

Eosinophil crystalloid granules: structure, function, and beyond

Valdirene S. Muniz,* Peter F. Weller,[†] and Josiane S. Neves*¹

*Institute of Biomedical Sciences, Federal University of Rio de Janeiro, UFRJ, Rio de Janeiro, Brazil; and [†]Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA

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ABSTRACT

Eosinophils are granulocytes associated with host defense against parasitic helminths with allergic conditions and more recently, with immunoregulatory responses. Eosinophils are distinguished from leukocytes by their dominant population of cytoplasmic crystalloid (also termed secretory, specific, or secondary) granules that contain robust stores of diverse, preformed cationic proteins. Here, we provide an update on our knowledge about the unique and complex structure of human eosinophil crystalloid granules. We discuss their significance as rich sites of a variety of receptors and review our own recent research findings and those of others that highlight discoveries concerning the function of intracellular receptors and their potential implications in cell signaling. Special focus is provided on how eosinophils might use these intracellular receptors as mechanisms to secrete, selectively and rapidly, cytokines or chemokines and enable cell-free extracellular eosinophil granules to function as independent secretory structures. Potential roles of cell-free eosinophil granules as immune players in the absence of intact eosinophils will also be discussed. *J. Leukoc. Biol.* 92: 281–288; 2012.

Introduction

Eosinophils are granulocytes associated with host defense against parasitic helminths, with the pathology of allergic conditions and more recently, with immunoregulatory responses. Eosinophils are distinguished by their dominant population of cytoplasmic crystalloid (also termed secretory, specific, or secondary) granules. Specific granules of human eosinophils are notable for their robust stores of diverse, preformed cationic proteins, such as EPO, MBP, and EARs, known as ECP and eosinophil-derived neurotoxin. In addition, these granules contain a diversity of preformed cytokines, chemokines, en-

zymes, and growth factors, including IL-2, -3, -4, -5, -6, -10, -12, and -13, IFN- γ , TNF- α , NGF, GM-CSF, stem cell factor, TGF- α , RANTES (CCL5), eotaxin-1 (CCL11), growth-related oncogene- α , and epithelial neutrophil-activating peptide-78/CXCL5 [1–3]. Mouse eosinophils house a similar content of basic protein in their granules, including MBP, EPO, and at least 13 mEARs. The mEARs present ~70% sequence identity among them and are estimated to constitute ~50% of the protein content within eosinophil granules. At least 6–8 mEARs are granule proteins [4–6]. Related to this point, mouse eosinophils and human eosinophils are not necessarily functionally equivalent [6]. Whereas EPO is highly conserved between mouse and human, the eosinophil ribonucleases and MBP are highly divergent. The facts that the human EARs and several mEARs are stored as preformed enzymes in granules and ready to be released indicates that regulated secretion of these eosinophil granule-derived, RNase-related proteins may contribute to the roles of eosinophils in host defense and/or immunopathogenesis. Mechanisms governing selective secretion of these granule-derived proteins underlie the biologic activities of eosinophils in health and disease.

Three different mechanisms are known to mediate the release of human eosinophil granule-stored proteins. Compound exocytosis, whereby the entire granule contents are released extracellularly following fusion of granules with plasma membranes, occurs when eosinophils interact with large targets, such as helminthic parasites. However, this process is not usually observed in vivo. Instead, secretion of granule contents from within intact eosinophils occurs primarily by a mechanism termed PMD. This is a process whereby granule contents are selectively mobilized into spherical and tubular vesicles that need to disengage from granules, transit through the cytoplasm, and fuse with the plasma membrane to release their specific granule-derived protein cargo at the cell surface [3, 7, 8]. A third mechanism of human eosinophil “degranulation” is associated with cytolysis. Following lysis of an eosinophil with loss of its plasma membrane integrity, there is a release and deposition of intact, cell-free, membrane-bound granules. Al-

Abbreviations: cysLT=cysteinyl leukotriene, EAR=eosinophil-associated RNase, ECP=eosinophil cationic protein, EPO=eosinophil peroxidase, IP₃K=inositol triphosphate kinase, LAMP=lysosome-associated membrane proteins, LT=leukotriene, MBP=major basic protein, mEAR=mouse eosinophil-associated RNase, PMD=piecemeal degranulation, RTK=receptor tyrosine kinase

