



Eosinophil Extracellular Traps and Inflammatory Pathologies—Untangling the Web!

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Eosinophils are an enigmatic white blood cell, whose immune functions are still under intense investigation. Classically, the eosinophil was considered to fulfill a protective role against parasitic infections, primarily large multicellular helminths. Although eosinophils are predominantly associated with parasite infections, evidence of a role for eosinophils in mediating immunity against bacterial, viral, and fungal infections has been recently reported. Among the mechanisms by which eosinophils are proposed to exert their protective effects is the production of DNA-based extracellular traps (ETs). Remarkably, DNA serves a role that extends beyond its biochemical function in encoding RNA and protein sequences; it is also a highly effective substance for entrapment of bacteria and other extracellular pathogens, and serves as valuable scaffolding for antimicrobial mediators such as granule proteins from immune cells. Extracellular trap formation from eosinophils appears to fulfill an important immune response against extracellular pathogens, although overproduction of traps is evident in pathologies. Here, we discuss the discovery and characterization of eosinophil extracellular traps (EETs) in response to a variety of stimuli, and suggest a role for these structures in the pathogenesis of disease as well as the establishment of autoimmunity in chronic, unresolved inflammation.

Keywords: eosinophils, degranulation, extracellular traps, ETosis, cytolysis, airways, sputum

INTRODUCTION

Eosinophils have intrigued physicians and scientists alike since the time of their first report in 1879. While lower numbers in systemic circulation ($<0.5 \times 10^9/L$) are generally considered healthy (1), increased peripheral numbers and, more importantly, tissue accumulation is associated with disease (2). Essentially, eosinophilia is best known in host responses to helminth infections, followed by the recognized pathological role of eosinophils as end-stage effector cells in allergic diseases such as asthma, atopic dermatitis, rhinitis, eczema, and related conditions. Eosinophils have also been implicated in non-allergic disease pathologies, such as Crohn's disease, chronic obstructive pulmonary disease (COPD), and more recently, non-atopic asthma. The common denominator underlying all these pathologies is the ability of eosinophils to secrete potent immunomodulatory factors stored as pre-formed mediators within their granules (cytokines, chemokines, growth factors), as well as *de novo* synthesized lipid mediators and oxidative metabolites (3, 4). Moreover, the presence of clusters of free intact, membrane-bound eosinophilic granules (FEGs) and cationic granule proteins [major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived

neurotoxin, and eosinophil peroxidase (EPX)] have been identified in inflammatory foci in eosinophilic diseases (5, 6). Indeed, presence of FEGs and cationic granule proteins is a better marker of disease activity than intact eosinophils as reported for asthma (5, 7). Despite their association with diseased states, granule proteins have been naturally (evolutionarily) selected for host defense against viruses, bacteria, fungi, and helminths [reviewed elsewhere in (8)]. The mechanisms of mediator release and tissue dispersal of eosinophil granules have therefore remained a topic of intense research.

EOSINOPHILS: DEGRANULATION, PRIMARY LYSIS, AND GRANULE RELEASE

In response to receptor stimulation, eosinophils typically release their granules (intact) or products of granules through degranulation. Degranulation is an umbrella term used to define processes where there is release of granule proteins from viable cells, or the release of intact/ruptured granules from “dying” cells, without reference to the specific underlying regulatory mechanism (6, 9). Four modes of degranulation have been reported for eosinophils using transmission electron, confocal, and super resolution microscopy studies, namely: (i) *classical exocytosis* (ii) *compound exocytosis* (10); (iii) *piecemeal degranulation* (PMD); and (iv) *cytolysis* [reviewed extensively, (9)].

The fourth physiologic form of degranulation is *cytolysis* which involves chromatolysis (disintegration of the chromatin of cell nuclei), followed by rupture of the cell’s plasma membrane, leading to release of FEGs (11). Research in the past decade has suggested that eosinophil cytolysis leading to the production of FEGs may be a major *modus operandi* of eosinophils *in vivo*, and is not a crush artifact of *in vitro* experimentation [release of cell granules due to mechanical damage to cells or inadequate tissue handling (12)]. Indeed, it is the second most commonly observed eosinophil degranulation mode after PMD in allergic tissues (13), ranging from 10 to 33% of all degranulation modes (13–15). Cytolysis has been observed as a dominant secretory mechanism in other diseases such as eosinophilic esophagitis (16). Cytolysis is also referred to as necrosis, where ruptured eosinophils release FEGs into the surrounding milieu (9). Whether cytolysis is synonymous to necrosis has been debatable ever since the early observations of FEG clusters in diseased inflammatory tissue. Persson and Erjefelt termed the phenomenon as “primary lysis” and concluded such events to be the fate of highly activated eosinophils (12). It is now considered that eosinophil cytolysis includes spilling of cellular contents, including nuclear materials such as histones and DNA, in addition to granular proteins onto the extracellular matrix, and can be from either live or lytic cells (17). The current review article focuses on recent observations of extracellular DNA traps associated with granule release from both live (18) and lytic eosinophils (19), largely

defined as eosinophil “cytolysis,” and their role in host defense and potential contribution to disease pathology.

