The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps

CARLOS HERMOSILLA[1]*, TAMARA MUÑOZ CARO[1], LILIANA M. R. SILVA[2], ANTONIO RUIZ[3] *and* ANJA TAUBERT[1]

[1] *Institute of Parasitology, Justus Liebig University Giessen, Giessen, Germany*
[2] *ICAAM – Instituto Ciências Agrárias e Ambientais Mediterrânicas, IIFA/Universidade de Évora, Portugal*
[3] *Department of Animal Pathology, Parasitology Unit, University of Las Palmas de Gran Canaria, Spain*

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SUMMARY

The capacity of polymorphonuclear neutrophils (PMN) and other leucocytes of the innate immune system to expel their DNA in a controlled process into the extracellular environment to trap and kill pathogenic microorganisms led to a paradigm shift in our comprehension of host leucocyte-pathogen interactions. Formation of neutrophil extracellular traps (NETs) has recently been recognized as a novel effector mechanism of the host innate immune response against microbial infections. Meanwhile evidence has arisen that NET formation is a widely spread mechanism in vertebrates and invertebrates and extends not only to the entrapment of microbes, fungi and viruses but also to the capture of protozoan and metazoan parasites. PMN produce NETs after stimulation with mitogens, cytokines or pathogens in a controlled process which depends on reactive oxygen species (ROS) and the induction of the Raf-MEK-ERK-mediated signalling pathway cascade. NETs consist of nuclear DNA as a backbone decorated with histones, antimicrobial peptides, and PMN-specific granular enzymes thereby providing an extracellular matrix capable of entrapping and killing invasive pathogens. This review is intended to summarize parasite-related data on NETs. Special attention will be given to NET-associated mechanisms by which parasites, in particular apicomplexa, might be hampered in their ability to reproduce within the host cell and complete the life cycle.

Key words: Neutrophil, neutrophil extracellular traps, innate immunity, parasite infection, DNA, histones.

INTRODUCTION

The main function of mononuclear phagocytes, such as polymorphonuclear neutrophils (PMN), monocytes and macrophages, in the innate immune defence has been classically understood as a variety of potent intracellular microbicidal mechanisms to kill invasive pathogens (Bainton *et al*. 1971; Borregaard and Cowland, 1997; Nathan, 2006; Brinkmann and Zychlinsky, 2007; von Kockritz-Blickwede and Nizet, 2009). Upon first contact with the pathogen, phagocytes engulf microbes and internalize them into their phagosomes. Efficient phagocytosis is enhanced by prior opsonization of the pathogens with complement factors or, in the re-exposed host, by specific antibodies recognizing epitopes on the pathogen surface. Subsequently, phagosomes must fuse with intracellular granules to form the phagolysosome, within which the pathogen will be killed by a combination of non-oxidative as well as oxidative mechanisms. The efficient non-oxidative killing mechanisms of phagocytes include antimicrobial peptides (AMPs) such as cathelicidins, defensins, cathepsins and proteases, whereas oxidative killing relies on the production of antimicrobial reactive oxygen species (ROS) via the NADPH oxidase complex (Nathan, 2006; von Kockritz-Blickwede and Nizet, 2009). PMN are the most abundant members of the phagocyte population, comprising between 50 and 80% of total white blood cells (Nathan, 2006; Hahn *et al*. 2013). PMN are highly mobile and short-lived leucocytes which are densely packed with secretory granules. They are able to respond to pathogens immediately after they have left the bone marrow. Therefore, PMN are considered a pivotal component of the host innate immune system representing the first line of defence against pathogens, as they are the first cells to be recruited to the site of infection (Brinkmann *et al*. 2004; Ermert *et al*. 2009; Brinkmann and Zychlinsky, 2012; Hahn *et al*. 2013).

Neutrophil extracellular traps

Beginning with the landmark study of Brinkmann *et al*. (2004), the paradigm of how PMN kill pathogenic bacteria has profoundly changed. The discovery of DNA-based antimicrobial neutrophil extracellular traps (NETs) has implications for our current knowledge concerning not only invasive pathogens but also the pathophysiology of infection

* Corresponding author: Institute of Parasitology, Justus Liebig University Giessen, Giessen, Germany. E-mail: Carlos.R.Hermosilla@vetmed.uni-giessen.de

