The expanding world of extracellular traps: not only neutrophils but much more

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INTRODUCTION

Extracellular traps (ETs) were first described in 2004 in a ground breaking publication by Brinkmann and colleagues who observed the released of web-like structures by neutrophils after stimulation with phospholipid murate acetate (PMA), lipopolysaccharides (LPS), interleukin 8 (IL-8), platelet-mediated neutrophil activation with phorbol myristate acetate (PMA), lipopolysaccharides and histones, ETs also comprise a number of molecules which impart an antimicrobial effect including elastase, cathespin G, proteases or defensins, bacterial permeability increasing protein (BPI), or myeloperoxidase (MPO; Brinkmann et al., 2004; Papayannopoulos et al., 2010). Extracellular traps are composed of DNA decorated with antimicrobial peptides, proteases, and histones. However, they also exhibit remarkable individual differences such as the type of sub-cellular compartments from where the DNA backbone originates (e.g., nucleus or mitochondria), the proportion of responding cells within the pool, and/or the molecular mechanism/s underlying the ETs formation. This review summarizes the knowledge accumulated in recent years regarding the complex and expanding world of ETs and their role in immune function with particular emphasis on the role of other immune cells rather than on neutrophils exclusively.

The release of extracellular traps (ETs) is a recently described mechanism of innate immune response to infection. Although ETs have been intensely investigated in the context of neutrophil antimicrobial effector mechanisms, other immune cells such as mast cells, eosinophils, macrophages and monocytes can also release these structures. The different ETs have several features in common, regardless of the type of cells from which they originated, including a DNA backbone with embedded antimicrobial peptides, proteases, and histones. However, they also exhibit remarkable individual differences such as the type of sub-cellular compartments from where the DNA backbone originates (e.g., nucleus or mitochondria), the proportion of responding cells within the pool, and/or the molecular mechanism/s underlying the ETs formation. This review summarizes the knowledge accumulated in recent years regarding the complex and expanding world of ETs and their role in immune function with particular emphasis on the role of other immune cells rather than on neutrophils exclusively.

Keywords: extracellular traps, neutrophils, mast cells, eosinophils, macrophages/monocytes, etosis

Table 1: the different cell types is a backbone composed of DNA decorated with antimicrobial molecules that is capable of snaring and killing a wide spectrum of microbes (Brinkmann et al., 2004; Fuchs et al., 2007; Urban and Zychlinsky, 2007; von Kockritz-Blickwede and Nizet, 2009). Nevertheless, it should be mentioned that ETs arising from different cell types also exhibit unique features, distinct from those originally described for neutrophils.

Much of the research on ETs has been conducted on neutrophils, most probably because these cells were the first to be associated with the production of such extracellular structures. This is also the reason why the mechanism of cell death leading to the formation of ETs was first termed Nettosis (Fuchs et al., 2007) and then later generalized to Ettosis. The differences between Ettosis and the other forms of cell death such as necrosis or apoptosis are summarized in Table 2. The intracellular signaling events reported to be involved in the induction of etosis include the activation of NADPH oxidase with the concomitant formation of reactive oxygen radicals (ROS; Papayannopoulos et al., 2010; Guimarães-Costa et al., 2012). There are also reports demonstrating that, in addition to chromosomal DNA, mitochondrial DNA could also be used by eosinophils (Yousefi et al., 2008) and neutrophils (Yousefi et al., 2009) to form ETs without induction of cell death. However, the mechanisms behind this unusual mode of ET formation remains a mystery. Although the primary function of ETs has been attributed to their antimicrobial effect, the overall role of ETs in host defense against pathogens remains a topic of debate.

