

Eosinophil extracellular DNA traps: molecular mechanisms and potential roles in disease

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Eosinophil extracellular traps (EETs) are part of the innate immune response and are seen in multiple infectious, allergic, and autoimmune eosinophilic diseases. EETs are composed of a meshwork of DNA fibers and eosinophil granule proteins, such as major basic protein (MBP) and eosinophil cationic protein (ECP). Interestingly, the DNA within the EETs appears to have its origin in the mitochondria of eosinophils, which had released most their mitochondrial DNA, but were still viable, exhibiting no evidence of a reduced life span. Multiple eosinophil activation mechanisms are represented, whereby toll-like, cytokine, chemokine, and adhesion receptors can all initiate transmembrane signal transduction processes leading to the formation of EETs. One of the key signaling events required for DNA release is the activation of the NADPH oxidase. Here, we review recent progress made in the understanding the molecular mechanisms involved in DNA and granule protein release, discuss the presence of EETs in disease, speculate on their potential role(s) in pathogenesis, and compare available data on other DNA-releasing cells, particularly neutrophils.

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Introduction

As is true for all hematopoietic cells, eosinophils differentiate from hematopoietic stem cells into a common precursor with the shared properties of both basophils and eosinophils, then entering the eosinophil lineage if in the presence of interleukin 5 (IL-5) [1•]. Other growth factors for eosinophils are granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3. These growth regulators are produced and secreted by stromal cells, but also by T cells, mast cells, and eosinophils. Cell surface receptors for IL-3, IL-5, and GM-CSF are detectable on

immature multipotent eosinophil progenitor cells, eosinophil-committed precursors, as well as mature eosinophils. Among these three cytokines, IL-5 is the most uniquely relevant to the eosinophil lineage and is responsible for selective differentiation to eosinophils.

Eosinophils are typically characterized by a bi-lobed nucleus with highly condensed chromatin. The cytoplasm contains two major types of granules, primary and secondary granules, as well as lipid bodies. Primary granules contain Charcot-Leyden crystal protein and eosinophil peroxidase (EPO). Secondary granules are bi-compartmental (matrix with a crystalline core), containing a number of cationic proteins, including eosinophil major protein (MBP), EPO, eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) that give the eosinophils their characteristic staining properties. MBP is confined to the central crystalline core, whereas the other cationic proteins are present in the matrix compartment.

The normal eosinophil count in the peripheral blood ranges between 50 and $500 \times 10^9/L$ [2•]. Besides being in blood and bone marrow, eosinophils are present in the gastrointestinal tract from the stomach through the large intestine as well as in thymus, spleen, and lymph nodes under physiological conditions. In other tissue and organs, however, eosinophil infiltration is observed only under pathological conditions.

Role of eosinophils in the defense against microorganisms

Eosinophils are associated with a number of infectious diseases, including parasitic helminthes, bacterial, fungal, and viral infections. Numerous studies have documented the presence of Toll-like receptors (TLRs) on the surfaces of eosinophils, suggesting that they can participate in the recognition and killing of pathogens [3].

The beneficial function of eosinophils has been primarily attributed to their ability to defend the host against parasitic helminths. This is based on several lines of evidence, including the ability of eosinophils to mediate antibody- (or complement-) dependent cellular toxicity against helminths *in vitro*. The observation that eosinophil levels increase during helminthic infections and that they aggregate and degranulate in the local vicinity of damaged parasites *in vivo* makes it likely that eosinophils participate in the protective immunity against selected helminths [1•]. Eosinophils may also exhibit a protective

role against RNA viruses, such as respiratory syncytial virus (RSV). Viral infections enhance mediator release from eosinophils and increase recruitment of eosinophils to the lung after antigen challenge [4].