EVOLUTION OF EXTRACELLULAR TRAPS: ETosis

The defining feature of our immune system is host defense involving recognition and elimination of pathogens that endanger our health. Typically, innate and adaptive immunity work in a continuum for achieving immune homeostasis. In the innate circle, polymorphonuclear neutrophils (PMNs) have been well-studied as the first line of defense against invading pathogens, while eosinophils are classically known for their proliferation and recruitment in response to helminthic parasites. Phagocytosis of microbial pathogens by innate cells (neutrophils and macrophages) followed by intracellular lysosomal degradation is the best-known innate immune mechanism for host defense against these microorganisms.

While anti-microbial mechanisms associated with phagocytosis have been very well-characterized, an unexpected observation was recently made demonstrating that neutrophils were capable of releasing nuclear DNA onto extracellular pathogens to control their growth and proliferation. These extracellular DNA deposits were termed neutrophil extracellular traps (NETs), and were first proposed in 2004 as a phagocytosis-independent anti-microbial pathway (20). Neutrophil extracellular traps (NETs) were found studded with high local concentrations of anti-microbial agents (peptides, proteases, reactive oxygen species), released into extracellular matrix to ensnare or degrade virulence factors and aid in the killing of bacteria (20, 21).

Similar extracellular traps (ETs) have subsequently been observed from other cells of the innate family members, for example, mast cells (22), monocytes (23), tissue macrophages (24), and eosinophils (18, 25–27). Recently, even lymphocytes, key effector cells of the adaptive immune system, were demonstrated to eject ETs *in vitro* when incubated with serum obtained from systemic erythematous lupus (SLE) patients (28). Based on such elegant observations, the release of ETs from immune cells that was associated with rupture of the cell membrane, was considered to be a novel cell death pathway (distinct from necrosis and apoptosis), and referred collectively as ETosis [extensively reviewed in (29)].

ETosis is proposed to be an immune-protective host defense mechanism, particularly at barrier sites, that has been conserved through evolution. Indeed, ET-like structures containing DNA have been discovered in plant root-tips that protect against fungal infections (30). Regulated release of chromatin ETs that ensnares microorganisms has also been demonstrated in several invertebrates (remarkably, an acoelomate), and essentially strengthens the notion that DNA traps are evolutionarily conserved as a defense weapon (31).

Notably, eosinophil-derived ETs have been observed in higher vertebrate forms. For example, both neutrophils and eosinophils undergo ETosis to trap and kill *Haemonchus contorta*, a gastrointestinal nematode in ruminant animals. Being trapped

Abbreviations: ANA, anti-nuclear antibodies; DAMPs, danger associated molecular patterns; EET, eosinophil extracellular traps; ECP, eosinophil cationic protein; EPX, eosinophil peroxidase; IF, immunofluorescence; MBP, major basic protein; SEM, scanning electron microscopy; LPS, lipopolysaccharide.

in ETs, the migration of the larvae to sites of infection is limited (32). In the case of the bovine respiratory pathogen *Manheimia haemolytica*, ETosis from innate immune cells including macrophages was shown to limit the spread of this bacterium (33).

EOSINOPHIL EXTRACELLULAR TRAPS: ARE EOSINOPHILS DEAD OR ALIVE?

Upon appropriate stimulation, eosinophils release intracellular DNA to form web-like ETs, embedded with granular proteins (refer to **Figure 1**). The deposition of extracellular DNA by immune cells is considered to be a double-edged sword, i.e., implicated both as a host defense machinery and in the development of certain pathologies. Compared to NETosis, coined by Steinberg and Grinstead, which is now accepted as a novel cell death pathway (21), the concept of extracellular trap formation with respect to eosinophils is conflicted. At present, there are two schools of thought regarding the formation of eosinophil extracellular traps (EETs) and the fate of cells releasing them (34, 35). In 2008, DNA net-like structures, similar to those reported for neutrophils, were observed for the first time by Yousefi et al. to be catapulted out of “live” eosinophils (18). Interestingly, the DNA present in these EETs was reported to be of *mitochondrial* origin, and DNA strands were embedded with granule proteins such as MBP and ECP, evident from colocalization studies both *in vitro* (18) and *in vivo* (27) (refer to **Figure 1**). Extracellular traps (ET) formation was independent of cellular cytoskeletal remodeling, since there was no inhibitory effect by cytochalasin D on EET release (18). The mitochondrial origin of EETs is yet to be confirmed in other reports (discussed in the next section).