1. Correspondence: Federal University of Rio de Janeiro, Institute of Biomedical Sciences, 373 Carlos Chagas Filho Ave., Centro de Ciências da Saúde (CCS), Room F14, 1st Floor, Ilha do Fundão, Rio de Janeiro, RJ, 21941-590, Brazil. E-mail: jneves@farmaco.ufrj.br

though PMD is considered the predominant mechanism underlying eosinophil degranulation and secretion, cytolysis has been recognized as a common mechanism for cell-free eosinophil granule release and deposition in tissues of eosinophilic diseases [9–12]. The presence of membrane-bound, cell-free granules extruded from eosinophils has been long recognized in tissues associated with eosinophilia, including allergic diseases and inflammatory responses to helminths. The first recognition of cell-free granules in sputum of asthmatics was reported in the early 1880s, but the relevance of these observations was unknown, and historically, finding of cell-free eosinophil granules in tissues was, at times, attributed to “crush artifacts”. However, increasingly, evidence for eosinophil cytolysis and the presence of membrane-bound, cell-free eosinophil granules localized in tissues has been associated with many eosinophilic disorders (reviewed in ref. [13]).

Unlike human eosinophils, the capacity of mouse eosinophils to secrete their granule contents, *in vitro* or *in vivo* in airway inflammation models, has been controversial [14–22]. Therefore, the applicability of mouse models to understand eosinophil-associated diseases on humans has been doubted [18]. Confirming previous *in vivo* studies [19], Shamri and collaborators [23], in a very recent publication, showed that secretion of granule proteins from mouse eosinophils in response to CCL11 was accompanied by ultrastructural changes, including reduced electron density, granule matrix coarsening, disassembled cores, or loss of the matrix or cores within granules, all of which are characteristics of PMD [19, 21]. In contrast, no evidence *in vitro* for other secretion mechanisms, such as exocytosis or cytolysis, was found in response to CCL11. Moreover, in a different study, Dyer and collaborators [22] demonstrated that the platelet-activating factor elicited EPO but not cytokines release from bone marrow-derived eosinophils, suggesting regulated and differential mouse eosinophil protein secretion.

Ultrastructurally, human eosinophil granules are trilaminar membrane-bound organelles that contain a crystalloid core surrounded by a matrix. Rather than being simple intracellular protein storage stations, the human crystalloid granules contain highly structured internal membranes within the granule organelles. Using TEM techniques, early and more recent ultrastructural evidence demonstrated within crystalloid granules the presence of intragranular membranes that exhibit points of continuity with the membranes that surround the granule [24]. In addition, TEM and electron tomography studies revealed that following agonist activation, mobilized eosinophil granule compartments become rearranged with intragranular vesiculotubular structures. This suggests that following activation of eosinophils by different stimuli, proteins might be segregated, selectively sequestered, and sorted within granule subcompartments [24, 25]. There are also studies suggesting that human eosinophil crystalloid granules may be sites of RNA and possibly DNA localization, indicating that protein synthesis might take place within eosinophil granules [26–28]. Moreover, we showed that human eosinophil crystalloid granules are rich sites of intracellular localization of many cytokine/chemokine receptors, which may facilitate the capability of the function of granules as independent secretory organelles [13, 29–32] (Fig. 1). Similar to humans, recent studies demon-

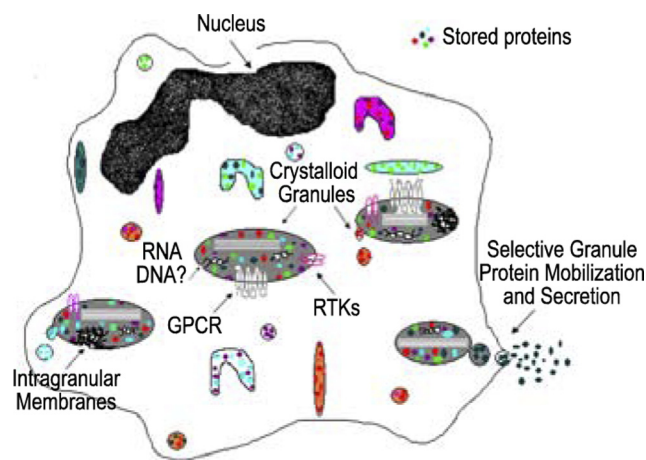


Figure 1. Eosinophils and their unique crystalloid granules. Eosinophil crystalloid granules express receptors on their membranes topologically oriented with ligand-binding domains displayed on the outer granule membrane and may be sites of RNA and possibly DNA localization. Eosinophil crystalloid granules contain intragranular membranes that might be involved in granule-stored protein sorting and selective secretion.