THE MOLECULAR BASIS OF EXTRACELLULAR TRAPS FORMATION

While significant progress has been made in unraveling the cellular processes that are taking place during the formation of ETs, many
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Table 1 | Differences between netosis, apoptosis, and necrosis.

| Membrane and organelle disintegration | Apoptosis | Necrosis |
|--------------------------------------|-----------|----------|
| Phosphatidylserine exposure during early steps of necrosis | Membrane blebbing | Vacuolization |
| Cellular swelling and bursting | Nuclear chromatin condensation without disintegration of the nuclear membrane | No exposure to Phosphatidylserine |
| Cell damage releasing the intracellular contents | Programmed cell death | Programmed cell death |

Table 2 | Cell types shown to release ETs and triggering stimuli.

| Cell type | Activating agent | Reference |
|-----------|------------------|-----------|
| Neutrophils | IL-8 | Ramos-Kichik et al. (2009) |
| Neutrophils, Mast cells | Lipopolysaccharide (LPS) | Brinkmann et al. (2004), von Kodolitsch et al. (2009), Ramos-Kichik et al. (2009) |
| Neutrophils, Mast cells | Phorbol-12-myristate-13-acetate (PMA) | Brinkmann et al. (2004), von Kodolitsch et al. (2008) |
| Neutrophils | Platelet via TLR4 | Clark et al. (2007) |
| Neutrophils, Eosinophils | Interferon (IFN)-γ | Yousei et al. (2008) |
| Eosinophils | Interferon (IFN)-α | Yousei et al. (2008) |
| Neutrophils | GM-CSF + C5a | Martinelli et al. (2004), Yousei et al. (2009) |
| Neutrophils | GM-CSF + LPS | Martinelli et al. (2004), Yousei et al. (2009) |
| Neutrophils | Lipopolysaccharide | Guimarães-Costa et al. (2009) |
| Neutrophils, Mast cells | M1-protein-fibrinogen complex | Lauth et al. (2009), Oehmcke et al. (2009) |
| Neutrophils, Mast cells, Eosinophils | Hydrogen peroxide | Brinkmann et al. (2004), von Kodolitsch et al. (2008), Oehmcke et al. (2009) |
| Neutrophils | Calcium | Wang et al. (2009) |
| Neutrophils, Mast cells | Glucose oxidase | Fuchs et al. (2007), von Kodolitsch et al. (2008) |
| Mast cells | IL-23 and IL-1β | Lin et al. (2011) |
| Neutrophils, Monocytes/Macrophages | Statins | Chov et al. (2010) |
| Neutrophils | Tumor necrosis factor (TNF)-α | Wang et al. (2009) |
| Neutrophils | Panton-Valentine leukocidin | Pfizenmaier et al. (2010) |
| Neutrophils | Platelet activating factor | Hakkim et al. (2011) |

aspects still remain unresolved. ET formation generally begins in stimulated cells with the loss of the tight organization of the nuclei followed by chromatin decondensation. The characteristic shape of the nuclei disappears and a gap between the inner and outer membrane of the nucleus emerges. Formation of vesicles in the nuclear membrane follows leading to widespread membrane disruption. At the same time, disruption of the granular membranes takes place in the cell cytoplasm facilitating the mixing of granular content with the chromatin leaking into the cytoplasm through the disrupted cellular membrane. Finally, eruption of the cell membrane follows and DNA mixed with the granular content is released into the extracellular milieu (Fuchs et al., 2007). This characteristic form of cell death, termed Netosis by Steinberg and Grinstein (2007), was described earlier by Taki et al. (1996) although without showing an association with the release of ETs. Netosis seems to be a process entirely independent of caspases and certain kinases such as RIP-1 and is not affected by the caspase inhibitor zVAD-fmk (Urban et al., 2009; Remijsen et al., 2011). Netosis is not associated with DNA fragmentation or phosphatidylserine (PS) exposure on the outer leaflet of the cellular membrane, which are distinctive aspects of apoptosis. The lack of PS impedes the clearance of cells undergoing netosis by phagocytic cells such as macrophages. An additional feature that distinguishes netosis from apoptosis and necrosis is the fact that both the nuclear as well as the granular membranes undergo fragmentation. A critical factor involved in etosis and ET formation is the production of ROS. In neutrophils, ROS produced by NADPH oxidases has been reported to inactivate caspase function thereby leading to the blockage of the apoptotic cell death pathway (Fadeel et al., 1998; Hampton et al., 2002). The importance of NADPH...
Although the molecular principles underlying the formation of ETs by mast cells (von Kockritz-Blickwede et al., 2008), eosinophils (Yousefi et al., 2008) and macrophages are also capable of releasing ETs, it should also be noted that in both cases where ET formation was non-associated with cell death, the cells needed to be primed first before stimulated to form ETs. In the case of neutrophils, cells were initially activated by granulocyte/macrophage stimulating factor (GM-CSF) followed by short-term toll-like receptor 4 (TLR4) or C3a stimulation (Figure 1). In these experimental conditions, viable neutrophils were able to release ETs that contained mitochondrial but no nuclear DNA.