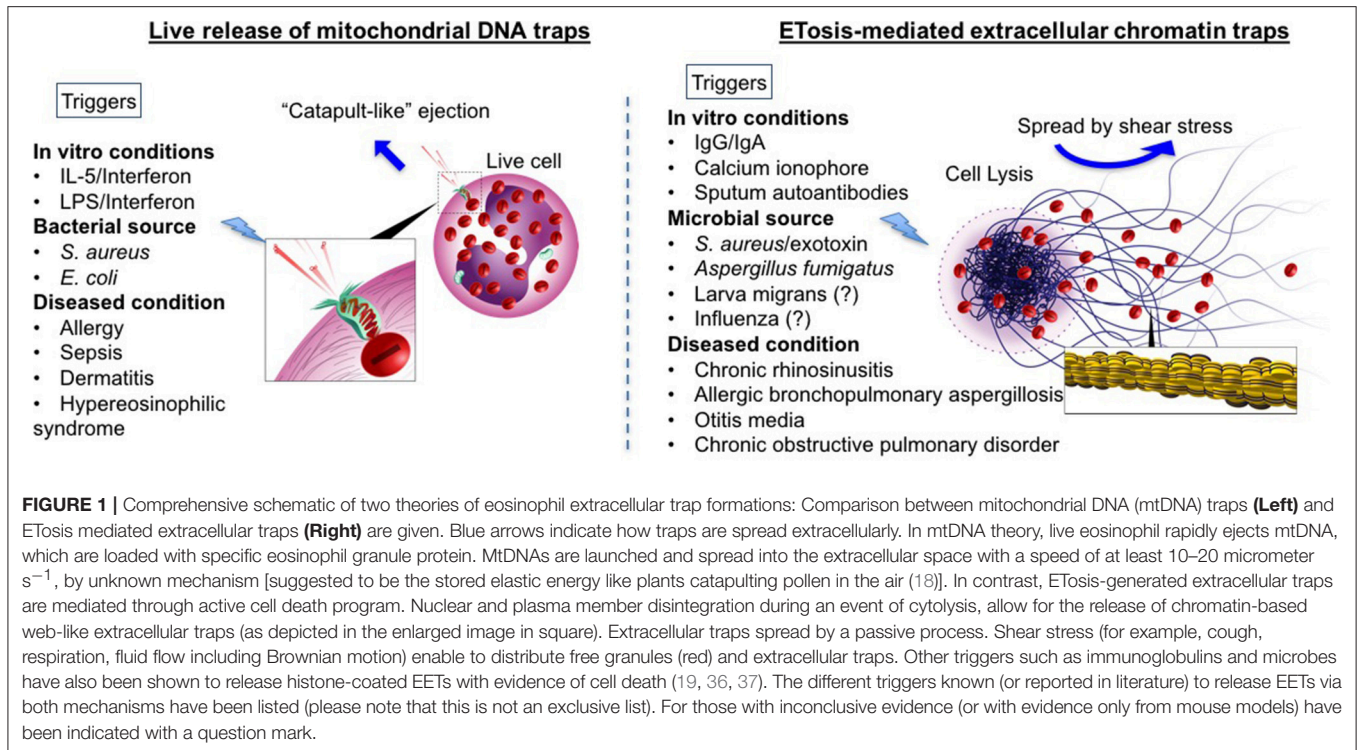
In contrast to the above report, Ueki et al. (19) elegantly demonstrated the release of similar web-like structures from lytic eosinophils upon multiple triggers (demonstrated previously to cause eosinophil cytolysis), such as immobilized IgG, IgA (1 mg/mL, 120 min) (38), and the calcium ionophore A23187 (1 μ M, 60 min) (39) (refer to **Figure 1**). These findings were obtained using transmission electron microscope and confocal (immunofluorescence) microscopy. Immunostaining with anti-histone antibody on non-permeabilized cells post-treatment confirmed that the DNA was *nuclear* in origin (histone-coated). To ascertain the difference between ETotic, apoptotic and necrotic eosinophils, anti-Fas activated eosinophils (undergoing apoptosis with high annexin V staining) and heat-inactivated eosinophils (with characteristic necrotic blebs) were compared with those undergoing ETosis. In ETotic eosinophils, surface annexin V staining was weak (indicative of low/no phosphatidylserine on the outer surface of the cell membrane, and suggesting these are not apoptotic) compared to Fas-activated cells. In addition, necrotic blebs were not observed. The casting of filamentous ETs was preceded by chromatolysis, involving rupture of both nuclear and cell membranes [events noted in primary lysis (11)].