strated for the first time that cell-free granules isolated from mouse eosinophils express functional CCR3 receptors and can secrete EPO and mEARS in response to CCL11 stimulation [23]. These findings support the import primacy of mouse eosinophils as a source of secreted mEARS, from within intact eosinophils and from their cytolytically released granules. These unique and novel abilities of eosinophil cell-free granules to remain secretory and competent extracellularly are evolutionarily common to humans and mice. This is important to comprehend how mouse models are relevant as a tool to understand human eosinophil cell secretion and allergic inflammation.

Here, we provide an updated review of the unique and complex structure of human eosinophil crystalloid granules. We discuss their significance as rich sites of a variety of receptors, as well as review our own recent research findings and that of others concerning the function of intracellular receptors and their potential implications in cell signaling. Special attention is paid to understanding how eosinophils use intracellular receptors as mechanisms to selectively and rapidly secrete cytokines/chemokines and the mechanism by which these intracellular receptors enable cell-free extracellular eosinophil granules to operate as independent secretory structures.

EOSINOPHIL CRYSTALLOID GRANULES ARE RICH SITES OF LOCALIZATION OF A VARIETY OF RECEPTORS

A series of subcellular fractionation and flow cytometry studies performed by our group revealed that crystalloid granules obtained from nitrogen-cavitated eosinophils are rich sites of localization for chemokine (e.g., CCR3), cytokine (e.g., IL-4 α -chain receptor), cysLT₁R and cysLT₂R, and purinergic receptors (P2Y₁₂R) [29–31] (Table 1). In Table 1, we provide

TABLE 1. Results Comparing Different Receptor Expression on Intact, Nonpermeabilized Eosinophils and on Isolated Eosinophil Granules under Resting Conditions

Receptors	Intact, nonpermeabilized eosinophils	Cell-free eosinophil granules	References
CysLT ₁ R ^a	+	+	[30]
CysLT ₂ R ^a	+	+	[30]
P2Y ₁₂ R ^a	+	+	[30]
CCR3 ^a	+	+	[29, 31, 33]
IFN- γ - α chain R	+	+	[29]
C5aR ^{a,b}	+	—	Upr
GM-CSF- α chain R ^c	+	—	Upr
GM-CSF/IL-5/IL-3- β chain R ^d	+	—	Upr
IL-5- α chain R ^e	+	—	Upr

^aGPCRs. ^bRabbit anti-human C5aR [5 μ g/ml (BD PharMingen, San Diego, CA, USA)]. ^cMouse anti-human GM-CSF- α chain R [5 μ g/ml (Chemicon, El Segundo, CA, USA)]. ^dMouse anti-human GM-CSF/IL-5/IL-3- β chain R [10 μ g/ml (Santa Cruz Biotechnology, Santa Cruz, CA, USA)]. ^eGoat anti-human IL-5- α chain R [5 μ g/ml (R&D Systems, Minneapolis, MN, USA)]. Upr, Unpublished results.

our most updated published and unpublished results comparing different receptor surface expression on intact nonpermeabilized eosinophils and on isolated eosinophil granules under resting conditions. Some receptors that are found on intact, nonpermeabilized eosinophils and on crystalloid granules (e.g., cysLT₁R, cysLT₂R, P2Y₁₂R, CCR3, IFN- α α -chain receptor) are topologically oriented with ligand-binding domains displayed on eosinophil plasma membranes and the eosinophil granule outer-delimiting membranes, respectively, as assessed after labeling with antibodies raised against amino terminal ligand domains. Intriguingly, the C5aR, the cytokine GM-CSF- α , and IL-5- α chain receptors and the common GM-CSF/IL-5/IL-3- β chain receptor were only found in intact eosinophils but not detected on the surface membranes of isolated crystalloid granules. These findings are remarkable, as they suggest that eosinophils and extracellular cell-free crystalloid granules might have different or complementary immunoregulatory roles during eosinophilic inflammation upon eosinophil cell lysis and subsequent cell-free granule release. The IL-5R data are particularly intriguing. Anti-IL-5 mAb therapies have been considered a modality for treatment of eosinophilic diseases [34, 35]. However, we were unable to find IL-5R α -chain expression on granule membranes. The impact that it might have in the anti-IL-5 therapies is currently unknown. Nevertheless, we might speculate that it would, in part, explain the lack of full protection of anti-IL-5 monotherapy in the control of asthma in some patients. More clinical trials are needed to better delineate this point.

