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Review

Extracellular traps and macrophages: new roles for the versatile phagocyte

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ABSTRACT

MΦ are multipurpose phagocytes with a large repertoire of well-characterized abilities and functions, including regulation of inflammation, wound healing, maintenance of tissue homeostasis, as well as serving as an integral component of the innate-immune defense against microbial pathogens. Working along with neutrophils and dendritic cells, the other myeloid-derived professional phagocytes, MΦ are one of the key effector cells initiating and directing the host reaction to pathogenic organisms and resolving subsequent responses once the threat has been cleared. ETs are a relatively novel strategy of host defense involving expulsion of nuclear material and embedded proteins from immune cells to immobilize and kill bacteria, fungi, and viruses. As research on ETs expands, it has begun to encompass many immune cell types in unexpected ways, including various types of MΦ, which are not only capable of generating METs in response to various stimuli, but recent preclinical data suggest that they are an important agent in clearing ETs and limiting ET-mediated inflammation and tissue damage. This review aims to summarize historical and recent findings of biologic research regarding ET formation and function and discuss the role of MΦ in ET physiology and associated pathologies. *J. Leukoc. Biol.* 97: 1023–1035; 2015.

Introduction

ETs are web-like structures composed of dsDNA, histones, antimicrobial peptides, and proteases, ejected by immune cells to ensnare microbes in a sticky matrix of extracellular chromatin and microbicidal proteins. They were first described in detail in neutrophils in 2004 and named as NETs [1]. The cytotoxic histone and granule-derived factor composition of ETs provides direct antimicrobial actions, while posing as a physical barrier, making microbes more vulnerable to phagocytosis and clearance by other immune cells (reviewed in refs. [2, 3]). Subsequent studies established that the formation of ETs by immune cells, dubbed "ETosis," is a process that is morphologically and functionally distinct from other forms of programmed cell death and necrosis (reviewed in refs. [4–6]). Some reports indicate that neutrophils may even retain their viability, as well as chemotactic and phagocytotic functions, even when deprived of their nuclei while undergoing NETosis (although they exhibit abnormal morphology and erratic crawling behavior) [7]. Hence, ETosis may serve as a first and last line of defense against microbes, with different manifestations, depending on the stimulus and local tissue environment. However, certain microbes have developed defenses against ETs, as various pathogenic species, including several *Streptococcus* species, *Staphylococcus aureus*, and *Vibrio cholera,* produce endonucleases to facilitate escape from ETs [8–12]. A recent paper demonstrated that *S. aureus* can also convert NET-derived exDNA into deoxyadenosine, a toxic metabolite that promotes caspase-3-mediated apoptosis of immune cells, thereby promoting persistent infection by excluding MΦ from the site of abscess formation [13].

Outside of the original observation of ET antimicrobial activity, recent studies have described an assortment of ET-associated immunologic processes and pathologies. ET formation plays a protective function during sepsis by ensnaring and neutralizing bacteria in the blood [14–16], and deficient ETosis may explain, in part, the susceptibility of neonates to infection and bacteremia [17]. Moreover, exDNA and histones are potential mediators of the immune response to trauma and related complications [18–21], and may limit gouty inflammation by promoting the degradation of sequestered inflammatory cytokines [22–26]. Despite the apparent protective abilities of ETs, they also contribute to many pathologic processes. For example, ETs play a role in the pathogenesis of certain vascular physiology disorders, including pre-eclampsia [27] and deep-vein thrombosis [28, 29]. The implications of ETs in disease resulted in hundreds of research manuscripts published in the last decade

Abbreviations: DAMP = damage-associated molecular pattern molecule, ESX-1 = 6 kDa early secretory antigenic target secretion system 1, ET = extracellular trap, exDNA = extracellular DNA, Hsp72 = heat shock protein 72, MΦ = macrophage(s), MET = macrophage extracellular trap, MPO = myeloperoxidase, NET = neutrophil extracellular trap, NLRP3 = nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3, PAD = peptidylarginine deiminase, PMN = polymorphonuclear leukocyte, ROS = reactive oxygen species, SLE = systemic lupus erythematosus

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exploring wide-ranging ET phenomena from the actions of exDNA in allergic asthma [30, 31] or potential consequences of medical therapy, such as transfusion-related lung injury [32], to the possible role of NETs in sequestering tumor cells, an unexpected ramification of ET physiology that may promote cancer-associated thrombosis and metastasis [33–35].

ETs can also trigger maladaptive immune responses by releasing self-antigens that elicit autoimmune reactions and antibody production (reviewed in refs. [36–38]). In SLE, the uptake of ET components, cathelicidin-derived antimicrobial peptide LL-37, IL-17, and citrullinated histones, complexed with self-DNA, stimulates APCs via TLR9 to produce type I IFNs [39, 40]. Failure to degrade or remove ET-derived factors can lead to B cell activation and the generation of autoantibodies against common SLE targets (such as α-LL-37 and α-dsDNA IgG) [41, 42]. Other autoimmune diseases of ET-mediated pathogenesis include psoriasis [43, 44], lupus nephritis [42], autoimmune vasculitis [45, 46], type I diabetes [47], and rheumatoid arthritis [48]. MΦ likely play an important but indirect role in this process by clearing ETs and associated debris, preventing further tissue damage and complications arising from the continued presence of potentially damaging factors.

