Functional Extracellular Eosinophil Granules: A Bomb Caught in a Trap

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Key Words

DNA nets · Eosinophil granules · Immunomodulation

Abstract

Eosinophils store a wide range of preformed proteins, including cationic proteins and cytokines, within their morphologically unique granules. Recently, we have demonstrated that cell-free eosinophil granules are functional, independent, secretory organelles and that clusters of cell-free granules are commonly found at tissue sites associated with various pathologic conditions. Cytolytic release of intact eosinophil granules produces extracellular organelles that are fully capable of ligand-elicited, active, secretory responses and are hence able to act as ‘cluster bombs’ that amplify the differential secretory properties of eosinophils. Herein, we review recent progress in elucidating the molecular mechanisms involved in the cytolytic release of intact cell-free functional eosinophil granules in a process associated with the liberation of eosinophil DNA traps (nets), a known aspect of the innate response recognized in various immune cells and pathological conditions. We also discuss the importance of clusters of cell-free eosinophil granules trapped in eosinophil DNA nets in disease and speculate on their potential role(s) in immunity as well as compare available data on DNA-releasing neutrophils.

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Introduction

Eosinophils are leukocytes that play important roles in host defense to parasites, allergic responses, immunoregulation and tissue remodeling [1]. Eosinophils store a wide range of preformed, biologically active proteins, including cationic proteins [such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)], cytokines, chemokines and growth factors [IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN- γ , TNF- α , NGF, GM-CSF, SCF, TGF- α , Rantes (CCL5), eotaxin (CCL11), GRO- α and ENA/78/CXCL5], within their morphologically unique population of cytoplasmic granules (also known as secondary, specific or crystalloid granules). Ultrastructurally, eosinophil crystalloid granules are membrane-bound organelles that contain a crystalloid core surrounded by a matrix [1, 2].

Eosinophil Cell-Free Granules as ‘Cluster Bombs’

Although intact, membrane-bound granules secreted by eosinophils have been widely observed at tissue sites associated with eosinophilic inflammation, their functional significance and ability to secrete preformed cat-

ionic proteins and cytokines have only recently become apparent [3–7]. Notably, it has been demonstrated that isolated human extracellular eosinophil granules are secretion-competent organelles. Purified cell-free eosinophil granules, isolated by subcellular fractionation from disrupted human peripheral blood eosinophils, have been shown to express several receptors, including CCR3, IFN- γ R α chain [4], two cysteinyl leukotriene receptors (CysLT1R, CysLT2R) [5] and the purinergic receptor P2Y12 [5], topologically oriented to engage their ligands. Stimulation can elicit intragranular signal transduction in purified cell-free granules, which causes differential secretion of granule-stored cytokines and other proteins [4, 5]. For instance, stimulation with IFN- γ leads to dose-dependent secretion of ECP, EPO, hydrolytic enzymes such as β -hexosaminidase (β -Hex) and cytokines such as IL-4 and IL-6, but not IL-13 [4]. IFN- γ -dependent release of effector proteins from the granules is prevented by intracellular kinase inhibitors, including tyrosine kinases, protein kinase C, and p38 mitogen-activated protein kinase (MAPK) [4]. In response to leukotriene (LT) C4 and the extracellularly generated LTD4 and LTE4, granules secrete ECP, but not cytokines [5]. Although the mechanism by which cell-free granule proteins are released is still unclear, evidence shows that the impressive intragranular membranous network plays a crucial role during cell-free granule protein mobilization and secretion [4].

These remarkable findings contribute to understanding the mechanism by which eosinophils modulate host and inflammatory responses. Cytolytic release of intact eosinophil granules produces extracellular organelles fully capable of ligand-elicited active secretory responses. These granules are able to act as functional ‘cluster bombs’ that amplify the differential secretory properties of eosinophils and likely contribute to the persistence and exacerbation of the inflammatory response.