DNA-releasing eosinophils have been primarily reported in the context of inflammatory diseases of the intestine (Yousefi et al., 2008) and skin (Simon et al., 2011). They seem to be less prominent, however, in the setting of infectious diseases despite the fact that these structures are also capable of snaring and killing bacteria (Yousefi et al., 2008). Furthermore, while induction of mtDNA associated with eosinophil granules has been reported to contribute to the increased survival of mice (up to 14 days) undergoing cecal ligation puncture (CLP; Yousefi et al., 2008), it is still not clear to what extent eosinophil ET formation contributes to host defense. In this regard, though evidence has been provided that hypereosinophilic transgenic animals are less susceptible to septicemia induced by CLP, the major role of eosinophils has been attributed to host defense against helminths (Blanchard and Rothenberg, 2009; Lüch et al., 2009). These granulocytic cells are able to infiltrate the gastrointestinal tract and have been associated with a variety of inflammatory conditions like inflammatory bowel disease (IBD) or eosinophil-associated gastrointestinal disorders (EGIDs; DeBrosse and Rothenberg, 2008; Wedemeyer and Voskuhl, 2008).

Besides eosinophils, mast cells, which also originate from bone marrow and contain different types of granules enclosing very potent biological effectors molecules, are also capable of releasing ETs following stimulation (Figure 2). Mast cells are located in close proximity to the host environment where they are most likely to encounter incoming pathogens. Although mast cells are largely known for their detrimental role in the context of allergic diseases, there is a growing body of evidence that suggests that they are also important contributors to host defense against pathogens (Galli and Wershil, 1996; Bischoff, 2007). Thus, mast cells are not only important for modulating the function of other immune cells (e.g., neutrophils) during infection but they also impart direct antimicrobial effects (Feger et al., 2002). Due to the limited phagocytic activity of mast cells, their antimicrobial activity is largely mediated by extracellular mechanisms including degradation and the concomitant release of highly potent antimicrobial peptides such as cathelicidins (CRAMP or LL-37), defensins (β-defensins) or proteases (tryptase, chymase). Mast cell degranulation occurs after exposure to pathogens and has been shown to be very efficient in inhibiting the growth of bacteria such as S. aureus (Abel et al., 2011). In addition, mast cells are also able to release ETs in a ROS-dependent manner. Mast cell ETs are composed of DNA and histones, which are the general components of most ETs, as well as mast cell-specific granule proteins like tryptase and CRAMP/LL-37 (von Kockritz-Blickwede et al., 2008). In contrast to neutrophils where NETs can be dismantled after treatment with only DNase, the complete disassembling of mast cell ETs requires treatment with DNase as well as the addition of enzymes degrading tryptase (e.g., MPO; von Kockritz-Blickwede...
FIGURE 1 | Schematic representation of the cellular processes involved in the formation of ETs. The process can be triggered by a number of stimuli including, PMA, LPS, C5a + GM-CSF, IFN-γ, LPS, bacteria, and viruses. IL-8 is also able to trigger ET release by interacting with the CXCL2/8 receptor and inducing H3 citrullination through PAD4 activation via Src kinases. Most pathways converge in the activation of the key enzyme NADPH oxidase. This enzyme is highly activated by PMA and formylated peptides. Induction of the fMLP receptor leads to a massive activation of protein kinase C (PKC) and NADPH oxidase activity. On the other hand, fMLP blocks autophagy via PI3K, Akt and mTOR activation, which is able to prevent Etoxis. NADPH oxidase activity results in ROS production and H3 citrullination leading to chromatin decondensation and nuclear collapse. Disintegration of the nuclear membrane and adsorption of antimicrobial granular proteins onto the decondensed chromatin network is the final step of Etoxis that precedes the release of ETs into the surrounding milieu after rupture of the plasma membrane.
et al., 2008). Another interesting feature is the recently reported involvement of the transcriptional hypoxia-inducible factor 1α (HIF-1α) in the modulation of ET release by human and murine mast cells (Brantzi-Heinemann et al., 2012). HIF is a well-known factor for its role in the regulation of the inflammatory and innate immune function of neutrophils and macrophages (Cramer et al., 2003; Peyssonnaux et al., 2005).