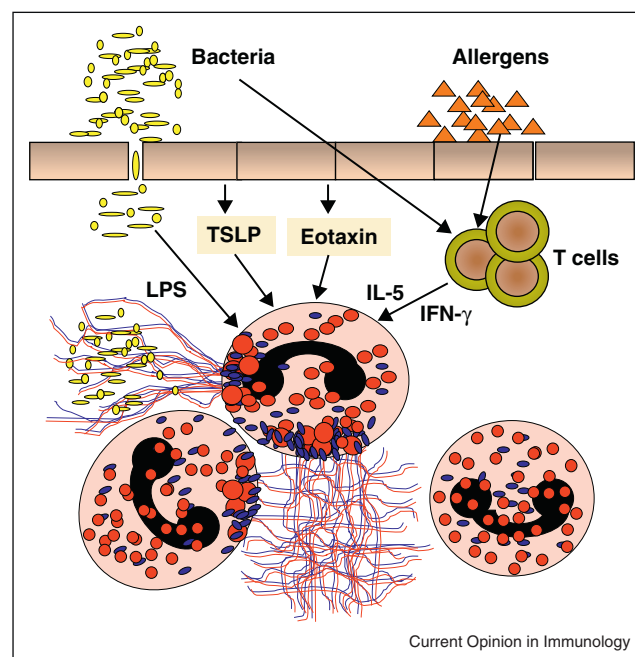
It is well known that eosinophil granules such as MBP, ECP have potent anti-microbial activity [5]. In addition, it was shown that isolated mouse eosinophils possess anti-bacterial properties both *in vitro* and *in vivo*. Interleukin-5 overexpressing transgenic mice, which had profound eosinophilia, demonstrated improved clearance of *Pseudomonas aeruginosa* introduced into the peritoneal cavity. Moreover, the findings of improved bacterial clearance following adoptive transfer of eosinophils, and, on the contrary, impaired bacterial clearance in mice with a congenital eosinophil deficiency established that the anti-bacterial effect was eosinophil-specific [6]. However, the phagocytic activity of eosinophils is largely limited compared with other phagocytic cells [7], suggesting that eosinophil-mediated bacterial killing might occur extracellularly. This assumption is supported by the observation that isolated eosinophil granules exhibit anti-bacterial properties *in vitro* and that administration of eosinophil granule extracts significantly improved bacterial clearance *in vivo* [6].

However, direct application of toxic eosinophil proteins likely causes immunopathology with major risks for organ dysfunction or even organ failure. Therefore, under normal physiological conditions, there should be a scaffold to ensnare the released eosinophil toxic granules, targeting only the infectious agents and not the host tissue. Such a clever defense mechanism by eosinophils should kill infectious agents like a professional killer, efficiently, quickly and quietly. Indeed, such an extracellular mechanism has recently been described whereby eosinophils eject DNA of mitochondrial origin, producing a sticky network that can capture both the granule proteins and bacteria, which are then killed in such extracellular DNA traps [8^{••}]. (Figure 1).

Mechanisms of extracellular DNA trap formation and its importance for anti-bacterial activity

Purified blood eosinophils generate extracellular EETs following cytokine priming (IL-5 or IFN- γ) and subsequent stimulation with lipopolysaccharide (LPS), complement factor 5a (C5a) or eotaxin [8^{••}]. Thymic stromal lymphopoietin (TSLP) stimulation of adherent eosinophils also results in the formation of EETs [9]. Upon formation of EETs, eosinophils remain viable and show no evidence for apoptosis or other types of cell death [8^{••},9]. A key intracellular signaling event appears to be the activation of the NADPH oxidase, since pharmacological inhibition as well as genetic defects of this enzyme are associated with impaired production of reactive oxygen species (ROS), completely abolishing the ability of

Figure 1



Shows a schematic representation of the formation of eosinophil extracellular traps (EETs) in eosinophil-infiltrated tissues. Eosinophils might be directly activated by invading bacteria and/or cytokines generated by epithelial cells and/or T cells. Adhesion molecules also play a role (not indicated here). Autocrine stimulation of eosinophils might also occur (not indicated). Mitochondria (blue) and granules (red) somehow release DNA and cationic proteins that co-localize in the extracellular space, forming the net-like structure called an EET. EETs are able to bind and kill bacteria, but also occur in non-infectious diseases, such as allergies. However, not all infiltrating eosinophils necessarily participate in EET formation.

