

Review Article

Far beyond Phagocytosis: Phagocyte-Derived Extracellular Traps Act Efficiently against Protozoan Parasites *In Vitro* and *In Vivo*

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Professional mononuclear phagocytes such as polymorphonuclear neutrophils (PMN), monocytes, and macrophages are considered as the first line of defence against invasive pathogens. The formation of extracellular traps (ETs) by activated mononuclear phagocytes is meanwhile well accepted as an effector mechanism of the early host innate immune response acting against microbial infections. Recent investigations showed evidence that ETosis is a widely spread effector mechanism in vertebrates and invertebrates being utilized to entrap and kill bacteria, fungi, viruses, and protozoan parasites. ETs are released in response to intact protozoan parasites or to parasite-specific antigens in a controlled cell death process. Released ETs consist of nuclear DNA as backbone adorned with histones, antimicrobial peptides, and phagocyte-specific granular enzymes thereby producing a sticky extracellular matrix capable of entrapping and killing pathogens. This review summarizes recent data on protozoa-induced ETosis. Special attention will be given to molecular mechanisms of protozoa-induced ETosis and on its consequences for the parasites successful reproduction and life cycle accomplishment.

1. Introduction

Professional mononuclear phagocytes, such as polymorphonuclear neutrophils (PMN), monocytes, and macrophages, are considered as the first line of defence of the early host innate immune response [1, 2]. Their main function has been classically understood to kill invasive pathogens by a variety of potent intracellular microbicidal effector mechanisms [3–7]. After the first contact with pathogens, mononuclear phagocytes engulf and internalize them into their phagosomes. By the fusion with intracellular granules and the formation of phagolysosomes the pathogens may be killed intracellularly by a combination of non-oxidative and oxidative mechanisms [1, 8]. Actions of potent antimicrobial peptides, such as defensins, cathelicidins, cathepsins, pentraxin, and lactoferrin, are parts of non-oxidative killing mechanisms, while oxidative killing relies exclusively on the production of

antimicrobial reactive oxygen species (ROS) via the NADPH oxidase (NOX) complex [5]. Within blood circulating phagocytes, PMN are by far the most abundant cell population representing 50–80% of the total white blood cells in different vertebrates [5]. Moreover, after being released from the bone marrow into the blood circulation, PMN are highly mobile and short-lived phagocytes, being densely packed with secretory granules [4, 8]. PMN granules are categorized into three different types based on their contents: primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules. The types of granules to be found in circulating PMN depend on their maturation stage. Thus, PMN maturation starts with the formation of primary granules, followed by secondary and tertiary granules [4, 9, 10]. The content of primary granules includes myeloperoxidase (MPO), neutrophil elastase (NE), cathepsin G, proteinase 3, defensins, and lysozyme;

secondary granules contain collagenase, gelatinase, cystatin, lysozyme, and lactoferrin; and tertiary granules comprise gelatinase, lysozyme, and arginase amongst others [10]. Following granule maturation, PMN will possess all three types of granules displaying full killing capacity not only in the blood but also in tissues/organs and gut lumen [10].

In addition, PMN act against pathogens by actively participating in complex inflammatory networks such as the release of a broad panel of proinflammatory chemokines, cytokines, and survival- and growth-factors which trigger both downstream proinflammatory effects and the transition into adaptive immune reactions. As such, several proinflammatory cytokines/chemokines were found enhanced in activated PMN in response to apicomplexan parasites, such as TNF- α , IL-1 β , CC, and CXC chemokines (e.g., IL-8, IP-10, GRO- α , RANTES, and MIP-1 α) [11–15]. Several of PMN-derived immunomodulatory molecules can augment the production of various chemokines and cytokines to further regulate phagocyte functions [16, 17]. More importantly, by this means activated PMN recruit not only other innate immune cells but also T cells to the site of infection [18–20] or even induce sterile inflammation [21, 22].

2. Neutrophil Extracellular Traps (NETs) and Phagocyte-Derived Extracellular Traps (ETs)

Beginning with the landmark study of Brinkmann et al. [31], the paradigm of how PMN fight and kill pathogenic bacteria has profoundly been changed. The discovery of DNA-based antimicrobial neutrophil extracellular traps (NETs) changed the current knowledge on innate immune reactions not only on the level of the pathogen killing but also on the pathophysiology of metabolic, autoimmune, reproductive, and inflammatory diseases, as well as cancer progression [32–37]. NETs are released by activated PMN by a novel cell death process, called NETosis [38], which can be stimulated by a variety of molecules and invasive pathogens. Microorganisms such as bacteria [31, 39–41], fungi [42–44], viruses [45–49], and parasites [50–55] were identified as NET inducers. Also different molecules or cellular structures such as GM-CSF/complement factor 5a [56, 57], activated platelets [40, 58], PMA and zymosan [24, 26, 31, 59], singlet oxygen [60], LPS [31, 61], and Fc receptor [42] trigger NETosis. In addition, IL-8 as well-known chemoattractant for PMN was demonstrated as NET inducer [31, 62]. Efficient NETosis requires mature PMN and in most cases NOX, MPO, NE, and peptidylarginine deiminase type IV (PAD4) activities [14, 24, 59, 63–65]. Furthermore, the process of NETosis obviously requires intracellularly signalling pathways of which Raf-MEK-ERK kinases as well as p38 MAPK are being the most frequently reported to be involved in this process [14, 23, 33, 66–69]. In addition, calcium release is needed for optimal NET formation in different vertebrate species [14, 23, 70–72]. Upon stimulation of PMN, the nuclear envelope disintegrates permitting the mixture of chromatin with granular proteins/peptides [38]. NE and MPO degrade histones (H1, H2A/H2B, H3, and H4) and promote chromatin decondensation [65], mediated by PAD4 via hypercitrullinating