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and inflammatory diseases (Logters *et al*. 2009; Hahn *et al*. 2013). Detailed analyses of these novel NET structures revealed that they consist of nuclear DNA as a backbone being decorated with histones, antimicrobial peptides and proteins derived from at least three PMN granule types (azurophilic, secondary and tertiary), such as neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin, lactoferrin, gelatinase, bacterial permeability-increasing protein (BPI), cathepsin G, peptidoglycan recognition proteins (PGRPs) and calprotectin (Bainton *et al*. 1971; Borregaard and Cowland, 1997; Brinkmann and Zychlinsky, 2007, 2012; von Kockritz-Blickwede and Nizet, 2009; Hahn *et al*. 2013). By concentrating these highly active components in a small area NETs provide a unique extracellular matrix capable not only of entrapping but also of killing invasive pathogens (Fuchs *et al*. 2007; Ermert *et al*. 2009; Abi Abdallah and Denkers, 2012; Hahn *et al*. 2013) with the advantage of minimized damage to the surrounding tissue (Logters *et al*. 2009; Hahn *et al*. 2013). NETs have been described so far in a wide range of different species such as humans (Gupta *et al*. 2005), mice (Beiter *et al*. 2006; Buchanan *et al*. 2006; Wartha *et al*. 2007; Ermert *et al*. 2009), horses (Alghamdi and Foster, 2005), cows (Lippolis *et al*. 2006; Behrendt *et al*. 2010), fish (Palic *et al*. 2007), cats (Wardini *et al*. 2010), chickens (Chuammitri *et al*. 2009) and insects (Altincicek *et al*. 2008). Furthermore, NETs are not exclusively involved in trapping pathogens (Urban *et al*. 2006; Brinkmann and Zychlinsky, 2007) but also in severe sepsis (Logters *et al*. 2009), preeclampsia (Gupta *et al*. 2005), reproduction disorders (Alghamdi and Foster, 2005) and autoimmune diseases (Logters *et al*. 2009). Recently, other types of leucocytes of the innate immune system, such as eosinophils (Yousefi *et al*. 2008), mast cells (von Kockritz-Blickwede *et al*. 2008) and macrophages (Aulik *et al*. 2012; Hellenbrand *et al*. 2013), have also been reported to extrude NET-like structures which are collectively entitled extracellular traps (ETs).

NETs are released by a novel 'suicidal' cell death pathway called NETosis, different from apoptosis and necrosis, which allows PMN to kill pathogens far beyond their lifespan (Brinkmann and Zychlinsky, 2007). Interestingly, a recent investigation demonstrated that certain PMN released NETs *in vivo* without undergoing cell death while maintaining their crawling and phagocytic activity (Yousefi *et al*. 2009; Yipp *et al*. 2012). Upon stimulation, PMN produce ROS, such as O_2^-, H_2O_2 and HOCl, which are antimicrobial and essential for NET formation (Brinkmann and Zychlinsky, 2007, 2012; Fuchs *et al*. 2007). Consequently, PMN from patients with chronic granulomatous disease (CGD), who lack functional NADPH oxidase, are not capable of forming NETs (Fuchs *et al*. 2007). During NETosis several nuclear and cytoplasmic events have to occur in order to initiate complete and proper NET extrusion. Firstly, NADPH oxidase-dependent ROS production leads to morphological changes such as delobulation of the PMN nucleus, disassembly of the nuclear envelope and degradation of the granule membranes (Fuchs *et al*. 2007). In addition, peptidylarginine deiminase (PAD)-mediated histone citrullination, followed by chromatin decondensation seem to be necessary for NET formation (Wang *et al*. 2009; Abi Abdallah and Denkers, 2012; Hahn *et al*. 2013). After the disassembly of nuclear and granule membranes, the mixture of both nuclear and granule content proteins, i.e. antimicrobial peptides and proteins, will occur prior to the extrusion of protein/histone-decorated NET structures into the extracellular space (Fig. 1). Most studies on NET formation strengthened the key role of a functional NADPH oxidase system. Nonetheless, myeloperoxidase (MPO) and NE also seem to be able to regulate proper NET release (Brinkmann and Zychlinsky, 2012). The signalling pathway involved in NETosis was shown to be Raf-MEK-ERK-dependent (Hakkim *et al*. 2011). Molecules known so far to induce NET formation include PMA, GM-CSF/LPS, LPS, IL-8, Ca^{2+} ionophores, thapsigargin, chemotactic complement-derived peptide complement factor 5 (C5a), TNF, IFN, lipophosphoglycan (LPG) of *Leishmania* spp. promastigotes, *Staphyloccocus epidermidis* δ-toxin, autoantibodies and LPS-activated platelets (von Kockritz-Blickwede and Nizet, 2009; Cogen *et al*. 2010; Guimarães-Costa *et al*. 2011; Abi Abdallah and Denkers, 2012; Brinkmann and Zychlinsky, 2012; Hahn *et al*. 2013). So far, data on NETosis appear to be focused on fungal and bacterial pathogens, such as *Aspergillus fumigatus*, *Aspergillus nidulans*, *Candida albicans*, *Cryptococcus neoformans*, *Escherichia coli*, *Helicobacter pylori*, *Histophilus somni*, *Listeria monocytogenes*, *Mannheimia haemolytica*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and on feline leukaemia virus among others (Brinkmann *et al*. 2004; Beiter *et al*. 2006; Urban *et al*. 2006; Grinberg *et al*. 2008; Bianchi *et al*. 2009; Ramos-Kichik *et al*. 2009; Urban *et al*. 2009; Aulik *et al*. 2010; Bruns *et al*. 2010; Wardini *et al*. 2010; Guimarães-Costa *et al*. 2011; Hakkim *et al*. 2011; Aulik *et al*. 2012; Hahn *et al*. 2013; Hellenbrand *et al*. 2013). In the present review, we focus on exciting recent NET-related research dealing with different parasite species.