Concurrently with EETs, intact membrane-bound granules were also observed to be released from cells. Since EET formation

occurred in parallel with eosinophil lysis, the term EETosis was coined, and cells were presumed to undergo necrotic death (19, 35). Later, this was disputed, since EET formation does not always accompany cell death (40). Eosinophil extracellular traps (EETs) were shown to be released from live eosinophils as depicted by Yousefi et al. (18) and Gevaert et al. (41), and it is interesting that EETosis was dependent on NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity. In this study, EETs were studded with granule proteins, suggesting degranulation; properties that resemble EETotic eosinophils observed by Ueki et al. (19). It is also perhaps compelling to emphasize that the number of EET⁺ live eosinophils reported by Gevaert et al. (41) in nasal polyps (~8.8% of the total eosinophils) was similar to the percentage of lytic eosinophils with FEG clusters (9.9%) reported by Erjefält and co-workers in nasal tissue (13).

In recent years, there have been reports of EETs accompanied with features of cell lysis. Charcot-Leyden crystals, the classical feature of eosinophil “activity,” were shown to closely associate with EETosis both *in vivo* and *in vitro* (42). EETosis was demonstrated *in vivo* in induced sputum plugs from COPD (chronic obstructive pulmonary disease) patients. Increased eosinophil free granules and cellular debris were found associated with EETs, and MBP was shown to co-localize with observed DNA-based ETs (25). Again, immobilized IgG (50 μ g/mL) and immunoprecipitated immunoglobulins from the sputum of severe eosinophilic asthmatics with increased anti-EPX IgG titers were shown to trigger extensive EETs *in vitro* from isolated peripheral eosinophils within 3–12 h of incubation (36). Sputum immunoglobulins with autoantibodies were more potent than affinity-purified IgGs from pooled healthy donors in inducing extensive histone-coated EETs in a time-dependent manner. Increased EETs over time was accompanied with an increase in extracellular lactose dehydrogenase (LDH) levels, indicative of membrane rupture and cell lysis (36).

According to a recent comment by Rosenberg and Foster (17), it is now realized that all EETs are not created equal, and some may be released from live cells while some emerge from lytic (necrotic) cells. However, the process of EET formation is morphologically and functionally distinct from any forms of programmed cell death. Looking at the current literature and ongoing discussion, it may be prudent to remark that the fate of eosinophils (whether dead or alive) during EET formation may be dependent on the stimuli either *in vitro* or *in vivo*. Specifically, *in vitro* experimental conditions such as priming, the nature of stimuli, the time-span involved, and the experimental technique used for assessment may have very distinct outcomes for EET formation from either live or dead cells. The incidence of live or dead eosinophils may also be dependent on the state of eosinophil activation itself at inflamed tissues/foci. For example, the same stimuli, such as *Staphylococcus aureus*, on isolated eosinophils *in vitro* caused EETs to catapult out of live cells within 15 min or less (37), while longer incubations with bacteria or other stimuli for >60 min allowed EET formation with concomitant FEG release (indicative of primary cytolysis) and reduced numbers of viable intact eosinophils (37). In contrast, LPS stimulation of IL-5/IFN γ -primed eosinophils with time-lapse automated confocal imaging confirmed that EETs from live eosinophils were released



within 1–11 s, with the kinetics of DNA spread estimated at 10–20 $\mu m s^{-1}$ (18). A later independent study demonstrated that EETs were released from activated eosinophils co-cultured with *S. aureus*/exotoxins (43), but the mechanism of EET release was not described.

SOURCE OF EOSINOPHIL EXTRACELLULAR DNA TRAPS: NUCLEAR OR MITOCHONDRIAL?