Most recently, monocytes/macrophages have also been reported to be capable of releasing ETs (Chow et al., 2010; Aulik et al., 2011). Macrophage ET production has been shown to be boosted by statins, which are inhibitors of the rate-limiting enzyme within the cholesterol biosynthesis 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase. In addition, increased production of ETs release by macrophages has been observed after inhibition of HMG-CoA reductase using siRNA or after treatment of macrophages with the downstream HMG-CoA reductase product mevalonate (Chow et al., 2010). Statins are also capable of inhibiting the release of ETs by neutrophils. The molecular mechanism mediating the effect of statins on phagocytes seems to be linked to the inhibition of the sterol pathway within the cell (Chow et al., 2010). Interestingly, bacterial components such as hemolysins of Escherichia coli or leukotoxin of Mannheimia haemolytica have been shown to induce the release of ETs by bovine macrophages (Aulik et al., 2011, 2012). However, the extent to which the molecular processes leading to the formation of ETs by monocytes/macrophages is comparable to the mechanisms already described for neutrophils, eosinophils, and mast cells, remains to be elucidated. Although little information is available regarding the molecular basis of ET release by macrophages, it seems that general mechanisms such as NADPH oxidase dependency and oxidative stress are involved (Chow et al., 2010).

**THE ANTIMICROBIAL EFFECT OF EXTRACELLULAR TRAPS**

Extracellular traps release is thought to be mainly an antimicrobial strategy used by host cells to control and eliminate pathogens (Brenkemann et al., 2004; von Kockritz-Blickwede et al., 2008; Linch et al., 2009; Saitoh et al., 2012). Thus, a number of bacteria, fungi, and parasites have been reported in the literature to be entrapped and killed by ETs (summarized in Table 3). Saitoh et al. (2012) provided the first report regarding the involvement of ETs in antiviral immunity. Their study showed that neutrophils are able to produce ETs in response to human immunodeficiency virus-1 (HIV-1) and, more interestingly, that these virus particles can be entrapped and neutralized by the ETs. By using blocking antibodies to MPO and α-defensin, it was possible to demonstrate that the viral neutralization was dependent on the presence of MPO and α-defensin in the NET structure. The production of NETs by neutrophils in this case was associated with TLR-7 and TLR-8 signaling as well as with ROS production (Saitoh et al., 2012). In addition, the investigators also showed that the anti-inflammatory cytokine IL-10 could reduce the release of extracellular DNA by neutrophils into the surrounding milieu. It is important to note, however, that these studies were carried out in vitro and, although this is an exciting new aspect of ET function in host immunity, it still remains to be demonstrated in the in vivo setting. Whether ETs produced by immune cells other than neutrophils are also capable of trapping and inactivating virus particles may be deserving of future investigation. The finding that different
pathogens are able to induce ETs in different innate immune cells argue for a general role of ETs in the innate immune response to pathogenic microorganisms and is supported by a number of in vivo studies revealing ET formation in necrotizing soft tissue infections caused by S. pyogenes (Buchanan et al., 2006), polymicrobial sepsis after cecal ligation and puncture (Vosselli et al., 2008) and S. pneumoniae infections in murine models (Buchanan et al., 2006).