PARASITE-INDUCED NET FORMATION

While most NET studies have focused on the effects of NET formation on bacterial and fungal pathogens, little attention has been paid to the role of NETs in the early host innate immune response against protozoan and metazoan parasites. As such, the first report on parasite-triggered NETosis was published in 2008, i.e. 4 years after the discovery of this

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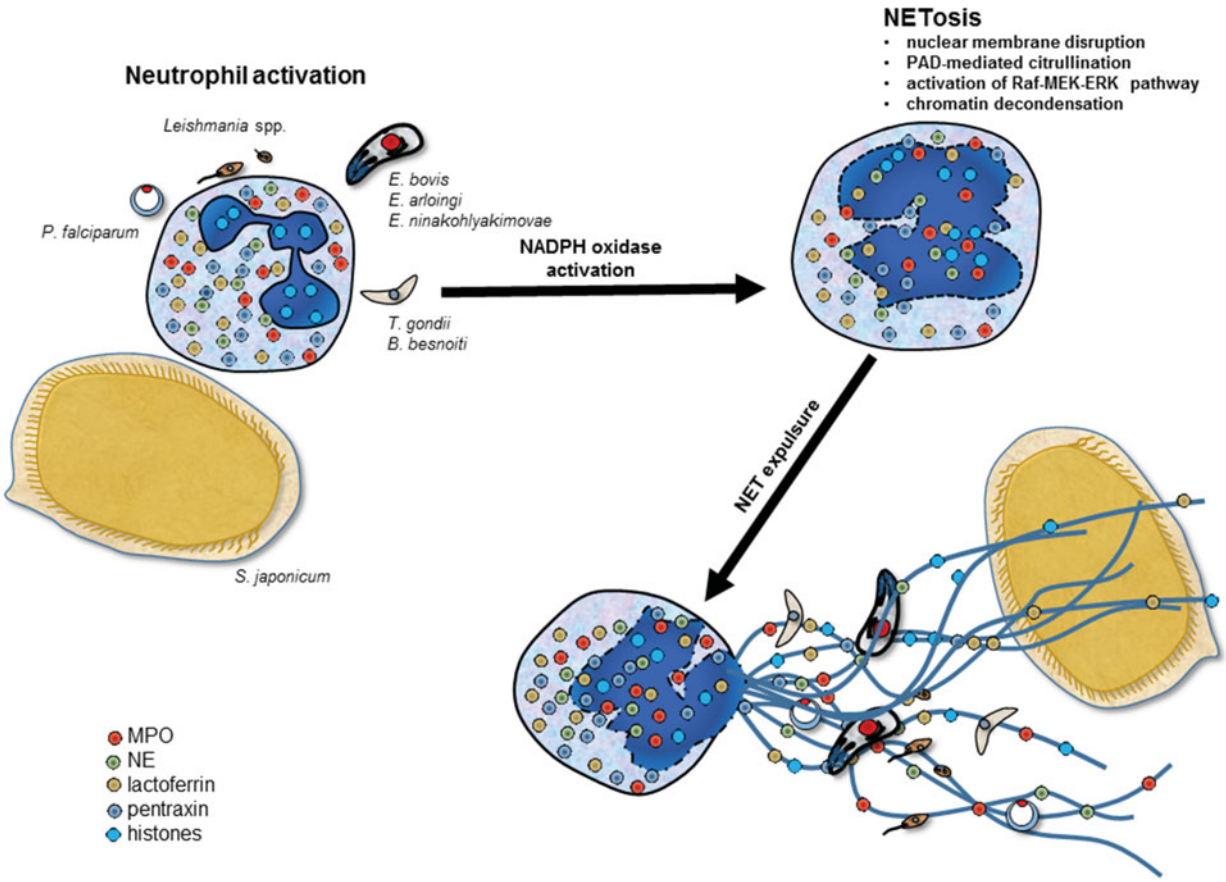

Fig. 1. Mechanisms of parasite-triggered neutrophil extracellular traps (NETs) release. PMN become activated by the contact with different protozoan parasite stages, such as trophozoites of *Plasmodium falciparum*, sporozoites of *Eimeria bovis*, *E. arloingi* or *E. ninakohlyakimovae*, tachyzoites of *Toxoplasma gondii* or *Besnoitia besnoiti*, amastigotes/promastigotes of *Leishmania* spp. and eggs of the metazoan parasite *Schistosoma japonicum*. Stimulation of PMN results in the activation of NADPH oxidase and the intracellular production of reactive oxygen species (ROS), PAD and Raf-MEK-ERK pathway activation. ROS molecules are required for the novel cell death pathway of NETosis, which is mainly characterized by the disintegration of the nuclear membrane envelope and granule membranes, chromatin decondensation, and the mixing of nuclear contents with cytoplasmic granular contents. As a final step, nuclear and granular components are expulsed by a cytoskeleton-dependent shrinkage of the dead PMN. Released NET structures studded with antimicrobial peptides, histones and proteases, have the capability to entrap, kill or immobilize the different parasite stages, whilst also initiating pro-immunoinflammatory innate immune reactions to recruit more leucocytes to the site of infection.