Although much of the early research on ETs primarily addressed neutrophil function in the context of microbial infection, emerging evidence suggests a wide-ranging and dynamic role for ETs of the MΦ lineage in health and disease. We focus this review on the primary roles of MΦ in ET physiology (illustrated in **Fig. 1**): the ETs produced by MΦ in response to pathogens and sterile inflammation and the role of MΦ in clearing ETs from tissue and regulating the local immune response.

EUKARYOTIC IMMUNE CELLS RELEASE ETS IN RESPONSE TO A WIDE RANGE OF MICROBES AND STIMULI

A variety of microorganisms are capable of stimulating immune cells to produce ETs, as listed in **Table 1**, along with isolated microbial byproducts, such as LPS [1] and bacterially derived toxins [49–51]. Nonmicrobial agents known to trigger ETosis in neutrophils are NO [81], PMA [1], ionomycin [55], and glucose oxidase [4], many of which directly form ROS or aid in their production. Furthermore, a wide range of inflammatory cytokines and chemokines can promote the production of NETs, including TNF-α [48, 82], IL-8 [1, 27, 83], IL-23 [43], and IL-1β

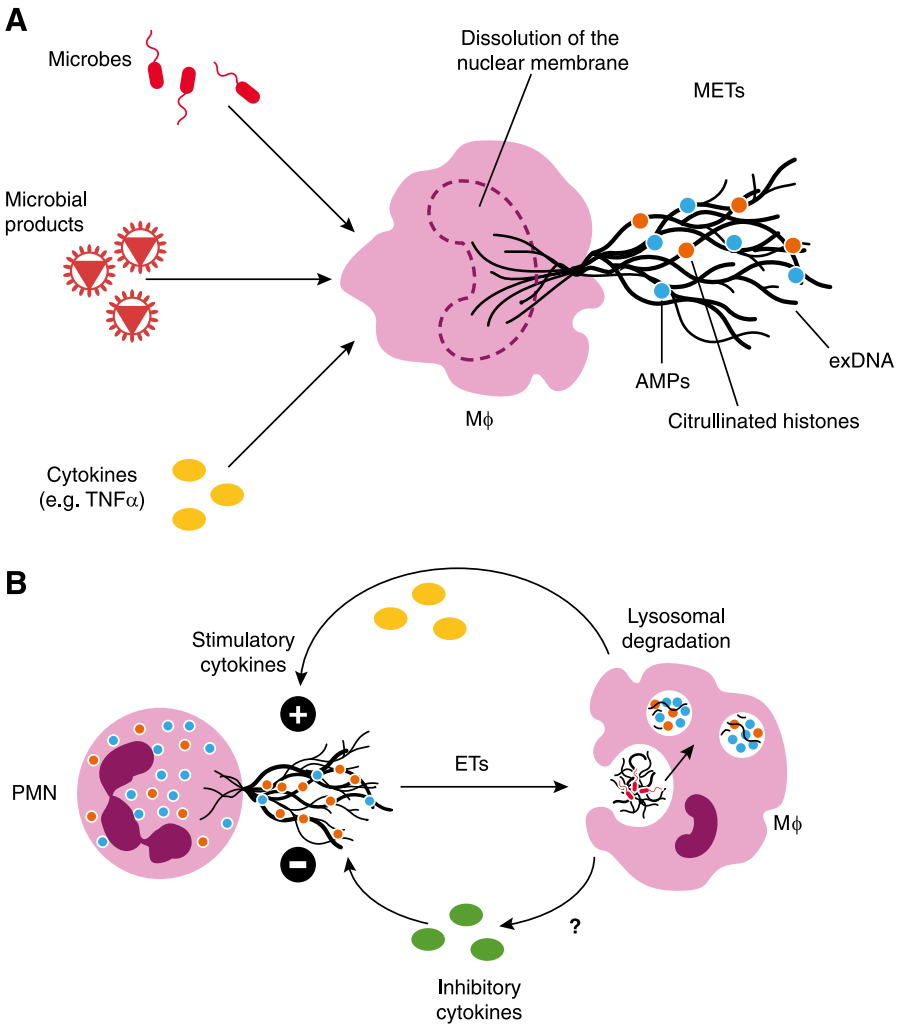

Figure 1. Interactions between MΦ and ETs. (A) A variety of stimuli can induce ETosis in MΦ, marked by dissolution of the nuclear envelope and ejection of METs into the extracellular space. (B) MΦ regulate the local tissue environment by secreting signaling molecules that can influence the activation states of other cells, such as neutrophils, and their ET formation. Subsequent phagocytosis of ETs by MΦ is an active endocytic process that results in intracellular degradation of ETs within lysosomes and may promote a proinflammatory response.