of specific histones to allow electrostatic coiling of the chromatin [64, 73, 74]. The total of the DNA complexes being decorated with granular proteins/peptides and specific histones (H1, H2A/H2B, H3, and H4) are finally extruded as NETs to the extracellular environment by dying PMN.

NET formation is primarily a NOX-dependent mechanism [14, 24, 59, 75, 76]. However, NOX-independent NETosis was also reported [29, 60, 67, 68, 77]. This mode of NETosis is accompanied by a substantially lower level of ERK activation and rather moderate level of Akt activation, whereas activation of p38 is similar in both kinds of NET formation [67, 68]. As an example, singlet oxygen can stimulate NETosis in a NOX-independent manner [60]. Irrespectively of NOX-dependency, pathogens may either be immobilised within sticky DNA fibres [55, 78, 79] or be killed via the local high concentration of effector molecules [31, 42, 51, 53].

Meanwhile, other types of leukocytes of the innate immune system, such as macrophages [80–83], monocytes [26, 28], mast cells [84, 85], eosinophils [55, 86, 87], and also basophils [88], have been reported to release NET-like structures which are now collectively entitled as extracellular traps (ETs).

Interestingly, Malawista et al. [89] described already many years ago that enucleated PMN may remain vital and are even capable of killing invasive microbes. More recent studies corroborated these findings proving that leukocytes do not necessarily die after ET extrusion [56, 68, 86]. In this context, Yousefi et al. [56, 86] demonstrated that eosinophils and certain PMN subpopulations release ETs of mitochondrial origin without dying. Furthermore, Yipp et al. [90] verified that PMN which had released NETs were still viable and retained their capability to engulf bacteria via phagocytosis. The precise mechanism of NOX-independent NET formation is not clear yet. However, it appears to be nonlethal for PMN and faster than NOX-dependent NET formation and to rely on a vesicular-based pathway releasing nuclear DNA [33, 68].

Additionally, different molecular pathways will lead in a stimulus-dependent manner to the extrusion of different types of ETs *in vitro* and *in vivo*. Different morphological forms of ETs were for the first time described in the human gout disease *in vivo* proving that monosodium urate crystals (MSU) induced aggregated (*agg*ETs), spread (*spr*ETs), and diffused (*diff*ETs) ET formation [91]. Consistently, also parasite-mediated ETosis resulted in different types of ETs. As such, the parasitic nematode *Haemonchus contortus* larvae triggered in ruminant PMN and eosinophils *agg*ETs, *spr*ETs, and *diff*ETs [55].

3. Apicomplexan Protozoa-Induced Formation of NETs and ETs

3.1. Plasmodiidae. While most NET- and ET-related studies focused on bacterial, viral, and fungal pathogens, little attention was paid to protozoan parasites. As such, the first ever published study on parasite-triggered NETosis was published in 2008 by Baker et al. [50] 4 years after the discovery of this novel effector mechanism [31] and reported on *Plasmodium falciparum*-triggered NET formation.

Plasmodium spp. parasites are mosquito-borne pathogens that cause malaria, a serious public health disease worldwide in the tropic and subtropics. Globally, an estimated 3.3 billion people are at risk of being infected with malaria of whom approximately 1.2 billion are at high risk (>1 in 1000 chance) of developing malarial disease [92]. The first report on *P. falciparum*-induced NETs referred to *P. falciparum*-infected children and demonstrated *in vivo* NET-entrapped trophozoite-infected erythrocytes in blood samples [50]. Moreover, Baker and colleagues [50] provided first evidence on the involvement of parasite-triggered NETs in the pathogenesis of malaria since the high levels of anti-dsDNA antibodies were above the predictive levels for autoimmunity. Interestingly, a recent study also indicates the capacity of *P. falciparum* to inhibit NET formation [93] which may be of relevance in immunopathogenesis. Thus, a mosquito-derived salivary protease inhibitor (agaphelin) induced by *P. falciparum* infection inhibited vertebrate elastase and NET formation [93]. Whether this represents a true anti-NET mechanism remains to be elucidated.

