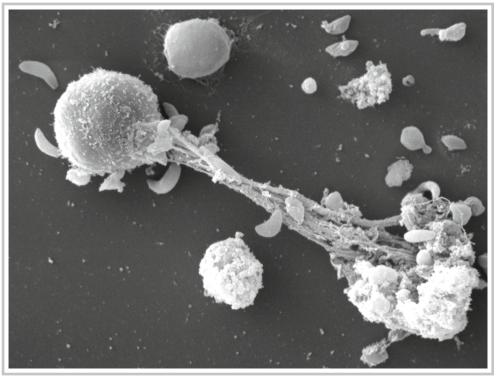
TAMARA MUÑOZ CARO

Analyses on Extracellular Trap (ET) formation against relevant coccidean parasites of ruminants



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submitted by

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9. **Muñoz Caro, T**; Hermosilla, C; Silva, L.M.R; Cortes H; Taubert, A (2013). Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite *Besnoitia besnoiti.* 6th Conference on Life Sciences of the International Giessen Graduate Center for Life Sciences (GGL). 11-12 September 2013, Giessen, Germany

10. Silva L.M.R; **Muñoz Caro, T**; Vila-Viçosa M.J.M; Cortes H; Hermosilla C; Taubert A. (2014) *Eimeria arloingi* stages trigger Neutrophil Extracellular Traps (NETs) as innate immune reaction. Annual Meeting of the Veterinary Medicine Immunology Group (VIA), 2nd-3rd May 2014, Jena, Germany

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15. **Muñoz Caro, T**; Hermosilla, C; Burgos, R.A; Mena, J; Conejeros, I; Hidalgo, M.A; Taubert, A. (2014). Molecular mechanisms involved in *Eimeria bovis* sporozoite-PMN-interactions and sporozoite-triggered NET formation. Meeting of the Parasitology Section of the German Veterinary Medical Society (DVG). 30 June to 2 July 2014. Leipzig, Germany

16. **Muñoz Caro, T**; Lendner M; Daugschies A; Hermosilla C; Taubert A. (2014) *Cryptosporidium parvum*-induced Neutrophil Extracellular Trap formation as early host innate immune reaction. Meeting of the Parasitology Section of the German Veterinary Medical Society (DVG). 30 June to 2 July 2014. Leipzig, Germany

17. **Muñoz Caro, T**; Hermosilla, C; Burgos, R.A; Mena, J; Conejeros, I; Hidalgo, M.A; Taubert, A. (2014). Molecular mechanisms involved in *Eimeria bovis* sporozoite-PMN-interactions and sporozoite-triggered NET formation. 26th Annual Meeting of the German Society for Parasitology. 16-19 July 2014 - Zurich, Switzerland

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19. **Muñoz-Caro, T**; Mena Huertas, SJ; Conejeros I; Alarcón P; Hidalgo MA; Burgos RA; Hermosilla C; Taubert A. (2015). Molecular mechanisms involved in *Eimeria bovis* sporozoite-PMN-interactions and NET formation. 7th International Giessen Graduate Centre for the Life Sciences (GGL) Annual Conference. 17-18 September 2014. Giessen. Germany

20. **Silva L.M.R; Muñoz Caro T**; Vila-Viçosa M.J.M; Cortes H; Hermosilla, C; Taubert, A. (2014) Establishment of a new *Eimeria arloingi* strain and first analysis on parasite-triggered neutrophil extracellular traps (NETs)*.* 11th International Coccidiosis Conference 26th-30th September 2014, Dresden, Germany

21. **Muñoz Caro, T**; Hermosilla, C; Silva, L.M.R.; Cortes, H; Taubert, A. (2014) Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite *Besnoitia besnoiti.* 11th International Coccidiosis Conference 26th-30th September 2014, Dresden, Germany

22. **Muñoz Caro, T**; Hermosilla, C; Burgos, R; Mena, J; Conejeros, I; Taubert, A. (2014) Molecular mechanisms involved in *Eimeria bovis* sporozoite-PMN-interactions and sporozoite-triggered NET formation*.* 11th International Coccidiosis Conference 26th-30th September 2014, Dresden, Germany

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24. **Muñoz-Caro, T**; Mena Huertas, SJ; Conejeros, I; Alarcón, P; Hidalgo, MA; Burgos, RA; Hermosilla, C; Taubert, A. (2015). Molecular mechanisms involved in *Eimeria bovis* sporozoite-PMN-interactions and NET formation. 25th WAAVP Conference of the World Association for the Advancement of Veterinary Parasitology.16 to 20 August 2015. Liverpool Convention Centre UK

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ABBREVIATIONS

AMPs - antimicrobial peptides
BPI - permeability-increasing protein
BUVEC - bovine umbilical vein endothelial cells
CR3- complement receptor 3
DNA - deoxyribonucleic acid
DNase - deoxyribonuclease
DPI - diphenylene iodondium
ECs - endothelial cells
EDN - eosinophil-derived neurotoxin
EpCAM - epithelial cell adhesion molecule
EPs - epithelial cells
ETs - extracellular traps
GM-CSF - granulocyte macrophage colony-stimulating factor
ICAM-1 - and intercellular adhesion molecule-1
IFN-γ - interferon gamma
iNOS - inducible nitric oxide synthase
MIF - macrophage inhibitory factor
MPO - myeloperoxidase
NADPH - nicotinamide adenine dinucleotide phosphate oxidase
NE - neutrophil elastase
NET - neutrophil extracellular trap
NO - nitric oxide
PAD4 - peptidylarginine deiminase type IV
PMN - polymorphonuclear neutrophil
PRRs - pattern recognition receptors
ROS - reactive oxygen species
TLRs - Toll-like receptors
SEM - scanning electron microscopy
SLE - systemic lupus erythematosus
VCAM-1 - vascular cell adhesion molecule-1

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1. INTRODUCTION

1.1. Relevant coccidian parasites of ruminants

1.1.1. Cyst-forming coccidia

Several apicomplexan parasites, such as *Toxoplasma gondii*, *Neospora* spp., *Sarcocystis* spp., *Hammondia* spp. and *Besnoitia* spp., represent closely related tissue cyst-forming parasites which belong to the family Sarcocystidae (Carreno et al. 1998; Mugridge et al. 2000). The life cycles of Sarcocystidae are complex and involve several parasite stages in definitive and intermediate hosts (Gondim et al., 2017). Since the present work exclusively dealt with two important cyst-forming parasites *Besnoitia besnoiti* and *T. gondii,* the current literature review was restricted to these species.

1.1.1.1. *Toxoplasma gondii*

T. gondii is the most studied species among all cyst-forming parasites. It belongs to the genus *Toxoplasma* (Nicolle and Manceaux 1909) and represents the only valid species of this genus (Frenkel 1977; Dubey and Beattie 1988; Tenter and Johnson, 1997). *T. gondii* is an ubiquitously occurring parasite with worldwide distribution. Life cycle of *T. gondii* is facultative heteroxenous and intermediate hosts are represented by a broad range of warm-blooded animals including livestock species (i. e. pigs, sheep, horses, cattle, goats, rabbits, camels, chickens, ducks and turkeys) and humans (Tenter et al., 2000). Definitive hosts are restricted to members of the family Felidae, such as domestic and wild cats (Dubey 1986, 1993; Frenkel 2000). In most cases, *T. gondii* parasitizes definitive and intermediate hosts without any clinical signs. However, infections can also lead to devastating disease in humans, as seen in congenitally infected children and in immunosuppressed individuals. In addition, *T. gondii* represents one of the most important causes of abortion in sheep (Dubey et al., 1998). Intermediate hosts become infected by uptake of sporulated oocysts or tissue cysts. Here, the endogenous development of *T. gondii* includes two phases of asexual replication. In the first phase, tachyzoites are formed intracellularly via stage conversion and multiply fast by repeated endodyogenies thereby lysing their host cells. Most probably upon immunological pressure, *T. gondii* initiates formation of tissue cysts. Here, bradyzoites multiply slowly within cysts by endodyogeny (Evans 1992). Tissue cysts are predominantly formed in neural and muscular tissues (Dubey et al., 1998; Tenter et al., 2000) and are therefore often found in in the central nervous system (CNS), the eye as well as skeletal and cardiac muscles. However, they may also be found in any tissue and visceral organs, such as lungs, liver, spleen, pancreas, testis, ovaries, endometrium and kidneys (Fleck et al., 1989, Dubey et al., 1998). Tissue cysts may persist in intermediate host species for their entire life and mechanisms of persistence are still unknown. However, it has been postulated that *T. gondii*-tissue cysts may break down periodically. Free-released bradyzoites then transform into tachyzoites which invade new host cells where new tissue cysts are formed (Weiss et al., 1988; Remington and Desmonts 1990; Evans 1992, Frenkel 2000). Overall, the sexual cycle of *T. gondii* is restricted to the feline intestine. When cats ingest tissue cysts, bradyzoites invade intestinal epithelial cells, reproduce asexually in several generations as tachyzoites and then perform the sexual phase (gamogony) with the formation of gamonts and oocysts. When cats ingest sporulated oocysts, sporozoites invade the intestinal wall, transform to tachyzoites and are spread throughout the body via the blood. The majority of these stages probably convert to bradyzoites and persists as cysts, but a small proportion returns to the gut and performs gamogony. Consequently, the prepatent period and frequency of oocyst shedding vary according to the infectious stage of *T. gondii* (Dubey 2006). Thus, infection via sporulated oocysts leads to a longer prepatent period of 18-36 days and to a higher shedding of oocysts than ingestion of tissue cysts (prepatency of 3-10 days) (Dubey 1996). Following oocyst shedding, sporogony occurs exogeneously and leads to development of infectious oocysts (Evans 1992). Thus, sporulated oocysts contaminate any environment and can also be found in drinking water or oceans (Isaac-Renton et al., 1998). In fact, infections with *T. gondii* in aquatic mammals have also been recorded indicating contamination and survival of oocysts in sea water (Cole et al., 2000). Moreover, *T. gondii* infections may cause severe disease in small ruminants leading to considerable economic losses especially in sheep industry. Infections with *T. gondii* may cause abortion in livestock, especially in sheep, New World camelids and goats (Buxton 1998). Small ruminants are highly receptive to *T. gondii* infection and often become infected via uptake of sporulated oocysts while grazing. Accordingly, seroprevalence is shown to increase with age, suggesting that most animals acquire infection postnatally (Dubey and Kirkbride, 1989).

Regarding human infections, toxoplasmosis represents one of the most common parasitic zoonosis worldwide (Jackson and Hutchison 1989). It has been estimated that up to one third of world human population has been exposed to this parasite (Sabin and Feldman 1948, Jackson and Hutchison 1989). Data on human *T. gondii* seroprevalence greatly vary among different geographical areas. A recent nationwide study in Germany (representative cross-sectional survey) concludes that *T. gondii* infections in Germany are highly prevalent and that age and eating habits (e. g. consumption of raw meat) of people appear to be of high epidemiological relevance (Wilking et al., 2016). Same authors concluded that 1.1% of adults and 1.3% of women aged 18–49 in Germany seroconvert each year. While infections with *T. gondii* in humans are very common, clinical disease is mainly related to certain risk groups (i. e immunocompromised humans, prenatally infected foetuses). In most cases, infected humans may show mild symptoms in which lymphadenopathy is the most significant clinical manifestation (Ho-Yen 1992, Bowie et al., 1997). However, severe manifestations, such as encephalitis, sepsis syndrome/shock, myocarditis or hepatitis, may also occur (Ho-Yen 1992). Meanwhile, congenital transmission of toxoplasmosis may lead to a broad spectrum of clinical manifestations, being subclinical in approximately 75% of infected newborns (McAuley 2014). The severity of clinical disease in congenitally infected infants is inversely related to gestational age at the time of primary maternal infection. As such, maternal infection in first trimester of gestation leads to more severe manifestations including neurodevelopmental disorders or ocular lesions, such as chorioretinitis, which is often reactivated later in life (Dunn et al., 1999; Black and Boothroyd 2000; McAuley 2014) than infections in last trimester. However, there are several recorded cases in which development of ocular signs, such as retinitis and retinochoroiditis, were also observed in acquired postnatal toxoplasmosis in humans (Bowie et al., 1997, Burnett et al., 1998). Recent studies also indicate that *T. gondii* infections may also be linked with neurological/behavioral disorders in animals and humans (House et al., 2011; Webster et al., 2013). In latter case, these disorders may vary from subtle behavioural and personality alterations to more severe ones, such as Parkinson (Miman et al., 2010a), cryptogenic epilepsy (Palmer 2007), schizophrenia (Torrey and Yolken 2003; Yolken et al., 2009), obsessive-compulsive disorder (Miman et al., 2010b) and dementia (McGeer et al., 2004; Webster et al., 2013).

1.1.1.2 *Besnoitia besnoiti*

Parasites belonging to the genus *Besnoitia* exhibit heteroxenous life cycles infecting different animal hosts including large and small mammals as well as reptiles. However, uncertainty exists on the biology of some of these species since only the life cycles of *B. darlingi* (final host: cats; intermediate hosts: opossums and lizards)*,* *B. wallacei* (final host: cats; intermediate hosts: rodents) and *B. oryctofelis* (final host: cats; intermediate hosts: rabbits) are currently known (Dubey and Lindsay 2003). *Besnoitia besnoiti* causes bovine besnoitiosis, often referred to as elephantiasis or bovine anasarque (Marotel 1912). Bovines are involved as intermediate hosts. So far, attempts to identify the final host(s) of *B. besnoiti* failed, although, analogous to species mentioned above, carnivorous definitive hosts are here suspected (Diesing et al., 1988; Basso et al., 2011). Besides the suspected infection route via oral uptake of sporulated oocysts, the parasite was proven to be transmitted mechanically by haematophagous insects of the genus *Glossina* and *Stomoxys* as well as by tabanids (Bigalke 1968; Schares et al., 2011; Liénard et al., 2011). This infection route may allow rapid transmission within a new geographical area (Álvarez-García et al., 2013). In addition, transmission of infection via mating (Esteban-Gil et al., 2014) and via iatrogen actions (e. g. by repeated use of hypodermic needles) (Basso et al., 2011; Álvarez-García et al., 2013) was hypothesized. Animal trade and movements throughout countries were identified as major risk factors for establishment of new bovine besnoitiosis foci in naive areas and countries (Álvarez-García et al., 2013). This parasitic disease is nowadays considered as emerging in Europe (EFSA, 2010). Within the acute phase of the infection asexual proliferation of tachyzoites occurs in host endothelial cells of different organs and vessels (Basson et al., 1970), in polymorphonuclear neutrophils (PMN) and monocytes *in vivo* (Basso et al., 2012; Álvarez-García et al., 2013). Often the acute phase is characterized by non-specific clinical signs including fever, oedema, enlarged lymph nodes, anorexia, weight loss, photophobia as well as swollen and painful testes (orchitis) in bulls that occasionally may not be noticed (Schulz 1960; Bigalke 1968; Gazzonis et al., 2017). Moreover, the most severe symptoms are observed in the chronic phase of infection based on the development of large tissue cysts in skin, mucosal membranes, sclera and conjunctiva (Pols 1960; Bigalke 1981). Massive tissue cyst formation in dermis and other areas leads to hyperkeratosis, alopecia, weight loss, decreased milk production, infertility in bull's (due to orchitis) and abortion (due to infection during pregnancy). This clinical status has a considerable economic impact on cattle industry (Kumi-Diaka et al., 1981; Cortes et al., 2005; Cortes et al., 2014; EFSA 2010). One important clinical risk factor being associated with *B. benoiti* transmission is linked to seasonality. Based on limited observations from two outbreaks in Europe, it was noted that emergence of clinical signs coincided with the summer period, when mixed herds shared pastures and blood-sucking arthropods, such as horseflies, are active (Alzieu 2007; Fernandez-García et al., 2009). Since no efficient treatments or vaccines are vailable, the control of bovine besnoitiosis entirely relies on herd management measures in combination with diagnostic means (Álvarez-García et al., 2014).

1.1.2 Non-cyst forming coccidia

Several apicomplexan parasites exhibit homoxenous life cycles and do not form tissue cysts in hosts. Within non-cyst forming coccidia relevant parasites are grouped, such as *Plasmodium* spp. as agents of human and animal malaria, *Eimeria* spp. as economically important pathogens of chicken, rabbits, goats, sheep and cattle, *Theileria* spp. as tick-borne parasites of cattle and small ruminants in Africa and Asia and *Cryptosporidium* spp., as opportunistic enteropathogens affecting animals and humans worldwide. Given that the current study focused on *Eimeria* and *Cryptosporidium* species, the review is restricted to these parasites.

1.1.2.1 *Eimeria* spp.

Eimeria species are highly prevalent protozoan parasites affecting farm animals (Chartier and Paraud 2012). The *Eimeria* genus belongs to the family Eimeriidae and includes obligate intracellular, host specific (monoxenous) enteropathogens affecting vertebrates (Witcombe and Smith 2014). *Eimeria* development occurs within specific host cells at distinct areas of the gut mucosa. The disease itself is known as coccidiosis or eimeriosis and occurs worldwide thereby representing a major livestock health problem in several domestic animal production systems (Foreyt 1990; Lima 2004; Daugschies and Najdrowski 2005; Witcombe and Smith 2014). Especially poultry (Chapman 2014), rabbits (Nosal et al., 2014) and ruminants (Daugschies and Najdrowski 2005; Ruiz et al., 2010; Silva et al., 2016) are affected by coccidiosis. The most pathogenic species in ruminants are *E. bovis* (cattle), *E. zuernii* (cattle), *E. ninakohlyakimovae* (goat), *E. arloingi* (goat), *E. christenseni* (goat) and *E. bakuensis* (sheep). In contrast to less pathogenic species, such as *E. intrincata*, *E. wyomingensis, E. weybridgensis, E. faurei, E. ellipsoidalis* and *E. alijevi,* they invade endothelial cells of the central lymph capillaries of the intestinal villi (Hermosilla et al., 2012) where they form macromeronts within a process which requires prolonged replication time and vast host cell modulation (Taubert et al., 2010; Lutz et al., 2011; Hermosilla et al., 2012).

Analogous to other apicomplexan parasites, the life cycle of *Eimeria* spp. includes three obligatory phases: sporogony, merogony and gamogony. Sporogony takes place exogenously in the environment under appropriate conditions (i. e. humidity, temperature and oxygen supply) and leads to the formation of the infective parasitic stage, the sporulated oocyst, containing fully formed sporozoites. After oral ingestion of sporulated *Eimeria* oocysts by the definitive host, sporozoites are released into the gut lumen and infect specific intestinal cells where first merogony occurs. In case of the ruminant pathogenic species *E. bovis* and *E. arloingi* (Ruiz et al., 2010; Hermosilla et al., 2012), sporozoites replicate in lymphatic endothelial host cells of central lacteals of the ileum, where they develop into large-sized macromeronts (up to 400 µm), releasing more than 170.000 merozoites of first generation. Thereafter, merozoites I migrate to ileum, caecum and colon (depending on the species). In case of *E. bovis,* second merogony takes place in host epithelial cells of the colon, being followed by gamogony in the same host cell type. Here new oocysts are formed and shed into the environment thereby completing the life cycle of *Eimeria* (Taylor and Catchpole 1994).

Pathogenesis of ruminant eimeriosis includes severe damage of intestinal mucosa leading to digestive and homeostatic dysfunction. Thus, concurrent diarrhoea results in plasmatic potassium imbalance, hypoproteinaemia, hyponatraemia and hypochloraemia in *Eimeria*-infected animals (Lima 2004; Daugschies and Najdrowski 2005). Consequently, negative effects on animal welfare and production performance are common, such as poor weight gain, dehydration, growth delay, low feed conversion and occasionally sudden death of infected animals (Koudela and Bokova 1998; Daugschies and Najdrowski 2005; Chapman 2014). Overall, coccidiosis often induces considerable economic losses (Daugschies and Najdrowski 2005).

In general, young (naïve) animals are most susceptible for eimeriosis and develop disease, especially under intensive rearing conditions (Soe and Pomroy 1992; Taylor and Catchpole 1994; Daugschies and Najdrowski 2005). In this context, stress is one of key risk factors being associated with clinical coccidiosis in ruminants. As such, animals are at a higher risk to develop clinical coccidiosis during weaning period, transports, re-grouping, inadequate feeding, as well as in parturition period or after concomitant infections (Faber et al., 2002; Lima 2004; Daugschies and Najdrowski 2005).

1.1.2.2 *Cryptosporidium*

Parasites of the genus *Cryptosporidium* cause cryptosporidiosis in a wide range of animals and - depending on species - also bear zoonotic potential. Molecular studies indicated that *Cryptosporidium* is more closely related to primitive gregarines than to coccidia (Carreno et al., 1999; Leander et al., 2003). Based on whole genome and further phylogenetic analyses, taxonomy was revised, and *Cryptosporidium* was transferred from coccidia to a new subclass, the cryptogregaria (Templeton et al., 2010; Cavalier-Smith 2015; Ryan et al., 2016). Specific features of *Cryptosporidium* include its epicellular localization within host cell, presence of a gregarine-like feeder organelle (Cavalier-Smith 2014), presence of large extracellular gamont stages, syzygy (end to end pairing for reproduction) and ability to undergo intra- and extracellular multiplication (Clode et al., 2015). Delimiting species within genus *Cryptosporidium* has also been controversial but currently 27 species infecting birds, reptiles, fish, and mammals are regarded as valid (Ryan et al., 2014; Ryan and Hijjawi 2015) with almost 20 *Cryptosporidium* species and genotypes also affecting humans (Tzipori 1983; Fayer and Ungar 1986; Xiao et al., 2004, Ryan and Hijjawi 2015). The most commonly reported species in humans are *C. hominis* and *C. parvum* (Xiao et al., 2010, Ryan et al., 2014). The latter species is also highly endemic in cattle worldwide (Blanchard 2012; Cho and Yoon 2014) causing one of most prevalent intestinal diseases of neonatal calves (Gillhuber et al., 2014). Infected animals may suffer from profuse watery diarrhoea and severe dehydration occasionally leading to death (Thomson et al., 2017).

Life cycle of *Cryptosporidium* includes ingestion of sporulated oocysts by susceptible host. Following excystation in the intestine, sporozoites infect host cells in the microvillous region of the intestinal mucosal epithelium and reside intracellular but extracytoplasmic (= epicellular) in these host cells. They develop into trophozoites within parasitophorous vacuoles which then undergo asexual divisions (merogonies I and II) forming merozoites I and II, the latter of which then perform gamogony and end-up with the formation of infective (sporulation of the oocysts already occurs within the gut), environmentally resistant thick-walled oocysts which are released in feces. Thin-walled oocysts which are formed in parallel, may also cause autoinfections of hosts. Overall, newborn calves shedding high numbers of oocysts are considered as major source of zoonotic *C. parvum* infections either via direct transmission or through oocyst contamination of water resources or agricultural land (Hunter and Thompson 2005). The economic impact of cryptosporidiosis in livestock is mainly due to costs of treatment, reduction in feed conversion as well as production inefficiency and animal losses (Thomson et al., 2017).

Regarding transmission of infection, surface water is considered as an efficient carrier for oocysts and contaminated water is one of the main causes of epidemic outbreaks of human cryptosporidiosis (MacKenzie et al., 1994). Especially in third world countries, *C. parvum* and *C. hominis* infections are a major cause of severe diarrhoea, developmental problems and death in young children and may also induce life-threatening chronic disease in immunocompromised and malnourished individuals (Guerrant et al., 1999; Snelling et al., 2007; Costa et al., 2011; Kotloff et al., 2013; Striepen 2013). Accordingly, a global enteric multi-center study (GEMS) revealed that *Cryptosporidum* spp. is the second leading cause of infant diarrhoea associated with toddler mortality in developing countries (Kotloff et al., 2013). So far, its eradication cannot be achieved since neither efficient therapeutics nor vaccines are available for humans (Thomson et al., 2017). In case of calves, metaphylactic halofuginon treatments may lower oocyst excretion and ameliorate coccidiosis (Joachim et al., 2003).

1.2 Innate immune cells and their effector mechanisms

Innate immune cells of mammals display a vast spectrum of defensive effector mechanisms against invasive pathogens, which are reviewed in the present chapter. The current doctoral thesis focused on the recently described effector mechanism of extracellular trap (ET) formation in response to coccidian parasite stages and included analyses of different leukocyte populations [i. e. polymorphonuclear neutrophils (PMN), monocytes and eosinophils] of different mammalian species.

1.2.1 Polymorphonuclear neutrophils (PMN)

PMN represent the most abundant leukocyte population and correspond to 50–80% (depending on the species) of blood immune cells. One main effector mechanism of these cells is the release of granules with antimicrobial properties (Segal 2005). Three types of granules are present in the cytosol of PMN. Azurophilic granules (primary granules) are the first ones to be formed during PMN maturation. These granules contain antimicrobial molecules, such as myeloperoxidase (MPO), defensins, lysozyme, bactericidal/permeability-increasing protein (BPI) and serine proteases [e. g. neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G] (Korkmaz et al., 2010). Therefore, azurophilic granules function as a primary repository for molecular weaponry of PMN (Campos-Rodríguez et al., 2016). Following azurophilic granule generation, so-called specific (secondary) granules are formed in PMN which contain lactoferrin and other antimicrobial compounds, such as neutrophil gelatinase-associated lipocalin (NGAL), human cathelicidin (hCAP-18) and lysozyme (Teng et al., 2017). The third granule population, i. e. gelatinase (tertiary) granules, are stores of several metalloproteases, such as gelatinase and leukolysin (Pei 1999). Besides these antimicrobial peptide populations, secretory vesicles serve as a reservoir for several important membrane-bound molecules necessary for PMN migration.

Another key effector mechanism of PMN is represented by their capacity of oxidative burst activities promoting killing of pathogens via oxygen-dependent pathways. Therefore, reactive oxygen species (ROS) are produced (**Fig. 1**) via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, a multicomponent enzymatic system formed by NOX enzymes that are rapidly assembled upon PMN activation (Robinson 2008). The complex comprises two membrane-bound subunits, gp91phox (Nox2) and p22phox, collectively known as cytochrome b558 (Cifuentes and Pagano 2006). The complex catalyzes electron reduction of oxygen using NADPH as an electron donor (Griendling 2004). The active oxidase transfers electrons from the cytosolic NADPH to molecular oxygen, thereby releasing superoxide anion (O_2-) into the phagosomal lumen (**Fig. 1**; Quinn and Gauss 2004). Superoxide is a highly reactive, free radical that generates hydrogen peroxide (H_2O_2; Cohen and Heikkila 1974) (**Fig. 1**). Both, hydrogen peroxide and superoxide anions are components of oxygen-dependent antimicrobial system of phagocytic leukocytes (Robinson and Badwey 1995) serving as substrates for MPO to generate additional ROS products, such as hypochlorous acid (HOCl; Klebanoff 2005; Ximenes et al., 2005; Williams and Griendling 2007). ROS efficiently kill microorganisms by protein and lipid oxidation. In general, ROS production varies between different

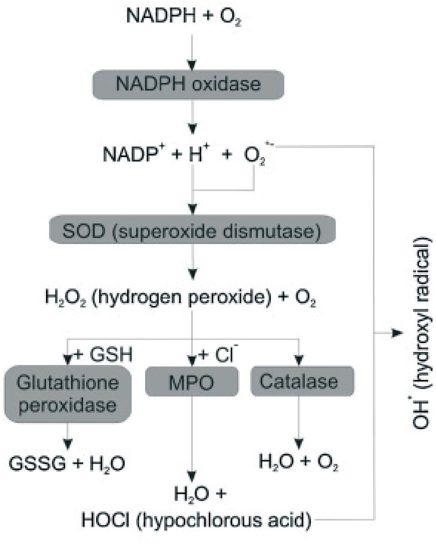

Fig. 1. Generation of reactive oxygen species (ROS) (according to Pruchniak et al., 2013).

leukocyte subpopulations. As such, PMN were demonstrated as more effective in this concern than other phagocytes, such as macrophages (Nathan and Shiloh 2000). In addition to ROS, several important antimicrobial proteins, such as defensins and cathelicidins, are present in PMN but are absent or scarce in tissue macrophages (Ganz 2003).

Besides the effector mechanisms of degranulation and ROS production, PMN also perform phagocytosis. A more detailed description of this mechanism is given in Chapter 1.2.2.

The initiation of innate immune responses and establishment of inflammatory reactions against pathogens are highly regulated processes mediated by various cytokines and chemokines being synthesized by professional phagocytes, such as PMN and monocytes (Cassatella 1999; Lacy and Stow 2011). PMN produce a broad range of immunoregulatory molecules, such as IL1α, IL-1β, IL-4, IL-6, IL-7, IL-10, IL-17, IL-18 (Tecchio et al., 2014). Cytokine release is mainly induced by immunoglobulin- or complement receptor-triggered signalling or by receptor-mediated pathogen recognition. In this process, pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), are often involved (McGettrick and O'Neill 2007; Iwasaki and Medzhitov 2010; Lacy and Stow 2011). Given that pro-inflammatory cytokines also mediate T lymphocyte and other immune cell recruitment and activation, these molecules participate in both, innate and adaptive immune responses, and also mediate a link between these immunological reaction types (Banyer et al., 2000). In addition to cytokines, chemokines also signify relevant innate effector molecules due to their ability to recruit distinct immune cell populations to site of infection (Scapini at al., 2000; Tecchio et al., 2014). As such, PMN-derived chemokines (e. g. CCL2, CXCL1, CXCL8, CXCL10) may act locally to induce PMN recruitment into peripheral tissues and at distance to mobilize PMN from bone marrow into blood stream (Sadik et al., 2011). These molecules also mediate recruitment and activation of other leukocytes in injured or inflamed tissue (Scapini and Cassatella 2014).

In addition, PMN are also able to kill pathogens by so-called neutrophil extracellular traps (NETs; Brinkmann et al., 2004). Classically, NETs are composed of extracellular DNA matrices being studded by several antimicrobial proteins that mediate extracellular trapping and eventual killing of pathogens (Brinkmann et al., 2004). The protein proportion of NETs accounts for approximately 70% and mainly consists of core histones (H1, H2A/H2B, H3, H4), microbicidal granular enzymes and peptides/proteins (Brinkmann et al., 2012), such as BPI, MPO, NE, cathepsin G, lactoferrin, gelatinase, peptidoglycan recognition proteins (PGRPs), calprotectin, PR3, high mobility group protein B1 (HMGB1) and LL37 (Brinkmann et al., 2004; Abi Abdallah and Denkers 2012; Hermosilla et al., 2014). NETs are extruded into the extracellular compartment via a novel cell death process meanwhile well-known as NETosis (Fuchs et al., 2007). Overall, NETosis-based cell death differs significantly from apoptosis, pyroptosis (**Fig. 2**) or necrosis. Thus, PMN-derived apoptosis is characterized by membrane blebbing, phosphatidylserine translocation and condensation of nuclear chromatin, while in necrosis the nucleus loses its structure and lobules fuse into a homogenous mass lacking segregation into eu- and heterochromatin (Fuchs et al., 2007). In contrast, during NETosis PMN undergo massive vacuolization and nuclear chromatin decondensation but do not show phosphatidylserine translocation. Furthermore, in contrast to apoptosis or pyroptosis, both, nuclear and granular membranes disintegrate whilst plasma membrane integrity is maintained during NETosis. This allows antimicrobial granular cargo to mix with nuclear chromatin (Remijsen et al., 2011a). Consequently, neither inhibition of caspases by zVAD-fmk 6 nor of RIP1 kinases by necrostatin-1 affects NETosis (Remijsen et al., 2011b) proving this cell death type as distinct from apoptosis, necrosis and pyroptosis.

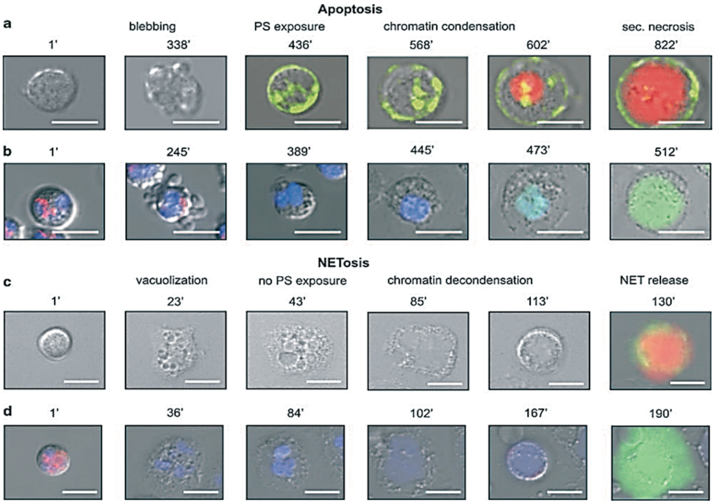

Fig. 2. Characterization of subcellular events during PMN apoptosis and NETosis by live cell imaging using four different channels (phase contrast, red, green and blue) and specific cellular markers. Apoptotic PMN (a, b) show membrane blebbing, phosphatidylserine (PS) translocation (visualized via Annexin V staining, green) and condensation of nuclear chromatin (a). Annexin V can only bind to PS of cells undergoing apoptosis, when PS is transferred to the outer leaflet, or after membrane rupture, when Annexin V can enter into the cell. PMN apoptosis is followed by chromatin condensation seen as positive signals from the cell-impermeable DNA dye propidium iodide (red) and the cell impermeable DNA dye Hoechst 33342 (blue; b). Here, PMN undergo secondary necrosis at latest time points. During NETosis (c, d) cells display massive vacuolization (c) and decondensation of nuclear chromatin as detected by Hoechst 33342 staining (d, blue). Here PS is not translocated before plasma membrane disruption and NET release. NET release is detected via staining of NETs-DNA with propidium iodide (red, c) and by the green signal coming from Annexin V (c, d) indicating membrane disruption. In addition, the mitochondrial membrane potential marker TMRM signal (red, d 1’) is lost during NET release. The loss of mitochondrial membrane potential is a characteristic event during initial stages of NETosis. Scale bars: 10 µm. (taken from: Remijsen et al., 2011a).

Effective NETosis requires mature PMN (Martinelli et al., 2004) and presence of enzymes MPO, NE and peptidylarginine deiminase type IV (PAD4) (Neeli et al., 2008; Wang et al., 2009; Papayannopoulos et al., 2010). For NETs release, sequential enzymatic and non-enzymatic actions must be achieved in PMN (illustrated in **Fig. 3**). Thus, the nuclear envelope disintegrates upon PMN stimulation and allows chromatin and granular protein mixture (Brinkmann et al., 2004; Fuchs et al., 2007). Then, NE and MPO degrade histones and promote chromatin decondensation (Papayannopoulos et al., 2010). PAD4 mediates chromatin decondensation by histone hypercitrullination leading to electrostatic coiling of the chromatin (Wang et al., 2009; Li et al., 2010; Leshner et al., 2012). These DNA-protein-complexes are then released as NETs into the extracellular compartment (**Fig. 3**).

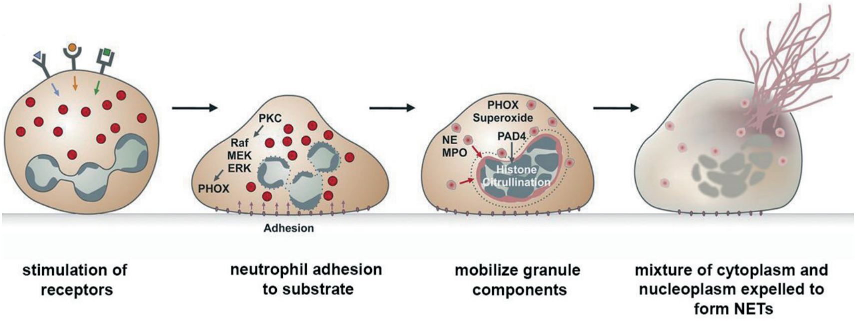

Fig. 3. Neutrophil extracellular trap (NET) formation: following receptor stimulation, PMN adhere to the substrate and mobilize granule components, such as NE and MPO. Nuclear chromatin is decondensed, histones are citrullinated and intracellular membranes disintegrate via PAD4 action. Finally, the cell membrane ruptures and a mixture of cytoplasmic and nucleoplasmic contents is released to form NETs. (taken from: Brinkmann and Zychlinsky 2012)

Classical NETosis requires the production of ROS and is mainly NOX-dependent (Brinkmann and Zychlinsky 2007, 2012; von Köckritz-Blickwede et al., 2010; Guimarães-Costa et al., 2012). However, NOX-independent NETosis was also described (Douda et al., 2015; Villagra-Blanco et al., 2017). Irrespective of NOX-dependency, pathogens may either be immobilized within sticky DNA fibres or be killed via the local high concentration of effector molecules (Brinkmann et al., 2004; Guimarães-Costa et al., 2009; Hermosilla et al., 2014).

In contrast to classical suicidal NETosis (Fuchs et al., 2007), a non-suicidal type of NETosis, called “vital NETosis”, was also reported allowing PMN to remain viable after NET release (Yipp and Kubes 2013). Here, nuclear chromatin expulsion is accompanied by release of granule proteins through degranulation. These components then assemble extracellularly and active anucleated cytoplasts remain which are still viable and continue to ingest microorganisms (Papayannopoulos 2017).

Up to date, a bunch of molecules have been identified as NET inducers, such as PMA, LPS, IL-8 (Brinkmann et al., 2004), zymosan (Silva et al., 2014; Reichel et al., 2015; Alarcón et al., 2017), hydrogen peroxide (Fuchs et al., 2007), platelet-derived TLR-4 (Clark et al., 2007), Ca^{2+} ionophores (Wang et al., 2009), thapsigargin (Gupta et al., 2010), chemotactic complement-derived peptide complement factor 5 (C5a; Martinelly et al., 2004), TNF-α (Keshari et al., 2012), Fc receptors (Urban et al., 2006), IFN-γ (Yousefi et al., 2008), lipophosphoglycan (LPG) of *Leishmania* spp. promastigotes (Guimarães-Costa et al., 2009), *Staphyloccocus epidermidis* δ-toxin, autoantibodies (Cogen et al., 2010) and antimicrobial peptide LL-37 (Neumann et al., 2014).