new effector mechanism. Until now, NET formation was described as induced mainly by protozoan parasite species, such as the euglenozoan *Leishmania amazonensis*, *Leishmania major*, *Leishmania braziliensis*, *Leishmania chagasi* and *Leishmania donovani* (Guimarães-Costa *et al*. 2009; Gabriel *et al*. 2010; Guimarães-Costa *et al*. 2011; Wang *et al*. 2011) and the apicomplexans *Plasmodium falciparum*, *Eimeria bovis* and *Toxoplasma gondii* (Baker *et al*. 2008; Behrendt *et al*. 2010; Abi Abdallah *et al*. 2012). So far, the only report of NET formation in response to a helminth parasite refers to the metazoan trematode *Schistosoma japonicum* (Chuah *et al*. 2013).

With the exception of *S. japonicum*, the parasites which are known to trigger NETosis are obligate intracellular parasites. This raises the question of how the extracellularly acting mechanism of NETs may have an impact on these pathogens. However, these parasites do not spend their entire life cycles inside the host cell. First, between entering the host and invading appropriate host cells intracellular parasites are in the extracellular space, and particularly sporozoites of *P. falciparum* and *E. bovis* have to move into host compartments by breaching cell plasma membranes to find and invade their final primary host cells (Mota *et al*. 2001; Behrendt *et al*. 2004). Second, the intracellular parasites must leave the primary host cell in order to successfully infect new cells. At both these points the parasites are vulnerable to leucocytes.

PLASMODIUM FALCIPARUM

Malaria in humans is an important febrile disease, caused by the genus *Plasmodium*. Annual cases

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worldwide are estimated to be in the range of 215–659 million (Breman and Brandling-Bennett, 2011).

The first evidence of *Plasmodium*-induced NET formation came from *P. falciparum*-infected children. In an African field study, blood samples of young patients with active malaria infections were tested for the presence of NETs (Baker *et al*. 2008). Baker *et al*. (2008) found that all children tested showed infected erythrocytes and trophozoites sticking to fibrous extracellular structures which were identified as NETs by DNA staining (Baker *et al*. 2008). These NET structures were circulating in the blood and often contained entrapped merozoite- and trophozoite-carrying erythrocytes. Furthermore, this investigation provided the first evidence of the potential involvement of NETs in the immunopathogenesis of malaria; patients had higher levels of antibodies against dsDNA which were above the predictive levels for autoimmunity (Baker *et al*. 2008). However, further studies clarifying the actual role of NETs in malaria immune defence or immunopathogenesis are lacking so far. Nevertheless, the concept that PMN-derived extracellular chromatin not only carries antiparasitic molecules, but may also carry molecules involved in autodestructive immune effector mechanisms, provides novel insights into the nature of innate immune responses against *P. falciparum* and other malaria parasite species. NETs may be considered as a double-edged sword, which functions not only as an effective antimicrobial first-line defence machinery but might also promote organ failure and even death in the absence of counter-regulation mechanisms (Logters *et al*. 2009).

EIMERIA BOVIS

Infections with different species of the apicomplexan genus *Eimeria* represent one of the most important parasitoses in livestock. Eimeriosis in cattle, also known as coccidiosis, is an important enteric parasitosis causing high economic losses and severe disease in calves (Faber *et al*. 2002; Daugschies and Najdrowski, 2005; Hermosilla *et al*. 2012). PMN appear to play a pivotal role in *E. bovis* defence. This leucocyte population was identified in parasitized intestine, of *E. bovis*-infected calves (Friend and Stockdale, 1980). PMN have been shown to interact directly with *E. bovis* stages and antigen, resulting in direct elimination (Behrendt *et al*. 2008) or production of pro-inflammatory cytokines (e.g. IL-6, IL-12, TNFα), chemokines (e.g. CXCL1, CXCL8, CXCL10) and iNOS upon encounter (Behrendt *et al*. 2008). Additionally, PMN were shown to adhere to *E. bovis*-infected endothelial cell layers (Hermosilla *et al*. 2006) and their phagocytic and oxidative burst activities were enhanced in response to *E. bovis* sporozoites *in vitro* or *in vivo* during infection (Behrendt *et al*. 2008). In 2010, NETs were discovered as an additional effector mechanism of PMN driven by encounters with *E. bovis* sporozoites (Behrendt *et al*. 2010). As also illustrated in Fig. 2A, scanning electron microscopy (SEM) analyses revealed that sporozoites of *E. bovis* were covered and entrapped within an extracellular network of long drawn-out and delicate fibres originating from dead and disrupted PMN. The DNA-based nature of *E. bovis*-induced NETs was shown by Sytox Orange staining and DNase treatment. Fluorescence images showing brightly stained fibres and the complete loss of fluorescence in DNase-treated samples corroborated the classical backbone structure of NETs (Behrendt *et al*. 2010). So far, no data are available on the parasite or PMN molecules involved in *E. bovis*-induced NETosis.