In addition, these findings support our previous subcellular fractionation characterizations, confirming that our eosinophil granule preparations were devoid of plasma membrane contamination [33]. Why some receptors are expressed on granule membranes and others not and whether these receptors traffic to and from granule membranes under specific stimulatory conditions remain unsolved.

Under conditions in which inflamed tissue sites are infiltrated by degranulating eosinophils, the extrusion of granules from eosinophils with receptors topologically oriented for ligand occupation suggests that granules might have secretory properties. Indeed, mechanisms responsible for the secretion

of preformed cationic proteins and cytokines have only been identified recently. Notably, studies demonstrated that isolated human eosinophil granules could exert extracellular functions as secretion-competent organelles after stimulation with ligands, including chemokines and cysLTs [29, 30]. This is a highly relevant observation in the context of eosinophil cell biology. Considering functional roles for these intracellular granule membrane-expressed receptors with ligand-binding domains displayed on the outer granule membranes, these findings not only allow granules to function extracellularly but also suggest that the granule-expressed receptors might potentially serve as intracrine mediators of eosinophil-derived granule secretion. For receptors, such as the lipid-mediator receptors (e.g., LTs and PGs), activated by hydrophobic ligands, which can be synthesized at the nuclear membrane or lipid bodies, it is possible to predict roles for these receptors in intracellular compartments, as they would be accessible to their ligands. For chemokine/cytokine receptors, it is also feasible that the ligands themselves are active intracellularly after their biosynthesis and/or based on specific cell uptake and internalization mechanisms.

PHYSIOLOGICAL IMPLICATIONS OF INTRACELLULAR RECEPTORS

RTKs (e.g., for cytokines) and GPCRs [e.g., receptors for CCL11 (eotaxin-1) and LTs] have traditionally been considered to transduce signals solely extracellularly from plasma membrane-expressed receptors to intracellular signaling molecules, thus evoking cellular responses. However, it is now more widely accepted that RTKs, as well as GPCRs, are capable of signaling through intracellular compartments, such as endosomes, the nucleus, the ER, the Golgi complex, the trans-Golgi network, and secretory granules [36–38]. Early evidence that receptors can signal from intracellular sites came from studies of RTKs signaling in endosomes [39–41]. In these studies, EGFRs and other RTKs internalized together with their ligands and remained phosphorylated and active in endosomes. Nevertheless, it has been challenging to demonstrate that this type of intracellular signaling produces specific effects in cell signal-

ing (such as full ERK activation) [42, 43] or behavior (proliferation or motility) [44]. Limited, prior experiments indicated that IL-10Rs are localized on the membranes of intracellular neutrophil granules [45] and that IFN- γ can signal intracellularly [46]. However, such signaling sites after receptor-mediated endocytosis have not been identified, with the notable exception of potential signaling at the nuclear membrane for IFN- γ [46]. Consistent with the finding regarding RTKs, in many cell types, most of the endogenous GPCRs are detected in intracellular sites or organelles. Recent findings necessitate revision of the traditional view of GPCR signaling and expand the diversity of mechanisms by which receptor signaling influences cell behavior in general. Studies have shown that a cell-permeable lipid estrogen has signaled through an ER-based GPCR [47]. Moreover, the chemokine receptor CCR5 (responsive to the chemokines RANTES and MIP) is largely retained in the ER and Golgi complex, even when observed in its natural context [48]. In addition to the ER and Golgi apparatus, other organelles, vesicular and nuclear intracellular sites of localization, and function for GPCRs have been identified.

Classically, upon ligand stimulation, GPCRs activate their respective G protein-effector pathways and are subsequently recruited to specialized domains at the plasma membrane, such as clathrin-coated pits. Most of the GPCRs show a rapid loss of responsiveness, followed by internalization from the plasma membrane. Endocytosed receptors then traffic to lysosomes for degradation or follow a recycling route (for review, see ref. [37, 38]). However, endocytic trafficking does not necessarily lead to signal termination. Recent data from three groups provide strong evidence for persistent signaling to adenylyl cyclase by endosomal-internalized GPCRs [49–51]. This phenomenon may be associated with the perpetuation of an effect initiated at the cell plasma membrane, even after removal of the agonist.