There are several possible reasons that could explain the discrepancy in findings regarding the source of EETs being either mitochondrial or nuclear in origin. In the former study suggesting a mitochondrial source for EETs (18), the concern is that the ejection of small particles of mitochondrial DNA may be physically challenging due to high fluid resistance (“drag”) within the cytoplasm. The amount of energy required to eject mitochondria through the cytoplasm is predicted to be very high, and is akin to throwing strings from underwater so that they fly up into the air. In contrast, the formation of “DNA clouds” and “DNA traps” as reported elsewhere were typically observed as deriving from the nucleus of cells (19). Perhaps most importantly, human eosinophils contain very few mitochondria (24–36/cell compared with ~1,300/cell for hepatocytes), suggesting that very little mitochondrial DNA can be released (44). Each mitochondrion contains ~2–10 copies of mitochondrial DNA, which is highly susceptible to reactive oxygen species-mediated damage because it lacks protective histones and has limited DNA repair mechanisms. Finally, the lack of nuclear DNA in eosinophil supernatants may be explained

by the highly adhesive properties of these DNA strands, which are likely to clump and adhere to cells and plate surfaces, particularly after shaking during incubation. This would precipitate the majority of nuclear DNA from supernatants while mitochondrial DNA, being smaller in its size, will remain soluble in the supernatant and more readily detected by PCR. In contrast, using sensitive molecular methods, increased release of dsDNA in the supernatant in an event of induced-EETs *in vitro* has been demonstrated (36). As described above, recent findings by Ueki et al. (19) suggest that most EETs consist of histone-bound DNA that is nuclear in its origins (26). Therefore, the ability of mitochondrial DNA to generate extensive and large DNA traps from eosinophils observed *in vitro* and *in vivo* is physically very challenging and conceptually difficult to apply. These discrepancies and subsequent speculations demand further investigation.

EETs AND HOST DEFENSE

As discussed in section Evolution of Extracellular Traps: ETosis, ET formation by innate cells is an evolutionarily conserved mechanism. Extracellular traps (ET) formation and function was first described in neutrophils, and these are well-known for their microbicidal properties both *in vitro* and *in vivo*, including mouse models of sepsis (45). In recent years, compelling evidence has accumulated suggesting similar roles for EETs and their possible role in host defense.

EETs and Parasites

Eosinophils are primarily implicated in helminthic parasite infections. ETosis has been proposed to be a novel extracellular

killing mechanism for pathogens that are too large to allow phagocytosis. There is limited evidence regarding EETs and parasites. Although a recent veterinary report showed that both neutrophils and eosinophils release ETs to trap *H. contorta* larva, a known pathogenic nematode in ruminant animals (32), the role of EETs is doubtful due to the low purity of isolated eosinophils (30%) using Percoll gradients in this study. EET⁺ eosinophils have been reported in the deep dermis of skin biopsies from patients with parasitic infections (larva migrans, ectoparasitosis) (46) however, *in vitro* evidence of parasite-induced EETs has not yet been obtained.

EETs and Bacterial Infection

As mentioned briefly in section Eosinophil Extracellular Traps: Are Eosinophils Dead or Alive?, primed eosinophils were demonstrated to cast EETs in response to a bacterial product trigger (LPS). Yousefi et al. (18) demonstrated that priming of eosinophils with IL-5 and/or IFN γ , or eotaxin, allowed catapult-like EET formation within 1–15 s on subsequent bacterial stimulation by *E. coli* (18) or *S. aureus* (41). Further, they demonstrated that activated eosinophils cultured with opsonized *E. coli* could kill approximately 90% of inoculated bacteria within 45 min by a phagocytosis-independent mechanism (18). The authors also demonstrated that in a model of post-caecal ligation and puncture, IL-5-transgenic and not wild-type mice showed demonstrable intestinal eosinophil infiltration and extracellular DNA deposition (indicative of EETs), which allowed protection against microbial sepsis (18). Prince et al. later demonstrated that EET release by lytic eosinophils, when co-cultured with *S. aureus*, was mediated by bacterial virulence factors (37). The first evidence of EETs embedded with eosinophilic granule proteins (ECP and MBP) *in vivo* was obtained in bacteria-infected caecum (*Spirochetosis*) (18). Caecal tissues in this model were infiltrated by EET⁺ eosinophils, rather than neutrophils (confirmed by the absence of a CD16⁺ population). It has also been demonstrated that EETs provide a larger adhesive surface that allows for encapsulation of fungi and bacteria (26).

EETs and Fungal Infection

Eosinophils are prominent cells in allergic bronchopulmonary mycosis and fungal-associated asthma (47), but their molecular interactions and consequent immunopathological roles in fungal infections are yet to be conclusively defined. The first evidence of EETs forming in fungal disease was reported in a recent clinical case (48), where a patient diagnosed with ABPA (allergic bronchopulmonary aspergillosis) showed CT evidence of bronchiectasis and mucus plugging. Bronchial secretions from this patient exhibited an intense accumulation of eosinophils in the mucus and chromatolytic nuclei aggregated to form filamentous structures that co-stained with anti-human histone 1 antibody and Hoechst 33342 DNA stain. Clusters of free eosinophil granules attached to DNA traps were also detected (48), in agreement with their previous report that EETosis releases intact membrane-bound free eosinophil granules (19). In addition, using scanning electron microscope, the same group demonstrated the capture of *Candida albicans* by EETs *in vitro* (19). Recent evidence from Muniz et al. (49) revealed that

Aspergillus fumigatus can induce EETs from isolated eosinophils *in vitro* in a ROS-independent manner, but occurred via CD11b binding (a receptor for fungal antigens) and activation of the Syk tyrosine kinase pathway (49). However, EETs were incapable of fungicidal activity. This is similar to findings relating to ETs released from neutrophils in response to *A. fumigatus*, where two independent studies failed to show any NET-related fungicidal activity (50), while there was some evidence that NETs could inhibit germination of fungal spores (51).