Interestingly, recent analyses doubt a strict species-specificity of *Eimeria*-induced NETosis and rather argue for a general phenomenon, since NET production was induced by (the strictly host specific) *E. bovis* sporozoites in caprine PMN, and bovine PMN also expelled NETs in response to a non-bovine *Eimeria* spp. (*Eimeria arloingi*; Muñoz Caro, unpublished data). Treatment with an NADPH oxidase inhibitor significantly reduced *E. bovis*-triggered NET formation, confirming the NADPH oxidase-dependence of NETosis, which is in agreement with data generated by other authors (Brinkmann *et al*. 2004; Urban *et al*. 2006; Brinkmann and Zychlinsky, 2007; Fuchs *et al*. 2007). *Eimeria bovis* sporozoites appear to be a potent inducer of NETosis since the degree and kinetics of NET production were much greater and faster, respectively, than NET formation induced by the generally used positive control, PMA. This observation was in accordance with data on *S. aureus* (Fuchs *et al*. 2007). Interestingly, the strongest NET formation occurred in response to viable sporozoites of *E. bovis* when compared with heat-inactivated sporozoites or their lysates. Similar findings were recently reported regarding NETosis in response to the closely related parasite *T. gondii* (Abi Abdallah *et al*. 2012) indicating that most probably not only parasite movement enhances NETosis but also certain molecules present at the surface or in excretory/secretory material can trigger this effector mechanism. However, so far no data are available on the nature of these molecules, neither in *Eimeria* nor in *Toxoplasma*.

In contrast to some bacterial pathogens, *E. bovis*-triggered NETosis resulted in the immobilization of sporozoites rather than having lethal effects since killing of these parasitic stages was not observed (Behrendt *et al*. 2010). Importantly, functional host cell inhibition assays using sporozoites pre-exposed to PMN indicated that NETosis significantly altered sporozoite infectivity (but not their viability) since subsequent infection rates were dramatically reduced (up to 65%). This effect alone may substantially affect

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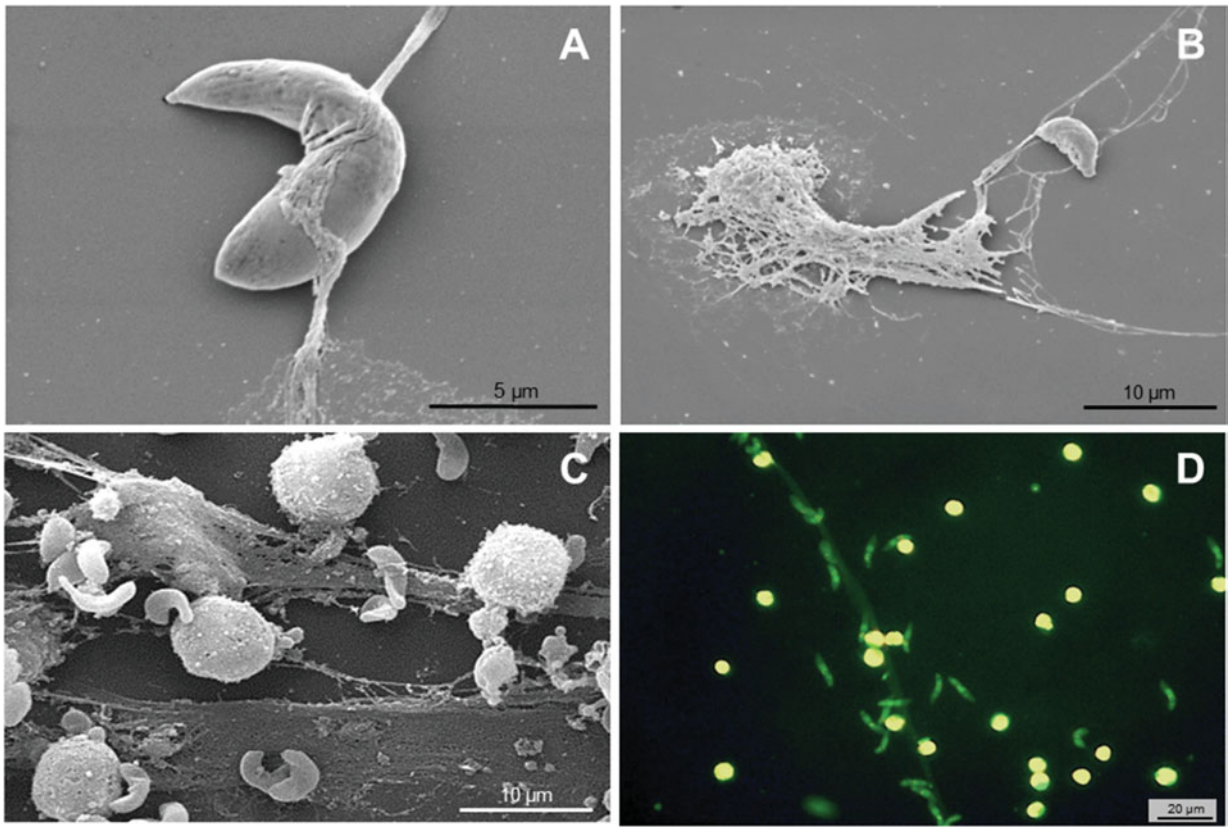