Following NET discovery, many NET-related studies focused on bacterial and fungal pathogens, such as *S. aureus* (Pilsczek et al., 2010), *Streptococcus* sp. (Beiter et al., 2006), *Escherichia coli* (Marin-Esteban et al., 2012), *Haemophilus influenzae*

(Juneau et al., 2011), *Klebsiella pneumoniae* (Papayannopoulos et al., 2010), *Listeria monocytogenes* (Munafo et al., 2009), *Mycobacterium tuberculosis* (Ramos et al., 2009), *Cryptococcus neoformans* (Rocha et al., 2015), *Mannheimia haemolytica* (Aulik et al., 2010), *Shigella flexneri* (Brinkmann et al., 2004), *Aspergillus fumigatus* (Bianchi et al., 2011), *Candida albicans* (Urban et al., 2006), *Yersinia* spp. (Brinkmann et al., 2004), and *Vibrio cholerae* (Seper et al., 2013). Furthermore, some viruses have also been demonstrated to induce NETs, such as feline leukemia virus (Wardini et al., 2010), influenza virus (Narasaraju et al., 2011) and immunodeficiency virus-1 (Saitoh et al., 2012).

When experiments of current dissertation started in September 2012, scarce information was available on parasites as NET inducers. Thus, NET formation had only been recorded in response to *Plasmodium* spp. (Baker et al., 2008), *Eimeria bovis* sporozoites (Behrendt et al., 2010), *Leishmania* spp. (Guimarães-Costa et al., 2009, 2011; Gabriel et al., 2010; Wang et al., 2011) and *Toxoplasma gondii* (Abi Abdallah et al., 2012) at that time point. Given that NETosis was and is a highly up-to-date topic, meanwhile a multiplicity of new parasite-related data was gathered. Thus, NET formation was identified as an important effector mechanism being directed against several protozoan and metazoan parasite species in the last years. As such, protozoan parasites *Besnoitia besnoiti* (current dissertation: Muñoz-Caro et al., 2014a), *Eimeria arloingi* (current dissertation: Silva et al., 2014)*, Eimeria ninakohlyakimovae* (Pérez et al., 2015), *Cryptosporidium parvum* (current dissertation: Muñoz-Caro et al., 2015b), *Trypanosoma cruzi* (Sousa-Rocha et al., 2015) and *Neospora caninum* (Wei et al., 2016; Villagra-Blanco et al., 2017) as well as metazoan parasites *Strongyloides stercoralis* (Bonne-Annee et al., 2014), *Haemonchus contortus* (current dissertation: Muñoz-Caro et al., 2015c), *Litomosoides sigmodontis* (Pionnier et al., 2016), *Brugia malayi* (McCoy et al., 2017), *Schistosoma japonicum* (Chuah et al., 2013), *Angiostrongylus vasorum, Aelurostrongylus abstrusus, Troglostrongylus brevior* (Lange et al., 2017) and *Trichobilharzia regent* (Skála et al., 2018) were proven as NET inducers.

1.2.2 Macrophages, monocytes, dentritic cells (DCs)

Mononuclear phagocytes represent an important innate immune cell population which display several key effector functions, such as phagocytosis, antigen presentation, and immunomodulation (especially via cytokine and chemokine release). Mononuclear phagocytosis mainly includes ingestion of particles > 0.5 µm and is performed to eliminate cellular debris and invading pathogens. Particle internalization is initiated by interaction of distinct phagocyte surface receptors, such as Fc-, complement-, TLR and scavenger-receptors with ligands on particles. This leads to polymerization of actin at site of ingestion, to phagocyte membrane extension around particles and actin-based particle internalization. However, phagocytosis of complement-opsonized particles also occurs without membrane extension with particle appearing to sink into the cell (Allen and Aderem 1996). Coordinated interactions of actin- and tubulin-based cytoskeletal elements are then required for endosome/lysosome trafficking and phagosome maturation. Moreover, binding to phagocytic receptors, such as Fc-receptors, lead to NADPH oxidase activation in macrophages or PMN (Fitzer-Attas et al., 2000). While a fraction of the intrinsic components of NADPH oxidase are present at plasma membrane during phagocytosis, most are delivered through fusion with intracellular storage sites. One of key features of mature phagosome in macrophages is the increase of phagosome acidity reaching a luminal pH ≤ 5 which is mandatory for optimal activities of proteases and lysosomal hydrolases being involved in pathogen killing. Phagosome acidification is also established by action of V-ATPase, a multi-subunit transmembrane protein that pumps protons into phagosome. Furthermore, antimicrobial capacity of macrophages is also achieved by the synthesis of nitric oxide (NO) which proved toxic for pathogens and tumour cells (MacMicking et al., 1997).

Referring to immunomodulatory molecules, activated monocytes and macrophages were reported to release pro-inflammatory cytokines (e. g. IL-1, IL-6, TNF, and INF-α/β) and chemokines (e. g. CCR1, CCR2, CCR5, and CXCR4) which are involved in the modulation of diverse biological processes (Nathan 2012).

Another important immune cell population is represented by mature DCs which are professional antigen-presenting cells and play a key role in the pathogenesis of allergic and neoplasic diseases (Soumelis et al., 2002). Mature DCs exhibit a limited capacity for lysosomal degradation of ingested material (Delamarre et al., 2005) and - in contrast to phagocytic immature myeloid DCs (Inaba et al., 1993; Steinman and Swanson 1995; Delamarre et al., 2005) - are not involved in direct pathogen clearance (Savina and Amigorena 2007).

In addition to these effector mechanisms, monocytes and macrophages are also capable to release extracellular traps (METs) via “METosis” (Doster et al., 2018). Alike NETs, characteristics of METs include DNase I-sensitive extracellular DNA fibres extending over the border of the cell (Aulik et al., 2012). In addition, elastase, often considered as a neutrophil-specific marker, was also identified in METs of human peripheral-blood monocytes and in THP-1 macrophage-like cells (Halder et al., 2016; Je et al., 2016). Furthermore, MPO was found in METs of diverse macrophage populations from human and non-human origin (Liu et al., 2014; O'Sullivan et al., 2015; Helder et al., 2016; Je et al., 2016; Pérez et al., 2016).

1.2.3. Eosinophils

Eosinophils are a key cell type in host responses to helminth infection and allergic disease (Weller and Spencer 2017). Their effector functions include release of cationic proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPX) and eosinophil-derived neurotoxin (EDN) which are stored in cytoplasmic granules and are released via degranulation upon eosinophil activation (Trulson et al., 2007). Eosinophils also utilize elastase activities and produce ROS (e. g. superoxide, peroxide, hypobromous acid) via peroxidases (Saito et al., 2004). In addition, eosinophils display immunoregulatory functions via release of cytokines (e. g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13, TNF-α; Hogan et al., 2008) and growth factors (e. g. TGF-β, VEGF, PDGF; Kato et al., 2005). Further, eosinophils produce lipid mediators, such as leukotrienes (e. g. LTC4, LTD4, LTE4) and prostaglandins (e. g. PGE2), acting as paracrine mediators pertinent for inflammatory processes (Luna-Gomes et al., 2011).

Another effector mechanism of eosinophils is represented by their capability to release eosinophil extracellular traps (EETs). This mechanism depends on NADPH-oxidase-mediated ROS production. Here, eosinophil granule proteins such as major

MBP and ECP are first released as intact structures binding then extracellularly to nucleic DNA structures (Yousefi et al., 2012; Ueki et al., 2013). Interestingly, eosinophils are also able to form extracellular structures out of mitochondria-derived DNA and released granule proteins. These ET-like structures are also able to bind and kill bacteria both, *in vitro* and *in vivo* (Yousefi et al., 2008).

1.2.4 Natural killer cells (NK)

NK cells are innate immune cells that control certain microbial infections and tumour growth (Vivier et al., 2008). They comprise up to 15% of peripheral blood lymphocytes and are found in peripheral tissues, including liver, peritoneal cavity and placenta (Trinchieri 1989). NK cytotoxicity is mediated by directed exocytosis of cytolytic granules to release perforins and granzymes, which perforate target cell plasma cell membrane and trigger apoptosis, respectively (van Dommelen et al., 2006). They also contribute to antibody-dependent cell-mediated cytotoxicity directed against tumour cells (Smyth et al., 2002). Cytokines involved in NK activation include IL-12, IL-15, IL-18, IL-2, and CCL5 (Stewart and Vivier 2007). NK cells also secrete IFN-γ and TNF-α for viral infection control (Vivier et al., 2008). Furthermore, it was demonstrated that this cell type bears antigen-specific memory functions which are fundamental for improved responses to re-exposure to same antigen (Watzl 2014). Additionally, it was recently demonstrated that NK cells are involved in induction of NET formation in venous thrombosis highlighting the role of NK cells in the development of this disease (Bertin et al., 2016).

1.2.5 Endothelial and epithelial cells

It is noteworthy that not only leukocytes but also other cell types, such as endothelial and epithelial cells, actively participate in orchestrating effective host innate immune responses. Due to their location, endothelial cells (ECs) are one of the first cells to interact with microbial components present in blood circulation. ECs coordinate recruitment of inflammatory leukocytes to sites of tissue injury or infection by expressing adhesion molecules like P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) which mediate leukocyte adhesion and transmigration across the endothelium to the site of inflammation (Ley and Reutershan 2006). They also act as antigen presenting cells by expressing major histocompatibility complex (MHC) I and II molecules (Mai et al., 2013). Furthermore, ECs produce enzymes with antimicrobial and immunoregulatory properties, such as inducible nitric oxide synthase (iNOS) or indoleamine 2, 3-dioxygenase (IDO) (Däubener et al., 2009). Pro-inflammatory cytokines, chemokines and growth factors, such as TNF-α, IL-1α, IL-1β, IL-3, IL-5, IL-6, IL-8, IL-10, IL-11, CSF1, CSF2, CSF3, CCL2, and CCL5 (Mai et al., 2013) serving for communicative processes with leukocytes (Muller 2003) are also released by activated ECs.

As described for ECs, also epithelial cells actively influence the development of inflammatory reactions by their capacity to synthesize a variety of cytokines (e. g. TNF-a, IL-1b, IL-6, IL-8) and chemokines (e. g. CCL2, CCL5, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL11) when being activated. Furthermore, they produce the epithelial cell adhesion molecule (EpCAM) which is exclusively expressed in epithelium and epithelial-derived neoplasms (Armstrong et al., 2003) and is involved in cell adhesion and many other important biological processes, such as cell signaling, migration, proliferation and differentiation (Trzpis et al., 2007).

1.3. Innate immune reactions against coccidian parasites

The innate immune system is the first-line of defence against pathogens and an evolutionary older system than the adaptive one (Alberts et al., 2002; Tschopp et al., 2003). Physical barriers, such as epidermis, mucosa, epithelial cells and endothelial cells form part of host innate immune system. Given that apicomplexan parasites are obligatory intracellular parasites, their free stages (e. g. sporozoites, merozoites, tachyzoites or bradyzoites) must invade specific host cells to continue their life cycle and to avoid extracellular immunological attacks (Morrissette and Sibley 2002). However, since all stages are also present in the extracellular compartment before invading host cells and when being released from oocyst/cyst stages or host cells, they also represent targets of leukocytes.

1.3.1 *Eimeria* species

Innate immune reactions represent an important part of effective *Eimeria* defence. As such, an accumulation of macrophages in gut and adjacent lymph nodes of primary and challenge *E. bovis*-infected calves or even in degenerating macromeronts was stated (Friend and Stockdale 1980; Taubert et al., 2009). Mucosal macrophage infiltration was also reported for *E. tenella*- and *E. acervulina*-infected chicken and for *E. separata*-infected rats (Trout and Lillehoi 1993; Vervelde et al., 1996; Shi et al., 2000). Furthermore, enhanced levels of monocytes or mononuclear cells were present in the blood of *E. maxima*-infected chicken and *E. nieschulzi*-infected rats (Rose et al., 1979). Interestingly, a macrophage inhibitory factor (MIF) was described for avian *Eimeria* species that is stage-specifically produced in merozoites, sporozoites or oocysts (Miska et al., 2007). Macrophages from immunized murine and avian species were also shown to display phagocytic activities against *Eimeria* sporozoites (Rose 1974; Rose and Lee 1977; Bekthi and Pery 1989) although complete elimination of parasites depended on presence of immune serum and complement (Bekthi and Pery 1989). In line with these data, *E. bovis* sporozoites were effectively phagocytized by bovine macrophages in presence of immune serum and peripheral blood monocytes from *E. bovis*-infected animals exhibited enhanced *ex vivo* phagocytic and oxidative burst activities (Taubert et al., 2009). Also for *E. tenella* and *E. maxima* infections, a macrophage-mediated phagocytosis of sporozoites was described with a peak-like increase of phagocytotic activity in the course of infection (Rose 1974; Rose and Lee 1977). Moreover, the role of macrophage-derived ROS or NO acting as effector mechanism against *Eimeria* species was reported (Liew and Cox 1991; Ovington et al., 1995). However, so far, no direct evidence of NO-mediated killing of *Eimeria* stages was given. Nevertheless, enhanced iNOS gene transcription was reported in *E. tenella* sporozoite-exposed magrophages and in intestinal mucosa of *Eimeria*-infected rats and chicken (Rose et al., 1992; Shi et al., 2001; Lillehoj and Li 2004; Dalloul et al., 2007). In addition, leukocytes from *Eimeria*-infected hosts produced increased levels of free oxygen metabolites, which negatively affected *Eimeria* stages (Ovington and Smith 1992; Prowse et al., 1992).

Referring to the production of immunomodulatory molecules, co-cultures of macrophages with *E. bovis* sporozoites induced the transcription of genes encoding for IFN-γ, IL-12, TNF-α, IL-6, CXCL1, CXCL8 and CXCL10 whilst in monocytes merely IFN-γ and CXCL10 gene transcripts were found up-regulated (Taubert et al., 2009). Monocytes also reacted upon *E. ninakohlyakimovae* sporozoite exposure by the upregulation of IL-12 and TNF-α, IL-6 and CCL2 gene transcription (Perez et al., 2016) and avian macrophages from *E. tenella*- and *E. maxima*-infected animals produced more IL-1 and TNF-α than control cells (Byrnes et al., 1993). In line, avian macrophage cell lines showed an increased TNF-α synthesis upon exposure to *E. tenella* sporozoites and merozoites (Zhang et al., 1995) and in avian macrophages an induction of various cytokine and chemokine gene transcripts (e. g. IL-1β, IL-6, CXCL8, and CCL3) after co-culture with *E. tenella*, *E. acervulina*, and *E. maxima* sporozoites was reported (Daugschies et al., 1986). It is worth noting that species-specific differences were apparent concerning respective transcriptional profiles. As such, species-specific induction of avian macrophage IFN-γ-mRNA by pathogenic species *E. tenella* may be of special interest (Dalloul et al., 2007). Furthermore, a specific *E. acervulina* antigen (antigen 3-1E) efficiently stimulated IL-12 release in DCs and led to upregulation of inflammatory modulators, such as CCL2, IL-6, TNF-α and IFN-γ (Rosenberg et al., 2005).

Referring to ETosis, Pérez et al. (2016) recently identified *E. ninakohlyakimovae* sporozoites as triggers of MET formation. Thus, caprine monocytes were shown to extrude METs following exposure to sporozoites, sporocysts and oocysts stages in a NADPH oxidase-dependent fashion. In addition, data of current dissertation delivered new knowledge on monocytes as potent ET releasers in response to *Eimeria* stages (Muñoz-Caro et al., 2015a); these data will be reviewed in chapter 2.

Besides macrophages, PMN are also involved in innate immune reactions against *Eimeria* infections. Histopathological studies demonstrated presence of PMN in mature *E. bovis* meronts (Friend and Stockdale 1980). In *E. maxima* (chicken) and *E. nieschulzi* (rats) infections a biphasic increase of PMN numbers in peripheral blood was observed during primary infection (Rose et al., 1979). In accordance, a biphasic upregulation of phagocytic and oxidative burst activities of PMN was detected during experimental *E. bovis* infections in calves (Behrendt et al., 2008). *In vitro*,

approximately one third of *E. bovis* sporozoites were eliminated by PMN in an antibody-independent manner (Behrendt et al., 2008). In line, an increased shedding of oocysts in PMN-depleted mice indicated an important role of PMN in *E. papillata* infections (Schito and Barta 1997). In agreement, Rose et al. (1984) detected an increased susceptibility to *E. vermiformis* infection in such mouse strains that exhibited defect PMN. As already reported for *E. falciformis* in the murine system (Bekhti et al., 1992), bovine PMN were reacted upon *E. bovis* sporozoite exposure via increased in ROS production and enhanced phagocytotic activities, that were even more enhanced after immune serum supplementation (Behrendt et al., 2008).
Exposure of bovine PMN to *E. bovis* sporozoites also resulted in a stage-specific induction of immunomodulatory molecules. Thus, exposure of PMN to sporozoites and merozoite I antigen led to enhanced IL-6/CCL2/CXCL1 and CXCL10/IL-12 gene transcription, respectively (Behrendt et al., 2008), which may be involved in the initiation of adaptive immune reactions (Taub et al., 1993; Gately et al., 1998). Interestingly, exposure of PMN to *E. bovis* sporozoites induced more iNOS gene transcripts than stimulation with merozoite-antigen. iNOS is essential for the generation of NO radicals, which are known to mediate killing of intracellular parasites (Ovington and Smith 1992; Ovington et al., 1995).
A first report on *E. bovis*-triggered NETosis in bovine PMN revealed sporozoites as potent NET inducers. Thus, sporozoites were entangled but not killed in DNAse I-soluble NET-like structures (Behrendt et al., 2010). Sporozoite-induced NET formation followed a rapid kinetic inducing DNA-release from PMN already within 30 min of incubation with sporozoites being hampered from host cell invasion (Behrendt et al., 2010). Moreover, further detailed molecular analyses were performed on *E. bovis*-triggered NETosis (Muñoz-Caro et al., 2015a) and NET formation was also identified as an effector mechanism directed against *E. arloingi* sporozoites (Silva et al., 2014). Since these findings were part of the current work, they will be presented in detail in chapter 2.

Data on other innate immune cells being involved in *Eimeria* defence are scarce. Studies *E. separata* infections in rats revealed an enhanced caecal infiltration with eosinophils and mast cells following repeated infections (Shi et al., 2000). In *E. papillata*-infected mice, NK cells caused an IFN-γ-mediated reduction of oocysts shedding (Schito and Barta 1997). The role of NK cells in *E. vermiformis*-challenged animals was also confirmed in NK cell-defective and antibody-depleted mice as well as in mice lacking IFN-γ synthesis (Smith et al., 1994; Rose et al., 1995) showing that at least in this experimental system, NK cells did not significantly contributed to control *Eimeria* infections (Smith et al., 1994). In accordance, by using SCID mice, the key role of NK cells was proven for *E. papillata* and *E. ferrisi*-, but not for *E. falciformis* or *E. vermiformis* infections (Schito et al., 1996). In avian *Eimeria* infections a strong cytotoxic activity of NK cells was detected; however this mechanism was restricted to re-infections (Lillehoj 1989). Meanwhile, human NK cells were found activated by *Eimeria* antigen exposure and stimulation with recombinant antigens induced intracellular IFN-γ and granzyme B synthesis (Aylsworth et al., 2013).

Referring to immune receptors being involved in innate *Eimeria* defence, little information is available, so far. During avian *E. praecox* infection TLR3, TLR4 and TLR15 were found highly expressed in duodenum and jejunum mucosa (Sumners et al., 2011). An upregulation of TLR4, TLR15 and MyD88 gene transcripts was also stated for heterophils and macrophages after *E. tenella* sporozoites exposure (Zhou et al., 2013). Interestingly, Fetterer et al. (2004) detected a profilin-like protein in *Eimeria* spp. sharing homology to *T. gondii* profilin which is recognized by TLR11 and known to trigger innate immune responses (Yarovinsky et al., 2005).

Besides classical leukocytes, also host cells are capable to perform innate immune reactions against *Eimeria* infections. As such, bovine endothelial cells reacted upon *E. bovis* infection during first merogony by selective chemokine gene transcription and by increased adhesion molecule gene transcription mediating PMN and PBMC adhesion to infected endothelium (Hermosilla et al., 2006; Taubert et al., 2006b, 2007). In agreement, microarray analyses of infected endothelial host cells revealed that *E. bovis* significantly alters host cellular immuno-associated reactions by the regulation of molecular networks being associated with leukocyte movement, immune cell trafficking and host cell inflammatory response (Taubert et al., 2010). Interestingly, the induction of these networks coincided with the time occurrence of parasite-specific antigens on the surface of infected host cells (Badawy et al., 2009). In addition, analyses on the proteome of *E. bovis* macromeront I-carrying host cells revealed parasite-induced alterations in distinct functional categories, such as host cell metabolism, cell structure, protein fate and gene transcription (Lutz et al., 2011). Moreover, in bovine colonic epithelial cells (BCEC) an upregulation of CXCL10 and

GM-CSF was stated during second merogony of *E. bovis* (Hermosilla et al., 2015). Referring to avian *Eimeria* infections, an enhanced gene transcription of IL-1β and chicken CC chemokine K203 was detected in jejunal and caecal mucosa of *E. maxima* and *E. tenella* infected chicken (Laurent et al., 2001). In addition, *E. tenella* infected epithelial cells also selectively showed an upregulation of IFN-γ, iNOS and chicken granulocyte colony-stimulating factor (CSF3) (Laurent et al., 2001).

1.3.2 *Cryptosporidium parvum*

Most studies on host immune reactions against *C. parvum* focused on adaptive immune responses and were mainly based on murine infection models (Blanshard et al., 1992; Aguirre et al., 1994; Schmidt et al., 2001; Chen et al., 2003; McDonald, 2008). However, some reports indicate a significant contribution of innate immune cells to development of protective inflammatory response against *C. parvum* (reviewed in Petry et al., 2010; MacDonald et al., 2013; Laurent and Lacroix-Lamandé 2017). Studies on humans and on *C. parvum*-infected BALB/c mice revealed a recruitment of macrophages and PMN to intestinal mucosa (Meisel et al., 1976; Genta et al., 1993; Boher et al., 1994). In addition, a moderate infiltration of eosinophils was found in abomasal mucosa of *C. andersoni*-infected cattle (Masuno et al., 2006). In a neonatal piglet model of *C. parvum* a significant increase of mucosal neutrophil MPO activity was detected and could be blocked via anti-CD18 antibodies (Zadrozni et al., 2006). Further studies revealed a cooperative role of macrophages and PMN in host antiparasitic resistance of mice acutely infected with *C. parvum* suggesting that *C. parvum*-induced macrophage activation was also influenced by PMN (Takeuchi et al., 2008). Furthermore, it was shown that DC numbers significantly increased following *C. parvum* infection in neonatal intestines (Lantier et al., 2013). In line, DC-attracting chemokines were produced by intestinal epithelial cells in response to *C. parvum* infection (Auray et al., 2007). In vitro, *C. parvum* sporozoite-exposed DCs expressed IFN-α and IFN-β within a few hours (Barakat et al., 2009) and vital sporozoites, soluble sporozoite antigens and recombinant parasite antigens equally induced DC maturation and subsequent release of cytokines such as IL-12, IL-1β and IL-6 (Bedi and Mead 2012). In the neonatal mouse model, mononuclear cells (especially macrophages and DCs) were proven to play a major role in mucosal innate immune reactions to *C. parvum* infections (reviewed in Laurent and Lacroix-Lamandé 2017). Respective details and a summary of cell actions are given in **Fig. 4**.

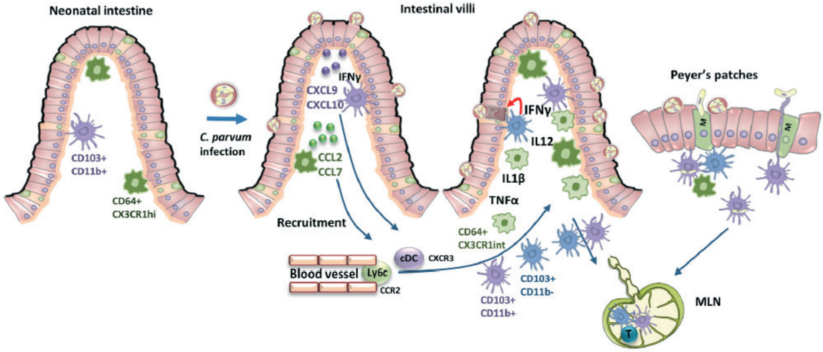

Fig. 4. Mucosal innate responses to *Cryptosporidium parvum* with a focus on mononuclear cells (neonatal mouse model). Few resident macrophages and dendritic cells are present in the neonatal mouse mucosa. After infection, the rapid release of chemokines by infected intestinal epithelial cells and those responding to the first wave of IFN-γ release by resident mononuclear cells allows the recruitment of multiple immune cell subsets to the lamina propria. Ly6c+CCR2+ inflammatory monocytes recruited by CCL2 and CCL7 quickly differentiate into CD64+CX3CR1int macrophages, producing proinflammatory cytokines including IL1-β and TNF-α, that decrease trans-epithelial resistance. CD103+ DCs are recruited by CXCL9 and CXCL10 (IFNγ-dependent chemokines) and produce high amounts of IL12 and IFNc, favouring control of the parasite in infected intestinal epithelial cells (red arrow) during the acute phase of the infection. CD103+CD11b-DCs produce the largest amounts of IFN-γ and therefore play a key role in the elimination process. Once activated, CD103+ DCs quickly migrate to the draining lymph node to activate specific T cells. *Cryptosporidium parvum* antigens and live parasites are also captured in specialised lymphoid tissues (Peyer's patches). Putative mechanisms of capture are represented (passage through M cells, capture by transepithelial dendrites). T-cell mediated adaptive immunity results in the definitive clearance of the infection. (Figure and legend taken from Laurent & Lacroix-Lamandé, 2017)

NK cells are also involved in innate immune reactions directed against *C. parvum* infections. Thus, activated human peripheral blood NK cells showed enhanced cytolytic activities against *C. parvum*-infected human intestinal epithelial cell lines (Dann et al., 2005). Activated NK cells were demonstrated early after infection in intestine of *C. parvum*-infected calves and were assumed to be involved in the innate response against this parasite (Olsen et al., 2015).

Referring to ET formation against *Cryptosporidium* parasites, no related data were available at the beginning of current work. As part of this dissertation, *C. parvum* sporozoites and oocysts were shown to effectively trigger NETosis (Muñoz-Caro et al., 2015b). The corresponding data are reviewed in chapter 2.

Given that *C. parvum* develops within intestinal epithelial cells of the small intestine, these host cells also participate in innate immune response against this pathogen (reviewed in Laurent and Lacroix-Lamandé, 2017). Intestinal epithelial cells express PRRs, such as TLRs, which might recognize *Cryptosporidium* antigens. As such, in a model of biliary cryptosporidiosis, a TLR4-mediated response was required for efficient eradication of *C. parvum* infection (Chen et al., 2005; O'Hara et al., 2011). Furthermore, the antimicrobial molecule β-defensin was identified in *C. parvum*-infected enteric mucosa (Tarver et al., 1998) and cathelicidin IL-37 was proven to exert parasitocidal effects on *C. parvum* stages and suggested to inhibit *in vitro* infection (Tarver et al., 1998; Giacometti et al., 1999; Zaalouk et al., 2004; Carryn et al., 2012). Several studied demonstrated an iNOS-mediated enhancement of NO synthesis in *C. parvum* infected epithelium (Leitch and He 1999; Gookin et al., 2004; Gookin et al., 2006). In line, the absence or inhibition of iNOS and the administration of antioxidants resulted in exarcerbation of epithelial infection and oocyst shedding (Leitch and He 1999; Huang and Yang 2002; Gookin et al., 2006). In addition, epithelial type I IFN expression is considered as a critical component of host cell invasion blockage (Pollok et al., 2001; MacDonald et al., 2013) and of immunity establishment (Barakat et al., 2009). Thus, *C. parvum* infection of intestinal epithelial cell lines was inhibited by type I IFN and IFN-γ but also TNF-α treatments (Lean et al., 2006; Barakat et al., 2009). *In vivo* studies revealed that synthesis of pro-inflammatory cytokines (e. g. TNF-α, IL-2, IL-12) in early stage of infection trigger production of NK cell-derived IFN-γ in *C. parvum*-infected mice (Codices et al., 2013). Other authors confirmed importance of these cytokines and of a Th1-type response for *C. parvum*-related immune control (Borad and Ward 2010; Petry et al., 2010; McDonald et al., 2013). Noteworthy, *ex vivo* studies on experimentally infected volunteers and chronically infected AIDS patients confirmed a *C. parvum*-induced expression of IFN-γ, IL-1β or IL-6 in jejunal biopsies (White et al., 2000). Moreover, an increased expression of CXCL-8 (syn. IL-8) and CXCL1 was detected in infected human intestinal epithelial cells *in vitro* (Laurent et al., 1997). In neonatal mouse model several chemokines (e. g. CCL2, CCL5, CXCL10 CXCL9) were produced during *Cryptosporidium* infection, all favouring immune cell recruitment (Lacroix-Lamande et al., 2002; Auray et al., 2007; Lantier et al., 2013). CXCL10 was also produced in epithelial cells of AIDS patients with active *Cryptosporidium* infection (Wang et al., 2007) and CCL5 was shown to play a crucial role in mediating immune responses against *C. parvum* in human intestinal cell line model (Laurent et al., 1997).

1.3.3 *Toxoplasma gondii*

Immunity to *T. gondii* infections is mainly based on IFN-γ-driven, T cell-dependent adaptive immune responses. However, innate immune cells play a pivotal role in shifting the immune responses towards effective T and NK cellular reactions and are therefore essential for the development of immunity (reviewed by Sasai et al., 2018, see also **Fig. 5**). Thus, inflammatory monocytes revealed as essential for controlling acute toxoplasmosis (Robben et al., 2005): following parasite recognition, innate immune cells (e. g. DCs, macrophages) release CCL2 and CXCL2 and thereby attract inflammatory monocytes to the site of *T. gondii* infection (Zlotnik and Yoshie 2000; Ma et al., 2014; Sasai et al., 2018). These monocytes appear to govern parasite control by pro-inflammatory molecule production (e. g. IL-1α, IL-1β, IL-6, iNOS, TNF, ROS) and also produced IL-10, a regulatory cytokine that prevents toxoplasmosis in brain (Biswas et al., 2015). Exposure of macrophages to *T. gondii* initiated MAP-Kinase- and NF-κB-dependent signalling pathways and led to distinct cytokine synthesis (e. g. IL-12, IL-1β, TNF-α) that subsequently stimulated IFN-γ production by NK and T cells (Philip et al., 1986; Nagineni et al., 1996). Macrophages became activated by IFN-γ and TNF-α leading to the upregulation of iNOS and specific GTPases, such as p47 GTPases, which afterwards limited parasite replication (Masek and Hunter 2013). An important parasitocidal mechanism is represented by iNOS-mediated production of NO in infected host cells (Adams et al., 1990). In agreement, *T. gondii*-infected iNOS-/- mice showed severe pathology associated with increased parasite burden in brain causing progressive toxoplasmic encephalitis (Scharton-Kersten et al., 1997; Khan et al., 1997). Besides NO, human macrophages also produce ROS to inhibit parasite replication (Murray et al., 1979;

Murray and Cohn 1979). Even though there is broad consensus that IFN-γ is the major mediator of resistance to *T. gondii*, there is also evidence on IFN-γ independent anti-microbial activities in macrophages (Masek and Hunter 2013). This is based on reports of patients with partial IFN-γ receptor (IFN-γR1) signaling deficiency who exhibit serological evidence of *T. gondii* infection, but are clinically asymptomatic (Janssen et al., 2002).

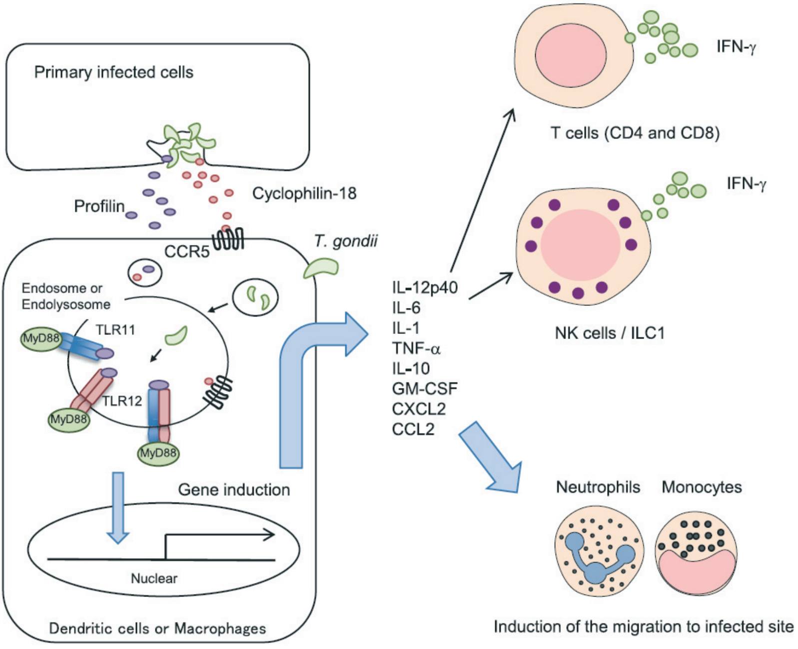

Fig. 5. Recognition of *T. gondii* by innate immune cells leads to activation of acquired immune reactions. Macrophages and DCs produce various inflammatory cytokines and chemokines to promote IFN-γ production in T cells, NK cells/Innate lymphoid cells (ILCs) mediating recruitment of neutrophils and inflammatory monocytes to sites of infection. ILCs are a newly identified lymphocyte subset related to innate immunity mainly producing IFN-γ and TNF-α in the small intestine in response to oral infection by *T. gondii* (Klose et al., 2014). (Figure and legend taken from Sasai et al., 2018).

Besides macrophages and monocytes, PMN are also involved in early *T. gondii* defence (Scharton-Kersten et al., 1997). As such, studies on PMN-depleted mice indicated that these animals are more susceptible for acute infections than wild-type mice (Sayles and Johnson 1996; Alexander et al., 1997; Scharton-Kersten et al., 1997). PMN were also shown to phagocytose and kill *T. gondii* tachyzoites *in vitro* (Wilson and Remington 1979; MacLaren and De Souza, 2002; MacLaren et al., 2004). Furthermore, stimulation of PMN with *T. gondii*-specific antigens resulted in the upregulation of several cytokines and chemokines, such as IL-12, CCL3, CCL4, CCL20, CCL5, CCL2 and TNFα (Bliss et al., 1999; Bennouna et al., 2003; Denkers et al., 2003, 2004). Recent studies also showed that human and murine PMN release NETs upon *T. gondii* exposure as demonstrated for the *in vitro* and *in vivo* situation (Abi Abdallah et al., 2012). Here, tachyzoites of three different *T. gondii* strain types (RH strain type I, II and III) were observed entrapped in NETs suggesting that extracellular traps were released in a parasite strain-independent manner likewise being dependent on the MEK-ERK-mediated pathway since chemical inhibition of ERK1/2 MAPK pathway in human PMN blocked *T. gondii*-induced NET formation (Abi Abdallah et al., 2012). In addition, we recently reported that *T. gondii* also triggers the formation of ETs in PMN and monocytes isolated from harbour seals (*Phoca vitulina*) (Reichel et al., 2015). Since these findings are part of the current, work they will be presented in chapter 2.

Also NK cells play a crucial role during acute *T. gondii* infection and are able to lyse both, infected cells and tachyzoite stages via cytotoxic mechanisms (Kamiyama et al., 1982; Hauser et al., 1982; 1983; 1986; Subauste et al., 1992). Their importance was also stated in mice models experiencing defective NK cell cytotoxicity or NK cell depletion since severe *T. gondii* manifestations were here observed (Hughes et al., 1988; Johnson et al., 1995; Hunter et al., 1995). The protective role of NK cells may be based on their capability to produce IFN-γ after contact with tachyzoites or parasite extracts (Denney et al., 1990; Sher et al., 1993; Scharton-Kersten et al., 1996). NK cells were also proven as a source of IL-10 in systemic *T. gondii* infection (Gigley 2016) and are therefore involved in the regulation of inflammation by this immunosuppressive cytokine (Perona-Wright et al., 2009). Studies using an *in vivo* mouse model demonstrated that, besides IL-12, NK cells to also produce IL-17 during toxoplasmosis contributing to the immune-mediated resistance to *T. gondii* infection (Passos et al., 2010).

Regarding cytokine responses induced by *T. gondii,* IFN-γ is well-accepted as key element for the development of effective host resistance (Nathan et al., 1983; Suzuki et al., 1988). Thus, this cytokine is the major regulator of cell-mediated immunity against this parasite with IFN-γ main sources being represented by T- and NK-cells (Sasai et al., 2018). Consequently, a lack of IFN-γ increases mortality in infected animals (Suzuki et al., 1988; Scharton-Kersten et al., 1996, Takács et al., 2012). IFN-γ production is mainly regulated by IL-12 secretion (Lieberman and Hunter 2002; see also **Fig. 5**). In line, the absence of IL-12 resulted in increased murine susceptibility to *T. gondii* infection (Gazzinelli et al. 1993; Lieberman et al., 2004). However, IL-12 is mainly produced by innate immune cells, such as PMN, DCs, macrophages and monocytes thereby playing a key role during acute phase of *T. gondii* infection (Hou et al., 2011). Interestingly, recent studies on human monocytes and DCs exposed to *T. gondii* tachyzoites showed that an efficient IL-12 response is rather triggered by phagocytosis of living parasites than by active host cell invasion (Tosh et al., 2016).

Referring to innate immune cell signalling, the TLR/IL-1R adaptor protein MyD88 was identified as a major regulator of host defence against *T. gondii* tachyzoites (Scanga et al., 2002). Overall, MyD88 is activated by almost all TLRs (O'Neill and Bowie 2007; Yarovinsky 2008) but TLR11 was identified as a key innate immune receptor that regulates IL-12-related response to *T. gondii* (Yarovinsky et al., 2005). Interestingly, the actin-binding protein profilin acts as a ligand of TLR11 (Plattner et al., 2008). In line, *T. gondii* profilin elicits TLR11- and MyD88-dependent IL-12 responses in DCs *in vitro* and *in vivo* (Yarovinsky et al., 2006, 2008). Additional, it was shown that blockage of profilin expression blocks *T. gondii*-induced DC-mediated IL-12 responses and abrogates the capacity of the parasite to infect host cells (Plattner et al., 2008).