3.2. Eimeriidae. Parasites of the genus *Eimeria* are worldwide of high veterinary and economic importance in livestock, especially in chicken [94], cattle and small ruminants [95–100]. Coccidiosis is a disease with high morbidity in animals of all ages, nonetheless inducing pathogenicity especially in young animals [101] and occasionally causing death of heavily infected animals [99, 102, 103].

Several studies showed that PMN infiltrate intestinal mucosa in response to *Eimeria* infections and are occasionally found in close contact to the parasitic stages *in vivo* [102, 104–107]. PMN have also been shown to directly interact with *E. bovis* stages and antigens *in vitro*, resulting in release of proinflammatory cytokines, chemokines, and iNOS [13]. Additionally, their phagocytic and oxidative burst activities were enhanced in response to *Eimeria* stages *in vitro* and *in vivo* [13]. First indications on *Eimeria* spp. as potent NET inducers came from Behrendt and colleagues who reported on sporozoites to be entangled by an extracellular network of delicate DNA fibres being extruded from PMN *in vitro* (Figure 1(a)) [52]. Using extracellular DNA measurements and DNase treatments the authors presented strong indications that these structures were NETs. Other studies confirmed typical characteristics of NETs, such as the colocalization of NE, MPO, and histones in the DNA backbone of *Eimeria*-induced NET-like structures [23]. Meanwhile, also other pathogenic ruminant *Eimeria* species were shown to induce NETosis, such as *E. arloingi* (Figures 2(a) and 2(b)) [24, 27] and *E. ninakohlyakimovae* (Pérez, personal communication). Importantly, Muñoz-Caro and colleagues proved NETs also to occur *in vivo* in *Eimeria*-infected gut mucosa [27]. The current data suggest that *Eimeria*-induced NETosis is a species- and stage-independent mechanism, since it was induced by sporozoites, merozoites I, or oocysts of different *Eimeria* species [23, 24]. Given that PMN were described to act even in the intestinal lumen via different effector mechanisms [27, 108, 109], it appears likely that interactions of luminal PMN with ingested *Eimeria* oocysts or newly excysted sporozoites may occur [6, 23, 24]. In particular, NET-related reactions

against oocysts would have a high impact on the ongoing infection since they may hamper proper excystation of infective stages (sporozoites) and, in consequence, dampen the degree of infection at the earliest possible time point in the host. Since *E. arloingi* sporozoites must egress from the oocyst circumplasm through the micropyle [24], NETs covering this area of the oocyst will have a detrimental effect on proper excystation [6, 24]. The same explanation seems feasible for *E. bovis* and *E. ninakohlyakimovae*, regardless of the fact that excystation occurs by rupture of the oocyst walls prior to sporozoites egress from sporocysts. Although all *Eimeria* species tested so far equally induced NETs, significant differences in entrapment effectivity were reported amongst different host species, parasite species, and stages. Thus, caprine NETs immobilised a high proportion of *E. arloingi* sporozoites (72%) [24], whilst in the bovine system considerably less parasite stages (*E. bovis* sporozoites: 43%, *B. besnoiti* tachyzoites: 34%) were found entrapped in NET structures [23, 59]. So far, it remains to be elucidated whether the varying effectivity of NETs is based on the PMN origin (goats are generally considered as strong immune responders) or on the parasite species.

The molecular basis of *Eimeria*-induced NETosis is not entirely understood, so far. Enzyme activity measurements and inhibition studies revealed a key role of NOX, NE, and MPO in *Eimeria*-triggered NET formation (see Table 1) which is in agreement to bacterial, fungal, and parasitic pathogens [14, 25, 59, 65, 75, 110]. Referring to signal cascades, analyses on the grade of phosphorylation revealed a key role of ERK1/2 and p38 MAPK in sporozoite-exposed bovine PMN. Since respective inhibitor experiments led to decreased parasite-mediated NET formation, Muñoz-Caro et al. [23] proved the relevance of this signalling pathway in sporozoite-triggered NETosis. This finding is in agreement with data on *T. gondii*-mediated NET formation [25]. Referring to Ca^{2+} influx, further inhibition experiments proved *E. bovis*-mediated NETosis as dependent on intracellular Ca^{2+} mobilization, since 2-ABP (inhibitor of store-operated Ca^{2+} entry) [23] and BAPTA-AM (binding intracellular Ca^{2+} ; Muñoz-Caro, unpublished data) but not EGTA (inhibitor of Ca^{2+} influx from the extracellular compartment; Muñoz-Caro, unpublished data) significantly blocked parasite-triggered NETosis. So far, little is known on PMN-derived receptors mediating parasite-triggered NETosis. Muñoz-Caro et al. [23] reported on enhanced CD11b surface expression on PMN following *E. bovis* sporozoite exposure. By antibody-mediated CD11b blockage leading to a significant reduction of parasite-triggered NETosis, the authors proved the relevance of this receptor in the NET formation process.