Fig. 2. NET formation triggered by different coccidian (*Eimeria bovis*, *E. arloingi*, *Toxoplasma gondii*, *Besnoitia besnoiti*) species. Bovine PMN were co-incubated with sporozoites of *E. bovis*/*E. arloingi* and tachyzoites of *T. gondii*/*B. besnoiti*, respectively, and thereafter analysed by scanning electron microscopy or fluorescence microscopy. (A) Detailed view of an *E. bovis* sporozoite firmly entrapped by thick bundles of NETs; (B) *T. gondii* tachyzoite entrapped in a network of long expulsed fibres originating from a dead PMN; (C) *B. besnoiti* tachyzoites captured within NETs; (D) co-localization of DNA and histone (H3) in caprine NETs capturing CFSE-stained *E. arloingi* sporozoites.

the success of ongoing infection and replication within the host and ameliorate the disease, since the pathogenicity of *E. bovis* infections mainly relies on later infection phases such as the gamogony. Overall, these data strongly suggest PMN to carry out their role as active leucocytes of early host innate immune responses against *E. bovis* by forming NETs in order to immobilize sporozoites and prevent them from invading host cells.

We have recently extended NET-associated analyses to other *Eimeria* species and have demonstrated that sporozoites of *E. arloingi* (Fig. 2D) and *Eimeria ninakohlyakimovae* (both caprine *Eimeria* species) also potently trigger NETosis in caprine PMN (Silva, unpublished data). The same phenomenon occurs with sporozoites of *Cryptosporidium parvum* (Muñoz Caro and Lendner, personal communication) and sporozoites of *T. gondii* (Muñoz Caro, unpublished data) suggesting NETosis as a general effector mechanism directed against this apicomplexan stage. Interestingly, the oocyst stages of *E. arloingi* and *C. parvum* have also been revealed as potent triggers of NETosis (Silva and Lendner, personal communication) indicating that NET formation may not represent a stage-specific defence mechanism. This has also been demonstrated for different *Leishmania* stages (Guimarães-Costa *et al*. 2009). Given that active PMN are localized in the mucus of intestinal mucosa (Szabady and McCormick, 2013), the inhibition of sporozoite release from oocysts through NET coverage may substantially prevent parasite infection at the earliest possible time point in the host. Consequently, further analyses on other stages, such as oocysts and merozoites, are urgently needed to clarify this question. The fact that merozoites (tachyzoites) of *T. gondii* also trigger NETosis (Abi Abdallah *et al*. 2012) provides further indications on a non-stage-specific mechanism.

TOXOPLASMA GONDII AND OTHER FAST PROLIFERATING TACHYZOITES

Toxoplasmosis is one of the most common parasitic zoonoses worldwide. Its causative agent, *T. gondii*, is a facultative heteroxenous, polyxenous protozoon that possesses the capability to infect almost all warm-blooded mammal hosts, including humans, domestic animals, wild mammals and marine

