such as TNF, and less obvious sorting – but certainly packaging – of soluble cytokines such as IL-6 and IL-10. Other cargo is also segregated at the TGN, including, glycosyl phosphatidyl inositol (GPI)-anchored proteins and lysosomal enzymes with mannose-6-phosphate tags.

Newly synthesized proteins, recycling, and endosomally derived proteins all converge at the TGN as cargo requiring packaging into carriers for post-Golgi transport. Several types of pleomorphic membrane-bound carriers, which can appear as tubules or vesicles and often do not have definable coats, exist within the constitutive secretory pathway. Carrier loading, formation, and transport from the TGN occur in distinct steps. Firstly, cargo must be segregated from resident Golgi proteins and sorted into budding carriers. At exit points on the TGN membrane, a complex array of machinery governs membrane curvature and fission of budding vesicles or tubules, which then use motors to attach to microtubules for transport. Imaging in live macrophages shows that cytokines at the TGN are loaded into tubular or pleomorphic vesicular carriers and the number of carriers forming can be enhanced to accommodate the increased flow of cargo, including cytokines, in activated cells (14, 15).

For many cytokines, the TGN sorting mechanisms are unknown or not well defined. In general, the sorting of transmembrane proteins occurs via adaptor recognition of sorting motifs in their cytoplasmic tails or by clustering of cargo proteins in distinct membrane domains such as lipid rafts. ARF and Rab GTPase family members, adaptors, golgins, and lipids, such as phosphoinositides, sphingolipids, and cholesterol, all contribute to cargo sorting and carrier loading at the TGN (17) (**Table 2**). How soluble cargo, including soluble cytokines such as IL-6 or IL-10, is sorted is still not clear. The transmembrane cytokine TNF is generally sorted into specific carriers, while soluble cytokines often appear in more than one carrier type and exit via more than one pathway. The latter is suggestive of less stringent or perhaps even no effective sorting, of soluble cytokines. While the transmembrane protein TNF is delivered to highly specific sites on the cell surface, the surface delivery sites for release of soluble cytokines may be less regulated. Tubular or vesicular carriers move along microtubule tracks to their destinations. In addition to their cargo, carriers must also be loaded with the necessary machinery for their docking and fusion at the target membrane – steps that frequently involve specific members of the Rab family of small GTPases and of the SNARE family of membrane fusion proteins (**Figure 2**).

Table 2 | Trafficking machinery that regulate cytokine secretion in macrophages.

Protein	TNF	IL-6	IL-10	Reference
iRhom2	+	NA	NA	(19)
p230	+	+	+	(14)
Golgin-97	–	–	+	(13)
CCTα	+	+	ND	(20)
PI3K p110δ	+	ND	–	(23)
Dynamin 2	+	ND	ND	(23)
Stx4	+	ND	+	(22)
Stx6	+	+	+	(12)
Vti1b	+	+	+	(12)
VAMP3	+	+	+	(11)
SNAP23	+	ND	+	(22)
SCAMP5	+	+	ND	(21)
Rab6	+	ND	ND	(15)
Rab11	+	ND	+	(11)
Rab37	+	ND	ND	(16)
Munc13-1	+	ND	ND	(16)
AP-1	+	ND	ND	(24)
Syt XI	+	+	ND	(25)
PKA	ND	ND	+	(13)
Rac1	+	ND	ND	(26)
CDC42	+	ND	ND	(26)

NA, not applicable; ND, not determined.

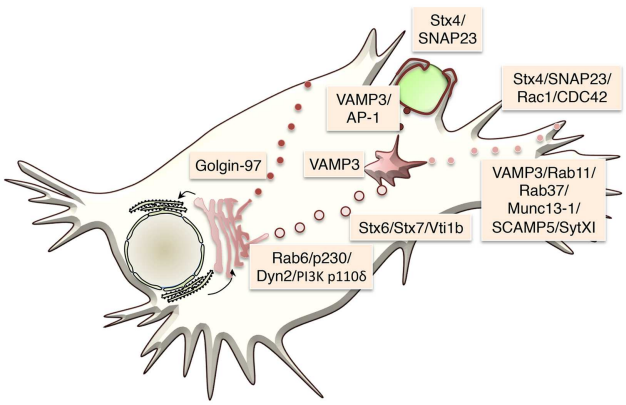

FIGURE 2 | Trafficking machinery regulating specific cytokine transport steps in macrophages is shown. The transport machinery that regulates the three major transport steps is shown.

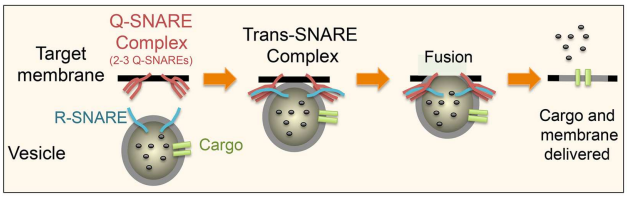

FIGURE 3 | Schematic showing the basic steps in SNARE-mediated secretion is shown. An R-SNARE on the donor membrane comes together with a Q-SNARE complex, consisting of two to three Q-SNAREs, on the target membrane to form a trans-SNARE complex. This brings the two membranes into close proximity leading to the formation of a fusion pore allowing the release of some or all of the contents of the vesicle. The pore can either close ("kiss and run") or as depicted above the membrane can fully fuse thus incorporating all of its membrane-associated proteins into the target membrane. This basic process is same in all cell types and at different stages of the transport pathways. What differs is the precise SNARE partners at these distinct stages.

MEMBRANE FUSION AND MAPPING PATHWAYS WITH SNAREs AND Rabs

Members of the SNARE family are in general ubiquitously expressed, with the exception of two neuronal specific SNAREs, Stx1, and SNAP25, and the immune specific SNARE Stx11 (27, 28). Each SNARE operates in one or more defined membrane locations, which can differ depending on cell type. They form the minimal machinery required for membrane fusion and loss of any one component of a specific *trans*-SNARE complex inhibits fusion at distinct sites in the cells (29, 30). For example, deletion or mutation of VAMP3, a recycling endosome SNARE, will inhibit secretion of pathways that require this SNARE for delivery of recycling endosome membrane to the cell surface (11, 31, 32). Thus, SNAREs have been employed to map a number of cytokine secretion pathways in macrophages and many other cell types.

SNARE proteins are mostly transmembrane proteins, with only a few of them existing as peripheral membrane proteins, anchored to membrane lipids by post-translational modifications and/or through protein–protein interactions. Membrane anchored heterologous SNAREs from opposing membranes form a tight four-helix bundle, where each helix is contributed by one of four SNARE motifs, which act to pull the two membranes into close proximity (30, 33) (**Figure 3**). The energy released when the membrane-bridging (trans-) SNARE complex forms initiates the formation of a fusion pore between the two opposing membranes. Cargo (or a portion of it) is discharge through this opening, which might subsequently close in a process known as "kiss and run exocytosis," or alternatively, the whole vesicle can fully fuse, merging transmembrane proteins and lipids into the target membrane and fully releasing its lumenal contents (34, 35). In the latter case, membrane and resident proteins – importantly including incoming SNAREs – are then retrieved by endocytosis, which in the case of macrophages is especially important as large quantities of membrane are turned over at cell surface during processes such as macropinocytosis and phagocytosis.

SNARE subtypes have been described based on their central amino acid, a glutamine (Q-SNARE) or an arginine (R-SNARE). Based on the position of their SNARE motif in the trans-SNARE four helix bundle, Q-SNAREs are further partitioned into one of four subgroups: Qa, Qb, or Qc or, in the case of SNAREs like SNAP23 that contribute two SNARE motifs, Qb,c (36, 37). Thus, a trans-SNARE complex consists of one R-SNARE and Q-SNARE complex, comprising two to three Q-SNAREs (Qa, Qb, and Qc or Qa and Qb,c), which are typically found on opposing membranes. The specificity of SNARE pairing to form individual complexes is in part dictated by the locations of family members.

After fusion, the SNARE complex then resides in the donor membrane and its subunits must be rapidly disassembled by the ATPase NSF with its cofactor SNAP, and recycled to their original sites for further use (38).

Over 60 mammalian members of the small GTPase family of Rabs help to regulate trafficking at multiple steps through trafficking pathways [reviewed in Ref. (39, 40)]. Like other GTPases, Rabs switch between an inactive GDP-bound form (in the cytoplasm) and an active GTP-bound form (on membranes). The Rab cycle and nucleotide switching is aided by a host of accessory proteins. Rabs have varied roles in trafficking through effectors that can include phosphoinositide-modifying enzymes, budding machinery, molecular motors, tethering factors, and SNAREs (41–43). Through tethering effectors, Rabs orchestrate the docking and tethering of vesicles prior to SNARE-mediated fusion on sequential organelles in transport pathways. Experimentally, Rabs can be assigned to transport steps through the actions of GDP- and GTP-bound functional mutants, through deletion or genetic mutation of Rab genes, including those associated with human disease. Macrophages have a rich array of Rabs and many of their immune functions include regulation by Rab cascades. Notably also, key Rabs are targeted by bacterial effectors of intracellular pathogens.

Molecular regulators, like the Rabs and SNAREs that are found throughout the different trafficking pathways, offer many potential targets for therapeutic intervention, although inflammation has not yet directly benefited from these approaches (44). SNAREs are notably inactivated by botulinum and tetanus toxins and the therapeutic potential for this approach in wide ranging conditions is actively pursued (45). Rab proteins are also considered as attractive therapeutic targets, including via prenylation inhibitors (46). Future studies will no doubt increasingly explore some of these solutions for controlling cytokine secretion.

TNF SECRETION

SORTING AND TRANSPORT OF TNF

TNF mRNA is constitutively expressed and controlled by mRNA stability and translation. Activation leads to its rapid transcription, generating a type II membrane protein that can be found in the macrophage Golgi complex soon after LPS-activation (47). TNF is then packaged exclusively into a population of TGN-derived tubules/vesicles labeled with the GRIP-golgin p230/golgin245 (14). The GRIP-domain golgins are located on distinct TGN domains and regulate trafficking to and from the TGN. Four mammalian GRIP-golgins exist on the TGN, namely p230, CCC88, GCC185, and golin-97, and all share a TGN targeting GRIP domain in their c-terminus (48). What is notable is that in the absence of LPS, similar numbers of p230 and golgin-97 labeled tubules/carriers exit the TGN but upon activation with LPS the p230-labeled tubules are selectively upregulated threefold to enhance cytokine transport (14).

The p230-labaled TNF carriers are also regulated by Rab6 and Rab6a′, which act to stabilize Arl1-recruited p230 on TGN membranes (15). The Rab6 isoforms are quintessential Golgi-associated Rabs and both participate in multiple steps of trafficking through several effectors. Myosin IIA is a Rab6 effector on the p230-labeled tubules that move TNF and other cargo from the TGN to recycling endosomes (49). Rab6, like other Rabs involved in the macrophage constitutive pathway, is upregulated by LPS, offering more transport capacity in activated cells.

Lipids and phospholipids at the TGN are also regulated for trafficking. Despite the preponderance of PI4P in Golgi membranes, a number of PI3 kinases regulate budding and vesicle exit from the TGN (50). Among them, PI3Kδ is found on TGN membranes, where it and dynamin II, regulate TNF trafficking and TGN exit (23). The GTPase dynamin II functions in fission of clathrin-coated and non-clathrin-coated vesicles and carriers at the Golgi and on other membranes (51). A number of lipid metabolic genes are also upregulated in response to LPS, including choline cytidylyltransferase alpha (CCTα), phospholipase D1 (PLD1), and the choline/ethanolamine phosphotransferase C/EPT (20). Lipids play an active role in the biogenesis of carriers and in organizing TGN exit domains (20). Thus, localized changes in lipid composition can greatly alter secretion by recruiting specific proteins and promoting membrane fission or fusion. CCTα, a key enzyme catalyzing PtdChol biosynthesis in macrophages, regulates vesicle formation and budding (20). Its inactivation reduces PtdChol synthesis leading to decreased TNF secretion suggesting that PtdChol and CCTα are also critical for transport from the Golgi complex (20). Thus, the upregulation of lipid machinery also enhances the export of TNF from the TGN.

TGN carriers transport TNF and other cargo to recycling endosomes as a second transit and sorting station. This transport is well-defined through identification of the relevant SNAREs (11, 12). The Q-SNARE complex Stx6/Stx7/Vti1b is packaged with TNF into TGN-derived vesicles and upon reaching the recycling endosome, this Q-SNARE complex pairs with the resident R-SNARE VAMP3 for fusion (11, 12). The members of this SNARE complex are upregulated and packaged into the TGN-derived vesicles in response to LPS to accommodate the increased number of p230 requiring fusion with the recycling endosome (11, 12). On-going transport to the cell surface requires the recycling endosomes, or tubular extensions of this compartment bearing VAMP3, to fuse with the plasma membrane, via the cell surface Q-SNARE complex of Stx4/SNAP23 (11, 22). This step of the constitutive pathway, like earlier stages, is also enhanced in activated macrophages by upregulation and increased levels of the cell surface SNAREs (11, 22). Loss or inactivation of any of these SNARE components blocks TNF secretion.

The location of the surface SNARE complex plays a key role where TNF secreted. Surface SNAREs, such as Stx4 are clustered in cholesterol-rich lipid rafts, which adorn filipodia and phagocytic cups on the surface of activated macrophages (52). Disruption of these lipid rafts reduces TNF secretion (52). Macrophages use their filopodia to explore their environment and capture pathogens; this binding leads to filopodia retraction and pseudopod formation from the underlying lamellipodia to form the phagocytic cup (53). Stx4 translocates and concentrates on these lipid rich pseudopods and regulates the focal fusion of the recycling endosome with the phagocytic cup (11, 52). This serves two purposes; providing extra surface membrane necessary for macrophages to engulf microbes while simultaneously delivering TNF to the pseudopod tips for rapid secretion (11, 52). Stx4 is then removed, and presumably recycled to the cell surface, after the phagosome is internalized (11, 52).

Two Rab GTPases, Rab11 and Rab37, have been found to regulate surface delivery of TNF (11, 16) and these Rabs are also upregulated after LPS activation of cells. Rab11 is a well-known marker of recycling endosomes, where it mediates transport to the cell surface and similarly participates in the delivery of TNF through recycling endosomes to the plasma membrane. Rab37 is also located on the TNF-loaded vesicles that fuse with the plasma membrane. Intriguingly, in macrophages Rab37 associates with Munc13-1, a diacylglycerol, calcium, and calmodulin activated SM family protein, known to be required for synaptic vesicle priming and for insulin release in pancreatic beta cells (16). Loss of Munc13-1 or Rab37 reduced TNF secretion (16).

Adaptor proteins regulate sorting of membrane proteins and the AP-I complex has been implicated in both phagocytosis and the trafficking of TNF from the recycling endosome (24). Typically, AP complexes recruit the coat protein clathrin to vesicles for their formation, but this is not the case during phagocytosis where AP-1 decorated endosomes amass below the phagocytic cup in an ARF-1-dependent, clathrin-independent manner (24). Depletion experiments suggest that AP-1 might act to sort TNF at the recycling endosome and a cleaved form of AP-1 accompanies carriers that bud off the recycling endosome to the surface for VAMP3- and ARF6-dependent delivery at the tips of pseudopodia during phagocytosis (54, 55).

The protein trafficking machinery described above largely regulates discrete stages of TNF secretion, but this may not be the case for all trafficking machinery. Secretory carrier membrane proteins (SCAMPs) are found in complexes with SNARE proteins and regulate exocytosis (21). In macrophages, SCAMP5 expression is upregulated by Toll-like receptors (TLR) agonists like LPS and by increases in intracellular calcium through stimulation with ionomycin (21). Both stimuli result in SCAMP5-dependent TNF secretion (21). SCAMP5 has the potential to regulate SNAREs at multiple points in the TNF secretory pathway and can form complexes with many of the SNAREs (Stx4, Stx6, SNAP23, and VAMP3) identified at the different stages of TNF secretion (21). In the absence of stimulus, SCAMP5 localizes predominantly to the Golgi complex along with Stx6 and after stimulation with ionomycin shifts to the recycling endosome and cell surface, where it colocates with Stx4/SNAP23 (21). This shift in localization occurs in parallel to TNF secretion (21). Although SCAMP5 forms a complex with SNARE proteins, this binding is not direct and occurs through the calcium binding transmembrane synaptotagmins (Syt) (21). Loss of the synaptotagmin-binding site in SCAMP5 inhibits ionomycin induced TNF secretion (21). SCAMP5 binds at least two synaptotagmins, Syt1 and Syt II, but their role in TNF has yet to be established.

However, other synpatotagmins play a role, or have been implicated in, TNF secretion. Syt V is located on recycling endosomes and filopodia-like structures and is recruited to the nascent phagosome (56). It has a role in the recruitment of recycling endosomes to the phagocytic cup, and its loss impairs phagocytosis suggesting that it might also positively regulate TNF secretion (56).

While Syt V's role in TNF secretion has not been tested directly, another synaptotagmin, Syt XI, located on recycling endosomes and recruited to phagosomes, has been found to inhibit TNF secretion. Its role in inhibiting cytokine secretion suggests that members of this family of proteins might play an important role in regulating different aspects of cytokine secretion (25). Syt XI is one of the few family members that does not bind calcium and unlike most family members, it inhibits vesicle fusion by an unknown mechanism but one that could be focused on inhibiting SNARE function. Syt XI also inhibits IL-6 secretion, whose trafficking diverges from TNF at the level of the recycling endosome, suggesting that Syt XI might function prior to surface delivery. Taken together, protein families, like SCAMPs and synaptotagmins, functioning alongside SNAREs, also clearly participate in cytokine release at or near surface domains.

Rho GTPases act as molecular switches, that cycle between GTP-bound inactive and GDP-bound inactive states, to regulate remodeling of the actin cytoskeleton in cellular processes such as adhesion, migration, and membrane trafficking. Rac1 and CDC42 regulate the cell surface leading to the formation of lamellipodia and smaller filapodia, respectively, and also play a role in regulating organelle movement (57, 58). In LPS stimulated macrophages, these two Rho GTPases are essential for efficient surface delivery of TNF (26). In the absence of Rac1 function in particular, TNF is synthesized and is held up in recycling endosomes along with other endosomal cargo such as transferrin (26). These results suggest that Rac1 is involved in the final stages of TNF transport to the cell surface, possibly through the positioning or surface delivery of TNF-loaded recycling endosomes.

TACE-MEDIATED RELEASE OF SOLUBLE TNF

Once incorporated into the cell surface, membrane anchored TNF is cleaved by the ADAM family metalloproteinase TNF-converting enzyme (ADAM17 or TACE) at a site after alanine 76, to release the soluble active TNF ectodomain. Both transmembrane proteins, TACE and TNF (proprotein) can be organized by lipid raft domains on the cell surface (52). In different cell types and contexts, TACE can be found sequestered in lipid rafts or in non-raft fractions. In macrophages, TACE appears primarily in non-raft fractions, whereas TNF is delivered initially to lipid rafts in the plasma membrane where the Stx4 Q-SNARE complex is enriched for membrane fusion (52, 59). TNF is delivered to nascent phagocytic cups, where TACE is also enriched (11, 52). One possible level of regulation for cytokine release is the movement of TNF out of lipid rafts to access TACE for cleavage.

The level of TACE at the cell surface is also modulated by LPS cell activation and by intracellular trafficking machinery (19). Recently, the intramembrane protease, iRhom2, has been identified as a key regulator of TACE. Although catalytically inactive, iRhom2 is a member of the rhomboid family of intramembrane serine proteases. At the ER, TACE is bound by iRhom2, which promotes its exit from the ER and loss of iRhom2 leads to a build up of TACE in the ER (19). Both the pro and mature forms of TACE bind to iRhom2 suggesting that it remains associated through multiple stages of the secretory pathway (60). Loss or inactivating mutations of iRhom2 in mice reduce the levels of TNF secreted in response to LPS challenge (61). These results together suggest that iRhom2 acts as a cargo receptor or chaperone to aid in the trafficking of TACE from the ER to the Golgi where furin activates TACE by cleaving its N-terminal inhibitory domain (19). Like many proteins that regulate TNF secretion, LPS stimulation upregulates iRhom2 levels,

permitting increased TACE transport to the cell surface for TNF cleavage and shedding (19).

INTERLEUKIN 6 SECRETION

Interleukin 6 (IL-6) is another proinflammatory cytokine and an example of soluble cargo transported through the constitutive secretory pathway. At the level of the TGN, some IL-6 can be segregated away from TNF into distinct carriers (10). The rest appears together with TNF in post-Golgi carriers and in recycling endosomes (10). Like TNF, IL-6 transport out of the TGN relies on PtdChol and CCTα (20). The R-SNARE VAMP3 on the recycling endosome regulates the delivery of Q-SNARE Stx6/Vti1b/Stx7 labeled vesicles containing IL-6 (10).

At the recycling, endosome IL-6 and TNF are segregated into distinct domains and transported to the plasma membrane through different routes (10). Exactly how they are sorted in the recycling endosome is unknown, but it maybe that TNF is actively sorted away from IL-6 or alternatively both are individually sorted. Not surprisingly, a number of trafficking machinery proteins that regulate TNF at the level of the recycling endosome, including SCAMP5 and Syt XI also regulate IL-6 secretion (21, 25). While both TNF and IL-6 are delivered to the cell surface, the transport route for IL-6 from the recycling endosome is multidirectional even during phagocytosis when TNF is specifically delivered to the tips of nascent phagosomes for secretion (10).

INTERLEUKIN-10 SECRETION

Interleukin-10 (IL-10) is also a soluble cytokine targeted to the lumen of the ER by a signal peptide. In RAW cells, IL-10 secretion begins later than that of TNF and IL-6 and persists for longer (13). Importantly, IL-10 is a regulatory cytokine and one of its primary effects is to decrease the secretion of TNF and other proinflammatory cytokines. From the ER, newly synthesized IL-10 is transported to the Golgi complex where at the TGN, it can be directed into at least two distinct pathways to the surface, one direct and the other indirect. The latter pathway carries the bulk (80%) of IL-10 in the same p230 labeled carriers as IL-6 and TNF to the recycling endosome, where it is sorted for transport to the cell surface (13). Depletion or inactivation of p230/golgin245 or other TGN machinery disrupts IL-10 secretion through this recycling endosome route (13) and the final surface delivery of IL-10 is enacted by Rab11 and VAMP3-positive recycling endosomes, which fuse with the plasma membrane (13). Thus, there is no separation of pro- and anti-inflammatory cytokines in terms of pathways utilized.

In the second secretory pathway, IL-10 is packaged with ApoE at the TGN into golgin-97 labeled tubules and transported directly to the cell surface, bypassing the recycling endosome (13). This ApoE transport pathway is dependent on microtubules and PKA (13). As a soluble cytokine, IL-10 is likely to be packaged into multiple routes for TGN exit and surface delivery. Redundancy in overall pathways, carriers, and molecular regulators for the secretion of different cytokines, such as IL-6 and IL-10, reflects the fact that many are soluble proteins, and therefore, handled as "bulk cargo" by the cell. As a deeper understanding of trafficking emerges, especially in post-Golgi steps, factors such as sorting receptors, membrane domains, and intraluminal pH, may well be found to differentiate the final release of soluble cytokines like IL-6 and IL-10.

INTERLEUKIN-1 BETA SECRETION

Members of the IL-1 cytokine family, particularly IL-1β, IL-18, and IL-37 are key inflammatory cytokines with important roles also in disease, and with the distinction of using non-classical or unconventional secretory pathways for their release (62). In macrophages activated through a TLR, the precursor pro-IL-1β is synthesized but to fully achieve cytokine release, a second danger signal, for instance extracellular ATP (63), activates the multisubunit signaling complex of the NLRP3/caspase-1 inflammasome. Activated caspase-1 proteolytically cleaves pro-IL-1β to produce the mature, active form of the cytokine that is secreted. Exactly how IL-1β and other such cytokines find their way out of cells remains a topic of conjecture and conflicting evidence (62).

Several routes have been championed for the release of cleaved IL-1β from monocytes/macrophages and in different circumstances any or all of these routes may apply. Earlier studies on human monocytes used inhibitors to implicate the ATP binding cassette transporter (ABC1) as an exit portal directly through the plasma membrane for the release of IL-1β (64). The ABC transporter route has also been demonstrated for other soluble, secreted mediators including macrophage migration inhibitor factor (MIF) (65). Cell death through pyroptosis and other mechanisms may also lead to leakage and release of leaderless cytokines (66). Unconventional vesicular or organellar pathways have also been touted. Exosomes, derived either by blebbing of the plasma membrane or by budding off endosomal/lysosomal compartments may enfold cytosolic IL-1β for transport out of the cell [reviewed in Ref. (67)]. Macrophages have secretory lysosomes (68), which equate in some respects to LROs as dedicated secretory compartments in some other cell types. There is evidence for the transfer (by an unknown transporter) of leaderless cytoplasmic proteins, including cytokines (69), into secretory lysosomes for release. Related organelles, autophagosomes, or autophagolysosmes, have also been implicated in IL-1β release. While autophagy is primarily a degradative process in the cytoplasm, secretory autophagy is proposed as a parallel function for unconventional secretion, in yeast (70) and mammalian cells (71). This secretory autophagy may contribute transiently to acute release of IL-1β in activated macrophages, via a pathway that interestingly is mediated by Atg5, inflammasome, the Golgi reassembly stacking protein 55 (GRASP55) and Rab8a (72). Knockdown of Rab8a reduced the secretion but not the synthesis of IL-1β (72); moreover, Rab8a and its close homolog Rab8b have distinct roles in secretory and degradative autophagy, respectively (71). Further studies are likely to reveal more of the machinery associated with these unconventional secretory routes, predictably more Rabs, and also possibly SNAREs for vesicular routes.

OTHER CYTOKINES

To date, little data are available on the trafficking machinery necessary for the secretion of many of the cytokines secreted by macrophages. For some, it has been shown specific subunits or receptors can chaperone cytokines through the secretory pathway. IL-15 and IL-12 are two such cytokines. IL-12 is a proinflammtory

cytokine that controls the differentiation of naïve T cells into IFN-γ-producing Th1 cells and plays key a role in the development of autoimmune diseases and in the pathology associated with inflammatory related disease such as psoriasis (73). The bioactive form of IL-12, known as p70, is composed of two subunits, P35 and p40, with the availability of p35 being the major determining factor in its secretion (74). P35 is regulated at both transcriptional and post-transcriptional levels, and like p40 contains a signal peptide that targets it to the ER (75). However, in contrast to p40 whose signal peptide is removed during translocation into the ER, the p35 signal peptide is cleaved in an unconventional manner (75). Within the ER, a primary intermediate cleavage occurs, followed by a second cleavage before or immediately after exit from ER (75). Once in the ER p35, an integral membrane protein, binds to soluble p40 subunit to form membrane anchored p70 heterodimer, which then leads to its transport through the Golgi complex to the cell surface (76).

IL-15 plays an important role in regulating the activity and persistence of a large variety of cell types. Much of the work on IL-15 secretion has been undertaken in other cell types where its secretion, via the ER and Golgi complex, is dependent on its cognate receptor, the IL-15 receptor α (IL-15Rα), which acts as a chaperone enabling IL-15 to exit the ER and traffic through the secretory pathway (77). In macrophages, IL-15Rα is upregulated by IFNγ treatment, leading to secretion of IL-15, suggesting that a similar chaperone mechanism might occur (77). IL-15Rα contains a nuclear export sequence and can also be transported to the cell surface via the nucleus along with IL-15 in a chromosomal region maintenance 1 (CRM1) dependent manner (78). ARF6 has also been shown to promote IL-15 secretion, although exactly how is not clear (78).

The DNA binding nuclear protein high-mobility group box 1 protein (HMGB1) has multiple roles including being a potent mediator of inflammation (79). Extracellular HMGB1 acts as a proinflammatory alarmin or DAMP (damage-associated molecular pattern) that regulates inflammation through its binding to TLR 2, TLR4, and TLR9, to CD42 and to the advanced gylcation end product receptor (RAGE) (79). It can be released passively during necrosis (80) or actively through at least one non-classical secretory pathway (72, 81). Secretion begins around 6 h post-stimulation with levels still increasing 30 h later suggesting that it has a "late" role in regulating inflammation (81). Lacking a secretory signal peptide, HMGB1 is not targeted to the ER and Golgi complex. It does possesses a nuclear localization signal (NLS) and two non-classical nuclear export signals (NES) that regulate its shuttling between the cytosol and nucleus, with its equilibrium almost completely shifted toward nuclear accumulation in unactivated monocytes and macrophages (82). On activation, phosphorylation and acetylation of HMGB1 target it to the cytosol and partially into lysosomes from where it can be secreted (81, 82). The trafficking machinery that regulate this pathway and its release from cells are unknown, although in cancer cells HMGB1 is found in a complex with Annexin A2, myosin IC isoform a, myosin 9, and Rab10, suggesting that these proteins might play a role in its release (83). In macrophages, the same autophagy-based unconventional secretion pathway that releases IL-1β can also secrete HMGB1 and accordingly its release has the potential to be regulated by Atg5, the inflammasome, the Golgi reassembly stacking protein 55 (GRASP55), and Rab8a (72). Thus HMGB1 might be released by two different routes, although whether this occurs simultaneously or under distinct circumstances is yet to be determined.

CONCLUSION

The sequential and temporally orchestrated nature of cytokine secretion limits the demand for increased secretion to distinct times of need. It is becoming increasingly clear that upon LPS-activation macrophages adapt to this mandate by resourcefully modifying the levels or location of specific trafficking machinery molecules. This bolsters the sorting of cargo at the two major sorting hubs – the TGN and recycling endosome – and accelerates the passage of cytokines through distinct transport pathways. Although cytokine trafficking has not directly been compared in macrophages under different activation regimes, the overlapping pathways used to traffic IL-10 compared to IL-6 for instance, would suggest that both M2 and M1 polarized cells have largely common secretory pathways.

The adaptations for some of the cytokine trafficking pathways are strategic, such as the routing of recycling endosome membrane containing TNF to the phagocytic cup during times of infection. This clever strategy allows the concomitant release of TNF and provides extra membrane for phagocytic cup formation processes. Other adaptations might allow the packaging of cytokines into more than one pathway for rapid release. Whether non-classical secretory pathways are similarly modified during infection is unknown, but answering this along with further elucidation of the classical pathways and machinery might in future allow the targeted intervention in inflammatory diseases where excessive cytokine secretion is a major determining factor.

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Granule protein processing and regulated secretion in neutrophils

Avinash Sheshachalam[1], Nutan Srivastava[2,3], Troy Mitchell[3], Paige Lacy[2,3] and Gary Eitzen[1]*

[1] *Department of Cell Biology, University of Alberta, Edmonton, AB, Canada*
[2] *Pulmonary Research Group, University of Alberta, Edmonton, AB, Canada*
[3] *Department of Medicine, University of Alberta, Edmonton, AB, Canada*

Edited by:
Uday Kishore, Brunel University, UK

Reviewed by:
Janos G. Filep, University of Montreal, Canada
Joana Vitte, Aix Marseille University, France

***Correspondence:**
Gary Eitzen, Department of Cell Biology, University of Alberta, 514 Medical Sciences Building, Edmonton, AB T6G 2H7, Canada
e-mail: gary.eitzen@ualberta.ca

Neutrophils are part of a family of granulocytes that, together with eosinophils and basophils, play an essential role in innate immunity. Neutrophils are the most abundant circulating leukocytes and are vital for rapid immune responses, being recruited to sites of injury or infection within minutes, where they can act as specialized phagocytic cells. However, another prominent function of neutrophils is the release of pro-inflammatory compounds, including cytokines, chemokines, and digestive enzymes, which are stored in intracellular compartments and released through regulated exocytosis. Hence, an important feature that contributes to rapid immune responses is capacity of neutrophils to synthesize and store pre-formed pro-inflammatory mediators in specialized intracellular vesicles and thus no new synthesis is required. This review will focus on advancement in three topics relevant to neutrophil secretion. First, we will examine what is known about basal level pro-inflammatory mediator synthesis, trafficking, and storage in secretory compartments. Second, we will review recent advancements in the mechanisms that control vesicle mobilization and the release of pre-formed mediators. Third, we will examine the upregulation and *de novo* synthesis of pro-inflammatory mediators by neutrophils engaged at sites of infection.

Keywords: exocytosis, Rho GTPase, protein sorting, secretion, cytokine

INTRODUCTION

Neutrophils are the most abundant leukocytes in blood, comprising 60–70% of all circulating white blood cells, and therefore, make up an essential part of both innate and adaptive immunity. Neutrophils are highly mobile and provide rapid responses to infection via phagocytosis of pathogens or release of potent pro-inflammatory mediators that chemically incapacitate pathogens and infected cells. Neutrophils are also recruited to the site of injury following trauma, resulting in an acute inflammatory response. Their short-life span allows for quick resolution of inflammation and can promote wound healing through stimulation of local tissue remodeling and chemoattraction of macrophage (1). Neutrophil immunity function depends on both the biogenesis of granules that store proteins with active antimicrobial activity, and on their ability to generate oxidative burst. Defects in either of these two processes results in severe immunodeficiency such as neutropenia, neutrophil-specific granule deficiencies, or chronic granulomatous disease when oxidative burst is lacking (2). In this review, we will consider the protein processing events that occur in neutrophils, resulting in their initial granulocytic morphology, and protein transitions that occur to activate neutrophil "attack" mode.

GRANULOCYTOPOIESIS

NEUTROPHIL DIFFERENTIATION FROM BONE MARROW

Mature neutrophils are differentiated from hematopoietic stem cells in the bone marrow in a process termed granulocytopoiesis or granulopoiesis (3). New neutrophils are produced daily in high numbers, up to 10^{11} in healthy individuals, which can increase several fold during infection (4). Consequently, neutrophil homeostasis is highly regulated to control their numbers. Granulocyte-colony-stimulating factor (G-CSF) is the principal cytokine regulating granulocytopoiesis. Lack of G-CSF production impairs granulocytopoiesis, resulting in neutropenia and severe immune deficiency (5, 6). Hematopoietic stem cell differentiation into granulocytes is regulated by the coordinated expression of three key myeloid transcription factors, GFI-1, PU.1, and members of the CCAAT enhancer binding protein family (C/EBPs). The balance between PU.1 and C/EBPα determines whether myeloblasts differentiate into granulocytes (high C/EBPα) or monocytes (high PU.1) (7).

The chemokines CXCL12 and CXCL2 and their neutrophil receptors, CXCR4 and CXCR2, respectively, regulate the release of neutrophils from the bone marrow. CXCR4–CXCL12 signaling promotes bone marrow retention, while CXCL2–CXCR2 promotes their release in what has been described as a tug of war model. The contribution of these chemokine signaling pathways to the regulation of neutrophil trafficking from bone marrow has been recently reviewed (8).

NEUTROPHIL DIFFERENTIATION FROM STEM CELLS

Neutrophils have been developed from human embryonic stem cells (hESCs) in culture. High yields were achieved by growing hESC in co-culture with semi-confluent OP9 stromal cells

to stimulate hESC differentiation (9). The OP9 stromal cell line, which is derived from the osteopetrotic mouse, was specifically selected since they lack production of macrophage-colony stimulating factor (M-CSF) and thus support neutrophil rather than macrophage differentiation (10, 11). The procedure was further improved using a unique cytokine cocktail to yield highly functional neutrophils capable of chemotaxis, phagocytosis, oxidative burst, and bacterial killing (12, 13). Recently, protocols have been reported that differentiated neutrophils can be prepared from induced pluripotent stem cells (iPSCs) (14–16). These are significant advancements since the ability to obtain functional neutrophils from hESCs, iPSCs, and even patient-derived iPSCs may progress toward their eventual use in the treatment of hematopoietic disorders (17, 18).

NEUTROPHIL GRANULARITY

SEQUENTIAL SYNTHESIS MODEL

Mature neutrophils emerge in the blood devoid of any proliferative capacity, but fully capable of launching an immune response. Ultrastructural images of mature neutrophils reveal a characteristic multi-lobed nucleus, very few mitochondria, a small Golgi structure, and a highly granular cytosol that is packed with vesicles (**Figure 1A**). The presence of different granule subsets is revealed by their varying size and intensity of stained with the peroxidase-reactive dye, 3,3′-diaminobenzidine (**Figure 1B**). Granule subsets are also distinguished by their protein content and their propensity for mobilization, which will be further discussed in following sections (19–21).

The difference in protein contents among neutrophil granule subsets is not driven by protein sorting. Instead, different granules are sequentially formed during neutrophil differentiation in what has been described as a targeting by timing model (23). According to this model, as different granule proteins are synthesized during different stages of neutrophil differentiation several granule subsets are generated (24–26). Neutrophils have at least three distinct granule subsets: (i) primary or azurophilic granules, which contain potent hydrolytic enzymes (e.g., elastase) and myeloperoxidases (MPO), (ii) secondary or specific granules, which contain

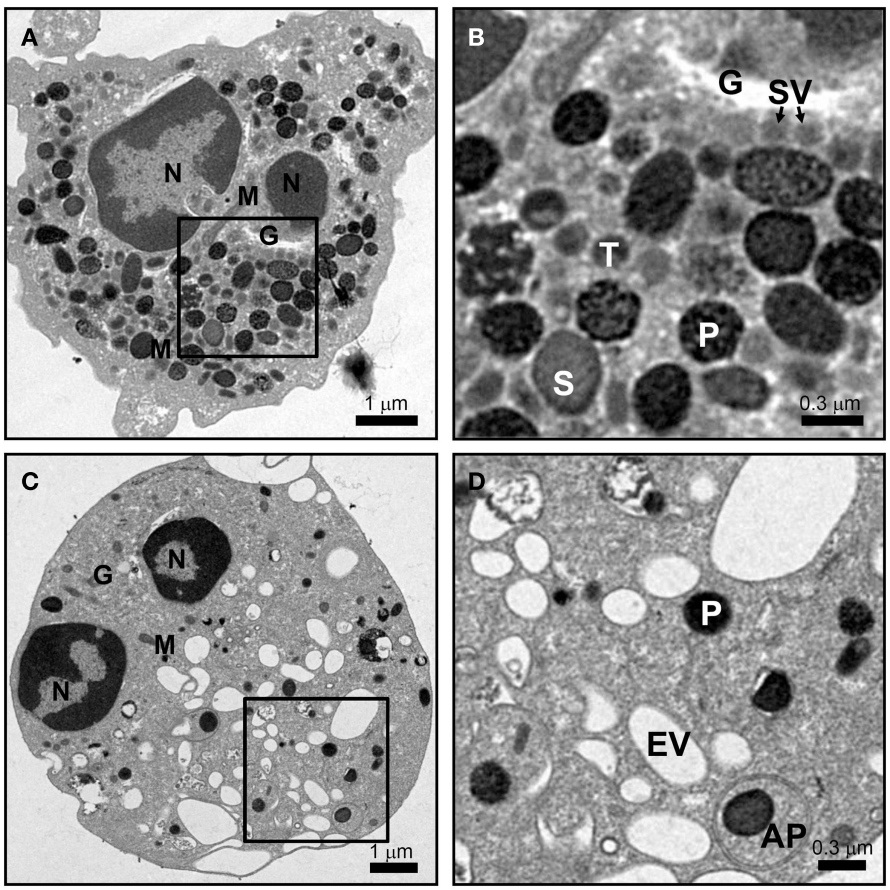

FIGURE 1 | Neutrophil morphology as observed by electron microscopy. Neutrophils were isolated from human peripheral blood, and processed for electron microscopy after incubation for 15 min at 37°C with vehicle **(A,B)** or 10 μM cytochalasin B and 5 μM fMLF **(C,D)** as described in Ref. (22). **(A,B)** The cytosol of a resting cell is filled with vesicles, with primary granules (P) staining intensely dark with DAB, while secondary (S) and tertiary (T) granules show more translucent staining. Secretory vesicles (SV) are in close proximity to the Golgi complex (G). Few mitochondria (M) are observed. **(C,D)** After stimulation only a few dense primary granules (P) remain and many empty vesicles (EV) appear. A double membrane autophagosome (AP) was observed to form around a primary granule; however, the relevance of this observation is unknown. Scale **(A,C)** = 8 μm × 8 μm, 9,100× magnification; **(B,D)** = 2.4 μm × 2.4 μm, 27,600× magnification.

high levels of the iron-binding protein lactoferrin, and (iii) tertiary or gelatinase granules, which contain matrix metalloproteinases (**Figure 1B**). Secondary and tertiary granules contain an overlapping set of proteins; however, all granules have distinctive buoyant densities and can be isolated by density gradient centrifugation (27). Recently, a fourth granule population has been described that was enriched in the microbial lectin ficolin-1. Ficolin-1 is found in tertiary granules; however, the authors found a second pool of ficolin-1-rich/gelatinase-poor granules with an elevated exocytosis propensity (21, 28). The importance of these granules may be to provide rapid release of pattern recognition molecules to activate the lectin complement pathway (28). Secretory vesicles (SV) also appear in density gradients that contain albumin indicative of their formation via endocytosis. SV can also be formed in mature neutrophils from a small Golgi structure (**Figures 2A,B**). These contain cytokines synthesized during immune activation indicative of an active biosynthetic pathway (29, 30), which will be discussed further in Section "Neutrophil *De Novo* Synthesis of Pro-Inflammatory Mediators."

GRANULE PROTEIN SORTING

Neutrophil granule proteins undergo a series of co- and post-translational processing and targeting events during sorting to their granule sub-compartment. Most, if not all, proteins packaged into neutrophil granules enter through the biosynthetic process as deduced by the presence of amino-terminal signal sequences for co-translational translocation into the endoplasmic reticulum (ER). The emergence of this "pre" sequence from the ribosome associates with the signal recognition particle, which drives co-translation into the lumen of the ER, followed by post-translational processing as proteins traverse the Golgi complex (e.g., protein maturation via glycosylation).

The precise mechanism by which proteins are targeted to neutrophil granules from the *trans*-Golgi network (TGN) is largely unknown, hampered by the fact that these cells are end-differentiated and do not synthesize granule proteins *de novo*. However, much information has been gained from analogous studies in other immune cells and the characterization of neutrophils from immunological disorders associated with sorting defects (31, 32). These studies confirm that neutrophils generate lysosome-like secretory granules both directly from the TGN and indirectly via a sorting endosomes (**Figure 3**). Post-TGN sorting is mediated by the multi-subunit adaptor protein complexes (APs) and the monomeric Golgi-localized γ-adaptin ear homology ARF binding (GGAs) protein. Both nucleate clathrin-coated vesicles and recruit luminal cargo for packaging into the vesicles (33). Three of the five known AP complexes function at the TGN and are likely involved in granule sorting since they have prominent roles in lysosomal sorting in other cell types (33, 34). AP-1 and GGAs direct the bulk sorting program from the TGN to sorting endosomes, while AP-3 and AP-4 shuttle cargo to granules from the sorting endosomes or directly from the TGN, bypassing the sorting endosomes (35). This distinction in pathways serves an important mechanistic role since soluble cargo utilizes receptors that require a dissociation step catalyzed in endosomes. This allows the receptor to be sorted back to the TGN while the cargo is transported into granules (**Figure 3**).

The sorting of granule membrane proteins, including cargo receptors, depends on dileucine or tyrosine-based sorting signals present in carboxy-terminal cytosolic domains (35). The consensus dileucine sequences, DXXLL or [DE]XXXL[LI], and tyrosine sequence, [G]YXXØ (where X represents hydrophobic residues and Ø a bulky hydrophobic amino acid), interact with GGAs/AP-1 and AP-3/AP-4 complexes, respectively.

The majority of soluble hydrolases and granzymes are modified with mannose-6-phosphate (M6P) in the Golgi, which is recognized by M6P-receptors to facilitate their exit from the TGN (33). Unlike hydrolases and granzymes, many soluble granule proteases are sorted via M6P-independent mechanisms (31, 36). Targeting of the major serine protease, neutrophil elastase, as well as other proteases requires AP-3 (37). Neutrophil elastase contains a tyrosine on the cytosolic face of it *trans*-membrane pro-domain, which interacts with AP-3 presumably for its sorting (38). However, neutrophil elastase mutants that lack a pro-domain still get sorted to

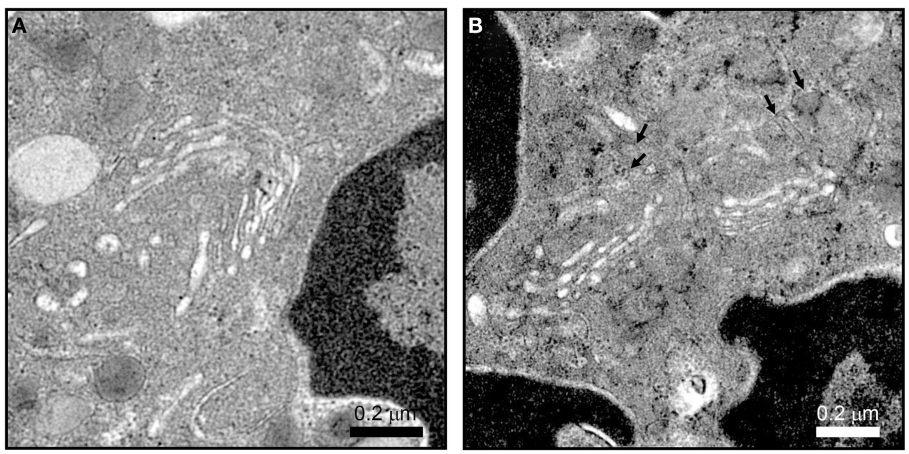

FIGURE 2 | Neutrophil Golgi complex as observed by electron microscopy. Neutrophils processed for electron microscopy as described in Ref. (22) show a small stacked Golgi with an increase in electron dense vesicle (*arrows*) after stimulation. Conditions were incubation with vehicle **(A)** or 10 μM cytochalasin B and 5 μM fMLF **(B)** for 15 min at 37°C. Scale, 2.4 μm × 2.4 μm, 27,600× magnification.

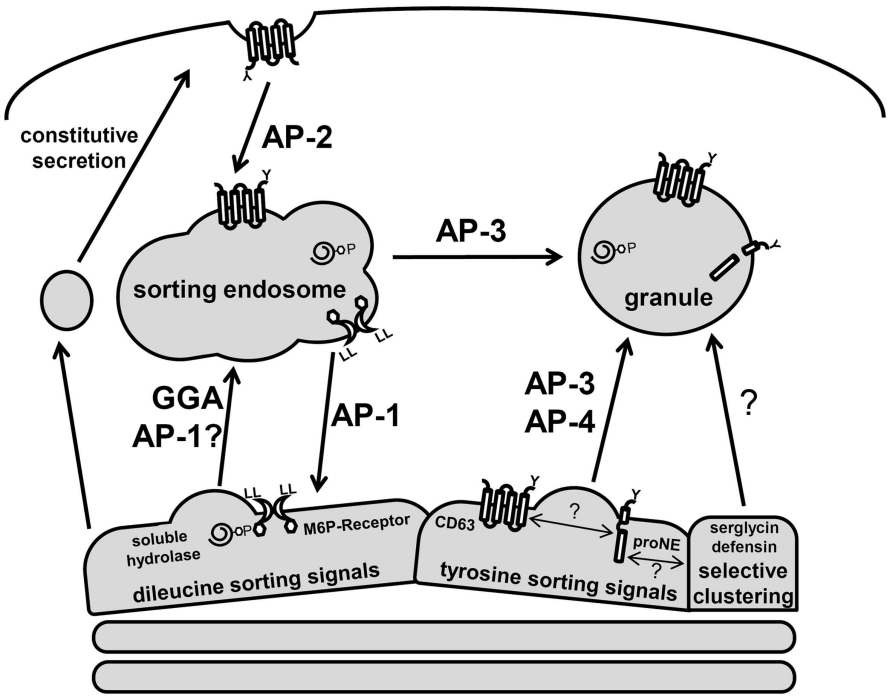

FIGURE 3 | Schematic of neutrophil protein sorting pathways. Three prominent trafficking pathways for granule proteins are depicted: AP-1/GGA-dileucine based sorting, AP-3/AP-4-tyrosine-based sorting, and selective clustering-based sorting. Soluble hydrolases contain M6P signals and are sorted via the M6P-receptors, which contain dileucine signals. These complexes are shuttle to the sorting endosome through GGA and AP-1-dependent pathways. In the endosome, a drop in pH results in dissociation of the receptor–cargo complex with the retrieval of the M6P-receptor by an AP-1 dependent pathway. Serine proteases (neutrophil elastase, proNE) and *trans*-membrane proteins (CD63) contain tyrosine signals and are sorted directly to granules via AP-3/AP-4. Many granule proteins contain neither dileucine nor tyrosine signals and are thought to sort to granules via a clustering-based pathway since this occurs for many granule proteins that are highly concentrated. CD63 trafficking is unique as it can be found on several neutrophil membranes and may act to selectively retrieve granule proteins that escape normal trafficking via constitutive secretion.

granules via a CD63-dependent mechanism (39). CD63, an abundant membrane protein of primary granules, is sorted by AP-3, and has been shown to play a role in granule protein quality control since it can be trafficked from many membranes to granules (40, 41). A recent study showed that CD63 and neutrophil elastase are present in a complex, highly indicative of a role for CD63 in the targeting of elastase to primary granules (42). Other serine proteases affected by CD63 granule sorting include proteinase-3 (39), and possibly the cathepsin family of proteases, which exhibit varying degrees of M6P-dependent/independent sorting (36, 43–45).

Neutrophils, as well as other granulocytes, may have acquired additional unique granule sorting mechanisms based on the fact that proteins in some granules can be tightly packed to the point of having a crystalline core. A selective aggregation of proteins destined for storage in granules would eliminate the need for distinct sorting motifs on each granule protein (24). Granule proteins, which are often cationic, may be clustered with serglycin, a major anionic proteoglycan of hematopoietic cells. Serglycin has been proposed to play a role in sorting and packing of several granule proteins including α-defensin and elastase (46, 47). Neutrophil elastase is absent from mature neutrophils in serglycin knock-out mice (48). Whether serglycin-induced clustering of granule proteins promotes granule localization via sorting or their retention in granules is still under investigation (49).

Most granule enzymes are synthesized as zymogens and undergo a proteolytic processing step that depends on the acidic pH levels within this lysosome-like compartment. The proteolytic cleavage of granule pro-domains converts these enzymes into their active forms and thus many granule proteins are maintained inactive until the proper compartment is reached. Some pro-domains have also been shown to have a role in sorting. The amino-terminal pro-peptide of pro-MPO has been shown to facilitate its targeting to primary granules (50). Unlike MPO, serine proteases (e.g., elastase and cathepsin G) are synthesized with both amino-terminal and carboxy-terminal pro-peptides that do not seem to be involved in their sorting (51).

IMMUNE DISORDERS FROM GRANULE PROTEIN SORTING DEFECTS

Granule protein trafficking disorders often manifest as lysosomal storage disorders (LSDs), which are a large group of metabolic diseases that result from deficiencies in specific lysosomal enzymes or defects in lysosome biogenesis (31, 52). For instance, defects in the ability to generate M6P sorting signals in the Golgi result in the LSDs mucolipidosis II (also referred to as I-cell disease) and mucolipidosis III. Mutations in GlcNAc-phosphotransferase are believed to be the primary genetic defect in mucolipidosis. GlcNAc-phosphotransferase activity is absent in MLII and altered in MLIII (53). Studies of patients with mucolipidosis revealed

neutropenia, with a major reduction in the number of neutrophils in MLII, but less severe in MLIII patients (53, 54). There have been no reports of neutrophil function studies from these patients, but presumably granules would lack most soluble hydrolases since these are sorted by the M6P pathway. However, studies of B lymphocytes showed that a portion of lysosomal enzymes were retained in granules with an unknown a back-up sorting event facilitating this partial sorting (36). The recent development of a mucolipidosis II mouse model might advance neutrophil functional characterizations (55, 56). These studies could reveal the prominence of M6P-independent sorting pathways in neutrophils and whether these can facilitate the partial retention of enzymes normally sorted by M6P.

Hermansky–Pudlak syndrome type 2 is also a LSD disorder that results from a mutation in AP3B1, which encodes a subunit of the AP-3 complex. The type 2 form includes immunodeficiency associated with neutropenia and partial albinism. The basis of this disease was first discovered as a similar autosomal recessive disease of dogs, canine cyclic hematopoiesis (38). The lack of AP-3 sorting resulted in reduced levels of neutrophil elastase and gelatinase in granules, but normal levels of other enzymes such as MPO and proteinase-3, which reside in the same granule fraction as elastase (57). This reveals the divergence in sorting pathways in neutrophils; AP-3 for neutrophil elastase versus GGA/AP-1 for MPO. Interestingly, AP-3 deficiency leads to congenital neutropenia in humans and cyclic neutropenia in dogs (38, 58), even though both are linked to the lack of neutrophil elastase sorting. Neutrophil elastase mutations cause a much more severe congenital neutropenia phenotype in humans (59). It has been suggested that increased apoptosis of myeloid precursors in patients carrying mutations in the elastase gene could lead to a maturation arrest of myelopoiesis and this triggers the more severe neutropenia phenotype (60).

Although neutrophil elastase is a soluble protein, there is evidence that in an intermediate stage of its processing it is a disulfide bonded *trans*-membrane protein (38). Upon enzymatic removal of the carboxy-terminal pro-domain, the protein maintains a *trans*-membrane conformation capable of associating with AP-3 complexes. Membrane-associated neutrophil elastase utilizes a tyrosine-based sorting signal that is located in the carboxy-terminal domain of the protein. Most elastase mutations associated with severe congenital neutropenia result in the removal of this sorting signal (38). Horwitz et al. have proposed that the localization of the enzyme in the lumen of granules or on the plasma membrane may regulate the differentiation pattern of myeloid progenitor cells into monocytes or granulocytes. This suggests that neutropenia may result from a defective differentiation switch between the monocytic and granulocytic lineage of myelopoiesis (61, 62). This is an appealing hypothesis that may account for the observation that granulocytes and monocytes reciprocally cycle in numbers during normal hematopoiesis and for the typical increase of monocyte blood counts observed in the majority of severe congenital neutropenia patients. Hence, not only granule sorting pathways are important for granule biogenesis but also in determining the population of cell types during hematopoiesis.