Bacteria and fungi NETosis was reported as a lethal effector mechanism [31, 42]. However, killing effects of NETs were not observed in the case of *Eimeria* spp. so far. Given that *Eimeria* spp. are obligate intracellular parasites, the main function of NETs rather seems to be the extracellular immobilisation of infective stages hampering them from host cell invasion. Accordingly, reduced host cell infections rates were reported for *E. bovis* and *E. arloingi* sporozoites when previously exposed to PMN [23, 24].

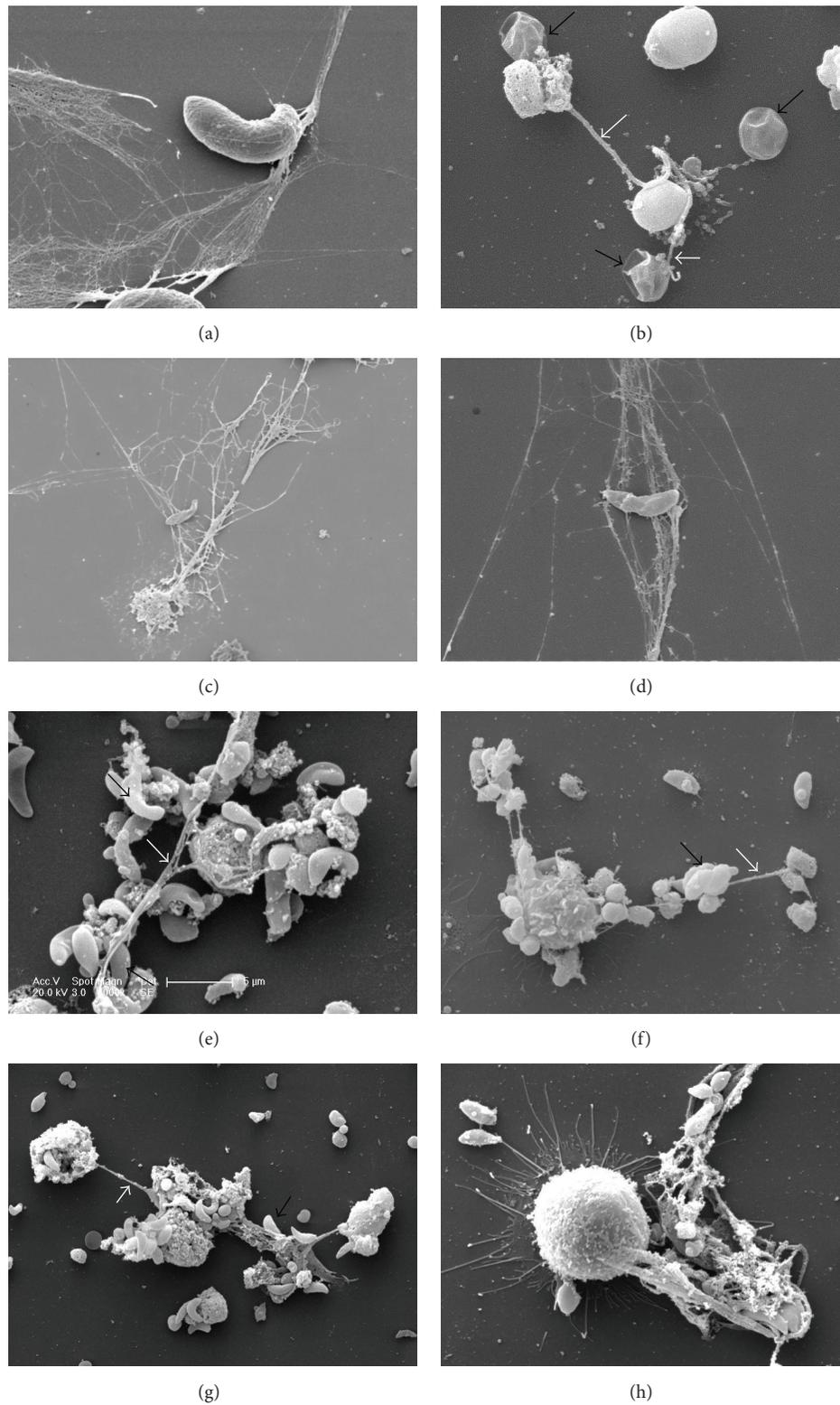


FIGURE 1: ETosis is not a parasite- nor a stage-specific cell death process (SEM analysis). (a) *Eimeria bovis* sporozoite-triggered bovine NETosis; (b) *Cryptosporidium parvum* oocysts (back arrows) induced NETosis (white arrows); (c) *Toxoplasma gondii* tachyzoites entrapped by a delicate DNA fibre derived from bovine PMN; (d) *Toxoplasma gondii* tachyzoite completely entrapped in filigree NET structures; (e) PMN-derived NETs (white arrow) after *Besnoitia besnoiti* tachyzoites encounter (black arrows); (f) *Neospora caninum* tachyzoites (black arrow) trapped in bovine NETs (white arrow); (g) monocyte-derived extracellular traps (METs) forming spread (white arrow) ETs entrapping *Besnoitia besnoiti* tachyzoites (black arrow); (h) *Besnoitia besnoiti* derived thick and thin METs.