EETs and Virus Infections

The role of eosinophil-induced ETs in limiting virus infections still remains to be elucidated. Interestingly, extracellular histones coated on EETs have recently been implicated in influenza-induced lung pathogenesis. Ashar et al. demonstrated a high accumulation of extracellular histones in mice infected with influenza virus that was associated with widespread pulmonary microvascular thrombosis (52). Increased accumulation of extracellular histones was also evident in nasal lavage from influenza-infected patients; however, this study did not investigate whether histones were generated due to EET formation. Neutrophil extracellular traps (NETs) were also observed to be induced by human respiratory syncytial virus *in vitro* (53), but there is no evidence of virus-induced EETs despite evidence of an anti-viral role for eosinophils (54). In fact, it is interesting that eosinophil recruitment to the lungs in response to *A. fumigatus* in a mouse model leads to active eosinophil degranulation and consequent protection from lethal respiratory virus infection (55).

EXTRACELLULAR TRAPS IN DISEASE PATHOLOGY

Despite their demonstrated and important role in host defense, ETs have also been associated with host damage, appearing in association with several pathologies including sepsis, diabetes, and autoimmunity (56). Most of our current knowledge about ETs is based on investigations of neutrophils and the phenomenon of NETosis [reviewed extensively (56)]. Neutrophil extracellular traps (NETs) are studded with self-antigens such as histones, dsDNA, and myeloperoxidase, all of which are implicated in a host of autoimmune diseases, and their role in autoimmune pathogenesis is well-defined (56–59). The association of free FEGs and eosinophil granule proteins with disease severity (5, 7, 15, 60), coupled with recent *ex vivo* evidence of EETs in diseased tissues (tabulated chronologically in **Table 1**) have led researchers to investigate and speculate possible contribution of EETs to disease etiology, sustenance, and progression in continuing studies of cell function, animal models, and clinical cases.

Delayed Resolution of Inflammation, Inefficient Clearance, and Autoimmunity

The presence of EETs, as shown in **Table 1**, has been reported in several inflammatory tissues obtained by biopsy—from bronchial, nasal, skin, esophageal, and intestinal sources, as well

TABLE 1 | *In vivo* evidence of eosinophil extracellular traps in human disease.

Year	Disease	Sample	Methodology used	References
2008	Crohn's disease	Paraffin-embedded intestinal sections	Confocal laser scanning microscope: anti-MBP polyclonal antibody, Sytox orange, and Mito tracker	(18)
2011	Asthma and allergic airways	Endobronchial biopsies	IF with propidium iodide and anti-MBP antibody	(27)
2011	Atopic dermatitis, allergic contact dermatitis, urticaria, bullous pemphigoid, hypereosinophilic syndrome, dermatitis herpetiformis	Skin biopsies	Confocal laser scanning microscopy with propidium iodide and anti-human ECP antibody	(46)
2013	Allergic sinusitis and hypereosinophilic syndrome	Tissue biopsies	Transmission electron microscopy	(19)
2015	Eosinophilic esophagitis	Esophageal biopsy	IF with propidium iodide and anti-human EPX antibody	(61)
2015	Eosinophilic cellulitis or Well's syndrome	Skin biopsies (flame figures)	IF with propidium iodide and anti-histone H2 antibody	(62)
2016	Eosinophilic otitis media and Chronic rhinosinusitis	Sinus and ear exudative secretions	Confocal IF with anti-human histone H1 mAb and Hoeschst 33342 DNA staining; SYTOX green	(26)
2017	Chronic obstructive pulmonary disease	Induced sputum plugs	DAPI staining colocalized with anti-MBP antibody and electron microscopy	(25)
2018	Eosinophilic otitis media (case studies)	Bronchial lavage fluid, bronchial secretions and mucus	Confocal IF with anti-human histone H1 mAb and Hoeschst 33342 DNA staining	(48) (63)
2018	Allergic bronchopulmonary aspergillosis	Bronchoscopic mucus plugs	Confocal IF with anti-citrullinated histone H3 mAb and Hoeschst 33342 DNA staining and SEM	(49)