In addition, GPCRs have been found to localize and signal on the cell nucleus [52]. For instance, GPCRs for lipid mediators, such as PGs and cysLTs, have been immunolocalized at nuclear membranes [52–54]. In an interesting study, Nielsen et al. [53] demonstrated that isolated intestinal cell nuclei express the cysLT₁R and responded to LTD₄ triggering ERK1/2 signaling. However, whether these nuclear localized receptors are involved in the cell cycle (survival or proliferation) is still unknown.

Moreover, in the context of cell secretory pathways, there are other physiological implications of intracellular receptors. In a previous study combining TEM with biochemical and immunological approaches, Spencer et al. [31] localized substantial cytokine and chemokine receptor expression at intracellular granules of blood-derived human eosinophils. Specific stimulation of intact eosinophils mobilized the IL-4 α chain receptor but not the common γ accessory chain from within eosinophil intracellular granules in association with emerging vesicles, carrying IL-4 within its binding domain [31].

Studies of eosinophil secretion by different groups have revealed different patterns of specific, stimulus-induced secretion of preformed, granule-derived cytokines [31, 55–61]. Observations of sorting and sequestering of preformed granule-derived cytokines suggest that specific mobilization of intragranular

cytokines and chemokines through binding to cognate receptors is a likely mechanism used by eosinophils to accomplish selective and rapid secretion from preformed intracellular granules stores. In accordance to that, Duitman et al. [62] described a critical role for the IL-15R in mediating secretion of IL-15 in different transfected cell lines and potentially in primary monocytes and macrophages. These findings help to explain, at least in part, how cytokines may be chaperoned and transported through cell secretory pathways. Binding of cytokines with their own cognate receptors might be the mechanism involved.

As mentioned previously, we showed that purified, cell-free eosinophil granules, obtained by subcellular fractionation of isolated disrupted peripheral human eosinophils, express receptors, topologically oriented to engage their ligands for CCR3, IFN- γ R α -chain [29], two cysLTRs (cysLT₁R, cysLT₂R), and P2Y₁₂R [30]. CCR3 and IL-10Rs have been demonstrated on intracellular mast cell [63] and neutrophil [45] granules, respectively. However, whether granules of other leukocytes likewise function extracellularly remains to be assessed. Extracellular eotaxin-1 (CCL11) or IFN- γ stimulation of free eosinophil granules elicited intragranular signal transduction that leads to the differential secretion of granule-stored cytokines, including IL-6 and IL-4 and other proteins [29]. In response to IFN- γ , cell-free granule ECP secretion was suppressed dose-dependently by tyrosine kinase, p38 MAPK, and PKC blockers but not by an IP₃K inhibitor. CCL11-initiated signaling within isolated granules was dependent on p38 MAPK, PKC, and also IP₃K. Our findings were the first evidence that purified eosinophil granules contained functional signal-transducing proteins [29].

This recent observation has been supported further by another study showing that the cysLT₁R, cysLT₂R, and purinergic P2Y₁₂R were expressed on eosinophil granule membranes. Stimulating cell-free eosinophil granules with the agonists LTC₄, -D₄, and -E₄ elicited secretion from granules of ECP but not eosinophil-derived cytokines or chemokines. Montelukast, a recognized inhibitor, principally of cysLT₁R, as well as the P2Y₁₂R antagonist MRS 2395, inhibited eosinophil granule ECP secretion after LTC₄/LTD₄/LTE₄ stimulation of eosinophil cell-free granules [30]. Whether the capacity of a cysLT₁R inhibitor, montelukast, to likewise inhibit secretion elicited by ligands (e.g., LTE₄) not active on cysLT₁R might suggest functional heterodimerization among cysLT₁R and other receptors expressed on eosinophil granule membranes remains to be ascertained.

Our observations support the notion that cell-free granules are organelles fully capable of ligand-elicited, active secretory responses. These observations are also important, as they expand our appreciation of the ability of eosinophils to contribute to immune system modulation after cell lysis. These findings suggest that the physiological implications of intracellular receptors might be uniquely related to enabling granules to function extracellularly as secretory-competent organelles and as regulators of granule secretion.