TABLE 1. Microbial inducers of NETs

Type	Species	References
Bacteria		
Gram-positive	*S. aureus*	[4]
		[10]
Gram-negative	*Streptococcus pyogenes*	[52]
		[11]
		[53]
	Streptococcus pneumoniae	[8]
		[54]
	Streptococcus sanguinis	[12]
	Streptococcus dysgalactiae	[55]
	Enterococcus faecalis	[55]
	Escherichia coli	[56]
	Shigella flexneri	[1]
	Salmonella typhimurium	[1]
	V. cholera	[9]
	Pseudomonas aeruginosa	[57]
	Klebsiella pneumoniae	[58]
	Haemophilus influenzae	[59]
	Listeria monocytogenes	[60]
	Mannheimia haemolytica	[51]
	Histophilus somni	[61]
	Helicobacter pylori	[62]
	Mycobacterium spp.	[63]
Fungi	*Candida* spp.	[64]
		[58]
	Aspergillus spp.	[65]
		[66]
	Cryptococcus neoformans	[67]
Parasites	*Plasmodium falciparum*	[68]
	Toxoplasma gondii	[69]
	Strongyloides stercoralis	[70]
	Eimeria spp.	[71]
		[72]
	Leishmania spp.	[73]
		[74]
Viruses	Influenza	[75]
		[76]
	HIV-1	[77]
	Feline leukemia virus	[78]
	Hantavirus	[79]
	Poxvirus	[80]

[43, 83]. Platelet-activating factor and activated platelets can also stimulate ETosis in neutrophils via TLR4 under certain circumstances [14, 32]. The extent of NET formation is greatly enhanced after priming with certain immunomodulatory molecules, resulting in noticeably more ETosis by granulocytes stimulated by LPS or complement component 5a (an anaphylatoxin generated during complement activation) and pretreated with GM-CSF, IL-5, or IFN-α and IFN-γ [84–86].

Early studies focused on NETs in human, rabbit, and mouse models [1, 4, 60], but more recently, ET research has grown to encompass a set of immune responses to various pathogens used across the entire eukaryotic domain. ET-like structures have also been documented in other vertebrate species, including cows [51, 55, 56], horses [87, 88], goats [72], cats [78], birds (chicken heterophils) [89], and fish [90, 91]. The phenomena is not just limited to vertebrates, as innate immune cells from a range of invertebrate species, such as insects [92], crustaceans (crab hemocytes) [93], and mollusks (mussel and oyster hemocytes) [93, 94] can produce similar products composed of extracellular chromatin, and even sea anemone cells can eject DNase-sensitive nuclear material in a similar fashion [93]. One of the most surprising findings of exDNA as a defense mechanism is the antifungal properties of extracellular chromatin secreted by root-defense cells in plants [95, 96]. Such results support the assertion that extracellular chromatin and exDNA-derived ETs are an evolutionarily conserved mechanism of host defense, further strengthened by the diversity of agents that can bring about the formation of ETs [97].

Although confined, so far, to cells of the hematopoietic lineage, ET formation has been documented in many human and murine immune cells, including eosinophils [84], basophils [24, 98], mast cells [43, 99], monocytes [100, 101], and MΦ of various subtypes (as described herein). These structures share the common components identified in NETs but exhibit unique features that can be distinguished based on properties, such as dependence on ROS production, origin of DNA backbone (nuclear or mitochondrial), and the stimuli that prime and activate the cells to undergo ETosis (reviewed in ref. [102]). Although many of the functions and components are the same, much more information is needed before the different types of ETs can be clearly delineated and characterized. New research suggests that MΦ are key players, involved directly and indirectly in the biology of ETs [102]. As MΦ are a multifunctional cell type with many abilities, including tissue repair, organ homeostasis, immune defense, and more (extensively reviewed in refs. [103–108]), it is not surprising that they are being included in the expanding ranks of leukocytes involved in this recently discovered phenomenon.

ETOSIS IS A COMPLEX, INTRACELLULAR PROCESS YET TO BE DEFINED CLEARLY

Although the molecular machinery and signaling pathways driving ET formation are beyond the scope of this review, there are several established aspects of ETosis that highlight their unique nature and function (reviewed in refs. [6, 109]), mostly examined in experiments by use of neutrophils as the model cell type for examination of intracellular mechanisms. Many stimuli are capable of triggering ET formation, confounding the search for a specific set of receptors and cellular sensors. Upon stimulation, opposition among the various isoforms of protein kinase C creates an intricate balance of signals controlling the downstream intracellular processes that initiate ETosis [110, 111]. In neutrophils, during the early stage of ETosis, the nuclear membrane disintegrates, granule contents are released into the cytoplasm, and the azurophilic granule proteases, neutrophil elastase and MPO, translocate to the nucleus, where they participate in degradation of histones, ultimately resulting in chromatin decondensation [58]. Concurrently, the N-terminal tails of histones 3 and 4 are post-translationally modified via the conversion of arginine residues to citrulline, a process catalyzed by a family of enzymes known as PADs (also known as protein arginine deiminases) [112]. Hypercitrullination facilitates local chromatin relaxation and allows the unfolding of nuclear

material necessary for generating ETs, and the PAD enzymes (particularly, PAD2 and PAD4, the isoforms expressed in leukocytes) are essential for the process in vivo and in vitro [113, 114].