Cytolysis as a Mechanism of the Release of Cell-Free Granules

Mechanisms that govern the selective secretion of intragranular proteins regulate eosinophil function in immunity. Eosinophils secrete the granule proteins through exocytosis, piecemeal degranulation (PMD) or cytolytic degranulation [8, 9]. Compound exocytosis, whereby granule contents are secreted following fusion of the granule and plasma membranes, occurs in response to large targets, such as helminthic parasites, but otherwise

is not commonly observed *in vivo*. Instead, eosinophils secrete granule contents mainly via PMD, whereby granule contents are selectively mobilized into spherical and tubular vesicles that disengage from granules, cross the cytoplasm and fuse with the plasma membrane to release their specific cargo at the cell surface.

A third pattern of eosinophil degranulation is associated with cytolysis. The loss of eosinophil plasma membrane integrity and resulting lysis lead to the release and extracellular deposition of intact, cell-free, membrane-bound granules. Although PMD is generally considered to be the predominant mechanism of eosinophil degranulation and secretion, evidence of eosinophil cytolysis and the presence of membrane-bound free eosinophil granules at tissue sites associated with eosinophilic disorders have been repeatedly described in the literature (for review, see [6]). The numerous *in vivo* observations of free extracellular eosinophil granules suggest a unique process by which human eosinophils undergo nonapoptotic cytolytic cell death that liberates intact extracellular granules. The mechanisms of eosinophil cytolytic release of intact granules are not well understood. However, a recent study provides the first evidence that human eosinophils are capable of undergoing an active cytolytic cell death, accompanied by the liberation of nuclear DNA and extrusion of intact functional granules, a process termed ETosis [10].

Extracellular DNA Trap Release: Lessons Learned from Neutrophils

The release of DNA nets by various cell types is part of the innate immune response and has been observed in multiple infectious, allergic and autoimmune eosinophilic diseases [11]. In addition to occurring across different cell types, release of DNA nets appears to be an evolutionary conserved mechanism: the release of extracellular DNA traps has been reported for neutrophils or related cells such as heterophils in a range of organisms, including fish, ox, horse, cat, mouse and chicken [12–16]. In 2004, Brinkmann et al. [17] demonstrated that upon stimulation with phorbol myristate acetate (PMA), lipopolysaccharide (LPS), Gram-positive and Gram-negative bacteria or IL-8 and TNF (mediators involved in sterile inflammation), neutrophils secrete chromatin associated with histones and proteins of neutrophil granules into the extracellular medium; this is as a result of an active mechanism not necessarily related to cellular disintegration, but that might represent an early event in

cell death. This process was named NETosis. Other cell types, such as mast cells [18, 19] and macrophages [20], can also die via this mechanism. Hence, the process of death accompanied by the release of extracellular DNA traps (ETs) was generally termed ETosis, with NETosis being used specifically for neutrophils by some authors. ETs associated with other proteins can kill microbes, thus preventing the spread of pathogens. Based mostly on neutrophil studies, it is possible to attest that ETosis is morphologically distinct from other classic cell death processes, including apoptosis and necrosis [21]. DNA fragmentation and phosphatidylserine exposure in the outer membrane leaflet, which are hallmarks of necrosis and apoptosis, respectively, do not accompany ETosis [22]. Moreover, ETosis appears to be independent of caspases [23]. Finally, rupture of the plasma membrane releases nuclear DNA that forms extracellular DNA nets, which, for neutrophils, have been shown to bind free antimicrobial molecules such as neutrophil granule proteins and histones. NADPH-oxidase-mediated production of reactive oxygen species (ROS) plays an essential role in activation of ETosis. ETs contain antimicrobial molecules and immobilize and kill pathogens, thereby preventing microbial spread and contributing to an antimicrobial environment [17, 24]. The molecular binding mechanism is still unknown, but electrostatic interactions between the anionic surface of the microorganisms and cationic components of the ETs have been discussed [25]. ETs are thought to be generated by cells undergoing cell death, thus allowing dying or dead cells to kill microbes. However, in 2009, Yousefi et al. [26] reported that GM-CSF-primed neutrophils release mitochondrial DNA via an oxidant-mediated process in response to LPS or C5a. This finding suggests that neutrophil mitochondrial DNA release and net formation do not require cell death. Subsequent studies have demonstrated that neutrophils respond uniquely to *Staphylococcus aureus* via a novel process of ET formation that does not require neutrophil lysis or breach of the plasma membrane [27]. This process involves the budding of nuclear DNA-containing vesicles from the nuclear membrane and their subsequent extrusion to the extracellular space [27]. Another study has demonstrated that neutrophil nuclear DNA release and ET formation do not involve cell lysis, nor do they limit the migratory and phagocytic function of these cells [28]. Altogether, these data suggest that, at least for neutrophils, it is not certain that cell death is associated with nuclear or mitochondrial DNA release.