Host cell interactions with *T. gondii* underly complex regulatory processes (Gazzinelli et al., 1993, 1994, 1996; Rettigner et al., 2004; Innes et al., 2005; Moore et al., 2005). As obligate intracellular parasite, *T. gondii* interferes with host signalling pathways to modulate innate immune responses (Denkers et al., 2003). However, several studies showed that host cells react upon *T. gondii* infection with the upregulation of several immunomodulatory molecules. Thus, adhesion molecules (e. g. E- and P-selectin, ICAM, VCAM-1) mediating immune cell attachment to infected cells (Taubert et al., 2006a) and chemokines (e. g. IL-8, CXCL2, CCL2, CCL3, CCL5, eotaxin) promoting immune cell recruitment were reported up-regulated in *T. gondii*-infected host cells (Deckert-Schlüter et al., 1999; Mennechet et al., 2002; Knight et al., 2005; Taubert et al., 2006a, b; Ju et al., 2009). In human intestinal epithelial cells, *T. gondii* infections induced MyD88-dependent IL-8 (CXCL8) secretion and ERK1/2 activation (Ju et al., 2009).

Besides chemokines, defensins are also reported to be enhanced in *T. gondii*-infected host cells (Foureau et al., 2010; Morampudi et al., 2011). These molecules bear chemoattractant properties on monocytes, T cells and DC (Lai et al., 2009) and elicit adverse effects on *T. gondii* tachyzoites (Tanaka et al., 2008; Morampudi et al., 2011). Interestingly, type I *T. gondii* parasites appear to suppress β-defensin 2 gene expression for immune evasion (Morampudi et al., 2011).

1.3.4 *Besnoitia besnoiti*

So far, hardly any data exist on innate immune reactions directed against *B. besnoiti* and most studies focused on epidemiological and clinical aspects of the disease. Applying hamster models, *in vivo* experiments showed that macrophage activation and specific cellular immune responses occurred during latent *Besnoitia* infections (Hoff and Frenkel 1974). *In vitro, B. besnoiti* infections led to endothelial host cell activation and promoted PMN adhesion and NET formation under physiological flow conditions (Maksimov et al., 2016).

At the beginning of the current doctoral thesis, no data on ET formation against *B. besnoiti* were available. Therefore, here presented data signify the first studies on *B. besnoiti*-triggered ETosis and show that both cell types, bovine PMN and monocytes utilize this effector mechanism to attack *B. besnoiti* tachyzoites. Respective data are described in more detail in chapter 2.

2. RESULTS AND DISCUSSION

The current work focused on the PMN-derived effector mechanism of NET formation in response to different parasitic species and stages. In addition, some digressions were made on other immune cells, such as monocytes or eosinophils, in respect to ETosis. As main outcomes of the current work, four new species were here identified as NET inducers. Furthermore, NET-specific molecules and signaling pathways being involved in parasite-triggered ETosis were here characterized and with CD11b a new PMN-derived receptor was proven as essential in *E. bovis*-triggered NETosis.

Regarding parasite-mediated ETosis, several protozoan parasite species were identified as ETs inducers, so far. As such, the euglenozoan parasites *Leishmania amazonensis, L. major*, *L. braziliensis*, *L. chagasi* and *L. donovani* (Guimarães-Costa et al., 2009, 2011; Gabriel et al., 2010; Wang et al., 2011) as well as the apicomplexan parasites *P. falciparum*, *E. bovis, N. caninum* and *T. gondii* have been reported to trigger ETosis in different host species *in vivo* and *in vitro* (Baker et al., 2008; Behrendt et al., 2010; Abi Abdallah et al., 2012; Wei et al., 2016; Villagra-Blanco et al., 2017, Yang et al., 2018). With the current work, new apicomplexan parasites were added to the list of NET inducers, such as *B. besnoiti, C. parvum* (now referred to as cryptogregaria) and *E. arloingi* (Silva et al., 2014; Muñoz-Caro et al., 2014a, b). Besides protozoa, nematode parasites were also proven to induce ETs, as documented for *S. stercoralis* (Bonne-Annee et al., 2014), the filarial parasites *B. malayi* (McCoy et al., 2017) and *L. sigmodontis* (Pionnier et al., 2016), the metastrongyloids *A. vasorum, A. abstrusus* and *T. brevior* (Lange et al., 2017) and trematode parasites *S. japonicum* (Chuah et al., 2013) and *T. regent* (Skála et al., 2018). Referring to *Haemonchus contortus*, the current data also added a new nematode species as potent trigger of NET formation (Muñoz-Caro et al., 2015c).

Since first discovery in 2004, knowledge on ET formation has immensely grown and nowadays comprises a set of immune responses to various pathogens across the entire eukaryotic domain (Boe et al., 2015). To date, two main mechanisms of ET release have been described. As a major route of ETosis, leukocytes release ETs via a slow lytic cell death mechanism that requires NADPH-oxidase complex activation and subsequent ROS generation (Papayannopoulos et al., 2010; Yang et al., 2016).

Since both factors were demonstrated to be crucial for parasite-induced NETs of the current studies, this type of NETosis is most likely to occur in the case of *E. bovis*-, *E. arloingi*-, *B. besnoiti*- and *H. contortus*-triggered NETosis (Muñoz-Caro et al., 2014a, b; Silva et al., 2014; Muñoz-Caro et al., 2015a, b, c; Reichel el at., 2015). Meanwhile, Pilsczek et al. (2010) and Yousefi et al. (2009) reported on a small proportion of leukocytes that rapidly expulse their nuclear content via vesicular secretion and thereby generate ETs in vital cell conditions. This form of ETosis may be accompanied with ongoing crawling and phagocytic activities (Yipp et al., 2012) and was suggested as NADPH oxidase-independent (Papayannopoulos 2017). However, this type of ETosis was not observed for any of examined parasites in the current work.

So far, NET formation has been documented in diverse vertebrate species, including cattle (Lippolis et al., 2006; Grinberg et al., 2008; Behrendt et al., 2010; Aulik et al., 2010; Muñoz-Caro et al., 2014, 2015a, b; Villagra-Blanco et al., 2017), horses (Alghamdi et al., 2005; Rebordão et al., 2014; Muñoz-Caro et al., 2015a), goats (Silva et al., 2014; Pérez et al., 2016; Villagra-Blanco et al., 2017; Yang et al., 2018), cats (Wardini et al., 2010), dogs (Wei et al., 2016), mice (Ermert et al., 2009), birds (chicken heterophils; Chuammitri et al., 2009) and fish (Palić et al., 2007; Pijanowski et al., 2013). With harbour seals we could add a new host species by the current work (Reichel et al., 2015).

In addition, this effector mechanism is not limited to vertebrates, since innate immune cells from a range of invertebrate species, such as insects (Altincicek et al., 2008), crustaceans (crab haemocytes; Robb et al., 2014), mollusks (mussel, oyster and gastropod haemocytes; Poirier et al., 2014; Lange et al., 2017, Skála et al., 2018) and earthworms (Annelida, coelomonocytes; Homa 2018) also release ET-like structures. Interestingly, even sea anemone cells can eject extracellular chromatin in a similar fashion (Robb et al., 2014). Accordingly, PMN of different host origins (bovine, caprine, canine, equine, seals) were here tested for NET formation against several parasite species and stages. Respective data showed that NETosis was equally performed by these PMN and did not significantly differ in the extent of the reaction thereby indicating host-independent reactions (Muñoz-Caro et al., 2015a). In line with these data, *T. gondii*-triggered NETosis was reported for both, human and murine PMN (Abi Abdallah et al., 2012). To indirectly confirm these data, we also tested bovine PMN against sporozoite stages of other, mainly non-bovine coccidian species (*Isospora suis*, *E. arloingi*, *T. gondii*) and proved host species-independent reactions since all parasites induced NETosis (Muñoz-Caro et al., 2015a).

Kinetic studies revealed *B. besnoiti* and *T. gondii* tachyzoite- as well *E. arloingi* and *C. parvum* sporozoite-triggered NETosis as a time-dependent process in the current work (Silva et al., 2014; Muñoz-Caro et al., 2014a; Reichel et al., 2015). These findings were here also confirmed for *H. contortus*-mediated NET formation (Muñoz-Caro et al., 2015c) and are in line with previously NET-related data on *E. bovis* (Behrendt et al., 2010), *T. gondii* (Abi Abdallah et al., 2012) and *T. cruzi* (Sousa-Rocha et al., 2015). Moreover, regarding the influence of pathogen doses on parasite-triggered NETosis, present data demonstrate a clear dose-dependency of *B. besnoiti*-, *C. parvum*-, *T. gondii*- and *H. contortus*-induced NET formation (Muñoz-Caro et al., 2014a; 2015b, c; Reichel et al., 2015). These data are in agreement with other NET-related studies on *E. bovis, N. caninum*, *L. amazonensis, L. major, L. braziliensis, L. mexicana* and *T. cruzi* (Behrendt et al., 2010; Wei et al., 2016; Villagra-Blanco et al., 2017; Guimarães-Costa et al., 2009; Wang et al., 2011; Sousa-Rocha et al., 2015).

Besides a time- and dose-dependency, current studies also showed that different stages of coccidian parasites are capable to induce NETs. As such, merozoite I stages of *E. bovis*, sporozoites of *I. suis*, *T. gondii*, *E. arloingi* and *E. bovis,* oocysts of *E. arloingi* and *C. parvum* and tachyzoites of *B. besnoiti* and *N. caninum* were shown to trigger NETosis (Silva et al., 2014; Muñoz-Caro et al., 2014a, 2015a,b; Rachel et al., 2015; Wei et al., 2016) suggesting commonly expressed surface molecules as ligands for PMN. In line, data on different *Leishmania* stages (Guimarães-Costa et al., 2009; Gabriel et al., 2010; Hurrell et al., 2015; Morgado et al., 2015) also indicated NETosis as a stage-independent defense mechanism and Abi Abdallah et al. (2012) also identified *T. gondii* tachyzoite stages to induce NETs. Interestingly, no strain-dependent differences were found for *T. gondii* (Abi Abdallah et al., 2012).

Besides *in vitro* evidence, *Eimeria*-triggered NETosis was also proven to occur *in vivo* by the current work. By analyzing histological gut samples, NETs were mainly found in close proximity to different *Eimeria* stages (i. e. meronts and gamonts) in the mucosa of *E. arloingi*-infected goats and *E. bovis*-infected calves (Muñoz-Caro et al., 2016). These *in vivo* data strongly suggest NETosis as an early host effector mechanism acting against intestinal coccidian stages as previously postulated (Hermosilla et al., 2014).

Current data indicated that *E. arloingi* sporozoite- and *B. besnoiti* tachyzoite-induced NETs rather mediate entrapment than killing of these parasite stages (Silva et al., 2014; Muñoz-Caro et al., 2014a). These data are in agreement with an earlier report on *E. bovis*-induced NETosis which also failed to demonstrate lethal effects of NETs (Behrendt et al., 2010). In contrast to these findings, certain lethal effects of NETs on tachyzoites of *T. gondii* (25% killing; Abi Abdallah et al., 2012) and on *L. amazonensis* promastigotes (53%; Guimarães-Costa et al., 2009) were recorded. However, for *Leishmania* parasites it was also shown that they may evade NET-mediated killing by different mechanisms, such as blocking oxidative burst activities of PMN, entering a non-lytic compartment within PMN or by resisting microbicidal activities of NETs (Gabriel et al., 2010; Chagas et al., 2014). Interestingly, other investigations on *Leishmania* infections demonstrated that salivary components from intermediate hosts (sand flies) being infected during blood meal may improve *Leishmania* survival in definitive hosts by affecting their innate immune system. As such, a *Lutzomya lingipalpis* salivary gland molecule, known as Lundep, was recently described to bear endonuclease activity with NET-destroying properties (Chagas et al. 2014). The capacity of NETs to entrap stages of coccidian parasites and to inhibit host cell invasion was first demonstrated for *E. bovis* by showing that exposure of sporozoites to bovine PMN hampered subsequent host cell invasion for up to 65% (Behrendt et al., 2010). Similar effects were here detected for *B. besnoiti* tachyzoites, *E. arloingi* and *C. parvum* sporozoites (Muñoz-Caro et al., 2014a; Silva et al., 2014; Muñoz-Caro et al., 2015b). Nonetheless, differences in the degree of parasite entrapment were observed. Thus, *E. arloingi* sporozoites were more effectively entrapped by caprine NETs (up to 72%) than *E. bovis* sporozoites (15%), *B. besnoiti*-tachyzoites (34%) or *C. parvum*-sporozoites (15%) by bovine NET structures (Behrendt et al., 2010; Silva et al., 2014; Muñoz-Caro et al., 2014a, 2015b) which may indicate host species- or parasite species-dependent differences. In all cases, treatments with DNase I (leading to NET resolution) restored parasite infectivity thereby confirming that blockage of parasite invasion could be attributed to NET formation (Muñoz-Caro et al., 2014a, b, 2015a, b; Silva et al., 2014; Reichel et al., 2015). DNase I treatments to resolve NETs have been used in both, animal- (Caudrillier et al., 2012) and human-derived experiments, so far (Pressler et al., 2008). Accordingly, whenever DNase I treatments were used to control for NET formation within the present dissertation, a significant diminishment of NETs was detected when compared to DNase I-free controls (Muñoz-Caro et al., 2014a, b; Silva et al., 2014; Muñoz-Caro et al., 2015a, b, c; Rachel et al., 2015) thus confirming the irrefutable fact that nuclear DNA is the main component of NETs (von Köckritz-Blickwede et al., 2009; Hakkim et al., 2010). The biological relevance of DNA-rich structures was also estimated for *in vivo* situations. Thus, *in vivo* data showed patients with systemic lupus erythematosus to exhibit high levels of serum DNase I-inhibitory activity that prevents degradation of NETs (Hakkim et al., 2010). Overall, dependent on extent and proper removal of NETs, these structures might have a considerable impact on pathophysiological processes *in vivo* and therefore play a role in autoimmune disorders (Lögters et al., 2009). In accordance, some reports showed increased levels of circulating free DNA (cf-DNA) in various human diseases (Swarup and Rajeswari 2007). However, this effect was attributed to tissue destruction and/or necrosis and etiology was related to autoantibodies raised against cf-DNA (Lögters et al., 2009). Aberrant levels of NETs or anti-NET antibodies may indeed lead to dramatic tissue damage as demonstrated for liver, endometrium and lung (Gupta et al., 2005; Beiter et al., 2006; Clark et al., 2007). In particular, extracellular histones (e. g. H2B) as NETs components can directly damage endothelial and epithelial cells by triggering cell death (Saffarzadeh et al., 2012). Moreover, impaired degradation and clearance of NETs was also linked to reproductive disorders, such as preeclampsia (Gupta et al., 2006; Hahn et al., 2012) or to a variety of autoimmune diseases, such as atherosclerosis (Döring et al., 2012), rheumatoid arthritis (Rohrbach et al., 2012), small vessel vasculitis (Kessenbrock et al., 2009), gout (Mitroulis et al., 2011) or systemic lupus erythematosus (Hakkim et al., 2010; Leffler et al., 2012; Liu et al. 2012).

Classical NET structures were shown to be composed of a DNA backbone being studded with several antimicrobial molecules, such as histones, NE or MPO (Brinkmann et al., 2004). As such, these effector molecules were consistently detected in fungi- and bacteria-induced NET structures (Urban et al., 2009; Brinkmann et al., 2004; von Kockritz-Blickwede et al., 2010). In line with those data, these classical characteristics were here confirmed for *B. besnoiti-, E. arloingi-, E. bovis-, T. gondii-, C. parvum-* and *H. contortus*-induced ETs by demonstrating the simultaneous presence of DNA with histones, NE and MPO in these structures. In addition to mere molecule detection, enhanced enzyme activities of NE and MPO were detected in case of *B. besnoiti*-, *E. arloingi*- and *E. bovis*-exposed PMN (Muñoz-Caro et al., 2014a, 2015a; Silva et al., 2014) confirming the key role of these enzymes in pathogen-induced NETosis as suggested by others (Papayannopoulos et al., 2010). As also reported for non-parasitic NET inducers (Papayannopoulos et al., 2010; Parker et al., 2012), functional inhibition experiments further confirmed biological importance of these enzymes since parasite-mediated NETosis was significantly blocked when NE and MPO inhibitors were applied (as proven for *E. bovis*, *E. arloingi*, *B. besnoiti*, *T. gondii*, *C. parvum* and *H. contortus* in the current work) (Muñoz-Caro et al., 2014a, 2015a, b, c; Silva et al., 2014; Reichel et al., 2015). Meanwhile, other closely related parasites, such as *N. caninum* have shown to trigger NE- and MPO-dependent NET formation in canine PMN (Wei et al., 2016). Furthermore, a significant role of NE and MPO enzymes has been proven by the use of respective knockout mice. Thus, it was shown that these animals are more susceptible to bacterial and fungal infections (Belaaouaj et al., 1998) as also reflected by severe impairment of MPO-deficient mice in their early host defense against *C. albicans* (Aratani et al., 1999). Besides histones, MPO and NE, several other antimicrobial components are present within ETs, such as bacterial BPI, pentraxin and lactoferrin (Brinkmann and Zychlinsky 2007, 2012; von Köckritz-Blickwede and Nizet 2009), which additionally contribute to pathogen killing (Brinkmann and Zychlinsky 2012).

Whilst NE and MPO originate from azurophilic PMN granules, other molecules, such as metalloproteinases (MMP), are contained in tertiary (gelatinase) granules. We here showed via zymographic analyses of PMN supernatants that MMP-9 is released from PMN upon *E. bovis* sporozoite exposure (Muñoz-Caro et al., 2015a). So far, the functional role of MMP-9 in NETosis is unclear. However, it is worth noting that the release of MMP-9 is regulated by the ERK 1/2 and p38 MAPK signaling pathway (Wang et al., 2002) which was proven to be involved in NET formation in the current work (Muñoz-Caro et al., 2015a) and by others (Hakkim et al., 2011; Abi Abdallah et al., 2012; Keshari et al., 2013; Cheng and Palaniyar 2013). Meanwhile, studies on the role of NETs systemic lupus erythematosus*,* revealed that activation of endothelial MMP-2 by NET-derived MMP-9 represents an important factor in impaired endothelial function (Carmona-Rivera et al., 2015).

During NETosis, NOX-dependent ROS production leads to morphological changes, such as delobulation of PMN nucleus, disassembly of nuclear envelope and degradation of granule membranes (Fuchs et al., 2007). In addition, PAD4-mediated histone citrullination, followed by chromatin decondensation appears obligatory for proper NET release (Wang et al., 2009; Abi Abdallah and Denkers 2012; Hahn et al., 2013). Most studies on NET formation strengthened pivotal role of a functional NOX system with subsequent ROS production. Accordingly, enhanced oxidative burst activities were demonstrated in bovine PMN exposed to *E. bovis* sporozoites both, *in vitro* and *ex vivo* (Behrendt et al., 2008). In addition, *T. gondii*-triggered oxidative burst activities were noted for macrophages and ROS production was linked to resistance of macrophages against this parasite (Murray and Cohn 1979). In accordance, current data revealed that PMN exposure to *B. besnoiti* tachyzoites, to *E. arloingi* and *E. bovis* sporozoites resulted in enhanced ROS production in these immune cells (Muñoz-Caro et al., 2014a, 2015a; Silva et al., 2014). Furthermore and in line with data on bacteriae- or fungi-induced NETosis (Fuchs et al., 2007; Bruns et al., 2010; Riyapa et al., 2012; Yoo et al., 2014), chemical NOX blockage via DPI treatments resulted in a significant diminishment of *B. besnoiti*-, *E. bovis*-, *E. arloingi*-, *T. gondii*-, *C. parvum*-and *H. contortus*-triggered NETosis thereby confirming NOX dependency of parasite-triggered NETosis (Muñoz-Caro et al., 2014a, b, 2015a, b, c; Silva et al., 2014; Reichel et al., 2015). In contrast, few studies recently showed that NET formation might also occur in a NOX-independent manner. Thus, *S. aureus* and *N. caninum* stages triggered NETosis independent of NOX activity (Pilsczek et al., 2010; Villagra-Blanco et al., 2017) and singlet oxygen (as a member of the ROS family) stimulated NETs in the absence of NOX activity (Nishinaka et al., 2011).

NOX-mediated ROS production was recently proven as Ca^{2+}-dependent in the bovine system (Conejeros et al., 2011). In addition, NET induction by thapsigargin, which mobilizes Ca^{2+} from intracellular pools, confirmed a key role of Ca^{2+}-influx in NETosis (Gupta et al., 2010) and human NET formation proved to be Ca^{2+-}dependent (Gupta et al., 2014). Given that store-operated calcium entry (SOCE) was shown to play a crucial role in Ca^{2+}-dependent ROS production (Hallett at al., 1990), the role of SOCE in *E. bovis*-, *C. parvum*- and *T. gondii*-induced NET formation was here analyzed. Since functional blockage of SOCE via 2-APB pre-treatments of PMN resulted in a significant diminishment of NETosis, parasite-triggered NET formation was here confirmed as a SOCE-dependent process (Muñoz-Caro et al., 2015a, b;

Reichel et al., 2015; Wei et al., 2016). SOCE is tightly regulated via the ERK1/2 MAPK-related signaling pathway, which was recently identified as essential for NET formation (Hakkim et al., 2011). In accordance to *T. gondii*-related reports (Abi Abdallah., 2012; Byrd et al., 2013), we demonstrated a significant *E. bovis* sporozoite-triggered up-regulation of ERK1/2 and p38 MAPK phosphorylation in bovine PMN (Muñoz-Caro et al., 2015a). In addition, a pivotal role of the ERK1/2- and p38 MAPK-dependent signaling pathways for parasite-induced NET formation was confirmed via functional chemical inhibition experiments showing that *E. bovis*-, *T. gondii*- and *C. parvum*-induced NETosis was significantly diminished in presence of ERK- and p38-specific inhibitors (Muñoz-Caro et al., 2015a, b; Reichel et al., 2015). These data are in line with a recent report on *N. caninum*-triggered NET formation in the canine system (Wei et al., 2016).

So far, little data are available on PMN-derived receptors being involved in pathogen-triggered NET formation. As such, a potential role of CD11b and CD18 was reported for *C. albicans* or *M. haemolytica*-mediated NETosis (Aulik et al., 2010; Byrd et al., 2013). Given that parasite-driven NETosis revealed as ROS-dependent in most cases (Behrendt et al., 2010; Muñoz-Caro et al., 2014a, 2015a, b, c; Silva et al., 2014; Reichel et al., 2015; Wei et al., 2016; McCoy et al., 2017) and that ROS production in cattle was described as a CD11b (CR3)-dependent mechanism (Conejeros et al., 2012), we here analyzed role of CD11b in parasite-triggered NET formation. Overall, a significantly enhanced CD11b expression was detected on bovine PMN surface via FACS-based analyses following exposure to *E. bovis* sporozoites. Blocking experiments proved key role of this receptor in *E. bovis*-mediated-NETosis since NET formation was significantly inhibited in presence of specific anti-CD11b-antibodies (Muñoz-Caro et al., 2015a). These findings were in accordance to Byrd et al. (2013) suggesting CD11b as a potential PMN-derived receptor being involved in NETosis since antibody-mediated blockage of this integrin inhibited fibronectin-dependent NET formation to *C. albicans* hyphae. So far, few other PMN-derived receptors, such as TLRs, have been described as participating in NET formation. Thus, a pivotal role of TLR4 was confirmed in *S. aureus*-triggered NETosis (Wan et al., 2017). TLR4, jointly with CR3, also played an important role in the induction of “vital NETs” *in vivo* (Yipp et al., 2012) and in *Wolbachia*-triggered NETosis (Tamarozzi et al., 2016). Referring to parasites, TLR2 and 4 appear to be involved in the induction of NETs by *T. cruzi* and its soluble antigens (Sousa-Rocha et al., 2015). Referring to NET-inducing viruses, it has been demonstrated that respiratory syncytial virus (RSV) fusion protein promotes TLR-4-dependent NET formation by human PMN (Funchal et al., 2015).

Recently, different morphological types of NETs were described: aggregated, spread and diffused NETs (Schauer et al., 2014). Interestingly, it was reported that aggregated NETs are capable to degrade cytokines and chemokines via serine protease activity in response to monosodium urate (MSU) crystals formation which commonly occurs at inflammatory sites during autoimmune gout disease (Schauer et al., 2014). In accordance, different morphological forms of NETs were also observed in response to different parasite species and stages in the current work. These were defined as follows: *i)* “diffuse” NETs (*diff*NETs) being composed of a complex of extracellular, decondensed chromatin decorated with antimicrobial proteins with globular and compact form and a size of 25–28 nm diameter; *ii)* “spread” NETs (*spr*NETs) consisting of smooth and elongated web-like structures of decondensed chromatin and antimicrobial proteins with a thin-fibered appearance and a diameter of 15–17 nm, and *iii)* “aggregated” NETs (*agg*NETs) being represented by large clusters of NET-like structures with a “ball of yarn”-like, clumpy and massive appearance typically involving a high number of PMN. As an interesting outcome, *B. besnoiti-, E. bovis-, E. arloingi-*, *T. gondii-* and *C. parvum*-triggered NETs almost exclusively showed *spr*NET and *diff*NET morphology (Muñoz-Caro et al., 2014a, 2015a, b; Silva et al., 2014, Reichel et al., 2015). In line, *B. besnoiti* tachyzoite-triggered monocyte-derived ETs also displayed a characteristic diffuse, non-filaroid type of NET structures (Muñoz-Caro et al., 2014b). In contrast to small protozoan stages, large-sized larval stages of the nematode *H. contortus* additionally induced the formation of *agg*NETs being allocated in close proximity or directly attaching the highly motile larvae and promoting their firm entrapment (Muñoz-Caro et al., 2015c). Occasionally, *agg*NETs led to an almost entire coverage of *H. contortus* larvae and to formation of anchor-like NET structures, that contacted *agg*NETs and hampered larvae to escape and to move on (Muñoz-Caro et al., 2015c). We therefore postulate that NET-related immune reactions may limit establishment of *H. contortus* larvae in the gastric glands *in vivo*. It may also be hypothesized that firm immobilization of L3 will not only dampen their migratory activity but also expose them more easily as targets for other leukocytes. Overall, we assume that formation of *agg*NET is more likely to be induced by large-sized parasites with both, size and high motility representing potential triggers for *agg*NETs release. In accordance, recent studies proposed that PMN are capable to sense size of pathogens and selectively release NETs in response to large-sized pathogens (Branzk et al., 2014). Interestingly in this respect, Warnatsch et al. (2017) recently showed that ROS molecules act as sensors of microbe size. Thus, small microbes induce formation of intracellular ROS whilst large microbes trigger ROS extracellularly and lead to recruitment of numerous PMN forming cooperative clusters (Warnatsch et al., 2017). The latter phenomenon may mirror the current observations on *H. contortus*-triggered *agg*NET formation. In addition, physical properties of particles, such as shape and rigidity, were proven to influence choice of effector mechanism of phagocytes (Wheeler and Underhill, 2014). Taken together, these findings may explain the high capacity of large-sized metazoan parasites, such as *S. japonicum* (Chuah et al., 2013)*, S. stercoralis* (Bonne-Année et al., 2014) and *H. contortus* (Muñoz-Caro et al., 2015c) to induce NETs. Obviously, a NET-based effector mechanism will also deliver a more effective defense since other strategies, such as phagocytosis, are most likely ineffective against multicellular pathogens.

Referring to ability of PMN to fight pathogens, different studies showed their capacity to actively transmigrate into the intestinal lumen and to attack pathogens within the luminal environment (Brazil et al., 2013; Seper et al., 2013; Szabady and McCormick 2013; Sumagin et al., 2014). In accordance, it was demonstrated in current work that PMN indeed interact with *E. arloingi* oocysts being present in intestinal lumen *in vivo* and that they release NETs which almost covered micropyle region besides other areas of oocysts (Muñoz-Caro et al., 2016). Given that *E. arloingi* sporozoites must egress through the micropyle (Jackson et al., 1964) instead of being set free by oocyst wall disrupture as seen in non-micropylic species (e. g. *E. ninakohlyakimovae, E. zuernii, E. bovis*), NET formation may hamper adequate oocyst excystation and sporozoite release.

Referring to NET-borne effects against coccidian stages, it has to be stated that these parasites mainly represent intracellular stages. However, all stages, i. e. sporozoites, merozoites I and tachyzoites, do not spend their entire life span inside host cells and will obviously become targets of immune cells in the extracellular compartment. Thus, *E. bovis* sporozoites have to traverse mucosal layer of the ileum to reach lymphatic capillaries for infection of adequate host cells, lymphatic

endothelial cells. Furthermore, *Eimeria* sporozoites are known to continuously leave inadequate host cells *in vitro* for other host cell invasion (Behrendt et al., 2004). Merozoites I and tachyzoites are released into the extracellular compartment via host cell lysis and then have to migrate for adequate host cell discovery. During their extracellular stay, these parasitic stages are vulnerable and become potential targets for leukocytes as previously postulated (Hermosilla et al., 2014; Silva et al., 2016). This is also reflected by the fact, that adaptive humoral and cellular immune responses are often directed against these different stages (Hermosilla et al., 2006; Taubert et al., 2006b; Behrendt et al., 2008; Taubert et al., 2008; Taubert et al., 2009; Hermosilla et al., 2014).
Besides directly affecting pathogens, NETs also contribute to the initiation or improvement of effective immune reactions, such as leukocyte recruitment (Hermosilla et al., 2006; Caudrillier et al., 2012; Narayana Moorthy et al., 2013; Luo et al., 2014), which may in turn, affect parasite survival as well. Given that high concentrations of antimicrobial molecules (e. g. histones) are reported to bear damaging capacities for tissues or cell layers (Saffarzadeh et al., 2012), we additionally postulate that NET-related molecules might also exhibit detrimental effects on infected host cells. Consistently to this assumption, present *in vivo* data showed that NETs were consistently found attached to surface of epithelial host cells carrying intracellular stages in heavily *Eimeria*-infected mucosa (Muñoz-Caro et al., 2016). Evidence on NET formation on *B. besnoiti*-infected primary BUVEC was recently given by Maksimov et al. (2016) describing a rapid attachment of PMN onto infected BUVEC under physiological flow conditions being followed by release of NETs. Maksimov et al. (2016) postulated that these events might serve as an orientation to attract other innate immune cells to site of infection in response to molecules present in NETs and to chemoattractant molecules released from *B. besnoiti*-infected endothelium thereby linking immune cell effector mechanisms with actions of infected endothelial cells. In accordance, previous reports already showed that coccidian infections lead to host cellular adhesion molecule and chemokine up-regulation thereby promoting adhesion of PMN and PBMC to apicomplexan-infected cell layers (Hermosilla et al., 2006; Taubert et al., 2006a, b).

Besides PMN, also other mammalian leukocyte populations (e. g. monocytes, eosinophils) were studied as potential ET-releasers in the current work. First evidence on monocyte-derived extracellular traps (METs) came from studies performed by Chow et al. (2010) proving that statins which are the rate-limiting enzymes in cholesterol biosynthesis were able to enhance the formation of ETs in human and murine monocytes/macrophages. Meanwhile, studies on bovine monocyte-derived macrophages being exposed to the bacteria *M. haemolytica* and its leukotoxin confirmed that this cell type is also able to release ETs in a NADPH-dependent process (Aulik et al., 2012). In accordance, bovine monocytes were also shown to release METs upon exposure to *B. besnoiti* tachyzoites in current work (Muñoz-Caro et al., 2014b). Compared to PMN-derived NETs, monocyte-derived METs consisted of thicker extracellular DNA-fibers, which, nonetheless, firmly entangled parasites as observed via SEM analysis (Muñoz-Caro et al., 2014b). Interestingly, we were also able to demonstrate the capacity of monocytes obtained from wild harbour seals (*P. vitulina*) to release METs against *T. gondii* tachyzoites (Reichel et al., 2015). In this study *T. gondii* tachyzoites were found tightly ensnared by harbour seal monocyte-derived ET structures which had a thicker appearance than those being released by ruminants (Reichel et al., 2015). These findings represented the first report of METs in marine mammals and therefore added a new species to the list. Recent works have also shown the formation of METs by caprine monocytes in response to *N. caninum* stages indicating that this early innate immune effector mechanism might be relevant during the acute phase of caprine neosporosis (Villagra-Blanco et al., 2017).

Finally, eosinophils were also identified as parasite-driven ET-releaser with the current work since EETs were shown to be formed by ovine eosinophils upon exposure to *H. contortus*-L3 (Muñoz-Caro et al., 2015c). These data proved that also eosinophils utilize this novel and efficient effector mechanism to attack parasites. This is in line to recent data demonstrating the role of EETs in pathogenesis of allergic diseases (Ueki et al., 2016) and in chronic inflammatory processes affecting humans, such as chronic obstructive pulmonary disease (COPD; Uribe Echevarría et al., 2017).

In summary, the current work presents a multitude of data on parasite-triggered (N)ET formation and consequently contributes substantially to the current knowledge on this early innate effector mechanism. As such, involvement of key molecular mechanisms, such as dependency on certain enzymes (NADPH oxidase, NE, MPO) and molecular pathways (MAPK and p38 kinases, intracellular calcium mobilization) or receptors (CD11b receptor) were demonstrated. Based on all findings, this novel effector mechanism of innate immune cells is rather considered as a species- and stage-independent process, leading to parasite entrapment and eventual immobilization which may affect infection rates and the outcome of disease *in vivo*. However, further studies tackling the *in vivo* relevance of this effector mechanism are urgently needed to better understand its role in pathogen defence.

3. ZUSAMMENFASSUNG

Die zum angeborenen Immunsystem gehörenden neutrophilen Granulozyten (PMN) verfügen über verschiedenen Effektormechanismen zur Bekämpfung invasiver Pathogene. Darunter fällt der erst seit 2004 bekannte Mechanismus der „Neutrophil Extracellular Trap“ (NET)-Bildung, der zur Attacke gegen Pathogene im extrazellulären Kompartiment genutzt wird. In der vorliegenden Dissertation wurden verschiedene Parasitenarten, wie die Protozoen *Besnoitia besnoiti*, *Eimeria arloingi*, *Cryptosporidium parvum*, *Toxoplasma gondii* sowie Stadien des Nematoden *Haemonchus contortus*, auf ihre Befähigung zur NET-Auslösung untersucht. Mit Ausnahme von *T. gondii* wurden die genannten Arten hier erstmalig als NET-Induktoren identifiziert. Über quantitative Analysen konnte gezeigt werden, dass die durch Apikomplexa ausgelöste NETose ein zeit- und dosisabhängiger Effektormechanismus ist. Des Weiteren zeigte sich, dass diese Abwehrreaktion weder einen parasitenspezifischen noch einen wirtsspezifischen Prozess darstellt, da Sporozoiten anderer verwandter, nicht-boviner Kokzidienarten (z. B. *Eimeria arloingi*, *Isospora suis*) gleichermaßen NETose bei bovinen PMN induzierten. Gleichfalls reagierten PMN anderer Spezies, wie Pferd, Hund und Ziege, mit einer NET-Freisetzung gegen den streng wirtspezifischen Parasiten *E. bovis*. Darüber hinaus induzierten nicht nur Sporozoiten-Stadien, sondern auch Tachyzoiten, Merozoiten und Oozysten die NET-Bildung, was auf einen stadienunabhängigen Mechanismus hindeutet. Somit scheint die NET-Bildung ein allgemein gültiger Effektormechanismus gegen Kokzidien zu sein.

Zur Charakterisierung der NETs wurden klassische Komponenten wie Histone, Neutrophile Elastase (NE) und Myeloperoxidase (MPO) mittels antikörperbasierter Untersuchungsverfahren in Ko-Lokalisierungsstudien zu NET-Strukturen nachgewiesen. Weiterführende Messungen von Enzymaktivitäten als auch funktionelle Inhibitionsexperimente bestätigten, dass NADPH-Oxidase, NE und MPO Schlüsselenzyme der über Kokzidien ausgelösten NETose sind. In Bezug auf sekundäre Botenstoffe, die an der NET-Bildung beteiligt sein könnten, konzentrierten sich die Untersuchungen auf den sog. „store operated calcium entry“ (SOCE). Die Verwendung spezifischer SOCE-Inhibitoren führte zu einer signifikanten Verringerung der parasiteninduzierten (*E. bovis, T. gondii* and *C. parvum*) NET-

Bildung, was einen Kalzium-abhängigen Prozess nahelegt. Da SOCE zudem in den über ERK1/2- und p38 MAP Kinase-vermittelten Signalweg involviert ist, wurde auch die Rolle dieser Enzyme überprüft und gezeigt, dass sie bei der *E. bovis*-induzierten NETose vermehrt phosphoryliert werden. Auch eine chemische Blockade der ERK-MEK- und p38-vermittelten Signalwege bestätigte die Schlüsselrolle dieser Enzyme bei der *E. bovis*-, *C. parvum*- und *T. gondi*-induzierten NETose. Neben Signalwegen wurde hier zudem erstmalig gezeigt, dass der PMN-assoziierte Rezeptor CD11b an der parasiteninduzierten NETose beteiligt ist. Entsprechend wurde eine signifikante Hochregulation der Oberflächenexpression dieses Rezeptors bei PMN in Reaktion auf *E. bovis*-Sporozoiten nachgewiesen. In Übereinstimmung führte eine Antikörper-vermittelte Blockade des CD11b zu einer signifikanten Abnahme der Sporozoiten-induzierten NET-Bildung.

Sowohl Scanning-elektronenmikroskopische Untersuchungen als auch Experimente zum Abfangen von Parasitenstadien zeigten die signifikante Fähigkeit von NETs, die untersuchten Parasitenstadien zu immobilisieren. Dementsprechend bestätigten Wirtszellinvasionstests, dass die extrazelluläre, NET-basierte Attacke zu einer wirksamen Verhinderung der aktiven Parasiteninvasion in Wirtszellen führt, wie für *E. arloingi*-, *C. parvum*- und *B. besnoiti*-Stadien nachgewiesen. Zusätzlich konnte hier auch unter Bearbeitung *ex vivo* gewonnener histologischer Präparate die *in vivo*-Relevanz von NETs über deren Nachweis in Darmpräparaten *Eimeria*-infizierter Tiere bestätigt werden.