One of the most well-recognized aspects of NET formation is the involvement of the oxidative burst and ROS production, and NADPH oxidase and MPO play a key role in regulating and generating these products [81, 115–119], as illustrated by findings that neutrophils isolated from NADPH oxidase-deficient human chronic granulomatous disease patients have impaired NET-forming capabilities [4]. The ETosis process is delayed by enzymatic inhibitors and in patients with genetic deficiencies in MPO activity, although minimal MPO function is still able to drive NET formation [117, 120]. The small GTPase Ras-related protein Rab27a, an important component of the azurophilic granule secretion system, is also involved in the process of ROS-dependent NETosis and activation of associated enzymes [121], along with the kinase mammalian target of rapamycin, via its regulation of the transcription factor hypoxia-inducible factor 1 α and autophagy pathways [122, 123]. Ultimately, the combination of decondensed nuclear material and protein cargo is ejected rapidly from the cell, creating the characteristic latticed web-like structure of ETs.

Evidence suggests that not all ETs are created equal, and immune cells can exhibit variations in ET formation and composition. For instance, mitochondrial DNA, alongside or in place of nuclear-derived chromatin, can serve as the structural backbone of ETs produced by eosinophils and neutrophils, without limiting the lifespan of the cells [84, 85]. Two forms of NET generation in response to microbes have also been described [49]: a classic, slow (3–4 h) lytic mechanism involving dissolution of intracellular membranes and vesicular release, as well as a rapid (5–60 min) mechanism of ET release independent of ROS generation [49, 124]. Furthermore, proteomics analysis revealed that different stimuli induce NETs with distinct compositions of protein cargo, sharing a core set of components common to most NETs [48]. Such discoveries suggest a possibility that ET generation is the result of several signaling pathways converging on a similar product exhibiting subtle differences based on the pathways involved. As researchers delve deeper into the precise characterization of ETosis and the structures generated by the process, the discovery of subtypes of NETs and their putative functions greatly expands the complexity of the subject. Unfortunately, most studies, to date, have focused on the molecular mechanisms underlying ETosis solely in neutrophils, leaving the possibility that ETosis could entail different intracellular mechanisms in other types of immune cells largely unexplored.

MΦ GENERATE EXTRACELLULAR, TRAP-LIKE STRUCTURES IN RESPONSE TO VARIOUS STIMULI

Although peripheral blood monocytes have been shown to undergo ETosis in response to bacterial pathogens and apicomplexan parasites [101, 125], the first detailed report of ET formation by mature, differentiated MΦ appeared in *Cell Host & Microbe* in 2010 [100], as part of a study linking the formation of ET to regulation of the sterol synthesis pathway. By dubbing the NET-like structures "METs," the authors found that the murine RAW 264.7 cell line and thioglycolate-elicited murine peritoneal MΦ produced ETs in the presence of *S. aureus* or when stimulated with PMA in vitro and identified typical ET components, including histone-DNA complexes and the antimicrobial peptides cathelicidins. Small interfering RNA-mediated silencing of the 3-hydroxy-3-methylglutaryl-CoA reductase gene, which encodes an enzyme important in cholesterol biosynthesis, resulted in a marked increase in ET formation but reduced microbial killing capacity. Similar results were observed when cells were treated with statin drugs, suggesting that loss of ET formation likely contributes to statin-induced reduction in bacterial phagocytosis by MΦ, as described in other studies [126]. Given the range of staphylococcal defenses that can impair immune cell function, such as neutralizing the oxidative burst [127, 128], interfering with complement factors [129–131], inhibiting cytokine production [132], and resisting phagocytosis [133–136], MET formation may be an alternative weapon to trap and eliminate virulent *S. aureus* strains that have developed defenses against the classic antimicrobial actions of phagocytes. When all other resources are exhausted, the self-destructive generation of ETs could function as a key, last-ditch effort to disable pathogenic microbes or possibly augment the efforts of other MΦ and immune effector cells.

Since the initial report of METs in 2010, other studies have come forth demonstrating the generation of METs by several MΦ subsets and cell lines in response to a wide range of microbes and stimuli [61, 70, 137, 138]. The molecules and microbes that have been used to stimulate MET formation are summarized in **Table 2**. Although most reports have identified common components among the integral constituents of METs, some results suggest that METs may actually consist of different subtypes and varieties. For example, Liu et al. [139] report the trapping of *E. coli* and *C. albicans* in MET-like structures with limited microbicidal activity produced by murine peritoneal MΦ and the murine J774A.1 MΦ cell line in an NADPH oxidase-independent manner distinct from cell lysis. Analysis of the structures by fluorescent in situ hybridization revealed that the METs were composed of nuclear and mitochondrial DNA, as well as MPO and lysozyme—proteins previously identified in NETs. The authors proposed that METs arising from mitochondrial or nuclear DNA may be the end results of separate cellular pathways [139], but thus far, the field of MET research only contains a handful of studies on METs, and it is unclear as to how many subtypes of METs exist or how they may differ in generation and composition.