In summary, despite our standard view of GPCRs or RTKs as cell surface recognition sites, there is increasing evidence of physiological roles for intracellular receptors for cytokines and

other ligands. Although these findings can represent a novel aspect of cell surface receptor signaling following their internalization, the hypothesis that the receptors can be retained within the biosynthetic pathway or localized to function on intracellular organelles cannot be discarded. It remains to be established to what extent these intracellular receptors represent immature or incomplete receptor systems, novel subcellular niches of signaling, or receptor stores for rapid mobilization. Functional implications of these intracellular receptors might include the persistence of responses initiated at the eosinophil's plasma membrane, signaling responses in specific intracellular subcompartments, the intracellular selective and rapid vesicular transport of cytokines and chemokines, and/or the enabling of granule organelles to function extracellularly as independent structures.

IN THE ABSENCE OF INTACT EOSINOPHILS: CELL-FREE GRANULES AS IMMUNE PLAYERS?

Prior studies identified eosinophils as end-stage effector cells, causing damage to parasitic pathogens in helminth infections and to injured tissues in allergic diseases. However, accumulating evidence suggests that eosinophils can perform various immune regulatory functions, such as regulating inflammation, maintaining epithelial barrier function, affecting tissue remodeling, and bridging innate and adaptive immunity [64, 65]. Many of these functions arise from the capacity of eosinophils to store a preformed array of Th1, Th2, and regulatory cytokines, chemokines, growth factors, and other immunomodulatory molecules, available for immediate release. Whereas eosinophils secrete low amounts of cytokines compared with T cells (following their activation of transcriptional- and translational-mediated synthesis of new cytokines), the fact that those mediators exist as preformed cytokines and are very rapidly secreted may indicate essential roles of eosinophils in the immediate innate immune response and in the regulation of the microenvironment at sites of pathogen penetration or injury. Importantly, several of these mediators fulfill additional functions through signaling interactions with other cells, including endothelial cells, DCs, T cells, and mast cells [2, 64–67]. In murine models, eosinophils migrate from tissues to draining LNs, where they colocalize with T cells. Within secondary lymphoid tissues, eosinophils may drive the developing immune response through eosinophil-expressed mediators and direct antigen-presentation functions [68–72].

As discussed previously, our studies suggest that cell-free granules might act as an extracellular source of cationic proteins, cytokines, and chemokines, likely contributing to the perpetuation of the inflammatory process in an affected organ, as well as immunomodulating the surrounding environment through the selective well-controlled secretion of cytokines and other immunomodulatory molecules [13, 29, 30].

The idea that subcellular components are capable of affecting immunity through interaction with neighboring cells has gained considerable scientific interest with the discovery of exosomes [73, 74]. Cells do not only use vesicular and

tubulovesicular transport carriers to deliver cargo between membranes within a cell but also generate vesicles that are secreted into the extracellular space. Exosomes are nanovesicles (50–100 nm) released from viable cells, constitutively or upon activation of cell secretion. Ultrastructurally, exosomes appear with a characteristic cup-shaped morphology or as round, well-delimited vesicles. Apart from their morphology, exosomes have a unique protein and lipid composition. As a consequence of their endosomal origin, nearly all exosomes contain proteins involved in membrane transport and fusion (RabGTPases, annexins, flotilin), in multivesicular body biogenesis (Alix and TSG101), and in processes requiring heat shock proteins (hsp70 and hsp90), integrins, and tetraspanins (CD63, CD9, CD81, and CD82). Whereas some of the proteins that are found in the proteome of many exosomal membrane preparations may merely reflect the cellular abundance of the protein, others are specifically enriched in exosomes and can therefore be defined as exosomal marker proteins (Alix, flotilin, TSG101, CD63). Another characteristic of exosomes is their enrichment in raft lipids, such as cholesterol, sphingolipids, ceramide, and glycerophospholipids with long and saturated fatty-acyl chains [73–75]. First characterized during reticulocyte maturation, exosomes play important roles as key players in immune responses. B cell-derived exosomes can present allergen-derived peptides and activate allergen-specific T cells [76]. More recently, it was demonstrated that exosomes functioned as vectors of mRNAs, microRNAs, and lipid mediators, capable of acting on targeted cells [77].