PROTEIN PROCESSING IN ACTIVATED NEUTROPHILS

Three functions that involve protein processing occur in neutrophils when activated for an immune response: (1) neutrophil migration up chemotactic gradients to sites of infection, (2) destruction of pathogens or injured/infected cells through oxidative burst and phagocytosis, and (3) mobilization of granules and release of inflammatory mediators. In this section, we review these functions with a particular focus on the mobilization of granules and the release of pre-formed inflammatory mediators.

NEUTROPHIL CHEMOTAXIS

Upon activation, neutrophils migrate out of blood vessels following gradients of chemoattractants in a process known as chemotaxis. Neutrophils respond to a wide range of "neutrophil-active" chemoattractants, including chemokines and cytokines (CXCL8, IFNγ), complement (C5a), eicosanoids (LTB_4), and pathogen-derived peptides such as formylated met–leu–phe (fMLF) (63, 64). Chemotaxis begins with neutrophil recruitment from circulation through physical interactions of neutrophil-specific adhesion molecules. The neutrophil adhesion cascade is the focus of recent reviews and therefore only briefly described here (65, 66). Neutrophils are initially tethered by selectin interactions, which can be upregulated on the surface of endothelial cells (E-selectin) and leukocytes (L-selectin). The lectin-like domains of selectins interact with sialylated carbohydrate groups present on surface proteins. This results in the characteristic rolling of neutrophils along the luminal side of the endothelium. Reduced velocity allows neutrophils to recognize chemokines on the surface of the endothelium, which activates integrins. Neutrophils will be released from low-affinity selectin interactions unless firm adhesive contacts are made between integrins and endothelial intracellular adhesion molecules (ICAMs). Integrin ligation triggers intracellular signaling for cytoskeletal rearrangement, which polarizes neutrophils and drives transmigration through the endothelium (diapedesis). Integrin signaling is essential as deficiencies result in leukocyte adhesion deficiency, which not only causes defects in chemotaxis and adhesion but also in phagocytosis and respiratory burst (2).

Regulation of the adhesion and chemotaxis requires the coordination of multiple protein signaling and processing events, which result in cytoskeletal rearrangements that generate cell polarity (67). The activation of distinct Rho proteins at the front (Rac1) and back (RhoA) of neutrophils create actin formations that drive movement (68). Rho proteins are central regulators of multiple intracellular processes, and it is interesting how signaling downstream from Rho has evolved to coordinate cytoskeletal remodeling in conjunction with other processes required for immune cell functions. These include many non-cytoskeletal-related functions such as regulation of gene transcription (69–72), calcium flux (73, 74), and oxidative burst (75–77).

Chemokines interact with G protein-coupled receptors (GPCRs) on the surface of neutrophils. One pathway activated by GPCRs is the lipid kinase pathway. PI3-kinase is activated, which converts phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] into phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] at the plasma membrane. $PI(3,4,5)P_3$ stimulates F-actin reorganization at the leading edge driving lamellipodia formation. The Rac

GEFs, Vav1, and P-Rex1, bind PI(3,4,5)P_3 via pleckstrin homology domains, activating Rac, which leads to the production of branched actin filaments at the leading edge (78–80). High levels of the phosphatase PTEN are present in the trailing edge of neutrophils, which consumes PI(3,4,5)P_3. Thus, a gradient of PI(3,4,5)P_3 is established toward the leading edge, which promotes polarization and directional motility (81). A recent report examined neutrophils derived from a conditional PTEN knock-out and showed it not only functions in polarization but also prioritizes chemoattractant signals. Normal neutrophils were shown to prefer bacterial chemoattractants over endogenous chemokines, but this preferential selection was lost in the PTEN knock-out (82).

Opposing roles for the two Rho proteins, RhoA and Cdc42, in the trailing edge (or uropod) were shown by examining neutrophils devoid of these proteins. In the absence of RhoA, neutrophil priming was unregulated, resulting in hyper-responsive activation and uncoordinated motility (83). Cdc42, on the other hand, was required specifically to maintain neutrophil polarity (84). WASp, a downstream Cdc42 effector, regulated polarity through the CD11b integrin, which recruits the microtubule end binding protein, EB1, to capture and stabilize microtubules at the uropod. This would allow the generation of "pushing" forces from the back of neutrophils. Cross-talk between Rho proteins has been an area of intense investigation (68) and these results show how Rho/Cdc42 signaling cross-talking can coordinately control neutrophil chemotaxis while at the same time minimize other immunity functions such as degranulation.

PHAGOCYTOSIS AND RESPIRATORY BURST

Neutrophil phagocytosis has been the focus of several reviews (85–87). For the purpose of this review, we will limit our consideration to the protein processes involved in NADPH oxidase assembly and how this is directed primarily to the phagosomal membrane.

The rapid immunological response of neutrophils not only depends partly on pre-formed granule proteins with antimicrobial activity but also on the ability to generate respiratory burst activity. Deficiency in respiratory burst results in a severe immunodeficiency called chronic granulomatous disease (2). Chronic granulomatous disease is linked to genetic abnormalities in any one of the five "phox" proteins, which comprise the NADPH oxidase complex. The small GTPase Rac is a sixth component that is physically part of the oxidase complex that regulates its function (75).

The NADPH oxidase complex generates reactive oxygen species (ROS) that are crucial for antimicrobial activity and antigen processing. However, excessive ROS production causes tissue damage and oxidative stress and therefore neutrophils must precisely control both the location and timing of NADPH oxidase activity. In unstimulated neutrophils, three oxidase subunits, p47-phox, p67-phox, and p40-phox, are cytosolic. Rac2 is also cytosolic, bound to its natural inhibitor RhoGDI. The remaining two subunits, gp91-phox and p22-phox, form the membrane-spanning catalytic core of cytochrome b558. Formation of the holoenzyme allows electrons to follow from NADPH bound at the cytosolic face of the complex, to FAD-bound cytochrome b558, which has two heme-bound oxygen groups that accept the electrons and produce superoxide.

The oxidase complex can be preferentially incorporated into the phagosome during pathogen endocytosis, which minimizes collateral tissue damage. However, residual oxidase complex at the plasma membrane or its activation in the absence of phagocytosis results in the release of ROS extracellularly. Three regulatory steps may facilitate the phagosomal-predominant spatial activation of the oxidase complex. p47-phox phosphorylation leads to a conformational change that exposes two amino-terminal SH3 domains, which interact with the proline-rich region of the membrane-bound p22-phox (88). Assembly that occurs on the plasma membrane is susceptible to in rapid dephosphorylation, resulting in unproductive oxidase assembly. Second is the regulation of the p40-phox subunit by PI3-kinase. This effect is mediated by the PX domain of p40-phox binding to PI(3)P, which results in an open conformation allowing NADPH access to the catalytic core (89, 90). PI(3)P is enriched on phagosome membranes, which facilitates the spatial restriction of oxidase activity to the phagosome rather than at the plasma membrane where the predominant phosphoinositol lipids are PI(4,5)P_2 and PI(3,4,5)P_3. A third regulatory step involves Rac. Rac is activated by guanine nucleotide exchange factors (GEFs) from the Vav or P-Rex families (78–80, 91). The function of these GEFs depend on membrane recruitment via increased levels of PI(3,4,5)P_3 (92). However, this create a spatial paradox since PI(3,4,5)P_3 is localized to the plasma membrane and not phagosomes. This is resolved by the oxidase complex showing preference for assembly with Rac2 over Rac1 (91, 93, 94). While Rac1 is predominantly localized to the plasma membrane, Rac2 has less affinity for the plasma membrane, likely due to fewer polybasic residues in its carboxy-terminus (95). This may result in spontaneous disassembly from the oxidase complex in the presence of high levels of PI(4,5)P_2 and PI(3,4,5)P_3, unless rapidly endocytosis occurs and the highly acidic phosphoinositides are metabolized.

Interestingly, the Rac GEF Vav has also been shown to affect oxidase assembly through a secondary affect involving the p40-phox subunit (96). This may involve other downstream effectors of Rac, which includes the serine kinase PAK1, which has been shown to phosphorylation p47-phox (97). In addition, RhoA has recently been shown to downregulate ROS production (98). The RhoA signaling pathway was linked to cytoskeletal remodeling, which would provide for an elegant way to downregulate oxidase activity during chemotaxis. Further studies of the convergence of multiple signaling pathways will be needed to reveal the full control of oxidase activity.

GRANULE MOBILIZATION AND REGULATION OF EXOCYTOSIS

Neutrophils, upon stimulation undergo a series of immediate changes without the need for *de novo* synthesis of proteins (compare **Figures 1A** and **1B** vs. **1C** and **1D**). Exocytosis, also known as degranulation in neutrophil, is the release pre-formed mediators from granules. Granule subsets are markedly different in their capacity for mobilization in response to stimulation (99, 100). Granules formed during the later stages of granulocytopoiesis are more prone to undergo exocytosis than granules formed during the earlier stages. A recent study reported exocytosis levels of 100% for SV, 38% for tertiary granules, 22% for secondary granules, and

only 7% for primary granules after stimulation (28). The specific steps of exocytosis involve granule translocation toward a target membrane via actin remodeling and microtubule assembly, followed by tethering and docking through the sequential action of the core fusion machinery of Rab and SNARE proteins (**Figure 4**) (101, 102).

Neutrophil granule contents, which include MPO, elastase, lactoferrin, and matrix metalloproteinases, possess potent antimicrobial activity but are also highly cytotoxic. Therefore, their release is highly regulated by binary signals to minimize aberrant degranulation. The first of these binary signals is adhesion-dependent while the second involves activation of immune receptors by ligand interactions. The adhesion-dependent step *in vivo* involves β2-integrins (100), which can be reconstituted *in vitro* by adhesion to biological surfaces or the addition of actin depolymerizing agents (22, 103, 104). The adhesion-dependent signaling cascade for degranulation operates through the Src kinases Fgr and Hck. Neutrophils from double knock-out $hck^{-/-}$ $fgr^{-/-}$, while still able to adhere to substrate, fail to undergo degranulation in response to tumor necrosis factor (TNF) (105). However, Src kinases are well known to be membrane proximal regulators of neutrophil degranulation during receptor-mediated activation, which comprises the second half of the binary signal (106, 107). For example, degranulation requires immune receptor–ligand interactions such as formylated peptides binding to GPCRs. This also triggers the activation of Src kinases, and hence it is curious why binary signals are needed that seemingly activate the same signaling cascade. Binary signals may be needed to reach an activation threshold for degranulation or perhaps distinct downstream pathways are activated. One downstream pathway specifically activated by the receptor-mediated kinase cascade is Ca^{2+} release from intracellular stores (107). Increasing concentrations of Ca^{2+} are responsible for the hierarchical release of neutrophil granules in the order of secretory vesicles > tertiary granules > secondary granules > primary granules (99).

Cross-talk between the binary degranulation signals makes it difficult to clearly define each signaling pathways. For example, recently a role for the highly abundant non-receptor tyrosine kinase, proline-rich kinase 2 (Pyk2), was described for degranulation (108). Pyk2 undergoes auto-phosphorylation after integrin ligation in calcium-dependent manner, allowing its association with the Src-family kinases. Furthermore, studies with $pyk2^{-/-}$ cells have shown its effect is mediated through paxillin and Vav, which are both phosphorylated by Pyk2 (108). Vav is a direct

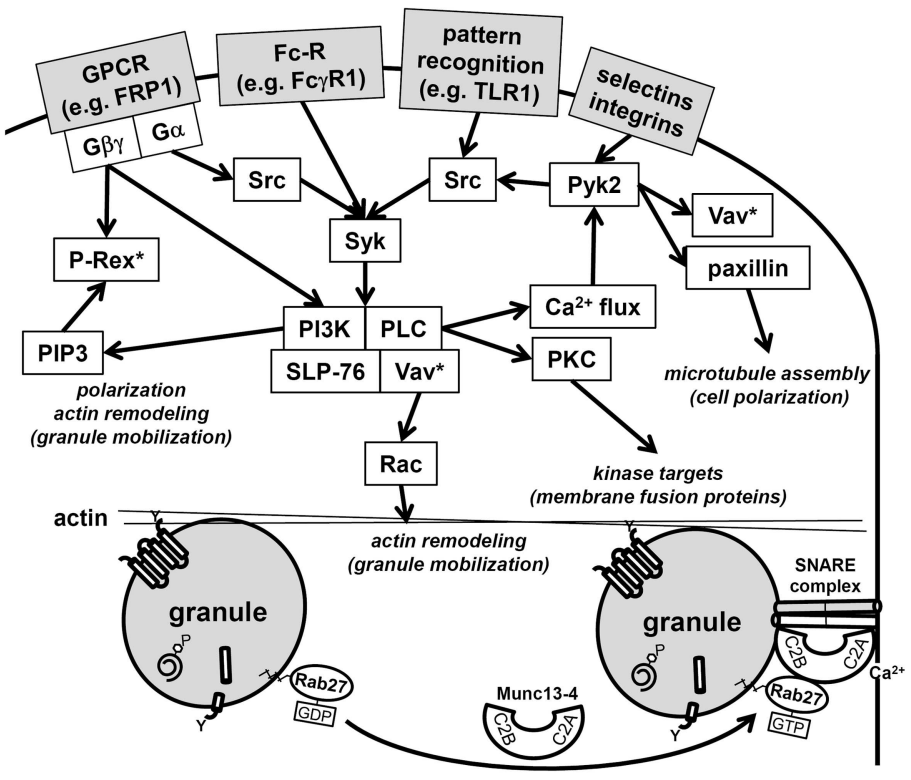

FIGURE 4 | Schematic of neutrophil signaling pathways regulating degranulation. Two pathways that regulate granule mobilization are depicted: upstream kinase cascade and downstream fusion machinery. Activation of neutrophils through surface receptors triggers the activation of a kinase cascade. Central downstream effectors of these kinases target cytoskeletal remodeling, these include Vav, which activates Rac, paxillin, which facilitates microtubule polarization, and the generation of PI(3,4,5)P3, which facilitates polarization and actin remodeling. Note that Rac is activated at multiple points in the signaling pathways (Vav*, P-Rex*) and hence may be needed for several immune cell functions in addition to degranulation. Granule tethering is regulated by Rab27 recruitment of Munc13-4. Munc13-4 is a calcium-sensitive link between Rab function and the fusion machinery of SNAREs. The Munc13-4:SNARE interaction requires calcium flux and is targeted by PKC, which are activated in the upstream kinase cascade.

activator of Rac, which remodels actin to facilitate degranulation (78), while paxillin serves as an important scaffolding protein and has a direct effect on microtubule assembly, which may contribute to polarized granule motility (109). Thus, Pyk2 signaling may be central to the transduction of multiple upstream signals into downstream granule mobilization and degranulation (**Figure 4**).

Downstream events are regulated by distinct core fusion machinery that drives granule docking and exocytosis at specific target membranes. Initially, Rab proteins tether vesicles to target membranes, then SNARE proteins catalyze fusion (102). Neutrophil granules fuse with target membranes when v-SNARES pair with their corresponding t-SNARES. VAMP-2 is predominantly localized to SV and tertiary granules, whereas VAMP-7 is predominantly localized to primary granules (110, 111). It seems that the membrane density of VAMP-2 provides a functional role in mobilization, docking, and fusion to the plasma membrane, while VAMP-7 redirects vesicles to fuse predominantly with phagosomes (110, 112).

Numerous additional regulatory proteins interact with the core fusion machinery of Rabs and SNAREs. Among these, the Munc family of proteins is important regulators of SNARE complex formation (101). Munc13-4 (also known as UNC13D) is highly expressed in neutrophils and interacts with Rab27, which is the Rab that direct granules to dock at the plasma membrane. Munc13-4 tethers granules to the plasma membrane for exocytosis via two calcium-sensitive lipid bind C2 domains (**Figure 4**) (113). Neutrophil subfractionation revealed that high levels of Munc13-4 rapidly translocate from the cytosol to granules upon stimulation with a secretagogue like fMLF (114). In this study, Munc13-4 was shown to associate with secondary and tertiary granules and down-regulation of Munc13-4 using small interfering RNA decreased their exocytosis (114). Recently, STK24 and CCM-3, two regulators of neutrophil exocytosis, were shown to interact with Munc13-4 (115). Lack of either STK24 or CCM-3 inhibited degranulation; however, they were shown to play opposing roles in the regulation of Munc13-4. Whereas STK24 inhibited Munc13-4 function by binding its C2B domain, CCM-3 counteracted STK24-mediated C2B inhibition (115). The STK24/CCM-3 complex seems provide an important additional control mechanism to halt aberrant degranulation.

NEUTROPHIL *DE NOVO* SYNTHESIS OF PRO-INFLAMMATORY MEDIATORS

Neutrophils are well characterized for their ability to synthesize and secrete over 70 different cytokines, chemokines, and growth factors. Although several of these have been characterized at the mRNA level only, 11 of these show controversial data for human neutrophils (116). The trafficking pathways that govern the synthesis, storage, and release of these factors are poorly understood. Several studies using immunogold staining analysis of transmission electron microscopy have revealed that neutrophils store pre-formed cytokines in secretory granules. Transforming growth factor-α (TGFα) is stored as a pre-formed mediator in secretory granules that are peroxidase-negative, suggesting localization to secondary or tertiary granules (117). Similarly, TNF-α was found as a pre-formed mediator in cytoplasmic vesicles following immunogold staining (118). However, the characteristics of these cytoplasmic vesicles were not further elucidated in this study. Other studies investigating cytokine and chemokine expression in neutrophils have indicated that mature peripheral blood neutrophils possess pre-formed IL-6, IL-12, and CXCL2 in their SV or tertiary granules (119).

In neutrophils, CD63 and CD68 are abundant membrane-bound proteins in primary and secondary granules, and both of these possess the YXXΦ motif, which interacts with AP-3/AP-4 complexes. AP-3 traffics cargo from tubular endosomes (recycling endosomes) to late endosomes, lysosomes, and related organelles, while AP-4 traffics protein from the TGN to endosomes or directly to lysosomes (33, 35). This indicates that CD63 and CD68 may be engaged in trafficking to primary and secondary granules through recycling endosomes and late endosomes. Conversely, cytokines that are secreted by neutrophils, including IL-1α/β, IL-6, CXCL8, IL-12, TNF, IFNγ, CXCL2, TGFβ, MIP-1α/β, and VEGF do not possess adaptor motifs, suggesting that they do not directly bind to adaptors in order to enter sorting pathways for their trafficking. Based on the absence of adaptor motifs in cytokines, it is likely that cytokine trafficking is mainly determined by adaptor motifs present in membrane proteins such as CD63 and CD68, which direct cytokine cargo to appropriate granular and vesicular compartments in cells.

Lipopolysaccharide (LPS) is major TLR agonist that is shed from the outer membranes of Gram-negative bacteria, which cause a significant burden of disease by inducing respiratory infections, gastrointestinal disorders, sepsis, pneumonia, and many other transmissible infections. LPS stimulation results in substantial production of TNF, a potent pro-inflammatory cytokine with cytotoxic and pyrogenic effects. Neutrophils stimulated by LPS have the ability to release TNF, potentially through granule stores (118, 120). However, the mechanisms used to secrete cytokines and chemokines has not been thoroughly investigated in neutrophils although it is evident that SNAREs and other trafficking machinery are required for exocytosis of cytokine and chemokine-carrying granules (110, 111, 121, 122).

CONCLUSION

Granule exocytosis and release of their cytotoxic contents at the plasma membrane is most often an undesirable effect of inflammation. We have highlighted many of the protein processes involved in pro-inflammatory mediator synthesis and granule biogenesis. Neutrophils possess several mechanisms that promote fusion of granules with phagosomes to minimize the effect of neutrophil-induced co-lateral tissue damage. However, in many inflammatory disorders, such as acute lung injury, ischemia/reperfusion injury, severe asphyxic asthma, rheumatoid arthritis, and septic shock, excessive neutrophil degranulation is a common feature. Insight into neutrophil protein processes holds much promise in the future treatment of these diseases.

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Neutrophil-derived cytokines: facts beyond expression

Cristina Tecchio[1]*, Alessandra Micheletti[2] and Marco A. Cassatella[2]*

[1] *Section of Hematology, Department of Medicine, School of Medicine, University of Verona, Verona, Italy*
[2] *Section of General Pathology, Department of Pathology and Diagnostics, School of Medicine, University of Verona, Verona, Italy*

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Eric Denkers, Cornell University, USA
Abhishek D. Garg, Katholieke Universiteit Leuven, Belgium

****Correspondence:***
Cristina Tecchio, Section of Hematology, Department of Medicine, Piazzale L. A. Scuro, Verona 37134, Italy
e-mail: cristina.tecchio@univr.it;
Marco A. Cassatella, Section of General Pathology, Department of Pathology and Diagnostics, Strada Le Grazie 8, Verona 37134, Italy
e-mail: marco.cassatella@univr.it

Polymorphonuclear neutrophils, besides their involvement in primary defense against infections – mainly through phagocytosis, generation of toxic molecules, release of enzymes, and formation of extracellular traps – are also becoming increasingly important for their contribution to the fine regulation in development of inflammatory and immune responses. These latter functions of neutrophils occur, in part, *via* their *de novo* production and release of a large variety of cytokines, including chemotactic cytokines (chemokines). Accordingly, the improvement in technologies for molecular and functional cell analysis, along with concomitant advances in cell purification techniques, have allowed the identification of a continuously growing list of neutrophil-derived cytokines, as well as the characterization of their biological implications *in vitro* and/or *in vivo*. This short review summarizes crucial concepts regarding the modalities of expression, release, and regulation of neutrophil-derived cytokines. It also highlights examples illustrating the potential implications of neutrophil-derived cytokines according to recent observations made in humans and/or in experimental animal models.

Keywords: neutrophil, cytokine, chemokine, human, mouse

INTRODUCTION

The immune system is well suited to a quick and specific response against foreign invaders, its ultimate objective being to protect an organism from injury and disease. Cytokines represent an integral component of the signaling networks among various cells, being, for instance, essential for the development and regulation of innate and adaptive immune processes. Cytokines constitute a large family of small proteins that are produced by immune and non-immune cells and that act locally among neighboring cells to direct important biological processes such as inflammation, immunity, repair, and angiogenesis (1). In this context, the relatively recent acquisition that also activated neutrophils, among leukocytes, are able to express and release a number of cytokines (2) has convinced researchers in the field to reconsider and thus reinvestigate neutrophil biological role not only in the context of inflammatory processes, but also in other conditions (3–5). By doing so, it has clearly emerged that, given the large array of cytokines that may potentially be produced (**Figure 1**), neutrophils can be functionally involved either in physiological processes such as hematopoiesis, angiogenesis, and wound healing (2, 6), or in pathological processes including inflammatory, infectious, autoimmune, and neoplastic diseases (2, 4, 7, 8). Needless to say that, based on the afore-mentioned considerations, there is an increasing interest in clearly identifying and characterizing all the cytokines that neutrophils may produce, as well as their precise role in diseases, with the purpose of identifying novel targets for therapeutic interventions.

Abbreviations: APRIL, a proliferation inducing ligand; BAFF, B-cell activating factor; BNDF, brain-derived neurotrophic factor; Bv8, bombina variegata peptide 8; HB-EGF, heparin-binding endothelial growth factor, (EGF)-like growth factor; FGF2, fibroblast growth factor-2; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage-colony-stimulating factor; HGF, hepatocyte growth factor; IL1-ra, interleukin 1 receptor antagonist; LIGHT, lymphotoxin-related inducible ligand that competes for glycoprotein D binding to HVEM on T cells; M-CSF, macrophage-colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIP2, macrophage inflammatory protein 2; NGF, nerve growth factor; NT4, neurotrophin 4; PBEF, pre-B-cell colony enhancing factor; RANKL, receptor activator of nuclear factor kappa-B (NFκB) ligand; SCF, stem cell factor; TGFα, transforming growth factor alpha; TGFβ, transforming growth factor beta; TRAIL, TNF-related apoptosis-inducing ligand; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.

CYTOKINE EXPRESSION IN NEUTROPHILS: TOOLS AND CAVEATS

Our knowledge of the production of cytokines by neutrophils mostly derives from studies on humans and mice. Human neutrophils are usually isolated from the peripheral blood, while murine neutrophils are traditionally isolated from the bone marrow or the peritoneal cavity. Similarly to other cell types, also in neutrophils the production of cytokines is usually preceded by an increased accumulation of the related mRNA transcripts, which can be detected by techniques such as quantitative reverse-transcription polymerase chain reaction (qPCR), Northern Blotting, ribonuclease protection assay, and *in situ* hybridization (9). The fact that, per-cell, neutrophils possess 10–20 times less RNA than other leukocytes (10), illustrates the need for using rigorous isolation procedures to allow the recovery of highly purified cell populations. In fact, a mononuclear cell contamination of neutrophils equal to only 1% (or even less) can translate into up to 20–30% RNA contamination (9): the latter, depending on the expression levels of the cytokine mRNA under study, may obviously produce false positive results attributed to neutrophils. Nowadays, reliable tools guaranteeing the isolation of highly purified $CD66b^+/C11b^+/CD16^-$ neutrophils (e.g., by immunomagnetic

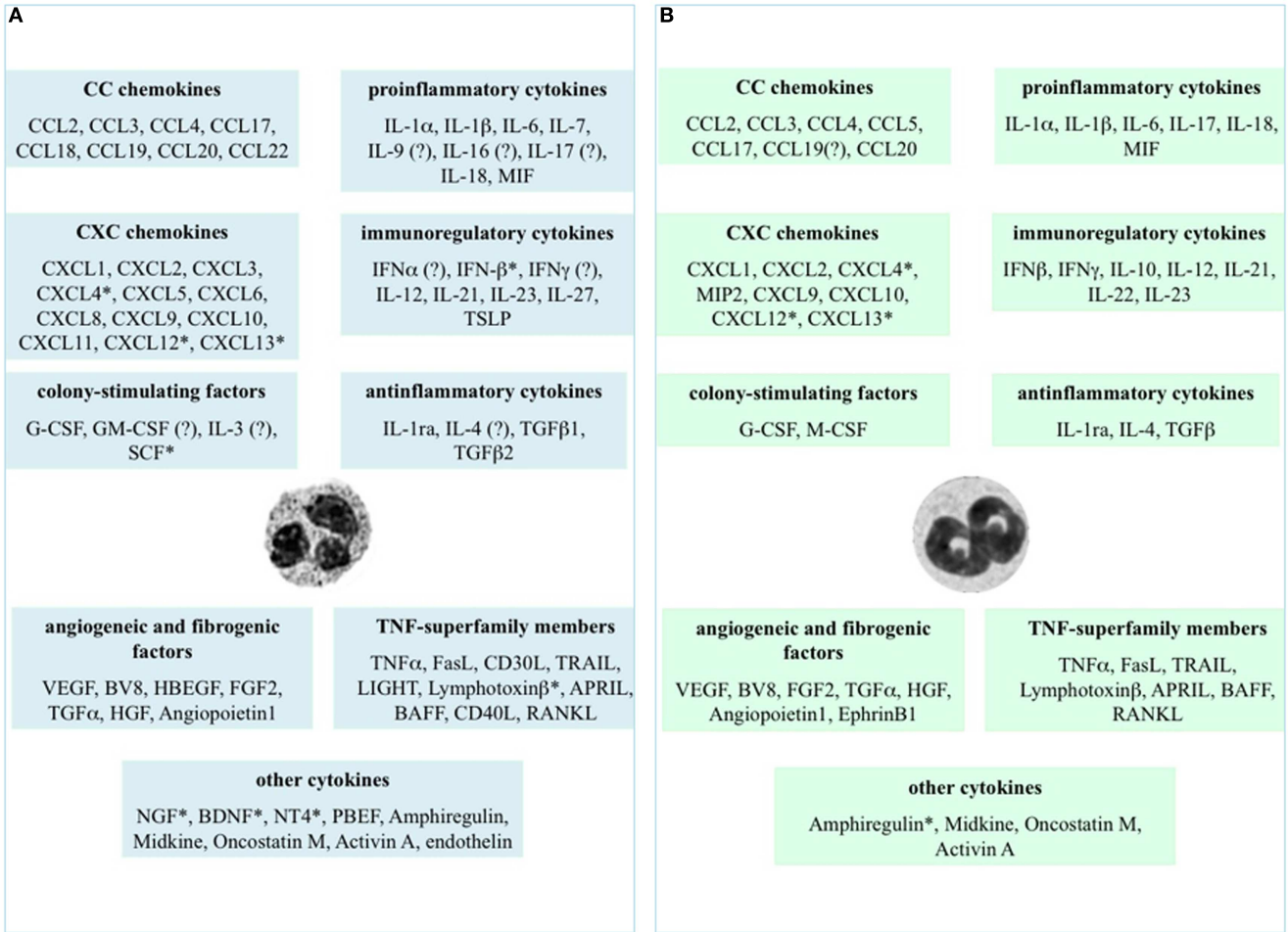

FIGURE 1 | Cytokines that neutrophils can potentially express and/or produce. Expression and/or production of the listed cytokines have been validated in human **(A)** and murine **(B)** neutrophils by gene expression techniques, immunohistochemistry, enzyme-linked immunoadsorbent assays (ELISAs), or biological assays. *Refers to studies performed at the mRNA level only. ? Indicates controversial data.

negative selection) are commercially available (11, 12). Even in mice, cytokine production by neutrophils should be carefully reevaluated by utilizing $Ly6G^{high}/CD11b^{high}$ positive cells only, as recently done (13).

It is also important to mention that, at least *in vitro* and with few exceptions, neutrophils usually make fewer molecules of a given cytokine than monocytes/macrophages or lymphocytes do on a per-cell basis (2, 10). *In vivo*, however, neutrophils constitute the majority of infiltrating cells in inflamed tissues and often outnumber mononuclear leukocytes by one to two orders of magnitude. Thus, the fact that neutrophils clearly predominate over other cell types under various *in vivo* conditions suggests that, under those circumstances, the contribution of neutrophil-derived cytokines can be of foremost importance. In any case, neutrophil-derived cytokines can be measured in cell-free supernatants or in cell lysates by using various methods, including enzyme-linked immunoadsorbent assays, radioimmunoassays, immunoprecipitation after metabolic labeling, bioassays, immunohistochemistry, intracellular staining by flow cytometry, or confocal microscopy. In our opinion, the latter two techniques should be used only to support other methods, due to potential artifacts consequent to antibody cross-reactivity or elevated neutrophil autofluorescence. Another important caveat for *in vitro* studies regards the necessity to always use endotoxin-free tissue culture media or reagents, since neutrophils respond to picomolar concentrations of lipopolysaccharide (LPS) (2, 10).

The literature demonstrates that neutrophils express and produce cytokines either constitutively or upon activation by microenvironmental stimuli (2). A variety of neutrophil receptors, including colony-stimulating factor and cytokine receptors, G protein coupled-, Fcγ- and complement receptors, or many pattern recognition receptors (PRR) (germline-encoded receptors recognizing structures in microorganisms and tissue damage products), have been shown to trigger cytokine production in neutrophils (2, 14). Among PRR, human and mice neutrophils are known to express almost all Toll-like receptors (TLRs), as well as to respond to their ligands [(15), and references therein]. TLR3 and TLR7 are actually the only TLRs that human neutrophils

do not express (16–18), unlike murine neutrophils that instead accumulate significantly high levels of TLR7 mRNA under inflammatory conditions (19, 20). Moreover, murine neutrophils do not basally express TLR3 transcripts (16, 21, 22) even though eventual accumulation has never been quantified under inflammatory condition. Interestingly, in human neutrophils, TLR4 activation by LPS fails to directly trigger the production of type I INFs and type I IFN-dependent genes as in other cell types (23, 24), due to its inability to engage the so called "TIR domain-containing adaptor protein inducing interferon β (TRIF)/TRIF-related adaptor molecule (TRAM)"-dependent pathway (23, 24).

Finally, an increasing number of studies have documented that TLR-induced cytokine expression by neutrophils can be positively/negatively influenced by immunomodulating factors such as IFNγ (25, 26) and IL-10 (27), respectively.

Following stimulation, neutrophils control their cytokine expression and production patterns by utilizing fine regulatory mechanisms acting at the transcriptional and/or post-transcriptional level (2, 4, 7, 28). Interestingly, recent studies have demonstrated that also microRNAs may regulate cytokine and chemokine production in neutrophils. For instance, miR9 was for the first time demonstrated to inhibit NFKB1/p50 transcripts in human neutrophils exposed to pro-inflammatory signals, operating in this manner as a feedback control for NFKB1/p50-dependent responses (29). More recently, miR-223 has been shown to negatively control the production of CXCL2, CCL3, and IL-6 by neutrophils, in a mouse model of *Mycobacterium tuberculosis* infection (30). These latter data have contributed in shedding light on the hitherto controversial role of neutrophils in tuberculosis (31), as they suggest that miR-223-dependent inhibitory effects may negatively control leukocyte chemotaxis at late stages of lung inflammation by means of developmentally accumulated miR-223 (30). Interestingly, examples of *de novo* synthesized cytokines that neutrophils store in significant amounts within intracellular pools also exist, and include B-cell activating factor (BAFF), TNF-related apoptosis-inducing ligand (TRAIL), CXCL8, CCL20, and interleukin (IL) 1 receptor antagonist (IL1-ra) (32, 33). However, very little is known on the precise intracellular localization and trafficking of these various cytokines and chemokines. Thus, much more work is needed to understand if, and how, the various neutrophil granules or other intracellular organelles contribute to cytokine metabolism and release (34).

CYTOKINE PRODUCTION BY NEUTROPHILS: FACTS

Figure 1 displays the cytokines that, to date, have been shown to be expressed or produced by, respectively, human (panel A) and murine (panel B) neutrophils, either constitutively or upon stimulation. It is evident that neutrophils express/produce cytokines belonging to various families, mostly including pro-inflammatory/anti-inflammatory cytokines, chemokines, immunoregulatory cytokines, tumor necrosis factor (TNF) superfamily members, and angiogenic/fibrogenic factors. At first sight, an analysis of the figure immediately suggests that the ability of neutrophils to produce such a variety of cytokines enables them to significantly influence not only the multiple aspects of the inflammatory and immune responses, but also antiviral defense, hematopoiesis, angiogenesis, and fibrogenesis. Importantly, numerous *in vivo* observations have confirmed and reproduced the *in vitro* findings, as well as often clarifying their biological meanings and implications (2). It can also be noticed that, in spite of a substantial conservation between the human and murine genomes (35), some differences in cytokine production exist between the two species (**Figure 1**), thus warning toward a *sic et simpliciter* extrapolation of data from experimental systems in animals to humans, or *vice versa*. In the case of IL-10, for instance, basal differences in the chromatin status of the *IL-10* locus, rather than a different responsiveness to activating signals, have been shown to account for the differential ability of human and murine neutrophils to switch on transcription of the IL-10 gene (36).

In the following paragraphs, some up-to-date findings illustrating potential biological roles that neutrophil-derived chemokines, pro-inflammatory/immunoregulatory cytokines, and TNF-superfamily members might have, under pathophysiological contexts, are briefly mentioned. For an extensive description on the role of neutrophil-derived cytokines in cancer and in angiogenesis, the reader may refer to our recent reviews (6, 8).

CHEMOKINES

Chemokines, amongst the cytokines produced by neutrophils, are of particular relevance because of their singular ability to selectively recruit discrete cell populations into sites of injury and thereby effectively regulate leukocyte trafficking (37). In addition, chemokines play fundamental roles in coordinating immune system responses, in regulating B- and T-cell development and in modulating angiogenesis (38). As displayed in **Figure 1**, both human and murine neutrophils may potentially produce several chemokines upon activation, including IL-8/CXCL8, GROα/CXCL1, MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3) and MIP-1β/CCL4 (37). Because the chemokines produced by neutrophils are primarily chemotactic for neutrophils, monocytes, dendritic cells (DCs), natural killer (NK) cells, and T-helper type 1 (Th1) and type 17 (Th17) cells, a potential role for neutrophils in amplifying their own arrival (39), as well as in orchestrating the sequential recruitment to, and activation of, distinct leukocyte types in the inflamed tissue, is plausible (37, 40). And in fact, in *in vitro* experiments, it has been demonstrated that: (i) human neutrophils activated by neutrophil-activating protein A from *Borrelia burgdorferi* recruit IFNγ- and IL-17-producing T lymphocytes *via* CCL2, CCL20, and CXCL10 release (41); (ii) LPS-activated neutrophils induce chemotaxis of immature and mature DCs, as well as adhesion of CCR6- and CCR7-expressing T cells *via* CCL19 and CCL20 (42); and (iii) IFNγ plus LPS-activated neutrophils induce chemotaxis of Th17 cells through CCL2 and CCL20 (11). Moreover, in different mouse models it has been proved that: (i) DCs are recruited to the *Leishmania* inoculation site by neutrophil-derived CCL3 (43); (ii) immature DCs are strongly attracted by neutrophil-derived CCL3, CCL4, CCL5, and CCL20 triggered by *Toxoplasma gondii* (44); and (iii) macrophage influx to granulomas is dependent on CCL3 and CCL4 released by activated neutrophils (45). More recently, CCL17, a chemokine that binds to CCR4, a chemokine receptor expressed in T-helper type 2 (Th2) cells and in regulatory T cells (Tregs) (46), has been

added to the list of neutrophil-derived chemokines (47, 48). Consistently, tumor-associated neutrophils (TANs) have been shown to chemoattractants Tregs in a mouse model of cancer, mainly *via* CCL17 (49). Because neutrophil depletion, in this model, was shown to reduce Tregs recruitment and, consequently, tumor growth, data provide, for the first time, a clear link between TANs and Tregs, acting together to impair antitumor immunity (49).

PRO-INFLAMMATORY AND IMMUNOREGULATORY CYTOKINES

As shown in **Figure 1**, neutrophils may become a significant source of pro-inflammatory and/or immunoregulatory cytokines. Among these cytokines, recent research has focused on neutrophil-derived IL-17 and IFNγ, as well as on their eventual role in inflammatory diseases and/or in protection against infections. However, since the data on the effective capacity of human neutrophils to express/produce IL-17 (11, 50) or IFNγ (51, 52) are still controversial in literature, we can only report on studies carried out in mice.

For instance, IL-23- and, indirectly, IL-12-, activated Gr-1$^+$-neutrophils, but not Th17 cells, have been found to be the predominant source of IL-17A in a mouse model of kidney ischemia-reperfusion injury (IRI) (53). Such neutrophil-derived IL-17 has been then shown to regulate natural killer T cells (NKT) activation, IFNγ production, neutrophil infiltration, ultimately inflammation, and tissue injury (53), thus establishing its requirement for kidney injury following IRI. In another, more recent study, researchers have identified a murine population of bone marrow neutrophils that constitutively express the transcription factor RORγt, and that rapidly produce and respond to IL-17A in a IL-23 plus IL-6-dependent manner (54). Autocrine activity of IL-17A on such neutrophil subset has been shown not only to induce the production of reactive oxygen species (ROS), but also to increase neutrophil-mediated fungal killing *in vitro* and, importantly, also in an *in vivo* model of *Aspergillus fumigatus*-induced keratitis (55). In a similar fashion, IL-17A produced by Ly6G$^+$-neutrophils, but not by NKT or γδT cells, was found to be important in providing protection against early pneumonic plague infection in mice (56). In this model, however, neutrophil-derived IL-17A did not significantly change neutrophil bactericidal activities, but was instead crucial for the IFNγ-mediated programming of M1 pro-inflammatory macrophages after *Yersinia pestis* challenge (56).

A number of *in vivo* experiments have reported that, in response to a variety of pathogens, including *Nocardia asteroides* (57), *Listeria monocytogenes* (58), and *Plasmodium berghei* (59), murine neutrophils secrete IFNγ, a crucial orchestrator for host defense against intracellular pathogen. Neutrophils have been found to be an important source of IFNγ also upon *Toxoplasma gondii* infection, in a model of genetically modified mice lacking all lymphoid cells due to deficiencies in Recombination Activating Gene 2 (RAG2) and IL-2Rγc genes (60). In these mice, although insufficient for complete host protection, neutrophil-derived IFNγ was found to be TLR11-independent and to significantly reduce pathogen load therefore extending mice survival (60). Moreover, other studies have shown that migrated neutrophils are responsible for the early production of IFNγ during pneumonia infections, in turn regulating bacterial clearance in mice (61). Interestingly, such IFNγ production does not require either IL-12, or CD11/18 complex, CD44, TLR2, TLR4, TRIF, and Nrf2, while it is nearly abolished in Nox2 deficient mice (62). Altogether, data not only underline the complexity of the neutrophil responses during pneumonia, but also highlight how tightly regulated is the process of IFNγ induction in neutrophils, as it likely involves interactions between multiple signaling pathways.

TNF-SUPERFAMILY MEMBERS

Human and murine neutrophils also express and produce many TNF-superfamily members (**Figure 1A,B**), although at variable levels (2). For example, human neutrophils synthesize – at least *in vitro* – very low amounts of TNFα (in the order of picogram per milliliter per million cells) in response to TLR agonists (2), which nonetheless exert potent autocrine effects in amplifying neutrophil-derived cytokines and chemokines [(63) and other unpublished observations from our group]. *In vivo*, neutrophil-derived TNFα has been recently described to either instruct skin Langerhans cells to prime antiviral immune responses (64), or to stimulate melanoma cells to migrate towards endothelial cells and metastasize to the lungs, in a mouse model of primary cutaneous melanoma undergoing repetitive ultraviolet (UV) exposure (65). Moreover, through application of confocal intravital microscopy to the mouse cremaster muscle, it has been very recently shown that chemoattractans-responding neutrophils release TNFα when in close proximity of endothelial cell junctions. Further, in TNF receptor (TNFR) (−/−) mice, neutrophils accumulated normally in response to chemoattractants administered to the cremaster muscle or dorsal skin, whereas neutrophil-dependent plasma protein leakage was abolished, suggesting that neutrophil-derived TNFα mediates microvascular leakage (66).

On the other hand, neutrophils have turned-out as a major source of both BAFF (67) and a proliferation inducing ligand (APRIL) (68), two TNF members that are critical for B-cell maturation, function, and survival. Accordingly, BAFF and APRIL (other than IL-21 and, possibly, CD40L) have proven to be fundamental mediators of the functions of a recently identified neutrophil subset in human spleen – the so called "B-helper" neutrophil subset – precisely for their ability to stimulate immunoglobulin diversification and production by splenic marginal zone B-cells (69). An involvement of BAFF-producing splenic neutrophils in the pathogenesis of murine lupus has been also demonstrated in a recent study, suggesting that neutrophils help to shape CD4$^+$ T cell responses *via* BAFF, which in turn contributes to the production of pathogenic autoantibodies (70).

Finally, another TNF-superfamily member that both human and murine activated neutrophils can produce and release is TRAIL, a trans-membrane/soluble molecule involved in tumor cell killing and autoimmunity (33). In humans, neutrophil-derived TRAIL has been classically detected *ex vivo* in the context of intravesical BCG infusion and systemic IFNα administration to treat, respectively, bladder cancer and chronic myeloid leukemia (71, 72). Since previous *in vitro* data had shown an effective TRAIL-mediated cytotoxicity of neutrophils towards leukemic cells (73, 74), it is conceivable to hypothesize further studies aimed at harnessing neutrophils against tumors, possibly *via* TRAIL induction. Nonetheless, our knowledge on the TRAIL production by neutrophils has been more recently extended to the mouse

system, as neutrophil-derived TRAIL has been shown to exert antiviral activities in a model of cytomegalovirus infection (75), as well as to mediate early bacterial killing in a model of pneumococcal pneumonia (76). Based on the afore-mentioned and other (2, 8, 42, 43) observations, it appears that TNF-superfamily members may contribute to a large extent to unexpected functions that neutrophils may exert.

CONCLUDING REMARKS

During the past decades, novel functions in homeostasis and pathology have emerged for neutrophils, mainly for their ability to represent a source for a variety of cytokines. It is plausible that with the development of very efficient cell isolation techniques and the increased availability of neutrophils purified from various compartments, such as spleen, peritoneal exudates, lungs, oral cavity, skin, bone marrow, cord blood, and placenta, our knowledge of the repertoire of cytokines produced by human and mouse neutrophils will expand.

Apart from what has been briefly summarized in this review, a number of issues remain to be better explored and/or clarified in this research area.

For instance, we need to elucidate all the stimuli that are able to induce cytokine synthesis in neutrophils. Such studies would be particularly helpful in understanding the pathogenesis of diseases in which neutrophils represent (or are presumed to be) the first cell type encountering, and interacting with, the etiologic agent. In fact, we know that the interaction of neutrophils with a given agonist produces a characteristic, stimulus-specific response (2). The recent findings that human neutrophils possess intracellular sensor systems that allow the recognition of foreign and potentially dangerous RNA and DNA, as well as the inflammasomes (17, 77–80), demonstrates that neutrophils, *via* cytokine/chemokine release, are in the position to act at the front-line of immunity not only toward extracellular, but also toward intracellular microorganisms, including viruses. Another aspect that needs to be more systematically dissected concerns the identification of the molecular mechanisms controlling cytokine expression in neutrophils. Such studies may lead to the identification of novel, maybe neutrophil-specific, transcription factors, or of neutrophil-specific chromatin organization programs (36). Finally, more information on the *in vivo* role of neutrophil-derived cytokines should be acquired in humans, since it mostly derives from experimental animal models. By doing so, it is tempting to predict that unanticipated functions of neutrophils can be discovered. Future challenges for scientists in the field will be to translate all these new insights into efficacious neutrophil-targeted therapies for the treatment of inflammatory conditions without compromising immunity.

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Mast cell mediators: their differential release and the secretory pathways involved

Tae Chul Moon[1], A. Dean Befus[1] and Marianna Kulka[2]*

[1] *Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, AB, Canada*
[2] *National Institute for Nanotechnology, National Research Council, Edmonton, AB, Canada*

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Frank A. Redegeld, Utrecht University, Netherlands
Axel Lorentz, University of Hohenheim, Germany

***Correspondence:**
Marianna Kulka, National Institute for Nanotechnology, National Research Council, 11421 Saskatchewan Drive, Edmonton, AB T6G 2M9, Canada
e-mail: marianna.kulka@nrc-cnrc.gc.ca

Mast cells (MC) are widely distributed throughout the body and are common at mucosal surfaces, a major host–environment interface. MC are functionally and phenotypically heterogeneous depending on the microenvironment in which they mature. Although MC have been classically viewed as effector cells of IgE-mediated allergic diseases, they are also recognized as important in host defense, innate and acquired immunity, homeostatic responses, and immunoregulation. MC activation can induce release of pre-formed mediators such as histamine from their granules, as well as release of *de novo* synthesized lipid mediators, cytokines, and chemokines that play diverse roles, not only in allergic reactions but also in numerous physiological and pathophysiological responses. Indeed, MC release their mediators in a discriminating and chronological manner, depending upon the stimuli involved and their signaling cascades (e.g., IgE-mediated or Toll-like receptor-mediated). However, the precise mechanisms underlying differential mediator release in response to these stimuli are poorly known. This review summarizes our knowledge of MC mediators and will focus on what is known about the discriminatory release of these mediators dependent upon diverse stimuli, MC phenotypes, and species of origin, as well as on the intracellular synthesis, storage, and secretory processes involved.

Keywords: granule, lipid body, exosome, lysosome, exocytosis, secretion

INTRODUCTION

Human mast cells (MC) are often characterized by their ability to release a variety of important mediators with a diversity of biological activities (1). The regulated release of peptides, amines, lipids, and even some gases depends on several molecular pathways: a prominent one of which releases large dense core vesicles (granules) through regulated exocytosis (degranulation); other pathways depend upon *de novo* production of mediators and complex vesicle trafficking and recycling, including constitutive secretion, exosomal and endosomal pathways; and other secretory pathways that are not dependent upon vesicles or membrane-bound moieties [e.g., gases such as nitric oxide by diffusion (2), lipid mediators from lipid bodies]. Although research is providing important new insights, we understand remarkably little about how the mediators are sorted into these secretory pathways and differentially released (**Tables 1** and **2**). Unanswered questions include: how are these pathways similar/dissimilar; how are mediators sorted into various compartments (e.g., progranules, granules, lysosomes, secretory vesicles, and exosomes); which stimuli activate these secretory pathways, and which proteins are involved; how do MC selectively release different cargo given different stimuli?

Constitutive exocytosis occurs in the absence of discernable stimuli for trafficking of secretory vesicles to the plasma membrane and can occur throughout the lifetime of a cell (3). Regulated exocytosis occurs after a clearly defined stimulus, either through changes in the extracellular environment [temperature (4, 5), pH (6), radiation (7), or osmolarity (8)] or ligation of a cell surface receptor (9). The pathways that control constitutive and regulated exocytosis have been extensively studied using powerful tools in high-resolution microscopy, molecular biology and animal model systems, and some of the molecules involved have been identified.

The terms degranulation, secretion, and exocytosis are often used interchangeably but have subtle variations in meaning. Degranulation refers to the loss of or release of granules and is most often associated with MC and basophils, both of which are characterized by their large intracellular granules. Secretion involves the release of a substance from one place of containment to another, i.e., from a cell to its extracellular environment or a gland to the skin's surface. Excretion is the elimination of a waste material from a cell or organ. Exocytosis is a process of cellular secretion or excretion in which substances contained in vesicles are discharged from the cell by fusion of the vesicular membrane with the outer cell membrane (10–12). MC exhibit all forms of these release events but MC are perhaps best known for their rapid secretion of granules (degranulation) that contain large stores of pre-formed mediators (9).

This review identifies our current understanding of the biogenesis of various mediator compartments, and the mechanisms of sorting and release of mediators from these compartments

Abbreviations: AND, anaphylactic degranulation; BMMC, bone marrow-derived mast cell; ER, endoplasmic reticulum; LAMP, lysosomal membrane protein; MC, mast cell(s); M6PR, mannose-6-phosphate receptor; PMD, piecemeal degranulation; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; STX, syntaxin; Syt, synaptotagmin; VAMP, vesicle associated membrane protein.

Table 1 | Mediators stored in human mast cell granules and their sorting mechanisms.

Mediator	Sorting mechanism(s)	Reference
Amines	Vesicular monoamine transporter (VMAT)-2-dependent	(64, 65)
	Serglycin proteoglycan-dependent electrostatic interaction	(66)
Histamine		(20, 28)
Polyamines		(71)
Proteoglycans	Unknown	
Heparin		(36, 37)
Chondroitin sulfates		(36)
Serglycin		(60, 66)
Proteases	[a]Serglycin proteoglycan-dependent electrostatic interaction	
Tryptases		(21–31)
Tryptase-α		
Tryptase-βI		
Tryptase-βII		
Tryptase-βIII		
Tryptase-γ		(25, 27)
Tryptase-δ		(26)
Chymase-1		(30)
Cathepsin G		(30)
Granzyme B		(31)
Carboxypeptidase A3		(29)
Lysosomal enzymes	[b]Fusion with secretory lysosome	
β-Glucuronidase		(20)
β-Hexosaminidase		(20)
Arylsulfatase		(20)
Cytokines	Unknown	
TNF	[c]Endosomal pathway	(32)
bFGF		(33)
IL-4		(34)
SCF		(35)

[a]*Human mast cells proteases may be sorted into granules by serglycin proteoglycan-dependent electrostatic interaction based on the mouse study (52).*
[b]*Lysosomal enzymes in human mast cell granules may be sorted by fusion of secretory lysosome and/or late endosome shown in RBL-2H3 cells (see **Figure 2**) (42).*
[c]*Human mast cells sort TNF into granules via endosomal pathway, but rodent mast cells do it via mannose-6-phosphate receptor (M6PR)-dependent pathway (see **Figure 2**).*

(**Figure 1**). We present some new postulates about exocytosis that may be particularly relevant to the MC, a highly specialized secretory cell (13). We also refer the readers some excellent recent articles for more details on various aspects of this subject (9, 14–19).

PRE-STORED MEDIATOR RELEASE FROM MC GRANULES

MEDIATORS STORED IN MC GRANULES

Mast cells are morphologically characterized by numerous, electron dense cytoplasmic granules which contain biogenic amines [histamine, serotonin] (20); several serine and other proteases {e.g., tryptase-α, -βI, -βII, -βIII, -γ [protease, serine S1 family member (PRSS) 31], -δ, chymase-1, cathepsin G, granzyme B, and carboxypeptidase A3} (21–31); lysosomal enzymes [β-glucuronidase (20), β-hexosaminidase (20), arylsulfatase (20)]; some cytokines [TNF (32), bFGF (33), IL-4 (34), and SCF (35)]; and proteoglycans [heparin (36, 37), chondroitin sulfates (36)] (**Table 1**). MC are able to overcome the large thermodynamic hurdle of storing high concentrations of these mediators in their granules by trapping them in an anionic gel matrix composed of chondroitin sulfates and heparin (38).

Subtypes of human MC are distinguished by the presence or absence of different serine proteases in their granules (i.e., tryptase$^+$/chymase$^-$: MC_T, tryptase$^+$/chymase$^+$: MC_{TC}, and tryptase$^-$/chymase$^+$: MC_C). MC activation has typically been measured by monitoring the release of granule mediators (degranulation), with a particular focus on histamine, β-hexosaminidase, or tryptase (39, 40). Pre-stored mediator release through MC degranulation can be an early and rapid event following stimulation, resulting in the release of large portions of stored histamine within 15–90 s. This release of pre-formed mediators enables not only rapid anaphylactic reactions and allergic responses but also initiates recruitment of leukocytes to sites of pathogen invasion, activation of innate immune processes, and inflammatory responses (1). Other longer term responses associated with granule-derived mediators include wound healing and tissue remodeling processes through multiple communications with other cells (e.g., fibroblast proliferation and extracellular matrix production by histamine and MC proteases) (41).

MC GRANULE HETEROGENEITY AND BIOGENESIS

Heterogeneity

Mast cells granules, also called secretory lysosomes, contain both lysosomal proteins such as acid hydrolases, e.g., β-hexosaminidase, as well as mediators such as histamine, and can secrete both together. MC also contain traditional lysosomes that can release enzymes such as β-hexosaminidase independently of histamine (42). Raposo et al. (43) distinguished three types of granules in mouse bone marrow-derived MC (BMMC) based on their contents of MHC class II, the lysosomal marker β-hexosaminidase, lysosomal membrane protein (LAMP)-1, LAMP-2 and mannose-6-phosphate receptors (M6PR), and the biogenic amine, serotonin: type I granules contain MHC class II, β-hexosaminidase, LAMP-1, LAMP-2, and M6PR but not serotonin (perhaps a classical lysosome); type II granules contain MHC class II, β-hexosaminidase, LAMP-1, LAMP-2, M6PR, and serotonin (perhaps a late secretory lysosome); type III granules contain β-hexosaminidase and serotonin but not MHC class II (**Table 3**) (42, 43). Baram et al. proposed a model wherein type II granules are generated by fusion of type I and type III granules,

Table 2 | Stimuli-selective mediator release from mast cells (some representative examples).