eosinophils to form EETs [8^{••}]. EETs contain mitochondrial DNA and granule proteins, but no cytosolic, mitochondrial, nuclear or membrane proteins [8^{••}]. The mitochondrial origin of the released DNA was established by a combination of microscopic and molecular biological techniques [8^{••}]. Enzymatic digestion of extracellular DNA results in the inability of eosinophils to kill bacteria in the extracellular space [8^{••}]. Thus, although DNA is unlikely to be toxic for bacteria, it is essential for the anti-bacterial effects mediated by eosinophils.

Similar extracellular DNA traps are formed by neutrophils [10^{••},11[•]], monocytes [12], and mast cells [13]. However, the views regarding the source of DNA and its mechanism of release differ. The current opinion held by most investigators is that the DNA is of nuclear origin and requires cell death. In neutrophils, the type of cell death leading to neutrophil extracellular traps (NETs) has been termed netosis [14]. However, for several reasons, a note of caution should be raised here: First, it has been shown that NETs can be generated by viable neutrophils both *in vitro* [11[•],15] and *in vivo* [16]. Live

microscopy movies of single cells convincingly demonstrated the ejection of DNA [11[•],16]. Second, NET formation occurs much more quickly than apoptosis or any other programmed cell death using stimuli known to delay apoptosis [17]. Third, unfortunately, in most reports, the authors had stimulated neutrophils with PMA, which is known to induce a rapid necrosis-like cell death [18]. The DNA leaking out in the extracellular space of PMA-stimulated neutrophils does not show the typical DNA-containing fibers of extracellular DNA traps [14]. When typical NETs were demonstrated, it could not be excluded that the structures were formed before cell death actually occurred. Fourth, cell death in association with NET formation does not necessarily represent a different and novel type of cell death. Fifth, it is hard to imagine how a dead cell can eject DNA, since energy would seem to be required.

In spite of this controversy regarding the origin and mechanism of DNA release in neutrophils, the antibacterial activity of the extracellular DNA structures has been characterized mainly using NETs, composed of DNA, histones, and multiple antimicrobial components, such as neutrophil elastase (NE), cathepsin G, proteinase 3, as well as defensins, myeloperoxidase (MPO), and Bacterial Permeability-Increasing (BPI) protein [10^{••},19]. The origin of the histones is not clear, but it may indeed originate from cells undergoing cell death in close proximity. Alternatively, histones might be released by an unknown active mechanism [10^{••}]. NETs, like EETs, immobilize at high concentrations granule proteins able to kill invading pathogens [19]. On the other hand, NETs or EETs may serve to avoid diffusion that might lead to unwanted tissue damage and subsequent organ dysfunction. Evidence for the further pathophysiological relevance of extracellular DNA traps is implicit in the observation that extracellular nucleases are present in several pathogenic bacteria, including *S. aureus*, *Clostridium perfringens*, and *S. pyogenes* [20]. Moreover, a *streptococcal* DNase has previously been implicated in disease progression [20]. Also, it should be noted that NETs have been implicated in autoimmunity [21].

Eosinophil extracellular traps in eosinophilic diseases

After the original description of EETs [8^{••}], their involvement has been demonstrated in multiple infectious diseases. For instance, EETs were present in *spirochetosis*, in which the binding of the bacteria by extracellular DNA has been demonstrated *in vivo* [8^{••}]. EETs were also evident in tissues infected with *schistosoma* [8^{••}]. In addition, EETs were present in infectious skin diseases, such as creeping disease and scabies [22]. In an experimental sepsis model, eosinophils contributed a protective effect associated with EETs [8^{••}].