Zusätzlich wurde gezeigt, dass „Extracellular Traps“ (ETs) nicht nur von PMN, sondern auch von anderen Immunzellen wie Monozyten und Eosinophile als Effektormechanismus gegen Parasitenstadien gebildet werden. Ein wichtiger Befund war zudem, dass Nematoden (*H. contortus)*-Larven verschiedene morphologische Erscheinungsformen der NETose wie *„diffuse“*, *„spread“* und *aggregated* NETs bei PMN induzieren.

Zusammenfassend wird hier postuliert, dass die effiziente NETose den Ausgang parasitärer Infektionen signifikant beeinflussen kann, indem obligat intrazelluläre Parasitenstadien daran gehindert werden, in geeignete Wirtszellen *in vivo* einzudringen.

4. SUMMARY

Polymorphonuclear neutrophils (PMN) belong to the innate immune system and own different effector mechanisms for invasive pathogen attack. Amongst these, “Neutrophil Extracellular Trap” (NET) formation is utilized to combat pathogens in the extracellular compartment. In the present dissertation several different parasites, such as the protozoans *Besnoitia besnoiti*, *Eimeria arloingi*, *Cryptosporidium parvum* and *Toxoplasma gondii* as well as the nematode *Haemonchus contortus* were investigated for their ability to induce NET formation, and, except for *T. gondii,* these species were here identified for first time as NET inducers. Quantative analyses demonstrated that coccidia-triggered NETosis is a time- and dose-dependent effector mechanism. Furthermore, this effector mechanism revealed as a parasite- and host species-independent process, since sporozoites of other, mainly non-bovine apicomplexan species (e. g. *Isospora suis, Eimeria arloingi*) equally induced NETosis in bovine PMN. In line, PMN from different host origin, such as equine, canine and caprine PMN, equally released extracellular traps in response to the strict host-specific parasite *E. bovis*. In addition, not only sporozoites but also tachyzoites, merozoites and oocysts induced NET formation suggesting a stage-independent mechanism and thereby proposing NET formation as a generally valid effector mechanism against coccidian parasites.

To further characterize parasite-triggered NETosis, classical components of NETs, such as histones, neutrophil elastase (NE) and myeloperoxidase (MPO), were detected in NET structures via antibody-based co-localization experiments. In addition, the key role of these enzymes were proven by both, measurements of enzymatic activities and functional inhibition experiments. Regarding second messengers that might be involved in NETs, we analyzed the role of “store operated calcium entry” (SOCE). Given that chemical blockage of SOCE resulted in a significant reduction of *Eimeria*-triggered NET formation, parasite-triggered NETosis revealed as a calcium-dependent process. Since SOCE is also involved in the ERK1/2- and p38 MAP kinase-mediated signalling pathways, the role of these enzymes was investigated via western blot analyses revealing enhanced enzyme phosphorylation during *E. bovis*-induced NETosis. In agreement, chemical inhibition of ERK-MEK- and p38-mediated signal transduction led to reduced NET formation

thereby indicating a key role of these signaling pathways for *E. bovis*-, *C. parvum*- and *T. gondi*-induced NETosis. Moreover, PMN-derived CD11b was here demonstrated for the first time to be involved in parasite-triggered NETosis. Thus, a significant upregulation of CD11b surface expression was detected upon PMN exposure to *E. bovis* sporozoites. In addition, antibody-mediated blockage of CD11b led to a significant decrease of sporozoite-triggered NET formation.

Scanning electron microscopic (SEM) analyses and parasite-entrapment assays highlighted the significant capability of NETs to entrap all parasite stages studied in the current dissertation. In accordance, host cell invasion assays confirmed that NET-triggered extracellular arrest of coccidian parasites effectively hampered these from active host cell invasion, as demonstrated for *E. arloingi-, C. parvum*- and *B. besnoiti*-stages. In addition, *in vivo* presence of NETs was here demonstrated via immunohistological analyses showing for the first time NET structures being in direct contact or close proximity to different *Eimeria* stages in intestinal tissue samples of *E. bovis*- and *E. arloingi*-infected animals.

Furthermore, other innate immune cells than PMN, such as monocytes and eosinophils, were here also proven to trigger ETs in response to parasitic stages. As an important finding, the formation of different morphological forms of NETs, such as “diffuse”, “spread” and “aggregated” NETs in response to nematode (*H. contortus*) larvae were here reported.

Overall and based on the current data, NETosis may significantly alter the outcome of coccidian infections via hampering parasitic stages from invading new host cells and thereby from proliferation *in vivo*.

5. DECLARATION/DEKLARATION

I declare that I have completed this dissertation without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principle of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen, Germany, in carrying out the investigations described in the dissertation.

Ich erkläre:

Ich habe die vorgelegte Thesis selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Thesis angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Thesis erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis niedergelegt sind, eingehalten.

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"If I have seen further it is by standing on the shoulders of giants".

Isaac Newton

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7. ORIGINAL PUBLICATIONS

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PLOS | ONE

Neutrophil Extracellular Traps as Innate Immune Reaction against the Emerging Apicomplexan Parasite *Besnoitia besnoiti*

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Abstract

Besnoitia besnoiti infection in cattle is an important emerging protozoan disease in Europe causing economic losses and severe clinical signs, such as generalized dermatitis, orchitis, and vulvitis in affected animals. Neutrophil extracellular trap (NET) formation was recently demonstrated as an important effector mechanism of PMN acting against several invading pathogens. In the present study, interactions of bovine PMN with tachyzoites of *B. besnoiti* were investigated in this respect *in vitro*. For the demonstration and quantification of NETs, extracellular DNA was stained by Sytox Orange or Pico Green. Fluorescent illustrations as well as scanning electron microscopy analyses (SEM) showed PMN-promoted NET formation rapidly being induced upon contact with *B. besnoiti* tachyzoites. Co-localization of extracellular DNA with histones, neutrophil elastase (NE) and myeloperoxidase (MPO) in parasite entrapping structures confirmed the classical characteristics of NET. Exposure of PMN to viable, UV attenuated and dead tachyzoites showed a significant induction of NET formation, but even tachyzoite homogenates significantly promoted NETs when compared to negative controls. NETs were abolished by DNase treatment and were reduced after PMN preincubation with NADPH oxidase-, NE- and MPO-inhibitors. Tachyzoite-triggered NET formation led to parasite entrapment as quantitative assays indicated that about one third of tachyzoites were immobilized in NETs. In consequence, tachyzoites were hampered from active invasion of host cells. Thus, transfer of tachyzoites, previously being confronted with PMN, to adequate host cells resulted in significantly reduced infection rates when compared to PMN-free infection controls. To our knowledge, we here report for the first time *B. besnoiti*-induced NET formation. Our results indicate that PMN-triggered extracellular traps may represent an important effector mechanism of the host early innate immune response against *B. besnoiti* which may lead to diminishment of initial parasite infection rates during the acute infection phase.

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Introduction

Bovine besnoitiosis is an endemic disease mainly in Africa and Asia caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti*. However, upcoming with reports on *B. besnoiti* infections in Portugal in 2005 [1], there is clear evidence for a spread of this disease in Europe since outbreaks were recently also described in Spain [2], France [3], Germany [4], Italy [5,6,7] and Switzerland [8]. Since all these European countries had previously been free of bovine besnoitiosis the European Food Safety Authority classified this parasitosis as an emerging disease in the EU in 2010 [9].

So far, no data are available on adaptive and innate immune reactions against the apicomplexan parasite *B. besnoiti*. PMN, which are the most abundant leukocytes in the bovine blood, play a fundamental role in innate host responses since they are the earliest immune cells to arrive at the site of infection. This cell type has previously been shown to interact with both *Eimeria bovis* [10] and closely related apicomplexan parasites such as *Toxoplasma gondii* [11,12,13] confirming an important role of PMN in innate immune reactions against these parasites.

Besides phagocytosis and the production of antimicrobial molecules, a major effector mechanism of PMN is the formation of neutrophil extracellular traps, called NETs [14], which lead to extracellular killing of bacterial and fungal pathogens [14,15,16]. NET were first described by Brinkmann et al. [14] showing that PMN are capable to release granular proteins and chromatin forming thin extracellular fibers that bind Gram-positive and-negative bacteria [15,16,17]. The major structural component of NETs is DNA which is studded with antimicrobial proteins composed of nuclear histones, granula-derived neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin, and gelatinase [14,18,19]. Overall, NET formation has been described as a novel form of cell death called ETosis which is distinct from apoptosis, autophagy and necrosis and depends on the generation of reactive oxygen species (ROS) by NADPH oxidase [17,20]. Whilst most studies have focused on bacterial and fungal pathogens, few attention has been paid on effects of NETs on apicomplexan parasites [21,22]. Thus, NET formation has been demonstrated for *Plasmodium falciparum* [23], *E. bovis* [24] and *T. gondii* [21,22]. In addition, NETosis was shown for different

Leishmania species [25,26]. The aim of this study was to describe for the first time that *B. besnoiti*-induced NET-formation which may represent an important host effector mechanism against the apicomplexan *B. besnoiti* during the acute phase of infection.

Materials and Methods

Host cell culture and *Besnoitia besnoiti* tachyzoite maintenance

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated as previously described by Taubert et al. [27]. Briefly, umbilical cords obtained from calves born by *sectio caesarea* were kept at 4°C in 0.9% HBSS–HEPES buffer (pH 7.4; Gibco, Grand Island, NY, USA) supplemented with 1% penicillin (500 U/ml; Sigma-Aldrich, St. Louis, MO, USA) and streptomycin (500 µg/ml; Sigma). For preparation of endothelial cells, 0.025% collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ, USA) was infused into the lumen of the isolated and ligated umbilical vein and incubated for 20 min at 37°C in 5% CO_2. After gently massaging the umbilical vein, the collagenase-cell suspension was collected and supplemented with 1 ml FCS (Gibco) to inactivate the collagenase. After two washings (400×*g*, 10 min, 4°C), the cells were resuspended in ECGM (endothelial cell growth medium; PromoCell, Heidelberg, Germany), plated in 25 cm^2 plastic culture flasks (Nunc, Roskilde, Denmark) and kept at 37°C in 5% CO_2.

B. besnoiti (strain Bb1Evora04) tachyzoites were maintained by serial passages in BUVEC. Tachyzoites were collected from BUVEC supernatants, centrifuged, washed thrice with PBS, counted and suspended in RPMI 1640 medium (Gibco) until further use.

Isolation of bovine PMN

Cattle ($n = 3$) were bled by puncture of the jugular vein. Heparinized blood was diluted in an equal amount of PBS containing 0.02% EDTA, layered on Biocoll Separating Solution (Biochrom AG) and centrifuged (800×*g*, 45 min). The pellet was suspended in 25 ml distilled water to lyse erythrocytes. Osmolarity was adjusted by adding 10× Hanks Salt Solution (HBSS, Biochrom AG). PMN were washed twice, re-suspended in RPMI medium, counted in a Neubauer haemocytometer chamber and incubated at 37°C and 5% CO_2 for at least 30 min before use. All animal procedures were performed according to the Justus Liebig University Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the State of Hesse (Regierungspräsidium Giessen) and in accordance to the current German Animal Protection Laws.

Scanning electron microscopy (SEM)

Bovine PMN were incubated with tachyzoites (ratio: 2:1) for 10, 30, 60, and 120 min on poly-$_L$-lysine (Sigma-Aldrich) pre-coated coverslips. Cells were fixed in 2.5% glutaraldehyde (Merck), post-fixed in 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold. Specimens were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

Quantification of NETs

NET formation was quantified using PicoGreen (Invitrogen). Therefore bovine PMN ($n = 3$) were incubated with tachyzoites (2:1 ratio) for different time spans (30–300 min, 37°C). In order to estimate the effects of the parasite viability or integrity on NET formation, tachyzoites were either attenuated by UV-light according to Zhao Y et al., 2013 [28] 60 min, 230 V/50–60 Hz), heat-inactivated (60°C, 30 min) or homogenized [three freeze and thaw cycles plus sonication (15 s, 50 kHz, ice bath)] (1:1 ratio; 5×10^5, 90 min, $n = 3$). To evaluate dose-dependent effects different PMN:tachyzoites ratios were used (1:1, 1:2, 1:3). For positive controls, zymosan (Invitrogen) was used (1 mg/ml). To estimate maximum values of extracellular DNA, PMN were lysed by Triton-X 100 treatment (0.1%; Sigma-Aldrich). To block NET formation, 90 U of DNase I (Roche Diagnostics) were used. NET inhibition assays were performed using diphenylene iodonium (10 µM, Sigma-Aldrich). After PMN/parasite co-cultures, micrococcal nuclease was added (5 U/well, New England Biolabs) (15 min, 37°C). Afterwards samples were centrifuged (300×*g*, 5 min). The supernatants were transferred (100 µl per 96-well) and PicoGreen (50 µl/well, diluted in 10 mM Tris/1 mM EDTA) was added. NET-formation was determined by spectrofluorometric analysis (484 nm excitation/520 nm emission) using an automated reader (Varioskan Flash; Thermo Scientific).

Visualization of NETs and detection of histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in *Besnoitia besnoiti* tachyzoites-induced NET structures

After incubation of bovine PMN with tachyzoites (ratio 1:1, 60 min) on poly-$_L$-lysine-treated coverslips and fixation of the samples [4% paraformaldehyde, Merck], NET structures were visualized by staining extracellular DNA with Sytox Orange (Invitrogen) according to Martinelli et al. [29] and Lippolis et al. [30]. For the visualization of tachyzoites within NET structures, tachyzoites were stained with CFSE (7.5 µM, 37°C, 30 min; Invitrogen) according to Hermosilla et al. [31] prior to PMN confrontation.

After fixation and three washings in PBS, samples were mounted in anti-fading buffer (Mowiol, Sigma-Aldrich). For the detection of histones, MPO and NE within NET structures the following antibodies were used: anti-histone (H3) monoclonal (DyLight, ab139848, Abcam], anti-MPO (Alexa Fluor 488, ABIN906866, Antibodies-online.com) and anti-NE (AB68672, Abcam) antibodies. Samples were washed thrice, blocked with BSA (1%, Sigma-Aldrch) and incubated in antibody solutions [1 h, room temperature (RT), for anti-histone; 24 h, RT, for anti-MPO and anti-NE antibodies]. The samples were washed in PBS and mounted in anti-fading buffer). Visualization was achieved using an invert Olympus IX81 fluorescence microscope.

Estimation of ROS, MPO and NE activities

ROS production was measured by oxidation of DCFH-DA (Sigma-Aldrich) to fluorescent DCF according to Conejeros et al. [32,33]. PMN ($n = 3$) were re-suspended in HBSS containing Ca^{2+} and incubated with *B. besnoiti* tachyzoites at 37°C in a 1:1 ratio (2.5×10^5 cells/well) in duplicates for 30 min of exposure. Thereafter, DCFH-DA (10 µg/ml) was added to each duplicate. For positive controls zymosan was used (1 mg/ml). The relative fluorescence units (RFU) were recorded at 15 min intervals for a period of 120 min applying 485 nm excitation and 530 nm emission wavelengths.

For the measurement of MPO activity, Amplex red reagent (Invitrogen) was used for peroxidase activity assessment. PMN and tachyzoites (1:1 ratio, $n = 3$) were incubated (30 min, 37°C) in HBSS-buffer without phenol red (Gibco). For positive controls zymosan was used (0.5 mg/ml). After incubation, 50 µM Amplex red was added to each well and peroxidase activity was measured every 10 min for 1 h in 571–585 nm fluorescence ranges.

NE activity was evaluated using the chromogenic substrate MeoSuc-Ala-Ala-Pro-Val-chloromethyl-ketone (Sigma-Aldrich). PMN ($n = 3$) were exposed to tachyzoites (1:1 ratio, 30 min, 37°C). For positive controls zymosan was used (1 mg/ml). After incubation, chromogenic substrate (3 mg/ml) was added to each sample just prior to measurement. NE activity was measured at 410 nm wavelength using an automated reader (Varioskan Flash; Thermo Scientific. NE- and MPO-inhibition assays were performed using the NE inhibitor chloromethyl ketone (CMK, 1 mM Sigma-Aldrich) and the MPO inhibitor ABAH (100 µM, Calbiochem) according to Parker et al. [34]. PMN were pre-incubated with the corresponding inhibitor (30 min, RT) prior to exposure to viable tachyzoites (1:1 ratio, 30 min, RT). Thereafter, NET formation was analyzed as described above.

Tachyzoite entrapment assay

Tachyzoite entrapment was quantified according to Nizet et al [35]. PMN were pre-activated by zymosan treatment (1 mg/ml, 30 min, 37°C). Meanwhile, tachyzoites were stained with CFSE (7.5 µM, 37°C, 30 min; Invitrogen) and washed twice in PBS. Thereafter, zymosan-stimulated PMN were exposed to CFSE-labeled tachyzoites (30 min, 37°C) in ascendant ratios (1:2; 1:3). Non-pre-exposed tachyzoites were used for controls. For inhibition, diphenylene iodonium (DPI; 10 µM) was added to PMN 30 min prior to exposure to CFSE-labeled tachyzoites as control of NET inhibition. The samples were washed twice in RPMI and measured for fluorescence intensities at 485/538 nm wavelengths. The percentage of entrapment was calculated as follows: [(A485/538 nm tachyzoites exposed to PMN)/(A485/538 nm non-exposed tachyzoites)] ×100%.

Host cell invasion assay

To test for the effect of parasite-triggered NETs on tachyzoite infectivity, three different experimental setups were chosen: 1) tachyzoites were incubated with PMN (1:2 ratio, 3 h, 37°C) allowing for effective NET formation. 2) For comparative reasons, an equal number of tachyzoites used in the setup 1 that were not pre-exposed to PMN were incubated in plain medium. 3) Similar to setup 1, equal numbers of tachyzoites were incubated with PMN (1:2 ratio, 3 h, 37°C) allowing for effective NET formation. Additionally, to resolve potential NET structures, DNase (90 U/well) was added 15 minutes before the end of the incubation period. In the next step, tachyzoites of setups 1–3 were transferred to confluent BUVEC (one 25 cm^2 flask for each setup) as host cells and incubated (1 h, 37°C, 5% CO_2). Overall three different BUVEC isolates were used in this experiment. After incubation, BUVEC layers were washed to remove PMN and dead/excrescent tachyzoites. Infection rates were estimated microscopically 24 h p. i. in ten randomly selected vision power fields (400× magnification).

Statistical analysis

By using normal distribution of data, co-culture/stimulation conditions were compared by one- or two-factorial analyses of variance (ANOVA) with repeated measures. Differences were regarded as significant at a level of $p \leq 0.05$.

Results

Tachyzoites of *Besnoitia besnoiti* exposed to bovine PMN trigger NET formation

SEM analyses revealed that exposure of live *B. besnoiti* tachyzoites to bovine PMN resulted in the formation of a delicate network of thicker and thinner strands of fibres originating from PMN and being firmly attached to the parasites, seemingly trapping them (Fig. 1). Kinetic analyses revealed different degrees of NETosis: after 10 min of exposure delicate PMN-derived filaroid structures being attached to tachyzoites were detected (Fig. 1A). Here, PMN still exhibited the morphology of intact cells. Later on, tachyzoites being trapped in a network of long drawn-

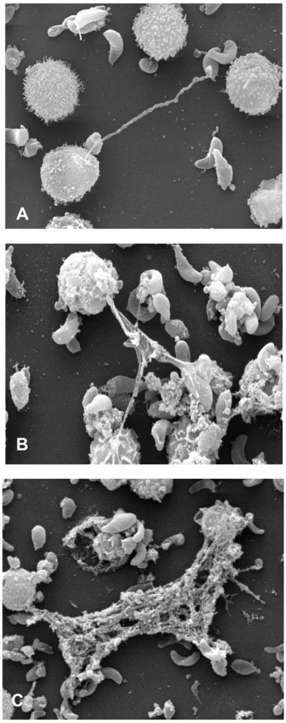

Figure 1. NETs formed by bovine PMN after confrontation with *B. besnoiti* tachyzoites. Scanning electron microscopy analysis revealed NETs being formed by bovine PMN co-cultured with *B. besnoiti* tachyzoites for different time periods [(A) 10 min (B) 30 min, (C) 60 min] in the absence of serum. (A) Delicate PMN-derived filaroid structure being attached to a tachyzoite, (B) Several tachyzoites being trapped in a network of long drawn-out fibres originating from dead and disrupted PMN. (C) Conglomerate of tachyzoites and a rather chunky meshwork of PMN-derived filaments.
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out fibres originating from disrupted PMN (Fig. 1B, 30 min) and conglomerates of tachyzoites and rather chunky meshworks of PMN-derived filaments (Fig. 1C, 60 min) were observed.

These parasite-induced NET-like structures were proven to contain DNA by Sytox Orange staining (Fig 2B, E, H). Tachyzoites were found in close proximity to NETs and presumably were trapped in these structures (Fig. 2F, I). Furthermore, co-localization of extracellular DNA with H3 (histone H3), NE and MPO in parasite entrapping structures confirmed the classical characteristics of NETs (Fig. 2). Furthermore, we observed the moment of NET extrusion of a NE-granule positive PMN capturing tachyzoites in NETs being decorated with NE-positive material (Fig. 2G–I).

Quantification of fluorescence intensities mirroring NET formation revealed that exposure of PMN to *B. besnoiti* tachyzoites significantly increased the amount of extracellular DNA when compared to parasite-free controls ($p<0.01$; Fig. 3). Furthermore, parasite-induced NET formation was dose-dependent, as increasing the amount of tachyzoites led to enhanced fluorescence intensities (Fig. 4).

Kinetic studies quantifying NET formation revealed fast and strong NET induction. Thus, strong reactions were observed already after 30 min of exposure, i. e. at the earliest time point measured in this assay. Notably, the values for NET formation were higher than those of the positive control after all time points tested indicating the strong capability of *B. besnoiti* tachyzoites to trigger NETosis. Given that Triton X100-treatment reflected lysis of all PMNs (= 100%), co-cultures of PMN and tachyzoites at a ratio of 1:2 led to 76.4±2.03% DNA release of the PMN, respectively. In contrast, in parasite-free negative controls 4.03±0.33% of the PMN contributed to extracellular DNA content of the samples (data not shown).

Since parasite entrapment was observed in SEM analyses, we established quantitative parasite-entrapment-assays using CFSE-stained parasites. Thus we could illustrate tachyzoite entrapment within NET structures (Fig. 5A). Furthermore, NET-formation led to a dose-dependent parasite capture revealing up to 34% of tachyzoites being immobilized in NET structures. (Fig. 5), when a ratio of 3:1 (tachyzoites:PMN) was applied. However, pre-activation of PMN did not significantly alter NET formation after tachyzoite exposure (Fig. 5). In order to validate that parasite

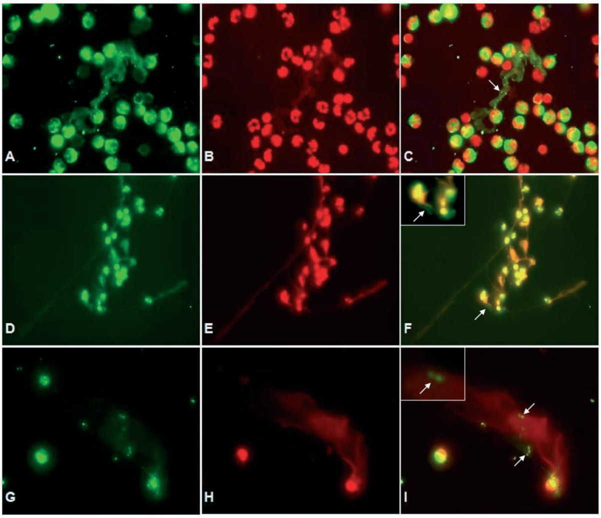

Figure 2. Co-localization of DNA with histones (H3), NE and MPO in tachyzoite-induced NET structures. Co-cultures of bovine PMN and *B. besnoiti* tachyzoites were fixed, permeabilized, stained for DNA using Sytox Orange (red: B, E, H) and probed for MPO (green: A), histones (green: D) and NE (green: G) using anti-MPO, anti-histone (H3) and anti-NE antibodies and adequate conjugate systems. Areas of respective co-localization (merges) are illustrated in C, F, I. The arrow in (C) indicates delicate globular structures within NETs. Arrows in (F) and (I) indicate tachyzoites being trapped in NET structures. Photomicrographs are of representative cells from 3 independent experiments. The time culture in this experiment was 60 min.

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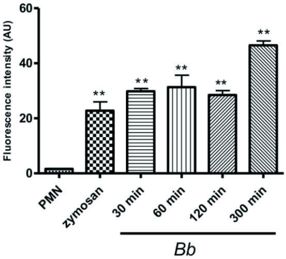

Figure 3. Kinetics of tachyzoite-triggered NET formation. PMN were incubated with *B. besnoiti* tachyzoites (ratio 2:1; 4×10^5 PMN: 2×10^5 tachyzoites), zymosan (1 mg/ml, positive control) or plain medium (negative control) for different time periods. After incubation, samples were analysed for extracellular DNA by quantifying PicoGreen-derived fluorescence intensities. Each condition was performed in triplicates. Arithmetic means of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \leq 0.05$.
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entrapment was caused by NET formation, PMN were preincubated with DPI (10 µM). As expected, a diminishment of fluorescence intensity derived from CFSE-stained parasites was observed.

Tachyzoite-triggered NETosis is accompanied by up-regulation of ROS, NE and MPO activities in PMN

Stimulation with zymosan serving as positive control significantly enhanced the enzymatic activities of NE as well as MPO, and of the ROS production in PMN (Fig. 6). Furthermore, exposure of bovine PMN to tachyzoites significantly induced NE and MPO enzymatic activities and ROS production ($p<0.001$), indicating these molecules as key factors in tachyzoite-induced NET formation.

Tachyzoite-triggered NET formation is diminished by treatments with DNase and inhibitors of NADPH oxidase, NE and MPO

The DNA-nature of *B. besnoiti*-induced NET-like structures was additionally confirmed by DNase treatment (Fig. 7A). A significant reduction of PicoGreen-derived fluorescence intensities after co-culture with tachyzoites was measured in DNase-treated samples ($p<0.001$). To further confirm the characteristics of NETs we performed inhibition assays with DPI, an inhibitor of the NADPH oxidase. Supplementation of DPI throughout the incubation period resulted in a significant reduction of parasite-induced NET formation ($p<0.05$; Fig. 7A). In addition, pre-incubation of PMN with NE and MPO inhibitors (CMK and ABAH respectively) resulted in a significant decrease of tachyzoite-triggered NET formation ($p<0.01$; Fig. 7A). In order to confirm NET characteristics after zymosan treatment, the experiment was performed with the positive control (zymosan, 1 mg/ml; Fig. 7B) and as expected we observed significant diminishment of NET formation after treatment with all inhibitors mentioned.

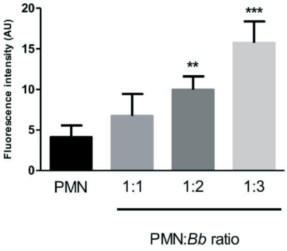

Figure 4. Dose-dependency of tachyzoite-triggered NET formation. PMN and *B. besnoiti* tachyzoites were incubated at different ratios (PMN:tachyzoites = 1:1, 1:2, 1:3). After incubation, samples were analysed for extracellular DNA by quantifying PicoGreen-derived fluorescence intensities. Each condition was performed in duplicates. Arithmetic means of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \leq 0.05$.
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Tachyzoite-induced NET induction only marginally depends on the parasite's integrity or viability

To analyze the role of tachyzoite viability and integrity in parasite-induced NETosis experiments were performed using either viable, attenuated (via UV-irradiation), dead (via heat-inactivation) or crushed (via homogenization) tachyzoites. Overall, all different tachyzoite formulations significantly induced NET formation when compared to non-exposed PMN ($p<0.001$; Fig. 8). Moreover, significant differences concerning NET formation were observed between all differentially treated tachyzoites exposed to PMN and the respective controls (vital $p<0.001$; attenuated $p<0.01$; heat inactivated $p<0.01$; homogenized $p<0.05$). However, diminished reactions were driven by homogenized parasites exposed to PMN. Consequently, these reactions resulted in significant differences compared to NET formation induced by vital tachyzoites ($p<0.001$; Fig. 8). It is noteworthy, that in case of heat-inactivated and homogenized parasites the DNA background in the controls was rather high and most probably resulted from free tachyzoite DNA. These overall results indicate that *B. besnoiti*-induced NET formation is at least partially dependent on the tachyzoite integrity.

Parasite-induced NET formation prevents tachyzoites from invading host cells

Host cell invasion is an indispensable requirement for successful survival and replication of the obligate intracellular parasite *B. besnoiti*. To determine the NET-triggered parasite-entrapment on subsequent tachyzoite infectivity, PMN-exposed tachyzoites were transferred to BUVEC as host cells and infections rates estimated thereafter. In parallel equal numbers of tachyzoites that had not been exposed to PMN before were used for BUVEC infection. As shown in Fig. 9, previous encounter of tachyzoites with PMN and, most probably, subsequent NET formation, significantly prevented the parasites from active invading host cells afterwards. Thus, infection rates decreased from 78.3±3.24%, resulting from non-exposed tachyzoites, to 41.06±4.26% achieved by PMN-pre-exposed tachyzoites, i. e. NET formation hampered tachyzoites from host cell invasion and led to a 40% reduction of the infection

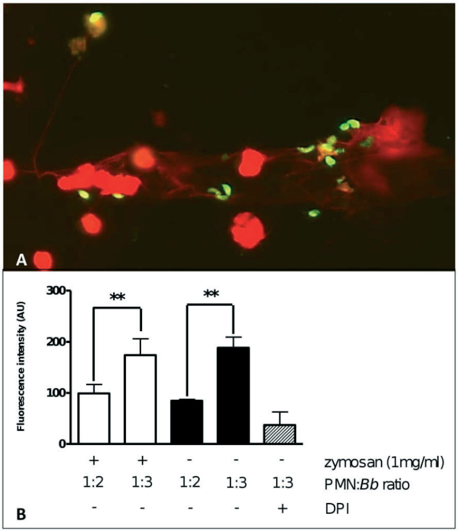

Figure 5. Quantification of *B. besnoiti* tachyzoite entrapment in NET structures. Entrapment of *B. besnoiti* tachyzoites within NET structures was illustrated after the exposure of CFSE- (7.5 μM, 37°C, 30 min) stained tachyzoites to bovine PMN and a subsequent DNA-staining by Sytox Orange (A). Quantification of tachyzoite entrapment in NETs (B) was performed after incubation of non-stimulated and zymosam-pre-activated PMN ($n = 3$; 2×10^5/96-well) with CFSE-stained tachyzoites (7.5 μM, 37°C, 30 min) for 30 min at ratios of 1:2 and 1:3. Thereafter, non-trapped tachyzoites were washed off and the resulting fluorescence intensities were calculated in relation to non-exposed CFSE stained tachyzoites. As an inhibition control DPI (10 μM) treatment was used. All experiments were performed as duplicates. Differences were regarded as significant at a level of $p \leq 0.05$.
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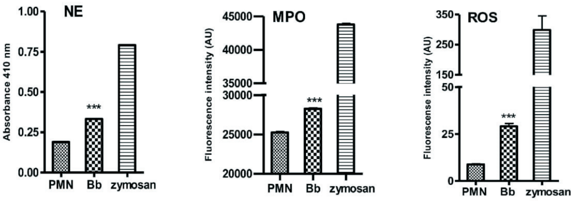

Figure 6. ROS production and enzymatic activities of NE and MPO in tachyzoite-exposed bovine PMN. Bovine PMN were exposed to *B. besnoiti* tachyzoites, zymosan (positive control) or plain medium (negative control) for 30 min. Thereafter, enzymatic activities of NE and MPO as well as ROS production were measured in the supernatants via the NE-chromogenic substrate MeoSuc-Ala-Ala-Pro-Val-chloromethyl ketone, Amplex red and the oxidation of DCFH-DA to fluorescent DCF, respectively. All experiments were performed in triplicates. Arithmetic means of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \leq 0.05$.
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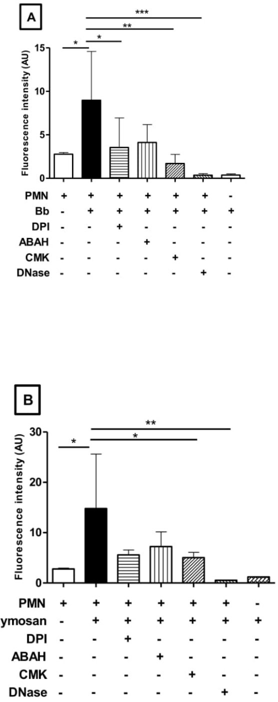

Figure 7. Inhibition of *B. besnoiti* tachyzoite-triggered NET formation. Fig. 7A: PMN were exposed to *B. besnoiti* tachyzoites in the presence or absence of inhibitors and DNase I (90 U). Cells were pre-incubated with DPI (10 μM), NE inhibitor (CMK, 1 mM) and the MPO inhibitor ABAH (100 μM) prior to exposure to tachyzoites (1:1 ratio, 30 min, RT). After an incubation period of 30 min with *B. besnoiti* tachyzoites NET-formation was determined by quantifying PicoGreen-derived fluorescence intensities (484 nm excitation/520 nm emission). The same experiment was performed with zymosan as positive control (1 mg/ml; Fig. 7B). Plain medium was used as negative control. Each condition was performed in triplicates for each PMN donor ($n = 3$). Differences were regarded as significant at a level of $p \leq 0.05$.
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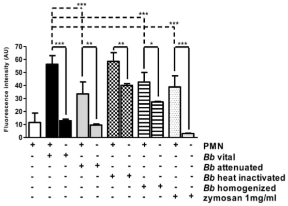

Figure 8. NET formation induced by differentially treated tachyzoites. Bovine PMN were exposed to vital, UV- attenuated, homogenized, and heat-inactivated *B. besnoiti* tachyzoites for 90 min. Stimulation with zymosan (1 mg/ml) served as positive control, plain medium was used as negative control. Samples were analysed for extracellular DNA by quantifying Pico Green-derived fluorescence intensities. Each condition was performed in triplicates. Arithmetic means of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \leq 0.05$.
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rate which may have a tremendous impact on subsequent parasite proliferation. To prove that this impairment was owed to NET formation, potential NETs were dissolved via DNase treatment being performed after 165 min of PMN-tachyzoite-exposure (i. e. after a time period that allowed for efficient NET formation) and such treated tachyzoites (used in equal numbers to the other setups) were used for BUVEC infection. As depicted in Figure 9, the infectivity of tachyzoites was completely restored by this treatment (74.27±0.25% infection rate) proving that *i*) the ensnarement of tachyzoites in NET structures hampered a large proportion of tachyzoites from host cell invasion, and *ii*) that NETs were not able to exhibit killing activities on tachyzoites within a time period of 3 hours.

Discussion

The results of this study show that bovine PMN strongly release NETs in response to the tachyzoite stage of *B. besnoiti*. The data emphasize the relevance of this effector mechanism in the defense of *B. besnoiti* as parasite-triggered NET formation actively interferes

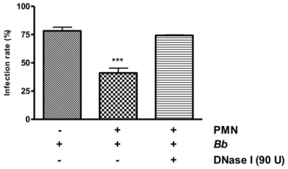

Figure 9. Infectivity of *B. besnoiti* tachyzoites after exposure to bovine PMN. Vital *B. besnoiti* tachyzoites were co-cultured for 3 h with bovine PMN (= PMN + *B.b.*) allowing for effective NET formation. To dissolve potential NET structures, DNase was supplemented 15 min before the end of the incubation period (= *B.b.* + PMN + DNase). Incubation of tachyzoites in plain medium served as PMN-free, infection control (= *B.b.* only). After incubation, samples were transferred to confluent BUVEC monolayers for 1 h. Thereafter, the cell layers were thoroughly washed and infection rates were estimated. Arithmetic means and standard deviations of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \leq 0.05$.
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with host cell invasion of tachyzoites thereby abrogating their further development which is ultimately linked to an intracellular lifestyle.

Since the first description of NETs as innate effector mechanism in 2004 [14], most studies focused on the effect of NETs on bacterial and fungal pathogens. However, there is increasing evidence on the relevance of NETosis as defense mechanism against protozoan infections. To date, NET-related data are only available on some apicomplexan species (*T. gondii*, *Plasmodium falciparum*, *E. bovis*) and *Leishmania* spp. [22,23,24,25,26]. With *B. besnoiti* we add a new apicomplexan parasite which is of high importance for cattle industry as envisioned by the declaration as emerging disease in the EU through the European Food Safety Authority in 2010 [9]. PMN-derived NET structures being firmly attached to *B. besnoiti* tachyzoites and subsequently leading to parasite entrapment were visualized by SEM as well as fluorescence imaging analyses. As equally described for *E. bovis* sporozoites [24] and *T. gondii* tachyzoites [22], quantitative assays revealed fast and strong induction of NETs by *B. besnoiti* tachyzoites. Overall, up to 76% of PMN were found to be involved in NET formation when compared to Triton-X lysis of PMN. In contrast to *E. bovis* sporozoites [24], tachyzoite-triggered NETs did not exhibit a clear time-dependency and induced reactions of almost equal strength irrespective of the time of incubation. Since other works reported on remarkable quantitative differences in extent and time course of pathogen- and phorbol 12-myristate 13-acetate (PMA) induced NET formation [17,24] and since PMA does not exhibit proper activation capacity on bovine PMN in contrast to human cells [36], we used zymosan for the stimulation of NETosis by bovine PMN in positive controls. In contrast to PMA, this molecule was demonstrated as potent activator of PMN in the bovine system [36]. Overall, stimulation of bovine PMN with zymosan turned out as reliable positive control inducing ROS-, NE- and MPO-activities as well as NET release.

NETs mainly consist of chromatin [14]. Thus, we confirmed the DNA-nature of tachyzoite-triggered NET by staining with Sytox Orange/PicoGreen. In addition, the resolution of parasite-induced NETosis by DNase treatments proved this typical characteristic of NETs. Besides chromatin/DNA, the major components of NETs are nuclear histones and granular components such as NE, MPO, lactoferrin, and gelatinase [14,18,19]. These molecules are of high relevance concerning the microbiocidal mechanism of NETs [14,37,38,39]. Applying co-localization analyses concerning extracellular DNA and histones (H3), NE or MPO in tachyzoite-entrapping structures we confirmed these classical characteristics of NETs. Furthermore, NE and MPO inhibitors treatments significantly reduced NETs formation in tachyzoite-exposed PMN revealing the essential role of these enzymes in *B. besnoiti*-induced NETosis.