Stimulus	Mechanism	Mediators	MC Types	References
DEGRANULATION AND *DE NOVO* SYNTHESIZED MEDIATOR RELEASE				
Antigen	FcεRI	Histamine	BMMC[a], RBL-2H3	(155–161)
		cysLTs, PGD_2, cytokines, chemokines, NO, ROS	hPBDMC, LAD2, HMC-1, rat PMC	
Neuropeptides (substance P, CGRP, capsaicin, etc.)	NKRs	β-Hexosaminidase, cytokines, chemokines	LAD2, hPBDMC	(162)
		cysLTs, PGD_2	BMMC	(163, 164)
		5-HT	Rabbit MC	(165)
Compound 48/80	MrgprX2	β-Hexosaminidase, cytokines, chemokines, PGD_2	BMMC	(164, 166)
Cathelicidin	GPCR	Histamine	Rat PMC	(167)
		Cytokines, chemokines, PGE_2, LTC_4	LAD2, hPBDMC	(168)
Defensins	GPCR	Histamine	Rat PMC	(167, 169, 170)
		Cytokines, chemokines, PGD_2, PGE_2, LTC_4	LAD2, hPBDMC	(168)
Pleurocidin	FPRL1 (GPCR)	β-Hexosaminidase, PGD_2, cysLTs, cytokines, chemokines	hPBDMC, LAD2	(171)
A23187	Ca^{2+} ionophore	Histamine	huMC, hPBDMC	(161, 172)
		β-Hexosaminidase	HMC-1	(173)
		Cytokines	FLMC	(174)
Morphine, codeine	Opioid receptors	β-Hexosaminidase, cytokines, chemokines	hPBDMC, LAD2	(175, 176)
Monomeric IgE	FcεRI	Cytokines,	BMMC	(177–179)
		β-Hexosaminidase	RBL-2H3	
			hCBDMC	
Nerve growth factor	Trk receptor	Histamine, PGD_2, PGE_2 cytokines	Rat PMC, BMMC	(180, 181)
***DE NOVO* SYNTHESIZED MEDIATOR RELEASE WITHOUT DEGRANULATION[b]**				
Zymosan, PGN, LTA	TLR2	GM-CSF, IL-1β, cysLTs	huMC	(88)
	Dectin-1 receptor	ROS	BMMC	(182)
PolyI:C, viral particles	TLRs	Cytokines	huMC progenitor	(183)
			LAD2, HMC-1, hPBDMC, BMMC	(184)
			KU-812	(185)
LPS	TLR4, CD14	Cytokines, chemokines	BMMC[c]	(186)
SCF	F-actin polymerization	Cytokines	hPBDMC	(187)
	MAP kinase kinase 3	Cytokines	BMMC	(188)
Lectins (ex: galectins)	TIM-3	Cytokines	HMC-1	(189)
DEGRANULATION WITHOUT *DE NOVO* SYNTHESIZED MEDIATOR RELEASE EXCEPT ROS[d]				
Complement peptides (C3a, C5a)	Complement receptors	Histamine	Human skin MC	(190–192)
Insect venoms	Guanylate cyclase	Histamine	Rat PMC	(193)

(Continued)

Table 2 | Continued

Stimulus	Mechanism	Mediators	MC Types	References
Pollutants (i.e. acrolein)		Histamine, ROS	RBL-2H3	(194)
Persulfate salts		Histamine, ROS	LAD2, KU-812	(195)
Advanced glycation endproducts (AGEs)		Histamine, ROS	Rat PMC	(196)
UV radiation		Tryptase	Human skin MC	(197)
Particulates (sodium sulfite, titanium dioxide nanoparticles, silver nanoparticles)	Non-FcεRI-mediated	Histamine, ROS	RBL-2H3 Rat MC	(198, 199) (200)
NEITHER DEGRANULATION NOR *DE NOVO* SYNTHESIZED MEDIATOR RELEASE EXCEPT ROS				
IgG	FcγRI, RIIA, RIII	ROS	BMMC, rat PMC, hPBDMC	(201)
Mercuric chloride ($HgCl_2$)		ROS	Rat PMC	(202)
Gold compounds		ROS	Rat PMC	(202)
D-penicillamine		ROS	Rat PMC	(202)
Mechanical stretch		??	RBL-2H3	(203)
Gamma radiation		??	BMMC, hPBDMC	(204)

[a] *BMMC, mouse bone marrow-derived mast cell; CGRP, calcitonin gene related peptide; cysLTs, cysteinyl leukotrienes; FLMC, fetal liver-derived mast cell; FPRL1, N-formyl-peptide receptor 1; GPCR, G-protein coupled receptor; hCBDMC, human cord blood-derived mast cell; hPBDMC, human peripheral blood-derived mast cell; huMC, human mast cell; MC, mast cell; MrgprX2, Mas-related G-protein coupled receptor member X2; NO, nitric oxide; RBL-2H3, rat basophilic leukemia-2H3; ROS, reactive oxygen species; rat PMC, rat peritoneal mast cell; TIM-3, T cell immunoglobulin and mucin domain-containing protein 3; TLR, toll-like receptor; Trk receptor, neurotrophic tyrosine kinase receptor.*

[b] *No detectable degranulation or very minimal degranulation detected at the time points and doses tested thus far.*

[c] *Reported in murine MC but not in human MC.*

[d] *None or minimal secretion of de novo synthesized mediators except ROS that have been tested thus far.*

which contain lysosomal proteins and secretory amines, respectively (42). However, there has been little experimental follow-up of this postulate and there is evidence that MC have more diverse types of granules than depicted by this model (**Figure 2**). Indeed, the relationship between this classification of granules and observations that serotonin and cathepsin D vs. histamine and TNF exist in distinct granule populations (see below) in mouse MC is unclear (44). It is likely that MC granules are more heterogeneous than the three types shown above (**Figure 2**; **Table 3**) and that this heterogeneity may depend on the tissue of residence and the species, health status, and even age of the individual (1, 45).

Biogenesis

The biogenesis of MC granules involves regulated fusion of what are called unit granules (small fusogenic granules) (46). These early unit granules buds from the trans-Golgi region and fuse to generate progranules in a region delimited by the outermost Golgi cisternae, rough endoplasmic reticulum (ER), and mature granules in the cytoplasm. The volumes of progranules are multiples of unit granules (i.e., volume of progranule created by three unit granules is three times unit granule volume). Progranules leave this zone as immature granules and become mature through a fusion process with other immature or mature granules. A process called "condensation" reduces the granule volume and organizes the contents, generating various sizes of mature granules (15). In addition to the homotypic fusion, which is postulated to form type III granules, immature granules or type III granules are also able to fuse to endosomes or lysosomes (Type I granules) in what might be the pathway that forms type II granules (secretory lysosomes) as Baram et al. proposed (see above and **Figure 2**) (42, 43). However, MC granules are likely more heterogeneous than the three types postulated, and our understanding of the later phase of granule biogenesis is thus depicted inside a black area in **Figure 2**.

Molecules involved in MC granule biogenesis

Several proteins involved in MC granule biogenesis and maturation have been identified (**Table 4**).

Rab GTPases. Rab3d and Rab5 play roles in the fusion of immature granules. MC from Rab3d knockout mice have granules that are larger than in MC from wild-type mice (47), while knockdown of endogenous Rab5 or expression of constitutively negative mutants of Rab5 significantly reduces the size of granules and increases their number (48). Moreover, Rab5 plays a role not only in homotypic granule fusion (type III granule biogenesis) but also in granule/endosome heterotypic fusion (type II granule biogenesis), and vesicle associated membrane protein (VAMP)-8 is involved in Rab5-mediated fusion of granules.

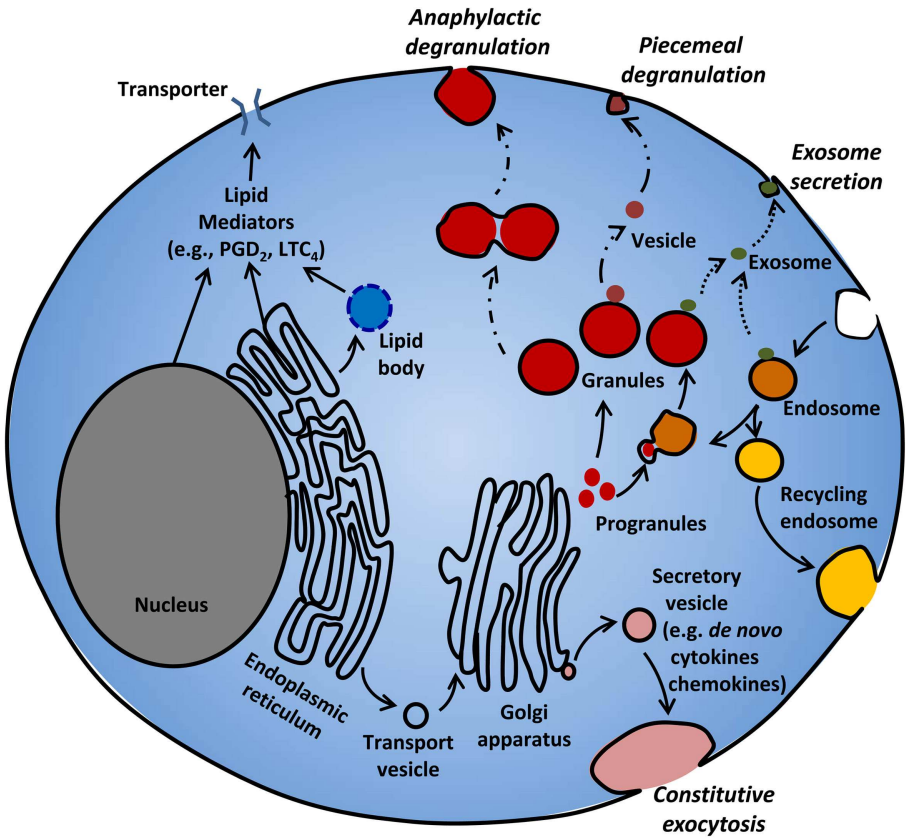

FIGURE 1 | Mediator release from MC. MC release various mediators from different compartments following different stimuli. MC rapidly release pre-stored granule contents by piecemeal or anaphylactic degranulation. Immature progranules and mature granules can fuse with endosomes, and store lysosomal proteins. Some mediators can be released from granules and endosomes through exosomal secretion. Lipid mediators such as PGD_2 and LTC_4 are synthesized in lipid bodies, nuclear and ER membranes, and released through active transporters. *De novo* synthesized cytokines and chemokines packaged in secretory vesicles are released through constitutive exocytosis.

Table 3 | Mast cell secretory granule subsets.

	Contents	Associated proteins	Reference
Type I	Cathepsin D	LAMP-2	(44)
	β-Hexosaminidase		(42)
		MHC-II	(43)
		M6PR	(72)
		LAMP-1 and 2	(43)
Type II	Histamine		(44)
	Serotonin	VAMP-8	(42, 44)
	β-Hexosaminidase		(42, 44)
		MHC-II	(43)
		M6PR	(72)
		LAMP-1 and 2	(43)
Type III	TNF (may be in type II as well)		(44)
	Serotonin	VAMP-8	(42, 44)
	β-Hexosaminidase		(42, 44)
		M6PR	(72)

Lysosomal trafficking regulator. Chediak–Higashi syndrome, a mutation of the lysosomal trafficking regulator (*LYST*) causes the formation of giant granules in many cells including MC, and can be studied using the orthologous lyst-deficient beige ($Lyst^{bg}/Lyst^{bg}$) mouse. In MC and pancreatic acinar cells of beige mice, there is giant granule formation, presumably the result of disordered fusion of granules, suggesting Chediak–Higashi syndrome (CHS)/*Lyst* plays a role in controlling granule fusion (15, 49).

Synaptotagmins. The synaptotagmins (Syts) are membrane-trafficking proteins with at least 15 members in mammals. The RBL-2H3 MC line expresses Syt II, III, V, and IX (42) and RBL-2H3 treated with antisense to Syt III showed enlarged granule size, impairment in granule maturation, and formation and delivery of internalized transferrin to the perinuclear endocytic recycling compartment involved in a slow recycling pathway (50).

Granin family. The granin family of proteins, first described in neuroendocrine cells, are also important in MC granule biogenesis and maturation. Secretogranin III, for example, is present in MC granules, depleted during MC degranulation and overexpression of this protein causes an expansion of the secretory

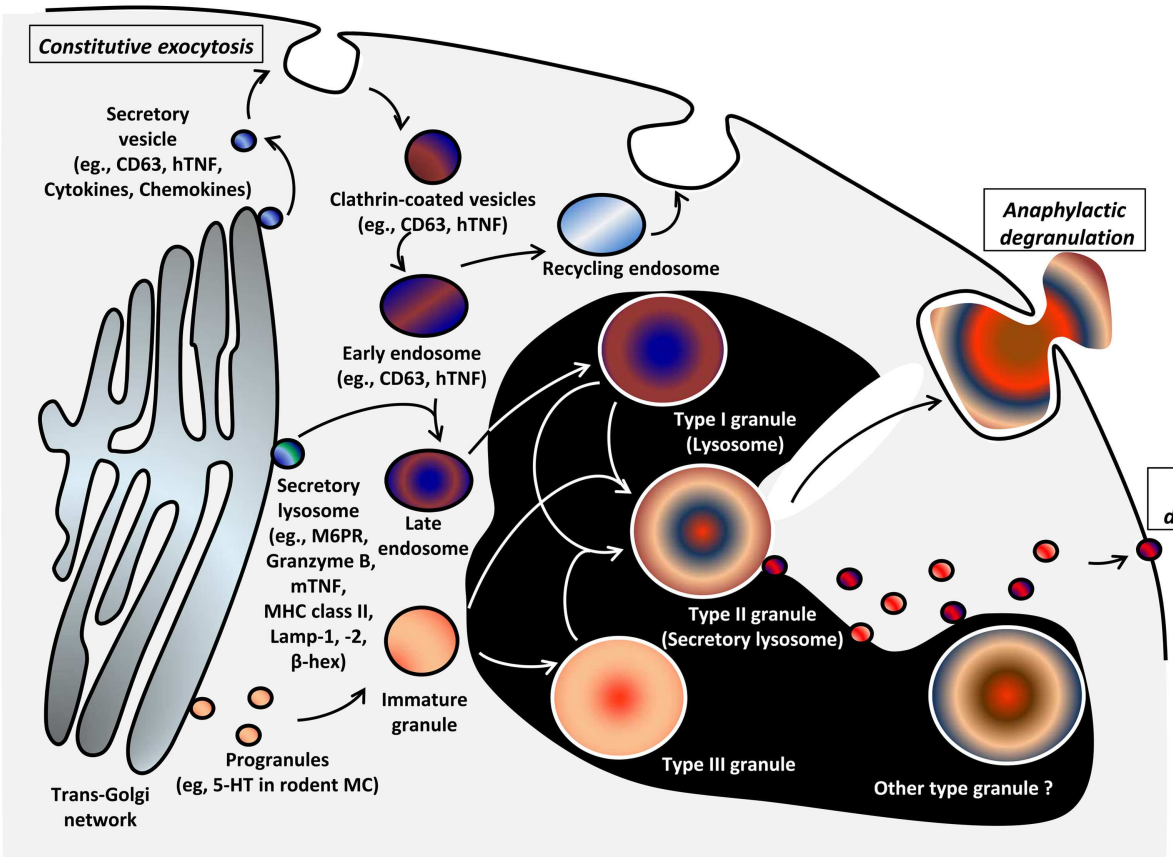

FIGURE 2 | Model of genesis of MC secretory lysosomes (granules) and their heterogeneity/plasticity [adapted from Raposo et al. (43)]. Type I granules and type III granules are formed from lysosomal/endosomal pathway and by unit granule fusion from the trans-Golgi region, respectively. Secretory lysosomes that bud from trans-Golgi network contain MHC class II molecules, mannose-6-phosphate receptor (M6PR), and the lysosomal markers LAMP-1, -2, and β-hexosaminidase. It is postulated that post-endosomal, type II secretory lysosomes arise through the fusion of Type I and III granules. The relationship of this model to observations of heterogeneity of secretory lysosomes with regard to histamine or 5-HT content and VAMP-8 expression is unclear and there likely exists more granule heterogeneity/plasticity than three types (44). The mechanism of genesis of granule types is poorly understood (black area).

vesicle compartment (51). Although the biologic role of this family of proteins is not well understood, they are involved in cholesterol sequestration, interact with chaperones of granule proteins, serve as precursors of granule cargo and function as calcium buffering proteins, making them potentially intriguing players in the life history of MC granules. Is it possible that some of their many functions are necessary to control progranule fusion and thereby aid their maturation of secretory lysosomes/granules? Could these proteins somehow regulate the core components of the fusion machinery and thereby determine which progranules fuse together? Further experimentation is required in this area.

Histamine and proteoglycans. Some granule mediators themselves such as histamine and the core proteoglycans such as serglycin are also important components of the granule maturation process. For example, BMMC from serglycin knockout mice have functional secretory granules but they are defective in dense core formation (52). Furthermore, these serglycin$^{-/-}$ BMMC are resistant to apoptosis associated with reduced release of proteases and defective caspase-3 activation (53). In addition, the lack of histamine or the enzymes that control its synthesis significantly alters the morphology and contents of granules. Peritoneal MC from histidine carboxylase knockout mice show abnormal granule morphology and contain fewer proteases and heparin (54). This is mainly due to the down-regulation of genes encoding granule proteases and enzymes involved in heparin biosynthesis. Interestingly, agonists of the H4 histamine receptor and exogenous application of histamine restored granule maturation. Therefore, histamine likely influences early steps in granule maturation but has little role in maintaining the integrity of fully formed mature granules since depletion of histamine from mature MC had no obvious effect on granule structure. It would be interesting to examine the effect of histamine depletion on immature MC (i.e., from CD34^{+} progenitors) as they progress through the MC differentiation process.

Adaptor-protein family. Components of our models of MC granule biogenesis can be extrapolated from other cells with complex secretory granule processes, such as pituitary lactotropes,

Table 4 | Molecules that are (or may be) involved in mast cell granule biogenesis and homeostasis.

Protein	Function	Cell type	Reference
DEMONSTRATED IN MAST CELLS			
Histidine decarboxylase	Promotes granule maturation	BMMC	(54)
Synaptotagmins	Membrane-trafficking	RBL-2H3	(42)
Secretogranin III	Regulates membrane dynamics of secretory vesicles via interaction with chromogranin A	RBL-2H3	(51)
Chromogranin A	Binds secretogranin and promotes granule biogenesis	RBL-2H3	(51)
Clathrin	May be involved in compensatory endocytosis following exocytosis and granule recycling	Mouse peritoneal MC	(205)
Polyamines	Regulate granule cargo storage and granule morphology	BMMC	(71)
Vesicular monoamine transporter 2 (VMAT2)	Transport of monoamines into secretory granules	Mast cells, megakaryocytes, thrombocytes, basophils, and cutaneous Langerhans cells from patients with mastocytosis	(206)
Serglycin	Retention of proteases in granules	BMMC	(52)
Nuclear receptor 4a3	Modifies granule contents	BMMC	(207)
DEMONSTRATED IN OTHER CELL TYPES BUT MORE WORK NEEDED IN MAST CELLS (POSSIBLE NEW PATHWAYS)			
V-ATPase	Hyperacidification of lysosomes	Many cell types	(208)
AP-1A	Transports cargo between the trans-Golgi network and endosomes	Corticotrope tumor cells	(209)
Rabs (32 and 38)	Trafficking enzymes into vesicles	Melanocytes	(210)

pancreatic β-cells, and transfected tumor cells. In these cells, progranules in the trans cisterna of the Golgi are covered with clathrin coats, which contain the adaptor-protein (AP) family of proteins that can bind cytosolic tails of transmembrane protein cargo, facilitating their entry into budding vesicles (55). Immature secretory granules are not responsive to secretagogues and it appears that one of the essential roles of the AP proteins is to facilitate their maturation (56). Could APs perform a similar function in MC? Clearly, additional studies are needed to further understand the genesis, heterogeneity, and plasticity of MC granules and uncover potential therapeutic opportunities within such knowledge; a frontier.

MECHANISMS OF SORTING AND STORAGE OF PRE-FORMED MEDIATORS IN MC GRANULES

The most unique and igneous feature of MC granules is their ability to store large concentrations of mediators in a small space for long periods. In theory, collecting a high concentration of such highly charged mediators in a membrane-enclosed space would require a large amount of osmotic work and create a thermodynamic disadvantage. However, MC trap the mediators in an anionic gel matrix composed mainly of heparin and chondroitin sulfate, which confers a huge thermodynamic advantage (38, 57). Upon cell activation, the polymer gel phase undergoes a transition and swells to release the mediators (58). This efficient solution is not-surprisingly conserved among living organisms and even phytoplankton use a similar packaging and degranulation process (59). Current understanding of sorting and storage mechanisms of pre-formed mediators are reviewed below and in **Table 1** but it is still poorly understood.

Proteoglycans

Proteoglycans, a core component of MC granules are heavily glycosylated proteins, consisting of a core protein and glycosaminoglycan side chains that are covalently attached to the core through glycosidic bonds. In MC, serglycin is a dominant core protein, and heparin and chondroitin sulfate are dominant glycosaminoglycans that can be used to distinguish some MC subpopulations (1). Sulfation of glycosaminoglycans imparts a negative charge on the proteoglycan, which is an important mechanism that helps to retain proteases and biogenic amines in MC granules (60). An ion exchange mechanism with the charged glycosaminoglycans is thought to be significant in mediator release from granules (61, 62). Moreover, as outlined above, proteoglycans are important in granule composition and maturation. However, details of the sorting mechanism of proteoglycans and other mediators into MC granules are poorly understood. Studies with rat pancreatic acinar cells provide clues and suggest that glycosaminoglycan side chains are necessary for proteoglycan sorting into granules, as deletion of serine–glycine repeat region of the serglycin core protein and treatment with p-nitrophenyl-β-D-xylopyranoside, an alternate substrate for glycosaminoglycan side chain attachment, prevented

sorting into granules and lead to accumulation of proteoglycans in Golgi (63).

MC proteases

Mast cell proteases are synthesized in the ER, modified in the Golgi complex, and sorted into progranules that bud from the trans-Golgi. Retention of MC proteases in granules depends on the serglycin proteoglycan in murine MC, as absence of mouse MC protease (mMCP)-5 (chymase) and carboxypeptidase, and reduction of mMCP-6 (tryptase) occurs in the granules of MC from serglycin deficient mice despite normal expression of protease mRNA and granule formation (52). Although limited information is available with other kinds of proteases, serglycin proteoglycan is involved in certain protease retention in MC granules. However, the mechanisms underlying the trafficking of MC proteases into the granule need to be elucidated and confirmed in human MC, although the storage of heparin and chondroitin sulfate proteoglycan in human MC granules has been shown (36).

Biogenic amines

Histamine and serotonin are biogenic amines stored in MC granules. There is evidence that transport of biogenic amines from cytosol into the MC granules occurs in a vesicular monoamine transporter 2 (VMAT2)-dependent manner (64, 65). Moreover, retention of biogenic amines and release from the granule is serglycin proteoglycan dependent (66). Whether retention of both MC proteases and biogenic amines are directly dependent on serglycin proteoglycan or involve only the glycosaminoglycan side chains, e.g., heparin, with their electrostatic charges (67–70), needs to be elucidated and extended to studies with human MC. Some polyamines (such as putrescine, spermidine, and spermine) are required for granule homeostasis and possibly aid in the native conformation and packaging of other granule molecules such as histamines and proteases (71).

Lysosomal enzymes

Many lysosomal enzymes (**Table 1**) are found in MC granules but the detail mechanisms of their sorting, trafficking, storage, and secretion are poorly understood. It is postulated that they are transported into type II MC granules when granules and endosomes fuse (**Figure 2**). Clearly, lysosomal enzymes can be found in both type II granules, as well as classical lysosomes (type I granules), and secreted from both compartments. Using MC from serglycin knockout mice, it was shown that storage and release of β-hexosaminidase is independent of serglycin (52).

Cytokines

Among the large number of cytokines and chemokines released after MC activation, TNF (32, 72), bFGF (33), IL-4 (34), and SCF (35) are known to be pre-stored in MC granules, and can be released by regulated exocytosis, as well as synthesized following MC activation and released through constitutive exocytosis (**Figure 1**) (73, 74). Many other cytokines and chemokines appear not to be stored [e.g., GM-CSF (75)], but are newly synthesized following MC activation and are secreted by constitutive exocytosis over the course of several hours/days (discussed below) (9, 76). For storage in the granule, there appears to be a different trafficking mechanism for TNF in rodent and human MC. In rodent MC, sorting of TNF from ER to granules occurs *via* a brefeldin A- and monensin-sensitive route, utilizing a M6PR-dependent pathway and N-linked glycosylation of asparagine at N86 (**Figure 2**) (72). By contrast in human MC, TNF does not utilize this pathway as the N-linked glycosylation motif NSS of rodent TNF is replaced by the RTP motif (32). By transfecting and chasing fluorescence-tagged TNF into human MC lines (HMC-1 and LAD2), Olszewski et al. showed that in human MC, TNF traffics to the plasma membrane transiently, but then is stored in MC granules by endocytosis (**Figure 2**) (32). These observations emphasize that evidence acquired from studies of rodent MC must be validated for human MC, as has been shown for several other examples of species differences among MC (1). Apart from TNF, the mechanisms of trafficking of bFGF, IL-4, and SCF, another cytokines stored in human MC granules have not been studied, and we still do not fully understand how these cytokines are sorted in granules and which secretion pathway(s) initiates their release. Based on the binding affinity of bFGF for heparin (77), the retention mechanism of bFGF in the granule is likely to be heparin dependent, although this needs to be confirmed. Immunohistochemistry has shown that IL-4 but not IL-5 are stored in MC secretory granules in the lung parenchyma and nasal mucosa of patients with active allergic rhinitis (34). Although the amount of IL-4 in the granules increases after FcεRI-mediated activation, it is unclear whether the majority of IL-4 released extracellularly is due to degranulation or constitutive exocytosis.

MECHANISMS OF SECRETION OF PRE-FORMED MEDIATORS FROM MC GRANULES

Two types of degranulation have been described for MC: piecemeal degranulation (PMD) and anaphylactic degranulation (AND) (**Figures 1** and **2**). Both PMD and AND occur *in vivo*, *ex vivo*, and *in vitro* in MC in human (78–82), mouse (83), and rat (84). PMD is selective release of portions of the granule contents, without granule-to-granule and/or granule-to-plasma membrane fusions. PMD in MC has been identified in numerous settings, ranging from chronic psychosocial stress (85) to estradiol (86), CCL2 (87) and TLR stimulation (88), and interactions with $CD4^+/CD25^+$ regulatory T cells (89). The granule morphology is relatively well retained following PMD, although ultrastructural changes are evident (79). It has been proposed that the mechanism of PMD involves the budding of vesicles containing selected mediators from granules and their transport to the plasma membrane, fusion, and mediator release (**Figures 1** and **2**) (90). Little is known about the molecular machinery involved in these processes.

In contrast to PMD, AND is the explosive release of granule contents or entire granules to the outside of cells after granule-to-granule and/or granule-to-plasma membrane fusions (**Figures 1** and **2**). Ultrastructural studies show that AND starts with granule swelling and matrix alteration after appropriate stimulation (e.g., FcεRI-crosslinking). Granule-to-granule membrane fusions, degranulation channel formation, and pore formation occur, followed by granule matrix extrusion (81). Granule-to-granule and/or granule-to-plasma membrane fusions in AND are mediated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (91). In human intestinal MC, protein expression of soluble *N*-ethylmaleimide-sensitive factor

Table 5 | Molecules involved in mast cell degranulation.

Protein	Function	Cell type	Reference
Munc 13-4	Positively regulates degranulation	RBL-2H3	(93, 104)
Munc-18-2	Controversial in degranulation, interacts with syntaxin-3	RBL-2H3	(94, 95)
Complexin II	Enhances Ca^{2+} mobilization and degranulation	RBL-2H3	(96)
VAMP-8	Controversial in degranulation	BMMC, RBL-2H3	(44, 48)
Synaptotagmin II	Controversial in degranulation	BMMC, RBL-2H3	(42, 62, 99)
Rab3a	Controversial in degranulation	RBL-2H3	(101, 102)
Rab3d	Negatively regulates degranulation	RBL-2H3	(101)
Rab27a	Negatively regulates degranulation, regulates cortical F-actin integrity	BMMC, RBL-2H3	(93, 105)
Rab27b	Positively regulates degranulation	BMMC	(105, 106)
Rac1	Positively regulates degranulation	RBL-2H3	(107, 108)
Rac2	Positively regulates degranulation, regulates Ca^{2+} mobilization	BMMC	(109)
Cdc42	Positively regulates degranulation, interacts with PLCγ1, increases IP_3 production	RBL-2H3	(107, 108)
DOCK5	Positively regulates degranulation, regulates microtubule dynamics, phosphorylation and inactivation of GSK3β	BMMC	(110)
MARCKS	Negatively regulates degranulation, delay of degranulation	BMMC, eHMC[a]	(111)

[a] *eHMC, embryonic hepatic-derived mast cells.*

attachment protein (SNAP)-23, syntaxin (STX)-1B, STX-2, STX-3, STX-4, VAMP-2, VAMP-3, VAMP-7, VAMP-8, and STX-6 have been reported (92). However, only VAMP-7 and VAMP-8 were found to translocate to the plasma membrane and interact with SNAP-23 or STX-4 upon activation. Moreover, inhibition of SNAP-23, STX-4, VAMP-7, or VAMP-8, but not VAMP-2 or VAMP-3, reduced histamine release mediated by FcεRI-crosslinking (92). Therefore, VAMP-7, VAMP-8, SNAP-23, and STX-4 are important SNARE molecules in human intestinal MC granule fusion and exocytosis.

Molecules involved in MC degranulation

Several proteins involved in MC degranulation were listed in **Table 5**.

Mammalian uncoordinated-18 proteins. The functions of SNARE proteins are regulated by several accessory proteins, but our knowledge is incomplete and at least in part, the information is controversial (**Table 5**). Munc 13-4 was shown to be a target of Rab27a and Munc 13-4-transduced RBL-2H3 release more histamine compare to the mock-transduced cells after IgE/Ag stimulation (93). In rodent MC, mammalian uncoordinated-18 (Munc-18)-2, located in the granule membrane, interacts with STX-3 and plays a role in granule-to-granule as well as granule-to-plasma membrane fusion (94, 95) whereas, Munc-18-3, located in the plasma membrane, also interacts with STX-4 (94). Following activation of RBL-2H3, another protein, complexin II translocates from the cytosol to the plasma membrane and interacts with a SNARE complex. Although translocation of complexin II to the plasma membrane did not induce membrane fusion, the reduction of degranulation after knockdown of this protein suggests that complexin II is a positive regulator of MC degranulation (96). The fusion of the SNAREs with the plasma membrane has been examined using transmission and freeze-fracture electron microscopy and biophysical modeling. About 30–60 s after activation, unetchable circular impressions about 80–100 nm in diameter were found on the E face (intracellular face) of the plasma membrane (97). These impressions are not permanent but are postulated to form the fusion sites for the granules directly preceding degranulation. Under certain conditions, these fusion sites can form rosettes and the coupling of this structure with the plasma membrane may then form a cup-shaped structure called a porosome (98). Due to the nanometer size of these SNARE docking sites, the MC degranulation complex has been called a nano-machine (97).

Vesicle associated membrane proteins. A study using BMMC from VAMP-8 deficient mice showed reduced serotonin, cathepsin D, and β-hexosaminidase release, but normal histamine and TNF release following IgE-mediated or PMA/ionomycin stimulation (44). By contrast, transfection of VAMP-8 in RBL-2H-3 did not affect the calcium ionophore/12-*O*-tetradecanoyl-13-acetate- or IgE-mediated release of fluorescent-labeled neuropeptide Y, which is stored in the same granules as serotonin and β-hexosaminidase (48). These conflicting data may be the result of different experimental systems (former using deficient mouse of VAMP-8 and latter using over-expression), or the fact that the latter study examined mediator release indirectly with fluorescent-labeled neuropeptide Y. Although further study is required, VAMP-8 is likely involved in granule biogenesis and degranulation of subsets of MC granules, which contain serotonin, cathepsin D, and β-hexosaminidase. Moreover, this suggests that there is heterogeneity

of MC granules (**Figure 2**), and that distinct mechanisms are involved in mediator release between subsets of granules.

Synaptotagmins. Synaptotagmin (Syt) II depresses Ca^{2+}-triggered secretion of β-hexosaminidase and MHC class II release (42), but increases cathepsin D release in RBL-2H3 and mouse BMMC (99). Moreover, in Syt II knockout mice there is a marked deficiency in degranulation and an impaired passive cutaneous anaphylaxis response (62). In RBL-2H3, Syt IX can regulate protein export from the endocytic recycling compartment to the plasma membrane and play a role in sorting proteins of secretory granules (100). Much remains to be learned about these proteins and MC function.

Rab GTPases. In addition to their role in granulogenesis mentioned above, the Rab family of GTPases is also involved in MC degranulation. Over-expression of Rab3a in RBL-2H3 showed no (101), or inhibitory effects (102) on FcεRI-mediated β-hexosaminidase release, while over-expression of Rab3d demonstrated that it translocates from the granule to the plasma membrane (103), and inhibits degranulation (101). Recently, it was established that Rab27a is located in histamine-containing granules in RBL-2H3 and that over-expression of constitutively active Rab27a reduced FcεRI-mediated histamine secretion (93). Munc 13-4 was found to be a target of Rab27a, and the Rab27a-Munc 13-4 complex was required for docking of granules to the plasma membrane and release of granule contents in RBL-2H3 cells (104). Moreover, Rab27a regulates cortical actin stability with its effectors melanophilin (Mlph) and myosin, as well as Rab27a/b/Munc13-4-dependent granule exocytosis (105). Rab27b knockout mice exhibited reduced passive cutaneous anaphylaxis and defects in FcεRI-mediated β-hexosaminidase release from BMMC (106). Although Rab5 is involved in MC granule biogenesis (see above), a transfection study showed that Rab5 is not involved in granule mediator release (48).

Rho GTPases. Among Rho GTPases, Rac and Cdc42 play a positive role in RBL-2H3 degranulation by regulating IP_3 production, upstream of Ca^{2+} influx and interacting with PLCγ1 (107, 108). More recently, the roles of Rac1 and Rac2, which have a 92% sequence identity, in MC degranulation were dissected using knockout mice (109). In BMMC from Rac2 knockout mice, FcεRI-, but not Ca^{2+} ionophore-mediated β-hexosaminidase release was defective because of a decrease in Ca^{2+} flux without changing F-actin remodeling and membrane ruffling, which are regulated by Rac1 (109).

Others. The cytoskeleton and the microtubule network is an essential component of the degranulation process in MC. Proteins such as DOCK5 (110), MARCKS (111), and myosin VI (112) regulate the progress of secretory granules through the cytoskeletal network and allow them to dock with the plasma membrane. When these proteins are disrupted, the degranulation process does not occur normally. Some of these pathways remain unexplored in MC, yet we know that some microtubule events facilitate granule fusion and are essential to degranulation. For example, myosin Va forms a complex with Rab27a and Mlph thereby regulating cortical F-actin stability upstream of Rab27a/b/Munc13-4-dependent granule exocytosis (105).

Although calcium flux is unequivocally an essential feature of the degranulation process [recently reviewed by Fahrner et al. (113) and Ashmole and Bradding (114)], other ion exchange complexes have also been implicated, such as potassium and chloride channels that facilitate and regulate Ca^{2+} signaling. Certainly, disruption of membrane potentials using cationic liposomes can impair Ca^{2+} flux and suppress the function of SNAP-23 and STX-4 (115). It has been suggested that inhibitors of these ion exchange pathways may be useful in the treatment of inflammatory diseases that are mediated by MC activation and degranulation (116).

Calcium flux is essential for degranulation but its role may be more extensive than first postulated and some theories have questioned the long-standing belief that granule membranes must fuse with the plasma membrane to facilitate exocytosis. There are new theories of exocytosis that have not yet been examined in MC, including the theory of porocytosis, or secretion without membrane fusion, in which Ca^{2+} ions form salt bridges among adjacent lipid molecules through which mediators would move according to mass action (13). This quantal secretion theory has been postulated to be important in neuromuscular junctions and the central nervous system and it offers an intriguing process for mechanisms underlying constitutive exocytosis. However, this mathematical model has not been validated experimentally in neurons or other secretory cells. Autophagy, an evolutionarily conserved bulk degradation system that facilitates the clearance of intracellular molecules, has also been shown to be an important regulator of MC exocytosis. A recent study by Ushio et al. has shown that proteins that normally control autophagy may also facilitate the fusion of small secretory vesicles and facilitate their fusion with the plasma membrane (117). In fact, many new regulatory pathways have been connected to degranulation and exocytosis. However, we need more sophisticated model system to validate these new theories. Given that most molecules known to be involved in MC degranulation have been studied in rodent models, their roles in human MC must be examined and potential distinctions between MC phenotypes in different species should be investigated.

SECRETION OF EXOSOMES

Exosomes are membrane-bound vesicles of ~30–100 nm that appear to bud from the internal surface of multivesicular bodies in the endosomal compartment of many cell types including MC (**Figure 1**) (118). They are important in cell–cell communications and a breadth of physiological and pathophysiological responses, notably antigen presentation and host defenses. The contents of exosomes include a richness of lipids such as ceramide, cholesterol, phosphatidylserine, and sphingomyelin; a great diversity of proteins (200–400) including MHC class II, phospholipases, heat shock proteins, co-stimulatory molecules (CD40, CD40L, and CD86), adhesion molecules, kinases, tetraspanins, cytoskeletal proteins, chaperones, aldolase A, TNF, FcεRI chains, processed peptides from antigens; and a plethora of mRNA (>1800) and microRNA (>100) species (119–122). Exosomes transfer diverse cargo and functional capacity among cells; for example, mRNAs and microRNAs in MC exosomes can be transferred between human and mouse MC or other cells and control gene expression

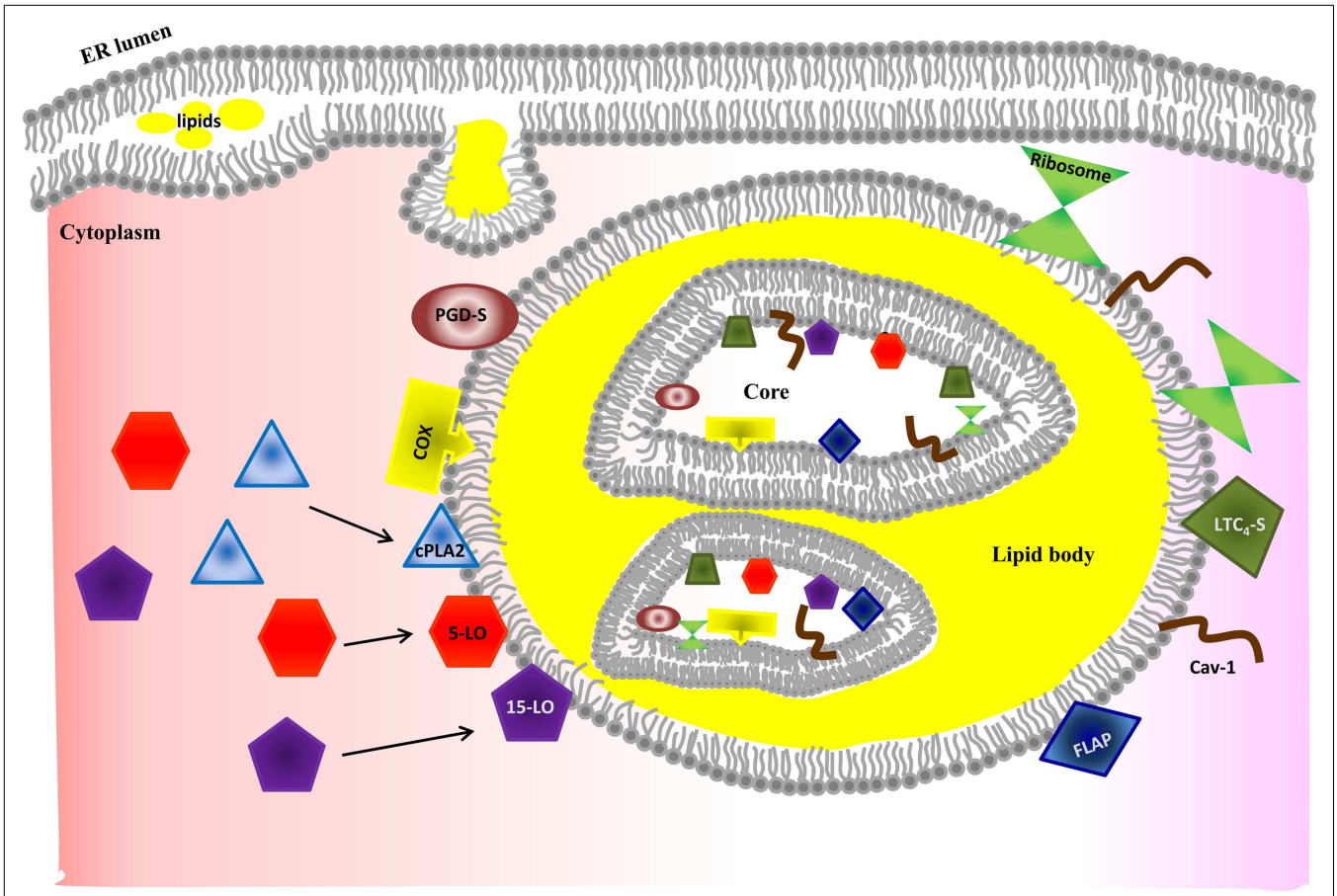

FIGURE 3 | Model of lipid body biogenesis and structure. Neutral lipids synthesized in the ER accumulate between bilayer of ER membrane and bud off as a lipid body. Lipid bodies have phospholipid monolayer on their outside. However, lipid bodies contain a bilayer core structure inside, which provides a hydrophilic area. The bilayer core can be created by incorporation of multiple loops of ER membrane and explains how ER membrane proteins (e.g., caveolin-1 and ribosome) are incorporated into lipid bodies. However, the exact mechanism of formation of the bilayer structure is poorly understood. Enzymes required for eicosanoid production have been found in both outer membrane and core of lipid bodies. Increased intracellular Ca^{2+} after MC stimulation induces activation and translocation of $cPLA_2$, 5-LO, and 15-LO to the lipid body membrane for eicosanoid synthesis. Further studies are required to unveil how MC control synthesis and secretion of arachidonic acid metabolites in a stimulus-specific fashion.

in recipient cells (119, 120). Carroll-Portillo et al. emphasized the potential functional significance of IgE-antigen and antigenic peptide–MHC class II MC–T cell–Dendritic cell interactions, as well as MC uptake of antigen-crosslinked receptor (16).

Mast cell exosomes can be secreted by both constitutive or regulated exocytosis (**Figure 1**) (16, 43) and the composition of protein constituents differs depending upon which pathway is employed (16). Knowledge is advancing rapidly about the molecular bases of exosome biogenesis, regulation of exosome loading, and the secretory pathways involved. The reader is referred to recent reviews of this intriguing and rapidly evolving subject (118, 123).

CYTOKINE/CHEMOKINE SECRETION FROM COMPARTMENTS OTHER THAN SECRETORY LYSOSOMES

In addition to regulated secretion of a limited repertoire of cytokines from stores in granules (above) through pathways of PMD or AND, MC secrete a diversity of cytokines and chemokines by other pathways [diagrammed by Lorentz et al. (14) in their Figure 3], including: constitutive exocytosis, better known in macrophages [e.g., Ref. (3, 9), etc.], and exosomal secretion (**Figure 1**). Frank et al. showed that involvement of SNAREs in chemokine release of human intestinal MC using their specific neutralizing antibodies. They showed that CXCL8, CCL2, CCL3, and CCL4 release after IgE/α-IgE stimulation were abrogated by inhibition of STX-3 or SNAP-23, but not by inhibition of STX-2 or VAMP-3. Moreover, inhibition of different SNARE subsets selectively reduce chemokine release (i.e., STX-4 or VAMP-8 inhibition selectively reduce CXCL8, and STX-6 inhibition reduces CXCL8 and CCL2) (124). However, other study using VAMP-$8^{-/-}$ BMMC showed that VAMP-8 does not affect cytokine/chemokine secretion (125). Therefore, there has been insufficient exploration of the molecular elements involved in these pathways in MC; careful analyses are needed in comparison to other cells such as macrophages, as well as among various MC subsets and between human and other MC.

LIPID MEDIATOR RELEASE

Activated MC release an abundance of arachidonic acid metabolites, notably leukotriene (LT) C_4, prostaglandin (PG) D_2, and

platelet activating factor (PAF) (126–128). These lipid mediators have bronchoconstricting and vasoactive properties, but also participate in host defense, inflammation, and allergic diseases through diverse activities such as effector cell trafficking, antigen presentation, immune cell activation, and fibrosis (129–131). Eicosanoids, lipid mediators derived from arachidonic acid, are *de novo* synthesized and released immediately from activated cells rather than stored, and exert autocrine and paracrine functions. However, recent observations of intracellular localization of the PGD_2 receptor, DP2/CRTh2, suggest intracrine functions of this mediator (132, 133). Eicosanoid synthesis can occur at several sites in the cell, including the ER (134), nuclear membrane (134, 135), phagosomes (136), and cytoplasmic lipid bodies (**Figure 1**) (81, 82, 137, 138), and the site of synthesis likely depends on the cell type, as well as the nature of the stimulation [see review in Ref. (139)]. Whether the enzymes and other proteins needed for eicosanoid synthesis are *de novo* synthesized following cell activation, or are dispersed in the cell and then translocated to a site of synthesis following activation, are under debate. In either case, how these proteins are targeted to a specific intracellular compartment of eicosanoid synthesis is unknown.

LIPID BODIES

Lipid bodies also referred to as lipid droplets are osmiophilic organelles surrounded by a monolayer of phospholipids and containing lipids with a unique fatty acid composition. Lipid bodies are composed of a core rich in neutral lipids and a variety of proteins depending upon the cell type and the conditions of stimulation (**Figure 3**) (137, 138, 140). These organelles are one of the major sites of eicosanoid generation. They are inducible during inflammatory processes and increase in size and number in several types of leukocytes, including MC (141–144). The numbers of lipid bodies in the cytoplasm of MC differ among MC phenotypes. For example, lipid bodies are rare in human skin MC in comparison to numbers in lung and gut MC (81, 82). In addition to eicosanoid synthesis, a few cytokines and chemokines are also found in lipid bodies of eosinophils (TNF), neutrophils (TNF), macrophages (TNF), and MC (TNF and bFGF) (33, 142). Much remains to be elucidated about the kinds of cytokines stored in lipid bodies, functions of these cytokines, and the mechanisms of their release.

LIPID BODY BIOGENESIS

Although the details of the biogenesis of lipid bodies in MC are poorly understood, they bud from the ER membrane where neutral lipids are synthesized (**Figure 3**) (145). Lipid bodies can also increase in size by fusion and this process occurs in a rapid regulated way (146).

MECHANISMS OF SYNTHESIS AND SECRETION OF LIPID MEDIATORS

Eicosanoids are made by oxidation of 20-carbon fatty acids. After activation, MC synthesize PGD_2 and LTC_4 from sequential enzymatic reactions of arachidonic acid metabolism of cyclooxygenase and lipoxygenase pathways, respectively. PAF also can be synthesized from lysophosphatidylcholine by PAF-acetyltransferase. Both arachidonic acid and lysophosphatidylcholine can be generated from membrane phospholipid (1-O-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine) by phospholipase A_2. Recently, studies on pro-resolving lipid mediators, such as resolvins, protectins, and maresins from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been advanced in macrophages and our understanding of their physiological, pathophysiological functions in resolution of inflammation are improved, but little is known their synthesis in MC (147).

Because eicosanoids synthesized intracellularly are negatively charged at physiological pH (148), they diffuse poorly across membranes. Thus, their secretion involves active transport through either organic anion transporters of the ATP-binding cassette type C family, also known as multidrug resistance-related proteins (149–152), or organic anion transporters of the solute carrier superfamily (153, 154). However, little is known of these mechanisms in MC and further study is needed to identify the mechanisms and their regulation.

CONCLUSION

Over several decades, there have been significant advances in our knowledge of MC biology that have transformed our understanding of this multifaceted immune cell from an effector cell in allergic inflammation to a major player in innate and acquired immunity. Because of the pivotal role of MC in allergic and other inflammatory reactions, therapeutic strategies to disrupt the action of MC mediators have been developed and used widely [e.g., antihistamines, anti-IgE (omalizumab), and Cys-LT1 receptor antagonist (montelukast)]. However, there remain large gaps in our knowledge about intracellular trafficking of MC mediators, particularly in the selective mechanisms of storage and secretion that are often dependent on the specific stimuli involved. Our knowledge of the biogenesis of MC cargos, the heterogeneity of granules, and the molecules involved in trafficking pathways of mediator secretion are rapidly evolving and likely to be a productive in helping to unravel the complexities of MC biology. A greater understanding of mediator specific trafficking pathways will provide opportunities to develop novel therapeutic targets for the treatment of increasingly wide-spread allergic and other inflammatory diseases.

AUTHOR CONTRIBUTIONS

Tae Chul Moon, A. Dean Befus, and Marianna Kulka equally contributed to writing this paper.

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Vesicular trafficking and signaling for cytokine and chemokine secretion in mast cells

Ulrich Blank [1,2,3]*, ***Iris Karina Madera-Salcedo*** [1,2,3], ***Luca Danelli*** [1,2,3], ***Julien Claver*** [1,2,3], ***Neeraj Tiwari*** [1,2,3†], ***Elizabeth Sánchez-Miranda*** [4], ***Genaro Vázquez-Victorio*** [4], ***Karla Alina Ramírez-Valadez*** [5], ***Marina Macias-Silva*** [4] ***and Claudia González-Espinosa*** [5]*

[1] *INSERM UMRS 1149, Paris, France*
[2] *CNRS ERL8252, Paris, France*
[3] *Université Paris Diderot, Sorbonne Paris Cité, Laboratoire d'excellence INFLAMEX, Paris, France*
[4] *Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, México City, México*
[5] *Departamento de Farmacobiología, Cinvestav, México City, México*

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Philippe Georgel, Strasbourg University, France
Jaya Talreja, Wayne State University, USA

***Correspondence:**
Ulrich Blank, INSERM UMRS1149, Université Paris-Diderot, Faculté de Médecine Site X. Bichat, 16 rue Henri Huchard, Paris 75018, France
e-mail: ulrich.blank@inserm.fr;
Claudia González-Espinosa, Departamento de Farmacobiología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Calzada de los Tenorios 235, Colonia Granjas Coapa, 14330 México City, México
e-mail: cgonzal@cinvestav.mx

[†]***Present address:***
Neeraj Tiwari, Department of Cell Biology, Yale University, New Haven, CT, USA

Upon activation mast cells (MCs) secrete numerous inflammatory compounds stored in their cytoplasmic secretory granules by a process called anaphylactic degranulation, which is responsible for type I hypersensitivity responses. Prestored mediators include histamine and MC proteases but also some cytokines and growth factors making them available within minutes for a maximal biological effect. Degranulation is followed by the *de novo* synthesis of lipid mediators such as prostaglandins and leukotrienes as well as a vast array of cytokines, chemokines, and growth factors, which are responsible for late phase inflammatory responses. While lipid mediators diffuse freely out of the cell through lipid bilayers, both anaphylactic degranulation and secretion of cytokines, chemokines, and growth factors depends on highly regulated vesicular trafficking steps that occur along the secretory pathway starting with the translocation of proteins to the endoplasmic reticulum. Vesicular trafficking in MCs also intersects with endocytic routes, notably to form specialized cytoplasmic granules called secretory lysosomes. Some of the mediators like histamine reach granules via specific vesicular monoamine transporters directly from the cytoplasm. In this review, we try to summarize the available data on granule biogenesis and signaling events that coordinate the complex steps that lead to the release of the inflammatory mediators from the various vesicular carriers in MCs.

Keywords: mast cells, signaling, vesicular trafficking, secretion, inflammation

INTRODUCTION

Mast Cells (MCs) are tissue cells that are well known effectors in IgE-mediated allergic or anti-parasitic responses, but research in the last two decades has revealed that they are also important participants in innate immunity and inflammation (1, 2). One of their prime functions is to release a large array of inflammatory mediators, which mediate tissue responses, participate in immunoregulatory and inflammatory processes as well as tissue remodeling (2–5). These mediators include compounds prestored in a release-ready form in their cytoplasmic secretory granules (SGs) such as histamine and MC specific proteases. MC also secrete newly synthesized lipid mediators such as leukotrienes or prostaglandines or a variety of cytokines, chemokines, and growth factors (3, 6). While synthesized lipid compounds freely diffuse across the membrane, the release of protein products depends on vesicular carriers and membrane fusion (7, 8).

Study of the biologic function and involved signaling pathways engaged by various membrane receptors has evidenced that MC can release either their full array of mediators or just some of them under particular conditions of stimulation (9). For example, while stimulation through the IgE Fc receptor (FcεRI) generally leads to a full response, stimulation through Toll-like receptor 2 and 4 (TLR2 and TLR4) or through some cytokine receptors induces the selective release of newly synthesized chemokines and cytokines in the absence of degranulation (9). The release characteristics also depend on the strength of stimulus as, for example, after weak stimulation (low antigen concentration in case of FcεRI) the release of chemokines over cytokines is favored (10). This is due to the engagement of different signaling pathways that at least in part imply engagement of distinct Src-related kinases (11).