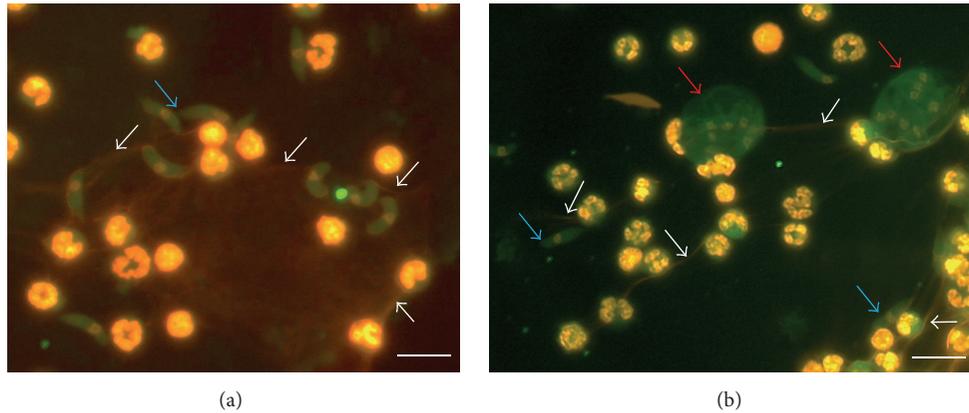


FIGURE 2: Colocalization of caprine NET-derived DNA and MPO. (a) Cocultures of caprine PMN and *Eimeria arloingi* sporozoites (blue arrows) were fixed, permeabilized, stained for DNA using Sytox Orange, and probed for MPO using anti-MPO along with adequate conjugate systems (white arrows); (b) cocultures of caprine PMN exposed to sporulated *E. arloingi*-oocysts (red arrows) and sporozoites (blue arrows). Filigree spread NET structures are indicated by white arrows. Bar scale = 20 μm .

The same feature was reported for monocyte-preexposed *E. bovis* sporozoites indicating that this leukocyte cell type also casts ETs in response to this parasite stage and that ETosis had an impact on parasite invasion [28]. Besides *E. bovis* [59], *E. arloingi* (Silva, unpublished data), and *E. ninakohlyakimovae* (Pérez et al., submitted manuscript) were also shown to induce monocytes-derived ETs. Furthermore, *E. ninakohlyakimovae*-induced monocytes-ETosis showed a rapid induction of ETs release upon viable sporozoites, sporocysts, and oocysts encounters, corroborating a stage-independent process in monocyte-derived ETosis. In addition, it was found that caprine monocyte-derived-ETosis is NOX-dependent. With the upregulation of the genes transcription encoding for IL-12 and TNF- α , relevant immunoregulatory cytokines with transition properties into the adaptive immunity [111] were also demonstrated in *E. ninakohlyakimovae*-exposed caprine monocytes (Pérez et al, submitted manuscript).

Since the reduction in infection rates early after infection automatically results in decreased proliferation of the parasite, this indirect ET-mediated effect should have a beneficial impact on the outcome of the disease. Despite advantageous properties of ETs, their ineffective clearance and/or poor regulation might also bear adverse pathological implications, leading to tissue damage in addition to enhanced local proinflammatory reactions [112, 113].

3.3. Sarcocystidae. Toxoplasmosis is caused by the facultative heteroxenous apicomplexan polyxenous protozoan *T. gondii* representing one of the most common parasitic zoonoses worldwide [114]. *Toxoplasma gondii* is well known to affect almost all warm-blooded mammals including a wide range of domestic animals, wild mammals, marine mammals, marsupials, and humans [115, 116]. In response to *T. gondii* infections, PMN are promptly recruited to the site of infection producing a variety of proinflammatory cytokines and chemokines [11, 117]. In addition, PMN are capable of killing *T. gondii* tachyzoites via phagocytosis [118, 119]. Besides this

effector mechanism, human, murine, bovine, and harbour seal (*Phoca vitulina*) PMN additionally perform NETosis in reaction to *T. gondii* tachyzoites (Figures 1(c) and 1(d)) [25, 26]. Abi Abdallah et al. [25] showed that NETosis was triggered by tachyzoites in a parasite strain-independent fashion as an invasion/phagocytosis-independent process. Interestingly, in the murine toxoplasmosis model, tachyzoites-induced NETs were not the result of a random cell lysis, but of a controlled DNA release process since lysozyme was still present in PMN after performing NETosis [25, 120]. In contrast to *Eimeria* spp., *T. gondii*-triggered NETosis had modest toxoplasmacidal effects by killing up to 25% of the parasites [25]. Considering the obligate intracellular life style of *T. gondii* and its enormous proliferative capacity in mammalian host cells, parasite entrapment via NETs might be of particular importance *in vivo* based on its interference with host cell invasion. Consistently, harbour seal PMN-promoted NETs significantly hampered host cell invasion of *T. gondii* tachyzoites *in vitro* [26]. *In vivo* evidence of *T. gondii*-induced NETosis was reported in a murine pulmonary infection model, revealing an increase of dsDNA contents in the bronchoalveolar lavage fluids of *T. gondii*-infected mice [25]. As equally reported for several other coccidian parasites [14, 23], *T. gondii*-induced NETs were also proven to be NOX-, NE-, MPO-, and Ca²⁺- (SOCE) dependent and to be mediated by an ERK 1/2-related signalling pathway in PMN (see Table 1) [25, 26]. Additionally, in earlier studies, not only the pivotal role of PMN but also the important role of monocytes in toxoplasmosis was clearly demonstrated [121–123]; however, their capacity to also induce ETs in response to tachyzoite stages was just recently demonstrated [26]. Exposure of harbour seal-derived monocytes to viable *T. gondii* tachyzoites resulted in a significant induction of monocyte-ETs and tachyzoites were firmly entrapped and immobilised within harbour seal monocyte-ET structures, hampering parasite replication [26].