Eosinophil Cell-Free Granules and ETs

In 2008, Yousefi et al. [29] provided the first evidence that eosinophils are capable of liberating ETs in response to bacteria and other stimuli. In that study, the analysis of colon biopsies from patients with either Crohn's disease, bacterial gastrointestinal infection or schistosoma infection revealed multiple extracellular DNA extrusions that were apparently generated by cells expressing ECP, most likely eosinophils. Notably, extracellular ECP colocalized with the DNA. They demonstrated that IL-5- or IFN- γ -primed human eosinophils *in vitro* can rapidly and non-cytolytically catapult mitochondrial but not nuclear DNA to form extracellular DNA nets in response to LPS from Gram-negative bacteria, complement factor C5a or CCL11. Moreover, this response was independent of eosinophil death [29]. Another study has demonstrated that eosinophils respond to thymic stromal lymphopoietin stimulation, a factor produced by epithelial cells at barrier surfaces, in a cell-adhesion- and ROS-dependent manner by releasing mitochondrial DNA and toxic granule proteins, thereby leading to ET formation (fig. 1) [30]. Hence, a mechanism of extracellular DNA release associated with eosinophil death was not originally recognized.

In a recent study, Ueki et al. [10] provided the first evidence that human eosinophils undergo extracellular DNA trap cell death or ETosis. Importantly, this study revealed a process by which eosinophils undergo cytolysis to liberate ETs concurrently with the release of intact, cell-free and secretion-competent granules. Specifically, the authors demonstrated that stimulation with immobilized IgG or IgA23, calcium ionophore (all three stimuli have been previously shown to elicit eosinophil cytolysis), PAF or PMA can elicit ETosis in peripheral human eosinophils in a NADPH-oxidase-dependent manner. Similar to neutrophils, one of the key signaling events required for ET release by eosinophils is the production of ROS and the activation of the NADPH oxidase [10] (fig. 1).

During ETosis, some eosinophil granules are released extracellularly as plasma-membrane-enveloped structures, followed by nuclear disintegration to form intracellular DNA nets. Subsequently, plasma membrane rupture releases both nuclear DNA-derived (histone-positive) nets and free eosinophil granules. ETosis-mediated cytolysis leads to the extracellular liberation of intact eosinophil granules that then bind to the DNA nets [10] (fig. 1). On the contrary, in neutrophils undergoing ETosis, the association between nuclear DNA and granule-contained protein appears to occur intracellularly before

Fig. 1. Eosinophils secrete ETosis-associated nuclear DNA (left panel) or mitochondrial DNA (right panel). During ETosis, some eosinophil granules are released extracellularly as plasma membrane-enveloped structures, followed by nuclear disintegration to form intracellular DNA nets. Subsequently, plasma membrane rupture releases both nuclear DNA-derived (histone-positive) nets and functional secretory eosinophil granules that might function as ‘functional immunoregulatory secretory bombs’. Eosinophils can also rapidly and noncytolytically catapult mitochondrial DNA independent of cell death. Both processes are dependent on ROS and are triggered by different stimuli as indicated. TSLP = Thymic stromal lymphopoietin.