The discovery of MET-like structures with mitochondrial-derived DNA as the primary structural backbone mirrors similar findings with neutrophils and eosinophils, in which specific stimuli were demonstrated to elicit generation of mitochondrial-derived DNA traps [21, 84, 85, 142]. Many of the components currently identified in METs (summarized in **Table 3**) are established as factors released with other, more well-characterized ETs [102]. Furthermore, elastases play a critical role in METosis, similar to the cellular mechanisms that regulate NET formation, as demonstrated by blocking the formation of

TABLE 2. Inducers of MET formation in various MΦ

Stimulating agent	MΦ Cell type	References
Microbes		
S. aureus	RAW 264.7 (murine) Murine peritoneal MΦ	[100]
E. coli, Candida albicans	J774.1 (murine) Murine peritoneal MΦ	[139]
M. haemolytica	Bovine MDMs Bovine alveolar MΦ	[137]
H. somni	Bovine MDMs	[61]
Mycobacterium tuberculosis	Human MDMs	[140]
Microbial products		
E. coli hemolysin	RAW 264.7 (murine) THP-1 (human)	[137]
M. haemolytica leukotoxin	Bovine MDMs	[137]
Other agents		
TNF-α	RAW 264.7 (murine)	[141]
PMA	RAW 264.7 (murine)	[100]
	Murine peritoneal MΦ	[137]
	Bovine MDMs	
Glucose oxidase	Bovine MDMs	[137]
Gold nanoparticles	Human MDMs	[138]

MDM = monocyte-derived MΦ.

METs with the use of elastase inhibitors [140]. These results lay down the foundation of MET research in ways that closely parallel NETosis, but subtle differences in the structure and function (e.g., trapping vs. killing activity, the reliance on ROS production, nuclear vs. mitochondrial origin) of different ET types may simply reflect the diversity in cellular physiology and differentiation of various leukocytes and their responses to microbial infection. For example, *M. haemolytica* elicits MET formation in alveolar MΦ but not in freshly isolated peripheral blood monocytes [137]. It should be noted that the proportion of MΦ-generating METs upon stimulation in vitro is relatively small (<25% of MΦ) in most experiments, regardless of stimulation [100, 137, 139]. METs are a supplementary strategy to disable microbes, but such a mechanism must necessarily be self-limiting and regulated to avoid interference with other essential MΦ functions. Indeed, it could be that different variants of ETs can contribute to immune defense and pathologies outside of the typical context and functions of ETs.

METS AND INTRACELLULAR PATHOGENESIS

An intriguing avenue of research is the role of ETs in infections by intracellular pathogens, such as the various *Mycobacterium* species, including the immune-evading bacterium *M. tuberculosis* and its less virulent relatives [63, 140, 143]. As a primary target and host-effector cell of tuberculosis infections, MΦ are highly susceptible to the invasive nature of microbes and modulations of cellular function [104, 144]. *M. tuberculosis* exploits IFN-γ to promote a form of nonprotective ETosis and necrosis in MΦ, ultimately facilitating bacterial aggregation and phagosomal rupture [140]. The structures of the mycobacteria-induced ETs exhibit morphologic similarities to the protective ETs generated in response to extracellular microbes. Furthermore, the process of MET formation was dependent on components of ESX-1, a mycobacterial virulence factor that is reported to activate the NLRP3 inflammasome and induce necrosis in heavily infected MΦ [145, 146]. However, it is unknown whether ETosis is

TABLE 3. Molecules associated with METs

Component	Putative roles	References
Histone-DNA complexes	ET backbone Cytotoxic	[100]
H4cit3	ETosis marker	[140] [141]
Mitochondrial DNA	Alternative ET backbone	[139]
LL-37	Microbicidal ET stabilizer	[100]
Lysozyme	Microbicidal	[139]
MPO	Chromatin decondensation	[139]
MIP-1α	Chemokine	[137]

a process independently induced by ESX-1 or a downstream effect of phagosomal rupture and NLRP3 activation, perhaps as a consequence of NLRP3 activation and other inflammatory responses to infection by *M. tuberculosis*.

Such manipulation of the innate-immune response can promote bacterial propagation by altering pathways common to or parallel with ET formation and may be an important part of the notorious hardiness and virulence of *M. tuberculosis* in human hosts. Indeed, IFN-γ has been implicated previously in caspase 1-independent necrosis and resulting bacilli dissemination from MΦ exposed to high intracellular bacterial burdens [147–149]. The implication of the ESX-1 system in infection-induced necrosis and ETosis is yet another example of how microbes can subvert host cellular physiology for their own ends. The virulence-promoting strategy is even more alarming when considering that IFN-γ not only plays an integral role in activating MΦ during intracellular infections, but also, evidence suggests that it prevents inflammation-mediated damage to lung tissue by regulating neutrophil accumulation in the lungs of tuberculosis-infected mice [150]. Furthermore, as a therapeutic tool in controlling tuberculosis, IFN-γ treatment is protective in low mycobacterial load-treated human MΦ [147], inhalation therapy for patients with active tuberculosis infection in several clinical trials [151–154], and established animal models of *Mycobacterium* infection [155–158]. With such a large set of studies supporting the medical value of IFN-γ treatment against *M. tuberculosis*, the targeting of virulence factors, such as ESX-1, could enhance the effectiveness of IFN-γ adjunct therapy by activating MΦ and maintaining an antimicrobial response without stimulating ET formation or immune cell necrosis.