Allergic diseases are often associated with blood and tissue eosinophilia, since EETs can be formed upon

stimulation of eosinophils with IL-5, TSLP, eotaxin, and C5a—all of which are present at allergic inflammatory sites (Figure 1). Therefore, it is not too surprising that EETs were found in bronchial asthma [23] as well as atopic dermatitis, contact dermatitis, and allergic drug reactions [22]. In all these diseases, the three-dimensional structure of EETs was demonstrated by confocal microscopy. In addition, in asthma, it was shown that the DNA is of mitochondrial origin [23]. EETs have so far not been reported in eosinophilic esophagitis, an allergic disease of the esophagus [24].

Interestingly, in bullous pemphigoid, an autoimmune disease with subepidermal blister formation, EETs have also been described, where they lined up along the dermal-epidermal junction in pre-bullous stages [22]. Moreover, in the epidermis covering these areas, the basal layer of keratinocytes showed signs of apoptosis [22]. These findings suggest that EETs might regulate, either positively or negatively, blister formation. This assumption is supported by the observation that large numbers of EETs are present in bullous delayed pressure urticaria [25].

Although EETs have not been reported in hypereosinophilic syndromes (HESs) [26], it is likely that they are formed under such pathological conditions. Whether EETs contribute to immunopathology remains to be shown, but it is likely that they contribute to thrombosis often associated with HESs [27], since NETs have been demonstrated to participate in thrombus formation [28].