The process of NET formation depends on the assembly/activation of the NADPH oxidase complex resulting in ROS production [40,17]. As reported for several other pathogens [14,17,22,24], *B. besnoiti*-triggered NET production also proved to be NADPH oxidase-dependent since it was significantly diminished by DPI treatment. Furthermore, the relevance of this enzyme complex in NET formation was confirmed by enhanced ROS production of tachyzoite-exposed PMN.

Parasite entrapment in NETs proved to be dose dependent. Overall, a third of tachyzoites were ensnared by NET structures when applying a ratio of 3:1 (tachyzoites: PMN). As previously reported for *E. bovis* sporozoites [24] NET-mediated parasite entrapment had an enormous implication on the infectivity of tachyzoites as shown in functional infection experiments. Thus, infection rates dropped dramatically when tachyzoites were pre-exposed to bovine PMN prior to endothelial host cell encounter, indicating that NETs were hampering the parasites from active host cell invasion. As expected, DNase treatment completely resolved this effect. This result furthermore suggested that NETs do not exhibit detrimental/lethal effects on tachyzoites as has been proposed for some bacterial pathogens. Since *B. besnoiti* is an obligate apicomplexan intracellular protozoan and since pathogenicity of the parasite is ultimately linked to continuous infection and proliferation cycles in endothelial cells *in vivo* [9,41] NET-mediated parasite entrapment and inhibition of host cell invasion will surely have an impact on the outcome of the disease.

So far, NET-triggering molecules originating from apicomplexan parasites are not known. To evaluate the influence of the parasites viability and/or integrity we used different formulations of tachyzoites. Overall, morphologically intact parasites (vital and UV-irradiated) all significantly triggered NET release in bovine PMN with the latter treatment leading to significantly reduced reactions. In contrast to *E. bovis* sporozoites [24], tachyzoites of *B. besnoiti* induced NETs irrespective of their viability. However, in accordance to *E. bovis* sporozoites [24] and *T. gondii* tachyzoites [22], soluble parasite lysates also induced significant NET release in PMN. In case of *B. besnoiti*, these reactions were significantly weaker than those induced by vital tachyzoites but still significant when compared to the negative control.

The results of this study demonstrated for the first time *B. besnoiti* tachyzoites as a strong inducer of NET formation. Considering the life cycle of *B. besnoiti* which includes active proliferation in endothelial cells during the acute phase of the disease, parasite entrapment via NET formation may be of particular importance *in vivo* since lysis of infected endothelial cells will lead to direct exposure of tachyzoites to blood components, such as circulating PMN and other leukocytes. It is worth noting that *B. besnoiti*-infected bovine endothelial cells display increased adhesion molecules gene transcription and enhanced PMN adhesion (Taubert, personal observation) allowing for close proximity of effector cells and parasites. However, so far the role of NET formation in *B. besnoiti*-infected cattle *in vivo* is not clear and will be difficult to determine. Nevertheless out data suggest parasite-induced NET formation as an effective and important effector mechanism in host innate immune reactions directed against *B. besnoiti*.

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Author Contributions

Conceived and designed the experiments: AT CH TM HC. Performed the experiments: TM CH LS AT. Analyzed the data: TM CH AT. Contributed reagents/materials/analysis tools: TM AT CH HC. Wrote the paper: TM AT CH LS HC. Blood obtention from cattle: CH LS TM AT HC. Isolation of tachyzoites from cysts: HC LS TM. Cell culture of bovine umbilical endothelial cells: AT TM CH LS. Besnoitia besnoiti in vitro cell culture: TM AT CH LS HC. Manuscript corrections: AT TM CH LS.

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ORIGINAL PAPER

Besnoitia besnoiti tachyzoites induce monocyte extracellular trap formation

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Abstract Extracellular trap (ET) formation has been demonstrated as an important novel effector mechanism of polymorphonuclear neutrophils (PMN), eosinophils, mast cells and macrophages acting extracellularly against pathogens. In the present study, we show that tachyzoites of the emerging apicomplexan parasite *Besnoitia besnoiti*, that have recently been reported as potent inducers of PMN-derived ETosis, also trigger the release of ETs in an additional cell type, namely in monocytes. Fluorescence illustrations as well as scanning electron microscopy analyses (SEM) showed monocyte-promoted ET formation to be rapidly induced upon exposure to viable tachyzoites of *B. besnoiti*. Classical characteristics of ETs were confirmed by the co-localization of extracellular DNA with histones (H3) or myeloperoxidase (MPO) in parasite-entrapping structures. Monocyte-derived ETs were efficiently abolished by DNase I treatment and significantly reduced by treatments with inhibitors of MPO and NADPH oxidase, thus strengthening the key roles of reactive oxygen species (ROS) and MPO in monocyte ET formation. For comparative reasons, we additionally tested sporozoite stages of the closely related parasite *Eimeria bovis* for their capacity to induce monocyte-derived ETs and showed that these stages indeed induce ETs. To our best knowledge, we here report for the first time on monocyte ETs against the apicomplexan parasites *B. besnoiti* and *E. bovis*. Our results indicate that monocyte-triggered ETs may represent an important effector mechanism of the host early innate immune response against *B. besnoiti* and add a new cell type to the list of cells capable to release ETs.

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Introduction

Bovine besnoitiosis is an endemic disease mainly occurring in Africa and Asia caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti* (Dubey et al. 2003, 2013; Olias et al. 2011; Schares et al. 2011, 2013). In Europe, bovine besnoitiosis occurred in a few restricted geographic areas in Portugal (Cortes et al. 2005), Spain (Fernandez-Garcia et al. 2009) and France (Jacquiet et al. 2009) since the first recorded cases in the beginning of the twentieth century. However, there is clear evidence for geographic expansion of this disease as outbreaks were recently described in Germany (Mehlhorn et al. 2009; Schares et al. 2009), Italy (Gollnick et al. 2010; Gentile et al. 2012; Rinaldi et al. 2013), Switzerland (Lesser et al. 2012) and Hungary (Hornok et al. 2014). Given that all these European countries had previously been free of bovine besnoitiosis, the European Food Safety Authority (EFSA) classified bovine besnoitiosis as an emerging disease in the EU in 2010 (EFSA 2010).

During its endogenous life cycle *B. besnoiti* bears stages that might become potential targets for professional phagocytes in vivo (Hermosilla et al. 2012; Muñoz-Caro et al. 2014). Thus, *B. besnoiti* tachyzoites infect host endothelial cells of blood vessels in different organs, polymorphonuclear neutrophils (PMN) and monocytes in vivo during the acute phase of disease and in all cases become exposed to the blood environment upon host cell lysis (Alvarez-Garcia et al. 2013). Professional mononuclear phagocytes, such as PMN, monocytes and macrophages, are considered as the first line of defence (von Kockritz-Blickwede and Nizet 2009; Branzk and Papayannopoulos 2013) and circulating blood monocytes are one of the earliest leukocyte cell types to be recruited to infection sites. Whilst no data are available on bovine monocyte-derived immune reactions in the case of *B. besnoiti* infections so far, this immune cell type has previously been shown to interact with sporozoite stages of the closely related parasite *Eimeria bovis* (Taubert et al. 2009) and with tachyzoites of *Toxoplasma gondii* (Harker et al. 2013; Quan et al. 2013; Ueno et al. 2014). Monocytes react upon stimulation to *E. bovis* merozoite I antigens by producing the pro-inflammatory cytokine IFN-γ and the chemokine CXCL10, confirming their relevant role in host immune reactions against this parasite (Taubert et al. 2009). Furthermore, peripheral monocytes of *E. bovis*-infected animals exhibited enhanced ex vivo phagocytic and oxidative burst activities resulting in reactive oxygen species (ROS) production (Taubert et al. 2009). In line with these results, enhanced levels of monocytes were also observed in *Eimeria maxima*-infected chickens (Rose and Hesketh 1979). In contrast to other coccidian species, very little is known on both adaptive and innate immune reactions in *B. besnoiti*-infected cattle. Recently, it has been shown that bovine PMN release neutrophil extracellular traps (NETs) in response to viable *B. besnoiti* tachyzoites in vitro, resulting in the efficient capture of these stages and the blockage of tachyzoite host cell invasion (Muñoz-Caro et al. 2014). However, hardly anything is known on the role of monocytes in innate immune responses against *B. besnoiti*, although these cells have even been reported to be infected in vivo during the acute phase of bovine besnoitiosis (Alvarez-Garcia et al. 2013).

In addition to phagocytosis and the production of antimicrobial molecules as well-known effector mechanisms of innate immune cells, the formation of PMN-derived extracellular traps (ETs) was recently discovered by Brinkmann et al. (2004). So far, eosinophils (Yousefi et al. 2008), mast cells (von Kockritz-Blickwede et al. 2008) and macrophages (Aulik et al. 2012) have been described as capable of ET extrusion against pathogens in addition to PMN. Released ETs can promote extracellular killing of bacterial, viral, fungal (Brinkmann and Zychlinsky 2007, 2012) (Papayannopoulos and Zychlinsky 2009) and parasitic pathogens (Abi Abdallah and Denkers 2012; Hermosilla et al. 2014).

Given that ETs may considerably capture *B. besnoiti* tachyzoites and hamper them from host cell invasion as hypothesized by Muñoz-Caro et al. (2014), the monocyte cell type may considerably contribute to innate immune reactions if it also proves to be ET-competent. Thus, the aim of this investigation was to characterize early innate immune reactions of bovine monocytes in terms of ET formation against viable tachyzoites of *B. besnoiti*. We show here that the exposure of bovine monocytes to these parasite stages indeed triggers the release of monocyte-derived ETs and furthermore characterize some features of this novel effector mechanism of monocytes.

Materials and methods

Animals

Holstein Friesian male calves ($n=3$) were purchased from a local farmer at the age of 2 weeks, treated with Baycox® (Bayer) and Halocur® (Intervet) in the second week after birth. Animals were assessed for parasitic infections, and when deemed parasite-free, maintained under parasite-free conditions in autoclaved stainless steel metabolic cages (Woetho) within a large animal facility being equipped with laminar flow lock entrance [Institute of Parasitology, Justus Liebig University (JLU)] until experimental *E. bovis* infection. They were fed with milk substitute (Hemo Mischfutterwerke) and commercial concentrates (Raiffeisen). Water and sterilized hay were given ad libitum. All animal experiments and procedures were performed according to the JLU Animal Care Committee guidelines, approved by the ethic commission for experimental animal studies of the State of Hesse, and in accordance to the current German Animal Protection Law.

Parasite maintenance

B. besnoiti (strain Bb1Evora04) was maintained in cell culture. Therefore, in vitro infections were performed using primary bovine umbilical vein endothelial cells (BUVEC) as host cells (Muñoz-Caro et al. 2014). BUVEC were isolated as previously described by Taubert et al. (2006) and cultured in complete endothelial cell growth medium (ECGM; PromoCell). Almost confluent BUVEC monolayers were infected with *B. besnoiti* tachyzoites (2.5×10^5 tachyzoites/25-cm^2 flasks) and maintained for 2–4 days until newly developed tachyzoites were set free via cell lysis. Tachyzoites were collected from BUVEC supernatants, washed thrice in PBS (400×*g*, 10 min), counted in a Neubauer chamber and suspended in RPMI 1640 medium (Gibco) until further use.

E. bovis (strain H) used in the present study was maintained by passages in Holstein Friesian calves as described by Fiege et al. (1992). For the production of oocysts, calves were infected at the age of 10 weeks with 5×10^4 sporulated oocysts each. Excreted oocysts were isolated from the faeces from 18 days p. i. onwards according to the method of Jackson (1964). Sporulation was achieved by incubation in a 2 % (*w*/*v*) potassium dichromate (Sigma) solution at room temperature (RT). Sporulated oocysts were stored in this solution at 4 °C until further use. Sporozoites were excysted from sporulated oocysts as described by Hermosilla et al. (2002). Briefly, sporulated oocysts were added to 4 % (*v*/*v*) sodium hypochlorite solution and magnetic stirred on ice for 20 min, before being washed twice with bi-distilled water. Oocyst solution was layered on Percoll^{TM} (GE Healthcare) 60 % gradients and centrifuged for 20 min at 1,500 rpm to remove the remaining debris. The oocysts band was collected and suspended in sterile 0.02 M L-cysteine/0.2 M $NaHCO_3$ (Merk) solution and incubated in a 100 % CO_2 atmosphere at 37 °C for 20 h. Afterwards, oocysts were resuspended in excystation medium [Hank's balanced salt solution (HBSS; Gibco) containing 0.4 % (*w*/*v*) trypsin (Sigma) and 8 % (*v*/*v*) sterile filtered bovine bile, obtained from the local slaughterhouse], incubated for up to 4 h at 37 °C and 5 % CO_2 atmosphere and microscopically controlled for sporulation. Freshly released sporozoites were washed twice (600×*g*, 15 min) with medium 199 (Gibco) containing 2 % (*v*/*v*) fetal calf serum (FCS), 1 % penicillin and streptomycin (both *v*/*v* 500 μg/ml; PS; Sigma-Aldrich) and finally suspended in tissue culture medium.

Isolation of bovine monocytes

Cattle (n=3) were bled by puncture of the jugular vein. For monocyte isolation, peripheral blood mononuclear cell (PBMC) was isolated according to Taubert et al. (2009). In brief, 18 ml of heparinized blood were mixed with 17 ml of 0.9 % NaCl and applied on the top of Biocoll®-paque (density=1.077 g/l; Biochrom, 12 ml) in sterile 50-ml centrifugation tubes (Nunc). After centrifugation (45 min, 400×*g*), the PBMC layer was collected and the cells were washed thrice (10 min, 400×*g*, 4 °C) in RPMI medium (Gibco). Thereafter, PBMC (7.5×10^7) were allowed to adhere (1 h, 37 °C) to 75-cm^2 tissue plastic flasks (Greiner), previously coated with 2 % sterile gelatine (2 h, 37 °C, thereafter dried) and autologous plasma (1 h, 37 °C, thereafter washed twice with RPMI 1640/1 % penicillin/1 % streptomycin, all Sigma-Aldrich). Monocytes were washed with pre-warmed RPMI 1640/1 % penicillin/1 % streptomycin to remove non-adherent PBMC. Monocytes were detached (5–10 min in 10 mM EDTA in Mg^{++} and Ca^{++}-free Hank's solution, RT), washed (10 min, 400×*g*, 4 °C) and resuspended in 4 °C RPMI 1640/1 % penicillin/1 % streptomycin. The cells were counted in a Neubauer haemocytometer and kept on ice until use. Trypan blue (Sigma) exclusion tests were applied to control the viability of the monocytes.

Scanning electron microscopy

Bovine monocytes were incubated with parasites (ratio 1:1) for 30, 60 and 120 min on poly-L-lysine (Sigma-Aldrich) precoated coverslips. Cells were fixed in 2.5 % glutaraldehyde (Merck), post-fixed in 1 % osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold. Specimens were examined using a Philips® XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

Quantification of monocyte-derived extracellular traps

Extracellular trap (ET) formation was quantified using PicoGreen®, a DNA-binding dye (Invitrogen) according to Muñoz-Caro et al. (2014). To assess parasite-induced ET formation, bovine monocytes of three blood donors were resuspended in serum-free RPMI 1640 (Gibco) medium and exposed to *B. besnoiti* tachyzoites or *E. bovis* sporozoites (37 °C, 1:1 ratio in 96-well plates, 30 and 60 min). For positive controls, stimulation with zymosan (1 mg/ml final concentration, Invitrogen) was used. To block ET formation, DNase I treatments (90 U/well, Roche Diagnostics) were applied. NADPH oxidase and myeloperoxidase (MPO) were inhibited via treatments with diphenylene iodonium (DPI, 5 mM, Sigma-Aldrich) and ABAH (100 μM, Calbiochem), respectively. Monocytes kept in plain RPMI 1640 medium served as negative control.

Following monocytes/parasite co-cultures, 50 μl of micrococcal nuclease buffer (New England Biolabs) containing 0.1 U/μl micrococcal nuclease (New England Biolabs) were added to each well and incubated (15 min, 37 °C). Afterwards, each sample was centrifuged at 300×*g* for 5 min and the supernatants were transferred to a 96-well flat-bottom plate (100 μl per well, in duplicates). Fifty microliters of PicoGreen® (1:200 dilution in 10 mM Tris base buffered with 1 mM EDTA) were added to each sample and the samples were incubated (4 min, in the dark). Monocyte-derived ET formation was determined by spectrofluorometric analyses of fluorescence intensities [excitation wavelength: 484 nm, emission wavelength: 520 nm, automated plate monochrome reader (Varioskan Flash® Thermo Scientific)].

In order to exclude both, parasite phagocytosis by monocytes and parasite invasion into monocytes, we treated monocytes or *B. besnoiti* tachyzoites with cytochalasin D (10 μg/ml, 30 min; Sigma-Aldrich) prior to co-cultures as published by Abi Abdallah et al. (2012). Furthermore, to assess the viability of parasites (tachyzoites and sporozoites) entrapped within monocyte-derived ETs, we used the Trypan blue exclusion test (Sigma-Aldrich) as reported by Muñoz-Caro et al. (2014) and Silva et al. (2014).

Visualization of ETs, histones (H3) and myeloperoxidase in parasite-induced ET structures

After co-culture of bovine monocytes and *B. besnoiti* tachyzoites (ratio 1:1, for 60 min) on poly-L-lysine-treated coverslips and fixation [4 % (*w*/*v*) paraformaldehyde, 20 min, in the dark; Merck], the samples were stained by Sytox Orange® dye (Invitrogen) according to Martinelli et al. (2004) and Lippolis et al. (2006). For the detection of histones and MPO within monocyte-ET structures, the following antibodies were here used: anti-histone (H3) monoclonal (DyLight, ab139848; Abcam) and anti-MPO (Alexa Flour 488 ABIN906866; Antibodies-online.com) antibodies. Samples were washed thrice, blocked with BSA (1 % Sigma-Aldrich) and incubated in antibody solutions [1 h, room temperature (RT), for anti-histone; 24 h, RT, for anti-MPO]. The samples were washed in PBS and mounted in anti-fading buffer (Mowiol; Sigma-Aldrich). Visualization was achieved using an invert Olympus IX81 fluorescence microscope.

Results

Tachyzoites of *B. besnoiti* trigger monocyte-mediated ET formation

Scanning electron microscopy (SEM) analyses revealed that exposure of live *B. besnoiti* tachyzoites to bovine monocytes resulted in the formation of a delicate network of thinner and thicker strands of fibres originating from single monocytes and often being firmly attached to the parasites, seemingly capturing them (Fig. 1a). Additionally, also clusters of monocytes simultaneously releasing ETs in response to parasites were detected. In general, monocyte-derived *B. besnoiti* tachyzoite-triggered ETs appeared thicker than the ones originating from *B. besnoiti*-exposed PMN (Muñoz-Caro et al. 2014). Kinetic analyses revealed different degrees of monocyte ETs: after 30 min of exposure, delicate monocytes-derived filaroid structures being attached to tachyzoites were detected (data not shown). Here, monocytes still exhibited the morphology of intact cells. Following 60 min of exposure, several tachyzoites being trapped in a network of long drawn-out fibres originating from dead and disrupted monocytes were detected (Fig. 1b–d, 60 min) and conglomerates of tachyzoites and rather chunky meshworks of monocyte-derived filaments were observed (Fig. 1d).

These parasite-induced monocyte ET-like structures were proven to contain DNA by Sytox Orange staining (Fig. 2). Moreover, co-localization of extracellular DNA with histone H3 and MPO corroborated the classical characteristics of ETs (Fig. 2a, b). In addition, we also observed the moment of ET extrusion of an MPO granule-positive monocyte capturing tachyzoites in monocyte-derived ETs being decorated with MPO-positive material (Fig. 2b).

Quantification of *B. besnoiti* tachyzoite-triggered monocyte-mediated ET formation and inhibition thereof by DNase I, DPI and ABAH treatments

The quantification of fluorescence intensities mirroring monocyte-derived ET formation revealed that the exposure of monocytes to viable *B. besnoiti* significantly enhanced the amount of extracellular DNA when compared to parasite-free controls ($p \leq 0.05$; Fig. 3). The DNA nature of parasite-induced monocyte-ET-like structures was confirmed by DNase I treatment (Figs. 3 and 4) leading to a significant reduction of PicoGreen® fluorescence intensities ($p \leq 0.05$; Fig. 3). To further corroborate the implication of NADPH oxidase in the monocyte-triggered ET formation process, we performed inhibition assays with DPI throughout the incubation period resulting in a significant reduction of *B. besnoiti*-induced monocyte ET formation ($p \leq 0.001$; Fig. 3). The latter results confirm ROS-dependency of *B. besnoiti*-triggered monocyte ET formation. The inhibition of MPO activity via ABAH treatments further underlined the key role of this enzyme in monocyte-mediated ETosis since ET formation was significantly blocked ($p \leq 0.01$; Fig. 3). As expected, we observed a significant increase in monocyte-derived ET formation after bovine monocyte stimulation with zymosan (see Figs. 4 and 5), proving this molecule as a reliable positive control for monocyte-mediated ETosis.

E. bovis sporozoites also trigger monocyte-mediated ETosis

Given that *B. besnoiti* tachyzoites but also sporozoites of the closely related coccidian *E. bovis* have been reported to induce ETs in PMN, we here extended the current experiments and additionally tested *E. bovis* sporozoites would also trigger ETs in the monocyte cell type. Notably, sporozoites of *E. bovis* even triggered stronger monocyte ET extrusion ($p \leq 0.001$; Fig. 4) than tachyzoites of *B. besnoiti*. DNase I treatments of sporozoite-exposed monocytes also resolved ET formation thereby proving the DNA nature of ETs.

Monocyte-mediated phagocytosis or parasite invasion do not interfere with monocyte ET formation

In order to exclude that monocyte-promoted phagocytosis of tachyzoites or active invasion of tachyzoites into monocytes would interfere with ET data, we either treated monocytes or tachyzoites with cytochalasin D, an inhibitor blocking the actin polymerization, which is required for phagocytosis as well as for the active parasite invasion process (Abi Abdallah et al. 2012) prior to monocyte-tachyzoite co-cultures. Even though both treatments

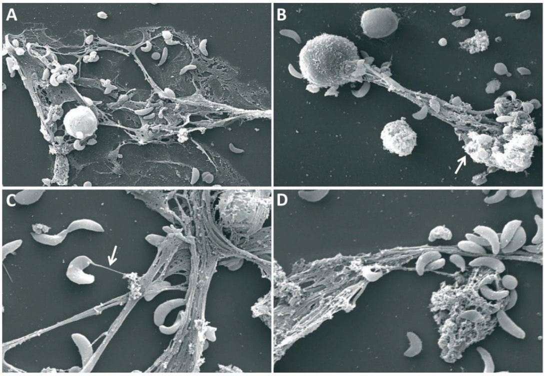

Fig. 1 ET formation by bovine monocytes after the exposure to viable *Besnoitia besnoiti* tachyzoites. Scanning electron microscopy analysis revealed ETs being formed by bovine monocytes exposed to viable *B. besnoiti* tachyzoites in the absence of serum (**a**–**d**). **a** Overview of streets of fibres composed of monocyte-derived ETs showing attached *B. besnoiti* tachyzoites to them and one intact monocyte, **b** moment of strong ETs release by a single dead monocyte (indicated by *white arrow*) with entrapped *B. besnoiti* tachyzoites, **c** captured *B. besnoiti* tachyzoites in a massive network of long drawn-out fibres originating from dead monocytes and one tachyzoite being attached to a thin ET fibre (indicated by *white arrow*), and **d** detailed view of thick bundles of monocyte-ETs fibres as well as a meshwork capturing a conglomerate of *B. besnoiti* tachyzoites

resulted in slightly diminished reactions, tachyzoite-triggered ET formation was highly significant ($p \leq 0.001$; Fig. 5) when compared to non-exposed monocytes. These data indicate that neither parasite invasion nor monocyte phagocytosis significantly interferes with parasite-induced monocyte ETosis.

Killing of ***B. besnoiti*** and ***E. bovis*** stages by monocyte ETs

Given that ETs are known to contain potent antimicrobial components, we next assessed the viability of *B. besnoiti* tachyzoites or *E. bovis* sporozoites being captured within extruded monocyte ETs. To address this issue, we used the

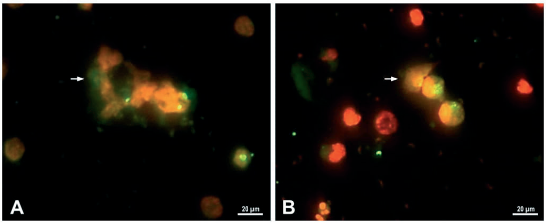

Fig. 2 Co-localization of DNA with histones (H3, **a**) and myeloperoxidase (**b**) in *Besnoitia besnoiti*-induced monocyte-derived ET structures. Co-cultures of bovine monocytes and viable *B. besnoiti*-tachyzoites were fixed, permeabilized, stained for DNA using Sytox Orange (*red*: **a**, **b**) and probed for bovine H3 (*green*, **a**) and MPO (*green*, **b**) using monoclonal anti-histone (H3) and anti-MPO antibodies and adequate conjugate systems. Merges (**a**, **b**) illustrate diffuse monocytes-derived ETs indicated by *white arrows* after the co-culture with vital tachyzoites of *B. besnoiti*

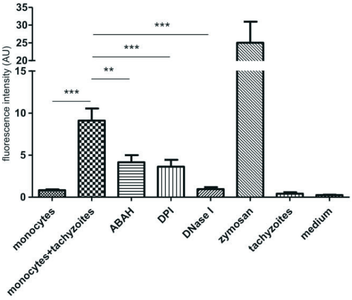

Fig. 3 Quantification of *Besnoitia besnoiti* tachyzoite-triggered monocyte-derived ETs and inhibition thereof. Bovine monocytes were exposed to viable *B. besnoiti*-tachyzoites in the presence or absence of different inhibitors and DNase I (90 U). Cells were preincubated with the NADPH oxidase inhibitor DPI (10 μM) and the MPO inhibitor ABAH (100 μM) prior to exposure to tachyzoites. After 30 min of incubation, monocyte-promoted ET formation was determined by quantifying PicoGreen-derived fluorescence intensities (484 nm excitation/529 nm emission). Stimulation of monocytes with zymosan served as positive control. Plain medium, monocytes and tachyzoites alone were used for negative controls. Each experimental condition was performed in triplicates for each monocyte donor ($n=3$). Differences were regarded as significant at a level of $p \leq 0.05$

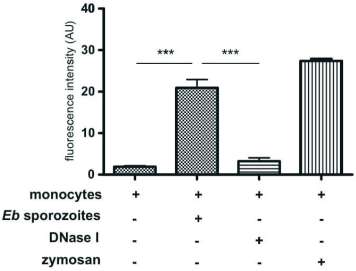

Fig. 4 Quantification of *Eimeria bovis* sporozoite-triggered monocyte-derived ET formation. Isolated bovine monocytes were exposed to viable *E. bovis* sporozoites in the presence or absence of DNase I (90 U). *E. bovis* sporozoite-induced monocyte-promoted ET formation was determined by the quantification of PicoGreen-derived fluorescence intensities (484 nm excitation/529 nm emission). Stimulation of monocytes with zymosan served as positive control. Monocytes in plain medium were used for negative controls. Each experimental condition was performed in triplicates for each monocyte donor ($n=3$). Differences were regarded as significant at a level of $p \leq 0.05$

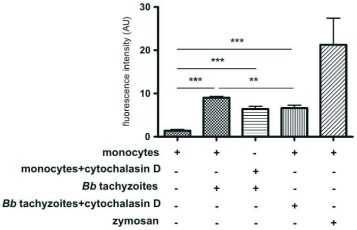

Fig. 5 Inhibition assays to evaluate monocyte phagocytosis and *Besnoitia besnoiti* cell invasion-dependent ET formation. Either vital monocytes or *B. besnoiti* tachyzoites were treated with cytochalasin D prior to co-cultures to block monocyte phagocytosis activity or tachyzoite invasion capacity, respectively After the incubation period (30 min), monocyte-triggered ET formation was determined by the quantification of PicoGreen-derived fluorescence intensities (484 nm excitation/529 nm emission). Stimulation of monocytes with zymosan served as positive control. Monocytes in plain medium were used for negative controls. Each experimental condition was performed in triplicates for each monocyte donor ($n=3$). Differences were regarded as significant at a level of $p \leq 0.05$

Trypan blue exclusion tests. The current experiments revealed that less than 2 % of both tachyzoites and sporozoites were stained by Trypan blue, indicating a very low proportion of dead specimens (data not shown).

Discussion

Formation of ETs has recently emerged as a powerful and efficient tool used by phagocytes, such as PMN, macrophages, eosinophils and mast cells, to combat microbial pathogens (von Kockritz-Blickwede and Nizet 2009). The data of the present study demonstrate that also the monocyte cell type undergoes ETosis in response to two apicomplexan parasites, *B. besnoiti* and *E. bovis*, that what have previously been demonstrated as inducers of PMN-derived ETs (Behrendt et al. 2010; Muñoz-Caro et al. 2014). Thus, we here add a new cell type to the group of ET-releasers by confirming classical characteristics of ETs, such as their DNA nature, morphological appearance, ROS- and MPO-dependency and co-localization of histones and MPO with DNA-rich fibres.

Monocyte-performed ET release rather seemed a non-parasite species-specific and non-stage-specific event since both *B. besnoiti* tachyzoites and *E. bovis* sporozoites significantly induced monocyte-derived ETs. This is in line to previous investigations on *Leishmania* spp.- and *Eimeria arloingi*-induced ET formation by PMN reporting on a stage-independent induction of PMN-promoted ETosis (Guimarães-Costa et al. 2009; Silva et al. 2014). Thus, *Leishmania* spp. amastigotes and promastigotes as well as *E. arloingi* sporozoites and oocysts all triggered NETs. The same feature was observed for *E. bovis* stages since oocysts, sporozoites and merozoites I all induced NET formation (T. Muñoz-Caro, personal observation).

ETs are mainly composed of DNA, histones and other antimicrobial peptides/proteins, forming an extracellular network, which may entrap and kill pathogens (Abi Abdallah and Denkers 2012; Brinkmann and Zychlinsky 2012; Hermosilla et al. 2014). Besides histones, several other antimicrobial components are present within ETs, such as the bacterial permeability-increasing protein (BPI), neutrophil elastase (NE), pentraxin, lactoferrin and MPO (Brinkmann and Zychlinsky 2007, 2012; von Kockritz-Blickwede and Nizet 2009), which contribute to pathogen killing (Brinkmann and Zychlinsky 2012; Branzk and Papayannopoulos 2013). By performing co-localization experiments, the current data confirmed the presence of histones (H3) and MPO in parasite-entrapping structures. In addition, inhibition of MPO via ABAH led to significantly reduced ETosis in tachyzoite-exposed monocytes confirming the pivotal role of this enzyme in ET formation. However, in contrast to data on *Leishmania* spp.- and *T. gondii*-induced NETs, monocyte-derived ET formation did not result in considerable parasite killing. A lack of ET-promoted lethal effects was also observed for other coccidian parasites, such as *E. bovis* and *E. arloingi* (Behrendt et al. 2010; Silva et al. 2014). Probing the same coccidian parasite and stage, Muñoz-Caro et al. (2014) also found only minimal killing effects of PMN-derived ETs on *B. besnoiti* tachyzoites. In conclusion, there is growing evidence that the main function of (N)ETs is the entrapment and subsequent immobilization of coccidian stages leading to host cell invasion blockage as has been demonstrated for *E. bovis*, *E. arloingi* and *B. besnoiti* stages (Behrendt et al. 2010; Muñoz-Caro et al. 2014; Silva et al. 2014). Given that all these parasites are obligate intracellular living protozoans, it is essential for them to establish themselves within an intracellular compartment to exploit the host cell metabolism for energy and essential nutrients, enabling parasite survival and further in vivo replication. Besides host cell invasion failure, any entrapped parasite might additionally become a potential target for other leukocytes of the host innate immune system to be recruited to the site of infection as previously discussed by Silva et al. (2014).

To date, two major ET release mechanism have been described. Firstly, leukocytes release ETs via a slow lytic cell death mechanism. This appears to be the major route of ETosis. Secondly, Pilsczek et al. (2010) and Yousefi et al. (2009) reported on a smaller number of leukocytes to rapidly expulse their nuclear content via vesicular secretion and to generate ETs in vital and intact condition as has been demonstrated by ongoing crawling and pathogen digestion activities (Yipp et al. 2012). Interestingly, in the current work, we also observed morphologically different kinds of ETs. Whilst most ETs consisted of rather strong fibres that even appeared to be thicker than those being illustrated in the case of NETs (Behrendt et al. 2010), fluorescence analyses additionally revealed a more diffuse, non-filaroid type of DNA-, MPO- and histone-rich structures present after tachyzoite exposure. However, it needs to be clarified whether these structures may represent 'small-sized' ETs being expelled by vital monocytes or if we just detected these ETs in the moment of extrusion.

Given that previous coccidian species and stages (Behrendt et al. 2010; Abi Abdallah et al. 2012; Muñoz-Caro et al. 2014; Silva et al. 2014) tested so far revealed as potent ET inducers, it is tempting to speculate that ETosis may represent a coccidian species-independent, stage-independent and generally acting ancient effector mechanism of mononuclear phagocytes against this particular protozoon group. We have recently demonstrated that even harbour seal (*Phoca vitulina*) PMN and monocytes release ETs in response to *T. gondii* tachyzoite exposure (M. Reichel, personal observation), thereby proving evidence on how conserved this ancestral innate immune effector mechanism appears to be as also demonstrated for several vertebrates and invertebrates species (Altincicek et al. 2008; Ng et al. 2013). However, given that it may represent a conserved mechanism and that many coccidian stages induce ETs in various leukocyte cell types of several host types, it is of main interest to identify leukocyte-derived receptors and parasite-derived ligands being involved in this process. It is tempting to assume that parasite ligands should share common molecular properties as being recognized by several types of ET-releasers. Unfortunately, no such parasite-derived molecules functioning as common ligands have been identified, so far.

The in vivo relevance of monocytes and their effector mechanisms in cattle besnoitiosis has not yet been investigated. However, we showed that *E. bovis* sporozoites also induced monocyte-derived ETs. This may have implications in the respective in vivo situation since *E. bovis*-infected calves showed significantly enhanced monocyte activities throughout infection and after in vitro exposure to *E. bovis* sporozoites and merozoite I antigen (Taubert et al. 2009), indicating that monocytes indeed contribute to *E. bovis* defence. Regardless, the current data demonstrate for the first time monocyte-promoted ET formation upon exposure to both tachyzoite- and sporozoite-stages of two apicomplexan species and reveal a new leukocyte cell type being able to extrude ET formation. Whether this mechanism indeed plays a role in vivo, particularly in the acute phase of bovine besnoitiosis, or might even contribute to the pathogenesis of the disease will have to be addressed in future experiments.

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ORIGINAL PAPER

The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps

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Abstract As a novel effector mechanism polymorphonuclear neutrophils (PMN) release neutrophil extracellular traps (NETs), which represent protein-labeled DNA matrices capable of extracellular trapping and killing of invasive pathogens. Here, we demonstrate for the first time NET formation performed by caprine PMN exposed to different stages (sporozoites and oocysts) of the goat apicomplexan protozoan parasite *Eimeria arloingi*. Scanning electron microscopy as well as fluorescence microscopy of sporozoites- and oocysts-PMN co-cultures revealed a fine network of DNA fibrils partially covering the parasites. Immunofluorescence analyses confirmed the co-localization of histones (H3), neutrophil elastase (NE), and myeloperoxidase (MPO) in extracellular traps released from caprine PMN. In addition, the enzymatic activity of NE was found significantly enhanced in sporozoite-exposed caprine PMN. The treatment of caprine NET structures with deoxyribonuclease (DNase) and the NADPH oxidase inhibitor diphenylene iodondium (DPI) significantly reduced NETosis confirming the classical characteristics of NETs. Caprine NETs efficiently trapped vital sporozoites of *E. arloingi* since 72 % of these stages were immobilized—but not killed—in NET structures. As a consequence, early infection rates were significantly reduced when PMN-pre-exposed sporozoites were allowed to infect adequate host cells. These findings suggest that NETs may play an important role in the early innate host response to *E. arloingi* infection in goats.