Bovine besnoitiosis caused by *Besnoitia besnoiti* is an endemic disease in Africa and Asia [124–126] and considered

TABLE 1: List of apicomplexan and euglenozoan protozoa capable of inducing ETosis, host species, professional phagocytes triggering ETs, and molecular mechanisms involved in this cell death process.

Parasites	Host species	Professional phagocytes	ETosis dependency	References
<i>Eimeria bovis</i>	Bovine	PMN	NOX NE MPO CD11b ERK 1/2 p38 MAPK SOCE	Muñoz-Caro et al. [23]
<i>Eimeria arloingi</i>	Caprine	PMN	NOX	Silva et al. [24]
<i>Eimeria ninakohlyakimovae</i>	Caprine	PMN	NOX	Pérez et al. (submitted manuscript)
	Mouse	Monocytes	NOX	Pérez et al. (submitted manuscript)
<i>Toxoplasma gondii</i>	Harbour seal	PMN	ERK 1/2 NOX NE MPO SOCE	Abi Abdallah et al. [25] Reichel et al. [26]
	Bovine	PMN	NOX NE MPO	Muñoz-Caro et al. [27]
<i>Besnoitia besnoiti</i>	Bovine	PMN	NOX NE MPO	Muñoz-Caro et al. [28]
	Bovine	Monocytes	NOX MPO	Muñoz-Caro et al. [28]
<i>Neospora caninum</i>	Bovine	PMN	NOX NE MPO ERK 1/2 p38 MAPK SOCE P2Y2 PAD4	Villagra-Blanco et al. (submitted manuscript)
<i>Cryptosporidium parvum</i>	Bovine	PMN	NOX NE MPO ERK 1/2 p38 MAPK SOCE	Muñoz-Caro et al. [14]
<i>Leishmania</i> spp.	Human	PMN	NOX NE PAD4	Rochael et al. [29]
<i>Trypanosoma cruzi</i>	Human	PMN	NOX TLR2 TLR4	Sousa-Rocha et al. [30]

as emergent in Europe [127]. During the acute phase of cattle besnoitiosis, *B. besnoiti* tachyzoites mainly replicate in host endothelial cells of different organs [28, 128] and, upon release, may be exposed to circulating leukocytes. *Besnoitia besnoiti* tachyzoites were recently reported as effective inducers of PMN- and monocyte-derived ETs (Figures 1(e), 1(g), and 1(h)) [28, 59]. In the latter case, ETosis was further reported to occur as an invasion- and phagocytosis-independent process [28]. A high proportion of PMN was found to be involved in NETosis, since up to 76% of encountered PMN were found to participate in NETosis leading to the immobilisation of approximately one-third

of the parasites [59]. *Besnoitia besnoiti*-triggered NETosis furthermore proved as vitality-independent process that was even induced by soluble parasite molecules (homogenates), though at lower levels [59]. Regarding PMN-derived effector molecules, NOX, NE, and MPO proved as essential for efficient *B. besnoiti*-triggered NETosis [59]. Thus, respective enzyme activities were encountered in tachyzoite-exposed PMN and chemical blockage of these enzymes via inhibitors blocked parasite-triggered NETosis [28, 59]. In contrast to tachyzoites of *T. gondii*, entrapped *B. besnoiti* tachyzoites were neither killed by NETs nor ETs since their host cell infectivity was entirely restored upon DNase I treatments [28, 59].

Given that *B. besnoiti* tachyzoites mainly proliferate within endothelial cells during the acute phase, these parasitic stages are released via cell lysis in close proximity to endothelium and are exposed to blood contents, such as leukocytes. Several reports have shown that NETs themselves interact with endothelium and may cause endothelial damage or dysfunction [129–131]. Since activated endothelial cells may produce a broad panel of immunomodulatory molecules with IL-8 or P-selectin having been identified as potent NET inducers [129, 132], interactions between infected endothelial cells, *B. besnoiti* tachyzoites, and NETs are quite likely. Accordingly, Maksimov et al. [15] recently reported on infection-induced upregulation of endothelial-derived IL-8 and P-selectin gene transcription and furthermore presented indications on NET formation occurring adjacent to infected endothelium after PMN adhesion assays being performed under physiological flow conditions as the ones present in small vessels.