In this context, the idea that eosinophilic granules contain secretory machinery that enables granules to selectively mobilize and secrete specific protein contents after eosinophil lysis may conceptually alter how we view basic cell biology. This also supports a new concept of organelles behaving as multifaceted structures with complex interactions with other cells and their local environment. In contrast to exosomes, eosinophil granules are larger in size (500–1000 nm) and express LAMP, such as LAMP-2 (CD 107b) and LAMP-3 (also known as CD63); vesicle-associated membrane protein-2 is not expressed on human eosinophil granules [33]. Despite the selective and well-controlled secretion of cytokines and cationic proteins, there is no evidence that cell-free eosinophilic granules can function as antigen-presenting structures or express MHC class II molecules. Little is known at present about eosinophil-derived exosomes. Compared with exosomes from other cells, less is understood about immunomodulatory functions of cell-free extracellular eosinophil granules.

Many questions regarding the nature and function of cell-free eosinophil crystalloid granules in tissues remain unanswered. Do eosinophil granules likewise mediate some of the immunomodulatory properties already known for intact eosinophils? Current knowledge is only beginning to explore the functional biology and responses of eosinophil granules. The molecular mechanisms involved continue to be intriguing and require further investigation.

LESSONS LEARNED AND AREAS OF UNCERTAINTY

Eosinophil granules are remarkable rich repositories of a variety of receptors and contain secretory machinery that enables granules to selectively mobilize and secrete specific protein content intracellularly and to function as secretory organelles extracellularly after eosinophil lysis. Whether these features of eosinophil granules are unique to eosinophils or are otherwise common, but as yet unstudied, in other granule containing leukocytes (e.g., mast cells, neutrophils, etc.) remains to be investigated. For eosinophil granules, outstanding questions remain, including delineation of the involvement of granule-expressed receptors on transport mechanisms that govern specific movement of vesicles trafficking proteins from granules to the cell surface. Moreover, regarding the extracellular secretory capacities of eosinophil granules, we are only now beginning to appreciate the biology and functional responses of eosinophil granules. For instance, we still do not know whether cytokine, chemokine, LT, and purinergic granule-expressed receptors are phosphorylated, desensitized, and internalized, as is the case with classic cell surface receptors. In addition, we do not know whether granule-expressed receptors are transported back to granule membranes or traffic to and from this subcellular compartment under specific stimulatory conditions. We also do not know whether granules are sites for de novo protein synthesis of granule-stored proteins.

One of the major uncertainties regarding extracellular cell-free granule function remains in our understanding of the mechanisms by which proteins are released. Whether intragranular vesicular structures fuse with the granule membrane and subsequently release the granule subcompartment content is currently unknown. Similarly, it remains unresolved whether or if there are vesicles coming out from the granule membrane, carrying granule cargo, as happens when granules function intracellularly.

However, we now appreciate that eosinophil involvement in diseases is not always signaled by the presence of intact cells. A recent report highlights the importance of recognizing that even in the conventional histopathologic absence of intact eosinophils, a more careful investigation of cell-free eosinophil granules or eosinophil granule protein deposition can be required for the definitive documentation of underlying eosinophil-mediated disease pathogenesis [78].

Other frequent questions regarding cell-free granules and their function concern the fates of eosinophil granules and whether they directly interact with other immune cells or for how long they remain functional in inflammatory tissues. To answer these complex questions, there are methodological obstacles to overcome. Visualizing cytokine secretion by cell-free eosinophil granules and tracking their interactions with other cells in vitro or in vivo has proven to be challenging. One of the reasons for this is because of their relatively small size, which makes granules sometimes difficult to visualize by regular confocal microscopy and instead, requires more powerful visualization tools, such as electron microscopy. Improvements in current imaging techniques will be required to determine

the full extent of cell-free eosinophil granule function and its fate in vivo.

In summary, recent investigations have provided insights into mechanisms of secretion of eosinophil granule contents, with eosinophils and as granules function extracellularly. Whether some of these insights into eosinophil granulocyte functionings are common to other granule-containing leukocytes remains to be investigated. More remains to be defined about underlying mechanisms responsible for mediating the selectivity of secretion of eosinophil granule contents. Through direct and indirect interactions with innate and adaptive immune cells and resident cells, eosinophils and their functional secretory granules might participate in complex networks affecting a multitude of physiological and pathological processes, expanding our appreciation of their specific effector and immunoregulatory roles in health and disease.

AUTHORSHIP

J.S.N. and P.F.W. designed the review. V.S.M., P.F.W., and J.S.N. wrote the paper.

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