Keywords *Eimeria arloingi* · Apicomplexa · NETs · Goats · Neutrophils

Introduction

Eimeria arloingi coccidiosis in goats is an important apicomplexan protozoan parasitosis, causing considerable animal health problems and economic losses in goat industry due to a severe clinical enteritis mainly in young animals (Soe and Pomroy 1992). Caprine coccidiosis might affect up to 100 % of 4–10-week-old goat kids (Mehlhorn and Armstrong 2001), depending on the type of management (Ruiz et al. 2006). So far, relatively little is known on the innate immune response against *Eimeria* infections in ruminants, although this immune system is considered to be older in evolutionary terms than the adaptive one (Tschopp et al. 2003). Particularly, polymorphonuclear neutrophils (PMN) play an important role since they are the most abundant cells in the blood, the first ones to be recruited to the site of infection, and dispose of a variety of effector mechanisms for pathogen killing such as phagocytosis, the production of reactive oxygen species (ROS), and the release of antimicrobial peptides/proteins. Additionally, the formation of neutrophil extracellular traps (NETs) has been identified as a further but extracellularly acting effector mechanism of PMN-mediated pathogen killing. NETs act efficiently against bacteria, virus, and fungi (Brinkmann et al. 2004; Brinkmann and Zychlinsky 2007; Fuchs et al. 2007; Hellenbrand et al. 2013; Jenne et al. 2013) and might represent a general ancient mechanism to eliminate invasive pathogens. So far, PMN have been demonstrated to extrude NETs in response to

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several molecular triggers (Abi Abdallah and Denkers 2012; Hermosilla et al. 2014) as well as to vital and dead pathogens (Behrendt et al. 2010). The most important molecular inducers currently known are lipopolysaccharide (LPS), phorbol-12-myristate-13-acetate (PMA), GM-CSF, IL-8, glucose oxidase, Ca^{2+} ionophore, thapsigargin, TNF, and LPS-activated platelets, among others (Brinkmann et al. 2004; Abi Abdallah and Denkers 2012; Hermosilla et al. 2014). Up to now, bacterial and fungal pathogens capable to induce NETs include the species *Staphylococcus*, *Streptococcus*, *Shigella*, *Salmonella*, *Escherichia*, *Mycobacterium*, *Listeria*, *Histophilus*, *Aspergillus,* and *Candida* among others (Brinkmann et al. 2004; Urban et al. 2006; Grinberg et al. 2008; Bianchi et al. 2009; Ramos-Kichik et al. 2009; Bruns et al. 2010; Hellenbrand et al. 2013). While most NET studies have focused in the past years on the effects of NETs on bacterial and fungal pathogens, little attention has been paid to the role of NET formation in protozoan infections (Hermosilla et al. 2014). Recent data show that protozoan parasites also induce NET release upon PMN activation (Behrendt et al. 2010; Abi Abdallah et al. 2012; Hermosilla et al. 2014). To date, NET formation has been described in response to apicomplexan parasites such as *Plasmodium falciparum*, *Eimeria bovis*, *Toxoplasma gondii*, and *Besnoitia besnoiti* (Baker et al. 2008; Behrendt et al. 2010; Abi Abdallah et al. 2012; Muñoz Caro et al. 2014) and euglenozoan parasites *Leishmania amazonensis*, *Leishmania chagasi*, *Leishmania donovani*, and *Leishmania major* (Guimarães-Costa et al. 2009; Gabriel et al. 2010). Although invasion strategies of apicomplexan and euglenozoan parasites may significantly differ, i. e., apicomplexan actively infect specific host cells to escape fast detrimental innate immune reactions, skin-delivered *Leishmania* spp. promastigotes search for professional phagocytes resulting in attachment and engulfment by phagocytosis, both parasite groups are capable to trigger strong NETs (Abi Abdallah and Denkers 2012; Guimarães-Costa et al. 2012). Recently, it was reported that eggs of *Schistosoma japonicum* can also trigger NETs in human and murine PMN thereby showing for the first time that metazoan parasites seem also able to trigger this relevant effector mechanism (Chuah et al. 2013). Consistently, it was also published that extracellular traps are associated with human and mice neutrophil- and macrophage-mediated killing of larval *Strongyloides stercoralis* (Bonne-Annee et al. 2014).

The aim of this study was to characterize early innate immune reactions of caprine PMN against *E. arloingi* with respect to NET formation. We show here that exposure of caprine PMN with viable sporozoites and oocysts of *E. arloingi* trigger the formation of NETs. Since this parasite-triggered effector mechanism occurs rapidly upon contact, it may have a high impact on sporozoite elimination in *E. arloingi*-infected animals. Consequently, these findings suggest that NETosis may play an important role in the early host innate immune responses against *E. arloingi* infections in goats.

Materials and methods

Parasites

Eimeria arloingi (strain A) used in the present study was initially isolated in 2012 from naturally infected goat kids, in Alentejo, Portugal. The *E. arloingi* (strain A) was maintained by passages in male White German goat kids for oocysts production. Therefore, three male White German goat kids were purchased from a local goat milk farmer at the age of 3 days, treated with Baycox® (Bayer) and Halocur® (Intervet), assessed for parasitic infections, and when found parasite-free, maintained under parasite-free conditions in autoclaved stainless steel metabolic cages (Woetho) within a large animal stable equipped with laminar flow lock entrance until experimental infection. Animals were fed with milk substitute (CombiMilk® Lämmermilch, Agravis) and commercial pellet concentrates (Lämmerpellets, Deuka). Water and sterilized hay were given ad libitum. Collection of oocysts and sporulation were performed as previously described (Hermosilla et al. 2002).

For the isolation of viable *E. arloingi* sporozoites, the following excystation protocol was used: sporulated oocysts were suspended in sterile-filtered 0.02 M L-cystein/0.2 M $NaHCO_3$ solution and incubated in a 100 % CO_2 atmosphere at 37ºC for 20 h. Afterwards, the oocysts were centrifuged (600×*g*, 15 min) and resuspended in Hank's balanced salt solution (HBSS, Gibco) containing 0.4 % (*w*/*v*) trypsin (Sigma-Aldrich) and 8 % (*v*/*v*) sterile-filtered bovine bile (obtained from the local slaughterhouse) and were incubated for 4 h at 37ºC and 5 % CO_2 atmosphere. Free-released sporozoites were then washed in Rosewell Park Memorial Institute (RPMI) 1640 medium (twice, 600×g, 15 min, Gibco), resuspended at concentrations of 2×10^6 sporozoites/ml until further use. All animal experiments were performed according to the Justus Liebig University (JLU) Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the JLU and in accordance to the current German Animal Protection Laws.

Isolation of caprine PMN

Healthy adult goats ($n=3$) kept at the Institute of Physiology (Faculty of Veterinary Medicine, JLU Giessen, Germany) served as blood donors. Animals were bled by puncture of the jugular vein, and blood was collected in 12 ml plastic tubes (Kabe Labortechnik) containing lithium-heparin as anticoagulant. Heparinized blood was diluted under sterile conditions in an equal amount of sterile phosphate buffered saline (PBS) containing 0.02 % ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), layered on Biocoll® separating solution (Biochrom AG), and centrifuged at 800×*g* for 45 min. After removal of plasma, lymphocytes, and monocytes, the cells were resuspended in 25 ml sterile distilled water and shaken for 40 s to lyse erythrocytes. Osmolarity was immediately readjusted by adding 3 ml sterile HBSS (10x, Biochrom AG). Caprine PMN were washed twice (10 min, 400×*g*, 4 °C) in RPMI 1640 medium without phenol red (Gibco), resuspended in the same medium, and incubated at 37 °C and 5 % CO_2 atmosphere for at least 30 min before use.

Scanning electron microscopy

Caprine PMN were incubated either with freshly isolated *E. arloingi* sporozoites or oocysts at a ratio of 1:1 for 1 h on poly-L-lysine precoated glass coverslips (Greiner). After incubation, cells were fixed (2.5 % glutaraldehyde in 0.1 M cacodylate buffer, 15 min) and afterwards washed in 0.1 M cacodylate buffer (Merck). The cells were then postfixed (1 % osmium tetroxide in 0.1 M cacodylate buffer, Merck), washed three times in distilled water, dehydrated in ascending ethanol concentrations, critical point-dried with CO_2, and thereafter sputtered with gold particles. Specimens were examined using a Philips XL30® scanning electron microscope at the Institute of Anatomy and Cell Biology at the JLU Giessen, Germany.

Co-culture of caprine PMN and *Eimeria arloingi* stages

Quantification of NETs

PMN (10^5 cells/200 μl) were placed in 1.5 ml reaction tubes (Eppendorf) and incubated for 30, 60, or 90 min after addition of parasites, inhibitors, or stimulants (37 °C, 5 % CO_2 atmosphere). All compounds used were diluted or suspended in RPMI 1640 medium (Gibco), while PMN in plain medium served as negative control. For positive controls, PMN were stimulated with zymosan (Invitrogen) at a final concentration of 1 mg/ml according to Muñoz Caro et al. (2014).

To test for *E. arloingi* sporozoite-induced NET formation, 10^5 vital sporozoites were added to caprine PMN. After incubation, 50 μl of micrococcal nuclease buffer (0.1 U/μl, New England Biolabs) was added to each sample and incubated (15 min, 37 °C). Afterwards, each sample was centrifuged (400×*g*, 7 min). To a 96-well flat-bottom plate (Nunc), 100 μl of each supernatant was transferred. Each sample was processed in duplicates. A 1:200 dilution of Pico Green® (Invitrogen) in 10 mM Tris base buffered with 1 mM EDTA was added to each well (50 μl). NET formation was determined using an automated plate monochrome reader (Varioskan Flash®, Thermo Scientific) at an excitation wavelength of 484 nm and an emission wavelength of 520 nm. NETs were quantified based on fluorescence intensity analyses.

Inhibition assays were performed by adding either diphenylene iodonium (DPI, 5 μM, Sigma-Aldrich) at the start of the incubation period or deoxyribonuclease (DNase, 90 U, Roche Diagnostics) 15 min prior to the end of the incubation period.

NET entrapment assays of Eimeria arloingi sporozoites and NET-mediated killing

The entrapment of *E. arloingi* sporozoites by NET formation was quantified as previously described (Chow et al. 2010), with some modifications. Briefly, caprine PMN (n=3, 10^5/sample) were stimulated with zymosan (1 mg/ml, 30 min, 37ºC, 5 % CO_2 atmosphere). For the detection of entrapped sporozoites within NET structures, freshly isolated sporozoites were meanwhile stained with the fluorescent dye 5(6)-carboxylfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) as previously described (Hermosilla et al. 2008). Sporozoites were suspended in the dye solution (2.5 μM CFSE in PBS) by gently shaking and incubated for 10 min (37 °C, 5 % CO_2 atmosphere). In order to stop the labeling process, an equal volume of PBS containing 10 % fetal calf serum (FCS, Gibco) was added, and CFSE-stained sporozoites (sporozoites^{CFSE}) were washed four times (400×*g*, 10 min) in PBS, resuspended again in PBS, and protected from light. Sporozoites^{CFSE} were exposed to zymosan-prestimulated PMN in a 1:1 ratio (10^5 PMN:10^5 sporozoites^{CFSE}) and incubated (60 min, 37ºC, 5 % CO_2). A total of 10^5 non-PMN-exposed to sporozoites^{CFSE} served as negative control and were used to establish a standard curve (data not shown). After incubation, co-incubated PMN and sporozoites^{CFSE} were gently washed to remove non-entrapped sporozoites^{CFSE} (400×*g*, 5 min), the supernatant was removed, and the cells/sporozoites were carefully resuspended in 100 μl RPMI 1640 medium. The content of each tube was transferred to a 96-well plate and the fluorescence intensity was measured in comparison to non-exposed sporozoites. The percentage of entrapment was calculated as [(492/517 nm experimental well)/(492/517 nm control well without PMN)]×100 % (Chow et al. 2010).

To determine the killing effect of NETs on sporozoites, the trypan blue exclusion test (trypan blue solution 0.4 %, Sigma-Aldrich) was performed to assess sporozoite viability. Freshly released *E. arloingi* sporozoites were co-cultured with caprine PMN (n=3), at a 1:1 ratio (2×10^5 cells, 60 min, 37ºC). Non-exposed sporozoites were maintained at 37ºC as negative control. For positive controls, non-exposed sporozoites were killed via heat inactivation (60ºC, 60 min). After the incubation period, trypan blue solution was added (1:10) to the samples (3 min, RT) and cell viability was estimated microscopically.

Estimation of enzyme activities: neutrophil elastase, NADPH oxidase, and myeloperoxidase

Neutrophil elastase (NE) activity was estimated using the NE-chromogenic substrate MeoSuc-Ala-Ala-Pro-Val-chloromethyl ketone (Sigma-Aldrich). Briefly, caprine PMN (n=3) were exposed to sporozoites in a 1:1 ratio (2×10^5 cells/well) in duplicates for 60 min at 37ºC. Zymosan was used as positive control (1 mg/ml). After the incubation period and immediately prior to the measurement of chromogenic substrate, 3 mg/ml final concentration was added to each sample. NE activity was assessed via absorbance at 410 nm wavelength, using an automated plate monochrome reader (Varioskan Flash®, Thermo Scientific).

NADPH oxidase activity was measured by the oxidation of 2′,7′-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich) to fluorescent DCF (Conejeros et al. 2011, 2012). In brief, PMN (n=3) were resuspended in HBSS containing Ca^{2+} and incubated with sporozoites in a 1:1 ratio (2×10^5 cells/well; 37ºC, 60 min, in duplicates). For positive control stimulation, zymosan was used (1 mg/ml). Afterwards, DCFH-DA (10 μg/ml) was added to each sample just prior to the measurement of the fluorescence intensities at 485 nm excitation and 530 nm emission wavelengths.

Myeloperoxidase (MPO) activity was evaluated via peroxidase activity assessment by the use of Amplex Red® reagent (Invitrogen). Caprine PMN were exposed to sporozoites in a 1:1 ratio (n=3, 2×10^5 cells/well) and incubated in HBSS buffer lacking phenol red (Gibco) for 30 min at 37ºC in duplicates. Zymosan in a concentration of 0.5 mg/ml was used as positive control. After incubation, 50 μM Amplex Red® (Invitrogen) was added to each sample and peroxidase activity was measured in 571–585 nm fluorescence ranges.

Visualization of NETs and detection of histones, myeloperoxidase, and neutrophil elastase as NET components

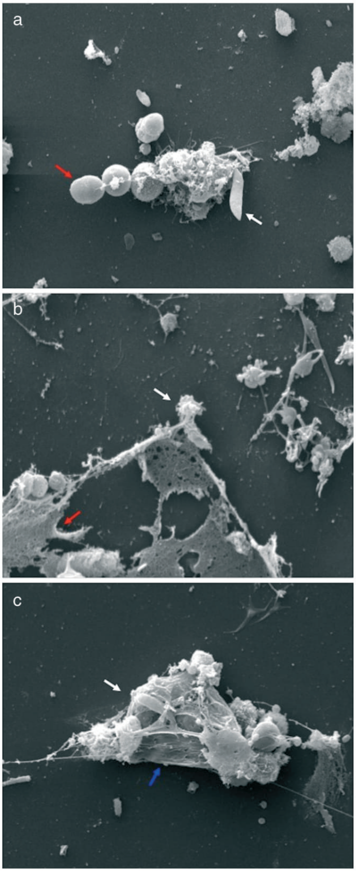
Illustrations of NETs being induced by *E. arloingi* stages were obtained with Sytox Orange® (S-11368, Invitrogen) nucleic acid staining. In brief, 10^5 PMN in serum-free RPMI 1640 medium (Gibco) were seeded on poly-L-lysine-treated glass coverslips in a six-well plate (Nunc) and exposed to sporozoites or oocysts at a 1:1 ratio (37 ºC, 5 % CO_2, 30 min). Each sample was fixed [4 % (*w*/*v*) paraformaldehyde, Merk, 37ºC, 20 min] and stored at 4ºC until further use. Before and after Sytox Orange® staining (5 mM Sytox Orange®, 5 min, RT, in the dark) (Martinelli et al. 2004), the samples were washed in PBS and covered with PBS to avoid drying of the cells. For the detection of histones (H3), MPO, and NE, the following specific antibodies were used: anti-histone monoclonal antibodies (rabbit monoclonal (E173) to bovine histone H3 phospho S10 DyLight® 488, 1:100; ab139848, Abcam), anti-MPO antibodies (rabbit polyclonal to MPO antibodies, Alexa Fluor 488, 1:200, ABIN906866, Antibodies-online.com), and anti-NE antibodies (rabbit polyclonal to human neutrophil elastase, 1:200, AB68672, Abcam). The samples were washed three times in PBS, blocked with bovine serum albumin (BSA) [1 % (*w*/*v*) in PBS, 30 min, RT, Sigma-Aldrich] and incubated with anti-histone, anti-NE, or anti-MPO antibodies (1 h, RT, in the dark for anti-histone, 24 h, RT, in the dark for anti-MPO and anti-NE antibodies). The samples were gently washed with PBS and mounted in anti-fading buffer (Mowiol®, Sigma-Aldrich). Visualization was achieved by using an invert Olympus IX81® fluorescence microscope.

Host cell invasion assay

To test the impact of NET formation on parasite host cell invasion capacity, vital sporozoites (10^5/well) were incubated with PMN (2×10^5/well, 90 min, 37 °C). In the case of DNase treatment, DNase (45 U/well) was added 15 min prior to the end of the incubation period. Non-exposed sporozoites were used for positive controls, and each sample was performed in duplicates. After incubation, PMN-sporozoite co-cultures were transferred to confluent bovine umbilical vein endothelial cell (BUVEC, n=3) monolayers (in 24-well plates) and incubated for 4 h (37 °C, 5 % CO_2 atmosphere) to allow sporozoites for host cell invasion. Thereafter, cell layers were washed thoroughly with prewarmed modified ECGM medium (ECGM, PromoCell) to remove PMN and remaining sporozoites. Infection rates were estimated microscopically 24 h post infection (p. i.) using ×400 magnification, and *E. arloingi*-infected host cells were counted in six randomly selected power vision fields per duplicate (n=18). The infection rate induced by non-PMN-exposed sporozoites was set as 100 %.

Statistical analysis

For statistical analyses, one- or two-factorial analyses of variance (ANOVA) with repeated measures were performed in order to compare co-culture/stimulation conditions. The Bonferroni method was used as a follow-up test to ANOVA. For comparing enzyme activities and ROS production, *t*-student tests were performed. All analyses were performed with the GraphPad



◀ **Fig. 1** *Eimeria arloingi* sporozoite- and oocyst-triggered NET formation. Scanning electron microscopy analyses revealed the presence of thicker and thinner filaments originating from PMN when co-cultured with *E. arloingi* stages. **a** Fine PMN-derived strands being attached to sporozoites (*white arrow*) and a non-activated PMN (*red arrow*, ×2,000 magnification); **b** sporozoites trapped in drawn-out fibers (*white arrow*, ×2,000 magnification); **c** *E. arloingi* oocyst (*blue arrow*) and a recently released sporozoite (*white arrow*) being entrapped in thicker and thinner extracellular fibers (×2,000 magnification)

Prism® 6 software. Differences were regarded as significant at a level of $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***); and $p \leq 0.0001$ (****).

Results

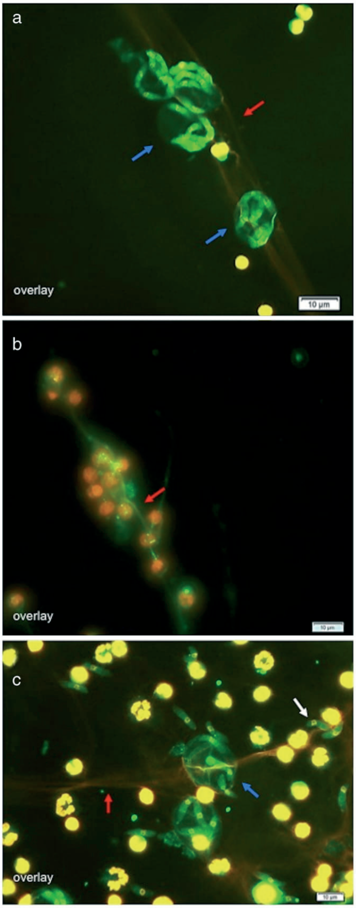
NET formation trigged by *Eimeria arloingi* stages—visualization and characterization

Scanning electron microscopy (SEM) analysis of caprine PMN exposed to *E. arloingi* revealed sporozoite-triggered formation of delicate networks of thicker and thinner filaments of fibers being extruded from PMN that were firmly attached to the parasites apparently trapping them (Fig. 1a, b). The majority of sporozoites presented a normal morphology; in contrast, caprine PMN exhibited different morphologies according to their stage of NETosis: non-activated PMN appeared as intact cells showing typical rounded cell morphology with sometimes irregular surface (Fig. 1a—red arrow); activated PMN showed up as dead or disrupted cells with drawn-out filaments trapping sporozoites (Fig. 1a, c) and, during the final stage of NETosis, presented as massively matted by PMN-derived fibers (Fig. 1b—red arrow). Moreover, we could also visualize the entrapment of *E. arloingi* oocysts by caprine NETs. As illustrated in Fig. 1c, a trapped oocyst (blue arrow) and a recently released sporozoite (white arrow) are completely covered by NET filaments originating from several activated, adjacent PMN, demonstrating that caprine PMN can react against both *E. arloingi* stages via NETosis.

Also, fluorescence analyses demonstrated the presence of NET-like structures proven to contain DNA by Sytox Orange® staining (Fig. 2a, b, c—red arrows, overlay). Sporozoites were located in intimate contact with NETs and were occasionally trapped in these structures (Fig. 1—white arrows). The classical characteristics of NETs were proven by co-localization studies on histones (H3), NE, and MPO, which were all detected in parasite-induced NET structures (green, Fig. 2a, b, c, respectively).

Quantification of caprine NET formation trigged by *Eimeria arloingi* sporozoites

For the quantification of caprine NET formation induced by *E. arloingi* sporozoites, kinetic and inhibition studies were performed, revealing fast and strong NET induction. Thus, significant NETosis was observed already 30 min after initial exposure, i. e. at the earliest time point of measurement.



◀ **Fig. 2** Co-localization of DNA with H3, NE, and MPO in sporozoite- and oocyst-induced NET structures. Co-cultures of caprine PMN and *E. arloingi* sporozoites and oocysts were stained for DNA using Sytox Orange® (*red*) and probed for histones (*green*, **a**), NE (*green*, **b**), and MPO (*green*, **c**) using anti-histone (H3), anti-NE, and anti-MPO antibodies and adequate conjugate systems. Merges (**a**, **b**, **c**) illustrate sporozoites (*white arrows*) and oocysts (*blue arrows*) being snared in NET structures (*green, red arrows*)

Overall, the amount of extracellular DNA was significantly higher in parasite-exposed samples when compared to parasite-free (negative) controls at all time points measured ($p<0.001$, Fig. 3). The process of parasite-triggered NET formation was time-dependent since the magnitude of reactions increased with the ongoing incubation period leading to the strongest reactions at 90 min of incubation time. It is noteworthy that at the latter time point, *E. arloingi*-induced NET formation even exceeded the reactions triggered by zymosan, which represents a very potent inducer of NETosis (Brown and Roth 1991; Muñoz Caro et al. 2014). Hence, *E. arloingi* sporozoites bear a strong capability to trigger NET formation.

The DNA nature of *E. arloingi*-induced NET structures was additionally confirmed by DNase treatment. Correspondingly, a significant reduction ($p<0.001$) of Pico Green®-derived fluorescence intensities were measured in parasite-exposed and DNase-treated samples (Fig. 3).

In order to confirm the necessity of the NADPH oxidase activity for the NET formation process, we performed inhibition assays with DPI, an inhibitor of the NADPH oxidase. Supplementation of DPI throughout the incubation period resulted in significant reduction of parasite-induced NET formation, independent of the incubation time (Fig. 3). These results also corroborated the hypothesis of ROS intervention in the process of *E. arloingi*-induced NETs.

Entrapment of *Eimeria arloingi* sporozoites

Entrapment of parasites was clearly observed in SEM and fluorescence images. Using CFSE-stained parasites, we established a quantitative assay for sporozoite entrapment. This assay revealed a strong induction of *E. arloingi*-triggered NETs entrapping up to 72 % of total sporozoites^{CFSE} (Fig. 4a, $n=6$). Fluorescence imaging illustrated the presence and immobilization of sporozoites^{CFSE} in NET structures (Fig. 4b, sporozoites^{CFSE}—white arrow; NETs—red arrow). Trypan blue exclusion test revealed that NET formation did not interfere with the sporozoite viability since 98 % of PMN-exposed sporozoites remained viable after exposure. The same rate of mortality was observed in non-exposed sporozoites. In contrast, heat-inactivated sporozoites showed 97 % mortality (data not shown).

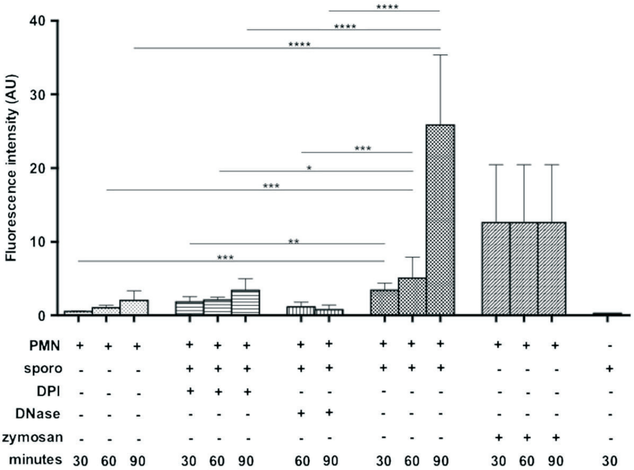


Fig. 3 Kinetic and inhibition assays on *E. arloingi* sporozoite-induced NETosis. PMN and sporozoites were incubated in a 1:1 ratio for 30, 60, and 90 min. Stimulation with zymosan (1 mg/ml) was used as positive control and sporozoites only as negative control. For NET inhibition, DNase- and DPI-treatments were applied. Extracellular DNA was quantified by Pico Green®-derived fluorescence intensities (AU)

Enzymatic activities of MPO, NE, and NADPH oxidase in NET formation

Sporozoite-triggered NET formation is accompanied by specific regulation of enzymatic activities in PMN. Incubation of caprine PMN and *E. arloingi* sporozoites significantly induced NE- and NADPH oxidase-activity (Fig. 5b, c), confirming the fundamental role of these molecules in NET formation. However, although MPO activity in PMN appeared to be higher in sporozoite-exposed PMN when compared to parasite-free controls, the differences were not significant (Fig. 5a). Stimulation with zymosan served as positive control and induced significantly enhanced enzymatic activities of NE and NADPH oxidase in PMN ($p<0.001$).

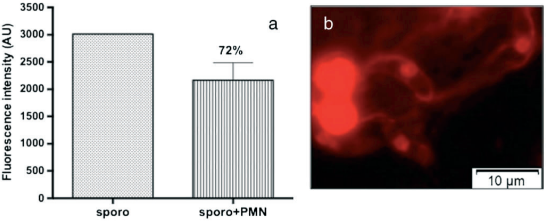


Fig. 4 Quantification of sporozoite entrapment in NET structures. Quantification of *E. arloingi* sporozoite entrapment in NETs (**a**) was performed after incubation of zymosan-stimulated PMN ($n=3$) with sporozoites^{CFSE}. Non-trapped sporozoites^{CFSE} were washed off, and the resulting fluorescence intensities were calculated in relation to non-exposed sporozoites^{CFSE}. **b** Entrapment of *E. arloingi* sporozoites^{CFSE} within NET structures (*red*) was illustrated by Sytox Orange®-derived DNA staining after the exposure of stained sporozoites to caprine PMN. All experiments were performed as duplicates

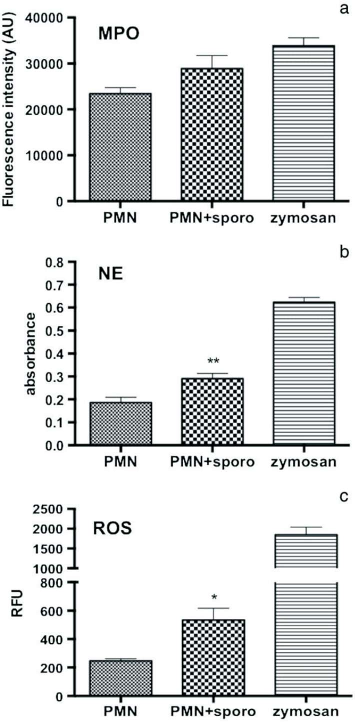


Fig. 5 Enzymatic activities of MPO, NE, and NADPH oxidase in caprine PMN after exposure to *E. arloingi* sporozoites. Caprine PMN were exposed to *E. arloingi* sporozoites, zymosan (positive control), or plain medium (negative control) for 60 min. Thereafter, enzymatic activities of NE and MPO as well as ROS production were measured in the supernatants using NE-chromogenic substrate MeoSuc-Ala-Ala-Pro-Val-chloromethyl ketone, Amplex red®, and the oxidation of DCFH-DA to fluorescent DCF, respectively. All experiments were performed in duplicates

Eimeria arloingi-induced NET formation hampers host cell invasion

To successfully complete their life cycle, free-released sporozoites have to invade host endothelial cells in vivo to continue their intracellular development. In order to estimate the influence of NET formation on sporozoite invasion capacities, PMN were preincubated with sporozoites for 90 min and directly transferred to confluent BUVEC monolayers as adequate host endothelial cells. Respective infection rates were estimated thereafter. Comparing to control parasites (non-PMN-exposed sporozoites, respective infection rates were set as 100 %), the pre-exposure of parasites with PMN significantly reduced the sporozoites invasion capacity for host cells ($p<0.0001$). Consequently, the infection rates decreased to 20 % of those induced by non-treated sporozoites, revealing the strong capability of caprine NETs to immobilize sporozoites and prevent them from host cell infection, a phenomenon that will exhibit a remarkable impact on subsequent intracellular parasite replication. This reaction was clearly reversible since DNase treatment applied toward the end of PMN pre-exposure (as such, the experimental set-up allowed for primary NET formation) significantly abolished this effect ($p<0.001$), proving NETs as to be the cause for invasion inhibition and confirming our previous results on non-lethal effects of NETs (Fig. 6).

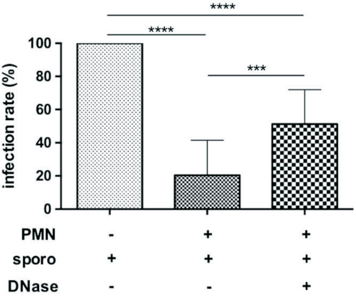


Fig. 6 Infectivity of *E. arloingi* sporozoites after exposure to caprine PMN. *Eimeria arloingi* sporozoites were co-cultured with caprine PMN for 90 min in the presence or absence of DNase. Incubation in plain medium served as PMN-free, positive control (the respective infection rate was set as 100 %). After incubation, samples were transferred to confluent BUVEC monolayers for 4 h. Thereafter, the cell layers were washed and infection rates were estimated (1 d. p. i.)

Discussion

PMN are considered as a fundamental component of the host innate immune system representing the first line of defense against pathogens and the first leukocytes to be recruited to the site of infection (Brinkmann et al. 2004; Ermert et al. 2009; Brinkmann and Zychlinsky 2012; Hahn et al. 2013). Besides phagocytosis- and oxidative burst-related defense actions, the formation of NETs is nowadays considered as one main effector mechanism of PMN (Brinkmann et al. 2004).

So far, NETs have been described in several species: humans (Gupta et al. 2005), mice (Ermert et al. 2009), horses (Alghamdi and Foster 2005), cows (Behrendt et al. 2010), fish (Palic et al. 2007), cats (Wardini et al. 2010), chickens (Chuammitri et al. 2009), insects (Altincicek et al. 2008), and crustaceans (Ng et al. 2013). With this work, we add a new species to the panel of NET-producing immune systems by demonstrating for the first time the capability of caprine PMN to produce NETs upon exposure to a non-specific stimulant (zymosan) and the specific caprine pathogen *E. arloingi*.

Most studies on pathogen-triggered NET formation focused on bacterial, viral, or fungal infections, so far (Urban et al. 2006; Fuchs et al. 2007; Aulik et al. 2010; Jenne et al. 2013), and only few protozoan parasites have been investigated as inducers of NETosis [*P. falciparum* (Baker et al. 2008), *Leishmania* spp. (Guimarães-Costa et al. 2009), *E. bovis* (Behrendt et al. 2010), *T. gondii* (Abi Abdallah et al. 2012) and *B. besnoiti* (Muñoz Caro et al. 2014)]. We here describe for the first time the release of caprine NETs in response to the goat-specific apicomplexan parasite *E. arloingi*, which is known as one of the most pathogenic species causing caprine coccidiosis worldwide. In agreement with observations in other *Eimeria* species (Behrendt et al. 2010), we documented NET-like structures being attached to the parasite via SEM and fluorescence-based assays. Staining of NET structures with the DNA stain Sytox Orange® and the resolution of parasite-induced filamentous structures by DNase treatments proved the DNA nature of sporozoite-triggered NETs. Besides chromatin, other components of NETs, such as nuclear histones, NE, cathepsin G, MPO, lactoferrin, and gelatinase, are described as pivotal for the microbiocidal effect of NETs (Brinkmann et al. 2004; Hermosilla et al. 2014). Via co-localization experiments showing the simultaneous presence of H3, NE, and MPO in caprine NETs, we confirmed these classical characteristics of NETs. Moreover, enhanced NE enzymatic activity in sporozoite-exposed PMN confirmed the key role of this molecule in the formation of NETs as suggested by others (Papayannopoulos et al. 2010; Muñoz Caro et al. 2014). The activation of the NADPH oxidase complex and subsequent production of ROS is a crucial step in NETosis (Brinkmann and Zychlinsky 2007; Fuchs et al. 2007). Since ROS production was significantly enhanced in sporozoite-exposed PMN and NADPH oxidase blockage via DPI treatment (Hosseinzadeh et al. 2012) diminished parasite-triggered NETosis, our results confirmed the relevance of this enzyme complex in pathogen-induced NET formation (Brinkmann et al. 2004; Fuchs et al. 2007; Behrendt et al. 2010; Abi Abdallah et al. 2012; Muñoz Caro et al. 2014). Findings of ROS-dependent NET induction in goats cannot be taken for granted since there have previously been differential regulation processes of inducible nitric oxide synthase production in bovine and caprine leukocytes (Adler et al. 1996) as well as differential lower phagocytic activities in caprine PMN after expose to *Candida albicans* when compared to bovine and buffalo PMN responses (Sahoo et al. 2000).

Overall, *E. arloingi* sporozoite-triggered NETosis was time-dependent as also previously reported by Behrendt et al. (2010) for *E. bovis*. Since zymosan was previously validated as potent and superior activator of PMN in the bovine system compared to PMA (Brown and Roth 1991), we used this molecule as positive control in NETosis assays. As expected, caprine PMN also reacted strongly upon zymosan by upregulating NADPH oxidase, NE, and MPO activities and NET release. Interestingly, *E. arloingi*-induced NET formation accounted for even higher values than zymosan stimulation after 90 min of incubation, pointing at a considerable NET-inducing capacity of sporozoites. Recent analyses doubt a strict species-specific *Eimeria*-induced NETosis and rather argue for a general phenomenon (Hermosilla et al. 2014), since NET production as induced by (strictly host-specific) *E. bovis* sporozoites in caprine PMN and bovine PMN also expelled NETs in response to the non-bovine *E. arloingi* sporozoites (Muñoz Caro et al. unpublished data).

While the sporozoite stage has been already demonstrated as potent NET trigger (Behrendt et al. 2010), we demonstrate here for the first time that also the oocyst stage of *E. arloingi* induces NETs. Similar findings were seen in leishmaniosis where *Leishmania*-triggered NETosis was not entirely stage-specific, since both promastigotes (*L. amazonensis*, *L. major*, *L. chagasi*) and amastigotes (*L. amazonensis*) promoted NETs (Guimarães-Costa et al. 2009, 2012). It appears noteworthy that *E. arloingi* oocysts are equipped with a prominent micropyle and polar cap. As such, all sporozoites being contained in the oocyst need to leave the oocyst through the micropyle after the polar cap has been removed (Jackson 1964) instead of being set free all at once during oocyst disrupture in non-micropylic species (e. g., *E. ninakohlyakimovae*, *E. zuernii*, *E. bovis*). In consequence, blockage of the micropyle via NETs immediately hampers all sporozoites inside the oocyst from excystation and effectively blocks the life cycle at a very early stage of host infection. Since PMN have been demonstrated to actively transmigrate into the intestinal lumen (Brazil et al. 2013; Seper et al. 2013; Sumagin et al. 2013; Szabady and McCormick 2013), they should also be able to interact with different luminal pathogen stages, such as ingested oocysts, in the in vivo situation. The SEM analyses performed in this study clearly showed oocysts and freshly excysted sporozoites being entrapped by PMN in NET-like structures in vitro.

As suggested for *E. bovis*, *T. gondii* and *B. besnoiti* (Behrendt et al. 2010; Abi Abdallah et al. 2012; Muñoz Caro et al. 2014), the relevance of NETosis as effector mechanism in the defense against *E. arloingi* rather lies in the entrapment and immobilization of the parasite resulting in the inhibition of host cell invasion than in pathogen killing. Accordingly, NETs did not vastly kill *E. arloingi* sporozoites as shown by trypan blue vital staining, but in agreement with reports on *E. bovis*-induced NET formation (Behrendt et al. 2010), NET-mediated parasite entrapment reduced the infectivity of *E. arloingi* sporozoites. In contrast to reports dealing with the *Eimeria* sporozoites, NETs appeared to exhibit certain lethal effects on tachyzoites of *T. gondii* (Abi Abdallah et al. 2012) and *L. amazonensis* promastigotes (Guimarães-Costa et al. 2009).

Interestingly, the capacity of caprine PMN to entrap sporozoite stages appears to be superior to that of bovine PMN since 70 % of sporozoites were ensnared in caprine NETs compared to 30 % in bovine NETs using the same co-culture ratios (unpublished data). However, the extracellular arrest of obligate intracellular-replicating parasites will obviously prevent sporozoites from host cell invasion and abrogate the life cycle of *E. arloingi*. Since the main replication step follows after endothelial cell infection via intracellular macromeront formation and subsequent second merogony and gamogony, every single sporozoite being immobilized in NETs may account for the outcome of the disease severity. Thus, NET formation should be considered as an important step in early innate immune response against caprine *E. arloingi* coccidiosis.

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Harbour seal (*Phoca vitulina*) PMN and monocytes release extracellular traps to capture the apicomplexan parasite *Toxoplasma gondii*

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ABSTRACT

Extracellular traps (ETs) are composed of nuclear DNA as backbone adorned with histones, cytoplasmic antimicrobial peptides/proteins which are released from a range of vertebrate and invertebrate host immune cells in response to several invading pathogens. Until now this ancient novel innate defence mechanism has not been demonstrated in any marine mammal. Interactions of harbour seal (*Phoca vitulina*)-PMN and -monocytes with viable tachyzoites of *Toxoplasma gondii* were investigated in this respect *in vitro*. For the demonstration and quantification of harbour seal PMN- and monocyte-derived ETs, extracellular DNA was stained with Sytox Orange. Fluorescence assays as well as scanning electron microscopy (SEM) analyses demonstrated PMN- and monocyte-promoted ET formation rapidly being induced upon contact with *T. gondii*-tachyzoites. The co-localisation of extracellular DNA decorated with histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in parasite entrapping structures confirmed the classical characteristics of PMN- and monocyte-promoted ETs. Exposure of harbour seal PMN and monocytes to viable tachyzoites resulted in a significant induction of ETs when compared to negative controls. Harbour seal-ETs were efficiently abolished by DNase I treatment and were reduced after PMN and monocytes pre-incubation with the NADPH oxidase inhibitor diphenilane iodondium. Tachyzoites of *T. gondii* were firmly entrapped and immobilised within harbour seal-ET structures. To our best knowledge, we here report for the first time on *T. gondii*-induced ET formation in harbour seal-PMN and -monocytes. Our results strongly indicate that PMN- and monocyte-triggered ETs represent a relevant and ancient conserved effector mechanism of the pinniped innate immune system as reaction against the pathogenic protozoon *T. gondii* and probably against other foreign pathogens occurring in the ocean environment.