plasma membrane rupture [31, 32]. On the molecular level, chromatin decondensation, a pivotal event during ETosis, has been shown to be accompanied by elastase migration from the primary granules to the nucleus, where this enzyme partially degrades histones [31]. Myeloperoxidase also migrates to the nucleus and assists elastase in chromatin decondensation by a still-unknown, enzymatic-activity-independent mechanism [31, 32]. Hence, diverse granule-derived proteins including elastase and myeloperoxidase are recognized to bind to neutrophil ETs as independent molecules. No evidence has shown that intact neutrophil granules bind to extracellular DNA nets. In contrast, concerning eosinophils, MBP or ECP have been observed to bind to extracellular DNA nets as free proteins, mainly in conditions where mitochondrial DNA was elicited [29, 30]. Ueki et al. [10] have demonstrated that free eosinophil granule proteins (MBP and EPO) do not predominantly bind to DNA nets, but remain within granules bound to them. The molecular binding mechanism of intact eosinophil granules to ETs remains unknown. In vivo, a different study has indicated that eosinophil granule proteins can be detected either in association with eosinophil extracellular traps or in extracellular granules in eosinophilic skin diseases [33].

Human [4, 5] and mouse [34] eosinophil granules, isolated by subcellular fractionation, have been demonstrated to express functional CCR3 chemokine receptors and

to secrete ECP in response to the ligand, CCL11, by utilizing their intragranular signaling and membranotubular network-based secretion systems. Part of the cell-free granules obtained during eosinophil ETosis exhibited secretion competency in response to eosinophil-activating CCL11 and secreted ECP [10], thus confirming that extracellular eosinophil granules might remain functional and capable of secreting stored proteins as ‘cluster bombs’. Curiously, ETosis also results in the release of eosinophil granules bound within plasma membrane. The plasma-membrane-enveloped granules did not exhibit CCL11-induced secretion. Whether the clusters of plasma-membrane-enveloped granules might exhibit delayed secretion competence and their in vivo fate are unknown.

Cluster Bombs Caught in DNA Traps: Roles in Innate Immunity

Secretory cell-free extracellular eosinophil granules function as ‘cluster bombs’, releasing cationic proteins and cytokines differentially in response to diverse stimuli independently of the cell. The findings that intact functional granules remain attached to the DNA nets after eosinophil ETosis might illuminate the regulation of eosinophil function in immunity. Eosinophils mediate the immune response in a number of infectious diseases, in-

cluding parasitic helminth infestation and bacterial, fungal and viral infections [1, 2, 35]. Eosinophils also cause tissue damage in helminth infections and bronchial asthma by releasing granule cationic proteins. More recently, eosinophils have been suggested to play an immunoregulatory role during parasitic infestation, in addition to their classic terminal functions [36–38]. Moreover, eosinophils are resident cells in the gastrointestinal tract (stomach through to the large intestine), thymus, spleen, lymph nodes and mammary glands, where they may contribute to organ immune homeostasis [2].

Fully activated human eosinophils appear to defend the host against large, nonphagocytosable organisms, most notably the multicellular helminthic parasites and fungi. However, defense mechanisms against these organisms may also have a detrimental effect on the host. Therefore, both physiological (e.g. gastrointestinal tract) and pathological (e.g. helminthic infection) eosinophil functions require a scaffold that would allow targeting secreted toxic granules only toward infectious agents and infected sites and not the normal host tissue. Indeed, the ability of eosinophils to secrete mitochondrial DNA [29, 30] or ETosis-associated nuclear DNA [10], thus producing a network that is capable of capturing granule proteins (MBP and ECP), secretory cell-free crystalloid granules and pathogens, illustrates a novel mechanism by which eosinophils trap microbes, prevent their dissemination throughout the organism and create a high local concentration of antimicrobial proteins. Functional cell-free secondary granules are known to differentially secrete preformed cytokines (Th1, Th2 and immunoregulatory cytokines) in response to different stimuli [8, 39], suggesting that, once trapped in DNA nets, granules might function in immunity beyond the innate response, for instance, by secreting their preformed regulatory cytokines such as IL-10 and/or IFN- γ .