The molecular mechanism/s responsible for the entrapment and killing of microorganisms within ETs is a matter of debate, though several hypotheses have been proposed. One such hypothesis is that entrapment is facilitated by the occurrence of electrostatic interactions arising from the cationically charged ET structure and the anionically charged bacterial surfaces (Brinkmann and Zychlinsky, 2007). The subsequent killing of the pathogen is postulated to arise from the ability of the ETs to increase the local concentration of certain antimicrobial peptides and therefore intensifying the contact between microorganisms and the antimicrobial agents (von Kockritz-Blickwede et al., 2008). Potential candidates being discussed to have antimicrobial properties within ETs are the histones. Several types of histones and histone-related peptides isolated from various organisms and cell types exhibit a broad spectrum of antimicrobial activities (Kawasaki and Iwamuro, 2008). In particular, the histone H2B displays antimicrobial properties against Gram-positive and Gram-negative bacteria and fungi (Li et al., 2007). An overview of the antimicrobial activities of histones is displayed in Table 4. In addition to histones, there are other cell specific components.

### Table 3 | Microorganisms able to trigger the release of ETs by specific cell types.

| Microorganism         | Cell type | Reference                      |
|-----------------------|-----------|--------------------------------|
| Aspergillus fumigatus | Neutrophils | Bruns et al. (2010), McCormick et al. (2010) |
| Candida albicans      | Neutrophils | Urban et al. (2006) |
| Cryptococcus gattii   | Neutrophils | Springer et al. (2010) |
| Cryptococcus neoformans | Neutrophils | Urban et al. (2009) |
| E. coli               | Neutrophils | Behrendt et al. (2010) |
| Enteroococcus faecalis | Neutrophils | Lippolis et al. (2006) |
| Escherichia coli      | Neutrophils | Lippolis et al. (2008), Grinberg et al. (2008), Webster et al. (2010) |
| Haemophilus influenzae | Neutrophils | Hong et al. (2009), Hakkim et al. (2011) |
| Helicobacter pylori   | Neutrophils | Hakkim et al. (2011) |
| Human Immunodeficiency Virus-1 (HIV-1) | Neutrophils | Saitoh et al. (2012) |
| Klebsiella pneumoniae | Neutrophils | Papayannopoulos et al. (2013) |
| Listeria monocytogenes | Neutrophils | Guimarães-Costa et al. (2009) |
| Listeria monocytogenes | Neutrophils | Ermert et al. (2009) |
| Mycobacterium canettii | Neutrophils | Ramos-Kichik et al. (2009) |
| Mycobacterium tuberculosis | Neutrophils | Ramos-Kichik et al. (2009) |
| Pseudomonas aeruginosa | Neutrophils | Lippolis et al. (2008) |
| Salmonella marcescens | Neutrophils | von Kockritz-Blickwede et al. (2008) |
| Shigella flexneri      | Neutrophils | Brinkmann et al. (2004) |
| Staphylococcus aureus  | Neutrophils, Mast cells | Brinkmann et al. (2004), von Kockritz-Blickwede et al. (2008) |
| Staphylococcus dysgalactiae | Neutrophils | Lippolis et al. (2008) |
| Staphylococcus pneumoniae | Neutrophils, Mast cells | Beiter et al. (2006), Crotty Alexander et al. (2010) |
| Streptococcus pyogenes | Neutrophils, Mast cells | Buchanan et al. (2006), von Kockritz-Blickwede et al. (2008) |