Concluding remarks and future prospects

EETs represent a common *in vivo* phenomenon associated with eosinophilic diseases. It has been demonstrated that they exhibit strong antibacterial activities. Their effects on fungi, helminthes, and viruses have not been tested so far. EETs are formed by viable eosinophils and contain mitochondrial DNA and granule proteins. Histones, although not demonstrated so far, may also associate with EETs, most likely due to cell death in close proximity. Whether EETs represent structures that limit immunopathology in the absence of infectious agents remains to be investigated. With the exception of the NADPH oxidase, the intracellular requirements for mitochondrial DNA release and subsequent EET formation are largely unknown. Future research on the subject promises to yield novel biomarkers and therapeutic targets in eosinophilic diseases.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Blanchard C, Rothenberg ME: **Biology of the eosinophil.** *Adv Immunol* 2009, **101**:81-121.
• The authors review the eosinophil biology and their contribution to inflammatory and adaptive immune responses.
2. Simon D, Simon HU: **Eosinophilic disorders.** *J Allergy Clin Immunol* 2007, **119**:1291-1300.
• A simple classification of eosinophilic disorders.
3. Wong CK, Cheung PF, Ip WK, Lam CW: **Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils.** *Am J Respir Cell Mol Biol* 2007, **37**:85-96.
4. Busse WW, Gern JE: **Viruses in asthma.** *J Allergy Clin Immunol* 1997, **100**:147-150.
5. Lehrer RI, Szklarek D, Barton A, Ganz T, Hamann KJ, Gleich GJ: **Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein.** *J Immunol* 1989, **142**:4428-4434.
6. Linch SN, Kelly AM, Danielson ET, Pero R, Lee JJ, Gold JA: **Mouse eosinophils possess potent antibacterial properties in vivo.** *Infect Immun* 2009, **77**:4976-4982.
7. Hatano Y, Taniuchi S, Masuda M, Tsuji S, Ito T, Hasui M, Kobayashi Y, Kaneko K: **Phagocytosis of heat-killed *Staphylococcus aureus* by eosinophils: comparison with neutrophils.** *APMIS* 2009, **117**:115-123.
8. Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E, Schmid I, Straumann A, Reichenbach J, Gleich GJ, Simon HU: **Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense.** *Nat Med* 2008, **14**:949-953.
• First description of mitochondrial DNA release by a cell. The mitochondrial DNA creates an extracellular structure containing eosinophil granule proteins that is able to bind and kill bacteria both *in vitro* and *in vivo*.
9. Morshed M, Yousefi S, Stöckle C, Simon HU, Simon D: **Thymic stromal lymphopoietin stimulates the formation of eosinophil extracellular traps.** *Allergy* 2012, **67**:1127-1137.
10. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: **Neutrophil extracellular traps kill bacteria.** *Science* 2004, **303**:1532-1535.
• Original description of neutrophil extracellular traps
11. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU: **Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps.** *Cell Death Differ* 2009, **16**:1438-1444.
• The results indicate that cell death of neutrophils is not required for neutrophil extracellular trap formation and that the release of mitochondrial DNA by eosinophils is not cell type-specific.
12. Webster SJ, Daigneault M, Bewley MA, Preston JA, Marriott HM, Walmsley SR, Read RC, Whyte MK, Dockrell DH: **Distinct cell death programs in monocytes regulate innate responses following challenge with common causes of invasive bacterial disease.** *J Immunol* 2010, **185**:2968-2979.
13. von Köckritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, Medina E: **Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation.** *Blood* 2008, **111**:3070-3080.
14. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A: **Novel cell death program leads to neutrophil extracellular traps.** *J Cell Biol* 2007, **176**:231-241.
15. Pilszczek FH, Salina D, Poon KKH, Fahey C, Yipp BG, Sibley CD, Robbins SM, Green FHY, Surette MG, Sugai M *et al.*: **A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*.** *J Immunol* 2010, **185**:7413-7425.
16. Clark SR, Ma AC, Tavenor SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD *et al.*: **Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood.** *Nat Med* 2007, **13**:463-469.
17. Geering B, Simon HU: **Peculiarities of cell death mechanisms in neutrophils.** *Cell Death Differ* 2011, **18**:1457-1469.
18. Takei H, Araki A, Watanabe H, Ichinose A, Sendo F: **Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis.** *J Leukoc Biol* 1996, **59**:229-240.
19. Papayannopoulos V, Zychlinsky A: **NETs: a new strategy for using old weapons.** *Trends Immunol* 2009, **30**:513-521.
20. Urban CF, Lourido S, Zychlinsky A: **How do microbes evade neutrophil killing?** *Cell Microbiol* 2006, **8**:1687-1696.
21. Dörner T: **SLE in 2011: deciphering the role of NETs and networks in SLE.** *Nat Rev Rheumatol* 2012, **8**:68-70.
22. Simon D, Hoesli S, Roth N, Staedler S, Yousefi S, Simon HU: **Eosinophil extracellular traps in skin diseases.** *J Allergy Clin Immunol* 2011, **127**:194-199.
23. Dworski R, Simon HU, Hoskins A, Yousefi S: **Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways.** *J Allergy Clin Immunol* 2011, **127**:1260-1266.
24. Straumann A, Aceves SS, Blanchard C, Collins MH, Furuta GT, Hirano I, Schoepfer AM, Simon D, Simon HU: **Pediatric and adult eosinophilic esophagitis: similarities and differences.** *Allergy* 2012, **67**:477-490.
25. Kerstan A, Simon HU, Yousefi S, Leverkus M: **Extensive accumulation of eosinophil extracellular traps in bullous delayed-pressure urticaria: a pathophysiological link?** *Br J Dermatol* 2012, **166**:1151-1152.
26. Simon HU, Rothenberg ME, Bochner BS, Weller PF, Wardlaw AJ, Wechsler ME, Rosenwasser LJ, Roufosse F, Gleich GJ, Klion AD: **Refining the definition of hypereosinophilic syndrome.** *J Allergy Clin Immunol* 2010, **126**:45-49.
27. Simon HU, Klion A: **Therapeutic approaches to patients with hypereosinophilic syndromes.** *Sem Hematol* 2012, **49**:160-170.
28. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD Jr, Wroblewski SK, Wakefield TW, Hartwig JH, Wagner DD: **Extracellular DNA traps promote thrombosis.** *Proc Natl Acad Sci USA* 2010, **107**:15880-15885.