Research in recent years on secretory mechanisms after stimulation has mostly focused on the degranulation process that concerns the release of mediators stored in cytoplasmic granules, which amongst many other compounds includes also certain cytokines (4). At present, little is known on the vesicular trafficking of newly synthesized cytokines and chemokines and its positive and/or negative regulation. Yet, it is clear that like secretion from cytoplasmic granules the release of newly synthesized

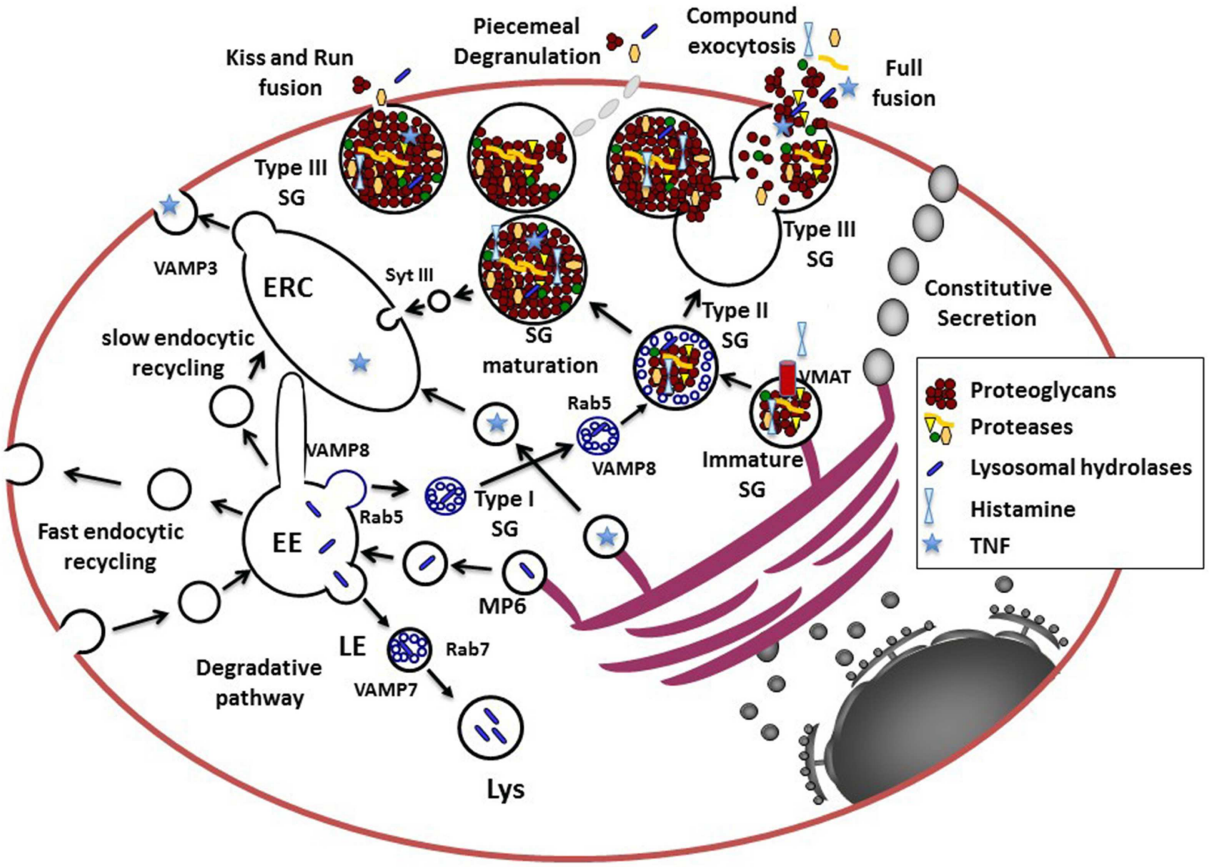

FIGURE 1 | Model for the secretory pathways in MC. Newly synthesized proteins destined for secretion enter the secretory pathway at the rough ER. After passage through the Golgi where posttranslational modifications occur, they reach the trans-Golgi network (TGN), which functions as a sorting hub depending on various structural motifs. For example, GPI-anchored or *N*-and *O*-glycosylated proteins enter the constitutive secretory pathway for trafficking to the cell surface. Proteins destined for the endosomal–lysosomal pathway are modified by Mannose-6 phosphate (MP6) for recognition by the Mannose-6-phosphate receptor that via an acidic cluster/dileucine motif is delivered to the early endosome (EE). Together with endocytosed proteins destined for degradation they are further sorted to late endosomes (LE) forming multivesicular structures and then fuse with lysosomes (Lys) via a VAMP-7 and Rab7-dependent pathway. Some proteins, like for example, newly synthesized TNF may be sorted into vesicles to reach the endocytic recycling compartment (ERC) from where it could be secreted. The ERC may serve as an additional sorting hub for the exocytosis and recycling of proteins via retrograde pathways (not shown in this figure). The cytoplasmic SG, which contain proteins destined for the regulated secretory pathway for release upon stimulation in MC and other hematopoietic cells are so-called mixed type organelles or secretory lysosomes carrying features of endosomes and lysosomes. Proteins destined for SG may leave the TGN at sites where larger protein aggregates are formed possibly via association to highly negatively charged proteoglycans. The immature SG formed may then rapidly fuse with carriers (type I SG) containing small intraluminal vesicles derived from the early endosome via a Rab5 and VAMP-8 dependent mechanism to form type II SG. Some inflammatory mediators like histamine incorporates into SG via specific vesicular monoamine transporters (VMAT). SG then undergo a maturation process, which generates mature type III SG by the retrieving cargo of missorted proteins and intraluminal vesicles to the ERC. Pre-formed inflammatory mediators can then be released via several types of fusion processes including transient kiss-and-run fusion, piecemeal degranulation, or multigranular/compound exocytosis, which involves full fusion and collapse of SG. It is possible that during stimulation all types of granules (type I, II, and III) are fusion competent as suggested by the fact that cultured cells with less mature granules can be stimulated for release.

chemokines, cytokines, and growth factors must be tightly regulated to ensure an appropriate biological response. Here, we review some of the important aspects of the vesicular trafficking mechanisms and their involvement in MC cytokine and chemokine secretion.

SECRETORY PATHWAYS IN MC

In MC, like in any other cell type, the secretory pathway (**Figure 1**) starts with the translocation of nascent signal-peptide containing proteins at the rough endoplasmic reticulum (ER) after recognition by the cytosolic signal-recognition particle (SRP) ribonucleoprotein complex, which binds to the ER-localized SRP-receptor (12). From the ER, the proteins sequentially travel through the Golgi stacks via a still not completely understood process involving either Golgi cisternal maturation or vesicular transport carriers (13). While passing the Golgi stacks posttranslational modifications occurs, such as for example, glycosylation, and proteins finally reach the Trans-Golgi network (TGN). The TGN functions as a sorting hub for protein trafficking (14–16). By default, via the constitutive secretory pathway, glycosylated or

glycosylphosphatidylinositol (GPI)-anchored proteins are delivered to the plasma-membrane (PM) via tubular–vesicular carriers that bud off from the ER by a well regulated process involving motor proteins and fission processes and then fuse with the PM (16). However, in the presence of specific sorting signals (sorting by exit) proteins may be routed to other intracellular compartments. For example, within the Golgi apparatus, lysosomal hydrolases acquire a mannose-6-phosphate (M6P) moiety (17). M6P is then recognized by the M6P receptor (M6PR) at the TGN. An acidic cluster/dileucine motif in the cytoplasmic tail of the M6PRs then serves as a sorting signal for specific adaptor proteins (such as AP-1) allowing packing into clathrin-coated transport vesicles targeting them to early endosomes (16). Within early endosomes, the acidic environment of the endosomal lumen releases hydrolases from the M6PR. After maturation into late endosomes a process that is accompanied by the generation of intraluminal vesicles via the endosomal sorting complexes required for transport (ESCRT) machinery (18) the late endosomes fuse with existing lysosomes, where hydrolases and other proteins like proteases accumulate and get activated to degrade proteins.

Another important pathway is the regulated secretory pathway. Specialized secretory cells such as endocrine, neuronal, exocrine, but also hematopoietic cells have the capacity of regulated secretion liberating secretory cargo stored in cytoplasmic granules following arrival of a stimulus (14). While endocrine, neuronal, and exocrine cells are known to form specialized SGs, cells from the hematopoietic lineage, but also some other cells such as melanocytes, possess mixed type organelles often called secretory lysosomes due to their close connection between the endocytic and exocytic pathway (19). They are characterized by the presence of lysosomal enzymes as well as markers of lysosomal origin such as CD63/LAMP-3, and LIMP IV/5G10 antigen (17). They often are also altered in patients with lysosomal storage diseases carrying mutations in a variety of genes involved in lysosome biogenesis (19).

The biogenesis of SG has been well worked out in neuroendocrine cells (15). In these cells, proteins targeted to the regulated secretory pathway become associated with specialized regions of the TGN forming larger aggregates that eventually associate with other soluble or membrane-localized cargo protein (sorting by entry) (14). After budding immature SG are formed that, via a series of homotypic fusion events, give rise to mature SG. This process is coupled to the removal of non-secretory missorted cargo via clathrin-coated vesicles (sorting by retention) and condensation, the latter being at least partially favored by the acidic environment in the granules maintained actively by proton pumps (5). In contrast to the well-characterized granule biogenesis in neuroendocrine cells, the biogenesis of secretory lysosomes is less clear. While melanosomes, for example, seem to derive from early or sorting endosomes with few small intraluminal vesicles (20), granules from lytic T cells are likely modified lysosomes containing a ring of multivesicular bodies, which derive from the fusion of late endosomes, surrounding a dense core that further matures upon T cell activation (19–21). The dense cores might derive, like for conventional SG, from the TGN. It is possible that proteoglycans may play an important role by their capacity to selectively aggregate and attract regulatory secretory proteins via charged interaction thereby serving as a nucleation point for sorting (22). In favor, experiments carried out with proteoglycan deficient hematopoietic cells, including MC, all show serious defects in granule biogenesis, protease content, and maturation (5). Additional studies showed that SG containing at least some of the granule proteases and mediators could still be formed even in the absence of proteoglycans leaving open the possibility of proteoglycan-independent formation of immature SG (23). However, the fact that all these SGs are morphologically altered lacking an electron dense core and crucial enzymes favor an important role of proteoglycans in physiological SG formation.

In MC, electron microscopy studies (24) have allowed to define three types of SGs: (1) type I SG likely representing matured endosomal/lysosomal organelles with numerous intraluminal vesicles that are rapidly accessible to endocytic tracers, (2) type II SG, which like in cytotoxic T cells contain a dense protein core surrounded by multivesicular bodies; these granules are also rapidly accessible to endocytic tracers, and (3) type III SG containing essentially electron dense material no more accessible to endocytic tracers. Likely, type III SGs are generated by a maturation process that may involve Synaptotagmin III (SytIII) regulating recycling and delivery of cargo to the endocytic recycling compartment (ERC) during granule maturation (25). This suggests that the ERC could be an important intermediate in granule recycling and maturation. The nature of the involved endocytic compartment is also not completely clear, but recent data have indicated an important role of the early endosomal marker Rab5 and VAMP-8 in granule size determination (26). This suggests that multivesicular bodies or type I SG may not derive from late endosomes but could directly form at the early endosome and fuse with Golgi-derived specialized SGs in homotypic fusion events (26). A possible role of SytIX in this process has been proposed as it excludes proteins destined for recycling to reach the SG (27). However, the fact that in human MC VAMP-7 serves as a soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) for release of SG may suggest that genuine late endosomes may also take part in the generation of SG (28). Granule maturation is a long process that can take several months in MC, as indicated by data (29) showing that MC SG get gradually enriched with incoming cargo such as proteoglycans, MC proteases, but also histamine and serotonin, the latter of which being transported via vesicular monoamine transporter (VMAT) systems directly from the cytoplasm (5). Interestingly, these positively charged molecules also contribute to granule homeostasis as in their absence SG maturation is affected as shown for histamine and MC proteases (5, 30). In addition to these classical sorting pathways, the ERC, initially characterized for its role in the slow pathway of Transferrin recycling, has also gained attention as a new sorting hub actively sorting cytokines for release as demonstrated in macrophages (31).

ANAPHYLACTIC DEGRANULATION

PRESTORED INFLAMMATORY MEDIATORS

MC are the effector cells of type I hypersensitivity reactions, which involves the release within a few minutes of cytoplasmic granular content into the surroundings after stimulation, a process often called anaphylactic degranulation (32). A potent stimulus for degranulation is the crosslinking of IgE bound to FcεRI by

multivalent antigens or allergens (3). Indeed, one distinctive features of MC is that they contain numerous SGs in their cytoplasm (32, 33). These granules are filled with different inflammatory compounds, many of which are bound to a highly charged anionic gel matrix composed of proteoglycans such as heparin or chondroitin sulfate, which differ among the different MC types and species (34). The presence of these proteoglycans attributes the specific staining properties of MC with cationic dyes such as toluidine blue enabling the classical metachromatic staining of MC in tissues, which have led to the discovery of these cells by Paul Ehrlich in the nineteenth century (35). Well-known mediators bound to these proteoglycans are cationic amines such as histamine or serotonin, but also proteases such as tryptase, chymase, and carboxypeptidase [for a complete review see for example (4, 36)]. The formation of such a gel matrix allows their tight packaging and is thermodynamically advantageous avoiding osmotic work (37).

When MCs are stimulated, up to 100% of granular content can be released in a single stimulation event within minutes enabling a maximal biological effect in its immediate vicinity, but also often with systemic effects as for example, occurring during a generalized anaphylactic shock (38, 39). This degranulation is made possible by a special type of exocytosis mechanism often called compound exocytosis involving the fusion of PM proximal granules with the PM followed by the sequential fusion with granules lying deeper in the cytoplasm (**Figure 1**). Although such sequential fusion events have been shown by electron microscopy studies (33), a multigranular fusion mode that leads to the fusion of large intracellular fused granules with the PM has often also been observed (40). Degranulation involves the extrusion of the whole condensed gel matrix, which is accompanied by a drastic swelling due to their hydration (41). The change in pH after release from the acidic milieu in the granule to the neutral pH in tissues allows the dissociation of cationic inflammatory mediators such as histamine and MC proteases (5). In addition to this anaphylactic degranulation mechanism, granular content can also be released in small portions by a mechanism called piecemeal degranulation (PMD), characterized by the gradual emptying of MC SG over much longer periods of time, but also kiss-and-run fusion involving only a short opening and contact with the external environment (see below).

While it was thought initially that cytokines are released only after new synthesis, in groundbreaking discoveries, Galli and coworkers (42) found that MC in addition to releasing newly synthesized TNF were able to store TNF in SG from where it could be released through anaphylactic degranulation. In particular, highly differentiated tissue MC such as peritoneal MC in mice were found to contain substantial amounts of TNF in their SG, while this was less the case in cultured cells. In fact, in some cultured MC lines, such as for example PT18 or RBL-2H3 (42, 43), no prestored TNF is detectable while this amount seems variable for other lines or primary cultured MC such as BMMC. Our own experiments in BMMC show that they contain detectable TNF that colocalizes with SG markers (**Figure 2**), but this concerns only a small fraction of cells, while other cells clearly express only classical granule markers such as serotonin or proteases. It is therefore possible that TNF can accumulate only in mature granules. As a possible mechanism a specific sorting signal based on N-glycosylation

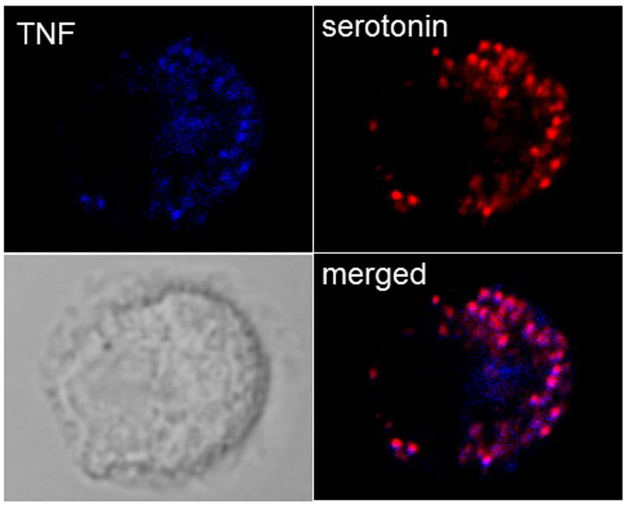

FIGURE 2 | Localization of TNF in SG in cultured BMMC. Colocalization of TNF (blue) with the SG markers serotonin (red) were analyzed in non-stimulated BMMC using confocal microscopy. Representative single optical sections and overlay (Merge) and the DIC images are shown. Note that in a single field only few cells show such TNF SG granular staining.

transporting TNF to the endosomal system was proposed (44). In human MC, where TNF is not glycosylated, TNF became enriched in SG after transient exposure on the PM and re-endocytosis (45). Besides TNF, a series of other cytokines including for example, IL-4, IL-5, IL-13, and vascular endothelial growth factor (VEGF) have been reported to be prestored in MC SG (4). Prestored cytokines have also be found in other cells of hematopoietic origin including eosinophils, platelets, and neutrophils (46–49).

EARLY SIGNALING PATHWAYS IN ANAPHYLACTIC DEGRANULATION

Anaphylactic degranulation can be induced via immunoreceptors, the FcεRI as a prototype (3), but MC can also be stimulated via IgG receptors under certain conditions (50). Furthermore, MCs express a whole variety of other receptors that can promote anaphylactic degranulation in particular certain G protein coupled receptors (GPCRs) such as receptors for complement peptides C5a and C3a, receptors for neuropeptides or receptor for certain inflammatory peptides such as for example endothelin (4). Independent of the receptor type, they all can induce a calcium signal and the activation of protein kinase C (PKC), in particular PKCα/β, which are necessary for anaphylactic degranulation to occur (3, 51). Signals which do not induce a substantial calcium response, such as for examples those generated via LPS and Toll-like receptors, although inducing the secretion of newly synthesized cytokines or chemokines do not induce anaphylactic degranulation.

Some recent reviews describe in detail the main molecular events following FcεRI crosslinking in MC (6, 51). Briefly, after antigen-dependent activation of FcεRI receptor, Src family kinases Lyn and Fyn are activated causing phosphorylation of FcεRIβ and FcεRIγ chains ITAMs (**Figure 4**). Receptor activation opens membrane calcium channels causing a receptor-operated calcium entry

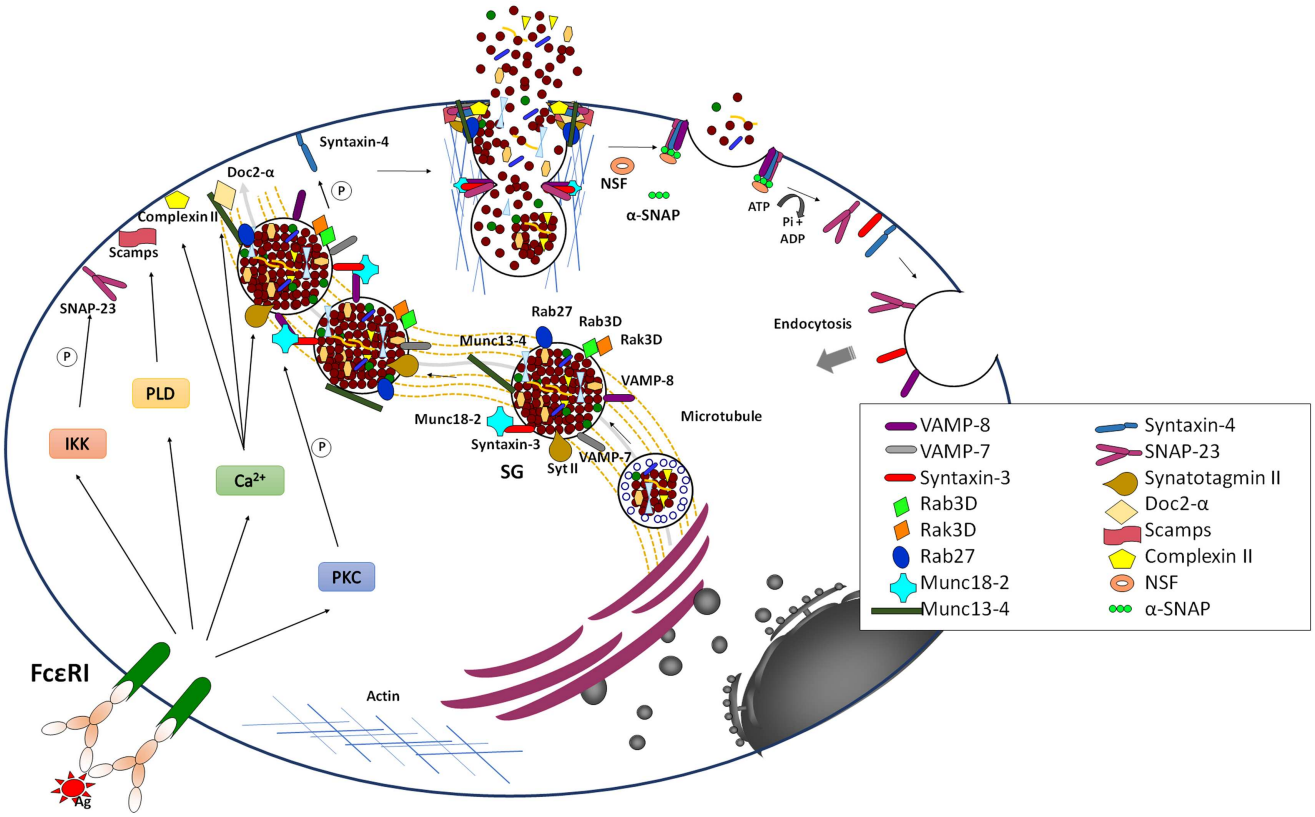

FIGURE 3 | Late signaling events in the anaphylactic degranulation. Aggregation of IgE bound to FcεRI leads to the generation of elevated levels of intracellular Ca^{2+} and the activation of PKC. In parallel, it also leads to the activation of PLD and IκB Kinase (IKK). Together they trigger anaphylactic degranulation, which involves SG–SG and SG–PM fusion events. Fusion is mediated by SNARE proteins that lie on opposing membrane. The formation of a stable well-characterized trans-SNARE complex in MC comprises the v-SNARES VAMP-8 that pairs with the two t-SNAREs, Syntaxin 4, and SNAP-23. During SG–SG fusion SNAP-23 relocates to the cytoplasm, which may be characteristic for the compound/multigranular fusion mode in MC. The SG-localized t-SNARE Syntaxin 3 may be specifically implicated in SG–SG fusion events and in a manner opposite to SNAP-23 translocates to the PM upon activation. Other v-SNAREs like VAMP-2 and VAMP-7 might also play a role in anaphylactic degranulation, which may indicate redundance or heterogeneity of the MC secretory compartment. The formation of SNARE complexes is modulated by multiple accessories proteins such as Munc18-2, Synaptotagmin II, Complexin, SCAMP, Doc2α, Rab27A and B, and Munc13-4 that allow to connect the fusion machinery to early signaling events to relieve existing fusion clamps and to coordinate the docking, thethering, and fusion events as well as the connection to cytoskeletal reorganization and transport mechanisms. Fusion is closely coupled to endocytosis of membrane lipids and proteins. The individual SNARE proteins are reconstituted by an energy-requiring step, which is mediated by the NSF ATPase via the α-SNAP adaptor protein.

(ROCE). Lyn kinase activation leads to the recruitment and activation of Syk kinase. Syk, in turn, phosphorylates the adapters LAT, SLP76, Gab2, and NTAL, among others. Those adapters participate in the assembly of large signaling complexes that include molecules such as Bruton's tyrosine kinase (BTK), which activates phospholipase Cγ (PLCγ) cooperatively with Syk. PLCγ activation induces diacylglycerol (DAG) production and inositol-1,4,5-trisphosphate (InsP3)-induced Ca^{2+} mobilization. Emptiness of intracellular calcium stores leads to the activation of a store-activated calcium entry (SOCE) to replenish internal stores. DAG and Ca^{2+} induce the activation of classical isoforms of PKC, PKCα, and β. On a complementary pathway, Fyn kinase phosphorylates Gab2 leading to the activation of phosphoinositide 3 kinase (PI3K). This results in activation of the PI3K-dependent protein kinase I (PDK1) and PKCδ. PI3K activation induces Phospholipase D (PLD) and Akt activation, the latter favoring MC survival and mTOR activation (51, 52). The Lyn pathway is essential for Ca^{2+} signaling, whereas the Fyn-dependent pathway is required for degranulation and maintenance of Ca^{2+} mobilization (53).

LATE SIGNALING EVENTS IN ANAPHYLACTIC DEGRANULATION

In addition to calcium and PKC, many different actors that are involved in the control of fusion during degranulation have been recently described (**Figure 3**). These include the highly conserved SNARE membrane fusion proteins (54–57). SNAREs can be divided into vesicular (v-SNARE) and target (t-SNAREs) localized, respectively, on opposing donor and acceptor membranes. They contain in their primary structure and about 60 aa α-helical SNARE motif, which upon arrival of the appropriate stimulus, can zipper to form a tight tetrameric trans-SNARE complex (composed of one v-SNARE and either two or three t-SNAREs, depending on the number of contained SNARE motifs) that drives the merger of lipid bilayers. After fusion SNAREs are disassembled under

energy consumption by the ATPase *N*-ethylmaleimide-sensitive factor (NSF), which becomes recruited to the SNARE complex via an adaptor called Soluble NSF Attachment Protein (SNAP).

While, SNARE complex formation and fusion can be reconstituted into liposomes in the absence of any additional protein (58), the process is extremely slow indicating additional control mechanisms. In living cells, numerous accessory proteins have been found to regulate membrane fusion including Sec1/Munc18 (SM)-, Munc13-, Rab-, complexin-, and Synaptotagmin (Syt)-family members (56, 59). Together, they are responsible for the priming, tethering, and docking to ensure specificity and efficiency of the fusion process. They also allow to connect the fusion machinery to cell stimulation relieving existing fusion clamps. Many were initially characterized in neuronal secretion at the synapse. Although, in general, the basic principles described may apply to secretion in other cells, differences clearly exist. In particular, vesicular release at the synapse is set up for an extremely fast (within milliseconds) release followed by a fast recycling process at specific active zones requiring likely additional proteins such as voltage-gated calcium channels or proteins involved in vesicle docking such as RIM (59). In other cells, such as MC the process is generally more dynamic and might require a more extensive crosstalk with the cytoskeleton for transport mechanisms that does not occur at specific active zones. Furthermore, as mentioned above, they involve also granule–granule fusion events to obtain a maximal release within the scale of a few minutes. Some of the differences are summarized in **Table 1**.

Several of the early studies in MC provided convincing evidence that, like in neurons, fusion implies SNARE proteins (60–63).

Table 1 | Comparison of exocytosis in mast cells and neurons.

Parameter	Mast cells	Neurons
Granules	Secretory lysosomes	Specialized secretory granules
Granule size	300–1000 nm	50 nm
Granule number	Up to 1000/cell	200–500/nerve terminal
Ca^{2+} requirements	1 μM	200 μM
PKC requirement	Yes	No
Time frame of exocytosis	Minutes	Milliseconds
Recycling time	Long (hours to days)	Very short (seconds to minutes)
Site of release	Multidirectional[a]	Active zone
Release characteristics	One single release, up to 100% of total granular content; multi-granular/compound mode	Multiple release one granule per fusion event

[a]*Note that under specific local conditions of stimulation, mast cells are able to deliver SG content within specific zones.*

Work from several laboratories has allowed to define a central SNARE complex composed of the PM-localized t-SNAREs SNAP-23 and Syntaxin 4 as well as the vesicular localized VAMP-8 (64–66). Interestingly, SNAP-23 was found to relocate to the cytoplasm in degranulation channels following activation, which could be a specific characteristic of the compound/multigranular mode of fusion as it enables granule lying deeper inside to get access to this PM SNARE (60). Furthermore, the implication of VAMP-8, which was initially described as an endosomal SNARE called endobrevin (67) was a clear indication for the close connection of the granular compartment with the endosomal/lysosomal system. In addition to these three SNAREs other possible SNAREs implicated include Syntaxin 3, VAMP-7, and VAMP-2 (28, 66, 68–70). In murine, but not in human MC (71), Syntaxin 3 is localized to SG and translocates to the PM, which might in a manner analogous to SNAP-23 be a characteristics of the compound/multigranular mode of fusion (70). However, the association with other SNARE proteins like Vti1b involved in vesicular fusion events is also possible (55). The fact that several types of SNARE complexes are implicated in membrane fusion in MC could be in agreement with the heterogeneity of the secretory compartment.

In addition to cognate SNARE proteins anaphylactic degranulation is regulated by an array of additional factors and signaling events. They include small GTPases known to regulate and coordinate discrete steps along the vesicular trafficking (72). A functional screen comprising 44 different Rab GTPases showed that many Rabs can affect either antigen and/or ionomycin/PMA triggered anaphylactic degranulation (73). The best characterized Rabs are the Rab27A and B isoforms. These studies indicate that while Rab27A may play a role in regulating the cortical actin disassembly limiting access of SG to the membrane, the role of Rab27B or the combined action of Rab27A and B facilitates degranulation by switching granules from microtubule-dependent movement to F-actin-dependent docking (74, 75). Indeed, degranulation is also accompanied by important remodeling of the actin cytoskeleton, which may favor the access to the PM (3). The docking step may imply another important factor, Munc13-4, that interacts with Rab27. Indeed, it was shown in MC that the interaction between both partners was required to correctly tether SGs at the PM and to prime them for fusion (75–77). Controversial data have been obtained with regard to the role of Rab3 isoforms. While initial data showed a potential role of Rab3A (78), we found that this isoform was not targeted to membranes in RBL MC and that its overexpression had no effect (79, 80). Rab3D, on the contrary, was SG-localized (81). Overexpression of wild-type and a constitutively active Rab3D mutant affected exocytosis (79). However, this was not confirmed in MC obtained from Rab3D knock-out animals although compensatory mechanisms by other Rab3 isoforms have not been examined (82).

Sec1/Munc18 protein family members are another type of fusion accessory proteins. They include three Munc18 isoforms implicated in exocytosis and some other family members involved in intracellular trafficking steps (56). Initial analysis of the neuronal isoform Munc18-1 suggested that it might be a negative regulator of fusion by its capacity to bind to particular syntaxin SNAREs preventing the binding of cognate SNARE partners. However, analysis of knock-out mice showed that its absence

completely blunted synaptic transmission advocating a positive regulatory function (56). Later on this was explained functionally by data showing that during exocytosis Munc18-1 switches its binding mode to bind the assembled SNARE complex thereby aiding the fusion process probably by electrostatic interactions with the membrane for fusion pore expansion (56, 83, 84). MC do not express protein of the neuronal isoform Munc18-1 but express ubiquitious Munc18-2 and Munc18-3 (85, 86) and Munc18-1 does not seem to play a role (87). No functional data have been obtained for Munc18-3, but several studies by different authors have clearly shown that the Syntaxin 3 binding protein Munc18-2 acts as a positive regulator of fusion as in its absence exocytosis is compromised in MC (70, 88, 89) but also in other hematopoietic cells (90). Further functional studies indicated that knock-down of both Syntaxin 3 and Munc18-2 yielded additive inhibitory effects on exocytosis suggesting that Munc18-2 besides fusion might regulate additional steps (70). Video imaging of SG movement indicated that Munc18-2 affected SG translocation to the membrane, likely through its dynamic interactions with the microtubule cytoskeleton (70). Munc18-2 may also represent a possible target for the action of protein PKC (8, 91). As data in chromaffin cells already suggested a docking effect (92), it may be possible that SG docking may not be static, but rather a dynamic process that needs to be maintained via cytoskeletal interactions. In agreement, the neuronal Munc18-1 isoform has also be found to bind to the Kinesin-1 adaptor protein, fasciculation, and elongation protein zeta-1 (FEZ1) (93).

Secretory carrier membrane proteins (SCAMPs), a family of ubiquitous membrane proteins of transport vesicles (94) has also been shown to regulate fusion. These tetraspanins contain a short conserved segment (E-peptide) between the second and third transmembrane domain. MCs express three SCAMP isoforms, with SCAMP1 and SCAMP2 being most highly expressed. Introduction of the E-peptide interfered with degranulation in permeabilized MC (95, 96). SCAMPs may act at the final fusion step in agreement with data showing that genetic deficiency of SCAMP2 causes an apparent defect in forming stable fusion pores that may depend on Arf6-stimulated PLD activity (94). PLD was indeed proposed as an effector in MC exocytosis (97).

Several effectors molecules have been described that connect the fusion apparatus to Ca^{2+} signaling. This includes Syt family proteins, which are single transmembrane proteins with tandem calcium-binding C2 domains (termed C2A and C2B). Calcium-binding promotes oligomerization and membrane phospholipid-binding as well as the interaction with the SNARE complex, allowing the formation of a quaternary SNARE-Syt-Ca^{2+}-phospholipid (SSCAP) complex driving of lipid bilayer mixing (98). While, neuronal expressed SytI was initially characterized as a possible sensor (99), recent studies in knock-out mice provided a clear evidence for the role of SG-localized SytII as the relevant Ca^{2+} sensor in MC (100). MC deficient in SytII showed a severe defect in both lysosomal β-hexosaminidase and histamine release and conferred to these mice a strongly decreased passive cutaneous anaphylaxis reaction (100). Although in addition to SytII, other Syt isoforms are expressed in MC, they do not seem to be directly involved in the fusion process but rather may regulate other intracellular trafficking steps (7). SytII may act in concert with ComplexinII (101, 102), a protein that binds to assembled SNARE complexes and that has been described in neurons to function as a fusion clamp. However, data from knock-out mice also support a positive action on exocytosis involving sequences adjacent to the SNARE-complex-binding domain. It seems that its interaction with the SNARE complex generates a metastable state that serves as a substrate for Syt following Ca^{2+}-influx with the induced rearrangement and cooperative interactions promoting fusion (56, 59).

In addition to Syt, Doc2 adaptors also contain tandem calcium-binding C2A and C2B domains the former of which exhibiting calcium-dependent phospholipid-binding activity thought to be important for regulated exocytosis through possible interactions with Munc13 isoforms as well as partly assembled SNARE complexes (103). MCs express the Doc2α isoform thought to be neuronal-specific, while the ubiquitous Doc2β isoform was not expressed (104). Doc2α-deficient MC showed a marked defect in degranulation and formed a tripartite complex with Munc13-4 and Rab27 suggesting that it could play a role in vesicle priming (104). Another possible Ca^{2+}-dependent process involves a kinase activity called Rak3D (from Rab3D-associated kinase) that was associated with Rab3D (105). Rak3D phosphorylated Syntaxin 4, but not Syntaxin 2 and 3 in its N-terminus, which prevented binding of the SNARE partner SNAP-23 suggesting that it could act as a fusion block. In agreement, the association with Rab3D decreased upon stimulation in a calcium-dependent manner. While phosphorylation of Syntaxin 4 was fusion inhibiting, phosphorylation of the other t-SNARE protein SNAP-23 rather seemed to be fusion promoting. This involved IκB kinase (IKK) also known to regulate the phosphorylation of IκB, which induces nuclear translocation of the NF-κB transcription factor (106). Indeed, in activated MC IKK phosphorylated a small fraction (~10%) of SNAP-23 on Ser95/Ser120 within its cysteine-rich linker region. In the absence of IKK, degranulation and anaphylactic responses were impaired. This was associated with decreased SNARE complex formation indicating a role for SNAP-23 phosphorylation in fusion.

OTHER FORMS OF DEGRANULATION

PIECEMEAL DEGRANULATION

Piecemeal degranulation is a mode of exocytosis characterized by loss of granule content in the absence of clearly observable granule–granule or granule–PM fusion events. PM is associated with the generation of small vesicles ranging from 30 to 150 nm budding from large SGs (107, 108) (**Figure 1**). Empty spaces of lost secretory particles are recognizable inside the SG, without evidence for changes in the size of the granule membrane. The mechanism behind PMD is known as the "shuttling vesicle" hypothesis (109). According to this, the transport of granule constituents out the cell is mediated by vesicles shuttling from the granule compartment to the PM. Electronic microscopy has shown, in fact, that vesicular transport granule content is associated with this type of secretion (110). Thus, an outwarding flow of cytoplasmic vesicles loaded with granule materials effects granule depletion during PMD. Vesicles containing part of the granule content buds from the granule membrane and move through the cytoplasm and fuse with the PM, leading to content discharge. In a closely coupled inward flow, endocytotic vesicles are retrieved from the PM and traverse the cytoplasm to fuse with granules. If the rate and amount

of vesicular traffic are balanced, granules and PM will maintain constant size. The most important characteristic of PMD is the fact that it seems to be modulated in different steps and by different stimulants. Strongly enhanced PMD was observed in mice overexpressing IL-4 resulting in eyelid lesions and enhanced fibrosis (111) or in human MC after stimulation with IL-1 (112). PMD has been studied in a number of different immune cells (47) but also enteroendocrine cells of the gastrointestinal tract, chromaffin cells of the adrenal medulla and chief cells of the parathyroid gland (108).

Alternatively to the shuttling vesicle hypothesis, PMD could also be the results of kiss-and-run fusion, which also result in partial vesicle emptying (see below).

KISS-AND-RUN EXOCYTOSIS

Kiss-and-run exocytosis refers to a special type of secretion in which a fusion pore opens and closes during vesicle fusion with the PM, allowing the release of some amount of granule content without full vesicle collapse (113). Kiss-and-run exocytosis (also known as "cavicapture") has been reported in PC12 cells (114), chromaffin cells (115), and insulin secreting pancreatic beta-cells (MIN6)-cells (116). In MC, initial classical patch clamp studies led to the observation of capacitance changes in the PM due to exocytosis of individual granule contents after GTP addition and, interestingly, this event seemed to be independent of Ca^{2+} mobilization (117). Later, a series of experiments using peritoneal MC from beige mice demonstrated "capacitance flickering" after intracellular administration of GTPγS, suggesting rapid and reversible steps in membrane fusion due to the opening and closing of a fusion pore, narrow enough to retard the escape of some molecules (118) but leading to the leakage of some soluble granule components. Besides these electrophysiological measurements kiss-and-run fusion in MC could also be observed directly in multicolor multiphoton fluorescence microscopy in histamine loaded cells through the pH cycling that occurs after a release event. In these studies using a tumor MC line the authors found that kiss-and-run fusion occurs actually very frequently accounting for about one-third of all fusion events in MC (119).

The choice between kiss-and-run and full fusion of vesicles may depend on several different mechanisms. One vision is that kiss-and-run fusion occurs when the threshold for full fusion is not reached. The threshold would be determined by the interplay between the regenerative recruitment of SNARE-mediated force and some kind of restraining force that counterbalance vesicle fusion. According to this "restraining force hypothesis" cytoskeleton-dependent restraining barriers exist that counterbalance SNARE-driven fusion mechanisms (113). Studies in neurons have shown that the relative incidence of kiss-and-run fusion is strongly regulated by key factors such as intracellular calcium accumulation, impulse frequency, and a previous history of activity. Calcium is important to switch the kiss-and-run mode of secretion to the full fusion events. High calcium shifts the mechanism from kiss-and-run to complete fusion (120). Furthermore, some lines of evidence suggest that the number of SNARE complexes might also be important in the balance between kiss-and-run and full fusion. In reconstituted systems (121), it has been calculated, for example, that one SNARE complex is sufficient for the bilayer fusion observed in kiss-and-run and three of those complexes are needed to prevent the nascent fusion pore from reclosing (a phenomenon needed in full fusion exocytosis). Also, the stability of the fusion pore can be altered by accessory proteins of SNARE function, including Syt (122), complexin (123), and Gβγ (124).

ENDOCYTOSIS–EXOCYTOSIS COUPLING

Regardless of the mechanism of exocytosis, the incorporation of membrane during exocytosis has to be closely coupled to endocytosis to retrieve the additionally inserted membrane. Several modes of endocytosis have been characterized (125). They include endocytosis after (i) full-collapse fusion, in which collapsed vesicles are retrieved by classical endocytosis involving membrane invagination and vesicle reformation, (ii) kiss-and-run fusion, in which the fusion pore just opens and closes without any significant membrane traffic, and (iii) after compound/multivesicular exocytosis one can also observe bulk endocytosis that retrieves giant vesicles from the membrane. In agreement with the multiple types of exocytosis in MC, a recent electrophysiological study has shown that, in addition to classical mode endocytosis, kiss-and-run as well as compound exocytosis events can be delineated (126).

Following exocytotic fusion the formed SNARE complexes are also reconstituted into the individual SNARE proteins. This is achieved by the NSF ATPase (127) and represents the energy consuming step within the SNARE cycle (98). The necessity of NSF in MC exocytotic event has been demonstrated by the fact that transfection of an inactive NSF mutant into RBL MC blocks degranulation (63).

DE NOVO SECRETORY PATHWAYS

Besides secreting mediators prestored in cytoplasmic granules, MC release also a whole array of *de novo* synthesized mediators. These include lipid compounds such as leukotrienes and prostaglandins, which are generated from arachidonic acid released from nuclear membrane phospholipids through the action of cytosolic phospholipase A2. These compounds are synthesized in the cytosol and then diffuse across the PM due to their lipid-derived nature and hence do not require lipid transport mechanisms (128).

MC also synthesize and release a large set of different cytokines, growth factors, and chemokines. An extensive list produced by MC can be found in a review by Galli and coworkers (4). Contrary to the lipid mediators, they are proteins and synthesized at the rough ER and released along the secretory pathway using vesicular carriers (31). As indicated certain cytokines and growth factors such as TNF and VEGF have also been shown to be present in cytoplasmic granules and thus can also be released by anaphylactic degranulation providing an immediate source available within minutes (4, 42). On the contrary *de novo* synthesized cytokines and chemokines require several hours to obtain maximal levels of secretion engaging complex signaling pathways. They involve transcriptional regulation through transcription factors, epigenetic control mechanisms, as well as post-transcriptional regulation through mRNA stabilization and microRNA (miRNA). Signaling pathways also exist at the level of vesicular trafficking regulating the selective sorting to specific small vesicles and tubovesicular organelles. The relative contribution of these control mechanisms

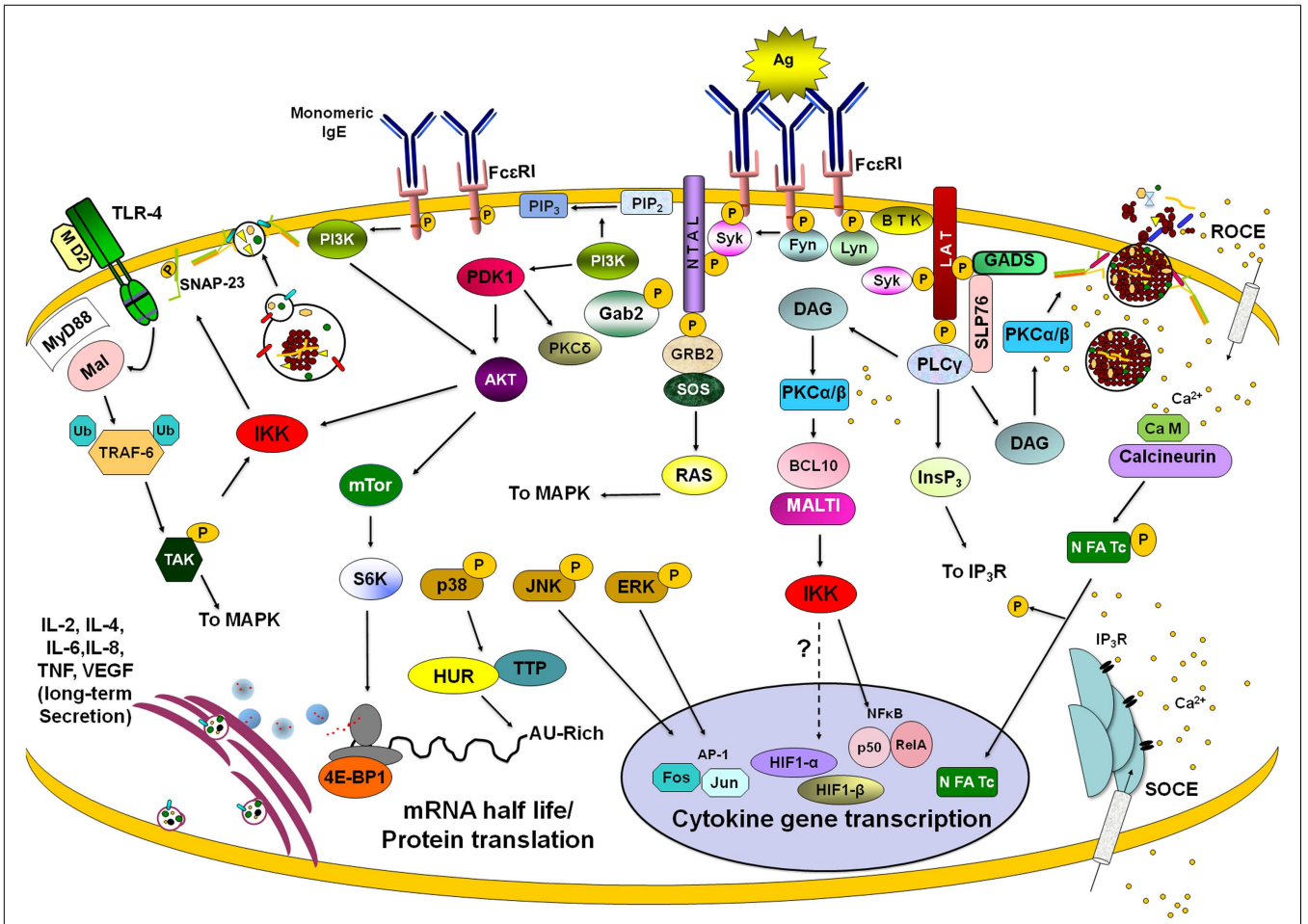

FIGURE 4 | Late signaling events in *de novo* cytokine/chemokine production. Stimulation of different membrane receptors lead to the activation of transcription factors, modulators of mRNA turnover, and effectors of ribosome activity that provoke long-lasting secretion of lymphokines in mast cells. After FcεRI triggering with IgE/antigen (Ag) complexes, Lyn and Fyn tyrosine kinases become activated and phosphorylate ITAMs from β and γ chains of the receptor. Further activation of Syk kinase leads to the phosphorylation of a number of adapters (such as LAT and NTAL) and enzymes (such as BTK and PLCγ). Enzymes and adapters form different macromolecular aggregates leading to the activation of different signaling pathways. PLCγ activation is connected with PKCα/β-dependent events through the generation of DAG and IP3. PKCα/β connect FcεRI triggering with IKK activation and IκB degradation, leading to nuclear translocation of NFκB. IP3-sensitive calcium release from intracellular stores lead to the activation of a store-operated Ca^{2+} entry (SOCE). IgE/Ag stimulation initiates also a receptor-operated Ca^{2+} entry (ROCE). Calcium increase allows calcineurin-dependent dephosphorylation and nuclear translocation of NFAT. On the other hand, Fyn-PI3K pathway leads to the activation of IKK and NFκB-dependent transcription but also triggers AKT, which controls mTor kinase and the initiation of protein translation by S6K and 4E-BP1. Activation of MAPK provokes the activation of AP-1 transcription factor but also modifies cytokine mRNA turnover through the modulation of mRNA-binding proteins such as HUR and Tristetraprolin (TTP). Binding of monomeric IgE to FcεRI induces mTor-dependent changes on 4E-BP1 phosphorylation leading to modifications on cap-dependent protein translation. Stimulation of toll-like receptor 4 (TLR4) leads to NFκB-mediated transcription by the MyD88/Mal-dependent pathway, which renders IKK activation after the TRAF-6/TAK1 axis. IKK, either activated by FcεRI or TLR4 receptors, phosphorylates SNAP-23 facilitating secretion. Also, IKK triggering (together with some calcium-dependent signals) modulates HIF1-α-dependent transcription. Once translated, cytokines and angiogenic factors are secreted for variable periods of time utilizing specific vesicle carriers. See text for details.

remains to be clarified but could largely differ between individual cytokines and chemokines. Some of the important signaling steps leading to their secretion are summarized in the following chapters.

TRANSCRIPTIONAL CONTROL BY THE ACTIVATION OF TRANSCRIPTION FACTORS

Figure 4 displays some of the important signaling pathways controlling *de novo* synthesis of cytokines in MC. Some of the details of the early signaling events leading to the activation of Ca^{2+} mobilization and PKC via PLCγ and DAG have already been described above. This PLCγ-DAG-Ca^{2+} signaling then initiates a signaling wave that culminates in the activation of different transcription factors for cytokine/chemokine production. Important transcription factors include nuclear factor of activated T cells (NFAT), nuclear factor-kappa B (NFκ-B), and activator protein-1 (AP-1), but many other transcription factors may also be involved depending on the cytokine/chemokine gene.

Nuclear factor of activated T cells are a family of four transcription factors (NFAT1–4) that in normal conditions are phosphorylated and reside in the cytoplasm (129). MCs have been shown to express NFAT1 and 2 (130). In stimulated cells, NFAT becomes dephosphorylated by calcineurin, a Ca^{2+}-calmodulin-dependent serine/threonine phosphatase. This results in a conformational change that now exposes a nuclear localization sequence (NLS), which binds importins, allowing NFAT translocation to the nucleus and initiation of the transcription of a number of pro-inflammatory and regulatory cytokine genes, such as IL-2, IL-4, IL-13, and TNF (130, 131). The rephosphorylation of NFAT then exposes a nuclear export sequence (NES) enabling transport back into the cytosol via the exportin Crm1.

Another essential transcription factor regulating cytokine expression in MC is NFκB (132). This family of proteins includes five members: NFκB1 (p50), NFκB (p52), RelA (p65), RelB, and cRel. Although they can form different homo and heterodimers, p50/cRel is the most common form of NFκB in immune cells (133). In MC, as in other immune cells, the activity of NFκB is controlled by inhibitor of kappa B (IκB) protein that binds to the heterodimer in the cytoplasm maintaining it in an inactive conformation. Activation of NFκB occurs when the IKK phosphorylates IκB on serine residues to target it to proteasomal degradation after polyubiquitination (133). FcεRI-dependent activation of NFκB in MC depends on the activation and lipid raft recruitment of the classical isoform of PKCβ (134) and on the formation of a complex formed by B cell lymphoma 10 (Bcl10) and mucosal-associated lymphoid tissue 1 (Malt1) (135). The PKCβ–Bcl10–Malt1 complex is necessary for NFκB activation and cytokine gene transcription, but not for secretion of pre-formed mediators in MC. Bcl10- and Malt1-deficient MC show normal MAPK (p38, ERK, and JNK), PKC and Akt activation after FcεRI crosslinking but IKK-induced phosphorylation of IκBα and its degradation is completely blocked (135). NFκB is essential for the synthesis of an important number of cytokines in MC including TNF and IL-6 (136).

Besides binding PKC DAG also binds to RAS-guanyl nucleotide-releasing protein (Ras-GRP). Together they activate MAPK and IKK, leading to the activation of the AP-1 (Fos-Jun) and nuclear factor κB (NFκB) transcription factors, respectively (137, 138). AP-1 consists of homo- and hetero-dimers of Jun family proteins, as well as heterodimers of Fos an Jun (139). Fos and Jun proteins are also synthesized and activated by phosphorylation (140). AP-1 acts together with NFAT and NFκB for synergistic activation of cytokine genes (129) for example after costimulation of MC with antigen and IL-33 (141).

Interestingly, NFAT, AP-1, and NFκB are optimally activated in response to different patterns of Ca^{2+} signaling in T cells. Transient high Ca^{2+} spikes lead to sustained activation of JNK and NFκB, but not NFAT, whereas, prolonged low increases in $[Ca^{2+}]i$, were sufficient to activate NFAT (142). Furthermore, as already mentioned above release characteristics depends on the strength of stimulus and this also holds true the activation of NFAT in MC (143).

POST-TRANSCRIPTIONAL CONTROL OF CYTOKINE PRODUCTION

Post-transcriptional regulatory mechanisms play an important role in the regulation of cytokine gene expression (144). They depend in part on specific mRNA sequences present in the 3′ untranslated region (3′ UTR). One major class consists of a conserved AU-rich sequence element (ARE) composed of several repeats of the pentanucleotide AUUUA present in mRNAs encoding growth factors, oncoproteins, or cytokines (145). The ARE has been associated with both an accelerated degradation of mRNA and interference with translation (146). Investigations in mice lacking the TNF ARE sequence in the mouse genome have clearly shown that the TNF ARE was required both for the alleviation and reinforcement of message destabilization and translational silencing in stimulated cells. Moreover, the mutant mRNA was no longer responsive to translational modulation by p38 and JNK kinases, demonstrating that the TNF ARE is a target for these signals (147). One major regulator in this mechanism is TTP, the prototype of a CCCH-zinc finger proteins able to bind to the ARE and to interact with a number of proteins able to regulate mRNA stability and translational control (148). In unstimulated cells, TTP associates with factors that mediate translational repression and mRNA decay, while in stimulated cells with high p38-mitogen-activated protein kinase (p38) and the c-Jun N-terminal/stress-activated protein kinase (JNK/SAPK) activity it associates with proteins that enhance mRNA stability and translation (148).

Cytokine synthesis is also controlled at the level of translation initiation (149). The key signaling pathway controlling the initiation of translation in eukaryotic cells has been shown to be commanded by the mTOR kinase (150) related to lipid kinases and an essential component of two distinct multiprotein complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (150). mTORC1, the rapamycin-sensitive complex, consists of mTOR, raptor, and LST8. The mTORC1 signals to inhibitory 4E-binding protein-1 (4EBP-1) and 40S ribosomal protein S6 kinase (S6K), which mediates efficient cap-dependent translation initiation (151). PI3K regulates the mTORC1 pathway via the activation of AKT (151). In MC, IgE-Ag stimulation induces PI3K-dependent activation of mTORC1, S6K1, and 4E-BP1 (52). Inhibition of mTORC1 with rapamycin had no effect on degranulation but inhibited IgE/Antigen-induced IL-6 and IL-8 production and MC survival (52). Interestingly, rapamycin also provoked the destabilization of TNF mRNA in a process that required the TNF ARE in RBL-2H3 cells (152).