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mammals (Tenter *et al*. 2000; Dubey, 2009). As descibed for other apicomplexan parasites, there is substantial evidence that PMN play a key role during *T. gondii*-infections, since they are rapidly recruited to the site of infection and produce a variety of pro-inflammatory cytokines and chemokines in response to this parasite (Bliss *et al*. 1999, 2000). Moreover, several data support evidence that PMN are capable of efficiently killing *T. gondii*-tachyzoites *in vitro* (Wilson and Remington, 1979; MacLaren and De Souza, 2002; MacLaren *et al*. 2004). First evidence of *T. gondii* tachyzoite-induced NET formation was suggested by NET-like structures (Fig. 2B) being observed in PMN/tachyzoite co-cultures via SEM analyses (Taubert, 2011). Abi Abdallah *et al*. (2012) then clearly proved that this effector mechanism occurs in different experimental set-ups. In this study thioglycollate-induced peritoneal murine PMN were used which underwent NETosis in response to *T. gondii* tachyzoites. By illustrating the co-localization of histones and DNA in filamentous structures the classical structures of NETs were demonstrated in this system. As described above for *Eimeria* sporozoites, NETosis appeared not to be host-specific since human PMN and murine PMN also responded via NET formation against *T. gondii* tachyzoites. Abi Abdallah *et al*. (2012) also showed that the release of murine NETs was a controlled process and not the result of random cell death by providing evidence that PMN retained intracellular lysozyme after NETosis induction. Interestingly, they also showed that NETosis occurred irrespective of the *T. gondii*-strain, since all three major genotypes of *T. gondii* induced NETs in a comparable manner. In order to exclude that NET formation was due to parasite invasion, the authors pre-exposed tachyzoites with cytochalasin D to inhibit this cytoskeleton-dependent process and showed that *T. gondii*-induced NET formation actually was a parasite phagocytosis-independent process (Abi Abdallah *et al*. 2012). In contrast to reports dealing with the sporozoite stage of apicomplexan parasites (Behrendt *et al*. 2010), NETs appeared to exhibit certain lethal effects on the tachyzoite stage, since 25% of tachyzoites within NET structures were killed (Abi Abdallah *et al*. 2012). The difference in the parasitocidal effects of NETs of different parasite stages may be based on the fact that the sporozoite stage is much larger in size and more resistant through its thicker pellicula when compared with tachyzoite stages. As such, it is well known that *T. gondii* tachyzoites do not survive gastric conditions when orally ingested (Tenter *et al*. 2000; Dabritz and Conrad, 2010), whilst sporozoites of *Eimeria* are more resistant to adverse conditions.

Besides *in vitro* data, Abi Abdallah *et al*. (2012) also provided indications on the *in vivo* relevance of *Toxoplasma*-triggered NETosis. A murine pulmonary model of infection was developed, in which parasites were applied intranasally. Besides significant PMN recruitment into the lungs, increased amounts of NETs were measured in the bronchoalveolar lavage fluids of *T. gondii*-infected mice.

Regarding the signalling pathways involved in *T. gondii*-induced NETosis, Abi Abdallah *et al*. (2012) demonstrated a key role of ERK1/2-mediated signal transduction, which is in agreement with previous results on bacteria-triggered NETosis (Hakkim *et al*. 2011). Accordingly, induction of (phosphorylated) ERK1/2-, AKT- and p38-expression was recently shown in *E. bovis*-exposed bovine PMN (Muñoz Caro, unpublished results). Overall, these data indicate a pathogen-independent and rather general involvement of this signalling pathway in NETosis.

Given that the tachyzoite stage of *T. gondii* significantly induced NET release in PMN we extended NET-associated analyses to a closely related apicomplexan parasite, *Besnoitia besnoiti*. In agreement with data on *Toxoplasma* (Abi Abdallah *et al*. 2012) *B. besnoiti* tachyzoites also strongly triggered NET formation in bovine PMN with a fast kinetics (Fig. 2C, Muñoz Caro *et al*. 2014). As described for other pathogens (Brinkmann and Zychlinsky, 2007), *B. besnoiti*-induced NET formation fulfilled all classical criteria of NETosis since it was inhibited by DPI and DNase treatments and proved to be dependent on PMN-derived ROS production and neutrophil elastase/myeloperoxidase activities (Muñoz Caro *et al*. 2014).

Given that all coccidian species tested so far have been revealed as potent NET inducers it is tempting to speculate that NETosis may represent a species-independent, stage-independent and generally valid effector mechanism of PMN against stages of this particular protozoan group, that are available only for a short period when in search for the adequate host cell. Thus it makes sense that coccidian-driven NETosis is a fast process to give PMN at least a chance to eliminate some stages or hamper them from host cell invasion, thereby reducing ongoing replication and parasite load in the final host.

LEISHMANIA SPP.

Leishmaniasis represents a major health problem and according to the WHO 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 (Ashford, 2000; Alvar *et al*. 2012). Leishmaniasis is a vector-transmitted zoonosis caused by more than 25 different euglenozoan obligate intracellular protozoan *Leishmania* species (Ashford, 2000; Alvar *et al*. 2012).

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Recent studies examined the potential role of NET formation during the early phase of leishmaniasis using promastigote stages of different *Leishmania* species. A study conducted by Guimarães-Costa *et al*. (2009) proved for the first time that promastigotes of *L. amazonensis*, *L. major* and *L. chagasi* were capable of inducing NET formation. Furthermore, they showed that NET-entrapped *L. amazonensis* promastigotes exhibited decreased viability, which was judged as an indication of leishmanicidal effects of NETs. Interestingly, *Leishmania*-triggered NETosis was not entirely stage-specific, since both promastigote stages (*L. amazonensis*, *L. major*, *L. chagasi*) and amastigote stages (*L. amazonensis*) promoted NET formation. Importantly, Guimarães-Costa *et al*. (2009) gave first indications on the nature of parasite ligands being involved in NET formation. Thus, parasite-derived lipophosphoglycan (LPG) was suggested as a trigger of NET release since this molecule also induced NETs in a purified form.