NB, only *in vivo*/tissue evidence. *in vitro* evidence is not given.

as sinus and airway secretions. Against the backdrop of host defense as discussed in the previous section, a plausible outcome of EETs in inflammatory pathology may involve exaggerated responses to microbial stimuli coupled with a reduced capacity in clearance of EET products, such as histones, dsDNA, and peroxidases. Eosinophil extracellular trap (EET) products are all known DAMPs (damage-associated molecular patterns) capable of activating both the innate and adaptive immune systems. Damage-associated molecular patterns (DAMPs) are capable of activating plasmacytoid dendritic cells that present these as self-antigens to cognate lymphocytes, triggering self/autoreactivity (64). For instance, EPX was shown to activate and mobilize dendritic cells to lymph nodes (65). Furthermore, inefficient degradation of DNA-EPX EET products or clearance of EPX released upon ETosis could allow accumulation and subsequent activation of the adaptive system to trigger autoimmune responses. The presence of EETs in tissues with pre-existing autoimmune responses could aggregate immune complexes between autoantibodies and their cognate antigens embedded on ETs. Clearance of such immune complexes by macrophages and dendritic cells could then potentially lead to proinflammatory cytokine secretion, which sustains inflammation (64), interferes with clearance, and prevents resolution, thereby contributing to a vicious cycle of inflammation.

An increased presence of EPX, and FEGs in the airways (sputa) of severe eosinophilic asthmatics were found to be positive predictors of sputum autoantibodies (anti-EPX IgG and anti-nuclear antibodies including anti-dsDNA, anti-histones

(36). Eosinophil extracellular traps (EETs) staining positive for DNA and MBP has been observed in bronchial biopsies obtained from allergic asthmatics that further correlated with infiltrating eosinophils (27). Peripheral blood eosinophils isolated from severe eosinophilic asthmatics compared to non-severe demonstrated higher potential of EET formations *in vitro* when stimulated with LPS and IL-5. The percentage of EET⁺ eosinophils negatively correlated with the lung function. Eosinophil extracellular traps (EETs) were shown to have autocrine effect on inducing further eosinophil degranulation and demonstrated a capability of activating epithelial cells to release pro-inflammatory cytokines (IL-6 and IL-8) (66). In a separate study, EETs were demonstrated to be released on stimulation with thymic stromal lymphopoietin (TSLP), a known Th2 alarmin (61). Thus, in a Th2 environment, EETs could be a potential source of self-antigens. Interestingly, *in vitro* experiments revealed that pharmacologically relevant concentrations of dexamethasone were incapable of reducing EET formation from autoantibody-activated eosinophils (36), hinting at a mechanism for a sustained inflammatory environment in steroid-resistant asthmatics. Furthermore, because of this sustained inflammatory environment, a delayed resolution of EET-induced inflammation may potentially trigger self-reactivity and initiate the production of autoantibodies against ET products such as DNA, histones, and granule proteins, as recently reported in severe asthmatic airways (36).

The concomitant presence of EETs and NETs in COPD sputum was reported recently (25). The authors concluded

that EETs may contribute to the severity of COPD. The accumulation of EET-related debris and subsequent phagocytosis by neutrophils may also serve to activate and trigger NETosis, an event that was extensively evident in the high exacerbation severe COPD group (25). It is important to note that in an earlier study, sputum derived from severe COPD patients had detectable anti-nuclear antibodies (ANAs), and an autoimmune pathology for COPD has been proposed (67). However, a direct role of EETs or NETs in the pathogenesis of COPD and any possible underlying autoinflammation has not yet been confirmed. Anti-nuclear antibodies (ANAs) were also reported in nasal exudative secretions from patients with sinus disease and Samster's triad (68), along with evidence of extensive histone-coated EETs in the nasal tissue (26).

Autoantibodies are potent triggers of EETs *in vitro* (36). Sputum with detectable titers of anti-neutrophil cytoplasmic antibody (ANCA), derived from patients diagnosed with eosinophilic granulomatosis and polyangiitis triggered extensive NETs and EETs *in vitro* (69). NETosis and pathogenesis of ANCA is well-known (58), but an avenue has now been opened to decipher the role of EETs in vasculitis and driving pulmonary complications in patients with eosinophilic granulomatosis and polyangiitis. EETs have also been demonstrated in skin biopsies from systemic autoimmune disorders such as Wegner's granulomatosis and bullous pemphigoid (46), as well as intestinal tissue from autoimmune disease such as Crohn's (18) (Table 1).