Besides cap-dependent translation, mRNAs for some angiogenic mediators, such as the VEGF, can be translated from a structured RNA element termed an internal ribosomal entry site (IRES) able to recruit the 40S ribosomal subunit (153). It has been found that in response to hypoxia and some other stimuli, 4E-BP1 and eIF4G participate in a regulatory pathway that switch protein translation from the normal cap-dependent to the IRES-dependent process (154) allowing selective protein synthesis to cope with an adverse environment. In MC, monomeric IgE induces the release of VEGF from a pre-formed pool but also importantly induces the production of VEGF mRNA by the long-lasting secretion of newly synthesized protein (155). IgE addition to BMMCs leads to dephosphorylation of 4E-BP1, indicating that low-level stimulation of FcεRI is able to induce IRES-dependent translation. The translational switch triggered by IgE was dependent on Fyn kinase activation and correlated with an increase in VEGF mRNA accumulation and VEGF secretion (156).

microRNAs AND CYTOKINE PRODUCTION IN MC

microRNAs are a large class of endogenous single-stranded small non-coding RNA molecules that control gene expression by binding to the 3′ untranslated region (3′ UTR) of mRNAs thereby reducing protein synthesis through repression of translation or induction of mRNA degradation. miRNAs control maturation, proliferation, migration, and activation of immune cells (157). MCs express a number of different miRNAs implicated in production of pro-inflammatory mediators. For example, miR-221, originally described to regulate the cell cycle of MC, was shown to favor MC adhesion and migration toward SCF or antigen. This improved IgE/Ag-mediated degranulation and IL-6 and TNF production (158). The miR-146a was found to negatively regulate NFκB signaling, blunting the elevated cytokine production after bacterial infections (159) and contributing to endotoxin tolerance. In MC, miR-146a expression in response to different stimuli is dependent on NFκB p50 (160). MiR-155 expression enhanced FcεRI degranulation and release of TNF, IL-6, and IL-13 related to the activity of the PI3K/Akt pathway (161). Recently, a systematic analysis of miRNA expression during the differentiation of BMMCs lead to the identification of 11 miRNAs that regulate the expression of specific transcription factors and 13 miRNAs that target transcripts of mMCP4 and mMCP6, regulating the synthesis of pre-formed inflammatory mediators (162).

EPIGENETIC CONTROL OF CYTOKINE PRODUCTION IN MC

Epigenetic modifications have been shown to influence the synthesis of transcription factors and to modify the sensitivity of promoters including in MC (163). For example, *in vitro* MC differentiation of MC was associated with decreased CD34 expression and increased HIF1A expression compared with bone marrow precursors. These changes were paralleled with changes in the methylation status of the promoters of those genes, suggesting that DNA methylation-dependent epigenetic regulation mediates the gene expression changes involved in maintaining the phenotype of mature MCs together with the differentiation of the HMC-1 cell line (164, 165). In another study, two constitutively DNAse I hypersensitivity sites (HSs) were described within the first intron of the IL-13 gene present in MC regulating the accessibility of the IL-13 locus for high level transcription (166). In the murine MC line derived from fetal liver CFTL-15, a cis-acting element in the second intron of the murine IL-4 gene has a dual function in regulating transcription in MC as well as chromatin accessibility of the IL-4 gene locus through the influence on the methylation state of the gene. MC-restricted transcription factors GATA-1/2 and PU.1 associate with the intron element and regulate its activity (167, 168).

Epigentic modifications are involved not only in the increase on gene transcription but also in the long-term inhibition of cytokine synthesis. In the THP-1 promonocytic cell line, during endotoxin tolerance, it was found that transcription of TNF gene in normal cells was preceded by dissociation of heterochromatin-binding protein 1α, demethylation of nucleosomal histone H3 lysine 9 increased phosphorylation of the adjacent serine 10, and recruitment of NFκB RelA/p65 to the TNF gene promoter. This was no more observed in tolerant cells and RelB, a repressor of transcription, remained bound to the promoter during silencing (169). Since MCs become tolerant after prolonged exposure to LPS, it is possible to speculate that the mentioned mechanisms of TNF gene silencing could also apply.

MOLECULAR TRAFFICKING EVENTS IN CYTOKINE/CHEMOKINE SECRETION

Contrary to the considerable advances made in the understanding of the regulation of fusion during anaphylactic degranulation, little is still known about the vesicular trafficking involved in MC cytokine/chemokine secretion. One problem is that endogenous cytokines and chemokines are not easily traceable for imaging studies due to the fact that they are secreted over a period of several hours. Thus, for example in macrophages one can observe protein accumulating in the Golgi, while vesicular structures are more difficult to delineate (170). Yet, Stow and coworkers have succeeded to image TNF secretory vesicles in macrophages, which produce considerable amounts of TNF that can be further enhanced by costimulating cells with IFNγ (171). Furthermore, in the case of TNF, addition of a TNF converting enzyme (TACE) inhibitor allows to block cleavage of transmembrane TNF at the PM and to detect its accumulation in the cell surface or in endocytosed vesicular structures (172). In some cases, the authors have also used overexpressed fluorescently tagged cytokines (TNF or IL-6) (173). Interestingly, while continuously produced from the viral promoter, their protein expression further increases after stimulation, which may in part be due to the fact that stimulation leads to enhancement of posttranslational control and vesicular trafficking mechanisms.

Accumulated data suggest that chemokine/cytokine secretion does not follow a unique trafficking pathway but rather could involve multiple pathways and organelles depending also whether they get delivered locally, such as for example at the T cell synapse (174) or the phagocytic cup in macrophages (31) or whether the release is more multidirectional on the cell surface. However, all studies agree that cytokine trafficking differs from the secretion of cytoplasmic SGs containing prestored mediators such as for example shown in NK cells (175).

Most of the vesicular trafficking events have been studied in macrophages. Here, after the initial transport through the Golgi stacks, TNF and IL-6 were found to bud off in highly dynamic tubular–vesicular structures (173). Interestingly, while TNF was generally found together with IL-6, the majority of IL-6 was found in independent carriers. All of them were finally delivered to the ERC, but they seem to reside in distinct non-overlapping areas. From the ERC, the individual cytokines are secreted independently with TNF largely fusing locally at the phagocytic cup, while IL-6 is secreted independently and not targeted to the phagocytic cup. This suggests that the ERC acts as a sorting hub for local secretion of cytokines. Interestingly, examination of another cytokine, IL-10, shows that only about half is delivered to the ERC, while the other half takes another transport route where it is directly delivered from the TGN to the PM via vesicular carriers containing the lipid binding protein ApoE (176). PMD has also been evoked as another mechanism for cytokine secretion. This concerns mostly cytokines prestored in cytoplasmic granules of eosinophils (177).

Concerning the SNARE fusion proteins necessary for constitutive secretion it was found that stimulation of macrophages

generally increases their expression, which could be a means to enhance the cytokine trafficking (171, 178). Similarly, some other regulatory proteins such as Rab and Munc18 isoforms are upregulated in LPS-stimulated macrophages (178). The fusion of vesicular carriers arriving from the TGN to the ERC seems to imply a SNARE complex containing the t-SNAREs Syntaxin 6 and Vti1b as well as the ERC-localized v-SNARE VAMP-3. The PM delivery implies a complex composed of SNAP-23 and Syntaxin 4 as well as VAMP-3 (31). Initial analysis of signaling events during such transport indicate that transport is not simply a default mechanism but to the contrary highly regulated. In particular, LPS allows recruitment of PI3Kδ to the TGN, where it allows recruitment of the GTPase dynamin involved in the fission of budding tubulovesicular structures (179). The phosphocholine cytidylyltransferase involved in lipid biogenesis seems also to get activated during this process (31).

Only a few studies have addressed the vesicular trafficking mechanisms in MC. Generally, they point to the fact that, with the exception of cytokines stored in cytoplasmic granules released by anaphylactic degranulation, they are released in a distinct manner that could resemble the mechanisms worked out in macrophages. Indeed, so far none of the SNARE accessory molecules involved in anaphylactic degranulation has shown an implication in cytokine trafficking. Neither the absence of Munc18-2 (70) nor of Syt II (100) has impacted on the secretion of cytokines such as TNF, IL-6, IL-4, or the chemokine CCL2 into the extracellular medium. The latter implies that calcium-mediated regulation may not play a major role in cytokine trafficking although other Syts expressed in MC (7) have not been tested. Yet, secretion of cytokines/chemokines in MC clearly implies SNARE fusion proteins. In human MC, it was shown that introduction of antibodies directed against PM localized t-SNAREs SNAP-23, Syntaxin 3, and Syntaxin 6 could block secretion in permeabilized human MC of all chemokines tested (CCL2, CCL3, CCL4, and CXCL8) albeit it was not always significant in the case of Syntaxin 6 (71). Anti-Syntaxin 4 and anti-VAMP-8 were able to selectively inhibit CXCL8, which could be explained by the fact that CXCL8 is stored in significant amounts in cytoplasmic granules of human MC and hence uses SNARE proteins involved in anaphylactic degranulation of human MC (28). No effects were seen neither for anti-Syntaxin 2, which localizes to the cytosol or the lysosomal marker VAMP-7 (71). In murine MC, functional data with SNARE proteins are not yet available, although the implication of VAMP-3 is suggested by the strong colocalization of VAMP-3 containing vesicles with TNF that had been protected against cleavage by a TACE inhibitor, while no colocalization with VAMP-8 containing vesicles was found (66, 180). This suggests that TNF may in a similar manner to macrophages take route through the ERC. Alternatively, it may also be delivered via the constitutive secretory pathway. Further studies in MC are however necessary to delineate the various vesicular trafficking events involved.

DISEASES ASSOCIATED WITH SG BIOGENESIS AND VESICULAR TRAFFICKING AND MAST CELL PHENOTYPE

In addition to the fundamental studies examining the secretory mechanisms of vesicular trafficking some important information has also been obtained from the study of inherited diseases in humans affecting SG biogenesis and secretory mechanisms in immune cells. Due to the associated defects of lymphocyte cytotoxic functions many of these diseases are characterized by a pathological condition called hemophagocytic lymphohistiocytosis (HLH) with defects in cytotoxic activity and expansion of polyclonal CD8-positive T cells and IFNγ-activated phagocytic macrophages, which infiltrate multiple organs and tissues including the nervous system causing also neurological manifestations [reviewed in Ref. (181)]. Amongst the familial forms of these diseases one can find familial lymphohistiocytosis 1 (FLH1) the most frequent form (50%) with still unknown genotype, FLH2 with a defect in the cytotoxic granule protein perforin, FLH3 with a defect in the SNARE accessory protein Munc13-4, FLH4 with a defect in the t-SNARE syntaxin 11, FLH5 with a defect in the SNARE accessory protein STXBP2 (Munc18-2) (181). Additional diseases showing signs of HLH include Griscelli Syndrome 2 (GS2) with a defect in Rab27A, Chediak–Higashi syndrome characterized by the presence of giant secretory lysosomes and a defect in the Lysosomal trafficking regulator (*Lyst*) gene (181). Another disease complex with secretory phenotypes is the Hermansky–Pudlak syndrome a heterogenous conditions leading to with different types of mutations in genes (*HPS1*, *AP3B1*, *HPS3*, *HPS4*, *HPS5*, *HPS6*, *DTNBP1*, *BLOC1S3*, and *BlOC1S6*) (182). Except for AP3B1 their protein products are part of the Biogenesis of Lysosome-related Organelles Complexes (BLOC), which regulates the traffic of vesicles in the endosomal system and also participate in endosomal membrane secretory lysosome fusion. Clinically the diseases are characterized by platelet secretory dysfunctions with altered granules and oculocutaneous albinism, pulmonary fibrosis, and granulomatous colitis (182). However, only defects in AP3B1, which is part of the AP-3 adaptor complex involved in the formation of new vesicles at the Golgi complex is also associated with lymphohistiocytosis and immune deficiency.

While the MCs phenotype has not been studied in human diseases some knowledge has been obtained from MCs obtained from mice carrying equivalent mutations. We have already described the effect of Munc13-4, Syntaxin 11, Munc18-2, and Rab27 mutations, which except for Syntaxin 11 all show a secretory phenotype. However, this generally concerns only anaphylactic degranulation, while for example cytokine/chemokine production seems not to be affected for example in Munc18-2 deficient cells (see above). Some earlier studies have also analyzed the effect of LYST mutations in MCs obtained from the beige mouse model. LYST codes for a ubiquitously expressed huge (425 kDa) cytosolic protein belonging to the BEACH (beige and Chediak–Higashi) family of proteins, which are proteins involved in vesicular trafficking and synaptic transmission, although their exact function is still unknown. Interestingly, LYST interaction partners include proteins involved, which are part of the ESCRT complex, suggesting a role of Lyst in the formation of intraluminal vesicles. Alternatively, data from the beige mouse model with a defect in LYST or LYST overexpression data indicate that LYST could be important in vesicle generation by fission (183). Indeed, MCs obtained from beige mouse are characterized largely increased giant granules (about 18× the volume), while granules of pancreatic acinar cells showed only a minor increase (23%) (184), suggesting that this may apply specifically to secretory lysosomes as granules from other hematopoietic cells are

also increased (183). Furthermore, granule composition appeared to be normal (185). Functional studies also showed that beige MCs were degranulation competent but seemed to show a higher frequency of granule–granule fusion events (186). Electrophysiological studies showed that membrane capacitance increases were about 10-fold higher in beige MCs in agreement with the large granule size (118).

Concerning Hermansky–Pudlak syndrome (HPS) only one elecrophysiological study has been performed with MCs from the ruby-eye mouse showing a defect in HPS6. HSP6 is part of the BLOC-2 complex composed of HSP3, HSP5, and HSP6. Patients with defects in BLOC-2 show a somewhat milder phenotype with absence of the development of pulmonary fibrosis. It interacts with BLOC-1 (*DTNBP1*, *BLOC1S3*, and *BlOC1S6)* required for cargo-specific sorting from early endosomes to lysosome-related organelles (187). In MCs derived from the ruby-eye mouse model a fusion pore phenotype was observed in electrophysiological studies with a threefold increase in the fraction and duration of transient fusion events (188). This suggests that HPS6 and potentially BLOC-2 may also play a direct role in secretion. The increased number of transient fusion events may also be an explanation for the observed platelet granule storage deficiency.

CONCLUDING REMARKS

The secretion of inflammatory mediators by MC through vesicular carriers is a highly regulated process starting with the biogenesis of SGs. Release is triggered by the activation of cell surface receptors initiating signaling processes culminating in the release from cytoplasmic granules by anaphylactic degranulation. As shown in this review many new signaling effectors have been described regulating the various steps involved in SG biogenesis and secretion. *De novo* production of cytokine requires additional control mechanisms at the level of transcriptional and post-transcriptional control of protein synthesis but as new data show may also be regulated at the level of vesicular trafficking, differing from the ones involved in the control of anaphylactic degranulation.

In addition, evidence has shown that MCs are receptive to a number of outside agents activating signaling pathways able to modulate the capacity of MC for secretion. These include, for example immunosuppressive cytokines like the members of the IL-10 family and the TGF-β superfamily (TGFβ and activins) (189). Certain Fc receptors have been also shown to negatively control signaling responses induced by MC (190, 191). Certain GPCRs coupled to Gs proteins that induce the production of cyclic AMP (such as β2-adrenergic, A2, and PGE_2 receptors), (192) or opiates acting through δ- or μ opiate receptors can also be potent inhibitors of MC secretion (193).

Together, this shows the necessity for exquisite control of the MC secretory processes in order to avoid the dangerous consequences it may have.

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Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity

Francis Davoine and Paige Lacy*

Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, AB, Canada

Edited by:
Uday Kishore, Brunel University, UK

Reviewed by:
Giovanna Schiavoni, Istituto Superiore di Sanità, Italy
Jaya Talreja, Wayne State University, USA

****Correspondence:***
Paige Lacy, Department of Medicine, 559 Heritage Medical Research Centre, University of Alberta, Edmonton, AB T6G 2S2, Canada
e-mail: paige.lacy@ualberta.ca

Eosinophils derive from the bone marrow and circulate at low levels in the blood in healthy individuals. These granulated cells preferentially leave the circulation and marginate to tissues, where they are implicated in the regulation of innate and adaptive immunity. In diseases such as allergic inflammation, eosinophil numbers escalate markedly in the blood and tissues where inflammatory foci are located. Eosinophils possess a range of immunomodulatory factors that are released upon cell activation, including over 35 cytokines, growth factors, and chemokines. Unlike T and B cells, eosinophils can rapidly release cytokines within minutes in response to stimulation. While some cytokines are stored as pre-formed mediators in crystalloid granules and secretory vesicles, eosinophils are also capable of undergoing *de novo* synthesis and secretion of these immunological factors. Some of the molecular mechanisms that coordinate the final steps of cytokine secretion are hypothesized to involve binding of membrane fusion complexes comprised of soluble *N*-ethylmaleimide sensitive factor attachment protein receptors (SNAREs). These intracellular receptors regulate the release of granules and vesicles containing a range of secreted proteins, among which are cytokines and chemokines. Emerging evidence from both human and animal model-based research has suggested an active participation of eosinophils in several physiological/pathological processes such as immunomodulation and tissue remodeling. The observed eosinophil effector functions in health and disease implicate eosinophil cytokine secretion as a fundamental immunoregulatory process. The focus of this review is to describe the cytokines, growth factors, and chemokines that are elaborated by eosinophils, and to illustrate some of the intracellular events leading to the release of eosinophil-derived cytokines.

Keywords: chemokines, growth factors, allergy, asthma, inflammation, helminths, cancer

INTRODUCTION

Eosinophils are granulocytic white blood cells that are rare in healthy individuals, but become elevated in both blood and tissue compartments in helminthic parasite infection as well as allergic inflammation, particularly in late-onset persistent eosinophilic asthma (1). Typically, the number of eosinophils generated from the bone marrow in healthy individuals are low, resulting in relatively few cells circulating systemically. Eosinophils that are produced in the healthy bone marrow predominantly home to the gut mucosa, where they may be involved in maintenance of homeostasis with gut microbiota (2). The numbers of blood and tissue eosinophils are markedly altered in a range of specific inflammatory and allergic responses, where eosinophils may be found in high densities in mucosal tissues. The recruitment of eosinophils is thought to be orchestrated by a complex series of events involving antigen-presenting cells (APCs), mast cells, T cells, B cells, along with their released cytokines as immune signals.

Along with responding to immune signals, eosinophils themselves are a source of over 35 cytokines, chemokines, and growth factors (3, 4). These have profound effects on the progression of immune and inflammatory responses (**Figure 1**). The purpose of this review is to evaluate the role that eosinophil-derived cytokines, chemokines, and growth factors, and how these may contribute to the propagation of immune responses.

CYTOKINES, CHEMOKINES, AND GROWTH FACTORS IN ALLERGIC INFLAMMATION AND ASTHMA

The role of eosinophils in immunity remains enigmatic. These granulated white blood cells are found, to varying degrees of similarity, in a wide range of invertebrates as well as vertebrates, including crustaceans, insects, mammals, fish, and birds (5, 6). Their expression in this wide variety of species suggests an important and evolutionarily conserved role in immunity. But what this role is, precisely, is still under intense scrutiny. While eosinophils have traditionally been implicated in maintenance of immunity against helminthic parasites, recent studies in transgenic mice that lack eosinophils suggest a more complex role for these cells than previously appreciated. In some cases, the absence of eosinophils actually inhibited parasitic growth (6), in contrast to the prevailing notion that eosinophils may be protective against helminthic parasites. More recent studies indicate that eosinophils may have a greater role in protection against viral infections, particularly respiratory viruses (7). Thus, the understanding of the role of

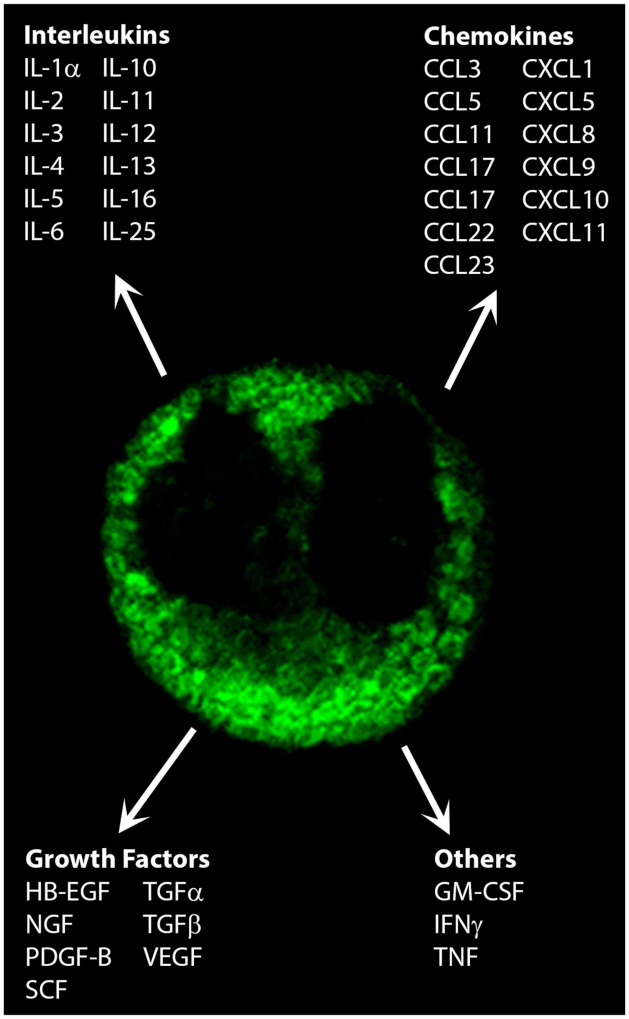

FIGURE 1 | Cytokines, chemokines, and growth factors secreted by eosinophils. Shown is the immunofluorescent staining pattern obtained for CCL5/RANTES in a human peripheral blood eosinophil by confocal microscopy. Original magnification × 100.

eosinophils in immunity remains a fascinating and evolving area of research.

From a clinical perspective, eosinophils are widely known for their tendency to increase markedly during allergic inflammation in tissues that normally harbor very few eosinophils, such as the lungs and upper airways. A key feature of allergic inflammation and asthma is the acute or chronic inflammatory cell infiltration at sites of allergen exposure in atopic subjects. Eosinophils co-migrate with inflammatory cells and are thought to contribute to bronchoconstriction, mucus secretion, edema, and tissue injury in the airways. The inflammatory processes that underlie allergic responses are orchestrated by an elaborate network of cytokines and chemokines that regulate IgE responses, bone marrow progenitor cell differentiation, and adhesion molecule expression. Infiltration of inflammatory cells can further exacerbate inflammation in target tissues by further secretion of cytokines, chemokines, and growth factors from tissue-migrated cells. The allergic response is often manifested as a biphasic reaction in asthma, consisting of an early phase response that involves APC-mediated activation of T cells to a Th2 phenotype and mast cell degranulation, followed by the late-phase response in which a secondary infiltration of inflammatory cells occurs in affected sites. The role of eosinophils in the biphasic allergic response is thought to be mainly associated with the late-phase response. Eosinophils recruited to inflammatory sites frequently undergo degranulation, releasing a range of cationic cytotoxic molecules, including major basic protein (MBP) and eosinophil peroxidase (EPX), as well as producing numerous cytokines, chemokines, and growth factors.

Several regulatory cytokines have been defined as belonging to two classes of $CD4^+$ T cells, which are involved in the initiation and maintenance of the allergic response. The first group of cytokines are those produced by T helper 1 (Th1) cells, which include interferon-γ (IFNγ), interleukin-2 (IL-2), and IL-12. The second group of cytokines are generated by Th2 cells, such as IL-4, IL-5, IL-9, and IL-13. More recent studies have suggested that Th17 and Treg cells are also important in the modulation of allergic responses by their production of immunosuppressive or regulatory cytokines including IL-10 and IL-17 (8). More recent studies have suggested an important role for IL-25 and IL-33 in the initiation of allergic responses, as these cytokines show a significant association with asthma in large cohort genome-wide association studies (9). In particular, IL-33 is important in the rapid induction of airway smooth muscle contraction by stimulating expansion of IL-13-producing type 2 innate lymphoid cells (10).

A substantial body of evidence from human and animal studies supports the hypothesis that allergic inflammation is an inappropriate response that arises from polarization of T cells toward a Th2 response, since greater expression of Th2 cytokines is seen in allergen-challenged individuals, along with a downregulation of Th1 cytokines. Enhanced expression of Th2 cytokines leads to the promotion of IgE switching of B cells, prevention of Th1 cytokine expression, increased tissue eosinophilia and eosinophil degranulation, and enhanced eosinophil survival. Prolongation of eosinophil survival, associated with delayed apoptosis, is thought to increase the amount of time that eosinophils actively release toxic mediators into tissues.

Against this background of Th2 responses in allergic inflammation, the cytokine network associated with allergy and asthma in humans is complex and not always associated with specific asthma phenotypes. Several studies suggest that although IL-4 triggers the polarization of T cells to a Th2 phenotype, it is not necessary for the manifestation of asthma (1). Moreover, the Th1 cytokine, IFNγ, may play a role in exacerbation of existing allergic inflammation as it is a potent activator of eosinophils *in vitro* (11–16). IFNγ has been found at elevated levels in the sera of patients with adult acute severe asthma (17, 18), and $IFN\gamma^+$ cells become upregulated in correlation with eosinophil infiltration in allergic subjects (19, 20). Th1 and Th17 cytokines are associated with activation of innate immune cells in the recently characterized phenotype of non-Th2 asthma, which is a late-onset form of asthma that is seen in women, obese patients, smoking-associated asthma,

and paucigranulocytic patients (1). Recent findings indicate that thymic stromal lymphopoietin (TSLP) may be a key target in airway hyperresponsiveness in allergic asthmatics (21). These observations suggest that Th2 cytokine responses alone are insufficient to promote asthmatic responses in the airways of human subjects.

However, the majority of asthma cases, although certainly not all, fit into the Th2 cytokine profile with varying degrees of eosinophilia (1). While the proportion of asthmatics exhibiting high numbers of eosinophils is not known, several studies of patients with mild to severe asthma suggest that it may be around 50% (1). Thus, eosinophils may be an important contributor to inflammatory responses at least half of asthma cases.

In summary, the substantial cytokine network underlying allergic inflammation is complex, with a Th2 cytokine profile and eosinophilia associating with some, but not all, asthma phenotypes. The way that eosinophil-derived cytokines contribute to immune defense or allergic diseases is not fully understood, although interestingly, recent discoveries have elucidated several novel functions for these cytokines in immunity and metabolism.

EOSINOPHILS AND THEIR DEGRANULATION RESPONSES

Eosinophils contain unique secretory granules known as crystalloid granules. These are so-called because of their characteristic crystalline cores, which appear electron-dense upon imaging by transmission electron microscopy. The crystalline core consists of highly concentrated, crystallized MBP, a cationic protein, which has cytotoxic effects on tissues upon its release (22). In addition to the MBP-rich crystalline core, crystalloid granules contain a matrix that is enriched in at least three other cationic proteins, which are EPX, eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN). The liquid phase of the matrix also contains many other enzymes and proteins, including cytokines, chemokines, and growth factors (**Figure 2**).

The contents of the crystalloid granule in eosinophils are released to the outside of the cell by at least four distinct mechanisms. These are (1) classical exocytosis (23); (2) compound exocytosis (24); (3) piecemeal degranulation (25), which is a form of exocytosis involving the fusion of small, rapidly mobilized secretory vesicles with the cell membrane; and (4) necrotic disintegration of the cell or "cytolysis," where whole, intact granules are released upon cell membrane rupture (26, 27). Piecemeal degranulation and cytolysis are most commonly observed in tissues obtained from patients with allergic inflammation (28, 29). Tissue damage associated with eosinophilic asthma and allergic inflammation is thought to be related to excessive release and tissue deposition of eosinophil granule proteins, particularly MBP, EPX, and ECP (22).

Several physiological agonists induce the release of eosinophil granule proteins by exocytosis, including platelet-activating factor [PAF; (30, 31)], opsonized surfaces (32), complement factors [C5a, (33)], immunoglobulin complexes (34), and cytokines and chemokines including granulocyte/macrophage colony-stimulating factor (GM-CSF), IFNγ, IL-3, IL-5, and CCL11/eotaxin (16, 35–37). Many of these factors are present in allergic inflammation and would be expected to contribute to activation of eosinophil degranulation responses.

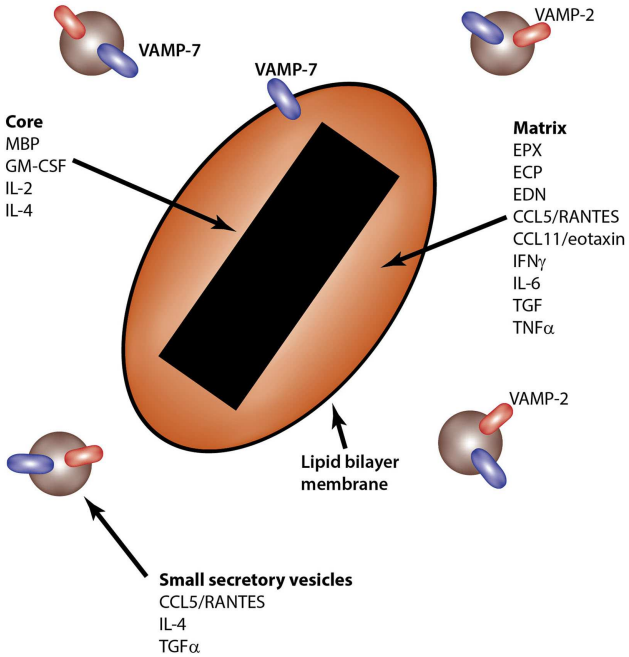

FIGURE 2 | Intragranular sites of storage for eosinophil-derived cytokines. The eosinophil crystalloid granule consists of two internal compartments: the core, enriched in MBP, and the matrix, which contains EPX, ECP, and EDN, among other granule components. Small secretory vesicles also transport cytokines, including CCL5/RANTES, IL-4, and TGFα. SNARE molecules are shown in the lipid bilayer membranes of granules and secretory vesicles.

HUMAN EOSINOPHILS AS A SOURCE OF CYTOKINES, CHEMOKINES, AND GROWTH FACTORS IN BLOOD AND TISSUES

Over 35 cytokines, chemokines, and growth factors have been characterized in eosinophils (**Table 1**). In the majority of cases, messenger RNA and protein for each product has been identified. Evidence for the synthesis and expression of nearly all eosinophil-derived cytokines, chemokines, and growth factors has been obtained from peripheral blood eosinophils purified from non-atopic as well as atopic subjects. A number of these have been found as stored, pre-formed mediators in crystalloid granules, giving eosinophils the ability to release these potent immunoregulatory factors rapidly (<1 h) into the surrounding milieu in response to activation.

In confirmation of observations with peripheral blood eosinophils, tissue eosinophils have also been characterized for their ability to synthesize and secrete cytokines, chemokines, and growth factors. Studies of tissue eosinophils from nasal polyps, bronchial biopsies, bronchoalveolar lavage (BAL) fluid, sputum samples, celiac mucosal biopsies, and skin biopsies from atopic individuals have shown that these cells are also capable of elaborating these immunomodulatory factors. For example, a significant percentage of eosinophils from subjects with allergic rhinitis express GM-CSF (41), IL-4 (50), IL-5 (108), CCL3/macrophage inflammatory protein-1α (MIP-1α) (71), CCL5/regulated on

Table 1 | Eosinophil-derived cytokines, chemokines, and growth factors.

Mediator detected in human eosinophils	Molecule detected	Mean quantity of protein stored (per 10^6 cells)	Release factors	Intracellular localization of stored protein	Reference
A. Cytokines					
A proliferation-inducing ligand (APRIL)	mRNA Protein	–	–	–	(38)
Granulocyte/macrophage colony-stimulating factor (GM-CSF)	mRNA Protein	15 pg	Ionomycin LPS	Crystalloid granules (core)	(39–42, 44)
Interleukin-1α	mRNA Protein	–	PMA	–	(45, 46)
Interleukin-1β	mRNA Protein	–	[Constitutively released]	–	(47)
Interleukin-2	mRNA Protein	6 pg	Serum-coated particles PHA	Crystalloid granules (core)	(48, 49)
Interleukin-3	mRNA Protein	–	Ionomycin IFNγ	–	(36, 40)
Interleukin-4	mRNA Protein	108 pg	Immune complexes Serum-coated particles Cytokines	Crystalloid granules (core)	(50–53)
Interleukin-5	mRNA Protein	–	Immune complexes	Crystalloid granules (core/matrix?)	(42, 54–57)
Interleukin-6	mRNA Protein	356 pg	Cytokines	Crystalloid granules (matrix)	(4, 15, 58, 59)
Interleukin-10	mRNA Protein	455 pg	Cytokines	Crystalloid granules	(4, 53, 60)
Interleukin-11	mRNA Protein	–	–	–	(61)
Interleukin-12	mRNA Protein	186 pg	Cytokines	Crystalloid granules	(4, 62)
Interleukin-13	mRNA Protein	4,596 pg	Cytokines	Crystalloid granules	(63, 64)
Interleukin-16	mRNA Protein	–	[Constitutively released]	–	(65)
Interleukin-17	Protein	–	–	–	(66)
Interleukin-25	mRNA Protein	–	[Constitutively released]	–	(67, 68)
Interferon-γ (IFNγ)	mRNA	997 pg	Cytokines	Crystalloid granules, small secretory vesicles	(4, 69)

(Continued)

Table 1 | Continued

Mediator detected in human eosinophils	Molecule detected	Mean quantity of protein stored (per 10^6 cells)	Release factors	Intracellular localization of stored protein	Reference
Tumor necrosis factor-α (TNF)	mRNA Protein	909 pg	Immune complexes TNF LPS Cytokines	Crystalloid granules	(4, 43, 53, 70, 71, 72, 73)
B. Chemokines					
CCL3/macrophage inflammatory protein-1α (MIP-1α)	mRNA Protein	–	–	–	(71, 74)
CCL5/regulated on activation, normal T cell expressed and secreted (RANTES)	mRNA Protein	7,000 pg	Serum-coated particles IFNγ	Crystalloid granules, small secretory vesicles	(16, 75)
CCL11/eotaxin	mRNA Protein	16–22 pg	C5a, immune complexes	Crystalloid granules	(76–78)
CCL13/monocyte chemoattractant protein-4 (MCP-4)	mRNA Protein	13 pg	Immune complexes	Crystalloid granules	(78)
CCL17/thymus activation regulated chemokine (TARC)	mRNA Protein	–	TNF + IFNγ or IL-4	–	(79, 80)
CCL22/macrophage-derived chemokine (MDC)	mRNA Protein	–	TNF + IFNγ or IL-4	–	(79, 80)
CCL23/myeloid progenitor inhibitory factor 1 (MPIF-1)	mRNA Protein	–	Cytokines	–	(81)
CXCL1/Groα	mRNA Protein	95 pg	Cytokines	Crystalloid granules	(82)
CXCL5/epithelial-derived neutrophil-activating peptide 78 (ENA-78)	mRNA Protein	1,500 pg	Cytokines	–	(83)
CXCL8/interleukin-8	mRNA Protein	–	C5a fMLP GM-CSF – RANTES or PAF Immune complexes TNFα LPS	–	(43, 53, 84–87)
CXCL9/monokine induced by gamma interferon (MIG)	mRNA Protein	–	TNF + IFNγ or IL-4	–	(79, 88)

(Continued)

Table 1 | Continued

Mediator detected in human eosinophils	Molecule detected	Mean quantity of protein stored (per 10^6 cells)	Release factors	Intracellular localization of stored protein	Reference
CXCL10/interferon γ induced protein 10 (IP-10)	mRNA Protein	–	TNF + IFNγ or IL-4	–	(79, 88)
CXCL11/interferon-inducible T cell alpha chemoattractant (I-TAC)	mRNA Protein	–	IFNγ	–	(88)
C. Growth factors					
Heparin-binding epidermal growth factor-like binding protein (HB-EGF-LBP)	mRNA	–	–	–	(89)
Nerve growth factor (NGF)	mRNA Protein	10 pg	–	–	(90)
Platelet-derived growth factor, B chain (PDGF-B)	mRNA	–	–	–	(91)
Stem cell factor (SCF)	mRNA Protein	9 pg	Chymase	Crystalloid granules	(92)
Transforming growth factor-α (TGFα)	mRNA Protein	–	Cytokines	–	(93–96)
Transforming growth factor-β (TGF-β)	mRNA Protein	–	Cytokines	–	(95–103)
Vascular endothelial growth factor (VEGF)	mRNA Protein	–	Cytokines	Crystalloid granules	(104–107)

activation, normal T cell expressed and secreted (RANTES) (109, 110), and transforming growth factor-β1 (TGFβ1) (98). As many as 44% of eosinophils present in nasal polyp tissues have been shown to be positive for IL-4 (50). Moreover, the majority of tissue-infiltrating eosinophils (84%) were found to be IL-4$^+$ during allergen-induced cutaneous late-phase reactions at 6 h (50). In another study, around 20% of tissue eosinophils were positive for IL-4 and IL-5 mRNA in skin biopsies of allergic individuals 24 h following challenge, which increased to 50–60% for protein expression of IL-4 and IL-5 (111). Eosinophils have also been shown to express IL-4 and IL-5 mRNA and protein in bronchial biopsies of atopic asthmatics as well as normal non-atopic subjects (112).

Other cytokines and chemokines have been shown to increase in tissue eosinophils during allergic inflammation. Eosinophils have been shown to exhibit greater expression of TGFβ1 than those of normal control subjects in bronchial biopsy tissue sections (101, 102). Nasal mucosal biopsies from seasonal rhinitis patients were found to contain elevated CCL5/RANTES$^+$ eosinophils, making up around 15% of the total CCL5/RANTES$^+$ population of cells (109). Eosinophils found in late-phase cutaneous reactions following allergen challenge in atopic subjects also express increased CCL5/RANTES mRNA and protein (75).

Endobronchial or segmental challenge with allergen consistently results in elevated eosinophil numbers in BAL fluid samples. Eosinophils accumulating in the airways following allergen challenge have been shown to express GM-CSF and IL-5 (42), as well as CXCL8/IL-8 (87), and CCL11/eotaxin (76). BAL-derived eosinophils that were recruited to the airways upon allergen challenge secrete significantly increased levels of CXCL8/IL-8 compared with those of normal controls during *in vitro* incubation (87). Supporting these observations is the study showing that sputum eosinophils also express GM-CSF as determined by immunocytochemistry (113).

The discovery of expression of cytokines by tissue eosinophils is not restricted to those found in the skin and airways. Eosinophils in the gut mucosa have also been found to express cytokines. In patients with active celiac disease, eosinophils from the gut mucosa were shown to be positive for IL-5 mRNA, and following treatment with a gluten-free diet, the numbers of IL-5$^+$

eosinophils declined (54). However, IL-5$^+$ eosinophils have not been detected in all gastrointestinal disorders, as intestinal mucosal eosinophils in Crohn's disease do not appear to be positive for IL-5 (56).

Other diseases also exhibit cytokine expression by eosinophils. IL-5$^+$ eosinophils have been detected in blood and tissue samples of individuals with eosinophilic cystitis, hypereosinophilic syndrome, and eosinophilic heart disease (55, 56).

Taken together, these and other *ex vivo* studies in humans have demonstrated that eosinophils derived from both the peripheral blood and tissue sources are capable of synthesizing, and in some cases releasing, cytokines, chemokines, and growth factors in eosinophilic diseases.

STORAGE AND SECRETION OF EOSINOPHIL-DERIVED CYTOKINES, CHEMOKINES, AND GROWTH FACTORS

As many as 10 cytokines, chemokines, and growth factors have been identified as pre-formed mediators that are stored within the crystalloid granules of eosinophils. Those found within granules are CCL5/RANTES (16), CCL11/eotaxin (77), GM-CSF (44), IL-2 (48), IL-4 (51, 52), IL-5 (56, 57), IL-6 (15), IL-13 (4, 63), transforming growth factor-α (TGFα) (114), and tumor necrosis factor-α (TNF) (70). The most abundant cytokine in eosinophils appears to be IL-13, followed by IFNγ and TNF (4). The techniques used to determine intracellular sites of cytokine storage include immunocytochemistry, subcellular fractionation, immunogold labeling, and immunofluorescence using confocal microscopy analysis.

Most cytokines that have been identified in eosinophil crystalloid granules appear to be stored within the matrix compartment surrounding the crystalline core, although a few may colocalized with the MBP-containing core. These are GM-CSF (44), IL-2 (48), and IL-4 (51). Some cytokines have not had their precise intragranular location determined, such as IL-5. These fascinating observations indicate that eosinophils are capable of storing pre-formed cytokines that may be released rapidly in response to inflammatory events.

Eosinophils appear to use a specialized tubulovesicular system to transport some of these cytokines and chemokines from the crystalloid granule to the cell membrane. This membrane transport mechanism, also known as piecemeal degranulation, allows the shuttling of granule contents to the cell surface through rapidly mobilizable secretory vesicles that bud from the surface of the crystalloid granule (115–118). This mechanism of cytokine transport was first identified with CCL5/RANTES, in which at least two intracellular compartments store pre-formed cytokine. The first is the crystalloid granule, and the second is a pool of small secretory vesicles that sediment at a higher buoyant density than granules when analyzed by subcellular fractionation (16).

Other studies have demonstrated that these small secretory vesicles are important in cytokine trafficking, particularly for TGFα (114) and more recently, IL-4 (119–122). The membrane trafficking mechanisms associated with piecemeal degranulation of cytokines and chemokines are described in more detail elsewhere in this issue (123).

Small secretory vesicles increase in numbers as well as in their content of cytokines and chemokines upon stimulation of eosinophils by immunoregulatory cytokines. For example, stimulation of peripheral blood eosinophils *in vitro* by IFNγ induces intracellular mobilization of IL-6 and CCL5/RANTES prior to their release (15, 16). IFNγ specifically induces the redistribution of CCL5/RANTES within eosinophils from crystalloid granules to small secretory vesicles within 10 min of stimulation (16). Intriguingly, the pool of CCL5/RANTES-containing small secretory vesicles was mobilized to the cell periphery within minutes of stimulation, leaving MBP$^+$ crystalloid granules behind in the cytoplasmic regions of cells, while crystalloid granule-associated CCL5/RANTES followed afterward. These findings suggest that granule proteins and cytokines/chemokines are selectively and differentially released in line with specific types of inflammatory responses in eosinophils.

Supernatants retrieved from IFNγ-stimulated eosinophils contained increased CCL5/RANTES and other crystalloid granule products (EPX), confirming the occurrence of degranulation (16). Following 16 h of stimulation by IFNγ, eosinophils were able to replenish their stores of CCL5/RANTES in their crystalloid granules. These findings have implications for sustained release of eosinophil-derived cytokines and chemokines in immune responses. An interesting possibility is that the eosinophil may have the potential to generate fine-tuned responses to immunological stimuli, with the release of cytokines/chemokines occurring separately from granule proteins.

CCL5/RANTES$^+$ small secretory vesicles were subsequently found to colocalize with the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE), VAMP-2 (vesicle-associated membrane protein-2) (124). SNARE proteins are universal fusion proteins that regulate the attachment (docking) of lipid bilayer-surrounded granules or vesicles to target membranes such as the inner leaflet of the plasma membrane (125). The fusion of CCL5/RANTES$^+$ vesicles upon docking with the inner leaflet of the plasma membrane is hypothesized to be dependent on binding to cognate target membrane-bound SNAREs, SNAP-23 and syntaxin-4 (117, 118, 126). Eosinophils depend on VAMP-7 for the release of granule proteins in response to intracellular secretagogues, GTPγS, and Ca^{2+} (127). However, we were unable to demonstrate whether CCL5/RANTES release was dependent on VAMP-7 as well, since negligible CCL5/RANTES was detected in supernatants of permeabilized eosinophils stimulated with these secretagogues. This suggests that the permeabilization process (using streptolysin-O) and/or the secretagogues used (GTPγS and Ca^{2+}) may not be optimal for inducing piecemeal degranulation, leading to cytokine secretion, in eosinophils.

Our recent findings indicate that eosinophil secretion may be evoked by the addition of PAF, a potent secretagogue for both human and mouse eosinophils (31). Degranulation was assessed by an EPX ELISA that was optimized in-house (128). Previously, mouse eosinophils were considered to be poor degranulators (129–132). Using these novel parameters for the assessment of mouse eosinophil degranulation, we determined that the guanosine triphosphatases (GTPases) Rac2 and Rab27a contribute to the secretion of EPX from eosinophils (133, 134). Rac2 regulates the assembly of the actin cytoskeleton network, which is essential for granule movement through the cytoplasm, while Rab27a acts through a family of Sec/Munc proteins that regulate SNARE

binding (135, 136). The role of GTPases in regulating cytokine secretion from eosinophils has not yet been defined.

Taken together, our studies suggest that cytokine trafficking and release in eosinophils may be mediated by VAMP-2 binding through its cognate SNAREs, SNAP-23, and syntaxin-4. Additional studies are required to understand how VAMP-2-mediated cytokine secretion may be regulated by GTPases.

POTENTIAL IMMUNOLOGICAL ROLES FOR EOSINOPHIL-DERIVED CYTOKINES, CHEMOKINES, AND GROWTH FACTORS

The ability of eosinophils to synthesize and secrete a large number of cytokines, chemokines, and growth factors suggest that these cells have the potential to regulate numerous immune responses, including allergic inflammation. The diversity of immunomodulators produced by eosinophils suggests that they may be able to orchestrate inflammatory processes in an exacerbating or modulatory manner. Many of the factors elaborated by eosinophils are likely to regulate immune responses, particularly CCL3/MIP-1α, CCL5/RANTES, CCL11/eotaxin, CXCL8/IL-8, GM-CSF, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF, and various growth factors. Other eosinophil-derived cytokines, chemokines, and growth factors, shown in **Table 1**, are likely involved in other types of reactions that apply to unique situations, such as atopic dermatitis, which may involve IL-12 released from tissue eosinophils following an initial phase of a Th2 response during cutaneous allergen challenge.

Eosinophil-derived chemokines may support the recruitment and maintenance of tissue eosinophils and lymphocyte infiltration during allergic inflammation. Eosinophils generate numerous chemokines including CCL3/MIP-1α and CCL5/RANTES (16, 71, 74, 75), both of which are major regulators of local inflammatory responses and chemoattractants for circulating leukocytes (137, 138). CCL5/RANTES exerts direct effects on eosinophils by elevating intracellular Ca^{2+}, triggering degranulation, and promoting superoxide release concurrently with enhanced chemotaxis (139–141).

CCL11/eotaxin is an important eosinophil-specific chemokine that is involved in chemotaxis of eosinophils into tissues, and is a highly potent agonist for inducing an influx of eosinophils during allergic responses (142). Gene knockout of CCL11/eotaxin markedly diminishes the tissue presence of eosinophils, which subsequently decreases allergic inflammation in the gut, skin, and airways (143). Like CCL5/RANTES, CCL11/eotaxin activates intracellular Ca^{2+} mobilization, degranulation, and respiratory burst in eosinophils, suggesting that it may act in an autocrine manner (144–146). Eosinophils express CCL11/eotaxin constitutively, apparently in association with their granules (77). This suggests a potential role for eosinophil-derived CCL11/eotaxin to function in a paracrine/autocrine manner for further recruitment of eosinophils at sites of inflammation.

Eosinophils release the neutrophil chemokine CXCL8/IL-8 (84), suggesting a role for eosinophils in recruitment of neutrophils at sites of inflammation. This chemokine is highly chemotactic and stimulatory for neutrophils and T cells (147), and eosinophils also have chemotactic responses to CXCL8/IL-8 following incubation with GM-CSF or IL-3 (148). However, IL-4 and IL-5 production in allergic inflammation may downregulate CXCL8/IL-8 expression, since these cytokines inhibit CXCL8/IL-8 release from monocytes (149). The overall role of CXCL8/IL-8 derived from eosinophils in inflammatory conditions is yet to be determined.

Eosinophils have been shown to synthesize and release abundant GM-CSF, which promotes degranulation and mediator release from these cells (37, 40, 44, 148). The activation of eosinophils by ionomycin, a calcium ionophore, induces GM-CSF release, which prolongs their own survival *in vitro* (40). Thus, GM-CSF is likely a critical eosinophil-derived cytokine that is important in maintaining the viability and effector function of eosinophils at inflammatory foci in allergic and immune responses.

The production of IL-1α from human eosinophils has been associated with cytokine induction of human leukocyte antigen DR (HLA-DR) expression (45). This suggests that the eosinophil has the capacity to function as an APC, as demonstrated in mouse models of allergic inflammation (150–153). Eosinophils have also been demonstrated to function as APCs during *Strongyloides stercoralis* infection (154). Secretion of IL-1α from eosinophils may be an important factor in allergen presentation to T cells in subjects with established eosinophilia during Th2-deviated immune responses to specific allergens.

A role in allergic inflammation is also implicated for eosinophil-derived IL-2, an essential growth factor for T cells that is likely to be critical for the initiation of the allergic phenotype following an early phase involving IL-4 stimulation (155). IL-2 also induces eosinophil chemotaxis via its receptor (CD25) expressed on a proportion of them (156).

IL-3 is a pluripotent growth factor required for the generation of a wide range of myelocytic cells and granulocytes, and is an important autocrine factor produced and used by eosinophils when stimulated (148, 157). Thus, tissue eosinophils that are actively secreting IL-3 are likely to prolong their own survival by autocrine signaling.

The role of IL-4 in allergic inflammation has been extensively studied and is among the most clearly defined of all known cytokines, with biologics development targeting the function of this cytokine (158). This cytokine is firstly important in the maintenance of Th2 responses (159, 160), and secondly, it is a critical factor in initiating the switch of B cells to IgE isotype production (161). IL-4 also has many other stimulatory roles in allergic inflammation, by inducing chemotaxis in eosinophils and enhancing the capacity of eosinophils to release granule proteins (35, 162). Eosinophil-derived IL-4 may be important in promotion of inflammation by increasing local IgE production, as well as upregulating vascular cell adhesion molecule (VCAM) expression on endothelial cells, which would increase leukocyte adhesion and transmigration into affected tissues (163). This would, in turn, increase eosinophil-specific migration into tissues by expression of very late antigen 4 (VLA-4) ligand (164).

Similarly to IL-4, IL-5 is also extensively investigated and well-defined for its role in allergic inflammation. While IL-5 is not necessary for skewing immune responses toward a Th2 phenotype, it is important in the downstream events that are typical of Th2 responses to allergens. IL-5 is essential for the terminal

differentiation of eosinophils from $CD34^+$ progenitors present in the bone marrow (165, 166). It also has numerous effects on eosinophils including prolongation of survival, induction of chemotaxis, priming, and degranulation so that their responses to agonists are enhanced (35, 167, 168). Finally, IL-5 prolongs the survival of eosinophils *in vitro* (169). These observations suggest that eosinophil-derived IL-5 is involved in exacerbations of the local allergic response following its release.

An additional cytokine derived from eosinophils that is likely to contribute to allergic responses is IL-6. This pleiotropic cytokine, generated during acute phase reactions, is important in regulating T and B cell function, as well as priming of granulocytes. IL-6 is an essential cofactor with IL-4 in isotype switching of B cells toward IgE production (170). In asthmatics, IL-6 is elevated in the serum and BAL in both baseline conditions and following allergen challenge along with IL-4 and IL-5 (171, 172). Whether IL-6 expressed from eosinophils is important in allergic inflammation is not known.

Eosinophils may also augment Th2 responses by secretion of IL-9, a potent T cell, and mast cell growth factor (173, 174). The expression of IL-9 mRNA and protein products were demonstrated in eosinophils along with mRNA encoding the IL-9 receptor α subunit, suggesting an autocrine role for this cytokine in eosinophils.

Eosinophils have the capacity to express and release the immunosuppressive cytokine IL-10 (53). A role for eosinophil-derived IL-10 is likely to enhance allergic inflammation, since this cytokine acts in concert with IL-4 to mediate the growth, differentiation, and isotype switching of activated B cells (175). However, IL-10 is classically known for its immunosuppressive role in immunity by decreasing cytokine secretion from inflammatory cells and preventing allergic inflammation (176, 177). A recent study showed that eosinophil-derived IL-10 has a novel immunoregulatory role for eosinophils in helminth infection (60). IL-10 generated from eosinophils induced the proliferation of myeloid dendritic cells and $CD4^+$ T lymphocytes, which inhibited expression of inducible nitric oxide synthase (iNOS), and protected intracellular *Trichinella spiralis* larvae. This striking observation suggests a protective role for eosinophils for intracellular *T. spiralis* larvae against NO-mediated killing, and further, that IL-10 derived from eosinophils drives this protective response. Further, it appears that *T. spiralis* exploits eosinophils to maintain its long-term survival in muscle tissue. These findings indicate a significant functional diversity of eosinophils that has not previously been appreciated until the advent of transgenic eosinophil-deficient mouse strains.

Among the more important cytokines released by eosinophils is IL-13, which is also stored in crystalloid granules as a pre-formed mediator (4, 63). IL-13 has many roles in the establishment of airway disease in asthma as well as pulmonary fibrosis, and also activates matrix metalloproteases in the airways (178). The activation of matrix metalloproteases by IL-13 is thought to protect against excessive allergic inflammation. IL-13 is also able to induce isotype switching of B cells to produce IgE (179), and has an important role in allergic inflammation (180). The expulsion of helminthic parasites from the gut of mice is also dependent on IL-13 (181). Eosinophils express IL-13 in inflammatory diseases (64), and this may have a role in the development of allergic inflammation, as discussed below.

Growth factors have an important role in inflammatory conditions, and those derived from eosinophils are likely to promote an inflammatory phenotype. Among these are heparin-binding epidermal growth factor (HB-EGF), which is a potent smooth muscle cell mitogen, and may contribute to pulmonary hypertension (89). Nerve growth factor (NGF) is elevated in subjects with allergic asthma, allergic rhinitis, and allergic urticarial–angioedema, with the largest increases observed in asthma (182). Eosinophils express NGF mRNA and protein, and this may contribute to the elevated levels seen in allergic inflammation (90). The most likely source of elevated serum NGF in allergy is from IgE-stimulated mast cells in tissues, since mast cells synthesize and secrete NGF (183). NGF stimulates T cells, B cell proliferation and differentiation, and eosinophil differentiation from peripheral progenitors (184), and is thus implicated in the pathogenesis of allergic inflammation.

Eosinophils also express the mast cell cytokine stem cell factor (SCF) (92). SCF may be associated with a positive feedback loop in tissue mast cells to maintain or exacerbate allergic inflammation, as well as inducing the growth and differentiation of mast cell progenitors residing in tissues (185).