Detailed analyses of cutaneous *Leishmania* lesions from biopsies of human patients in Brazil proved *in vivo* evidence of *Leishmania*-induced NETosis demonstrating the simultaneous presence of extracellular DNA and histones (Guimarães-Costa *et al*. 2009). Guimarães-Costa *et al*. (2009) suggested that histones are involved in the parasite inactivation/killing process, since anti-histone-antibodies significantly reduced the lethal effects of NETs. The leishmanicidal effect of histones was proven in promastigote co-cultures with purified H2A histones leading to parasite killing. In agreement, Wang *et al*. (2011) demonstrated that the histone H2B also has lethal effects on *Leishmania* promastigotes.

In contrast to reports on coccidian species, Gabriel *et al*. (2010) showed that in the case of *L. donovani* the induction of NETosis was a stage-specific event. In agreement, it was a ROS-dependent process that was equally triggered in human and murine PMN. In contrast to previous findings on *Leishmania*-LPG-dependent NET induction (Guimarães-Costa *et al*. 2009), Gabriel *et al*. (2010) observed an LPG- and GP63- (promastigote surface metalloprotease) independent pathway of NETosis by using genetically modified *L. donovani* promastigotes. However, in this infection system, LPG appeared to be involved in the resistance to NET-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. The different and partially adverse functions of LPG in different *Leishmania* species may be attributed to the wide variation of the LPG composition that might occur not only within one *Leishmania* species but also within strains or even sub-strains.

SCHISTOSOMA JAPONICUM

Schistosomiasis is a chronic parasitic snail-borne disease of humans and animals mainly in tropical and sub-tropical areas. Caused by digenean trematodes of the genus *Schistosoma*, the disease affects about 200 million people worldwide (Ross *et al*. 2002). The disease is characterized by an active granulomatous cellular immune response that eventually leads to severe chronic hepatic fibrosis. In contrast to *Schistosoma mansoni* infections, PMN are known to play a key role in schistosomiasis due to *S. japonicum* infections (Hsu *et al*. 1972; Von Lichtenberg *et al*. 1973; Chuah *et al*. 2013), but their precise role in limiting or promoting hepatic pathology remained unclear until Burke *et al*. (2010) clearly demonstrated that PMN are localized within the core (adjacent to *S. japonicum* eggs) and the periphery of mature granulomas induced by *S. japonicum*. In a recent study analysing the spatial and temporal transcriptomics of *S. japonicum*-induced hepatic granuloma formation, Chuah *et al*. (2013) found an up-regulation of PMN-derived molecules associated with the production of NETs (e.g. NGP, S100A8/A9, ELA2, LTF and MMP9). *In vitro* incubation of murine and human PMN with *S. japonicum* eggs led to NET formation. *In vivo* evidence on *S. japonicum*-promoted NETosis was obtained microscopically within granulomas isolated from the livers of infected mice. Co-localization studies on DNA and NE within these structures confirmed NET existence within the core of *S. japonicum*-induced hepatic granulomas. In contrast to these findings, there were no indications of any NETs structures present in the core of *S. mansoni*-induced hepatic granulomas as a result of the absence of PMN in this region (Chuah *et al*. 2013). However, there was no indication that *S. japonicum* eggs were killed by NETs during an *in vitro* assay of 4 h duration, as the nuclei of the schistosome embryos remained intact after egg entrapment. The authors speculated that although NETs may not exhibit direct killing effects on *S. japonicum* eggs, the antimicrobial properties of NETs might have restrictive effects on their motility. Chuah *et al*. (2013) hypothesize that the *in vivo* release of NETs in the core of *S. japonicum* granuloma may lead to initial trapping and containment of the eggs attributing a dual role to PMN during the progression and pathogenesis of *S. japonicum*-promoted hepatitis.

CONCLUSIONS

Since the first discovery of neutrophil extracellular traps almost 10 years ago much knowledge has been gained concerning this interesting and extracellularly acting effector mechanism of PMN. However, research mainly focused on fungal and bacterial pathogens. Consequently, the first evidence of parasite-induced NETosis was presented only

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5 years ago and research in this field still seems to be under-represented, although PMN are known to play a pivotal role in several parasitic infections. Nonetheless, it appears undeniable that several parasites, mainly protozoans so far, trigger this newly discovered effector mechanism of PMN *in vitro* and *in vivo*. The complex composition of the parasites may not always allow for immediate killing via NETs; however, as proven for some coccidian species, NETs may significantly alter the outcome of infection via hampering certain stages from invading their host cells. So far it is not known whether parasites have also evolved counter mechanisms to resolve NETs, as is known for some bacterial species. In addition, almost no data are available on the molecules involved in PMN-parasite-interactions during NETosis. We therefore call for more studies on the role of NETs in the innate host defence against protozoan and metazoan parasites.

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