### Table 4 | Short overview of histones and their antimicrobial properties.

| Histone   | Origin                        | Antimicrobial spectrum                          | Reference                                    |
|-----------|-------------------------------|-------------------------------------------------|----------------------------------------------|
| Histone H1 | macrophages, epithelial cells, liver, intestine, skin | S. aureus, L. monocytogenes, S. typhimurium, E. coli | Hemmila et al. (1993), Rose et al. (1998) |
| Histone H2A | Placenta, skin | E. coli, L. monocytogenes, S. aureus, B. subtilis, S. pneumonia, C. albicans | Kim et al. (2003), Cho et al. (2002), Fernandes et al. (2002), Li et al. (2007) |
| Histone H2B | Placenta, skin | S. aureus, L. monocytogenes, S. typhimurium, B. subtilis | Li et al. (2007) |
The beneficial or detrimental effect of ETs can be determined by the extent of the response. Moderate release of ETs during productive infection remains a subject of debate. Furthermore, in certain circumstances, the production of ETs can be detrimental for the host. For example, the release of high quantities of DNA and histones can induce autoimmune reactions that may be involved in the development of autoimmune diseases like lupus erythematosus or rheumatoid arthritis (Mohan et al., 1993; Zhong et al., 2007). Preeclampsia, a severe disorder of late pregnancy characterized by an increasing level of cell free DNA in the maternal plasma, is another pathological disorder in which ET formation may also be involved (Clark et al., 1998). In this disorder, a massive release of DNA probably in response to high levels of inducing factors (e.g., IL-8 or microdebris of the placenta) has been observed (Gupta et al., 2005, 2007). Similarly, the release of ETs by platelet-activated neutrophils under blood flow conditions can result in reduced blood perfusion of the tissue and ischemia (Clark et al., 2007). The beneficial or detrimental effect of ETs can be determined by the extent of the response. Moderate release of ETs during infection can contribute to pathogen killing and control of the infection, thus conferring a beneficial effect. Conversely, massive release of ETs during pathological conditions can induce autoimmunity as well as organ damage and is thus highly deleterious for the host.

PATHOGEN EVASION OF EXTRACELLULAR TRAPS

Successful pathogens have evolved intricate countermeasures to subvert the mechanisms of host defense. Shortly after ETs were discovered, a number of studies reported the ability of certain pathogens to circumvent the antimicrobial activity of these structures. One of the main strategies used by pathogenic bacteria to escape the ETs is through the production of DNases that cleave DNA and therefore dismantle their DNA backbone. This mechanism has been described for S. pyogenes, which produces a very potent bacteriophage-encoded DNase designated Sda1. Strains of S. pyogenes producing Sda1 are more resistance to ET-dependent killing than strains lacking the Sda1 gene (Sunby et al., 2005; Buchanan et al., 2006). A similar strategy has been reported for S. pneumoniae (Buchanan et al., 2006) and S. aureus (Udo et al., 1999; Berends et al., 2010). Changes in the composition of the bacterial cell wall can also help to avoid the antimicrobial activity of ETs. Thus, S. pneumoniae mutant strains lacking positively charged di-alanyl residues on their lipoteichoic acid (LTA) have been shown to be more susceptible to ET killing than the corresponding wild-type strain (Wartha et al., 2007a,b). D-alanylation of LTA by bacterial species harboring a homolog of the dlt operon like S. pyogenes (Kristian et al., 2003) or S. aureus (Kraus et al., 2008) are known to be much more resistant against the antimicrobial activity of cathelicidins. An indirect strategy of microbes to avoid the antimicrobial effect of ETs is to reduce the recruitment of immune cells involved in the production of ETs. This is achieved by the blocking or cleaving of chemotactic mediators bound to the DNA backbone of the ETs still retain their antimicrobial capacity.

Although the antimicrobial effect of ETs has been extensively demonstrated in many experimental settings, the extent to which these structures contribute to pathogen killing during productive infection remains a subject of debate. Furthermore, in certain circumstances, the production of ETs can be detrimental for the host. For example, the release of high quantities of DNA and histones can induce autoimmune reactions that may be involved in the development of autoimmune diseases like lupus erythematosus or rheumatoid arthritis (Mohan et al., 1993; Zhong et al., 2007). Preeclampsia, a severe disorder of late pregnancy characterized by an increasing level of cell free DNA in the maternal plasma, is another pathological disorder in which ET formation may also be involved (Clark et al., 1998). In this disorder, a massive release of DNA probably in response to high levels of inducing factors (e.g., IL-8 or microdebris of the placenta) has been observed (Gupta et al., 2005, 2007). Similarly, the release of ETs by platelet-activated neutrophils under blood flow conditions can result in reduced blood perfusion of the tissue and ischemia (Clark et al., 2007). The beneficial or detrimental effect of ETs can be determined by the extent of the response. Moderate release of ETs during infection can contribute to pathogen killing and control of the infection, thus conferring a beneficial effect. Conversely, massive release of ETs during pathological conditions can induce autoimmunity as well as organ damage and is thus highly deleterious for the host.

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