Other growth factors produced by eosinophils include TGFα and TGFβ, which may modulate wound-healing and tissue remodeling (93, 102). TGFβ is specifically recognized for its role in chronic inflammation and fibrosis, and eosinophil-derived TGFβ may exert a role in tissue repair by inducing fibroblast growth and differentiation into myofibroblasts (186). Thus, TGFβ released from eosinophils may have a role in extracellular matrix protein deposition, particularly collagen, which contributes to structural abnormalities observed in severe allergic inflammation, including stromal fibrosis and basement membrane thickening.

Purified peripheral blood eosinophils from atopic individuals have been demonstrated to spontaneously release the pro-inflammatory cytokine TNF upon culture, and normal eosinophils stimulated with immobilized immunoglobulins or TNF express mRNA for this cytokine (53). TNF is a highly potent activator of monocytes, T cells, neutrophils, and endothelial cells, and enhances eosinophil adhesion and cytotoxicity (187, 188). This particular cytokine has numerous roles in inflammatory conditions, as well as helminthic infection and neoplasia associated with eosinophilic infiltration.

The Th1 cytokines, IL-12 and IFNγ, are also expressed in eosinophils. These are typically associated with inflammatory conditions that are distinct from the Th2 profile in allergic inflammation, and usually downregulate allergic inflammation following their release (189). However, Th2 cytokines can paradoxically promote Th1 responses in immune cells. IL-12 produced from eosinophils treated with IL-4 has been demonstrated to promote IFNγ mRNA expression in human Th1 cells (62). Allergen patch test reactions in atopic patients have shown that a Th1-like T cell activation occurs following the initial phase of increased local expression of Th2 cytokines associated with eosinophil infiltration after allergen challenge (190). Eosinophil-derived IL-12 was proposed to induce a switch from Th2 to Th1 responses commonly seen in late-phase allergic skin reactions (191). Eosinophils also express IFNγ in normal and atopic individuals (4, 69). Stimulation

of eosinophils by IFNγ has been postulated to enhance allergic inflammation during viral infections, suggesting an autocrine role for this cytokine (192).

Taken together, these findings show that eosinophils have the capacity to generate numerous immunoregulatory cytokines, chemokines, and growth factors. However, while the discovery that eosinophils can synthesize and secrete these immunomodulatory factors is important, it is essential to determine whether eosinophil-derived cytokines, chemokines, and growth factors have bioactive roles in immunity. In this way, we may learn more about the specific immunological function of eosinophils in the regulation of inflammatory processes.

DO EOSINOPHIL-DERIVED CYTOKINES, CHEMOKINES, AND GROWTH FACTORS HAVE BIOACTIVE ROLES IN THE IMMUNE SYSTEM?

Although the studies described above have presented evidence for the expression and release of a plethora of cytokines, chemokines, and growth factors from eosinophils, around a third of these have been shown to have a direct bioactive role. These include a proliferation-inducing ligand (APRIL), CCL5/RANTES, GM-CSF, IL-1β, IL-4, IL-10, IL-12, IL-13, IL-16, TNF, and TGFβ, which have been shown to have direct bioactive effects on other cells or in mouse models.

Recent studies indicate that eosinophils express APRIL, along with IL-6, which promoted the survival of plasma cells in the bone marrow in mice (38). This important finding suggests that eosinophils may have a role in enhancing and maintaining immunoglobulin production from plasma cells, which was shown to be true in a later study where eosinophils increased IgA^+ plasma cell numbers and the secretion of IgA in the gut mucosa (193). These studies suggest that eosinophils are essential for maintaining the integrity of the gut mucosal immunity, which is in agreement with their usual tissue homing under homeostatic conditions.

Other studies have shown that eosinophil-derived CCL5/RANTES and IL-16 have direct effects on lymphocytes in culture by inducing chemotactic activities (62). Thus, human eosinophils have the ability to alter the function of $CD4^+$ lymphocytes and memory T cells (194, 195). Finally, eosinophil-derived CCL5/RANTES has been shown to exert bioactive effects in eosinophil chemotactic assays as determined by the inhibitory effects of antibody to CCL5/RANTES, suggesting a role for this chemokine in autocrine signaling to enhance eosinophil migration.

The prolongation of eosinophil survival by GM-CSF is sensitive to inhibition by cyclosporin A, suggesting that this drug may modulate allergic inflammation by preventing autocrine cytokine signaling in eosinophils (40). Thus, eosinophil-derived GM-CSF is likely to have biological effects on survival of tissue eosinophils as well as newly recruited cells.

A recent study has demonstrated a role for eosinophil-derived IL-1β in inducing synthesis and secretion of IL-17 in activated $CD4^+$ T cells (47). As Th17 cells are implicated in the pathogenesis of allergic airway inflammation (196), this may be a key mechanism by which eosinophils promote Th17 cell differentiation.

Perhaps the most well studied cytokine elaborated by eosinophils is IL-4. Eosinophils are among the most abundant IL-4-expressing non-T, non-B cell populations in the lung and spleen of mice infected with the helminthic parasite *Nippostrongylus brasiliensis* (197). In the IL-4 reporter 4get mice, eosinophils were the most prevalent IL-4-producing cells infiltrating the lungs of mice infected with *N. brasiliensis*, with an up to 1000-fold increase (198). IL-4 expression in eosinophils is constitutive and programmed at an early stage of ontogeny (199). Moreover, instillation of IL-4 led to expansion of IL-4-producing eosinophils *in vivo*, suggesting that IL-4 is a potent factor in promoting the differentiation of bone marrow progenitor cells into Th2 cytokine-producing eosinophils, using mice expressing IL-4 with GFP (4get) (200). The production of IL-4 by eosinophils also occurs in other infections; when mice are infected with the fungal *Cryptococcus neoformans*, the majority of cells expressing IL-4 in the airways are eosinophils (201).

Adjuvant stimulation of B cell responses has been linked to IL-4 production from eosinophils in the spleen, based on findings in Δdbl-GATA eosinophil-deficient mice that were administered with alum (153). When alum was injected intraperitoneally into eosinophil-deficient animals, early B cell priming and IgM production was attenuated. This suggests a pivotal and previously unrecognized role for eosinophils in modulating the adaptive immune response to vaccines.

Interestingly, recent findings have indicated that eosinophil-derived IL-4 is also essential for the biogenesis of beige fat, a type of brown adipose tissue that is found in abundance in newborns that promotes non-shivering thermogenesis (202). Eosinophil-derived IL-4 was demonstrated to switch monocytes to the alternatively activated macrophage phenotype (203, 204), leading to the conversion of beige fat precursor cells to beige adipocytes (205). In a separate study, the satiety hormone leptin was found to be a potent secretagogue for eosinophils, and induced the expression and secretion of IL-1β, IL-6, and CXCL8/IL-8, as well as other chemokines, from eosinophils (206). This observation suggests that eosinophils may have an important role in improving the metabolic phenotype, by promoting insulin responsiveness and decreasing the incidence of obesity.

Eosinophil-derived IL-4 may be important in liver regeneration by promoting hepatocyte proliferation (207). In this study, liver injury induced by partial hepatectomy or carbon tetrachloride (CCl_4) in 4get mice resulted in the rapid recruitment of eosinophils, which secreted IL-4 and induced the proliferation of hepatocytes and liver growth.

The secretion of IL-4 from eosinophils in local tissues during allergic reactions may serve as a major initial source of IL-4 required for switching tissue-infiltrating naïve T cells to the Th2 phenotype. Several mouse models of Th2 inflammation support this notion. Mice infected intraperitoneally with eggs from the trematode *Schistosoma mansoni*, which generates a strong Th2 phenotype, exhibited early IL-4 increases derived from degranulating peritoneal eosinophils. Eosinophil-derived IL-4 induced priming of naïve T cells and activation of mast cell IL-5 release (154). The significance of IL-4 generation from eosinophils in human studies is yet to be discovered.

Stimulation of eosinophils with the Th2 cytokine IL-4 can also promote the development of Th1 responses. As described in Section "Potential Immunological Roles for Eosinophil-Derived Cytokines, Chemokines, and Growth Factors" above, IL-4-treated

eosinophils release the Th1 cytokine IL-12, which in turn induced the expression of IFNγ from Th1 cells during the culture of T cells with eosinophil-conditioned media (62). The finding that eosinophils may release Th1 cytokines suggests a more nuanced control of cytokine responses in allergic inflammation that these cells may possess. In summary, IL-4 production from eosinophils may be important in a variety of immune and metabolic responses that are only just beginning to be understood. These observations will continue to shape our understanding of the biological role of eosinophils in immunity.

A fascinating recent study using a computational model demonstrated that eosinophil-derived IL-13 is required for allergic airway responses (208). This was determined by the use of IL-13$^{-/-}$ eosinophils that were adoptively transferred by intravenous injection into Δdbl-GATA mice, which exhibited low airway hyperresponsiveness. IL-13 is important in inducing many of the characteristics of allergic airway disease, including airway hyperresponsiveness, goblet cell hyperplasia, and mucus secretion (180). In the computational model, it was found that eosinophil-derived IL-13 could not sustain an allergic asthma response in the absence of T cell (or other cell type)-derived IL-13, but that IL-13 production by eosinophils was integral to the development of allergic asthma (208).

Co-culture of human eosinophils and their conditioned media with the colon carcinoma cell line Colo-205 led to the production of TNF, which was involved in tumor cell killing along with granzyme A (73). This suggests a novel function for eosinophil-derived TNF in regulating tumor cell growth, and that tumor cells may be directly recognized by eosinophils.

Eosinophil-derived TGFβ has been shown to regulate fibroblast proliferation and differentiation (209). This is indicative of a potential role for eosinophils in wound-healing. Indeed, eosinophils were found to express both TGFβ and IL-13 following intradermal allergen challenge, which resulted in increased repair and remodeling events in human atopic skin (210).

OTHER PHYSIOLOGICAL OR PATHOLOGICAL ROLES FOR EOSINOPHIL-DERIVED CYTOKINES

Although earlier studies in mice suggested that eosinophil-derived IL-4 was required for mounting a Th2 response during immune reactions to intraperitoneally injected *Schistosoma mansoni* eggs (211), more recent studies have shown that ablation of eosinophils from mice had negligible outcomes, or even inhibitory effects, on helminth larval or egg expulsion in Δdbl-GATA or PHIL mice (212). These experimental findings imply that eosinophil-derived cytokines, chemokines, and growth factors have no specific role in the regulation of parasitic worm diseases, in contrast to the classically held notion that eosinophils are important in parasitic worm expulsion.

In other studies, certain types of lymphoid and solid tumors have been associated with the infiltration of eosinophils into cancerous tissues (213), particularly specific lymphomas and Hodgkin's disease. Although tumor-related eosinophilia was considered to be an epiphenomenon arising from the spontaneous elaboration of IL-5 from tumor cells, or overproduction of T cells during chemotherapy with IL-2 (214), there is evidence of eosinophil activation as a result of IL-2 anticancer therapy (215, 216). In some cases, tissue eosinophilia is considered to be a positive prognosis for head and neck cancers (217) and advanced bladder cancer (218). Specifically, in oral squamous cancer, eosinophils are a positive prognosis for early stages of disease (Stages II and III), but an unfavorable prognosis for advanced cases (Stages III–IV) (219, 220). The involvement of eosinophil-derived cytokines, chemokines, and growth factors in neoplasias associated with eosinophilic infiltration is partially understood. In oral squamous cell carcinoma with tumor-associated tissue eosinophilia (TATE), the source of CCL11/eotaxin is apparently eosinophils (221). The cultured oral squamous cell carcinoma cell line SCC9 secretes chemotactic prostaglandin D_2, which promotes eosinophil transmigration, thus providing a signal to recruit eosinophils into tumor masses (222). Eosinophils that infiltrate into tumors/lymphomas also express IL-6, TGFβ, and CCL24/eotaxin-2 (223).

However, while numerous studies have highlighted a role for eosinophil recruitment and activation in many types of cancers, and that eosinophils express receptors and mediators shared with cytotoxic T cells (224), there are few that implicate eosinophil-derived cytokines, chemokines, and growth factors in the regulation of cancer growth. Interestingly, as described above, a recent study showed that human eosinophils possessed tumoricidal activity toward a colon cancerous cell lines in culture by releasing TNF (73). The tumor-killing effects of eosinophil-derived TNF were the first description of a cytokine, chemokine, or growth factor elaborated by eosinophils implicating a role for these immunomodulators in cancer.

Injection of IL-17E increases the efficiency of chemotherapy and results in eosinophilia (225). Eosinophil-derived IL-17 may be implicated in antitumor activity, since IL-17E (IL-25) exerts antitumor activity against many types of cancerous cell lines, at least in xenograft models using human cancer cells in mice (226). Eosinophils express IL-17 as determined by immunocytochemistry and Western blot analysis (66). Thus, the production of IL-17 from eosinophils may be important in protection of the host against cancers.

Moreover, since eosinophils produce numerous growth factors such as vascular endothelial growth factor (VEGF), as well as a number of other factors that promote angiogenesis, it is possible that eosinophilic inflammation is implicated in tumor neovascularization (227). Hypothetically, eosinophil-derived cytokines, chemokines, and growth factors may be involved in enhancing T cell-mediated tumor killing, particularly at the level of the local tissue environment where large numbers of infiltrating eosinophils accumulate and are actively degranulating onto tumor cells.

Taken together, these findings suggest that eosinophils may serve as important components of natural immunity, and their cytokines, chemokines, and growth factors may contribute to augment inflammatory responses in allergy and other conditions. Further studies are awaited to understand the involvement of eosinophil-derived immunomodulatory factors in the regulation of allergic inflammation and other inflammatory conditions, as well as the extent to which the release of these factors may be manipulated for therapeutic benefit.

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Eosinophil secretion of granule-derived cytokines

***Lisa A. Spencer*[1], *Kennedy Bonjour*[2], *Rossana C. N. Melo*[2†] *and Peter F. Weller*[1*†]**

[1] *Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA*
[2] *Laboratory of Cellular Biology, Department of Biology, Federal University of Juiz de Fora (UFJF), Juiz de Fora, Brazil*

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Roma Sehmi, McMaster University, Canada
Nives Zimmermann, Cincinnati Children's Hospital, USA

****Correspondence:***
Peter F. Weller, 330 Brookline Avenue, E/CLS Rm 940, Boston, MA 02215, USA
e-mail: pweller@bidmc.harvard.edu

†Rossana C. N. Melo and Peter F. Weller have contributed equally to this work.

Eosinophils are tissue-dwelling leukocytes, present in the thymus, and gastrointestinal and genitourinary tracts of healthy individuals at baseline, and recruited, often in large numbers, to allergic inflammatory foci and sites of active tissue repair. The biological significance of eosinophils is vast and varied. In health, eosinophils support uterine and mammary gland development, and maintain bone marrow plasma cells and adipose tissue alternatively activated macrophages, while in response to tissue insult eosinophils function as inflammatory effector cells, and, in the wake of an inflammatory response, promote tissue regeneration, and wound healing. One common mechanism driving many of the diverse eosinophil functions is the regulated and differential secretion of a vast array of eosinophil-derived cytokines. Eosinophils are distinguished from most other leukocytes in that many, if not all, of the over three dozen eosinophil-derived cytokines are pre-synthesized and stored within intracellular granules, poised for very rapid, stimulus-induced secretion. Eosinophils engaged in cytokine secretion *in situ* utilize distinct pathways of cytokine release that include classical exocytosis, whereby granules themselves fuse with the plasma membrane and release their entire contents extracellularly; piecemeal degranulation, whereby granule-derived cytokines are selectively mobilized into vesicles that emerge from granules, traverse the cytoplasm and fuse with the plasma membrane to release discrete packets of cytokines; and eosinophil cytolysis, whereby intact granules are extruded from eosinophils, and deposited within tissues. In this latter scenario, extracellular granules can themselves function as stimulus-responsive secretory-competent organelles within the tissue. Here, we review the distinctive processes of differential secretion of eosinophil granule-derived cytokines.

Keywords: secretion, eosinophil, granule, degranulation, piecemeal degranulation, cytolysis, cytokine

INTRODUCTION

EOSINOPHILS ARE DISTINGUISHED BY THEIR EOSIN-LOVING SPECIFIC GRANULES

Paul Ehrlich's discovery of eosinophils in 1879 was based on the distinctive "eosin-loving" property of eosinophil intracellular granules. The characteristic dark pink punctate staining seen in standard hematoxylin and eosin (H&E) preparations is due to the high cationic protein content of eosinophil granules reacting with the acid dye eosin (1). The most abundant (and most cationic) of the eosinophil granule-derived proteins is major basic protein (MBP), and it is MBP that forms the crystalline lattice structure of the eosinophil granule core, an identifying ultrastructural feature of eosinophils (**Figure 1**). Eosinophils store their hydrolytic enzymes and cationic granule proteins, including MBP, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), within the core and surrounding matrix of eosinophil specific granules (**Figure 1**), and it has been long appreciated that secretion of these granule-derived proteins can exert toxic effects on parasites, microbes, and host tissue cells [reviewed in Ref. (2)].

More recently appreciated is that in addition to cationic proteins and hydrolytic enzymes, eosinophils are sources of numerous (over three dozen identified to date) cytokines and chemokines, with a range of biological functions (3, 4). It is now recognized that along with the cationic proteins, many, if not all, of these cytokines are stored within eosinophil specific granules, available for very rapid secretion without the need for *de novo* synthesis (5). A recent study demonstrated co-expression of at least seven immunomodulatory cytokines preformed within specific granules of human blood eosinophils (6), and a number of physiological stimuli have been identified that elicit differential secretion of granule-stored cytokines from eosinophils (7–10). Therefore, it is fitting that the distinguishing morphological feature of eosinophils (i.e., their specific granules) should also represent a functional distinction for these cells.

VAST ARRAY AND BIOLOGICAL RELEVANCE OF EOSINOPHIL GRANULE-DERIVED MEDIATORS AND MECHANISMS OF SECRETION

With the growing awareness of the diverse repertoire of eosinophil granule-derived cytokines has come an evolution in understanding the varied roles eosinophils play in biology. Previously considered strictly end-stage effector cells in parasitic helminth infections and allergic diseases such as asthma, eosinophils, and their secreted products are now regarded as participants in organ development (11, 12), metabolism (13), maintaining (14–16) and/or recruiting (17) lymphocyte populations, anti-microbial (18–22)

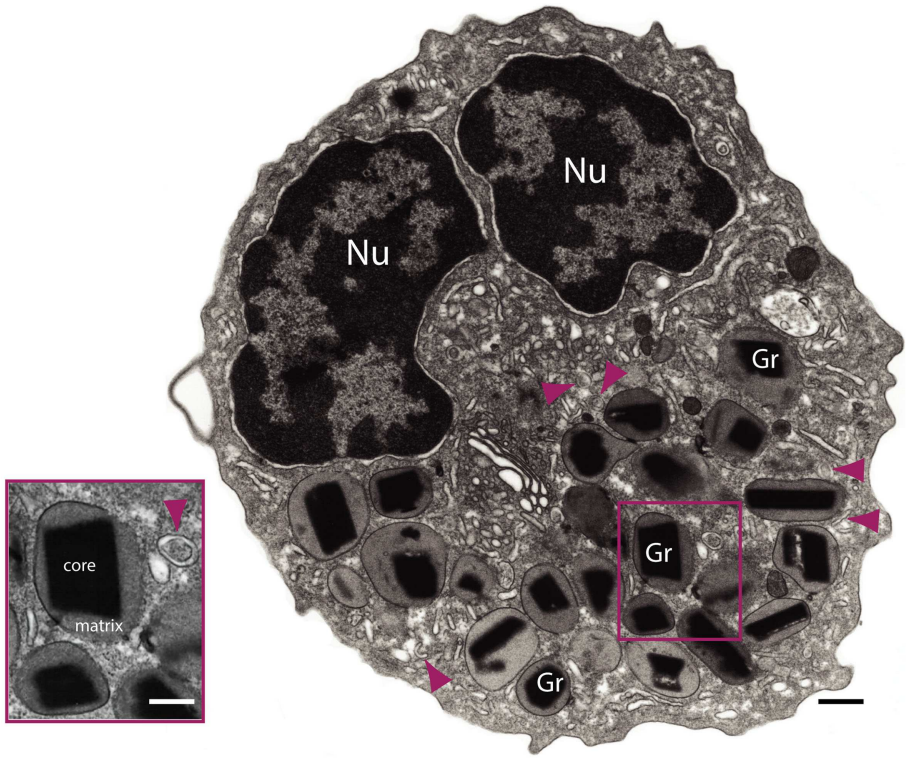

FIGURE 1 | Transmission electron microscopy of a human eosinophil. This cell is characterized by a major population of specific granules (Gr) with a unique morphology – an internal often electron-dense crystalline core and an outer electron-lucent matrix surrounded by a delimiting trilaminar membrane. Note the typical bilobed nucleus (Nu) and large tubular carriers (arrowheads). The inset shows secretory granules and a tubular vesicle at higher magnification. Bars: 500 nm; 300 nm (inset).

and fungal (23–25) immunity, tissue repair and regeneration (26–31), immunomodulation (32–37), and tumor immunity (38), and reviewed in Ref. (39).

How does the eosinophil accomplish the highly selective process of secretion of its granule-derived proteins? Basic Immunology textbooks often define degranulation from granulocytes such as eosinophils to occur by a process of classical exocytosis, whereby intracellular granules fuse with the plasma membrane and engage in a wholesale release of granule contents, or in more extreme instances compound exocytosis, whereby intracellular granules fuse together prior to fusion with the plasma membrane and release of their combined contents (**Figure 2**). Although degranulation via classic and compound exocytosis is observed upon interaction with very large metazoan parasites, in most other physiologically relevant scenarios eosinophils either (1) differentially and progressively secrete their granule-stored contents through a vesicle-dependent process termed piecemeal degranulation (PMD) or (2) deposit intact granules directly into the tissue through a distinctive mode of cell death, termed eosinophil cytolysis (**Figure 2**). To appreciate the extensiveness of PMD and cytolysis in tissue eosinophils *in situ*, we would refer the reader to Erjefalt et al. (40), and Saffari et al. (41), wherein using morphological criteria the authors quantify the number of tissue eosinophils undergoing PMD and/or cytolysis in association with allergic diseases (i.e., allergic rhinitis and asthma), eosinophilic esophagitis (EoE), or inflammatory bowel diseases (IBDs). In the former study, nearly all eosinophils from tissue sections of allergic disease patients exhibited evidence of PMD, and 27% of the eosinophils showed signs of cytolysis. In IBD samples, greater than 50% of the tissue eosinophils exhibited signs of PMD, while 14% were cytolytic (40). In the latter study, approximately 93% of esophageal eosinophils in EoE exhibited two or more features of degranulation, including loss of cell membrane integrity (marker of cytolysis), cytoplasmic vesiculation (PMD), or reversal of granule core staining (PMD). A total of 70% of esophageal eosinophils exhibited all three features (41). In the remainder of this manuscript, we will explore these two distinct modes of eosinophil secretion of granule-derived proteins.

PIECEMEAL DEGRANULATION

The process of PMD was first identified ultrastructurally through electron microscopy studies of mast cells, basophils, and eosinophils in the mid 1970s (42). PMD is characterized by a progressive emptying of granule contents without granule to plasma membrane fusions, rather PMD is accomplished by numerous spherical and tubular secretory vesicles that shuttle granule-derived proteins from the granule to the plasma membrane for secretion. Within the past decade, biochemical and advanced microscopic techniques have enabled an unprecedented look into the process of PMD, and are revealing eosinophil intracellular

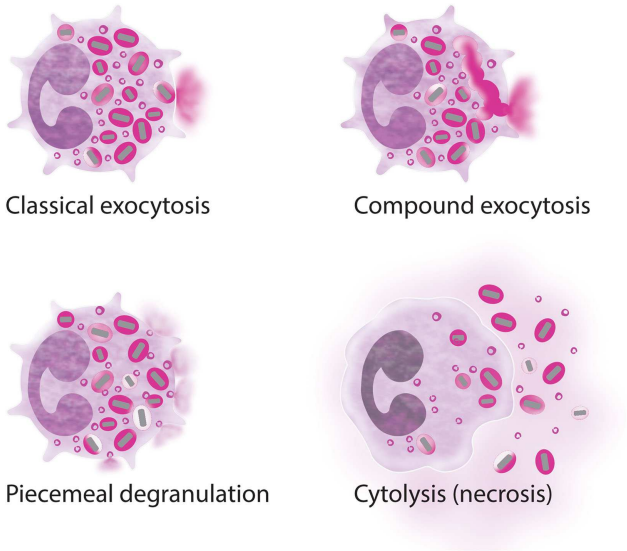

FIGURE 2 | Processes of eosinophil secretion. Eosinophils may secrete their granule proteins by classic exocytosis (individual granule fusion with the plasma membrane and release of the total granule content); compound exocytosis (intracellular granule–granule fusion before extracellular release); piecemeal degranulation (vesicular transport of small packets of materials from the secretory granules to the cell surface); and/or cytolysis (extracellular deposition of intact granules upon cell lysis). More than one process can be involved in inflammatory responses.

granules to be dynamic organelles, which undergo protein sorting and vesicle formation.

EOSINOPHIL GRANULES ARE DYNAMIC INTRACELLULAR ORGANELLES

Among the earliest ultrastructural indications of PMD are alterations within granules; the crystalline core may become less sharply defined, and variations in the electron density of the core and surrounding matrix occur, representative of disassembled matrices and cores and a reorganization of granule contents (**Figure 3**). Small spherical vesicles and elongated tubule carriers [eosinophil sombrero vesicles (EoSVs), discussed in the next section] are seen budding from emptying granules, and numbers of spherical vesicles and tubular carriers increase within the cell cytoplasm [(43) and **Figures 4A–C**]. Immuno-electron microscopy using antibody Fab fragments conjugated to very small nano-gold particles confirm budding vesicles contain granule-derived cytokines and cationic proteins [(43–45) and **Figures 4D–F**]. Vesicles fuse with the plasma membrane and secrete cytokines extracellularly in discreet packets (**Figures 4G,H**).

Several lines of evidence support the conclusion that these vesicles are actually generated by and emerge from the granules themselves. Vesicular structures are apparent within emptying granules in the early stages of PMD, and treatment with the vesicular transport inhibitor brefeldin-A (BFA) inhibits the number of cytoplasmic vesicles (**Figure 4B**) and cytokine secretion by PMD, and causes depositions of membrano-lipid deposits within granules (43). Eosinophils undergoing PMD were further studied by dual-axis automated electron tomography, allowing for tracking of granule structures and content in three dimensions. Tomographic reconstructions demonstrate that granule contents within emptying granules are rearranged into intragranular vesiculotubular compartments and mobilized to the granule membrane. Moreover, both elongated tubular and small spherical vesicles containing granule contents were observed emerging directly from the eosinophil granules in tomographic reconstructions (43, 44, 46).

TUBULAR CARRIERS AND RECEPTOR-MEDIATED TRAFFICKING OF GRANULE-DERIVED CYTOKINES

As noted above, eosinophils contain over three dozen preformed cytokines; most, if not all, of these cytokines are stored within intracellular granules. Eosinophil granule-derived cytokines are differentially secreted in response to exogenous stimulation, indicating that mechanisms must exist to sort granule-stored cytokines into granule-derived secretory vesicles. One mechanism has been described for the specific mobilization of IL-4. Analysis of eosinophil lysates after subcellular fractionation revealed IL-4 receptor alpha chains (IL-4Rα) are enriched within granule- and vesicle-containing fractions. Upon stimulation of eosinophils with eotaxin-1, a chemokine known to elicit PMD of eosinophil granule-stored IL-4, complementary approaches based in immuno-electron microscopy and flow cytometry demonstrated granule-expressed IL-4Rα is mobilized into secretory vesicles in parallel with IL-4 (47). Importantly, antibodies that compete with IL-4 for binding to IL-4Rα failed to detect vesicle-mobilized IL-4Rα, suggesting that the receptor engages IL-4 during loading into secretory vesicles, and remains engaged while traversing the cytoplasm. This conclusion is supported by immuno-EM studies wherein IL-4 detected within secretory vesicles appears to be membrane-bound (47), in contrast to the free luminal expression pattern exhibited by vesicle-contained MBP (48) (**Figures 4D–F**). Vesicle-carried TGF-α also exhibits a membrane-associated expression pattern (49), and eosinophils express receptors for most (if not all) of the cytokines that they also store, suggesting that receptor-mediated chaperoning of cognate cytokines might be a universal method of regulating eosinophil-derived cytokine secretion.

This observation of receptor-mediated transport of granule-derived cytokines also provides a function for the large tubular carriers (EoSVs) characteristic of eosinophils undergoing PMD. EoSVs represent a distinct vesicle population, distinguishable from smaller spherical vesicles by morphology and subcellular density. EoSVs observed within the cytoplasm by electron microscopy are viewed as elongated tubes, or when curled, may appear to take on the shape of a "c" or a donut ring (**Figures 4A,C**). A key aspect of EoSV morphology is the large surface area:volume ratio, a conformation that is optimal for a receptor-mediated transport mechanism (44).

Although the number of cytoplasmic vesicles increases in eosinophils undergoing PMD, spherical vesicles and EoSVs are also observed in non-stimulated eosinophils (see **Figure 1**). For example, a substantial pool of MBP-loaded vesicles can be observed in intimate association with secretory granules in unstimulated eosinophils (48). It is unclear whether cargo-laden cytoplasmic vesicles are vestiges of a previous round of PMD, and/or whether eosinophils might utilize secretory vesicles as another, rapidly mobilizable, depot for intracellular cytokine storage. Of note, this

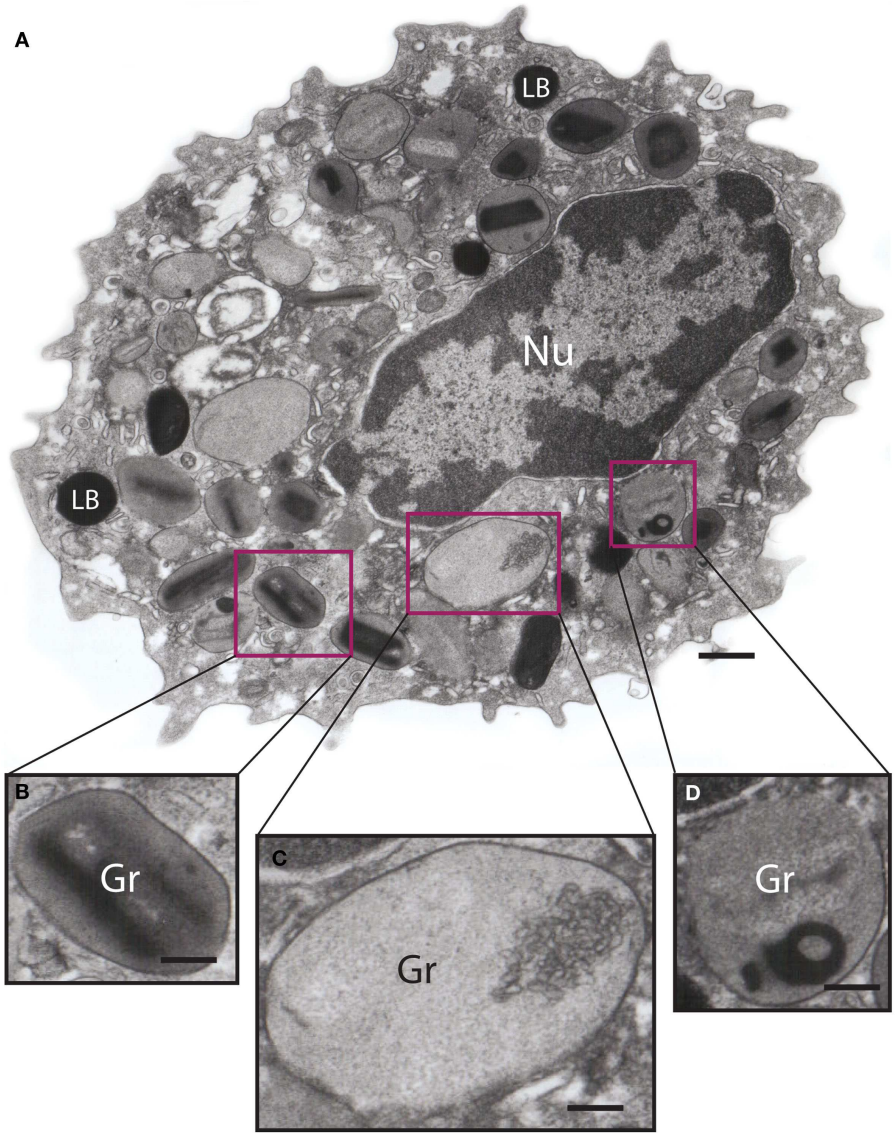

FIGURE 3 | Ultrastructure of an eotaxin-activated human eosinophil showing piecemeal degranulation (PMD). **(A)** After stimulation, specific granules (Gr) exhibit different degrees of emptying of their contents and morphological diversity indicative of PMD, such as **(B)** lucent areas in their cores, **(C)** enlargement and reduced electron density, and **(D)** residual cores. Eosinophils were isolated by negative selection from healthy donors, stimulated with eotaxin-1 for 1 h, immediately fixed and prepared for transmission electron microscopy as before (43). Nu, nucleus; LB, lipid body. Scale bar: 500 nm **(A)**; 170 nm **(B–D)**.

issue might be relevant to eosinophil postmortem function as well, as we will later see that EoSVs are released along with cell-free granules from cytolytic eosinophils (see Cytolysis below).

INTRACRINE REGULATION OF EOSINOPHIL PMD

Despite large strides in delineating dynamic intragranule vesiculation and receptor-mediated cytokine sorting, how exogenous signals are transmitted to and decoded by intracellular granules remain unclear. Exposure of eosinophils to a number of physiological stimuli, such as chemotactic lipids and chemokines, cytokines (e.g., IL-3, IL-5, and GM-CSF), and complement components can result in their priming, effectively lowering the signaling threshold for inducing subsequent stimulus-induced cytokine secretion. Intracellular mechanisms that drive eosinophil priming upstream of enhanced secretion are not fully understood. However, inside-out signaling that upregulates the expression, affinity, and/or valency of eosinophil-expressed integrins (e.g., $\alpha_M\beta_2$) appears to play a significant role [reviewed in Ref. (50)] and may be mediated through a pathway involving PKCβII-dependent phosphorylation of the actin bundling protein L-plastin (51).

In addition to a role for priming in eosinophil secretion, data suggest the existence of intracrine mediators that act on intracellular receptors, possibly expressed on granules. For example, eotaxin-1-induced secretion of IL-4 is dependent upon an

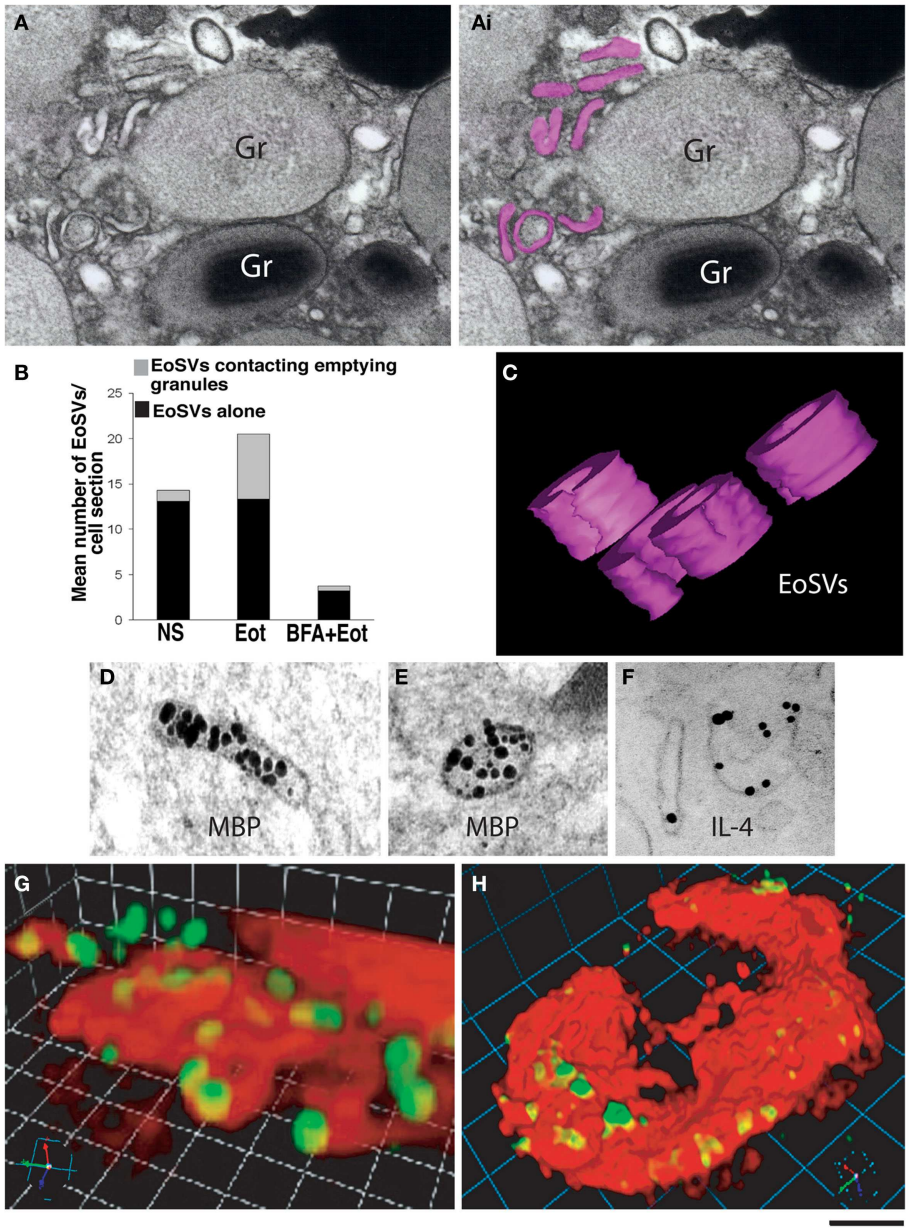

FIGURE 4 | Vesicular trafficking of granule-derived products from human eosinophils. **(A)** Eosinophil sombrero vesicles – EoSVs – [highlighted in pink in **(Ai)**] are observed in the cytoplasm surrounding an emptying, enlarged secretory granule (Gr). An intact granule (Gr) with typical morphology is also observed. **(B)** Quantification of EoSV numbers revealed significant formation of these vesicles and association with granules undergoing release of their products, after eotaxin-1 (EOT) stimulation (45). Brefeldin-A (BFA) pretreatment suppressed all EoSV numbers dramatically ($P < 0.05$). NS, not stimulated. **(C)** Three-dimensional (3D) models obtained from electron tomographic analyses show EoSVs as curved tubular and open structures surrounding a cytoplasmic center. **(D–F)** As demonstrated by immunonanogold electron microscopy, major basic protein (MBP) **(D,E)** is transported within the EoSVs lumen, while IL-4 mobilization is associated with vesicle membrane **(F)**. In **(G,H)**, human blood eosinophils suspended in an anti-IL-4 capture antibody-containing agarose matrix were stimulated with eotaxin-1. 3D reconstructed images demonstrate released and captured IL-4 as focal fluorescent green spots at the outer surface of the cell membrane (stained in red). **(B,F–H)** were reprinted from Ref. (45) and **(C–E)** from Ref. (46) with permission. Scale bar: 250 nm **(A)**; 150 nm **(C–F)**; 4 μm **(G)**; 6 μm **(H)**.

intracrine pathway involving the lipid mediator leukotriene C_4 (LTC_4). Eotaxin-1 stimulation of eosinophils elicits the generation of LTC_4 from intracellular lipid bodies; this LTC_4, acting via an intracellular receptor, is necessary for subsequent IL-4 release (52, 53). Eosinophil intracellular granules express leukotriene receptors on their outer granule membranes (54). Further studies are necessary to determine the specific intracellular target(s) of the LB-generated LTC_4.

In addition to the intracrine signaling mediators, cytoskeletal elements, GTPases, and membrane-associated proteins further co-ordinate granule and vesicle trafficking, and membrane fusions in PMD. For example, specific SNARE proteins [Soluble NSF Attachment Protein (SNAP) receptors] expressed by granule, vesicle, and plasma membranes within eosinophils co-ordinate membrane tethering, docking, and fusions [reviewed in Ref. (55)]. SNARE-mediated membrane interactions are discussed in more detail in another article within this thematic issue.

CYTOLYSIS

Intriguingly, secretion of eosinophil granule-derived mediators does not necessarily cease upon cell death. The realization that eosinophils can undergo a distinct mode of cell death that results in the expulsion of intact intracellular granules has been a long time in coming. Structures resembling eosinophil cell-free granules appeared in drawings and stainings of asthmatic sputum as early as the 19th century, and in the early 20th century, free eosinophil granules were observed within pulmonary tissues from fatal asthma, a portion of which were attributed to eosinophil death [reviewed in Ref. (56)]. Importantly, in the late 1990s, Persson et al. helped to validate these earlier descriptions by demonstrating the existence of eosinophil cell-free granules in guinea pig trachea after provocation (i.e., epithelial shedding), using a methodology that could not be discounted on the basis of mechanical artifact, that of performing deep tissue staining of whole mounts (57). However, it is only within the last decade that cytolysis has been more widely appreciated as a physiologically significant mode of eosinophil activity, defined ultrastructurally, and evaluated within the context of specific diseases.

EOSINOPHIL CYTOLYTIC CELL DEATH DEPOSITS GRANULES, BOTH FREE AND ASSOCIATED WITH NUCLEAR DNA NETS, INTO SURROUNDING TISSUE

In addition to eosinophils exhibiting morphological evidence of PMD, micrographs of diseased tissues reveal eosinophils undergoing a cytolytic process of cell death morphologically distinct from both apoptosis and necrosis (58, 59). In contrast to the chromatin condensation and fragmentation of apoptotic nuclei, cytolytic eosinophils are characterized by dissolution of the nuclear membrane and DNA de-condensing into the surrounding cytoplasm (**Figure 5**). Membrane blebbing characteristic of necrotic cells also does not occur, rather cytolytic eosinophils are typified by a loss of membrane integrity, and release of intracellular contents, including eosinophil specific granules, into the surrounding tissue. Of note, EoSVs are also expelled from cytolytic eosinophils and deposited within the tissue alongside cell-free granules [**Figure 5** and (41)]. Tissue-deposited, eosinophil cell-free granules are observed both within the spatial limits of the original cell and also scattered, independently or in clusters, outside of the confines of the originating cell.

The morphological sequelae associated with eosinophil cytolysis are elicited *in vitro* by a number of stimuli, including exposure to a Ca^{2+} ionophore, immobilized IgG or IgA, PMA, and GM-CSF or IL-5 in combination with PAF (58, 60, 61). Closer examination of eosinophil death induced by cross-linking Siglec 8 (62) or exposure to *Staphylococcus aureus* supernatant (63) might implicate these as eosinophil cytolytic stimuli as well (64). By analyzing eosinophils undergoing cytolytic cell death *in vitro* induced by the calcium ionophore A23187, Ueki et al. recently reported a sequence of events that included, chronologically (1) alterations in nuclear shape and density, (2) expulsion of single or small clusters of granules from the cell, (3) decondensation of nuclear contents into the cytoplasm, and (4) loss of membrane integrity, accompanied by the release of single granules or granule clusters (58).

Ueki et al. also demonstrated that under these conditions, eosinophil cytolysis was accompanied by extrusions of nuclear DNA nets. Cell-free granules liberated from cytolytic eosinophils were observed both incorporated into the DNA net-like lattices, and also standing alone as DNA-free granule clusters (58). One might speculate that this so-called "DNA trap cell death" serves a protective function by bringing the anti-microbial power of eosinophil granule-derived proteins into close proximity to pathogens immobilized by a DNA trap. Of note, eosinophil cytolytic DNA trap cell death is reminiscent of the anti-microbial DNA traps described by Yousefi et al. (22) and Morshed et al. (65), wherein mitochondrial DNA is catapulted from live eosinophils along with granule-derived proteins (i.e., ECP and MBP), forming extracellular nets with demonstrated microbicidal functions. However, two important distinctions exist between the DNA nets elicited through eosinophil cytolytic death and the DNA traps described by Yousefi and Morshed. First, in contrast to eosinophil cytolysis-generated nets, the DNA traps described by Yousefi and Morshed emerge from eosinophils that remain viable. Second, the origin of the catapulted DNA in the latter case is mitochondrial, while cytolytic eosinophils extrude DNA nets of nuclear origin. Stimuli causing the expulsion of mitochondrial DNA traps from viable eosinophils include brief stimulation of IL-5-, or IFN-γ-primed eosinophils with LPS, C5a, or eotaxin (22), or stimulation of non-primed eosinophils with TSLP (65). It has yet to be seen how, if at all, the processes of DNA net extrusion from cytolytic eosinophils and the expulsion of mitochondrial DNA nets from viable eosinophils might relate to one another.

SOME EOSINOPHIL GRANULES EXTRUDED FROM CYTOLYTIC EOSINOPHILS REMAIN SECRETORY-COMPETENT ORGANELLES

One might predict that the consequence of extracellular granule release through eosinophil cytolysis, whether in association with or distinct from DNA nets, would be the continued capacity of eosinophils to deliver their granule contents postmortem. Micrographs of diseased tissues reveal eosinophil extracellular granules exhibiting varying degrees of dissolution of their delimiting membranes (56), suggesting some fraction of cell-free granules with compromised granule membrane integrity might "leak" their protein content within tissues. However, very recent studies now indicate that a portion of extracellularly deposited granules retain the integrity of an intact granule membrane (58). Moreover, extracellularly deposited granules express chemokine, cytokine, and lipid receptors on their outer membrane, such that the ligand binding domains are outwardly oriented and thereby available to interact with exogenous stimuli within the tissue [reviewed in Ref. (66)]. Neves et al. demonstrated outwardly oriented granule-expressed receptors to be functional; in response to exogenous

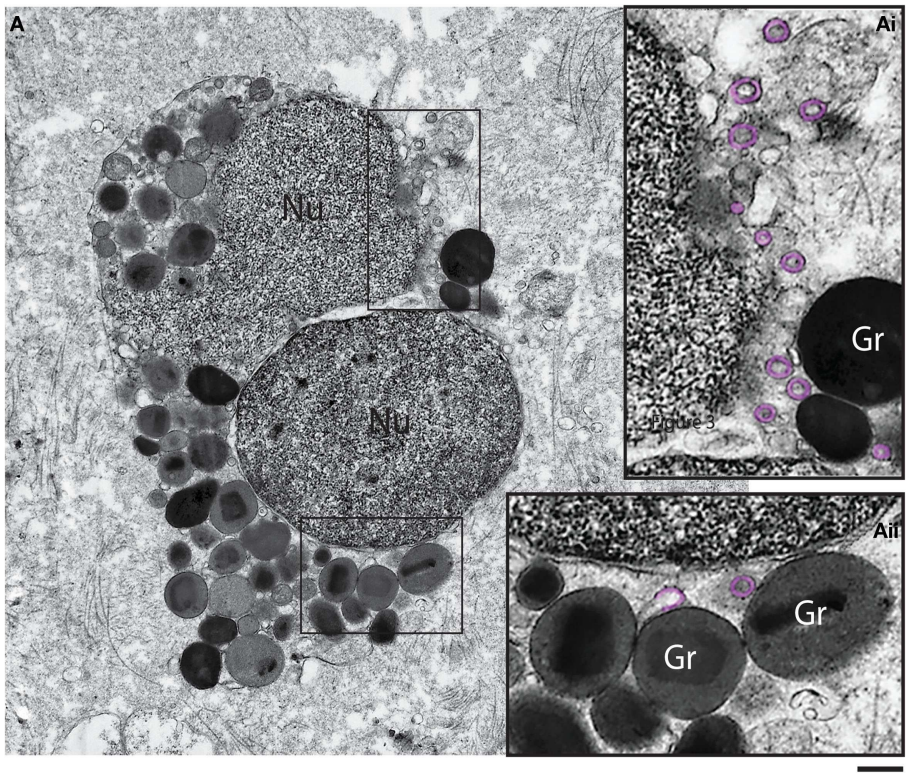

FIGURE 5 | Ultrastructure of a tissue human eosinophil undergoing cytolysis. Note the disintegrating nucleus (Nu) and extracellular free secretory granules (Gr) in the surrounding tissue. **(Ai, Aii)** are boxed areas of **(A)** seen at higher magnification. Note the presence of free, intact eosinophil sombrero vesicles (EoSVs – highlighted in pink) in the tissue, after cell lysis. Tissue eosinophils were present in a biopsy performed on a patient with inflammatory bowel disease. Scale bar: 800 nm **(A)**; 300 nm **(Ai, Aii)**.

eotaxin-1, IFN-γ, or leukotrienes, eosinophil cell-free granules exhibited kinase phosphorylation suggestive of activation of signal transduction pathways within the granule, and differentially released cationic proteins and cytokines in a stimulus dose- and kinase-dependent manner (54, 67). The implication of these findings is that cell-free granules liberated from cytolytic eosinophils function as stimulus-dependent, secretory-competent organelles within tissues.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Biological functions of eosinophil-derived cytokines are a burgeoning field. Earlier views of eosinophils as strictly end-stage effectors in parasitic diseases are now being expanded to encompass a new understanding of eosinophils as multifunctional leukocytes participating in developmental, metabolic, and immune cell functions. Keeping pace with newly appreciated eosinophil functions in heath and disease is a growing understanding of the dynamic complexities involved in the stimulus-dependent, differential liberation of cytokines from eosinophil intracellular granules, both through the vesicular transport-based process of PMD in viable eosinophils, and postmortem through tissue-deposited eosinophil cell-free granules and EoSVs elicited from cytolytic eosinophils. These cutting edge mechanistic insights will be critical to the next generation of therapeutic approaches in targeting eosinophil-associated diseases, where one must now consider the manner by which eosinophils die when devising anti-eosinophil strategies, and must measure contributions of tissue-deposited eosinophil cell-free organelles when evaluating cytokine-mediated functions of eosinophils *in situ* in disease.

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A role for syntaxin 3 in the secretion of IL-6 from dendritic cells following activation of toll-like receptors

Laura E. Collins[1], Joseph DeCourcey[1], Keith D. Rochfort[2], Maja Kristek[1] and Christine E. Loscher[1]*

[1] *Immunomodulation Research Group, School of Biotechnology, Dublin City University, Dublin, Ireland*
[2] *School of Biotechnology, Dublin City University, Dublin, Ireland*

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Loredana Saveanu, Institut National de la Sante et de la Recherche Medicale, France
John P. Vasilakos, 3M Company, USA

***Correspondence:**
Christine E. Loscher, Immunomodulation Research Group, School of Biotechnology, Dublin City University, Collins Avenue, Dublin 9, Ireland
e-mail: christine.loscher@dcu.ie

The role of dendritic cells (DCs) in directing the immune response is due in part to their capacity to produce a range of cytokines. Importantly, DCs are a source of cytokines, which can promote T cell survival and T helper cell differentiation. While it has become evident that soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptors (SNAREs) are involved in membrane fusion and ultimately cytokine release, little is known about which members of this family facilitate the secretion of specific cytokines from DCs. We profiled mRNA of 18 SNARE proteins in DCs in response to activation with a panel of three Toll-like receptors (TLR) ligands and show differential expression of SNAREs in response to their stimulus and subsequent secretion patterns. Of interest, STX3 mRNA was up-regulated in response to TLR4 and TLR7 activation but not TLR2 activation. This correlated with secretion of IL-6 and MIP-1α. Abolishment of STX3 from DCs by RNAi resulted in the attenuation of IL-6 levels and to some extent MIP-1α levels. Analysis of subcellular location of STX3 by confocal microscopy showed translocation of STX3 to the cell membrane only in DCs secreting IL-6 or MIP-1α, indicating a role for STX3 in trafficking of these immune mediators. Given the role of IL-6 in Th17 differentiation, these findings suggest the potential of STX3 as therapeutic target in inflammatory disease.

Keywords: dendritic cells, SNAREs, STX3, IL-6, MIP-1α

INTRODUCTION

Since the discovery of soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptors (SNAREs) in the 1980s they have been defined to have an essential role for the trafficking of molecules and membranes within cells (1). To date, there are 38 members of the SNARE family and the subcellular locations and differential combinations of SNARE proteins differ between cell types. The pairing of SNARE proteins is selective, which limits trafficking and membrane fusion between intracellular organelles or membrane fusion (2). Studies investigating the expression of individual SNAREs and their subcellular locations have led to the mapping of intracellular pathways. More advanced work has begun to assign functions to SNAREs in specific cellular immune responses such as the role of STX6 and Vti1b in the secretion of TNF from activated macrophage (3).

Dendritic cells are essential for the generation of a functional immune response. They do so by the capture and processing of antigens, expression of surface co-stimulatory molecules, migration to lymphoid organs, and secretion of cytokines and chemokines (4). Understanding these actions, such as their ability to drive T helper cell responses makes the dendritic cells (DC) a powerful tool for manipulating the immune system. Furthermore, the cytokines they secrete are associated with the pathogenesis of a wide range of inflammatory diseases (5). For example, blockade of TNF-α has been clinically successful in a number of immune mediated pathologies such as rheumatoid arthritis, Crohn's disease, and psoriasis (6). Abolishment of IL-23 by either utilizing IL-23 knockout mouse models or subjecting wild-type mice to anti-IL-23 treatment has been shown to render mice resistant to certain inflammatory and autoimmune diseases such as experimental autoimmune encephalitis (EAE), collagen-induced arthritis (CIA), and inflammatory bowel disease (IBD) (7–9). As DCs act as a link between innate and adaptive immunity and secrete cytokines, which are important in inflammatory diseases, investigating how DCs secrete these cytokines may provide us with new therapeutic targets in these diseases.

To date, there has been little work exploring the expression or function of SNAREs in DCs. The most notable studies include a profile of SNARE expression in DCs of Vti1a, Vti1b, VAMP3, VAMP8 and STX8, which were up-regulated in response to acetylsalicylic acid (ASA) (10). Furthermore, Ho et al., in 2008 attributed a functional role to VAMP8 in DCs. They demonstrated that overexpression of VAMP8 resulted in significant inhibition of phagocytosis and VAMP8$^{-/-}$ DCs had significantly increased phagocytic ability indicating that VAMP8 could inhibit phagocytosis from DCs (11).