1. Introduction

The harbour seal (*Phoca vitulina*), also known as common seal, is found along temperate and arctic marine coastlines of the Northern Hemisphere. Their population size in the Wadden Sea as estimated in 2012 (Wolff, 2013) is 26,220 individuals and has increased continuously since 1990. Nevertheless, harbour seals are still considered as threatened by various factors such as pollution and disturbances caused by humans (Wolff, 2013; Wolff et al., 2010). Investigations on the adaptive immune system of this pinniped species are still scarce in literature (Cavagnolo, 1979; De Swart et al., 1993; Ross et al., 1993, 1994) and even less is known on studies related to the pinniped innate immune system (Frouin et al., 2010), although leukocytes of this former system represent the first line of defence and the first ones to be recruited to sites of infection.

Main function of mononuclear phagocytes, such as polymorphonuclear neutrophils (PMN), monocytes and macrophages, has been understood as a variety of potent intracellular microbicidal mechanisms to kill invasive pathogens (Bainton et al., 1971; Borregaard and Cowland, 1997; von Köckritz-Blickwede and Nizet, 2009). Nonetheless, detailed knowledge on pinniped mononuclear phagocyte molecules and effector mechanisms is still in its infancy (Frouin et al., 2010), and needs to be addressed in the near future as this system is complex and able to mount a rapid, specific and protective response against pathogens (Borregaard and Cowland, 1997; Brinkmann and Zychlinsky, 2007, 2012; Hahn et al., 2013; von Köckritz-Blickwede and Nizet, 2009).

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The two main effector mechanisms displayed by professional phagocytes against pathogens are the phagocytosis and the release of antimicrobial molecules such as reactive oxygen species (ROS) and antimicrobial peptides/proteins (Borregaard and Cowland, 1997; Brinkmann and Zychlinsky, 2007; Hermosilla et al., 2014; Nathan, 2006; von Köckritz-Blickwede and Nizet, 2009). Besides phagocytosis and the production of ROS, a major effector mechanism of PMN is the formation of neutrophil extracellular traps, called NETs (Brinkmann et al., 2004), which lead to extracellular killing of bacteria, fungi (Brinkmann and Zychlinsky, 2007, 2012; Hahn et al., 2013), virus (Wardini et al., 2010) and parasites (Abi Abdallah and Denkers, 2012; Hermosilla et al., 2014). NETs were described for the first time with the landmark study of Brinkmann et al. (2004) demonstrating that PMN were capable of killing pathogens far beyond their lifespan by releasing granular proteins, and chromatin forming thin extracellular fibres which bind and kill bacteria. Detailed analysis of NETs revealed that they consist of nuclear DNA as a backbone being adorned with histones and antimicrobial peptides/proteins, such as neutrophil elastase (NE), myeloperoxidase (MPO), pentraxin, lactoferrin, gelatinase, bacterial permeability-increasing protein (BPI), cathepsin G, peptidoglycan recognition proteins (PGRPs) and calprotectin among others (Brinkmann and Zychlinsky, 2007, 2012; Hermosilla et al., 2014; von Köckritz-Blickwede and Nizet, 2009).

Besides PMN, other leukocytes of the innate immune system, such as eosinophils (Yousefi et al., 2008), mast cells (von Köckritz-Blickwede et al., 2008), macrophages (Aulik et al., 2012; Bonne-Année et al., 2014; Hellenbrand et al., 2013), monocytes (Muñoz-Caro et al., 2014a, 2014b), avian heterocytes (Chuammitri et al., 2009), insect oenocytoid cells (Altincicek et al., 2008), crustacean hemocytes (Ng et al., 2013) and several other invertebrate species (Poirier et al., 2014; Robb et al., 2014) have additionally been reported to extrude NET-like structures which are now collectively entitled as extracellular traps (ETs). Regardless of whether ETs are released by activated PMN or any other leukocytes, ETs like NETs are mainly composed of DNA adorned with antimicrobial molecules and histones (Brinkmann and Zychlinsky, 2012; Guimarães-Costa et al., 2012; von Köckritz-Blickwede and Nizet, 2009). In most cases, the source of released DNA is decondensed chromatin from the cell nucleus (Brinkmann and Zychlinsky, 2012; Guimarães-Costa et al., 2012) but mitochondrial DNA can also serve as source for ETs as demonstrated for eosinophil- and mast cell-derived ETs (von Kockritz-Blickwede et al., 2008; Yousefi et al., 2008; Yousefi et al., 2009).

Overall, ET formation has been described as a novel 'suicidal' cell death pathway called ETosis, distinct from apoptosis, autophagy and necrosis (Brinkmann and Zychlinsky, 2007, 2012; Fuchs et al., 2007; Hermosilla et al., 2014). ETosis depends on the generation of ROS by NADPH oxidase and the induction of RAF-MEK-ERK-mediated signaling pathway cascade (Hakkim et al., 2011; Muñoz-Caro et al., 2015). Additionally, peptidyl-arginine deiminase (PAD)-mediated histone citrullination, followed by chromatin decondensation seems to be essential in ET extrusion (Hahn et al., 2013; Wang et al., 2009). Known molecules to trigger ETs are PMA, zymosan, hydrogen peroxide, 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., *Staphilococcus epidermidis* δ-toxin, autoantibodies and LPS-activated platelets among others (Brinkmann and Zychlinsky, 2012; Cogen et al., 2010; Guimarães-Costa et al., 2012; Muñoz-Caro et al., 2014a, 2014b; von Köckritz-Blickwede and Nizet, 2009).

Until now, most data on ETosis appear to be focused on fungal, bacterial and viral pathogens, such as *Aspergillus fumigatus*, *A. nidulans*, *Candida albicans*, *Cryptococcus neoformans*, *Escherichia coli*, *Helicobacter pilori*, *Histophilus somni*, *Listeria monocytogenes*, *Mannheimia haemolytica*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pyogenes* and feline leukemia virus among others (Hahn et al., 2013; von Köckritz-Blickwede and Nizet, 2009). In contrast, little attention has been paid on parasite-triggered ETosis (for reviews see Abi Abdallah and Denkers, 2012; Hermosilla et al., 2014). Until now, the induction of ETs have only been demonstrated for apicomplexan protozoa *Plasmodium falciparum*, *Eimeria bovis*, *Eimeria arloingi*, *Toxoplasma gondii* and *Besnoitia besnoiti* (Abi Abdallah et al., 2012; Baker et al., 2008; Behrendt et al., 2010; Muñoz-Caro et al., 2014a, 2014b, 2015; Silva et al., 2014; Taubert, 2011) and euglenozoan protozoa *Leishmania* spp. (Gabriel et al., 2010; Guimarães-Costa et al., 2009, 2012, 2014; Wang et al., 2011). The only reports of ETs induction by metazoan parasites refer to *Schistosoma japonicum* (Chuah et al., 2013) and *Strongyloides stercoralis* (Bonne-Année et al., 2014).

The species-spectrum of ETs-inducing vertebrates includes humans (Bonne-Année et al., 2014; Brinkmann et al., 2004; Gupta et al., 2005), mice (Beiter et al., 2006; Wartha et al., 2007), horses (Alghamdi and Foster, 2005), cows (Behrendt et al., 2010; Lippolis et al., 2006), goats (Silva et al., 2014), fish (Palic et al., 2007), cats (Wardini et al., 2010), chickens (Chuammitri et al., 2009), insects (Altincicek et al., 2008), crustaceans (Ng et al., 2013) and molluscs (Poirier et al., 2014; Robb et al., 2014). To date however, ETs have never been studied in any wild marine mammal as ancient effector mechanism against parasites. In the present study using the harbour seal as an animal model for pinnipeds and other closely related marine mammals, we therefore investigated the formation of ETs by PMN and monocytes against the pathogenic apicomplexan protozoa *T. gondii*. This parasite is a facultative heteroxenous, polyxenous protozoa which possess the capability to infect virtually all warm-blooded animals, including marine mammals (Dubey et al., 2011; Tenter et al., 2000), such as seals (Cabezón et al., 2011; Fujii et al., 2007; Simon et al., 2011), sea otter (Conrad et al., 2005; Goldstein et al., 2011; Miller et al., 2008), dolphins (Dubey et al., 2008) and whales (Mazzariol et al., 2012). To our best knowledge, we here report for the first time on *T. gondii*-triggered ET formation in harbour seal-PMN and -monocytes. Our results indicate that PMN- and monocyte-triggered ETs may represent an important ancient conserved effector mechanism of pinnipeds against *T. gondii* and most probably against other pathogens occurring in the ocean environment.

2. Material and methods

2.1. Experimental animals

Six to eight months old-aged wild harbour seals (*P. vitulina*) originating from the Northern Dutch Wadden Sea, which had been submitted 3 months before to the Seal Rehabilitation and Research Centre (SRRC) in Pieterburen, The Netherlands, for rehabilitation purposes, were used as blood donors. All harbour seals ($n = 6$) used in the present study were clinical healthy and in good body conditions at the moment of blood collection. Animals were kept in individual enclosures with a small integrated sea water tank, in which sea water was changed every day, situated in a quarantine restriction facility unit at the SRRC. Animals were fed twice a day exclusively with previously frozen (−20 °C) and thawed Atlantic herring (*Clupea harengus*) to avoid infections, supplemented with vitamins (Akwavit®; Kasper Faunafood). Individual seal enclosures were sanitised daily at the SRRC.

2.2. Isolation of harbour seal-PMN and -monocytes

Harbour seals ($n = 6$) were bleed by puncture either from the posterior tarsal sinus vein (*sinus tarsalis*) or the pelvic plexus veins (*sinus plexus pelvis*). Thereafter, 10 ml of harbour seal blood was collected in lithium-heparinised tubes (Kabe Labortechnik), kept at 4 °C, and immediately transferred by car (within 5 h) to the Institute of

Parasitology, Justus Liebig University Giessen, Germany, for further PMN- and monocyte-isolation and subsequent ETs experiments.

The isolation of harbour seal PMN was performed according to Muñoz-Caro et al. (2014a, 2014b) with some slight modifications due to strong distilled water-resistant haemolysis displayed by harbour seal erythrocytes. Briefly, heparinised harbor seal blood was diluted with PBS (1:3 ratio) containing 0.02% EDTA, layered on Biocoll® separating solution (density = 1.077 g/l; Biochrom AG) and centrifuged (800 × g, 45 min, 4 °C). The pellet was suspended in 25 ml distilled water in order to lyse erythrocytes for 40 s. Osmolarity was adjusted by adding 10× Hanks buffer salt solution (HBSS, Biochrom AG). PMN were washed twice, re-suspended in RPMI 1640 culture medium (Gibco), counted in a Neubauer haemocytometer chamber and incubated at 37 °C and 5% CO_2 atmosphere for at least 30 min before experimental use.

For harbour seal monocyte isolation we followed the protocol of Taubert et al. (2009) and Muñoz-Caro et al. (2014a, 2014b). After the Biocoll® separating centrifugation (800 × g, 45 min, 4 °C) the lymphocyte/monocyte layer was collected and the cells were washed three times (10 min, 400 × g, 4 °C) in RPMI 1640 medium (Gibco). In brief, 7.5×10^7 peripheral blood mononuclear cells (PBMC) were allowed to adhere (1 h, 37 °C) to 75 cm^2 tissue plastic flasks (Greiner), previously coated with 2% sterile gelatine solution (2 h, 37 °C, thereafter dried) and autologous harbour seal plasma (1 h, 37 °C, washed twice with RPMI 1640/1% penicillin/1% streptomycin, all Sigma-Aldrich). Non-adherent PBMC were removed and monocytes were washed with pre-warmed RPMI 1640/1% penicillin/1% streptomycin. Harbour seal monocytes were detached (10 min in 10 mM EDTA in Mg^{++}- and Ca^{++}-free HBSS solution, RT), washed (10 min, 400 × g, 4 °C) and re-suspended in 4 °C RPMI 1640/1% penicillin/1% streptomycin. Harbour seal monocytes were kept on ice until use and counted in a Neubauer haemocytometer chamber for further monocyte ET-experiments.

All the blood samples used for the present study were provided by the Seal Rehabilitation and Research Centre (SRRC), and the SRRC gave permission for using them for this study to both the Institute of Parasitology and the Institute of Anatomy and Cell Biology of Justus Liebig University in Giessen, Germany. The SRRC is allowed by the Government of The Netherlands to rescue and rehabilitate wild seals (application number FF/75/2012/015). The samples used were primarily collected for routine diagnostic control during rehabilitation.

2.3. Host cell culture and Toxoplasma gondii *tachyzoite maintenance*

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated as previously described by Taubert et al. (2006). Umbilical cords obtained from calves born by *sectio ceasarea* were kept at 4 °C in 0.9% HBSS-Hepes buffer (pH 7.4; Gibco) supplemented with 1% penicillin (500 U/ml; Sigma-Aldrich) and streptomycin (500 µg/ml; Sigma-Aldrich). For isolation of endothelial cells, 0.025% collagenase type II (Worthington Biochemical Corporation) was infused into the lumen of the isolated and ligated umbilical vein and incubated for 20 min at 37 °C in 5% CO_2 atmosphere. After gently massaging the umbilical vein, the collagenase-cell suspension was collected and supplemented with 1 ml FCS (Gibco) in order to inactivate the collagenase. After two washings (400 × g, 10 min, 4 °C), the endothelial cells were re-suspended in complete endothelial cell growth medium (ECGM; PromoCell), plated in 25 cm^2 plastic culture plates (Nunc) and kept at 37 °C in 5% CO_2 atmosphere.

Toxoplasma gondii (strain RH) tachyzoites were maintained by serial passages in BUVEC monolayers according to Taubert et al. (2006). Free released *T. gondii*-tachyzoites were harvested from BUVEC supernatants, centrifuged thrice (200 × g, 1 min), washed thrice with sterile PBS, counted and suspended in RPMI 1640 medium (Gibco) until further use. For comparative reasons and to elucidate parasite species-specific ETs-induction, we also conducted experiments with the closely related apicomplexan protozoa *Besnoitia besnoiti.* Tachyzoites of *B. besnoiti* (strain Bb1Evora04) were maintained in BUVEC as previously reported elsewhere (Muñoz-Caro et al., 2014a, 2014b), collected from supernatants as described earlier for *T. gondii*-tachyzoites, and exposed to harbour seal-PMN and -monocytes to assess ET formation thereafter.

2.4. Scanning electron microscopy (SEM)

Harbour seal-PMN and -monocytes were incubated with viable *T. gondii*- as well as *B. besnoiti*-tachyzoites (ratio: 1:1) for 10, 30, 60 and 90 min on poly-L-lysine (Sigma-Aldrich) pre-coated coverslips. Then cells were fixed in 2.5% glutaraldehyde (Merck), post-fixed in 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold particles. Specimens were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

2.5. Quantification of ETs

ET formation was quantified using Pico Green® (Invitrogen). Therefore, harbour seal-PMN and -monocytes ($n = 6$) were incubated with either tachyzoites of *T. gondii* (ratio 1:1) or *B. besnoiti* (ratio 1:1) for different time spans (30–90 min, 37 °C, 5% CO_2). To evaluate dose-dependent effects different PMN:tachyzoites ratios were used (1:0.5, 1:1, 1:2, 1:3). For positive controls, zymosan (Invitrogen) was used (1 mg/ml) according to Muñoz-Caro et al. (2014a, 2014b). To estimate maximum values of extracellular DNA, PMN and monocytes were lysed by Triton-X 100 treatment (0.1%; Sigma-Aldrich). To degrade produced ET formation, 90 U of DNase I (Roche Diagnostics) were here used as reported elsewhere (Behrendt et al., 2010). After PMN- and monocyte-parasite co-cultures, micrococcal nuclease was added (5 U/well; New England Biolabs) for 15 min at 37 °C. Afterwards samples were centrifuged (300 × g, 5 min). The supernatants were transferred (100 µl per 96-well) and Pico Green® (50 µl/well, diluted in 10 mM Tris/1 mM EDTA) was added. ET-formation was determined by spectrofluorometric analysis (484 nm excitation/520 nm emission) using an automated multi-plate reader (Varioskan Flash; Thermo Scientific).

2.6. Visualisation of ETs and detection of histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in tachyzoite-triggered harbour seal ET structures

After incubation of harbor seal PMN/monocytes with tachyzoites (ratio 1:1, 60 min) on poly-L-lysine-treated glass coverslips (Greiner) and fixation of the cell samples (4% paraformaldehyde; Merck), ET structures were visualised by staining extracellular DNA with Sytox Orange® (Invitrogen) according to Lippolis et al. (2006).

For the detection of histones, NE and MPO within ET structures the following antibodies were used: anti-histone (H3) monoclonal (DyLight, ab139848; Abcam), anti-NE (AB68672; Abcam) and anti-MPO (Alexa Fluor 488, ABIN906866; http://Antibodies-online.com). Samples were washed thrice, blocked with BSA (1%; Sigma-Aldrich) and incubated in antibody solutions (1 h, RT, for anti-histone; 24 h, RT, for anti-MPO and anti-NE antibodies). In case of harbour seal monocyte-derived ET assays, exclusively antibodies against MPO were used with the same incubation conditions as described earlier. The samples were then washed in PBS and mounted in anti-fadding buffer (Mowiol®; Sigma-Aldrich). Visualisation of ETs was achieved by using an inverted Olympus IX81 fluorescence microscope equipped with a digital camera.

2.7. Inhibition assays of NADPH oxidase, NE and MPO for investigating their role in harbour seal PMN-derived ETs

For the NADPH oxidase-, NE- and MPO-inhibition assays, the following enzyme inhibitors were used: the NADPH-oxidase inhibitor diphenylene iodonium (DPI, 10 µM, Sigma-Aldrich); the NE inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone (CMK; 1 mM, Sigma-Aldrich) according to Muñoz-Caro et al. (2014a, 2014b); the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH; 100 µM, Calbiochem) according to Parker et al. (2012). In brief, isolated harbour seal PMN and tachyzoites of *T. gondii* (1:1 ratio, $n = 6$) were incubated (60 min, 37 °C) in a HBSS-buffer without phenol red (Gibco). In parallel, seal PMN were pre-incubated with the corresponding inhibitor for 30 min at RT for subsequent exposure to viable *T. gondii*-tachyzoites (1:1 ratio, 60 min, 37 °C). Thereafter ET-formation as quantification of extracellular DNA was determined by spectrofluorometric analysis (484 nm excitation/520 nm emission) using an automated multi-plate reader (Varioskan Flash; Thermo Scientific). As positive control, zymosan (Sigma-Aldrich) was used in a final concentration of 1 mg/ml.

2.8. Store operated calcium entry (SOCE) inhibition by using 2-aminoethoxydiphenyl borate (2-APB) on harbour seal PMN exposed to Toxoplasma gondii tachyzoites

Harbour seal PMN ($n = 6$) were pre-incubated with 2-APB for 15 min in concentrations of 100 µM in HBSS medium for subsequent exposure to *T. gondii*- tachyzoites in 1:1 ratio (3×10^5 PMN:3×10^5 tachyzoites) for 60 min in duplicate according to Muñoz-Caro et al. (2015). As negative control, PMN were used in plain medium. As positive control, zymosan (Sigma-Aldrich) was used in a final concentration of 1 mg/ml. After the incubation period, ET formation was quantified based on fluorescence intensity analyses as previously explained.

2.9. Host cells invasion assay

To test the impact of parasite-induced harbour seal NETs on *T. gondii*-tachyzoite infectivity we followed the host cell invasion assay previously published elsewhere with slight modifications (Behrendt et al., 2010; Muñoz-Caro et al., 2014a, 2014b; Silva et al., 2014). In brief, two different experimental setups were chosen: (1) vital *T. gondii* tachyzoites were incubated with harbour seal PMN (2×10^5/well, 1:1 ratio, 60 min, 37 °C) allowing them for effective NET formation. (2) For comparative reasons, an equal number of tachyzoites used in the setup 1 that were not pre-exposed to harbour seal PMN were incubated in modified ECGM medium (PromoCell). Each setup was performed in duplicate. In the next step, tachyzoites of setup 1 and 2 were transferred to confluent BUVEC layers cultured in 24-well tissue culture plates (Greiner) as host cells and incubated (4 h, 37 °C, 5% CO_2 atmosphere) to allow active tachyzoite host cell invasion. Overall, two different BUVEC isolates were used in this host cell invasion experiment. After incubation, BUVEC monolayers were washed thoroughly with pre-warmed modified ECGM medium (PromoCell) to remove PMN and dead/excrescent tachyzoites. Infection rates were then estimated microscopically 24 h p.i. in six randomly selected vision power fields (400× magnification).

2.10. Statistical analysis

By using normal distribution of data, co-culture/stimulation conditions were compared by one- or two-factorial analyses of variance (ANOVA) with repeated measures. Differences were considered as significant at a level of $p \leq 0.05$.

3. Results

3.1. Toxoplasma gondii tachyzoites induce harbour seal PMN- and monocyte-derived ET formation

SEM analyses revealed that the exposure of live *T. gondii*-tachyzoites to harbour seal PMN and monocytes resulted in the formation of a delicate network resulting in outspread and diffused ETs of thicker (1.8 µm) and thinner (0.5 µm) strands of fibres originating from dead PMN and being firmly attached to the tachyzoites, seemingly trapping them (Fig. 1). Similar findings were observed for harbour seal PMN exposed to *B. besnoiti*-tachyzoites (Fig. 1B). Kinetic studies revealed different amounts of ETosis as after 10 min of exposure rather delicate PMN-derived filaroid structures being attached to tachyzoites were detected (Fig. 1A, B). Here, single PMN still exhibited the morphology of an intact cell (Fig. 1A, B). Later on, tachyzoites of *T. gondii* being entrapped in a network of long drawn-out fibres originating from disrupted harbour seal PMN (Fig. 1C; 30 min) and conglomerates of *T. gondii*-tachyzoites and rather chunky meshworks of PMN-derived ET filaments (Fig. 1C, D) were observed.

Corresponding ET structures were revealed by SEM analyses of harbour seal monocytes exposed to viable tachyzoites of *T. gondii*. In general, monocyte-derived ETs were rather thicker (1 µm) than the ones originating from PMN (data not shown). These parasite-triggered monocyte ET-like structures were proven to be composed of DNA by Sytox Orange® staining. Tachyzoites of *T. gondii* were found in close proximity of ETs and presumably entrapped within these structures. Moreover, co-localisation of extracellular DNA with H3, NE and MPO confirmed the typical structures of ETs in parasite entrapping structures (Fig. 2). Furthermore, we also observed the moment of ET expulsion of NE-granule positive PMN capturing tachyzoites in ETs being adorned with NE-positive material (Fig. 2). The histone H3-, NE- and MPO-positive signals in harbour seal ETs indicate a role of these molecules in *T. gondii* tachyzoite-triggered ETosis.

Kinetic studies quantifying harbour seal PMN-derived ET formation after parasite exposure revealed fast and strong time-dependent ET induction. Thus, ET reactions were observed already after 30 min of exposure, i.e. at the earliest time point measured in this kinetic assay. It is noteworthy to mention that values of harbour seal ET formation were significantly higher already after 60 and reaching highest levels at 90 min of exposure when compared to negative controls, indicating the strong capability of *T. gondii* tachyzoites to trigger ETosis (Fig. 3; 60 min $p \leq 0.01$; 90 min $p \leq 0.001$). *Toxoplasma gondii* tachyzoite-induced ETs were dose-dependent, as increasing the amounts of tachyzoites led to significant enhanced ET reactions (Fig. 4; $p \leq 0.05$).

3.2. Toxoplasma gondii-induced seal ETosis is diminished by DNase I-, NADPH oxidase-, NE- and MPO-inhibitor treatments

The DNA-nature of *T. gondii*-triggered ETs was additionally confirmed by DNase I treatment (Fig. 5). A significant reduction of Pico Green®-derived fluorescence intensities after the co-culture with tachyzoites was measured in DNase I-treated samples ($p \leq 0.01$). To further corroborate the classical characteristics of harbour seal ETs we performed inhibition assays of NADPH oxidase, NE and MPO by using the enzyme inhibitors DPI, CMK and ABAH, respectively. The pre-exposure of harbour seal PMN with DPI, CMK and ABAH before *T. gondii*-tachyzoite co-culture confirmed the applicability of these inhibitors in the pinniped system and led to significant inhibition of NADPH oxidase, NE and MPO enzymatic activities. To address the role of these enzymes in tachyzoite-triggered NET formation, ETosis was quantified in harbour seal PMN samples that had first been exposed to the inhibitors and thereafter to *T. gondii*-tachyzoites. As

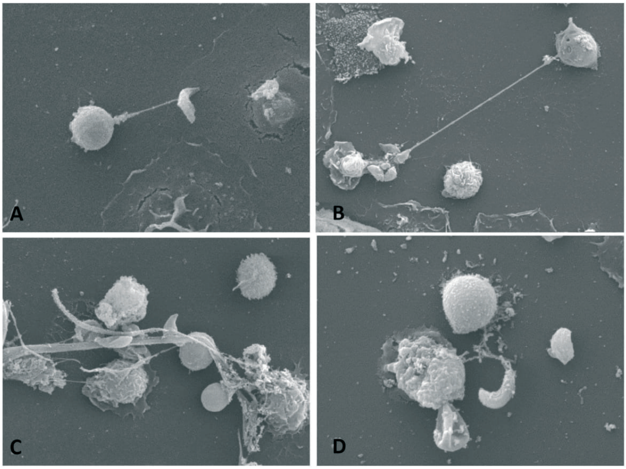

Fig. 1. ET formation by harbour seal (*Phoca vitulina*)-PMN after the exposure of vital *Toxoplasma gondii*- and *Besnoitia besnoiti*-tachyzoites. Scanning electron microscopy analysis revealed ETs being formed by harbour seal PMN co-cultured with *T. gondii* or *B. besnoiti* tachyzoites for different time points [(A, B) 10 min, (C) 30 min, (D) 60 min]. (A) Delicate PMN-derived filaroid structure being attached to a *T. gondii*-tachyzoite, (B) delicate and long PMN-derived ET-structure being attached to a *B. besnoiti*-tachyzoite, (C) several *T. gondii*-tachyzoites being captured in a network of long drawn-out fibres originating from dead PMN (D) activated and flattened PMN.

shown, a significant reduction of parasite-triggered ETosis was observed in case of enzyme inhibition (Fig. 5; $p \leq 0.05$ for NADPH oxidase; $p \leq 0.05$ for NE and MPO), highlighting the key role of all these enzymes in tachyzoite-induced ET release. In general, zymosan stimulation of harbour seal PMN was used in each experiment according to work published by Muñoz-Caro et al. (2014a, 2014b) and Silva et al. (2014), and proved to be a reliable positive control in the pinniped immune system, as zymosan stimulation revealed to be a potent inducer of ETs when compared to untreated negative controls.

3.3. Toxoplasma gondii*-induced harbour seal ET formation is a store-operated calcium entry (SOCE)-dependent process*

As previously demonstrated for other mammalian PMN, ROS production is a Ca^{++}-dependent process (Burgos et al., 2011; Conejeros et al., 2011). Since tachyzoite-triggered ETosis in seal PMN was proven to be ROS-dependent we further analysed the role of Ca^{++} and SOCE in *T. gondii* tachyzoite-triggered ET formation. Therefore, we evaluated whether the presence of the potent SOCE inhibitor 2-APB (Burgos et al., 2011; Conejeros et al., 2011; Muñoz-Caro et al., 2015) would influence *T. gondii*-mediated ETosis. The fact that addition of 100 μM 2-APB resulted in a significant reduction of *T. gondii* tachyzoite-induced NETs when compared with non-treated tachyzoite-exposed PMN ($p \leq 0.05$, Fig. 5) strongly suggests a Ca^{++}/SOCE-dependent ET-process.

3.4. Tachyzoite-triggered harbour seal ETosis is not parasite species-specific

To account for parasite-specificity in harbour seal ETs induction, we additionally tested tachyzoites of the closely related apicomplexan *B. besnoiti* as inducers of ETs. As demonstrated in Fig. 6, viable tachyzoites of *B. besnoiti* in principal were also able to induce ET formation in harbour seal PMN thereby reaching high significant differences in the strength of ET reactions ($p \leq 0.001$) when compared to negative controls (Fig. 6). Furthermore, SEM analyses illustrated the formation of fine extracellular networks of thinner and of thicker strands of fibres originating from dead PMN and being firmly attached to *B. besnoitia*-tachyzoites (Fig. 1). These data clearly argue against a parasite-specificity of ETosis in case of *P. vitulina*-PMN.

3.5. Toxoplasma gondii *tachyzoite-induced harbour seal monocyte-derived ETs*

Quantitative fluorescence intensities mirroring monocyte-derived ETs revealed that the exposure of *T. gondii*-tachyzoites with harbour seal monocytes resulted in significantly enhanced amounts of extracellular chromatin when compared to tachyzoite-free controls (see Fig. 7; $p \leq 0.001$). As shown for PMN-derived ETs in SEM analyses, monocyte-released ETs also captured efficiently *T. gondii*-tachyzoites although monocyte-derived ETs were rather thicker than PMN-triggered ETs.

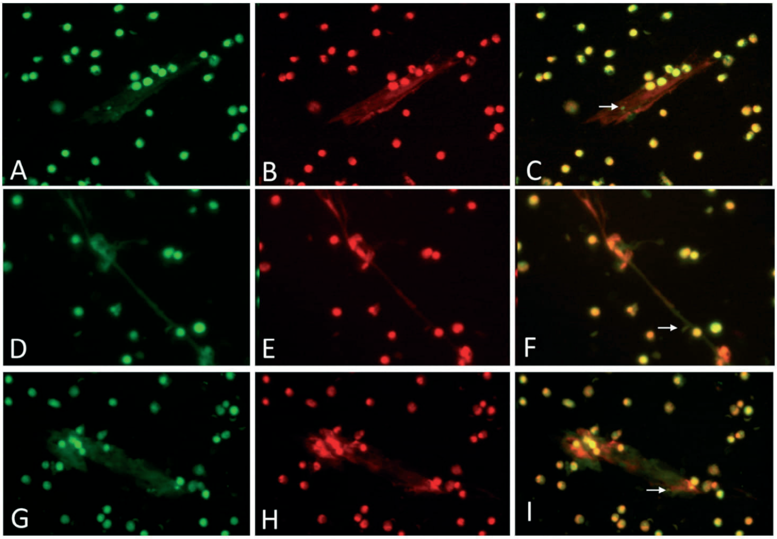

Fig. 2. Co-localisation of DNA decorated with histones (H3), NE and MPO in *Toxoplasma gondii* tachyzoite-triggered PMN-derived ET structures. Co-cultures of harbour seal (*P. vitulina*) PMN and tachyzoites of *T. gondii* were fixed and stained for DNA using Sytox Orange (red: B, E, H) and probed for NE (green: A), histones (green: D) and MPO (green: G) using anti-NE-, anti-histones (H3)- and anti-MPO-antibodies and adequate conjugate systems. Areas of respective co-localisation (merges) are illustrated in C, F and I. The arrows in C, F and I indicate tachyzoites being entrapped in ET-derived from PMN (C, F, I). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

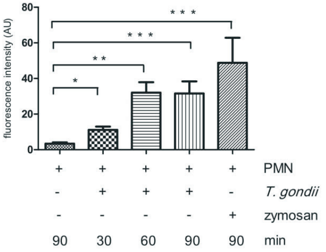

Fig. 3. Kinetics of *Toxoplasma gondii* tachyzoite-triggered ET formation in harbour seal (*Phoca vitulina*) PMN. PMN were incubated with *T. gondii*-tachyzoites (ratio 1:1; 3×10^5 PMN: 3×10^5 tachyzoites), zymosan (1 mg/ml, positive control) or plain medium (negative control) for different time points. After incubation, samples were analysed for extracellular DNA by quantifying Pico Green-derived fluorescence intensities. Each condition was performed in duplicate. Arithmetic means of six PMN donors, minimum and maximum were calculated. Differences were regarded as significant at a level of $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

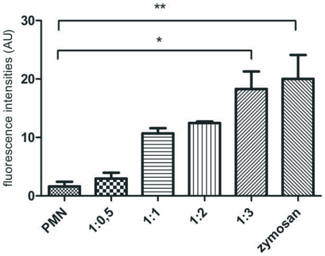

Fig. 4. Dose-dependency of *Toxoplasma gondii* tachyzoite-triggered ETs in harbour seal (*Phoca vitulina*) PMN. PMN and *T. gondii* tachyzoites were incubated at different ratios (PMN:tachyzoites = 1:0.5, 1:1, 1:2, 1:3) for 60 min at RT. After the incubation, samples were analysed for extracellular DNA by quantifying Pico Green-derived fluorescence intensities. Each condition was performed in duplicate. Arithmetic means of six donors, minimum and maximum values were calculated. Differences were regarded as significant at a level of $^*p \leq 0.05$, $^{**}p \leq 0.01$.

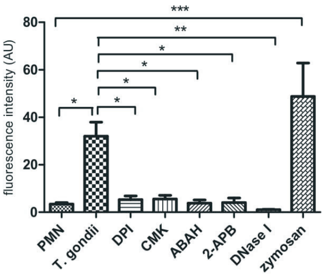

Fig. 5. Inhibition of *Toxoplasma gondii* tachyzoite-triggered ET formation in harbour seal (*Phoca vitulina*) PMN. PMN were exposed to *T. gondii* tachyzoites in the presence or absence of inhibitors and DNase I (90 U). Cells were pre-incubated with DPI (10 µM), NE inhibitor CMK (1 mM), MPO inhibitor ABAH (100 µM) and the SOCE inhibitor 2-APB (100 µM) prior to exposure to tachyzoites (1:1, 60 min, RT). After the incubation period of 60 min with tachyzoites of *T. gondii* ET formation was determined by quantifying Pico Green-derived fluorescence intensities (484 nm excitation/520 nm emission). Zymosan (1 mg/ml) was used as positive control and plain medium was used as negative control. Each condition was performed in duplicate for each PMN donor ($n = 6$). Differences were considered as significant at a level of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

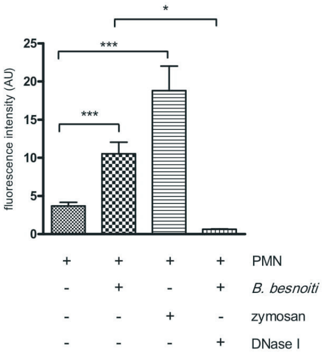

Fig. 6. Quantification of *Besnoitia besnoiti* tachyzoite-triggered ET formation in harbour seal (*Phoca vitulina*) PMN. PMN were incubated with *B. besnoiti*-tachyzoites (ratio 1:1; 3×10^5 PMN: 3×10^5 tachyzoites), zymosan (1 mg/ml, positive control) or plain medium (negative control) for 60 min. Additionally, PMN were incubated with *B. besnoiti*-tachyzoites in the presence or absence of inhibitors and DNase I (90 U). After incubation, samples were analysed for extracellular DNA by quantifying Pico Green-derived fluorescence intensities. Each condition was performed in duplicate. Arithmetic means of six PMN donors, minimum and maximum were calculated. Differences were regarded as significant at a level of $^{*}p \leq 0.05$, $^{***}p \leq 0.001$.

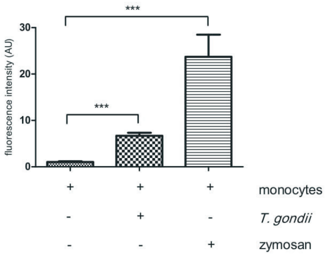

Fig. 7. Quantification of *Toxoplasma gondii* tachyzoite-triggered ET formation in harbour seal (*Phoca vitulina*) monocytes. Monocytes of harbour seals (*P. vitulina*) were incubated with *T. gondii*-tachyzoites (ratio 1:3; 1×10^5 monocytes: 3×10^5 tachyzoites), zymosan (1 mg/ml, positive control) or plain medium (negative control) for 60 min at RT. After incubation, samples were analysed for extracellular DNA by quantifying Pico Green-derived fluorescence intensities. Each condition was performed in duplicate. Arithmetic means of six PMN donors, minimum and maximum were calculated. Differences were regarded as significant at a level of $^{***}p \leq 0.001$.

Additionally, DNA-, H3- and MPO-fluorescence co-localisation assays corroborated the typical structures of monocyte-derived ETs against *T. gondii* tachyzoites (data not shown).

3.6. *Toxoplasma gondii tachyzoite-induced harbour seal ETs are able to reduce host cell invasion*

Host cell invasion is an indispensable requirement for successful survival and replication of any obligate intracellular parasite. Therefore, in order to determine the NET-triggered parasite-entrapment effects on subsequent tachyzoite infectivity, ET-exposed tachyzoites were transferred to BUVEC as host cells and infection rates estimated thereafter. In parallel equal numbers of tachyzoites that had not been exposed to PMN before were used for BUVEC infection. As shown in Fig. 8, previous encounter of *T. gondii* tachyzoites with PMN and subsequent NET formation significantly prevented active parasite host cell invasion. Thus, ET-exposed *T. gondii* tachyzoites resulted in significant reduced infection rates when compared to positive controls (non-ET exposed tachyzoites; see Fig. 8; $p \leq 0.001$).

4. Discussion

PMN and monocytes of the innate immune system represent the first line of defence and the first ones to be recruited to site of infection (Bainton et al., 1971; Borregaard and Cowland, 1997; Hahn et al., 2013; von Köckritz-Blickwede and Nizet, 2009). The harbour seal innate immune system efficiency is of critical importance to the long-term survival of these animals in order to cope with neozoans or any other emerging foreign pathogens encountering in the ocean environment. Typically considered terrestrial occurring parasites, such as *T. gondii* and *Neospora caninum*, behaved in the past decades as neozoan parasites within the marine environment (Fujii et al., 2007), and have emerged as relevant pathogens for several pinnipeds (Cabezón et al., 2011), dolphins (Dubey et al., 2008), whales (Mazzariol et al., 2012) and sea otters (Conrad et al., 2005; Miller et al., 2008). Toxoplasmosis in marine mammals is intriguing and at present indicative of contamination of the ocean environment and coastal waters with sporulated oocysts (Conrad et al., 2005; Dubey, 2009; Dubey et al., 2008).