In contrast to studies of extracellular pathogens, the putative function of ETosis in intracellular infections is a largely unexplored field of research, and many questions remain to be answered. Neutrophils are the predominant cell type infected by mycobacteria in the airways of patients with active tuberculosis [159], and accordingly, *M. tuberculosis* can exploit similar mechanisms and pathways in PMNs to induce cell death via NETosis [63, 143]. With the consideration of the wide range of bacterial species that are obligate or facultative intracellular parasites of phagocytes and granulocytes, such as certain species of *Ehrlichia*, *Chlamydia*, and *Legionella*, it seems very likely that ETs will be associated indirectly or directly in a wider range of pathologies. The results of Wong and Jacobs [140] highlight the complex interactions between host cells and intracellular parasites and illustrate how the relevance of cellular processes, such as ETosis, can often extend beyond the conventional roles established by prior research. Along with the explicit implications of METs in extracellular and intracellular infections, it is also becoming apparent that ET formation is involved in more than just microbial pathogenesis and the subsequent host response.

STERILE INFLAMMATORY CONDITIONS CAN PROMOTE MET GENERATION

In the absence of an active infection, the induction of METs can be triggered by inflammatory conditions in sterile tissue environments and may even contribute to persistent inflammation. Although many studies illustrated the ability of cytokines to induce ET formation directly in vitro [83, 160], the physiologic role of direct cytokine-induced ETosis is difficult to assess in vivo. Recently, Mohanan et al. [141] presented data suggesting that high levels of inflammatory cytokines (i.e., TNF-α) can induce the generation of ETs from MΦ in vitro. With the identification of exDNA and hypercitrullinated histones in MET-like structures isolated from adipose tissue, the authors demonstrated further that lesion-associated MΦ express the PAD isoform PAD2, as well as a lower expression of PAD4 (essential for NET formation [113]), a finding also observed in stimulated RAW264.7 cells. These results further support the idea that the sterile inflammation-induced formation of MET-like structures is highly analogous to the well-studied mechanisms of ETosis in neutrophils, although it remains to be seen if the molecular mechanisms of ETosis in MΦ differ in response to varying sterile stimuli, as has been demonstrated in generation of NETs [5, 161].

It is also currently unknown whether METosis serves any beneficial role in sterile inflammation or is a negative consequence of other inflammatory processes. Nonetheless, these data place MΦ and ETs together at the center of obesity-induced inflammation [141]. The in vitro studies suggest that high levels of TNF-α released by dying and inflamed adipose tissue are able to stimulate MET formation in the absence of microbes. The release of extracellular chromatin could cause more cell death and tissue damage and in a feedforward cycle, contribute to the release of additional inflammatory cytokines and potential autoantigens into the local environment. TNF-α is firmly established at the center of many inflammatory disorders, suggesting that MET-like structures remain to be discovered as components of other sterile inflammatory processes and diseases.

REGULATION AND CLEARANCE OF ETS BY MΦ PLAY A CRITICAL ROLE IN CONTROLLING INFLAMMATION

MΦ fulfill a key position in the resolution of inflammation and the early stages of tissue repair by clearing apoptotic cells and debris (reviewed extensively in refs. [162–166]), and now evidence is surfacing that these functions may include the removal of ETs and immobilized microbes. The established capability of excessive or persistent ET formation to induce inflammation, tissue destruction, and even autoimmune responses, as illustrated in **Fig. 2**, supports recent studies establishing a critical role for MΦ in the neutralization and clearance of ETs before irreversible tissue damage occurs [36, 167]. For example, when alveolar MΦ were depleted from the lungs of influenza virus-challenged mice, the animals' lungs showed signs of excessive tissue inflammation and neutrophil invasion, concurrent with an accumulation of NETs in damaged alveoli and pulmonary microvasculature [76]. In contrast, neutrophil-depleted animals exhibited mild pathology in the lungs, suggesting that the MΦ are the key cell type, limiting excessive pulmonary inflammation and tissue damage during viral infection. Furthermore, the inflammation was abrogated with the treatment of antibodies directed against factors essential