To investigate the role of SNAREs in DC secretion we profiled the expression of mRNA following activation with a panel of Toll-like receptors (TLR) ligands using the established JAWS II DC cell line. We hypothesized that an up-regulation of particular SNAREs in DCs following activation may be indicative of a role for them in the secretion of cytokines or chemokines. This is the first study to demonstrate that activation of DCs with TLR ligands results in the differential expression of SNARE proteins, which correlates with the profile of cytokines and chemokines being secreted by the cell. Furthermore, we confirmed a role for STX3 in the secretion

of IL-6 using RNAi. This study suggests that SNARE proteins may provide new therapeutic targets in inflammatory disease.

MATERIALS AND METHODS

ANIMALS AND MATERIALS

C57BL/6 mice were purchased from Charles River at 6–8 weeks of age. Animals were housed in a licensed BioResource Unit (Dublin City University), according to Health Products Regulatory Authority (HPRA) regulations and had *ad libitum* access to animal chow and water. LPS *E. coli* serotype R515 was purchased from Enzo Life Sciences. Loxoribine and PGN from InvivoGen, rGMCSF from Sigma Aldrich, and STX3 siRNA from Life Technologies.

DENDRITIC CELL CULTURE

A murine DC line JAWS II (CRL-11904) was purchased from ATCC and maintained in fully supplemented α-MEM supplemented with 10% (v/v) fetal bovine serum (FBS), 2% (v/v) Penicillin–Streptomycin, and 5 ng/ml rGM-CSF. Bone marrow dendritic cells (BMDCs) were harvested from the femurs and tibia of mice and were also cultured in fully supplemented α-MEM. On day three, supernatants were removed from BMDCs and replaced with fresh fully supplemented α-MEM. On day seven BMDCs were used for subsequent experiments.

QUANTITATIVE POLYMERASE CHAIN REACTION

JAWS II DCs were cultured for 7 days in the presence of murine recombinant GM-CSF (Sigma Aldrich) and plated at 1×10^6 cell/ml. Cells were then stimulated with 5 μg/ml PGN, 100 ng/ml LPS (*E. coli* serotype R515), or 1 mM Loxoribine over a time-course. Total RNA was extracted, converted to cDNA, which was then subjected to quantitative real-time PCR. $2^{-\Delta\Delta Cq}$ formula of the delta–delta Cq method was used and a fold change in expression of SNARE genes was normalized against that of endogenous control S18. SNARE mRNA levels were determined relative to expression in the absence of TLR stimulation, which is given an arbitrary value of 1. All primer sequences are listed in **Table 1**.

DC STIMULATION AND MEASUREMENT OF CYTOKINES AND CHEMOKINES

JAWS II DCs or BMDCs were stimulated with LPS (100 ng/ml), Loxoribine (1 mM) or PGN (5 μg/ml) at timepoints between 0 and 12 h. Culture supernatants were removed and stored at −80°C until further analysis. IL-1β, IL-6, TNFα, MIP-1α concentrations in cell culture supernatants were analyzed by DuoSet ELISA Kits (R&D Systems) according to manufacturer's instructions.

RNAi OF STX3

JAWS II DCs were plated and left to rest overnight. GeneSilencer® (Genelantis) reagent and serum free media were added to tube one. siRNA diluent, specific STX3 siRNAs (Invitrogen™) (1 nM) (**Table 2**) or 1 nM scrambled non-silencing siRNA or 1 nM of fluorescently labeled Cy™ 3 labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Invitrogen™) and serum free media were added to tube two and left to incubate at RT for 5 min. The two tubes were then mixed together and incubated for 10 min. This mixture was then added directly to the plate and left for 24 h. Medium was then removed, replenished with fresh media with and without LPS 100 ng/ml and left for another four hours.

Table 2 | STX3 siRNA sequences.

Sequence (5′–3′)	Sense	Antisense
STX3 #1	CAGCCUUCAUGGACGAGUUtt	AACUCGUCCAUGAAGGCUGtg
STX3 #2	GGAAGAUGAGGUUCGGUCAtt	UGACCGAACCUCAUCUUCCtc

Table 1 | Primers used for PCR and their sequences.

Gene	Ref Seq ID	Forward [5′–3′]	Reverse [5′–3′]
SNAP-23	NM_009222	GTTCTTGCTCAGGCTTCC	CCAACCAACCAATACCAATAATG
STX-2	NM_007941	GGTGGCAAAGGTGATGTT	CAGGTATGGTCGGAGTCA
STX-3	NM_001025307	CCACAACCACTAGCATCATAA	CTCAAGAGATATTCCGCCTTAA
STX-4	NM_009294	GGTGTCAAGTGTGAGAGAG	AACCTCATCTTCATCGTCTG
STX-5	NM_001167799.1	GCAAGTCCCTCTTTGATGAT	TTCAGATTCTCAGTCCTCACT
STX-6	NM_021433	CAAGGATTGTTTCAGAGATGGA	CCTGACAATTTGCCGAGTA
STX-7	NM_016797	CACAACGCATCTCCTCTAAC	TAATCGGTCTTTCTGTATCTTTCTC
STX-11	NM_001163591.1	ATCACGGCAAAATGAAGGA	GGTCGGTCTCGAACACTA
STX-12	NM_133887	CGCAAGAAGATGTGTATCCT	CTCTGAGGCAAGCACTTC
STX-16	NM_001102432.1	GAGCAGTACCAGAAGAAGAAC	CAAGTCCTATCACCAATAATCCA
Vti1a	NM_016862	GAATGTATAGCAACAGGATGAGA	CCGTGTTATCCAGCAGATG
Vti1b	NM_016862	TACCTTGGAGAACGAGCAT	TGGACATTGAGCGAAGAATC
VAMP-1	NM_001080557	CCCTCTGTTTGCTTTCTCA	CGTTGTCTTCGGGTAGTG
VAMP-2	NM_009497	CTCCTTCCCTTGGATTTAACC	TGAAACAGACAGCGTATGC
VAMP-3	NM_009498	TTGTTCTTGTTGTATATCACTCCTAA	GGCTCGCTCTCACAGTAT
VAMP-4	NM_016796	GTATGCCTCCCAAGTTCAAG	TGTAGTTCATCCAGCCTCTC
VAMP-7	NM_011515	GATGGAGACTCAAGCACAAG	GACACAATGATATAGATGAACACAAT
VAMP-8	NM_016794	GGCGAAGTTCTGCTTTGA	CTTGACTCCCTCCACCTC
S18	NM_011296	CTGAGAAGTTCCAGCACATT	GCTTTCCTCAACACCACAT

Supernatants were removed and analyzed for the levels of IL-1β, IL-6, TNF-α, and MIP-1α. Western blot was performed to verify STX3 knockdown.

CONFOCAL MICROSCOPY

0.13–0.16 mm thick coverslips were placed in a six-well plate. BMDCs harvested from C57BL/6 mice and JAWS II DCs were added and left over-night to adhere. Cells were then stimulated with LPS (100 ng/ml), Loxoribine (1 mM), and PGN (5 μg/ml) for 1 h. Cells were washed and fixed with 3% Paraformaldehyde (PAF) and this reaction was then quenched with 50 mM Ammonium Chloride. Cells were permeabilized with 0.1% saponin, 0.25% fish gelatin and 0.02% sodium azide in PBS. STX3 primary antibody was added to the coverslips and incubated overnight at 4°C. Corresponding fluorescently conjugated secondary antibody (AlexaFluro 488 or AlexaFluro 546) was then added to the coverslips and incubated for 1 h at room temperature. Nuclei were stained with propidium iodide dye (PI) and samples mounted using DAKO anti-fade medium. Slides were then viewed using a Zeiss 710 confocal microscope and analyzed using LSM software.

STATISTICS

The parametric student's *t*-test was used using Excel (Microsoft) to determine significant differences between two samples. The level of statistical significance was indicated by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

RESULTS

SNARE mRNA EXPRESSION IN DCs FOLLOWING ACTIVATION WITH A PANEL OF TLR LIGANDS

Using RT-quantitative polymerase chain reaction (qPCR) we analyzed the expression of Qa SNAREs (STX2, STX3, STX4, STX5, STX7, STX11, STX12, and STX16) (**Figure 1**), Qb SNARES (Vti1a and Vti1b), Qbc SNARE (SNAP23), Qc SNARE (STX6)(**Figure 2**), and R SNAREs (VAMP1, VAMP2, VAMP3, VAMP4, VAMP7, and VAMP8)(**Figure 3**) in JAWS II DCs following stimulation with 100 ng/ml LPS, 1 mM Loxoribine, or 5 μg/ml PGN. RT-qPCR was normalized with S18 levels. This housekeeping gene showed smallest standard deviation compared to β-actin and GAPDH between the technical replicates and LPS, Loxoribine and PGN stimulation. Thus was used for all subsequent experiments (Figure S1 in Supplementary Material).

The most significant fold change increases in mRNA expression in the Qa SNAREs over time were that of STX3 and STX11 (**Figure 1**). Expression of STX3 mRNA in JAWS II DCs significantly increased following activation with LPS and Loxoribine post 1 and 4 h stimulation and conversely was significantly down-regulated following PGN activation ($p \leq 0.01$ and $p \leq 0.05$). STX11 mRNA expression in JAWS II DCs increased significantly following activation with LPS, Loxoribine and PGN at 1 and 4 h compared to control cells ($p \leq 0.001$) (**Figure 1**).

In the assessment of expression of Qb SNARES (Vti1a and Vti1b), Qbc SNARE (SNAP23), and Qc SNARE (STX6) there was a significant up-regulation of Vti1a mRNA at 4 and 12 h post LPS and Loxoribine stimulation ($p \leq 0.01$, $p \leq 0.001$, $p \leq 0.01$) in comparison to control cells (**Figure 2**).

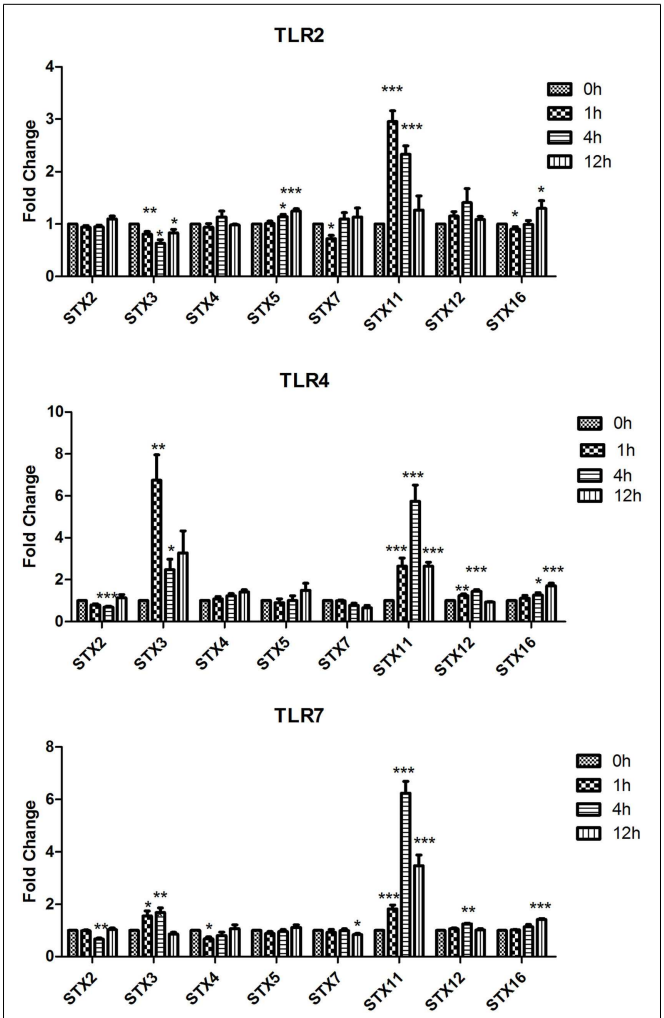

FIGURE 1 | Effect of TLR ligand stimulation on Qa SNAREs (STX2, STX3, STX4, STX5, STX7, STX11, STX12, and STX16) mRNA expression in JAWS II DCs. JAWS II DCs were plated 1×10^6/ml and stimulated with 100 ng/ml LPS, 1 mM Loxoribine, or 5 μg/ml PGN at 1, 4, and 12 h. The amount of specific SNARE protein was quantified by reverse transcription followed by RT-qPCR and normalized with S18 levels. Fold differences were calculated relative to SNARE levels at time zero (assigned value of 1). Results are mean ± SEM of quadruplicate assays. A two sample, two-tailed student's *t*-test comparing ΔCts of control and 1, 4, or 12 h stimulated sample $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

While VAMP1 was significantly down-regulated following stimulation with LPS, Loxoribine, and PGN; VAMP2 was significantly increased at 12 h post stimulation with these ligands (**Figure 3**). Following Loxoribine stimulation VAMP8 expression was significantly down-regulated at 12 h ($p \leq 0.05$) and up-regulated following PGN stimulation after 4 h. LPS stimulation did not have any significant effect on VAMP8 expression (**Figure 3**).

Our data demonstrate that SNAREs are regulated in JAWS II DCs in response to TLR ligation and that their expression differed depending on the type of TLR ligand used to activate the cell (**Figures 1–3**).

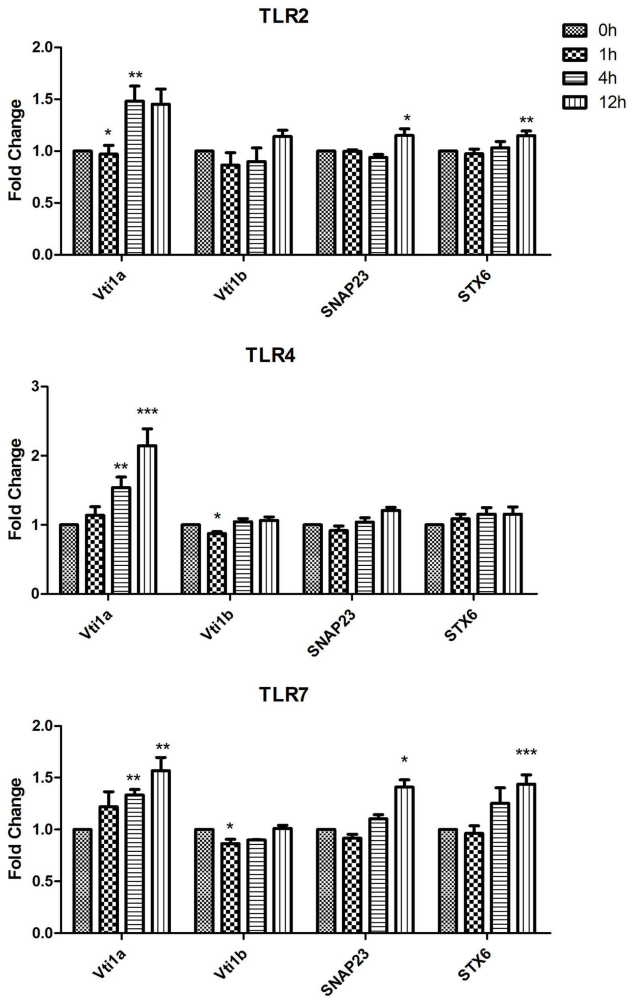

FIGURE 2 | Effect of TLR ligand stimulation on Qb SNARES (Vti1a and Vti1b), Qbc SNARE (SNAP23), Qc SNARE (STX6) mRNA expression in JAWS II DCs. JAWS II DCs were plated 1×10^6/ml and stimulated with 100 ng/ml LPS, 1 mM Loxoribine, or 5 μg/ml PGN at 1, 4, and 12 h. The amount of specific SNARE protein was quantified by reverse transcription followed by RT-qPCR and normalized with S18 levels. Fold differences were calculated relative to SNARE levels at time zero (assigned value of 1). Results are mean ± SEM of quadruplicate assays. A two sample, two tailed student's *t*-test comparing ΔCts of control and 1, 4, or 12 h stimulated sample $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

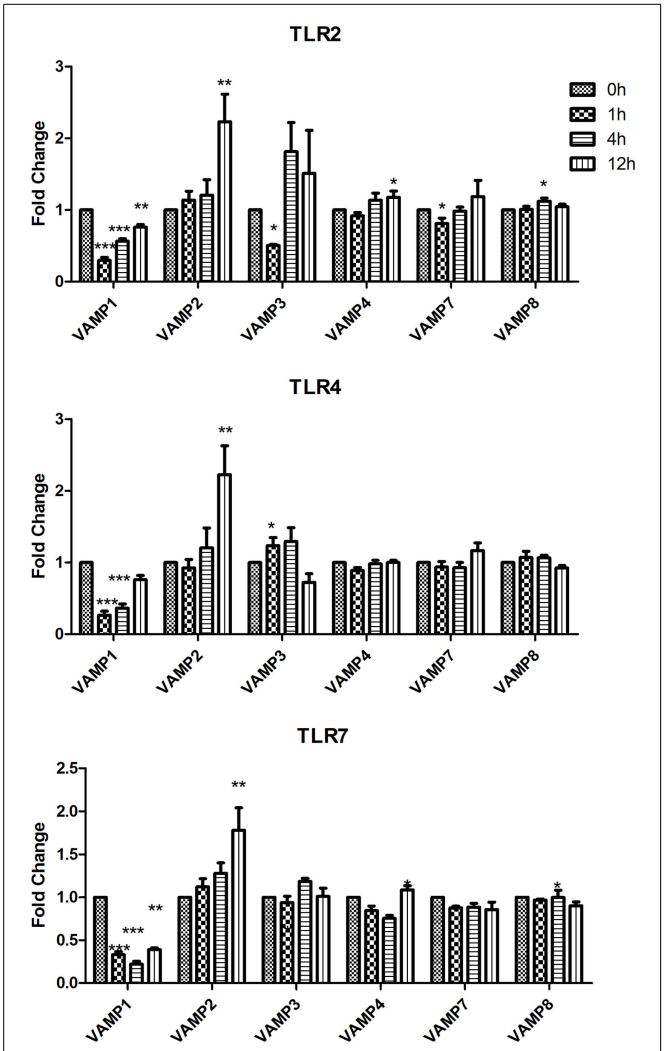

FIGURE 3 | Effect of TLR ligand stimulation on R SNAREs (VAMP1, VAMP2, VAMP3, VAMP4, VAMP7, and VAMP8) mRNA expression in JAWS II DCs. JAWS II DCs were plated 1×10^6/ml and stimulated with 100 ng/ml LPS, 1 mM Loxoribine, or 5 μg/ml PGN at 1, 4, and 12 h. The amount of specific SNARE protein was quantified by reverse transcription followed by RT-qPCR and normalized with S18 levels. Fold differences were calculated relative to SNARE levels at time zero (assigned value of 1). Results are mean ± SEM of quadruplicate assays. A two sample, two tailed student's *t*-test comparing ΔCts of control and 1, 4, or 12 h stimulated sample $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

SECRETION OF CYTOKINES AND CHEMOKINES BY DCs FOLLOWING ACTIVATION WITH A PANEL OF TLR LIGANDS

To identify patterns in cytokine and chemokine secretion that may correlate with the expression of SNAREs, we analyzed the secretion of IL-6, TNF-α, and MIP-1α over the same time-course of 1, 4, and 12 h (**Figure 4**). IL-6 secretion was significantly up-regulated from JAWS II DCs following LPS stimulation at 4 h and 12 h and Loxoribine at 12 h ($p \leq 0.01$, $p \leq 0.001$) but remained unchanged following PGN stimulation. TNF-α secretion was significantly up-regulated at 4 and 12 h following LPS, Loxoribine, and PGN stimulation ($p \leq 0.001$, $p \leq 0.05$ and $p \leq 0.01$) and MIP-1α secretion was significantly up-regulated following LPS stimulation at 4 and 12 h ($p \leq 0.01$ and $p \leq 0.001$) and Loxoribine stimulation at 12 h ($p \leq 0.001$) but not with PGN stimulation (**Figure 4**). The trend of expression of IL-6 and TNF-α in response to LPS and Loxoribine stimulation at 4 and 12 h and MIP-1 α at 4 h post correlated with up-regulation of STX3 mRNA expression at 1 h following LPS and Loxoribine stimulation but not PGN stimulation (**Figures 1** and **4**).

KNOCKDOWN OF STX3 SIGNIFICANTLY DECREASES THE SECRETION OF IL-6 AND MIP-1 ALPHA FROM JAWS II DCs

As previous studies have indicated a role for STX3 in secretion of chemokines (12) and we have demonstrated that increased

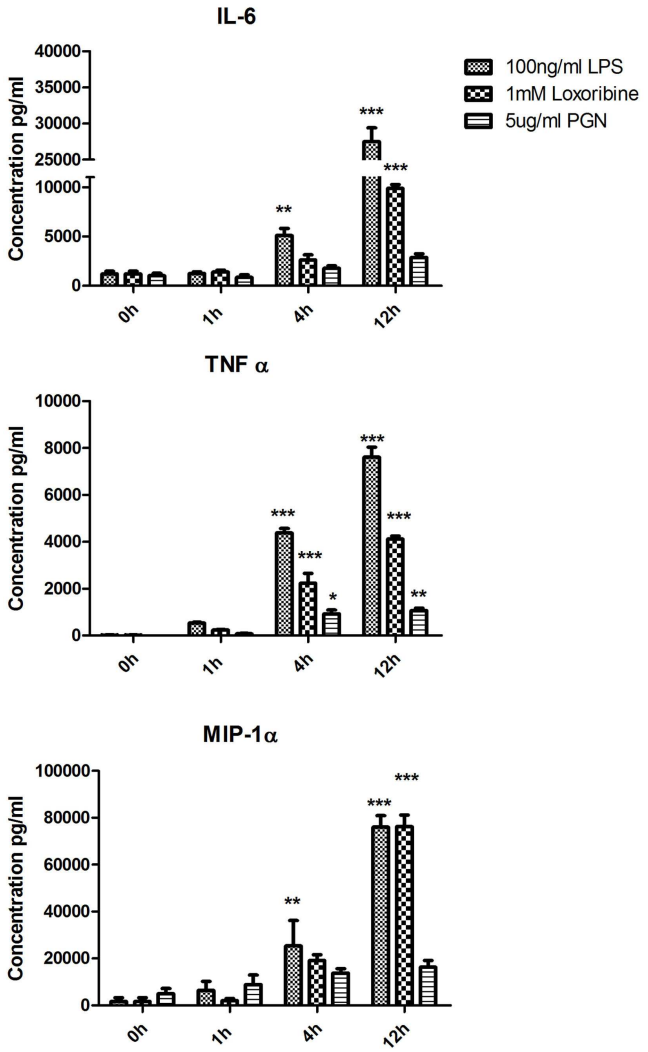

FIGURE 4 | Time-course of cytokine and chemokine secretion from JAWS II DCs in response to TLR4, TLR7, and TLR2 activation. JAWS II DCs were cultured in r-GM-CSF for 7 days and subsequently were plated (1×10^6 cells/ml) and left to rest overnight. Cells were then stimulated with 100 ng/ml LPS, 1 mM Loxoribine, and 5 μg/ml PGN. Supernatants were recovered after 1, 4, and 12 h and assessed for levels of cytokines using specific immunoassays. Results are ±SEM of quadruplicate assays and represent three independent experiments. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ comparing control versus stimulated JAWS II DCs as determined by a two-tailed *t*-test.

expression of STX3 correlated with cytokine and chemokine secretion we examined whether abolishing STX3 expression would have any effect on cytokine or chemokine secretion from DCs. STX3 was knocked down using STX3 specific siRNAs (Invitrogen™). Transfection efficiency was confirmed with Cy3 GAPDH (Figure S2 in Supplementary Material) and STX3 knockdown by both siRNAs was confirmed at the protein level by using Western blot (**Figure 5**). Supernatants were subsequently removed 4 h later and analyzed for basal levels of IL-1β, IL-6, TNF-α, and MIP-1α (**Figure 5**). In the absence of STX3, secretion of IL-6 and MIP-1α were significantly decreased ($p \leq 0.05$ and $p \leq 0.01$) whereas IL-1β was significantly increased ($p \leq 0.05$), with no significant changes in TNF-α secretion (**Figure 5**).

STX3 TRANSLOCATES TO THE PLASMA MEMBRANE ONLY IN IL-6 SECRETING JAWS II DCs AND BMDCs

We next used primary BMDCs to confirm our findings from the JAWS II DC line. We analyzed secretion of IL-6, TNF-α, and MIP-1α from BMDCs activated with LPS, Loxoribine, and PGN and demonstrated that IL-6 is significantly secreted from BMDCs when activated with all three TLR ligands (**Figure 6A**). mRNA of STX3 was then examined in BMDCs and was up-regulated in all instances where TLR activation of BMDCs resulted in IL-6 secretion, which supports our findings in the JAWS II cell line (**Figure 6B**).

Given that we had shown correlation between secretion of IL-6 and increased expression of STX3 in both JAWS II DCs and BMDCs we next wanted to confirm this relationship by examining the expression of STX3 in IL-6-secreting and non-secreting DCs using confocal microscopy. STX3 was detected in the cytoplasm of control JAWS II DCs and following stimulation with LPS and Loxoribine STX3 translocated to the plasma membrane after 1 h. PGN stimulation in JAWS II DCs did not result in translocation of STX3 to the plasma membrane. In BMDCs, which do respond to PGN and secrete IL-6, activation with PGN resulted in STX3 translocation (**Figure 7**). Unstained cells and cells stained with primary STX3 antibody (1°) alone were visualized for auto-fluorescence while secondary antibodies alone were imaged for non-specific binding in every experiment (Figure S3 in Supplementary Material). An overview of our finding on STX3 in BMDCs and JAWS II DCs are summarized in **Figure 8**.

DISCUSSION

Several studies have addressed the role of SNAREs in cytokine release from immune cells, however, little is still known about the specific SNAREs involved in cytokine or chemokine secretion by DCs. Indeed, expression of SNAREs in immune cells, especially at the mRNA level has also not been well characterized. Investigating their expression in immune cells, such as DCs may provide valuable insights into their involvement in cytokine and/or chemokine secretion thereby uncovering potential new targets in inflammatory disease. Previous studies have used the correlation between SNARE expression and cytokine secretion as a way of elucidating candidate SNAREs involved in the secretion of these immune mediators (13). We took a similar approach and used the JAWS II DC cell line to profile the expression of SNAREs and subsequent secretion of cytokines and chemokines in DCs following exposure to ligands for TLR2, TLR4, and TLR7. We demonstrate that activation of DCs with these TLR ligands results in differential expression of SNAREs and subsequent secretion of cytokines and chemokines and describe a role for STX3 in secretion of IL-6.

Here, we have shown for the first time, the expression of SNARE proteins in DCs following activation with a range of TLR ligands. Interestingly, many of the SNAREs we examined did not change in expression over the 12-h time points we assessed. This was of interest as some SNAREs have been well reported to play a role in other immune cells. For example, an up-regulation of STX6

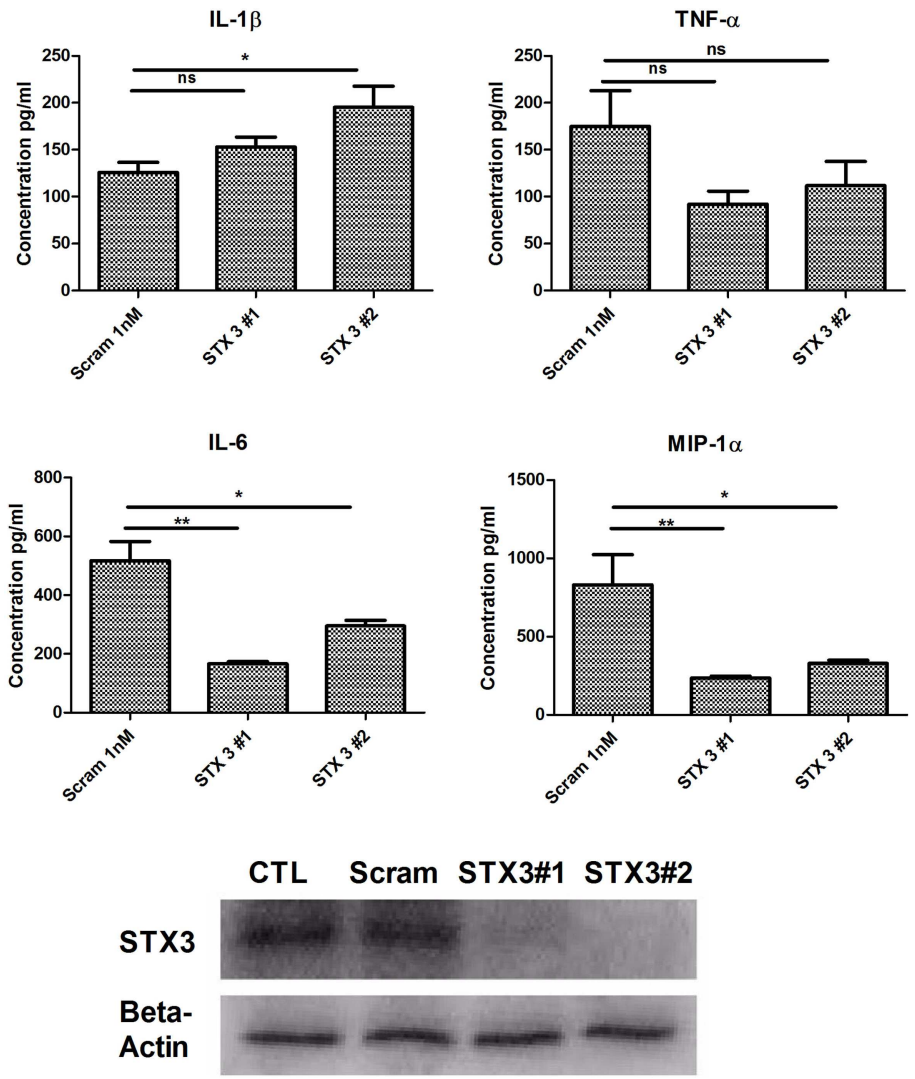

FIGURE 5 | Knockdown of STX3 by siRNA. JAWS II DCs were transfected with siRNAs against STX3 (STX3 #1 and STX3 #2) or a negative control non-silencing siRNA. At 24 h after the transfection, new media was put on the cells and left for 4 h. Lysates and media were then harvested. Basal levels of cytokines or chemokines were assessed using specific immunoassays and knockdown was confirmed at protein level by western blot. $*p < 0.05$ and $**p < 0.01$ comparing control versus STX3 siRNA transfected JAWS II DCs as determined by a two-tailed *t*-test.

mRNA 2 h post stimulation with LPS has been reported in RAW 264.7 macrophage cells (3) and SNAP23 mRNA from the skeletal muscle of patients with type II diabetes was up-regulated (14). As SNAREs are used up during the fusion reactions and are recycled for numerous rounds of transport (15), we propose that along with caspases, other post-transcriptional regulation and recycling of SNAREs may be another reason for the lack of changes of SNARE mRNA expression in DCs.

We also did not detect any change in expression of VAMP8, which is one of the only SNAREs, which has been assigned a functionality in DCs to date. It has been previously indicated to be involved in phagocytosis in DC. Ho et al. reported that an over-expression of VAMP8 significantly inhibited phagocytosis suggesting that VAMP8 negatively regulates this process (11). The same group in another study notes that although they have indicated a role for VAMP8 in DCs, VAMP8 mRNA was not significantly elevated during DC maturation (16). This correlates with our data and Ho et al. accredited this to regulation at the mRNA level of VAMP8 by caspases (16). This may be indicative for a role for caspases and other molecules involved in post-transcriptional regulation of SNARE mRNA in DCs as VAMP8 mRNA has been shown to be up-regulated in other cells types such as over-reactive human platelets. Indeed hyper-reactive platelets have a higher fold expression of 4.8 compared to hypo-reactive platelets (17).

Our data does demonstrate significant up-regulation of SNAREs such as STX3, STX11, Vti1a, and Vti1b in DCs following activation. There was a significant up-regulation of Vti1a mRNA at 4 and 12 h post LPS and Loxoribine stimulation. It has

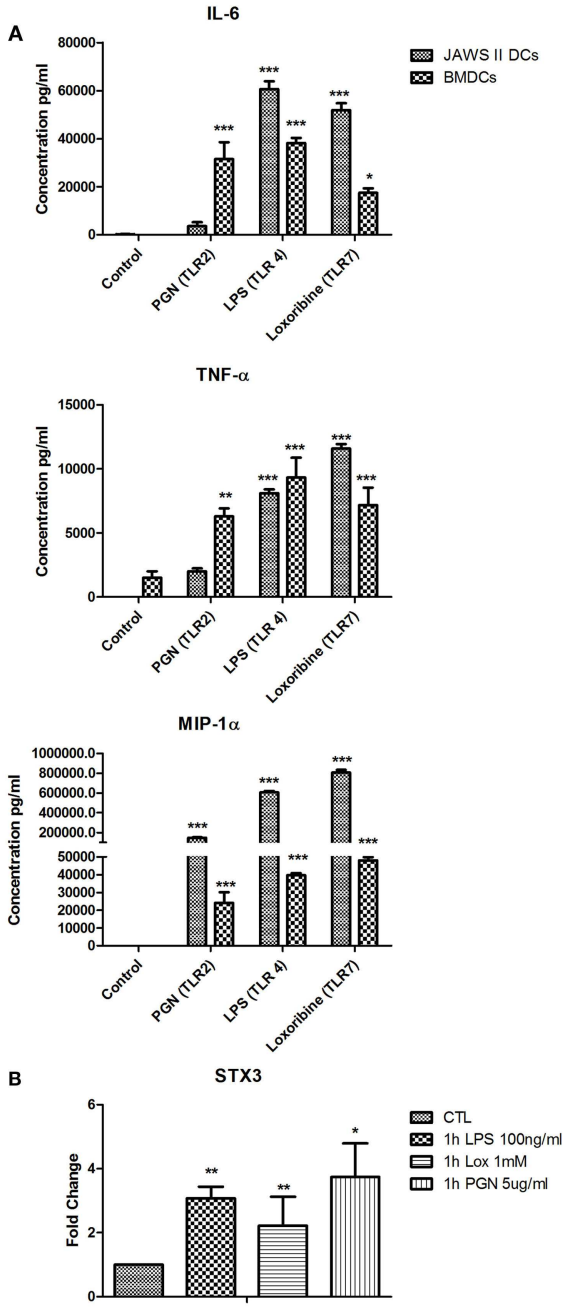

FIGURE 6 | (A) Cytokine and chemokine secretion from BMDC versus JAWS II DCs in response to TLR2, TLR4, and TLR7. **(B)** Effect of TLR ligand stimulation on Qa SNARE, STX3 mRNA expression in BMDCs. DCs were cultured in r-GM-CSF for 7 days and subsequently were plated (1×10^6 cells/ml) and left to rest overnight. Cells were then stimulated with 100 ng/ml LPS, 1 mM Loxoribine, and 5 μg/ml PGN and mRNA and supernatants were recovered after 1 or 24 h Supernatants were assessed for levels of cytokines using specific immunoassays and the amount of STX3 was quantified by reverse transcription followed by RT-qPCR and normalized with S18 levels. Fold differences were calculated relative to SNARE levels at time zero (assigned value of 1). Results are ±SEM of quadruplicate assays and represent three independent experiments. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ comparing control versus stimulated JAWS II DCs or BMDCs as determined by a two-tailed *t*-test.

previously been reported that up-regulation of Vti1a and Vti1b inhibits phagocytosis in DCs (10). As mature DCs are known to have reduced phagocytic ability (18) it may suggest that Vti1a was up-regulated to inhibit phagocytosis as the DC matured. There was also significant up-regulation of STX11 mRNA expression in response to LPS Loxoribine and PGN compared to control cells. STX11 is highly expressed in cells of the immune system and interestingly has an already established role in immune disease. Loss or mutation of the STX11 gene results in an autosomal recessive disorder known as, familial hemophagocytic lymphohistiocytosis type-4 (FHL-4), which causes immune dysregulation. This disorder is characterized by high levels of inflammatory cytokines and defective function of T cells and natural killer (NK) cells (19). STX11 has been reported to be up-regulated in DCs following LPS activation but *Stx11* deficiency did not appear to affect DC function (20). STX11 has also been indicated to regulate other cells of the immune system such as NK, $CD8^+$ T cells, macrophage, platelets, and in human blood neutrophils where up-regulation of STX11 mRNA expression during differentiation has been reported (19–21). While it is clear from our study that STX11 regulation in DCs may be important we did not find a definitive correlation for this SNARE with the profile of cytokines or chemokines being secreted by the DCs.

One of most significant increases in mRNA expression over time was that of STX3. Expression of STX3 mRNA in JAWS II DCs significantly increased following activation with LPS and Loxoribine 1 and 4 h post stimulation and conversely was down-regulated following PGN activation. A role for STX3 has been documented in mast cells and epithelial cells but not yet in DCs. STX3 and VAMP7 have been shown to be important for apical transport of trans-membrane and secretory proteins in epithelial cells (22). Interestingly, a role for STX3 has been recently documented in the secretion of chemokines from mast cells. This study demonstrated that blocking STX3 activity with neutralizing antibodies inhibited the secretion of chemokines following IgE stimulation. The chemokines inhibited included IL-8, MCP-1, MIP-1α, and MIP-1β (12). As the JAWS II DCs secreted large amounts of MIP-1α it suggested that STX3 also has a role in chemokine secretion in DCs.

To elucidate a role for these up-regulated SNAREs we assessed the secretion of IL-6, TNF-α, and MIP-1α from JAWS II DCs following activation with TLR4, TLR7, and TLR2 ligands over the same time-course that the SNARE mRNA was profiled. IL-6 and MIP-1α were significantly secreted when JAWS II DCs were stimulated with LPS and Loxoribine but not with PGN. This was of particular interest to us as STX3 expression in JAWS II DCs was up-regulated significantly in response to LPS and Loxoribine but not to PGN stimulation. The concept of SNARE up-regulation paralleled with cytokine up-regulation has proven useful in other studies. Murray et al. correlated SNARE protein levels with TNF secretion from macrophage following LPS stimulation, which ultimately lead to the elucidation of STX4 functionality in macrophage secretion of TNF-α(3). The different roles of each SNARE in different immune cells is highlighted by the fact that in contrast to the role of STX4 in macrophage, we demonstrate a key role for this SNARE in secretion of IgE in B cells (23). With these factors in mind we examined STX3 in further detail to elucidate its functional roles in DCs.

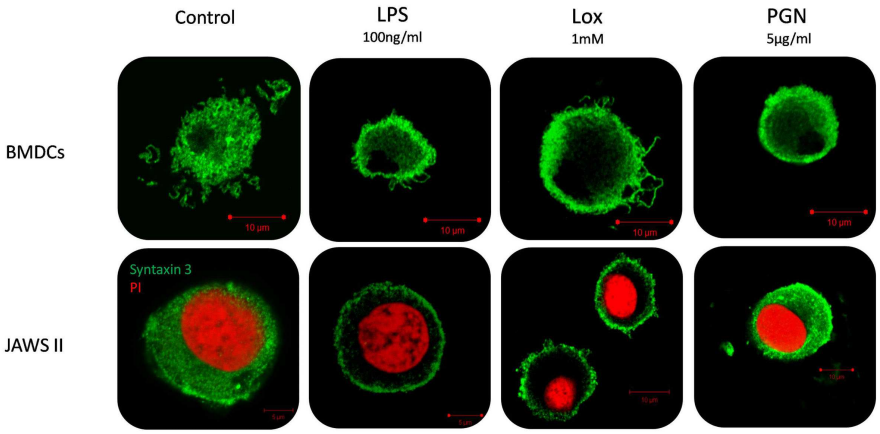

FIGURE 7 | Localization of STX3 to the plasma membrane in JAWS II DCs and BMDCs. JAWS II DCs and BMDCs were plated 1×10^6/ml and stimulated with 5 µg/ml PGN, 100 ng/ml LPS, and 1 mM Loxoribine for 1 h. Immunofluorescent indirect double staining of STX3 (Green) and nuclei (red) in TLR4, TLR7, and TLR2 stimulated JAWS II DCs and BMDCs to show localization of STX3 within the cells.

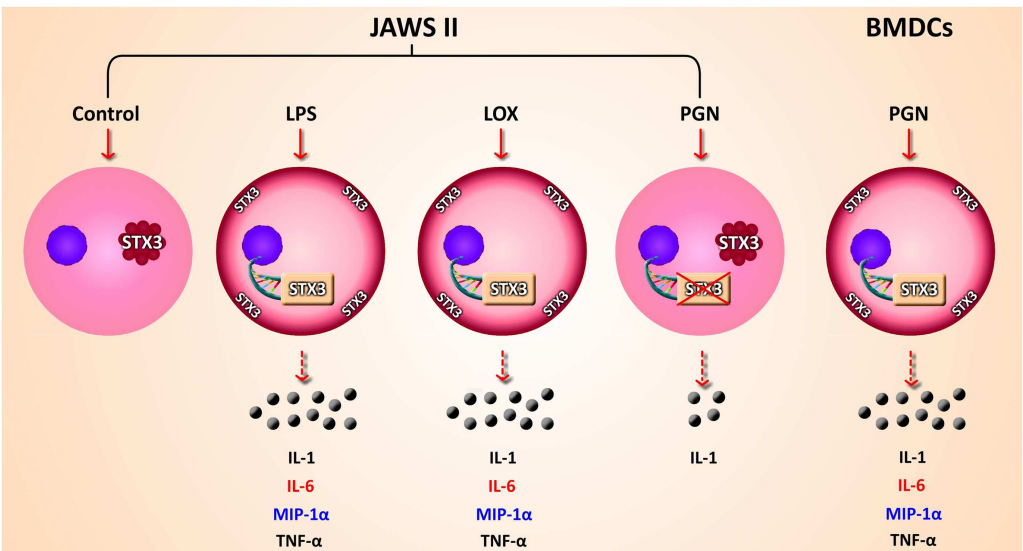

FIGURE 8 | Overview of findings. Schematic representation of secretion of cytokines and chemokines secreted from JAWS II DCs and BMDCs in response to TLR activation. mRNA expression up-regulation and translocation of STX3 in DCs only secreting IL-6 (red) while MIP-1α (blue) was also secreted in these cells, not to the same level as in BMDCs

We firstly depleted the levels of STX3 by means of a siRNA specific to STX3. In these experiments, IL-6 secretion was significantly down-regulated in the absence of STX3. MIP-1α was also down-regulated when STX3 was depleted by using RNAi. This correlates with recent data from mast cells where STX3 was reported to be involved in chemokine release from mast cells (12). Using confocal microscopy we assessed the cellular location of STX3 during activation with TLR ligands in both JAWS II DCs and BMDCs, and showed that translocation of STX3 only occurred in cells where IL-6 was secreted. This data indicates that STX3 has a role in IL-6 secretion (and possibly MIP-1α) from DCs and is an important SNARE in DC function.

IL-6 levels were not completely abolished from the JAWS II DCs and this may be explained as loss of SNAREs can be compensated by other members of the SNARE family, which have only 30% amino acid similarity (24). However, cytokines such as IL-6 are critical mediators of the autoimmune diseases and IL-6 is implicated in several disease processes. Increased levels of IL-6 have been observed in IBD, RA, systemic-onset juvenile chronic arthritis (JCA), osteoporosis, and psoriasis (25). IL-6 blockade has also been reported to be successful in autoimmune diseases and humanized anti-IL-6 receptor antibody is now routinely used in the clinic for the treatment of RA and JCA (26). In the past decade, IL-6 has been implicated with TGF-β in the differentiation of the

Th17 subset, therefore its blockade may potentially improve these diseases at the pathogenic initiation phase (27).

The importance of future work in identifying SNARE complexes in immune function is highlighted by the award of a Nobel prize to Randy W. Schekman, Thomas C. Südhof and James E. Rothman who identified and functionally characterized these proteins and their involvement in trafficking. The potential to develop therapeutics that target SNAREs and suppress the secretion of cytokines may have significant advantages over therapies that target the effects of these cytokines post release. Indeed SNAREs may represent novel therapeutic targets in a wide range of disease states.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00691/abstract

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An emerging role for SNARE proteins in dendritic cell function

Laura E. Collins†, Joseph DeCourcey†, Mariana Soledad di Luca, Keith D. Rochfort and Christine E. Loscher*

Immunomodulation Research Group, School of Biotechnology, Dublin City University, Dublin, Ireland

Edited by:
Paige Lacy, University of Alberta, Canada

Reviewed by:
Koichi S. Kobayashi, Texas A&M Health Science Center, USA
Shahram Salek-Ardakani, University of Florida, USA

****Correspondence:***
Christine E. Loscher, Immunomodulation Research Group, School of Biotechnology, Dublin City University, Collins Avenue, Dublin 9, Ireland
e-mail: christine.loscher@dcu.ie

†Laura E. Collins and Joseph DeCourcey have contributed equally to this work.

Dendritic cells (DCs) provide an essential link between innate and adaptive immunity. At the site of infection, antigens recognized by DCs via pattern-recognition receptors, such as Toll-like receptors (TLRs), initiate a specific immune response. Depending on the nature of the antigen, DCs secrete distinct cytokines with which they orchestrate homeostasis and pathogen clearance. Dysregulation of this process can lead to unnecessary inflammation, which can result in a plethora of inflammatory diseases. Therefore, the secretion of cytokines from DCs is tightly regulated and this regulation is facilitated by highly conserved trafficking protein families. These proteins control the transport of vesicles from the Golgi complex to the cell surface and between organelles. In this review, we will discuss the role of soluble *n*-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNAREs) in DCs, both as facilitators of secretion and as useful tools to determine the pathways of secretion through their definite locations within the cells and inherent specificity in opposing binding partners on vesicles and target membranes. The role of SNAREs in DC function may present an opportunity to explore these proteins as novel targets in inflammatory disease.

Keywords: dendritic cells, SNAREs, IL-6, IL-23, IL-12

INTRODUCTION

Dendritic cells (DCs) were first described by Ralph Steinman and Zanvil Cohn as a rare murine splenocyte with dendrite-like protrusions that displayed phagocytic abilities (1). Following their discovery, work carried out by Steinman's group demonstrated that DCs play a vital role in orchestrating immunity. In their immature state in the peripheral tissue, DCs are characterized by a high capacity for antigen capture and processing via phagocytosis. They display distinct chemokine receptor expression, chemokine responsiveness, and low T cell stimulatory capabilities (2). The ability of DCs to regulate immunity is dependent on their maturation, which is induced by the interaction of DCs with antigens that are subsequently presented to T cells. During this process, DCs secrete distinct profiles of cytokines and chemokines and express co-stimulatory molecules required to drive specific T cell responses (3). The control of these secretory pathways is an essential factor in regulating innate and adaptive immune homeostasis and controlling responses to infection (4). In this review, we will discuss the factors that induce DC maturation, the cytokines secreted by DCs that drive adaptive immunity, and the secretory pathways that have been identified and their potential as immunotherapeutic targets.

DENDRITIC CELL ACTIVATION

For maturation to occur, foreign antigens must be identified by DCs. DCs express pattern recognition receptors (PRRs) that allow them to recognize microbes that display pathogen-associated molecular patterns (PAMPs). PRRs can be divided into toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) and retinoic acid-inducible gene-I (RIG)-like receptors (RLRs) (5). Following engagement of these PRRs, signaling cascades are activated that induce the production of inflammatory cytokines (6). Inflammatory responses can also be triggered by endogenous damage-associated molecular patterns (DAMPs). These are intracellular markers that are also recognized by PRRs, and are released during tissue damage or when a cell is necrotic releasing intracellular material into its surroundings (7). Following PRR ligation and subsequent maturation, DCs become antigen presenting cells (APC) (8). The mature DC travels to the local lymph node (9), which facilitates the presentation of antigen to antigen-specific lymphocytes to prime naive T cells to become effector T cells. Three signals are required for this: antigen presentation, up-regulation of co-stimulatory markers, and secretion of cytokines; this review will focus on the third signal, cytokine secretion and their effects (10).

CYTOKINE POLARIZATION OF T CELL SUBSETS

Clonal immunity involves DC activation of T cells through presentation of processed antigen to naïve T cells in an immunogenic form and the secretion of immune mediators called cytokines. Cytokines are classified into interleukins (IL), interferons (IFNs), and colony-stimulating factors. Cytokine profiles secreted from DCs polarize naïve T cells into different phenotypes of $CD4^+$ T cells. These differential cytokine secretion patterns are dependent on the PRR activation, and subsequently are the key signals required for the differentiation into different T helper (Th) cell phenotype subsets that are required to clear the antigen that trigger the immune response (11).

In order to clear bacterial and viral infections, naïve $CD4^+$ T-cells require cytokine signaling from DCs to initiate differentiation into a Th1 phenotype. TLRs present on DCs recognize PAMPs, such as lipopolysaccharide and unmethylated dsDNA, through TLR4 and TLR9 respectively. Engagement of these TLRs results in secretion of the cytokines IL-1, IL-6, TNF-α, and IL-12p40 (10). Dimerization of IL-12p40 with IL-12p35 results in an active IL-12p70 heterodimer. IL-12p70 together with IFN-γ, which is produced in large amounts by activated T-cells, leads to differentiation of T-cells in to an IFN-γ producing Th1 cells subset (12). Th2 cells produce high levels of IL-4, IL-5, and IL-13. They are responsible for initiating the humoral immune response and are required to clear helminth infections. Th2 cells have been generated *in vitro* via stimulation of the TCR and the addition of IL-4 (13). IL-4 induces the induction of Th2 cells via STAT6, given the fact that DCs do not produce IL-4, the mechanisms through which Th2 cells initiate this subset remain unclear. However, cytokine signaling from DCs can indirectly affect the differentiation. DCs activated through TLR2, DC-SIGN, and OSCAR (an FcγR-associated receptor) lead to the activation of Erk1 and Erk2 pathways and phosphorylation of c-Fos (14). This leads to the inhibition of IL-12 and increased production of IL-10 in DCs, which is sufficient to induce a strong Th2 response in naïve T-cells. Cytokines produced by other cells can also influence DCs to drive a Th2 subset through chemokine production that recruits IL-4 producing cells and the activation of cytokine receptors (11).

Th17 cells were first described in 2006 and identified as a T helper subset distinct from Th1 and Th2 cells that produced the cytokine IL-17 (15). Th17 cells have been indicated to confer protection against extracellular bacteria and fungi by the secretion of effector molecules such as IL-17, IL-21, IL-22, GM-CSF, and CCL20 (16). However, Th17 cells have also been implicated in many autoimmune and inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis, psoriasis, and inflammatory bowel disease (IBD) (17). The conditions for differentiation of Th17 cells is more complex than Th1 and Th2 subsets, and there is evidence to support a role for a number of cytokines produced by DCs, including IL-1, IL-18, IL-6, and IL-23. These cytokines are produced by DCs in response to PRR recognition of bacteria and fungi.

In the case of Th17 differentiation, it is not just TLR-mediated activation that promotes the secretion of these cytokines. Dectin-1 and dectin-2 activate CLRs on DCs to promote section of IL-6 and IL-23, cytokines that play a major role in differentiating and stabilizing the Th17 response respectively (18). T-regulatory cells are essential for negative regulation of the immune response, as they possess immunosuppressive abilities that confer protection to the host from autoimmunity and prevention of excessive immunopathology. If this response becomes dysregulated, it can lead to autoimmune disorders due to loss of homeostasis. IL-27, IL-10, and TGF-β1 secretion by DCs have been heavily indicated in the induction of Treg cells (19).

TARGETING CYTOKINES IN INFLAMMATORY DISEASE

Cytokines are essential in driving the adaptive immune response; thus, targeting cytokines associated with inflammatory disease has become a significant area for drug development (20). Indeed, secretory pathways are attractive to study as potential targets to block critical cytokines with the possibility of switching off inflammation without any detrimental side effects (21). A number of therapeutics currently exist that target key cytokines such as IL-23 and IL-6, which are associated with inflammatory disease.

IL-23, essential for stabilizing Th17 cells, has been implicated in the pathogenesis of multiple sclerosis, arthritis, and IBD. Using mouse models of diseases, the essential role of IL-23 has been established (16, 22, 23). A human monoclonal antibody, Ustekinumab, has been developed and licensed to target the IL-12/23 pathway. This antibody targets the p40 subunit of IL-12 and IL-23, and was originally developed to target IL-12 induction of Th1 cells, as IL-23 had still not yet been identified or recognized in its function of driving Th17 differentiation. These applications include psoriatic arthritis, multiple sclerosis, and Crohn's disease, and the drug has displayed good efficacy except in the case of multiple sclerosis (24).

IL-6 has also been implicated as an important cytokine in driving the Th17 subset. Aberrant expression of IL-6 has been indicated in inflammatory diseases such as RA and juvenile idiopathic arthritis (JIA), leading to the development of a humanized IL-6 receptor (25). Tocilizumab blocks IL-6 receptor in both soluble and membrane bound forms with the result of blocking IL-6 function. In the clinic, tocilizumab has demonstrated amelioration of inflammatory diseases such as RA and JIA with improvement in symptoms such as joint destruction and a reduction in the number of flare incidents in JIA (26).

SECRETION OF IMMUNE MEDIATORS

It is clear that DC cytokine release is essential and tailored to clearing specific infections while also controlling immune homeostasis. Regulation of protein trafficking and release of immune mediators such as cytokines, chemokines, and lysosomal enzymes into the immediate environment from DCs is therefore highly regulated in order to prevent autoimmunity and inflammation. Indeed, a number of mutations or deficiencies resulting in dysregulated trafficking can have major implications in immune syndromes (27).

Depending on the required function of an immune mediator, different pathways exist to transport newly synthesized proteins out of the cell. These pathways are usually shared by a number of factors and their release can be constant (constitutive release) or triggered (regulated release) (4). Proteins produced in the endoplasmic reticulum (ER) can be soluble and contained within vesicles or membrane bound and associated within the plasma membrane of transported vesicles.

Thus, a number of proteins share transport to the plasma membrane to conserve energy (27). The release of these proteins can be basal or dependent on their transcription at gene level in response to cellular signaling (28).

SNARE PROTEINS

The transport of vesicles between organelles, endosomes, and the cell surface plasma membrane is controlled by a complex network of cellular machinery of membrane associated proteins, lipids, and cytoplasmic proteins. Soluble-N-ethylmaleimide–sensitive factor accessory protein receptor proteins (SNAREs) are a large conserved family identified in the docking/fusion machinery that

allow membranes to overcome opposing forces and fuse docked lipid bilayers (27).