Heightened EET Response in Infection and Associated Inflammatory Pathology

Airway epithelial damage by exaggerated EET anti-bacterial response and collateral tissue damage could possibly explain epithelial (barrier) dysfunction and inflammatory pathology in diseases such as eosinophilic chronic rhinosinusitis (CRS) (70), atopic dermatitis (71), and asthma (72). With respect to intestinal inflammatory diseases, Yousefi et al. (18) demonstrated that increased numbers of eosinophils infiltrated the intestinal lining in *IL-5* transgenic mice after caecal ligation and puncture compared to wild type animals. Increased tissue eosinophilia was associated with an increased level of detectable EETs deposited in the tissue, possibly as a consequence of an exaggerated response to intestinal bacteria. Moreover, the authors showed the presence of EETs in inflamed intestinal tissue from patients with Crohn's disease (18). Again, within eosinophilic asthmatics, there is a subset of patients who suffer from recurrent infective bronchitis (with mixed granulocytic sputum) and low lung function that exhibit autoantibodies in their sputum samples (73). Interestingly, ANAs and anti-EPX IgGs in the sputa of these patients also has the ability to induce EETs *in vitro* (36). It is possible that in addition to NETs, EETs in response to bacterial infection (discussed earlier in section EETs and Bacterial Infection) could be associated with a breakdown of local tolerance as well as a source of potential self-antigens.

Mucus Plugging and Sticky Secretions

Neutrophil extracellular traps (NETs) have been known to contribute to the viscosity of airway secretions in cystic fibrosis

patients (74), and DNase treatment was shown to have clinically relevant improvement (75). Dornase alfa [recombinant human deoxyribonuclease I (rhDNase)] is used therapeutically to cleave the DNA in mucus plugs which improves mucociliary clearance, and leads to improved pulmonary function. Recent evidence suggests that EETs add substantially to the viscosity of eosinophil-rich exudates of patients with CRS, eosinophilic otitis media and ABPA [reviewed in (35)]. Muniz et al. (49) and Ueki et al. (19) demonstrated the presence of abundant nuclear histone-bearing EETs in mucus secretions obtained from the airways of ABPA patients and nasal secretions of CRS patients, respectively. Compared to NETs, EETs assembled to form more stable aggregates that entrapped fungi and bacteria through hydrophobic interactions, demonstrated *ex vivo* in nasal exudates from CRS patients. When compared to NETs, the EETs exhibited thicker fibers coated by higher number of histone molecules and were less susceptible to proteolytic degradation (19).

Cunha et al. showed that ovalbumin sensitization and challenge in mice led to the accumulation of EET⁺ EPX⁺ live eosinophils in lung tissues and induced increased extracellular DNA in bronchoalveolar lavage (76). In subsequent years, the same group showed that recombinant DNase treatment significantly decreased airway resistance, concomitantly with goblet cell hyperplasia and reduced EET formation (77). Eosinophil extracellular trap (EET) secretions also increased mucin/airway secretions in these mice post-allergen challenge. Recent observations of Dunican et al. (78) showed that mucus plugs in asthmatics correlated with increased airway eosinophilia and EPX content, which occurred in 58% of asthmatics compared to 4.5% of healthy volunteers, and was also associated with reduced lung function. It may be speculated that in asthmatics with increased eosinophilic activity (indicated by increased EPX), there may be a loss of immune tolerance, increasing presence of local autoantibodies, and generation of EETs that contribute to goblet cell hyperplasia and IL-13-related mucin overproduction, both of which lead to the production of highly viscous airway-blocking mucus plugs that are unique to eosinophilic disorders for their exceptionally thick peanut butter-like consistency.

CONCLUSIONS

We now have incontrovertible evidence that EETs form in response to many different stimuli deriving from microbes and their products, as well as non-infectious stimuli such as DAMPs, complement proteins, and immunoglobulins. These traps are important in protection of the host in response to invasive pathogens such as bacteria, and potentially, viruses, fungi, and parasites. However, in the case of chronic, unresolved inflammation, it is apparent that EETs can cause health complications by promoting the production of highly viscous mucus secretions, and potentially setting the stage for the development of autoantibodies. The localized presence of autoantibodies in the lungs (79) is proposed to further exacerbate EET formation, setting up a vicious cycle of inflammation that is not readily ameliorated by glucocorticosteroid treatment.

Further studies are anticipated in this area to understand more about how EETs may be prevented in patients with severe inflammatory conditions including steroid-dependent asthma and sinus disease.

AUTHOR CONTRIBUTIONS

MM prepared the first draft. SU and PL contributed to the development of the manuscript. All authors have read and agreed to the submitted manuscript.

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