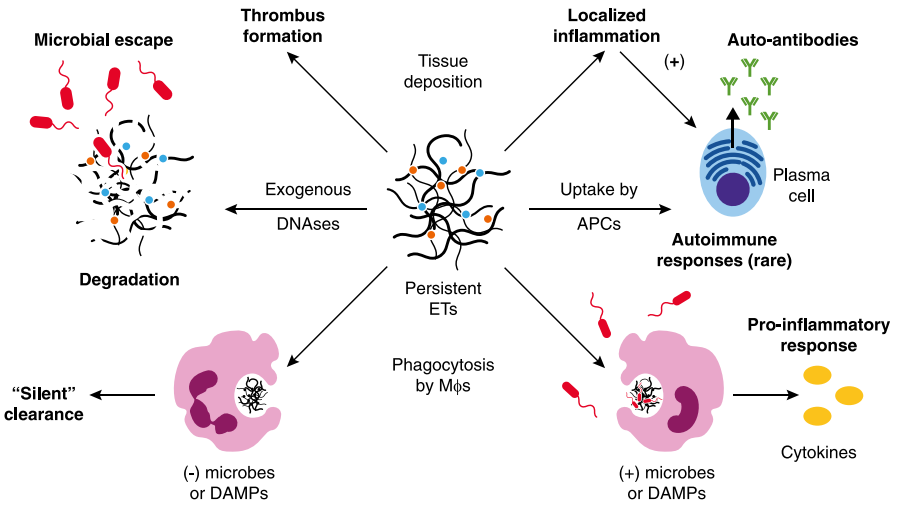

Figure 2. The fates of ETs. Persistence of intact ETs within tissue can lead to a number of deleterious effects, including tissue damage, local inflammation, thrombus formation, or the development of an autoimmune response by the adaptive immune system. ETs can also be degraded by extracellular nucleases produced by pathogens, releasing trapped microbes and neutralizing the microbicidal actions of ETs. Upon phagocytosis by MΦ, ETs can be cleared in an immunologically silent manner or may provoke an inflammatory response depending on the local environment and the presence of microbial pathogens or DAMPs.

for NETosis, such as MPO, placing ETosis at the center of the observed pathology. Such studies suggest that MΦ can regulate ET generation during the innate immune response, but whether these processes are indirectly influencing PMN function or are a direct result of MΦ-mediated ET clearance was unclear.

Detailed investigation by Farrera and Fadeel [168] established that MΦ engulf NETs and NET fragments actively and directly via cytochalasin D-dependent phagocytosis, ultimately resulting in lysosomal degradation. They also discovered that clearance was facilitated by the nucleic acid-degrading functions of DNAse I and by opsonization of NETs by complement factor C1q, both proteins previously established to play key roles in the removal of extracellular chromatin and dying cells [169–171]. However, physiologic concentrations of DNAse I were insufficient to degrade NETs completely, signifying that other mechanisms, such as complement opsonization or MΦ phagocytosis, are essential for effective clearance. Incomplete degradation of ETs may also activate complement and further inhibit the action of serum nucleases, exacerbating autoimmune responses and compounding the problem of NET-mediated tissue damage [170]. In the context of the lungs, other immune-related proteins may facilitate the clearance and uptake of ETs by tissue-resident MΦ. For instance, surfactant proteins enhance the removal of apoptotic cells by alveolar MΦ in the airways and may limit the generation of anti-DNA antibodies [172–174]. The deciphering of the mechanisms controlling ET neutralization and removal and the understanding of the consequences when these processes fail will be important for characterizing the contribution of ETs to inflammation and disease.

ENGULFMENT OF ETS BY MONONUCLEAR PHAGOCYTES CAN INFLUENCE CELLULAR RESPONSES

An important question that arises from research on the clearance of ETs by phagocytosis is whether the process has an effect on the cellular and immunomodulatory functions of MΦ and other phagocytic cells. A key finding by Farrera and Fadeel [168] was that exposure to PMA-induced ETs alone did not evoke an inflammatory response upon uptake by monocyte-derived MΦ, occurring in an immunologically silent manner without coinciding cytokine release. However, upon stimulation with LPS, the presence of NETs enhanced MΦ secretion of proinflammatory cytokines IL-1β, IL-6, and TNF-α. Perhaps the presence of ETs and microbes or DAMPs together provokes a more robust inflammatory response, preparing MΦ for an active infection or injury in the immediate vicinity. Internalization of the antimicrobial peptide, LL-37, a common component of ETs, is known to promote MΦ differentiation toward the M1-polarized proinflammatory state [175]. Neutrophil-derived LL-37 is able to recruit a variety of leukocytes to sites of inflammation [176, 177], including inflammatory monocytes [178, 179], and has been demonstrated (along with intact NETs) to activate the NLRP3 inflammasome in human and murine MΦ, resulting in increased production of IL-1β and IL-18 [180]. IL-18 can further trigger the release of more NETs from neutrophils, resulting in a positive-feedback loop among PMNs, ETs, and activated MΦ to heighten the local inflammatory environment. The observation that the inflammasome-mediated response was heightened in MΦ derived from lupus patients [180] provides additional evidence that ET-related dysfunction may play an important role in chronic inflammatory disorders arising from dysregulation of immune signaling and that the components of ETs can influence the balance through changes in MΦ function.