Recent NET-related investigations on the closely related cyst-forming apicomplexan protozoa *Neospora caninum* have shown that bovine PMN exposed to viable tachyzoites also result in strong NETosis (Figure 1(f)). With regard to molecular mechanisms, *N. caninum*-triggered NETosis seems to be P2Y2-, NOX-, SOCE-, MPO-, NE-, ERK1/2-, p38 MAPK-, and PAD4-dependent (Villagra-Blanco et al., submitted manuscript).

3.4. Cyptosporiidae. *Cryptosporidium parvum* is an euryxenous apicomplexan parasite with worldwide distribution and high zoonotic potential, mainly affecting young children, immunocompromised humans, and neonatal livestock [133]. Typically, cryptosporidiosis is a water- and food-borne enteric disease that causes diarrhoea, dehydration, weight losses, and abdominal pain and leads to significant economic losses in the livestock industry [133, 134]. After ingestion, sporozoites are released from oocysts into the intestinal lumen and infect small intestine epithelial cells [135]. Recent studies reported on a significant contribution of PMN and macrophages to inflammatory responses in cryptosporidiosis *in vivo* [136, 137]. Muñoz-Caro and colleagues reported on NETs being cast by both bovine and human PMN in response to *C. parvum* stages [14]. Parasite-triggered NETosis proved stage-independent since it was induced by both sporozoites and oocysts (Figure 1(b)). Especially in the latter case parasite stages were occasionally entirely covered with NET structures thereby most probably hampering proper sporozoite excystation [14]. Given that PMN were shown as active even within the intestinal lumen [108, 109, 138, 139], these reactions should have a significant impact on ongoing *in vivo* infection. *In vitro* infection experiments additionally showed the negative impact of NETs on host cell invasion since infection rates were significantly reduced when using PMN-preexposed *C. parvum* stages [14]. The fact that these reactions were entirely reversible via DNase I treatments rather argued against any cryptosporidicidal effects of NETs [14]. The colocalization of NE, histones, and MPO with DNA in parasite-mediated extracellular fibres proved classical characteristics of NETs and inhibitor experiments emphasized the key role of NE, NOX, and MPO in efficient NET formation [14].

In agreement with findings on *Eimeria*-induced NETosis, inhibition experiments revealed *C. parvum*-triggered NET formation as dependent on intracellular Ca^{2+} release and ERK 1/2 and p38 MAPK-mediated signalling pathways [14]. Interestingly, *C. parvum* sporozoite-exposed bovine PMN showed increased gene transcription of proinflammatory molecules, some of which were recently shown as potent NET inducers (e.g., IL-8 and TNF- α) [140, 141] and may have potentiated NET reactions.

4. Euglenozoan Protozoa-Induced NETs

4.1. Trypanosomatidae. Infections with *Leishmania* spp. represent a major health problem and according to the WHO [92] 10% of the human world population is at risk of infection, meaning that approximately 12 million people in 98 countries are infected, and 2 million new cases occur each year [142, 143]. Leishmaniasis is a vector-transmitted zoonosis caused by more than 25 different obligate intracellular protozoan *Leishmania* species [142–144]. Particularly PMN have been implicated in the immunopathogenesis of leishmaniasis [145–149] and recent studies examined the potential role of NETs during the early phase of the disease of different *Leishmania* species. Guimarães-Costa et al. [51] showed for the first time that promastigotes of *Leishmania amazonensis*, *L. major*, and *L. chagasi* were capable of triggering NET formation. Additionally, *Leishmania*-triggered NETosis seems not entirely stage-specific, since both promastigotes (*L. amazonensis*, *L. major*, *L. chagasi*, *L. donovani*, *L. mexicana*, and *L. braziliensis*) and amastigotes (*L. amazonensis*, *L. braziliensis*) promoted NET formation *in vitro* and *in vivo* [51, 147, 150–152]. More importantly, Guimarães-Costa et al. [51] provided first indications on possible parasite-specific ligands being responsible for *Leishmania*-mediated NETosis. Thus, *Leishmania*-derived lipophosphoglycans (LPG) were suggested as the main trigger of NET release since these molecules also induced NETs in a purified form. The former authors showed that NETs possessed detrimental effects on parasites as NET-entrapped *L. amazonensis* promastigotes exhibited decreased viability [51]. Authors also demonstrated that the extracellular DNA and histones found on NETs were involved in the parasite inactivation/killing process [51]. The leishmanicidal effects of histones were proven in promastigotes cocultures with purified H2A histones leading to the killing of parasites and by a significant reduction of leishmanicidal effects when cocultured in the presence of anti-histone antibodies. Additionally to H2A histone killing effects, Wang et al. [153] demonstrated that also the histone H2B could directly and efficiently kill promastigotes of *L. amazonensis*, *L. major*, *L. braziliensis*, and *L. mexicana*.