SNARE proteins are all identified by a common conserved SNARE motif of 60–70 amino acids arranged in heptad repeats typical for coiled coils (29). Most SNAREs are type II integral membrane proteins that possess a single trans-membrane domain at their carboxyl (C-terminal) end that is connected to the SNARE motif by a flexible linker, with the majority of the protein extending into the cytoplasm (30). The conserved coiled-coil SNARE motif is necessary for complex formation and membrane fusion (31). When SNAREs form in a tight helical bundle, they form a conserved structure of an elongated coiled coil of four intertwined α-helices that correspond to the four interacting SNARE motifs occupying specific positions in the complex (32). There is a highly conserved layer of interacting amino acids in the central hydrophobic core of the helical bundle consisting of three glutamines and one arginine (31). Each of these contributing motifs contributes an amino acid and their structures are divided into sub-families used to further classify the SNARES as R-SNAREs or Q-SNARES (33).

R-SNARES are usually found on the membrane and have arginine (R) as the central functional residue in the SNARE motif. Q-SNARES are mainly found on the target membrane and are defined by a central glutamine (Q) residue. In order to form a complex and mediate membrane fusion, one R-SNARE and two or three Q-SNARE motifs are required to form a stable RQabc four helical bundle (30, 34). The SNARE proteins on opposing membranes interact to form the α-helix bundle that "zippers" together from the N-terminus to the C-terminus forming a trans-SNARE complex (or SNAREpin). The energy provided by the zippering complex formation pulls the membranes together displacing the water and hydrostatic pressure keeping them apart, thereby facilitating the mixing of hydrophobic lipids (35). Once the membranes have fused, the trans-SNARE conformation is converted to a highly stable inert cis-SNARE conformation on the resulting fused membrane. In order to dissociate this stable structure, metabolic energy is required and this is provided by two chaperone proteins; *N*-ethylmaleimide-sensitive factor (NSF), and Soluble NSF attachment protein (SNAP) (36, 37). This dissociation facilitates the recycling of SNARE proteins and prevents accumulation of SNAREs on the membrane. Many SNAREs operate predominantly in a specific subcellular compartment with their partners and some Qa SNAREs such as Syntaxin (STX)2 and STX4 are found on the plasma membrane and involved in fusion of vesicles and secretion of their contents from the cell. Other Qa SNAREs including STX5 and the R-SNARE vesicle-associated membrane protein (VAMP)4 are localized in the Golgi apparatus (38). These SNAREs are therefore found in the membranes of the donor compartment, acceptor compartment, and the intermediate trafficking vesicles (39).

THE ROLE OF SNAREs IN DENDRITIC CELLS

As the immune system consists of a plethora of cells that have specific functions, an understanding of the mechanism of trafficking is extremely important for when these functions become dysregulated and lead to disease. Cytokine signaling from DCs, in particular, is one of the most important types of signaling for the regulation of the adaptive immune response. However, while the role of SNAREs in secretion of key immune mediators has been addressed in many immune cells, the role of SNAREs in DCs function is poorly understood. Examples of SNAREs regulating critical function in other immune cells include STX11 which has been implicated in neutrophil degranulation and interferon-γ (IFN-γ) secretion from natural killer cells (40). Inhibition of SNAP23 or STX3 abolished chemokine release from mast cells and has been attributed to STX11. (41). While these individual SNAREs and their function have been identified in these cell types, a full elucidation of these trafficking pathways and/or SNARE partners have yet to be identified. However, in the last 10 years, Stow and colleagues have mapped the secretion of TNF-α from macrophages and fully elucidated an original secretion pathway through recycling endosomes that is implicitly linked to their phagocytic activity (42–44).

Furthermore, loss of STX11 in macrophages resulted in increased phagocytosis and TNF-α secretion, suggesting that this SNARE plays its part as a negative regulator (45). This hypothesis is supported in patients with the hyper-inflammatory disease, familial hemophagocytic lymphohistiocytosis type 4 (FHL-4), which results from a deletion or a mutation in STX11 (46). These patients possess high levels of pro-inflammatory cytokines (IFN-γ, IL-6, TNF-α, and IL-18) and have a higher number of macrophages that become over-activated and have increased phagocytic activity (47, 48). From their findings in macrophage, Offenhäuser et al. (45) accredited STX11's regulatory ability due to it how it binds and inhibits other SNARE proteins via its conserved SNARE sequence and lack of a trans-membrane domain (45). A role for STX11 in regulation of DCs has also been suggested. STX11 was examined in Bone marrow-derived dendritic cells (BMDCs) by D'Orlando et al. (40), and it was reported that although mRNA levels were up-regulated in BMDCs, STX11 deficiency did not affect BMDC activities or TLR-induced secretion of IL-12, IL-6, or TNF-α (41). Work by our own group, in a paper published in this issue of *Frontiers*, shows a correlation of up-regulation of STX11 expression in BMDCs in response to TLR ligands. Further work is required to confirm the role of STX11 in DCs although it is possible that the role of STX11 in phagocytic APCs is conserved (49).

VAMP8 is an R-SNARE that has also been reported to significantly inhibit phagocytosis in DCs (50). In this study, the authors show that VAMP8 associates with lysosome-associated membrane protein-2 (LAMP2) at the phagosome in dendritic cells, following bacterial exposure. Use of *VAMP8*$^{-/-}$ DCs significantly increased phagocytic ability. Furthermore, over-expression of VAMP8 in a DC2.4 cell line inhibited phagocytosis. Ho et al. also suggest that VAMP7 also negatively regulates phagocytosis in the same manner, as both VAMP7 and VAMP8 can form SNARE complexes with STX7, STX8, and Vti1b (50). This complex has been show to mediate homotypic fusion of early and late endosomes (51).

SNARE expression in DC has been reported in some detail in a paper describing differential expression following treatment with acetylsalicylic acid (ASA), the active ingredient in aspirin. Cai et al. reported that SNARE expression of Vti1a, Vti1b, VAMP3, VAMP8, and STX8 were all up-regulated following treatment with ASA (52). The authors describe a decrease in phagocytosis in response to ASA, and attribute it to the up-regulation of Vti1a and Vti1b

on phagosomes, and inhibition of SNARE complex formation. This study also describes the up-regulation of VAMP8 in cells that have decreased phagocytic ability, corroborating the findings by Ho et al. (50). Vti1b can also form a SNARE complex with VAMP 8 in the previously described STX7/STX8/Vti1b SNARE complex identified on endosomal membranes (51).

Cross-presentation is another essential function of DCs that is regulated by SNARE proteins. Sec22b was identified by Cebrian et al. (2011) as a specific regulator of cross-presentation. Sec22b is localized to the ER-Golgi intermediate compartment which interacts with STX4 on the phagosome. In the absence of Sec22b, DCs were unable to transfer antigen from phagosomes to the cytosol and degradation of the antigen in the phagosome increased (53). It was recently shown that while Sec22b-mediated phagosomal recruitment is required for cross presentation, it is independent of TLR signaling and cannot deliver MHC class I (MHC-I). Nair-Gupta et al. have shown that large quantities of MHC-I are stored in the endosomal recycling compartment (ERC) (which possess Rab11a, VAMP3, and VAMP8 on its surface). TLR signaling through the myeloid differentiation (MyD) marker, MyD88, results in phosphorylation of SNAP-23 present on the phagosome. This leads to stabilization of SNARE complexes, fusion of ERC and the phagosome, and ultimately cross-presentation (54). These findings regarding cross-presentation are summarized in **Figure 1**.

Work in our own group is currently identifying the mechanisms of secretion of key immune mediators involved in inflammatory

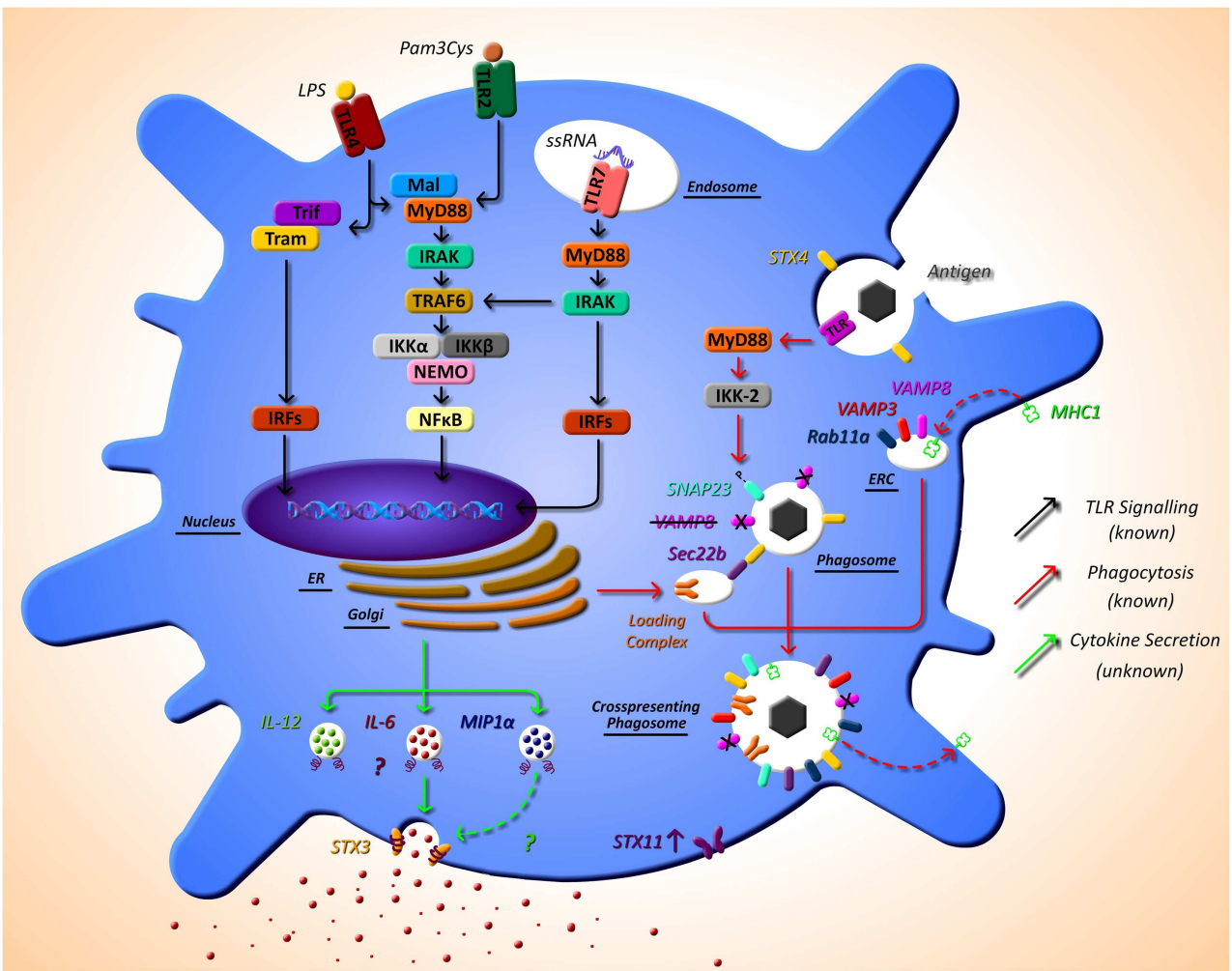

FIGURE 1 | The as yet unknown side of DC secretion post-TLR activation, and trafficking molecules currently identified in DC function. Following TLR activation with TLR ligands, such as LPS, DCs activate intracellular pathways that lead to the transcription of inflammatory mediators, such as cytokines. While this pathway is extremely well defined (black arrows), very little is known of the post-transcriptional pathway and how proteins, in particular cytokines, are trafficked out of the DC. It is evident from other cell types that SNARE proteins play a role in secretion of cytokines; however, to date, their specific role has not yet been described. SNARE importance in DCs has been highlighted by studies that demonstrate that STX4 on the phagosome interacts with Sec22b on the ER-Golgi intermediate compartment, which is required for cross-presentation and MyD88-dependent TLR signaling, and results in phosphorylation of SNAP23 present on the phagosome. This leads to fusion with endosomal recycling compartment (ERC) and ultimately cross-presentation. Furthermore, VAMP8 has been shown to be a negative regulator of phagocytosis (red arrows). Our recent work has indicated a role for STX3 in IL-6 and possibly MIP1-α secretion (green arrows). This work and others also suggests a role for STX11 in DC function as it is highly up-regulated post TLR2, TLR4, and TLR7 activation.

disease and we have recently identified a role for STX4 in IgE secretion from plasma cells (55). Due to the role of DCs in the direction of adaptive immune responses via secretion of cytokines, we aim to elucidate these pathways in detail in these cells. Another finding from the research paper published in this issue of *Frontiers*, we have described the effects of different TLR ligands on DCs and measured the cytokine profiles secreted by these cells and their concurrent SNARE expression (49). Specific TLR pathway activation allows DCs to respond as needed to specific challenges. For example, when TLR4 is activated by lipopolysaccharide (LPS), this in turn activates DCs to produce the cytokines necessary to orchestrate an adaptive immune response to clear a bacterial infection, as described above. Depending on the TLR agonist stimulation, different cytokine profiles are secreted by DC, and we demonstrate that these profiles correlate to differential expression of SNAREs. Assessing the secretion of cytokines from JAWSII DC's following activation with TLR ligands revealed that IL-6 and MIP-1α secretion was significantly increased following stimulation with LPS (TLR4) and Loxoribrine (TLR7) but not PGN (TLR2). This correlated with a change in expression of STX3. Furthermore, knockdown of STX3 using siRNA against STX3 resulted in a significant decrease in IL-6 and MIP-1α secretion with no effect on IL-1β or TNF-α, indicating a role for STX3 in IL-6 secretion. To our knowledge, this is the first study to profile SNARE expression patterns following different TLR stimulations in DCs and demonstrates the correlation between cytokine secretion patterns and expression of SNAREs (49). This is overviewed in **Figure 1**, indicated by the green arrows.

CONCLUSION

Identifying specific SNARE complexes and their regulators will lead to a better understanding of DC function during inflammation and may present potential therapeutic targets in a wide range of inflammatory diseases. Current therapeutic monoclonal antibodies target aberrant and subsequent damaging cytokine production by blocking their activities. It is well known that prevention is better than a cure and the current therapeutic strategies do not meet this requirement, highlighting the need for more targeted therapies. Monoclonal antibodies treatments are also prone to off-target effects such as drug induced liver damage and the generation of anti-idiotypic antibodies, rendering them ineffective (56). While a number of combined antibody therapies and personalized medicine methodologies are being investigated, targeting trafficking proteins and pathways to inhibit cytokine release from the cell may prove to be a more effective therapeutic approach.

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Airway epithelium interactions with aeroallergens: role of secreted cytokines and chemokines in innate immunity

Vivek D. Gandhi and Harissios Vliagoftis*

Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, AB, Canada

Edited by:
Charles Dudley Mills, BioMedical Consultants, USA

Reviewed by:
Masaaki Murakami, Hokkaido University, Japan
Yuri Sykulev, Thomas Jefferson University, USA
Jian Zhang, The Ohio State University, USA
Rakesh K. Kumar, University of New South Wales, Australia

***Correspondence:**
Harissios Vliagoftis, Pulmonary Research Group, Department of Medicine, University of Alberta, 550A Heritage Medical Research Centre, Edmonton, AB T6G 2S2, Canada
e-mail: hari@ualberta.ca

Airway epithelial cells are the first line of defense against the constituents of the inhaled air, which include allergens, pathogens, pollutants, and toxic compounds. The epithelium not only prevents the penetration of these foreign substances into the interstitium, but also senses their presence and informs the organism's immune system of the impending assault. The epithelium accomplishes the latter through the release of inflammatory cytokines and chemokines that recruit and activate innate immune cells at the site of assault. These epithelial responses aim to eliminate the inhaled foreign substances and minimize their detrimental effects to the organism. Quite frequently, however, the innate immune responses of the epithelium to inhaled substances lead to chronic and high level release of pro-inflammatory mediators that may mediate the lung pathology seen in asthma. The interactions of airway epithelial cells with allergens will be discussed with particular focus on interactions-mediated epithelial release of cytokines and chemokines and their role in the immune response. As pollutants are other major constituents of inhaled air, we will also discuss how pollutants may alter the responses of airway epithelial cells to allergens.

Keywords: airway epithelium, proteinases, pollutants, airway inflammation, HDM allergens, cockroach allergens, fungal allergens

INTRODUCTION

Allergic asthma is a complex disease that involves interactions of genetic and environmental factors (1). Genetic factors predispose to atopy, but the development of allergic sensitization is also dependent on environmental factors with exposure to a particular allergen being one of the most important. When sensitized individuals are exposed to the same allergen, they develop allergy symptoms from target organs; these symptoms can be organ specific in conditions like allergic rhinitis and asthma, or generalized, as in the case of anaphylaxis.

Aeroallergens are the major triggers for respiratory allergy (2), although foods, drugs, and other allergens can also trigger disease. The major mode of entry of aeroallergens in the body is through inhalation, although these allergens can also affect the immune system following skin exposure. The airway epithelium is the first line of defense against inhaled aeroallergens (3). The epithelium acts as a structural barrier to prevent invasion of inhaled particles carrying aeroallergens, but it is also the first innate immune cell type that interacts with antigens and other components of the inhaled particles. Studies on these interactions support the idea that epithelial innate immune functions may be instrumental for the development of an immune response through pro-inflammatory mucosal responses, following interactions with inhaled antigens and chemicals found in pathogens, allergens, and pollutants.

The theme of the review will be the role of epithelium as innate immune cells in allergic airway inflammation. Our discussion will focus primarily on cytokines and chemokines released upon interaction of the airway epithelium with major aeroallergens and how these mediators shape the immune response toward the inhaled allergen. We will also discuss the role of air pollutants in modifying the results of these interactions.

AIRWAY EPITHELIUM: AN IMMUNOLOGICALLY ACTIVE BARRIER

Airway epithelial cells express tight junction (TJ) proteins such as occludin, claudin, and zonula occludens, which give epithelial cell monolayers their barrier property. Apart from being a barrier, the airway epithelium plays multiple roles, such as maintaining airway surface liquid (ASL) levels, the mucociliary escalator, and also epithelium restitution upon injury (4, 5). To fulfill these roles, the airway epithelium is comprised of a number of specialized cell types that work in harmony to maintain homeostasis in the airways. The characteristics of the various epithelial cell types found in the lungs and their specific roles are summarized in **Table 1**.

Identification of pattern recognition receptors (PRRs) (6) and proteinase-activated receptors (PARs) (7) highlighted the potential of airway epithelial cells to sense/interact with allergens. Further secretion of immune mediators through activation of these receptors gave new insight to airway epithelial cells being an immunologically active innate immune cell. Similar to the airway epithelium, epithelium in other organs, such as gut (8, 9) and skin (10, 11), play the same multiple roles in tissue homeostasis and in the development of immune responses to foreign antigens.

Allergen–airway epithelium interactions and their effects on epithelial properties as well as on the immune system will be discussed below.

Table 1 | Different airway epithelial cell types and their characteristics.

	Cell type	Characteristics/functions
Bronchial Epithelium	Basal cells	• Only cells that express hemidesmosomes, thus firmly attached to the basal membrane via integrins • Self-renewal capacity • Act as progenitor for goblet and ciliated cells • Produce variety of bioactive molecules including cytokines
	Columnar ciliated cells	• Terminally differentiated cells that arise from either basal or goblet cells • Possess cilia that clear mucus from the airways
	Goblet cells	• Secrete mucus into the airways to trap foreign particles • Self-renewal capacity • Transdifferentiate into ciliated cells
	Club cells	• Produce bronchiolar surfactant and specific antiproteinases, such as secretory leukocyte proteinase inhibitor and other enzymes • Progenitor for goblet and ciliated cells
Alveolar Epithelium	Type I cells	• Very thin cells that cover 97% of alveolar place • This thin structure is important as it allows easy gas exchange between alveoli and blood
	Type II cells	• Produce pulmonary surfactants that are important for keeping alveolar space open and thus allow gas exchange • Progenitor cells for alveolar epithelium

ALLERGEN–AIRWAY EPITHELIUM INTERACTIONS

The airway epithelium and allergen–epithelium interactions play a pivotal role in airway immune responses. Allergen–airway interactions start with the recognition of an allergen by receptors present on the airway epithelium. These interactions result in partial loss of epithelial integrity and/or release of inflammatory mediators from epithelial cells. Inflammatory mediators can activate the innate immune system at the same time that the allergen can interact and activate dendritic cells (DCs) that are present below the epithelial monolayer. The default response of immune system to most of these interactions is the development of immune tolerance, which means that subsequent interactions of the organisms with the same allergen will not lead to pathology. In certain individuals, however, the same interactions can lead to the development of allergic sensitization. The exact circumstances and factors responsible for the decision between tolerance and allergic sensitization are not well understood. In mouse experimental models, it has been shown that activation of PRRs (12) or PAR-2 (13) can bias the system toward sensitization. It is also known that inflammatory mediators produced by epithelial cells after interactions with allergens may bias the immune response toward allergic sensitization through their effects on allergen-DC-T cell interactions (3). A subsequent exposure of an individual with an allergic sensitization to the same allergen results in IgE-mediated activation of mast cells (14). However, even in sensitized individuals, inflammatory mediators released from epithelial cells following interactions with allergens play an important role in recruiting inflammatory cells and mediating allergic inflammation. Since the aim of the review is to discuss the role of allergen–epithelium interactions and its immune outcomes, we will focus on the epithelial responses that play role in allergic sensitization, the first part of the process described above, and allergic inflammation, the second part of the process.

The airway epithelium–allergen interactions and the functional consequences of these interactions are affected by the structural and functional state of the epithelium at the time of these interactions. For example, decreased TJ protein expression in atopic asthmatics (15) could compromise the epithelial barrier function (16) allowing allergen invasion. Moreover, there is evidence that mucociliary clearance is not efficient in asthmatic airways (17, 18). It is not clear whether this impairment is the result of environmental factors, including the effects of inhaled allergens, or driven by genetics, but ineffective airway clearance may result in prolonged presence of the allergens in the airways allowing them to have protracted effects. In addition allergen can directly alter epithelial properties, as will be discussed below.

In vitro, *ex vivo*, and *in vivo* systems have been used to study the outcomes of direct allergen–epithelium interactions. *In vitro* approaches have generated interesting results about epithelial responses to allergens, but the biological relevance of these results is questionable as these interactions happen under artificial conditions and in the absence of other cells/factors, such as other immune cells that may modulate the effects. *Ex vivo* and *in vivo* approaches on the other hand suffer from the inability to determine that the effects seen are mediated by direct epithelium–allergen interactions and are not due to the indirect effects of other immune cells–allergens interactions on the airway epithelium. However, the two approaches give complementary and very important results and we will review them separately below.

In vitro research to understand allergen–epithelial interactions has been carried out using both alveolar and bronchial airway epithelial cells. A549 is the most utilized cell line to study interactions with alveolar epithelium while various cell lines, such as BEAS-2B, 16HBE14o-, Calu-3, NCI-H292, and primary airway epithelial cultures, from healthy and asthmatic patients, have been used to study interactions with the bronchial epithelium. In addition, both immersed cultures and air–liquid interface (ALI)

cultures have been used in studies performed with bronchial epithelial cells. ALI cultures mimic a physiological airway epithelium and facilitate *in vitro* study of epithelial functions such as barrier property, mucus secretion, and mucociliary escalator, which is not possible with immersed cultures. ALI cultures also provide a unique opportunity to study cellular interactions between epithelial cells and other immune cells by using co-culture systems. However, this variety of experimental settings and cells used has generated often inconsistent and difficult-to-explain results.

In the following section, we will review studies focusing on interactions of airway epithelial cells with some of the most common aeroallergens including house dust mite (HDM), cockroach, fungal, and pollen allergens. A schematic of the mechanism of these interactions and the biological effects of interactions are shown in **Figure 1**. Allergens include proteins with very different structures and activities and can interact with epithelial cells through a variety of mechanisms. *In vitro* studies have shown the proteinase activity of allergens can alter epithelial cell morphology and cause epithelial cell detachment (19, 20). Moreover, proteinase activity of allergens has been implicated in allergic sensitization and allergic inflammation in animal models (21). In this review, we will classify the allergen–epithelial interactions into proteinase-dependent and proteinase-independent interactions.

EPITHELIAL TIGHT JUNCTION DISRUPTION

Most of the direct effects of allergens on the immune system require allergens to penetrate through the epithelium into deeper tissues. The main mechanism by which allergens cross the epithelial barrier is through the degradation of epithelial TJs. A HDM allergen with cysteine proteinase activity can degrade the adhesion protein occludin and allow allergens to penetrate through the epithelial monolayer (22). Similarly, cysteine and serine proteinases from pollen grains (23, 24) and fungi (25) can induce TJ degradation.

In addition to direct degradation of TJ, HDM proteinases can also have an indirect effect on TJ integrity. HDM proteinases activate PAR-2 (26), a pro-inflammatory receptor on epithelium, which in turn transactivates epidermal growth factor receptor leading to E-cadherin destabilization and loss of epithelial barrier integrity (27). Thus, although proteinases associated with allergens or allergen particles are prime candidates to mediate TJ degradation, this may not always be the case. Another study comparing various HDM extracts from different manufactures showed that the extract with the lowest proteinase activity caused maximum barrier dysfunction (28). The study also showed that this extract was the most potent inducer of inflammatory mediator release indicating that these mediators could contribute to epithelial barrier dysfunction. The idea was supported by the observation

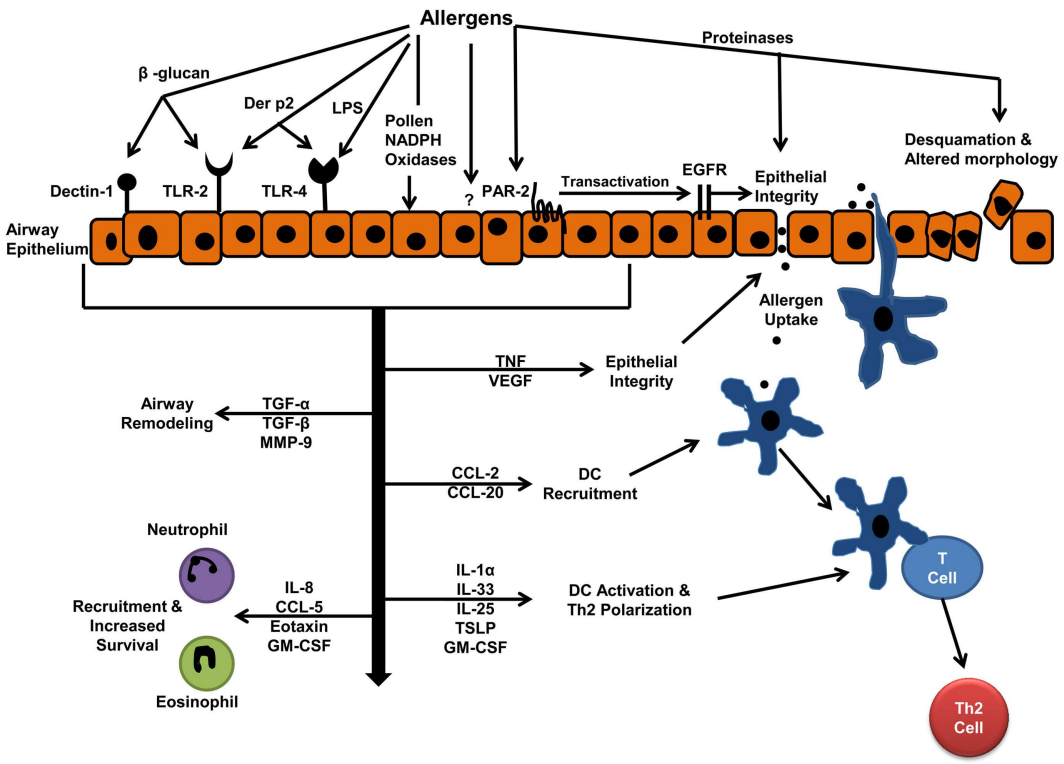

FIGURE 1 | Allergen–epithelial interactions: effects on allergic sensitization, and allergic airway inflammation. Aeroallergen interactions with epithelium result in increased epithelial permeability, which facilitates allergen entry and uptake by dendritic cells (DCs). The mediators released by epithelium, upon allergen interactions, drive DC-T cell interactions toward Th2 immune response. These mediators also develop inflammation by recruiting inflammatory cells to the airways.

that cockroach allergens affect epithelial permeability indirectly through the release of vascular endothelial growth factor (VEGF) from epithelial cells (29). Chronic exposure to tumor necrosis factor-α (TNF) also causes barrier dysfunction by disrupting TJs (30). The observation that fungal allergens can induce TNF release from airway epithelial cells (31) indicates that this may be another indirect method utilized by allergens to increase airway epithelial permeability.

Finally, TJ degradation by allergens may also be specific to epithelium from asthmatics. For example, a study has shown that epithelium from only asthmatic individuals and not from healthy individuals was sensitive to fungal allergens-mediated increased epithelial permeability (31). However, it is not clear whether asthma-induced epigenetic changes make the airways of asthmatic individuals sensitive to allergens or it is genetic defects in the TJ proteins that make an individual more sensitive to allergen penetration, sensitization, and asthma development.

EPITHELIAL RELEASE OF INFLAMMATORY MEDIATORS: CYTOKINES AND CHEMOKINES

Allergens interact directly with airway epithelial cells and induce the release of inflammatory mediators, including cytokines and chemokines. In most cases, however, the exact type of epithelial cell responsible for the release of mediators is not described. Many of the studies discussed below utilize immersed cultures of airway epithelial cells, which are comprised primarily of basal cells (32); basal cells have been shown to express receptors that can be activated by allergens (6, 33). Thus, we speculate that basal cells may be the main cell type releasing cytokines and chemokines in response to aeroallergen stimulations, although other epithelial cell types may also be involved. There is more information on the responses of epithelial cells, from different anatomical locations, to interactions with allergens and these will also be discussed below. We also discuss interactions of allergens with alveolar epithelial cells. Although allergen particles may not be able to reach the alveolar space, individual allergenic proteins may bind to other smaller particles [i.e., diesel exhaust particles (DEPs)] and reach this area.

House dust mite

House dust mite allergens induced the release of CCL20 from human airway epithelial cells, while cockroach allergens do not have the same effect (34); CCL20 release in this study was dependent on the interaction of β-glucan from HDM with dectin-1 on epithelial cells and independent of proteinase activity. β-glucan from HDM also induced CCL20 release from human nasal epithelial cells, but in this case through interactions with TLR-2 (35). Even though HDM-induced CCL20 release from human airway epithelial cells was proteinase-independent, in mouse models of allergic airway inflammation CCL20 release from airway epithelial cells was shown to be proteinase and PAR-2-dependent (36).

Der p2, an HDM allergen without proteinase activity, induced cytokine release from the airway epithelium through TLR-2 (37) and TLR-4 (38) activation. Der p2 is shown to induce reactive oxygen species (ROS) and ROS-mediated nerve growth factor (NGF) release from airway epithelium (39), and NGF was found to be important for the development of asthma features (40). An *in vivo* study, using a model of tissue specific TLR-4 knockout, showed that HDM-mediated allergic inflammation was dependent on the interaction between HDM and TLR-4 on airway structural cells (41). However, in this model, HDM-induced IL-25 and thymic stromal lymphopoietin (TSLP) were not TLR-4 dependent. IL-25 and TSLP are vital mediators for the development of allergic sensitization and are released primarily by epithelial cells. Thus the presence of these mediators, in the absence of epithelial TLR-4, shows a requirement of other receptors on airway epithelial cells for the development of inflammation.

In line with this argument, HDM has shown proteinase-dependent release of IL-25 from bronchial epithelial cells (42). Moreover, a study also demonstrated that the inflammatory property of HDM was mainly dependent on the proteinase activity of HDM extract (19). Der p3 and Der p9, HDM allergens with serine proteinase activity, induced chemotactic mediators CCL11 and granulocyte macrophage colony-stimulating factor (GM-CSF) release from alveolar cells through PAR-2 (43).

Cockroach allergens

Cockroach proteinases can also activate PAR-2 (44) and PAR-2-mediated release of CXCL8 from human alveolar (45) and bronchial epithelial cells (46) as well as CCL20 and GM-CSF from mouse tracheal epithelial cells (36). Cockroach allergens can also induce the release of IL-33 (47) and IL-25 (42) from human bronchial epithelial cells, but the role of proteinases in this instance is not clear.

These studies have primarily used HDM and cockroach whole body extracts to study their interactions with epithelial cells. However, humans are exposed to HDM and cockroach frass in their daily lives. It is true that majority of the allergens that are present in frass are also found in whole body extract (48, 49), allowing us to consider results from crude extracts. Antigen characteristics and abundance may also vary to some degree between species (50, 51) and the composition of allergen extracts varies from manufacturer to manufacturer (28, 49). These differences make at times the studies difficult to interpret and impossible to compare.

Fungal allergens

Alternaria alternata is the best studied fungal species for interactions with epithelial cells. It has been shown to induce proteinase-dependent IL-6 and CXCL8 release from alveolar (20) and bronchial epithelial cells (52); cytokine release from bronchial epithelial cells was shown to be PAR-2-mediated. Apart from these mediators, *A. alternata* has also been shown to induce PAR-2-mediated release of GM-CSF (52) and TSLP (53) from bronchial epithelial cells, as well as release of IL-33 (47) and IL-25 (42). Involvement of proteinases in the release of IL-33 and IL-25 is not clear. *Aspergillus fumigatus* (54) and *Cladosporium herbarum* (20) also induced proteinase-dependent release of IL-6 and CXCL8 from an alveolar cell line. Purified serine proteinase Pen c13 from *Penicillium citrinum* showed to induce CXCL8 in alveolar cell line and primary cells through PAR-1 and PAR-2 activation (55). Similarly, another purified serine proteinase Pen ch13 from *Penicillium chrysogenum* induced CXCL8, prostaglandin E_2 (PGE_2), and transforming growth factor-β (TGFβ) from A549 and primary human bronchial epithelial cells (25). Fungal extracts, similarly with HDM and cockroach extracts, possess chitin and it has been

shown that chitin can induce CCL2 from a mouse airway epithelial cell line (56) but the effect of chitin on human airway epithelial cells have not been studied.

As discussed, allergens from HDM, cockroaches, and fungi are able to activate PAR-2 receptors, which are expressed throughout the airways. *In vitro* PAR-2 activation has been shown to release mediators from cells of different phenotypes, which are present in the airways, for example, IL-25 (42) and PGE_2 from bronchial epithelial cells (7), matrix metallopeptidase 9 (MMP-9) from small airway epithelial cells (57), and CCL2 from alveolar epithelial cells (58).

Pollens

Pollen grains are complex structures containing proteins with and without enzymatic activity. We saw in the previous section that proteinases released from pollen grains can alter epithelial integrity; however, pollen-mediated release of mediators has been found to be proteinase-independent (19). Pollen extracts induced release of IL-6, CXCL8, GM-CSF (19), and TGF-β (59) from airway epithelial cells was independent of enzyme activity or lipopolysaccharide (LPS). Pollens also induced IL-25 from bronchial epithelial cells (42), but the mechanism of release is not known. Finally, pollen grains contain NADPH oxidases, which increase intracellular ROS in epithelial cells (60), and thus increase oxidative stress.

A number of the studies discussed here have been carried out with purified allergens and others with crude extracts that contain a great number of different allergens. An *in vitro* study compared inflammatory mediators induced by a purified grass pollen allergen Phl p1 (61) and a crude grass pollen extract (GPE) (62) in a human epithelial cell line. Both stimuli induced many common mediators; however, purified Phl p1 induced the release of granulocyte-colony stimulating factor (G-CSF) while GPE induced CCL3, CCL4, and CCL5. In addition to this differential regulation, the most important difference was that the purified Phl p1 upregulated CCL28 and downregulated TSLP, but the opposite regulation was evident for GPE (63). The authors suggested that the whole extract can have other inhibitory or stimulatory components which could be crucial to overcome/maintain the disease phenotype. This observation was supported by an *in vivo* study, which showed that a purified HDM allergen induced mild late asthmatic responses compared to complete allergen extracts that induced severe responses (64). Thus, it is advisable to use whole allergen extract for studies, which will mimic real life conditions.

Furthermore, the concentrations of allergens used for *in vitro* and/or *in vivo* studies may not be close to physiological concentrations encountered by human airway epithelial cells *in vivo*. Since biological responses are often dependent on the concentration of a protein in addition to the affinity of the ligand for its receptors, results seen in studies may not be similar with what would happen *in vivo* in humans.

Synergy with other inflammatory mediators

An important question is why aeroallergens do not induce inflammation in non-asthmatic individuals if they can directly activate epithelial cells. One possibility is that the tissue microenvironment where allergen–epithelial cell interactions take place influences the final outcome of these interactions. Asthmatic airways, in contrast to healthy airways, are characterized by the presence of chronic allergic inflammation. The levels of Th2 inflammatory cytokines such as IL-4 (65), IL-13 (66, 67), and fibrogenic mediators, such as TGF-β (68), are increased in asthmatic airways. There is evidence of synergy for HDM allergens with IL-4 and TGF-β to induce release of the Th2 chemoattractant CCL17 from airway epithelial cells (69). HDM also showed synergy with IL-4 and IL-13 for induction of CXCL8 and GM-CSF release from airway epithelial cells (70). Interestingly, IL-4 also enhanced TSLP release in response to *A. alternata* (53), while the Th1 cytokine interferon gamma (IFNγ) inhibited TSLP induction. TSLP plays an important role in allergic sensitization and thus this observation reflects the importance of the tissue microenvironment in the development of immune response. Cockroach allergens acted synergistically with TNF to induce PAR-2-mediated CXCL8 (71) and MMP-9 (72) release from airway epithelial cells. Finally, PAR-2 activation has also shown synergy with IL-4 for TSLP release (53) and with LPS for CXCL8 release and PAR-2 mRNA expression (73).

In addition, cytokines released by epithelial cells after direct activation by allergens may subsequently activate epithelial cells to release other mediators. For example, direct interaction of epithelial cells with allergens caused TSLP release (53), which could in turn activate epithelial cells to release IL-13 (74). Further, IL-13 acted on airway epithelium to induce periostin (75) and CCL11 (76) release. It was also shown that HDM allergens induced TLR4-mediated IL-1α release from human airway epithelial cells, which acted in an autocrine manner to induce dendritic cell (DC) chemoattractants IL-33 and GM-CSF from airway epithelial cells (77). Inflammation could therefore be perpetuated by this "loop effect" that allergens and cytokines/chemokines exert on the airway epithelium.

Asthmatic vs. non-asthmatic airway epithelial cells

A second important question is whether there is a difference in the allergen-induced response between asthmatic and healthy airway epithelia. Attempts to address this question have used cells isolated from the airways of asthmatic and non-asthmatic individuals; these cells retain their characteristics after culture *in vitro* (78). According to one study, untreated cells from HDM-allergic patients showed higher basal expression for genes regulating cytokines, chemokines, and growth factors compared to cells from non-allergic subjects (79). This upregulated expression in the absence of stimulus could be because of asthma-induced epigenetic changes in the epithelium, which could result in constant increased amounts of mediator release. Other studies have also shown that HDM allergen-mediated activation of airway epithelial cells from asthmatic patients, and not from healthy individuals, release CCL20 (80) and transforming growth factor-α (TGFα) (70), which can cause DC chemotaxis and cell proliferation, respectively. The latter manuscript has also shown that cells isolated from asthmatic individuals demonstrate a tendency for increased release of CXCL8 and GM-CSF upon HDM stimulation. These differences could be responsible for the altered immune response to allergens seen in the airways of asthma patients.

Effect of epithelial phenotype

We have already discussed the phenotypic and functional heterogeneity of airway epithelial cells. This heterogeneity may explain different epithelial responses to allergens depending on the part of the airways where the interaction takes place. There is substantial experimental evidence showing that cells from different compartments of the airways may respond differently to allergens. The HDM allergens Der p2 (81, 82) and Der f2 (82), which have no proteinase activity, induced IL-6, CXCL8, G-CSF, GM-CSF, CCL2, and CCL20 from bronchial epithelial cells, but not from alveolar epithelial cells, while HDM allergens with proteinase activity induced IL-6, CXCL8, and CCL2 release from alveolar epithelial cells (19) and CCL2 release from bronchial epithelial cells (80), but not from nasal epithelial cells (19). In addition, *A. fumigatus* (54) as well as timothy grass and birch pollens (19) showed CCL2 release from alveolar epithelial cells, but not from bronchial epithelial cells. This information may indicate that in the case of inhalation of multiple allergens, different allergens act on the upper and lower airway epithelial cells causing release of a variety of inflammatory mediators. Also receptors activated by allergens show heterogeneity in their pro-inflammatory responses. Small airway epithelial cells demonstrated PAR-2-mediated CCL11 release (83), while PAR-2-mediated activation of bronchial epithelial cells did not induce the release of CCL11 (52, 84).

In conclusion, interactions between aeroallergens and airway epithelial cells are complex events that are influenced by a number of factors as discussed above. A plethora of mediators are released from airway epithelium as a result of these interactions. In the subsequent sections, we will discuss the effects of these mediators on different immune cells, and thus on the immune response.

ROLE OF RELEASED MEDIATORS IN SHAPING THE IMMUNE RESPONSE

As we mentioned above, allergen–airway epithelial cell interactions participate in both the development of allergic sensitization, and in the development of allergic airway inflammation in sensitized individuals. Here, we will describe the role of various epithelial-derived mediators in these two processes.

ROLE IN ALLERGIC SENSITIZATION

Tight junction disruption induced by allergens, as has been discussed above, may facilitate antigen penetration and uptake by DCs, an important first step toward allergic sensitization. However, there is evidence regarding mechanisms, other than TJ disruption, which aid allergen uptake by DCs (85, 86). Another crucial factor required for the development of allergic sensitization is the presence of a favorable microenvironment for DCs. Allergen–airway epithelium interaction results in release of mediators, which provide a favorable environment that supports DC maturation, activation, and also directs DCs interaction with CD4 T cells toward Th2 phenotype, resulting in allergic sensitization and allergic inflammation (87). Most of these studies were done with HDM and cockroach allergens. However, these mechanisms could also be applied to pollen and fungal allergens as they also activate TLR-2, TLR-4, Dectin-1, and PAR-2 receptors that are important for HDM and cockroach effects. Some of the studies showing this effect will be reviewed here.

Allergen-mediated activation of PAR-2 on airway epithelial cells could induce CCL2 release (58), while dectin-1 (34) and TLR-2 (35) activation resulted in the epithelial release of CCL20; CCL2 (88) and CCL20 (89) are chemoattractants for immature DCs. This will result in the recruitment of the immature DCs to the airways, facilitating the first step toward the allergic sensitization, i.e., allergen–DC interaction and allergen uptake by DCs. CCL2 has been shown to play a role in the generation of Th2 responses in mouse models of allergy (90) and also of airway hyper-reactivity (91), a consequence of allergic sensitization. It is interesting that bronchial lavage from allergic asthmatics showed increased CCL20 (80), which indicates that this mechanism may be also important *in vivo* in humans.

Mediators released by the epithelium upon allergen interactions, such as TSLP (92), IL-25, and IL-33 (93), act on DCs to induce OX40L expression. This expression of OX40L on DCs is vital for the development of T cells into the Th2 phenotype (94). In mouse models, interaction of OX40L expressing DC with OX40 on T cells resulted in increased expression of IL-4 (95), which induces T cell differentiation to Th2 cells. Recent evidence showed the inability of DCs to induce Th2 responses in the absence of GM-CSF, another epithelial-derived mediator (96), suggesting a role of GM-CSF in the development of a DC phenotype that promotes Th2 response. In addition to its role in the development of antigen-specific Th2 cells, IL-33 may also play a more direct role in the development of humoral immune responses to inhaled aeroallergens, although the exact mechanism is not clear (97). Finally, IL-33 may also have other pro-inflammatory effects in allergic airway inflammation by supporting eosinophil survival (98) and IL-4 and IL-13 release from basophils (99).

In conclusion, proteinase activity of allergens may facilitate entry and uptake of allergens by DCs, while components, with and without proteinase activity, induce a cytokine/chemokine milieu that supports DCs maturation and polarizes their interactions with T cells toward development of Th2 responses.

ROLE IN ALLERGIC AIRWAY INFLAMMATION

Interactions of allergens with the airway epithelium are the first events that take place after allergen inhalation by an allergic individual. The mediators released from these interactions play an important role in the development of airway inflammation by acting as chemotactic or survival factors for inflammatory cells.

As mentioned previously, CCL2 (88, 100) and CCL20 (89) recruit monocytes or immature DCs to the site of inflammation. DCs can also act as inflammatory cells and promote inflammation in sensitized individuals (101). GM-CSF increases eosinophil survival (83), while CCL11 acts as chemoattractant for eosinophils to the airway (102). Airway epithelial cells also release neurotrophins under allergic inflammatory conditions that increase eosinophil survival (103).

Allergens mediate release of TGF-β (59). TGF-β is increased in the asthmatic airways and its release was further increased after allergen challenge (68). TGF-β has been shown to induce more extensive epithelial-mesenchymal transition (EMT) *in vitro* in airway epithelial cells isolated from asthmatic individuals compared to cells from healthy individuals (104). In addition, TGF-β-treated, but not untreated, airway epithelial cells undergo EMT

upon HDM exposure *in vitro* (105). These observations raise the possibility of differential effect of allergens on airways of asthmatics vs. non-asthmatics but their significance has to be validated *in vivo*. Moreover, TGF-β has found to be involved in airway remodeling by inducing airway smooth muscle cell proliferation and increased mucus production (106). Other released mediators from the airway epithelium, such as IL-25 (107) and TSLP (108), are also involved in airway remodeling. Finally, a recent human study showed that neutralizing TSLP not only prevents the allergen-induced increase in exhaled nitric oxide and blood and sputum eosinophils, but also decreases their levels in mild allergic asthmatics (109).

Allergen-induced epithelial release of MMP-9 has not been reported; however, we have shown that activation of PAR-2, which is a target of allergen-proteinases, causes MMP-9 release from airway epithelial cells (57). Increased presence of MMP-9 in sputum has been observed in patients with severe asthma (110). The same study showed that after allergen challenge, MMP-9 activity was significantly increased in severe and mild asthmatics. MMP-9 could be responsible for airway remodeling by degrading extra cellular matrix (ECM).

As discussed, the activation of airway epithelial cells by allergens also releases CXCL8 and IL-6 (19, 20, 45, 81). CXCL8 may be a chemoattractant for eosinophils in allergic individuals (111), but it may also contribute to the neutrophilia seen in the airways in cases of acute asthma exacerbations or severe asthma (112). IL-6 has been found to be upregulated in severe asthma (113), but its role in asthma is uncertain as it possesses both pro- and anti-inflammatory properties (114).

The majority of these mediators released by the epithelium are also released by other immune cells in the airways. Thus, it would be an overstatement to conclude that the epithelial cell is the sole cell type responsible for the above mentioned responses. However, when HDM allergen-mediated epithelial NF-κB activation was inhibited *in vivo*, allergen-mediated inflammatory mediator release, inflammation, and remodeling was significantly reduced (115). This observation depicts that the mediators release by airway epithelium upon allergen-airway epithelium interactions contribute significantly in the development of allergic sensitization and allergic inflammation.

INTERACTIONS BETWEEN ALLERGENS AND POLLUTANTS

Pollutants are a major pro-inflammatory component of inhaled air and constitute a major health concern (116, 117). Pollutants are present in indoor and outdoor environments and can be gaseous such as ozone and nitrogen dioxide or particulate matter such as DEPs and cigarette smoke (CS). Direct interactions of these inhaled pollutants with airway epithelial cells have been discussed quite extensively elsewhere (118–121). More interestingly, the simultaneous presence of pollutants and aeroallergens in the air results in complex interactions between the two. Pollutants exert direct effects on aeroallergens but also alter the host responses to inhaled aeroallergens.

POLLUTANT EFFECTS ON AEROALLERGENS

Indoor and outdoor airborne particles carry aeroallergens. HDM and cockroach allergens are found in general on particles with a median diameter of 10–30 μm (122), while cat and dog allergens are found on particles with 5 μm mass median diameters (123, 124). Suspended particulate matter in homes can carry dog, pollen (125), and cat allergens (126). DEPs, a major outdoor particulate pollutant, have also been shown to bind pollen, dog, cat, and HDM allergens (125, 127). Because of their very fine size, DEPs can facilitate penetration of these allergens into the lungs and therefore increased numbers of pollution particles may increase the amount of allergen interacting with the epithelium.

It has also been shown that DEPs can disrupt pollen particles causing release of allergenic sub-pollen particles. Interestingly, detailed analysis of pollen obtained from areas with pollution showed increased presence of allergenic proteins (128, 129), which resulted in a higher allergenic property of the allergen (128). This may be another mechanism by which pollution increase allergen–epithelial interactions.

Finally, the effect of climate change and pollution on the allergenicity of pollen has been studied. Comparing recent and a decade-old pollen extract, the authors showed that the allergenic potency of the recent pollen extract was higher (130). It was further shown that the recent pollen extract harvested from the urban area had a higher allergenic potency than the recent pollen extract from suburb. Recently, the same authors also demonstrated that the recent pollen extract from an urban area, which has faced the climate change and increased pollution, was more effective at inducing transepithelial permeability and ROS production in the cultured airway epithelial cells (131).

POLLUTANT EFFECTS ON AEROALLERGEN-INDUCED RESPONSES

The effects of various pollutants on the development of allergic sensitization and allergic airway inflammation have been an area of intense research in both animal and human systems. The first evidence 30 years ago showed that inhalation of ozone (132, 133) and DEPs (134) increase sensitization to inhaled allergens as measured by the presence of antigen-specific IgE (132) and subsequently resulted in increased anaphylactic sensitivity upon the allergen challenge. Inhalation of suspended particulate matter along with an allergen has shown to act as an adjuvant and increase IgE production (135). Similarly, in a human study, DEPs, when inhaled with allergen, promoted Th2 inflammation and allergen specific IgE (136).

The mechanisms of this priming/sensitization effect of pollutants have been studied in detail. Interestingly, mice exposed to the pollutant nitrogen dioxide prior to allergen exposure developed TLR2, MyD88, and NF-kB dependent sensitization to the allergen, resulting in Th2 inflammation and airway hyper-responsiveness (137). Pollutants such as ozone (138) and nitrogen dioxide (139) can induce maturation of CD11c+ myeloid DC and increase antigen uptake and antigen-presenting activity of DCs. DEPs and ambient particulate matter upregulated TSLP in bronchial epithelial cells (140) and DEP-induced TSLP can promote myeloid DCs maturation (141) and increase OX40L expression (142), favoring Th2 inflammation. These, and possibly others, are some of the mechanisms of increased allergic sensitization in the presence of pollutants.

The effects of pollutants on allergen-induced responses in sensitized individuals have also been studied *in vivo*. Motorcycle

exhaust particles (143) and DEPs (144, 145) can increase airway hyper-responsiveness to allergen and allergen-mediated early and late inflammatory responses in different animal models of allergy. Similarly human studies have shown that prior exposure to pollutants such as ozone, nitric dioxide alone or in combination with sulfur dioxide increased bronchial responsiveness to pollen (146) and HDM allergens (147–149) and also amplified airway inflammation (150, 151).

The *in vivo* studies discussed above, however, do not allow us to identify the cells involved in pollutant–allergen interactions. The airway epithelium would be expected to play a significant role in these effects, especially since pollutants can directly activate airway epithelial cells. Nitrogen dioxide increased epithelial permeability and induced leukotriene C_4 synthesis (152). Moreover, nitrogen dioxide (153), DEPs (154), and ozone (155) induced CXCL8, GM-CSF, and TNF from cultured airway epithelial cells. As was shown for allergen–epithelial cell interactions, ozone and nitrogen oxide released more inflammatory mediators from epithelial cells from asthmatics than those from healthy donors (156). This observation indicates that pollutants induce a different pro-inflammatory environment in the airways of asthmatics compared to non-asthmatics.

Limited data exist regarding the effect of pollutants on allergen–airway epithelium interactions. These studies have been performed primarily with alveolar cell line A549; because of the fine size of allergen-carrying pollutant particles, these particles can reach to alveolar space and therefore may affect alveolar epithelial cells. Studies have shown that exposure to submicron particles and allergen individually or in combination induced alterations in cellular morphology (increased microvilli), functions (increased lysozyme and surfactant-producing multilamellar bodies) (157), and metabolic activity (damage to mitochondria, tonofilaments, and rough endoplasmic reticulum) in lung epithelial cells (158). Sodium sulfite and HDM acted synergistically for detachment of the cells (159), which could cause inflammation and decrease epithelial barrier integrity. Further, exposure of these cells to the combination of pollen grain-Pb (160) and pollen allergens-DEPs (161) caused significant increase in IL-5 mRNA and Th2 cytokines release, respectively, from airway epithelial cells.

In summary, apart from having inflammatory effects on their own, pollutants can increase the concentration, exposure, and allergenic property of aeroallergens. Thus, pollutants exert a priming effect on immune system for allergens and also increase inflammatory responses to allergens.

Apart from these pollutants discussed so far, CS is a major factor influencing allergic sensitization and asthma. Smoking is a major source of indoor particles (162). Maternal smoking has been shown to increase the risk of asthma development in children (163). Using animal models, it has been shown that passive smoking acts as an adjuvant to increase allergen-mediated allergic immune responses (164) and airway remodeling (165). Effects of CS on airway epithelial cells are similar to other pollutants, i.e., increased allergen-mediated epithelial permeability and inflammatory properties, which have been reviewed in detail (166, 167).

CONCLUSION

Current literature establishes the airway epithelium as an innate immunity organ that senses inhaled allergens through an armory of receptors, and initiates innate and adaptive immunity. This potential has been established clearly through *in vitro* studies, although more detailed *in vivo* studies are still needed to validate these results.

Two approaches could improve our understanding regarding the role of airway epithelium in allergic inflammation. *In vitro* co-culture of epithelial cells grown in ALI with one or more of the other immune cells that may play a role in allergic inflammation, such as DCs and T cells, in the presence of particular allergens may clarify the sequence of events leading to the development of allergic airway inflammation. These studies should be coupled with *in vivo* models utilizing airway epithelium-specific strains of knockout mice. These studies should start with tissue-specific knockouts of epithelial receptors interacting with allergens and continue with similar knockout strains of signaling molecules. These studies may reveal common links between major allergens for their interactions with the airway epithelium and improve our understanding of the basic mechanisms leading to allergen-specific sensitization and inflammation.

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