Recent observations by Braian et al. [143] bring to light the capacity of NETs to activate MΦ when the ETs are triggered by intracellular pathogens, such as *M. tuberculosis*. In contrast to NETs generated upon PMA treatment, mycobacteria-induced NETs up-regulated the production of IL-6, TNF-α, IL-1β, and IL-10 by MΦ upon phagocytosis of the ETs. Furthermore, infection-induced NETs contained Hsp72, which was absent in PMA-activated NETs, a finding in accordance with prior observations that Hsps expressed by infection-induced apoptotic neutrophils can activate MΦ toward a proinflammatory phenotype [181–184]. Although Hsp72 alone was capable of stimulating inflammatory cytokine production, the observed response was much greater when Hsp72 was bound in NETs, whether

triggered by *M. tuberculosis* infection or by pretreatment of PMA-induced NETs with rHsp72. The presence of externalized Hsps in NETs can thus spread the danger signal of mycobacterial infection from neutrophils to MΦ, preparing the cells to deal with an active infection in the vicinity.

Local signaling mechanisms may attune the behavior of MΦ to the surrounding environment, enhancing inflammation when the phagocytes encounter the remains of ETs and microbes or initiating MΦ-mediated resolution of the inflammatory response and subsequent tissue repair postinjury or infection. In the absence of pathogens or DAMPs, silent clearance of ETs would avoid sterile inflammation and maintain tissue homeostasis, similar to the removal of apoptotic neutrophils [165, 166]. Although complement-aided removal of apoptotic cells influences phagocytes toward an anti-inflammatory phenotype [185–187], and opsonization of C1q facilitates uptake of NETs by MΦ [168], there is no published evidence to suggest that complement-aided clearance of ETs can also result in anti-inflammatory signals. In accordance with their position as adaptable regulators of local homeostasis, MΦ may therefore use ETs and entrapped materials sense danger or damage in their immediate surroundings and then, gauge the appropriate time to initiate subsequent immune responses and resolution. For example, alveolar MΦ, not often exposed to ETs in healthy airways, can be stimulated by NETs in vitro to produce proinflammatory CXC chemokines and TNF-α [188], a potentially useful response should they encounter ETs and microbes in infected lungs.

ETs play a direct role in clearing infectious pathogens through immobilization and cytotoxic factors but may also supplement the killing capacity of phagocytes in other ways. For example, ingestion of antimicrobial and granule-derived proteins from healthy and apoptotic neutrophils enhances the ability of MΦ to clear extra- and intracellular microbes [189–191]. Furthermore, a key NET component, neutrophil-derived elastase, stimulates *Leishmania*-infected MΦ via TLR4 and aids in the elimination of intracellular protozoa [192], whereas purified MPO, another common component of NETs, also enhances MΦ microbe-killing ability upon uptake by MΦ [193, 194]. Together, these data suggest that the protective role of ETs goes beyond that of simply trapping and killing pathogens and involves signaling mechanisms and antimicrobial factors that strengthen MΦ phagocytic responses. If so, the interactions and cooperation between PMNs and MΦ could be much more complex than previously anticipated, involving extracellular chromatin and antimicrobial peptides in novel ways.

CONCLUDING REMARKS

It is becoming apparent that developing tools to control ETosis at the molecular level could impact our understanding of inflammatory processes and leukocyte physiology. Inhibitors specific to the different PAD isoforms have been developed [195–197] (some for potential anti-cancer activity [198]) and demonstrated to be efficacious in controlling sterile inflammatory pathologies in mouse models, raising the possibility that isoform-specific PAD inhibitors could be used to further research on ETs and to control ET-mediated inflammation therapeutically in disorders ranging from multiple sclerosis [199, 200] to vascular disease [201] and others [195, 202]. Additionally, several well-characterized pharmaceutical agents modulate NET formation, including vitamin C [203], nitroxides [204], flavonoids, *N*-acetyl-L-cysteine, and 5-aminosalicylic acid (mesalamine) [205]. Despite the relative newness of the field, there are already a wide range of methods and techniques with which researchers and clinicians may manipulate ET formation and function, ensuring continued progress and innovation in the field.

Until a larger selection of animal models is established or modified to focus on the in vivo functions of METs, it will be incompletely understood how they influence the pathogenesis and resolution of infectious and immune-mediated disease and the extent to which the roles of the ETs generated by different immune cell types have overlapping or distinct functions. As MΦ serve diverse roles in the immune response and tissue homeostasis, it would not be surprising that METosis could have far-reaching ramifications in human health and immunity. With the consideration that NETs are implicated in a wide range of diseases related to immune dysfunction [36], METs may play similar roles in promoting excessive inflammation or autoimmunity. Barely over 1 decade ago, ETs were an exciting, novel mechanism with intriguing antimicrobial functions. As the field of immunobiology grows larger, ETs have become a prominent subject of research that draws the various biologic disciplines of immunology, medicine, microbiology, biochemistry, and more into the context of a multifaceted cellular phenomenon. The current knowledge on ET formation and clearance by MΦ is only the beginning of our knowledge; as research advances, future studies will undoubtedly reveal the mechanisms behind the complex role of MΦ and ETs in immune defense and disease.

AUTHORSHIP

D.M.B. outlined the review and wrote the manuscript. B.J.C., M.M.C., and J.A.I. edited and revised the text. J.A.I. edited the figures and tables. E.J.K. oversaw manuscript preparation and edits.

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DISCLOSURES

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The authors declare no conflicts of interest.

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