Isolated eosinophil granule proteins such as MBP and ECP have potent antimicrobial activities [35, 40, 41]. In addition, isolated mouse eosinophils have been shown to possess antibacterial properties. For instance, IL-5-overexpressing transgenic mice exhibit profound eosinophilia and improved clearance of *Pseudomonas aeruginosa* introduced into the peritoneal cavity [42]. Moreover, cecal ligation and puncture result in intestinal eosinophil infiltration and extracellular DNA deposition in association with protection against microbial sepsis in these mice, but not in wild-type controls [29]. However, the phagocytic activity of eosinophils is low compared to other phagocytic cells [43], suggesting that eosinophil-mediated bacterial killing might occur extracellularly. In this

context, the existence of eosinophil ETs associated with clusters of secreting granules might represent a novel mechanism of eosinophil-mediated innate immune response that may be crucial for maintaining the intestinal barrier function after inflammation-associated epithelial cell damage and to prevent uncontrolled bacterial invasion. Moreover, it is described that eosinophils catapult their mitochondrial DNA in response to thymic stromal lymphopoietin, known to be produced by epithelial cells at barrier surfaces such as the intestine, airways and skin [33]. There is no evidence that intact cell-free granules are extruded from eosinophils during the noncytolytically elicited release of mitochondrial DNA. However, it is possible to speculate that eosinophils, after catapulting their mitochondrial DNA, might undergo the ETosis process. Another possibility is that both processes occur simultaneously or in a stimuli-dependent manner, especially in vivo. However, more studies are needed in order to better clarify this point.

Eosinophils are also known to recognize aspartate protease activity and cysteine protease activity produced by the fungus *Alternaria alternata* [44] and by cockroaches [45], respectively, and to respond by releasing granule proteins and cytokines. Eosinophils may also exhibit a protective role against RNA viruses, such as respiratory syncytial virus. Viral infections enhance mediator release from eosinophils and increase recruitment of eosinophils to the lung after antigenic challenge [46]. However, the presence of eosinophil ETs and the effect of this on fungi and viral infections remain to be investigated.

Pathogen recognition receptors such as complement receptors and Toll-like receptors have been identified in eosinophils. The beneficial function of eosinophils has been primarily attributed to defense against parasitic helminths, as eosinophils are able to mediate complement-dependent cellular toxicity against helminths in vitro [47]. Although eosinophils express receptors for complement fragments (e.g. C5aR) on their membranes, C5aR is not expressed in cell-free granules [3]. Thus, complement activation can activate intact eosinophils, but would not (at least in the case of the C5a fragment) induce secretion in free granules, suggesting that intact eosinophils and free granules may have differential functional roles. Furthermore, cell-free granules express receptors for CCL11 (i.e. CCR3), IFN- γ , cysteinyl leukotrienes and nucleotides [4, 5], all of which are produced or released during cell injury and death.

The role of eosinophil ETs associated with secretory-competent granules can be supported by different in vivo findings. ETs are abundantly found at sites of infection

and in noninfectious models of inflammation. Accordingly, ETs have been reported in inflammatory diseases of the intestine (Crohn's disease and bacterial and schistosoma infections) and in various eosinophilic skin diseases [11, 29, 33, 48]. In one of these articles, the authors have indicated that eosinophil granule proteins might remain within extracellular granules, but can also be detected in association with eosinophil extracellular traps [33]. Interestingly, eosinophils in biopsies from the sinuses and skin of allergic sinusitis and hypereosinophilic syndrome subjects, respectively, exhibit sites of ETosis but not apoptosis or necrosis as well as revealing the liberation of free extracellular granules [10].

Concluding Remarks

Cell-free eosinophil granules function extracellularly as secretion-competent organelles, similar to 'cluster bombs' that selectively release their cationic proteins and cytokines. Recent findings provided evidence that human eosinophils are capable of undergoing an active and cytolytic cell death accompanied by liberation of histone-

bearing nuclear DNA extracellular nets and extrusion of intact cell-free functional granules – a process termed ETosis. Eosinophil ETosis is similar to that in neutrophils, but notably differs in that some functional secretion-competent eosinophil granules are released extracellularly. Whether these granules selectively release eosinophil cationic proteins, inflammatory cytokines or help to immunomodulate the microenvironment by the release of their preformed regulatory cytokines such as IL-10 or IFN- γ is a question for the future. More studies in the field are still needed; however, it is reasonable to speculate that the presence of 'functional immunoregulatory bombs' trapped in DNA nets plays a role that might go beyond the prevention of infection or the delimitation of infected and inflamed areas. The findings might illuminate the mechanisms that underlie the role of eosinophils in immunity.

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