In case of *L. donovani*, Gabriel et al. [150] reported NETosis as a ROS-dependent process which was equally triggered in human and murine PMN (see Table 1). However, *Leishmania*-lipophosphoglycan- (LPG-) dependent NET induction reported by Guimarães-Costa et al. [51] was not observed with *L. donovani*. When using genetically modified *L. donovani* promastigotes Gabriel et al. [150] observed a lipophosphoglycan- and GP63-independent (promastigote surface metalloprotease) NETosis pathway. Nonetheless, in

this infection system, LPG appeared to be involved in the resistance to NETs-mediated killing, since the wild type of *L. donovani* maintained its viability in the presence of NETs, whilst mutant parasites lacking LPG were efficiently killed by these extracellular structures [150].

A more recent study revealed that *Leishmania* parasites trigger not only the classical ROS-dependent NETosis as previously demonstrated but also a ROS-independent form, named as early/rapid vital NETosis [29]. During this early/rapid *Leishmania*-triggered NETosis, in which NET formation takes place after 5–15 min of activation without affecting PMN viability [29, 68], the parasites are also being efficiently entrapped.

Regarding NET-related evasion strategies of Trypanosomatidae parasites, *Leishmania* spp. seem capable of evading NET killing by firstly blocking the oxidative burst activity of PMN or even by resisting microbicidal activity of NETs [145, 150]. Moreover, Guimarães-Costa et al. [152] showed that *L. infantum* promastigotes express the enzyme 3'-nucleotidase/nuclease which was previously described to be involved in parasite nutrition and infection and was proven to be part of the ability of promastigotes to escape NET-mediated killing. A recent investigation has shown that a salivary component of the sand fly insect that transmits leishmaniasis may also play a role in the survival of *Leishmania* in the definitive hosts, by modulating their innate immune system. A molecule named Lundep from the salivary gland of *Lutzomyia longipalpis* was recently described as an endonuclease with NET-destroying properties in humans [145]. In the presence of Lundep, human NETs were disrupted, thus increasing *L. major* survival rates [145]. Furthermore, Chagas et al. [145] measured the NE release from NETs as an indicator of NET destruction, since NE is normally decorating NETs backbone structures and found at low concentrations in culture supernatants, as previously demonstrated [39]. Lundep was responsible for the significant increase of NE concentration in the supernatants when compared to negative controls [145]. In conclusion, these experiments showed degradation of DNA scaffold of NETs, destroying their functional integrity, and increasing promastigote survival and exacerbating *L. major* infection.

American trypanosomiasis or Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*. Approximately eight million people are affected by this tropical disease in the Americas and an average of 12,000 deaths per year is known to occur due to American trypanosomiasis [154]. It is well known that macrophages, eosinophils, monocytes, and PMN are implicated in the control of early infection [30, 155]. Recently, Sousa-Rocha et al. [30] demonstrated *in vitro* that *T. cruzi* is able to trigger NETs in a dose-, time-, and ROS-dependent manner. In agreement with reports on *Eimeria* spp. and *B. besnoiti* [23, 24, 59] but in contrast to observations on *T. gondii* and *Leishmania* spp. [25, 51], the viability of *T. cruzi* stages was not affected by NETs, but NETosis significantly impaired the parasite host cell infectivity. In fact, NETs components as NE may affect *T. cruzi* infectivity, since this enzyme appears to be involved in increased trypanocidal activity and in the reduction of trypomastigote release by prestimulated infected macrophages [30, 156]. Additionally,

the authors showed via antibody-mediated blockage that *T. cruzi*-triggered NETosis is a TLR2- and TLR4-dependent process. Moreover, the study showed that not only viable *T. cruzi* trypomastigote forms but also soluble antigens and killed *T. cruzi* parasites induced NET release in human PMN. *In vivo* murine studies indicated the relevance of NETosis for the outcome of trypanosomiasis since significantly decreased parasites numbers were found in the blood system of those animals which had previously been infected with NETs-pretreated parasites [30].

5. Conclusions

During the last years a vast amount of data on protozoan-mediated ETosis was published strengthening the role of this effector mechanism in the defence of parasitic infections. Several *in vivo* data have now proven the existence and importance of this early host innate effector mechanism. However, there is still a total lack of information on parasite-derived ligands triggering ETosis. Taking into account that in most cases ET formation is considered as a species- and stage-independent process, rather ubiquitous occurring molecules may represent parasite-derived target molecules of ETs. Moreover, recent data revealed that other leukocytes such as monocytes, macrophages, basophils, mast cells, and eosinophils also perform ETosis upon pathogen encounter. However, respective data on parasite-induced mechanism are scarce. Furthermore, ET-related research mainly focused on the leukocytes aptitude to impact the parasites life cycle, but not on the propensity of parasitic stages to develop counter mechanisms for ETs avoidance. While a bunch of data is available on bacterial nucleases or other counter mechanisms, parasites have been neglected on this topic. Taken together, we call for more parasite-related studies in the exciting field of ETosis.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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