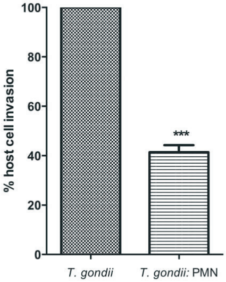

Fig. 8. Inhibition of *Toxoplasma gondii* tachyzoite host cell invasion by harbour seal (*Phoca vitulina*) PMN derived ET formation. Vital *T. gondii* tachyzoites were co-cultured with harbour seal (*P. vitulina*) PMN for 60 min (ratio 1:1, n = 3) allowing for effective NET formation. In addition, incubation of tachyzoites in plain medium served as PMN-free infection control. After incubation, samples were transferred to confluent BUVEC monolayers for 4 h incubation (37 °C, 5% CO_2 atmosphere) to allow tachyzoites for host cell invasion. Thereafter, the cell layers were thoroughly washed and infection rates were estimated after 24 h. Arithmetic means and standard deviations of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of *** $p \leq 0.001$.

Since the first description of ETs as important novel innate effector mechanism in 2004 by Brinkmann et al. (2004), it was not investigated up to this point whether ETs are released by leukocytes of marine mammals. The results of this study show that harbour seal-PMN and -monocytes strongly extrude ETs in response to tachyzoites of *T. gondii*. The data emphasise the relevance of this ancient innate effector mechanism in the defence against endogenous parasites as *T. gondii*-triggered ETs actively entrapped and immobilised tachyzoites. Thus, PMN- and monocyte-derived ET structures being firmly attached to tachyzoites and subsequently leading to parasite entrapment were visualised by SEM as well as fluorescence imaging assays. In addition, quantitative assays revealed fast and strong induction of ETs in harbour seal-PMN as well as -monocytes as recently new reported phagocyte capable of ET formation after parasite exposure (Muñoz-Caro et al., 2014a, 2014b). However, in contrast to *B. besnoiti* (Muñoz-Caro et al., 2014a, 2014b), *T. gondii* tachyzoite-triggered ETs did exhibit evident time-dependency by reaching highest levels at the end of the observation period (90 min). Time-dependent ET formation has been previously reported for *E. bovis*- and *E. arloingi*-induced reactions in bovine and caprine PMN, respectively (Behrendt et al., 2010; Silva et al., 2014).

We used zymosan for the stimulation of ETosis by harbour seal-PMN and -monocytes in positive controls as this molecule has been demonstrated before as potent activator of PMN (Conejeros et al., 2011; Muñoz-Caro et al., 2014a, 2014b; Silva et al., 2014). Overall, the stimulation of harbour seal-PMN/monocytes with zymosan turned out as reliable positive control inducing marked ET formation.

Released ETs mainly consist of DNA (Brinkmann and Zychlinsky, 2012; Brinkmann et al., 2004; Fuchs et al., 2007). Thus, we confirmed the DNA nature of tachyzoite-triggered ETs by staining with Sytox Orange®. In addition, the resolution of *T. gondii*-induced ETosis by DNase I treatments proved this typical characteristic of ETs. Alongside chromatin/DNA, the major components of ETs are nuclear histones and granular proteins such as NE, MPO, lactoferrin, pentraxin, and gelatinase (Brinkmann and Zychlinsky, 2012). These molecules are of high relevance concerning microbiocidal mechanism of ETs (Brinkmann and Zychlinsky, 2012; Fuchs et al., 2007; Urban et al., 2009). Via co-localisation analyses of extracellular DNA adorned with histones (H3), NE and MPO in tachyzoite-entrapping structures we proved the classical components of ETs. Moreover, NE- and MPO-inhibitor treatments significantly reduced ET formation in tachyzoite-exposed PMN revealing the key role of these two enzymes in *T. gondii*-induced ETs. In case of monocyte-derived ETs we also confirmed classical molecules of ETs such as DNA backbone adorned with H3 and MPO, respectively.

The process of ETs depends on the assembly/activation of the NADPH oxidase complex resulting in ROS production (Fuchs et al., 2007). As reported for several pathogens, *T. gondii*-triggered ET release also proved to be NADPH oxidase-dependent since it was significantly diminished by DPI treatment. Since SOCE was proven to play a crucial role within Ca^{++}-dependent ROS production (for review see Burgos et al., 2011) we here used with 2-APB a well-known inhibitor of SOCE for functional assays. The pre-incubation of harbour seal PMN with 2-APB prior to *T. gondii* tachyzoite exposure resulted in a significant diminishment of ETosis and therefore confirmed the assumption that parasite-triggered ET formation is Ca^{++}-(SOCE)-dependent. Consistently with these findings, *E. bovis*-triggered ETosis in bovine PMN seems to be a Ca^{++}-(SOCE)-dependent (Muñoz-Caro et al., 2015) as well as in cyclosporin A-mediated ETosis (Gupta et al., 2014). Moreover, *T. gondii* tachyzoite-induced ETs proved to be dose dependent as highest reactions were detected when applying a ratio of 3:1 (tachyzoites:PMN). Nonetheless, even lower ratios (1:1; 2:1) resulted in a strong increase of ETs when compared to negative controls.

Results of these study show that both harbour seal-PMN/monocytes strongly extrude ETs in response to *T. gondii*-tachyzoites. Interestingly, harbour seal ETosis was not parasite species-specific since tachyzoites of *B. besnoiti* equally triggered strong ET formation. Extended ETosis-associated analyses to other sporozoites of *Eimeria* spp., *Isospora suis*, *T. gondii* and *Cryptosporidium parvum* further support species-specific independent ETosis process as a general effector mechanism directed against apicomplexan parasites (Hermosilla et al., 2014; Muñoz-Caro et al., 2015). In case of *T. gondii* and also true for *B. besnoiti*, *T. gondii*-tachyzoites exhibit an enormous replication capacity, and therefore efficient intervention against this parasite stage will have an impact on the outcome of toxoplasmosis. Considering the life cycle of *T. gondii*, which includes active proliferation of tachyzoites in endothelial cells of all vessels and organs during the acute phase of infection, parasite-entrapment via ETs may be of particular importance *in vivo* since lysis of infected endothelial cells will lead to direct exposure of *T. gondii* tachyzoites to blood components, such as circulating PMN and monocytes. In this context, we here demonstrate that infection rates dropped dramatically when *T. gondii* tachyzoites were pre-exposed to harbour seal PMN prior to endothelial host cell encounter, indicating that NETs were hampering the parasites from active host cell invasion. According to this finding, other investigations using either *Eimeria*-sporozoites (Behrendt et al., 2010; Silva et al., 2014) or *B. besnoitia*-tachyzoites (Muñoz-Caro et al., 2014a, 2014b) also clearly indicated that ETosis significantly altered parasite infectivity. It is worth noting that *T. gondii*-infected endothelial cells display increased adhesion molecule gene transcription (E- and P-selectin, ICAM1, VCAM1) and enhanced PMN adhesion (Taubert et al., 2006), allowing close proximity of effector cells and parasites. Nonetheless, until now the role of monocyte- and PMN-derived ET formation in *T. gondii*-infected pinnipeds *in vivo* is not clear and will be difficult to determine. However, our data suggest parasite-induced ETs as an effective and relevant effector mechanism in harbour seal innate immune reactions directed against *T. gondii.* In summary, this is the first study which shows that marine mammals are capable of extruding ETs against pathogens and provide further evidence that this phenomenon is an ancestral effector mechanism of the innate immune system.

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VETERINARY RESEARCH

RESEARCH Open Access

Eimeria bovis-triggered neutrophil extracellular trap formation is CD11b-, ERK 1/2-, p38 MAP kinase- and SOCE-dependent

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Abstract

Eimeria bovis is an important coccidian parasite that causes high economic losses in the cattle industry. We recently showed that polymorphonuclear neutrophils (PMN) react upon *E. bovis* sporozoite exposure by neutrophil extracellular trap (NET) formation. We focused here on the molecular mechanisms that are involved in this process. The sporozoite encounter led to an enhanced surface expression of neutrophil CD11b suggesting a potential role of this receptor in *E. bovis*-mediated NETosis. Antibody-mediated blockage of CD11b confirmed this assumption and led to a significantly decreased sporozoite-triggered NET. In addition, *E. bovis*-induced NETosis was found to be Ca^{2+}-dependent since the inhibition of store-operated calcium entry (SOCE) significantly diminished NET. Furthermore, NADPH oxidase, neutrophil elastase (NE) and myeloperoxidase (MPO) were confirmed as key molecules in sporozoite-triggered NETosis, as inhibition thereof blocked parasite-triggered NET. PMN degranulation analyses revealed a significant release of matrix metalloprotease-9 containing granules upon sporozoite exposure. We further show a significantly enhanced phosphorylation of ERK1/2 and p38 MAPK in sporozoite-exposed PMN indicating a key role of this signaling pathway in *E. bovis*-mediated NETosis. Accordingly, ERK 1/2 and p38 MAPK inhibition led to a significant decrease in NET formation. Finally, we demonstrate that sporozoite-induced NETosis is neither a stage-, species-, nor host-specific process.

Introduction

Eimeria bovis is an important intracellular apicomplexan parasite of cattle, causing severe haemorrhagic diarrhoea (typhlocolitis) especially in calves leading to high economic losses worldwide [1]. Polymorphonuclear neutrophils (PMN) appear to play a pivotal role in *E. bovis* defense. This leukocyte population was identified in parasitized intestine of *E. bovis*-infected calves [2] and PMN have been shown to interact directly with *E. bovis* stages and antigen, resulting in direct elimination or production of pro-inflammatory cytokines, chemokines and iNOS upon encounter [3]. Additionally, PMN were shown to adhere to *E. bovis*-infected host endothelial cells [4] and their phagocytic and oxidative burst activities were enhanced in response to *E. bovis* sporozoites in vitro or ex vivo during infection [3]. Furthermore, we recently identified sporozoites of *E. bovis* as potent inducers of neutrophil extracellular traps (NET) [5]. Overall, NET formation has been described as a novel form of cell death called ETosis which is distinct from apoptosis, autophagy and necrosis leading to extracellular entrapment and eventually the killing of pathogens [6,7]. NET have been shown to be involved in several infections caused by bacteria, viruses and fungi [8-11]. However, little attention has been paid to the role of NET in the early host innate immune response against protozoan parasites [12,13]. By now, NET formation has been described to be induced by some protozoan parasites, such as *Leishmania* spp. [14], *E. bovis*, *Toxoplasma gondii*, *Plasmodium falciparum*, *E. arloingi*, and *Besnoitia besnoiti* [5,12,15-17].

NETosis is an NADPH oxidase-mediated ROS-dependent mechanism [7-11] which causes the expulsion of a mixture of nuclear and cytoplasmic granule contents leading to the formation of fiber-like structures being decorated with

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histones and granular molecules, such as neutrophil elastase (NE) and myeloperoxidase (MPO) amongst others [7,10,11]. On the mechanistic level, parasites are immobilized within these DNA-rich structures and may be killed via high local concentrations of antimicrobial molecules [12,13]. Consequently, some cases of parasite-triggered NETosis have been described as ROS-, NE- or MPO-dependent [5,12,16,17].

So far, few pathogen-derived molecules triggering NETosis have been identified such as bacterial toxins [18], virus [19] or surface lipophosphoglycans of *Leishmania* [14]. In addition, little data are reported on NET-associated PMN-derived ligands and Ca^{2+}-mediated signalling pathways [20]. As such, CD11b and CD18 are described as being involved in *Candida albicans* [21] or *Mannheimia haemolytica*-mediated NETosis [18]. Recently Ca^{2+}-dependent ETosis has been reported [20]. In the case of parasite-triggered NETosis, no data on PMN ligands or Ca^{2+}-dependency are available, so far. This work focuses on molecular mechanisms involved in *E. bovis*-induced NET formation. We here demonstrate that respective NETosis depends on granulocyte NE and MPO activities and on the activation of an ERK1/2- and p38-related signaling pathway. Furthermore store-operated Ca^{2+} entry and CD11b receptor binding is involved in *E. bovis*-triggered NETosis.

Materials and methods

Parasite

The *E. bovis* (strain H) used in the present study was maintained by passages in Holstein–Friesian calves for oocyst production as described by Hermosilla et al. [22]. Calves were infected orally with 5×10^4 sporulated *E. bovis* oocysts. Collection of oocysts, oocyst sporulation and excystation of sporozoites were performed as previously described [22]. Free-released sporozoites were washed three times with sterile PBS ($400 \times g$, 10 min), counted in a Neubauer haemocytometer chamber and thereafter suspended at final concentrations of $2.5 \times 10^5 – 10^6$ sporozoites/mL in culture medium (RPMI 1640, 1% penicillin/streptomycin, v/v; Gibco) free of fetal calf serum (FCS) until further experimental use. In addition, sporulated *E. arloingi* oocysts (strain A) [16], *Isospora suis* oocysts (kindly provided by Anja Joachim, University of Veterinary Medicine of Vienna, Austria) and *Toxoplasma gondii* oocysts (kindly provided by Anja Joachim, University of Veterinary Medicine of Vienna, Austria and Peter Deplazes, Faculty of Veterinary Medicine, University of Zurich, Switzerland) were used for comparative analyses on parasite species-specific NET induction. Sporozoite excystation of the latter parasite species was performed according to Pinckney et al. [23] as well as Freyre and Falcon [24].

Isolation of bovine PMN

Adult dairy cows ($n = 3$) were bled by puncture of the jugular vein and blood was collected in 50 mL plastic tubes (Nunc) containing 0.1 mL heparin (Sigma-Aldrich) as anticoagulant. Heparinized blood was diluted in an equal amount of PBS containing 0.02% EDTA (Sigma-Aldrich), layered on Biocoll Separating Solution® (Biochrom AG) and centrifuged at $800 \times g$ for 45 min. After removal of the plasma and PBMC layer, the pellet was suspended in 25 mL distilled water and gently shaken for 40 s to lyse erythrocytes. Osmolarity was immediately re-adjusted by adding 3 mL of Hanks Salt Solution (HBSS 10 x, Biochrom AG). PMN were washed twice, re-suspended in RPMI 1640 medium (Gibco), counted in a Neubauer haemocytometer chamber and subsequently incubated at 37 °C and 5% CO_2 atmosphere for at least 30 min before use.

Flow cytometric analysis of *Eimeria bovis* sporozoites-induced CD11b surface expression on bovine PMN

PMN were incubated with CFSE-stained *E. bovis* sporozoites (1:1 ratio, 30 min, 37 °C) in HBSS containing 0.9 mM $CaCl_2$. For positive controls, PMN were treated with platelet activating factor (PAF 100 nM, 15 min, 37 °C; Calbiochem). For negative controls, non-treated and non-exposed PMN were used. After incubation, cells were pelleted ($300 \times g$, 20 °C, 6 min), resuspended in 200 μL HBSS and incubated with anti-CD11b antibodies coupled to allophycocyanin (APC; clone M1/70; 20 min, in the dark; BD Pharmingen) and washed at 300 × *g*, 20 °C, 6 min. Thereafter, the cells were analyzed using a FACS Canto II cytometer (BD Biosciences, San Diego, CA, USA). The data were displayed as plots of forward versus side light scatter. The mean fluorescence of APC was determined from a minimum of 10^4 cells using BD FACS Diva 6.1 software (BD Biosciences, San Diego, CA, USA).

Inhibition of store-operated calcium entry (SOCE)

Bovine PMN were pre-incubated with the SOCE inhibitor 2-aminoethoxydiphenyl borate (2-APB) for 15 min at concentrations of 50 and 100 μM in HBSS medium prior to exposure to *E. bovis* sporozoites in a 1:2 ratio (2×10^5 PMN: 4×10^5 sporozoites, 60 min, 37 °C).

For NET quantification, 50 μL of micrococcal nuclease buffer containing 0.1 U/μL micrococcal nuclease (both New England Biolabs) were added to each well and incubated (15 min, 37 °C). Afterwards the samples were centrifuged ($300 \times g$, 5 min) and the supernatants were transferred into a 96-well flat-bottom plate (100 μL per well in duplicates). Fifty microliters of Pico Green® (1:200 dilution in 10 mM Tris base buffered with 1 mM EDTA) were added to each sample and the samples were incubated (4 min, in the dark). NET formation was determined by spectrofluorometric analysis at an excitation wavelength of 484 nm and an emission wavelength of 520 nm using an automated plate monochrome reader (Varioskan Flash®; Thermo Scientific). For negative controls, PMN in plain medium were used. For positive controls, stimulation with zymosan (1 mg/mL) was used.

Immunoblotting for the detection of phosphorylated ERK1/2 and p38 MAPK

PMN (5×10^6 in HBSS containing 0.9 mM $CaCl_2$) were exposed to *E. bovis*-sporozoites (5×10^6) for 15 and 30 min at 37 °C in a final volume of 500 µL. For positive controls, PMN were stimulated with PAF (100 nM). Thereafter, total protein was extracted as described previously by Hidalgo et al. [25] and 40 µg total protein were analyzed by a 10% SDS-PAGE. Immunoblotting was performed according to a protocol previously described by Hidalgo et al. [26]. Antibodies directed against phospho-p38 MAPK and phospho-ERK1/2 (Cell Signalling, Beverly, MA, USA) were used according to the instructions provided by the manufacturer. Anti-mouse HRP-conjugated antisera (Santa Cruz Biotechnology, USA) were used as secondary antibodies (2 h of incubation, RT, in constant agitation). Signals were detected using an enhanced chemiluminescence system (Western Lightning® Plus-ECL; Perkin-Elmer, USA). After signal detection and documentation, the bound antibodies were removed by stripping the membranes (100 mM 2-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl, pH 6.7, for 2 h at 50 °C with agitation, followed by several washes with TBS-Tween 0.1%) according to Hidalgo et al. [25] and each membrane was re-probed with an antibody recognizing total p38 MAPK (p38 MAPK anti rabbit antibody; Cell signaling technology) and ERK1/2 (rabbit polyclonal IgG; Santa Cruz Biotechnology, USA). The samples were then further processed as described above. The intensities of each band were analyzed using the Software Image J and the signals were normalized to total ERK1/2 for p-ERK1/2 and to total p38 for p-p38.

NET inhibition assays using ERK1/2, p38 MAPK inhibitors and functional CD11b monoclonal antibodies

For the inhibition of ERK 1/2 and p38 MAPK the following inhibitors were used respectively: UO126 (50 µM; Sigma-Aldrich) and SB 203580 (10 µM; Sigma-Aldrich). Therefore, PMN were preincubated with the inhibitors for 30 min at RT in HBSS-buffer without phenol red (Gibco). CD11b was blocked via pre-incubation in mouse anti bovine CD11b monoclonal antibodies (MCA1425, diluted 1:5 in PBS; AbD Serotec). For antibody control, we used an irrelevant antibody at an identical concentration (anti-bovine CD4, AbD Serotec). Then, PMN were exposed to viable *E. bovis*-sporozoites (1:2 ratio: 2×10^5 PMN + 4×10^5 sporozoites) for 60 min at 37 °C. Thereafter extracellular DNA was quantified as described above. All measurements were achieved using an automated monochrome reader (Varioskan Flash; Thermo Scientific). For positive controls, zymosan (Sigma-Aldrich) was used in a final concentration of 0.5 mg/mL.

Visualization of NET and detection of histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in NET structures

Bovine PMN were incubated with *E. bovis* sporozoites (ratio 1:1; 30 min) on poly-$_L$-lysine-treated coverslips and fixed [4% (w/v) paraformaldehyde, Merck, 20 min in the dark]. NET structures were visualized by staining extracellular DNA with 5 mM Sytox Orange dye (Invitrogen) for 10 min at RT according to Martinelli et al. [27]. For the visualization of sporozoites within NET structures, sporozoites were stained with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, 7.5 µM, 37 °C, 30 min; Invitrogen) according to Hermosilla et al. [28] prior to PMN exposure. After fixation of the cells and three washings in sterile PBS, the samples were mounted in anti-fading buffer (Mowiol®, Sigma-Aldrich) and stored (4 °C, in the dark) until further use. For the detection of histones, MPO and NE within NET structures the following specific antibodies were used: anti-histone monoclonal antibodies [rabbit (E173) anti-bovine histone H3, phospho S10 DyLight® 488, 1:100; ab139848, Abcam], anti-MPO antibodies (rabbit anti-bovine MPO, Alexa Fluor 488, 1:200; ABIN906866) and anti-NE antibodies (rabbit anti-human NE, 1:200; AB68672, Abcam). Therefore, the samples were washed three times with PBS, blocked with BSA [1% (w/v) in PBS, 30 min, RT, Sigma-Aldrich] and reacted with anti-histone, anti-NE or anti-MPO antibodies [1 h, RT, in the dark for bovine anti-histone (H3); 24 h, RT, in the dark for both anti-MPO and anti-NE antibodies]. The samples were then gently washed in PBS and mounted in anti-fading buffer (Mowiol®, Sigma-Aldrich). Visualization was achieved using an inverted Olympus® IX81 fluorescence microscope equipped with a digital camera.

Measurements of NADPH oxidase, NE and MPO enzymatic activities and inhibition of these enzyme activities

For NADPH oxidase-, NE- and MPO-inhibition assays, the following inhibitors were used: the NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 µM, Sigma-Aldrich); the NE inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone (CMK; 1 mM, Sigma-Aldrich), according to Scapinello et al. [29] and the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH; 100 µM, Calbiochem), according to Parker et al. [30]. In brief, PMN and sporozoites (1:1 ratio, $n = 3$) were incubated (30 min, 37 °C) in HBSS-buffer without phenol red (Gibco) for positive controls. In parallel, PMN were pre-incubated with the corresponding inhibitors for 30 min at RT prior to exposure to viable *E. bovis*-sporozoites (1:1 ratio, 30 min, 37 °C). To estimate maximum values of extracellular DNA, PMN were lysed by Triton-X 100 treatment (0.1%; Sigma-Aldrich). To block NET formation, 90 U of DNase I (Roche Diagnostics) were supplemented 15 min before the end of incubation period. NET were quantified via Pico Green®-DNA staining as described above.

NADPH oxidase, NE and MPO enzymatic activities were measured using respective substrates: 10 µg/mL DCFH-DA (Sigma-Aldrich); 3 mg/mL of the NE chromogenic substrate MeoSuc-Ala-Ala-Pro-Val-chloromethyl-ketone (Sigma-Aldrich) and 50 µM Amplex red (Invitrogen), respectively. ROS production was measured by oxidation of DCFH-DA to fluorescent DCF according to Conejeros et al. [31]. The relative fluorescence units (RFU) were recorded at 15 min intervals for a period of 30 min applying 485 nm excitation and 530 nm emission wavelengths. NE activity was measured every 10 min for 30 min at 410 nm wavelength and MPO-peroxidase activity was measured every 10 min for 30 min in 571-585 nm fluorescence ranges. All measurements were achieved using an automated monochrome reader (Varioskan Flash; Thermo Scientific). As positive control, zymosan (Sigma-Aldrich) was used at a final concentration of 0.5 mg/mL.

Determination of matrix metalloproteinase 9 (MMP-9) activities in PMN supernatants

PMN (10^6/500 µL HBSS/0.9 mM $CaCl_2$) were exposed to equal numbers of *E. bovis* sporozoites (15 and 30 min, 37 °C). Stimulation of PMN with PAF (100 nM, 5 min, 37 °C) was used for positive controls. PMN cultivated in plain medium were used for negative controls. After incubation, the cells were centrifuged (600 × *g*, 6 min) and the supernatants were assayed for gelatinase activity by zymography. Therefore, substrate gel electrophoresis was performed using the method described by Conejeros et al. [32]. Briefly, 10 µL of supernatant/slot were loaded on polyacrylamide gels (10%, 0.75 mm thickness) containing 0.28% of gelatin (Sigma-Aldrich). In parallel a recombinant MMP-9 standard (Sigma-Aldrich) and a molecular mass marker (Fermentas International Inc., Canada) were loaded as reference samples. The gels were run at 200 V for 1 h in a Bio-Rad Mini Protean II chamber (Bio-Rad Laboratories, CA, USA). Thereafter, the gels were incubated twice in Triton X-100 (2.5%, under constant shaking, RT, 30 min) and overnight at 37 °C in reaction buffer (100 mM Tris, pH 7.5; 10 mM $CaCl_2$). The gels were stained in Coomassie Brilliant Blue R-250 (Winkler, Santiago, Chile; 0.5% in acetic acid : methanol : water = 1 : 3 : 6). MMP-9 (gelatinase B) enzymatic activity present in the test samples were determined according to the degree of gelatin degradation (visible as clear bands of 82 kDa) relative to the MMP-9-control by means of band intensity measurements (applying ImageJ 1.35 s software).

Host cell infection and production of viable *Eimeria bovis* merozoites I

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated from umbilical cord veins, according to the method of Hermosilla et al. [4]. Confluent BUVEC monolayers ($n = 3$) were infected with 2.5×10^5 freshly excysted *E. bovis*-sporozoites suspended in modified ECGM [ECGM (PromoCell) supplemented with 70% (v/v) M199 (Gibco), 2% FCS (Gibco) and 1% penicillin (Sigma-Aldrich)] per 25 cm^2 flask (Greiner). The cells were fed 24 h after infection and thereafter every third day with modified ECGM. To control for *E. bovis* macromeront development and the release of merozoites I, infected BUVEC cultures were analyzed daily over a period of 22 days using a phase-contrast inverted microscope (IX81 microscope®, Olympus). Free-released merozoites I were harvested from the supernatant of infected BUVEC monolayers (400 × *g*, 5 min) and washed twice in PBS. The merozoites I were counted in a Neubauer chamber haemocytometer.

Scanning electron microscopy (SEM)

Bovine PMN were incubated with *E. bovis* sporozoites, merozoites I or oocysts at a ratio of 1:1 for 90 min on poly-$_L$-lysine (Sigma-Aldrich) pre-coated coverslips. After incubation, the samples were fixed (2.5% glutaraldehyde in 0.1 M cacodylate buffer, 15 min, all Merck) and washed with 0.1 M cacodylate buffer. The samples were post-fixed in 1% osmium tetroxide (Merck) in 0.1 M cacodylate buffer, washed three times in distilled water, dehydrated in ascending ethanol concentrations, critical point dried by CO_2-treatment and sputtered with gold. Specimens were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

Quantification of NET derived from PMN and parasites from different origins

NET formation was quantified using Pico Green® (Invitrogen), a DNA-binding dye. In order to quantify *E. bovis* sporozoite- or merozoite I-induced NET formation, bovine PMN ($n = 3$-5) were re-suspended in serum-free medium RPMI 1640 and incubated (all at 37 °C) with *E. bovis*-sporozoites in a 1:2 ratio (2×10^5 PMN: 4×10^5 sporozoites, 60 min), with *E. bovis*-merozoites I in a 1:1 ratio (1×10^5 PMN: 1×10^5 merozoites, 90 min), with oocysts (5×10^4, 60 min, 1:1) and with sporozoites of *E. arloingi* ($n = 3$; 1×10^5 PMN: 1×10^5 sporozoites, 60 min), *Isospora suis* ($n = 3$; 1×10^5 PMN: 1×10^5 sporozoites; 60 min) and *T. gondii* ($n = 5$; 1×10^5 PMN: 1×10^5 sporozoites; 60 min). In addition, PMN isolated from different host species origin (caprine, horse and dog) were incubated with *E. bovis* sporozoites in a 1:2 ratio (2×10^5 PMN: 4×10^5 sporozoites, 60 min at 37 °C). For positive controls, zymosan was used at a final concentration of 0.5 mg/mL. Plain RPMI 1640 medium served as negative controls. NET-formation was determined based on Pico Green®-DNA staining as described above.

Eimeria bovis sporozoite entrapment assay

For the quantification of sporozoite entrapment within NET structures we followed the method described by Köckritz-Blickwede et al. [8] with slight modifications. Briefly, PMN (2.5×10^5/96-well) were pre-activated by zymosan treatment (30 min, 37 °C). Meanwhile, *E. bovis*-sporozoites were stained with FITC dye (0.2 mg/mL, 30 min, on ice, in the dark, Invitrogen) and washed twice in PBS ($3000 \times g$, 10 min). Thereafter, zymosan-stimulated PMN were exposed to FITC-labeled *E. bovis* sporozoites ($512 \times g$, 10 min; subsequent incubation for 30 min at 37 °C) in ascendant ratios (0.5:1;1:1 and 1:2). Non-exposed sporozoites were used for controls (1:1; 1:2). The samples were washed twice in RPMI 1640 medium and measured for fluorescence intensity at 485/538 nm wavelength. The percentage of entrapment was calculated as follows: [(A485/538 nm sporozoites exposed to PMN)/(A485/538 nm non-exposed sporozoites)] × 100%.

Ethics statement

All animal procedures were performed according to the Justus Liebig University Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the State of Hesse (Regierungspräsidium Giessen) and in accordance to the current German Animal Protection Laws. Identification number of animal care and project licence: GI 18/10-Nr.A51/2012 544_AZ952 (*Eimeria bovis*-oocyst production); GI 18/10-Nr.A9/2012 521_AZ877 (Bovine blood samples). European Animal Welfare Legislation: ART13TFEU.

Statistical analysis

Co-culture/stimulation conditions were compared by one-or two-factorial analyses of variance (ANOVA) with repeated measurements in order to compare co-culture/stimulation conditions. All analyses were performed with the GraphPad Prism® 6 software. Differences were regarded as significant at a level of $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).

Results

Exposure to *E. bovis* sporozoites up-regulates neutrophil CD11b surface expression on bovine PMN and triggers NET formation in a CD11b-dependent manner

Since no data are available on PMN-derived receptors being involved in *E. bovis*-mediated NETosis, we here analyzed whether sporozoite exposure would affect CD11b

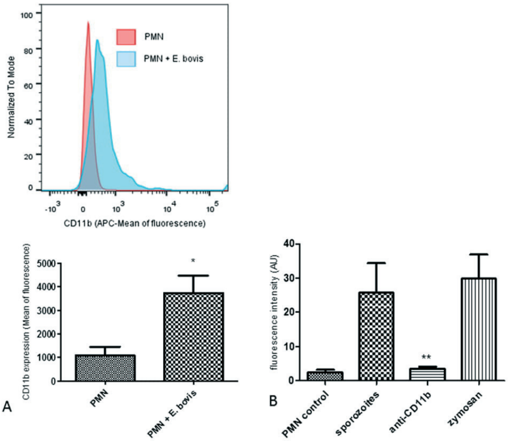

Figure 1 CD11b surface expression and CD11b-dependent-NET formation of bovine *E. bovis* sporozoite-exposed PMN. (A) Bovine PMN were exposed to CFSE-labeled sporozoites (1:1) and subjected to FACS analysis probing with an antibody directed against CD11b. Non-exposed PMN in plain medium served as negative controls. The histograms represent the shift in the mean fluorescence of the PMN population analyzed and are representative of the experiments performed. **(B)** NET formation in the presence of anti-CD11b antibodies. Bovine PMN were pre-treated with anti-CD11b prior to sporozoite exposure. Stimulation with zymosan served as a positive control; PMN in plain medium were used for negative controls.

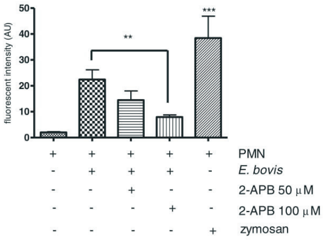

Figure 2 Influence of store-operated calcium entry (SOCE) on *E. bovis* sporozoites-induced NET formation. PMN were treated with the SOCE-inhibitor 2-APB prior to exposure to *E. bovis* sporozoites. For negative controls, non-treated PMN were used. For positive controls, zymosan stimulation was used. NET formation was quantified based on DNA-derived fluorescence intensities using an automate plate reader.

surface expression of bovine PMN. Applying FACS methodology, a significant parasite-triggered increase of CD11b surface expression was shown when compared to non-exposed PMN ($p \leq 0.05$, Figure 1A). In addition, blockage of CD11b via specific antibodies led to a significant reduction in sporozoite-mediated NET formation when compared to antibody-free controls ($p \leq 0.01$; Figure 1B). We also did not observe any significant NET induction using our irrelevant antibody control. Thus, these results suggest CD11b as a potential candidate for neutrophil receptors being involved in NET formation.

Eimeria bovis-induced NET formation is store-operated calcium entry (SOCE)-dependent

As previously shown in the bovine system, ROS production is a Ca^{2+}-dependent process [32]. Since sporozoite-triggered NETosis was recently proven to be ROS-dependent [5], we here analyzed whether the presence of the Ca^{2+} (SOCE) inhibitor 2-APB [31,32] would influence sporozoite-mediated NETosis. The fact that treatment with 100 µM 2-APB resulted in a significant reduction of *E. bovis* sporozoite-induced NET when compared with non-treated but sporozoite-exposed PMN ($p \leq 0.01$, Figure 2), suggests a Ca^{2+}/SOCE-dependent process. Overall, stimulation of PMN with zymosan revealed to be a potent SOCE inducer compared to the negative control ($p \leq 0.001$; Figure 2).

Sporozoite-induced NETosis is a ROS-, NE- MPO-dependent process

Experiments applying specific antibodies against H3, NE and MPO alongside with DNA-staining proved NET structures (estimated via extracellular DNA-positive fibres) as co-localizing with histone H3-, NE- and MPO-positive signals indicated a pivotal role of these molecules in

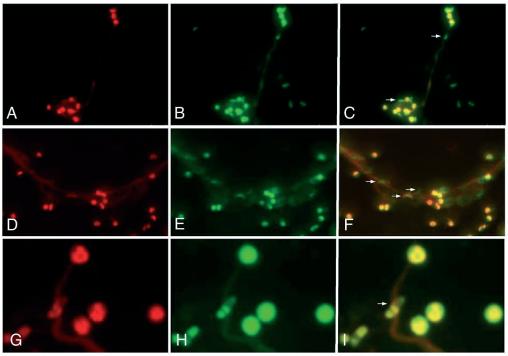

Figure 3 Co-localization of DNA with histones (H3), neutrophil elastase (NE) and myeloperxodase (MPO) in *E. bovis* sporozoite-induced NET structures. Co-cultures of bovine PMN and *E. bovis* sporozoites and oocysts were fixed, permeabilized and stained for DNA using Sytox Orange (red: **A**, **D**, **G**) and probed for histone 3 (H3) (green: **B**), MPO (green: **E**) and NE (green: **H**) using anti-H3, anti-NE and anti-MPO antibodies jointly with adequate conjugate systems. Areas of respective co-localization (merges) are illustrated in **C**, **F**, **I**. Photomicrographs are of representative cells from 3 independent experiments.

sporozoite-triggered NETosis and confirmed classical NET characteristics (Figure 3). In addition, PMN exposure to *E. bovis* sporozoites resulted in a significant up-regulation of NADPH oxidase-, NE- and MPO- enzymatic activities ($p \le 0.05$ for NADPH oxidase; $p \le 0.01$ for NE and MPO; Figures 4A-C). Furthermore, we confirmed the key role of these enzymes since inhibition of NADPH oxidase, NE and MPO led to significantly reduced sporozoite-triggered NETosis when compared to non-treated controls (Figure 4D; DPI and ABAH: $p \le 0.05$; CMK: $p \le 0.01$). As expected, DNase I treatments led to a significant NET resolution ($p \le 0.001$, Figure 4D). Total DNA release from PMN was controlled by Triton X treatments revealing a proportion of up to 43% of PMN to be involved in sporozoite-mediated NETosis. Overall, these data confirm the relevance of NADPH oxidase, NE and MPO in *E. bovis* sporozoite-induced NET formation.

E. bovis sporozoite exposure enhances matrix metalloprotease 9 (MMP-9) release of bovine PMN

Given that tertiary granule contents of PMN also contribute to NET formation based on their antimicrobial peptide/protease activities as described by Brinkmann et al. [6], we additionally analyzed PMN supernatants for the enzymatic activity of MMP-9. Co-cultures of PMN with *E. bovis* sporozoites led to a significant increase of MMP-9 release ($p \le 0.05$; Figure 5) at both time points tested (15 and 30 min) indicating that tertiary granules or MMP-9 may also be involved in sporozoite-triggered NETosis. As expected, stimulation with PAF revealed to be a reliable positive control for MMP-9 release in the bovine system ($p \le 0.05$).

ERK1/2- and p38 MAPK-signaling pathways are involved in *E. bovis* sporozoite-triggered NET extrusion

We analyzed whether the ERK1/2 and p38 MAPK signaling pathway is involved in sporozoite-triggered NETosis. Exposure of *E. bovis* sporozoites to PMN resulted in a fast and significant phosphorylation of ERK1/2 ($p \le 0.05$ after 15 and 30 min of incubation, Figure 6A) and p38 ($p \le 0.05$ at 15 min, Figure 6B) MAPK. Furthermore, experiments applying inhibitors of ERK1/2 and p38 MAPK confirmed the relevance of this signaling pathway since treatments with both inhibitors resulted in a significant reduction of sporozoite-mediated NET formation (UO126: $p \le 0.01$ and SB 203580: $p \le 0.05$, Figure 6C).

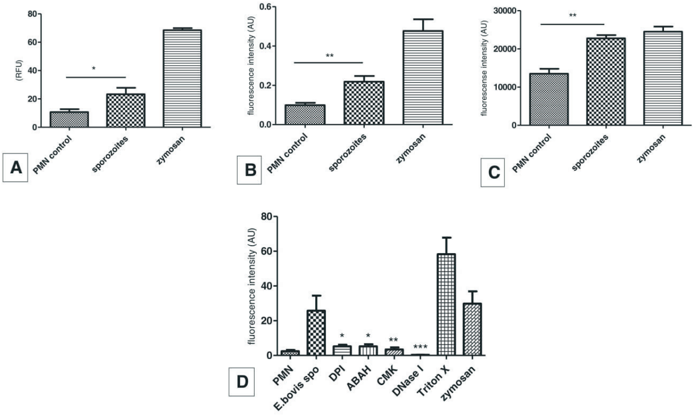

Figure 4 ***E. bovis*** **sporozoite-induced NETosis in bovine PMN is a NADPH oxidase-, NE- MPO-dependent process. (A-C)** NADPH oxidase, NE and MPO enzymatic activities in *E. bovis* sporozoite-exposed PMN. PMN were exposed to *E. bovis* sporozoites and enzymatic activities of NADPH oxidase **(A)**, NE **(B)** and MPO **(C)** were measured using via DCFH-DA oxidation, NE-chromogenic substrate analysis and using Amplex red reagent, respectively. **(D)** Sporozoite-triggered NET formation after inhibition of NADPH oxidase (via DPI), NE (via CMK) and MPO (via ABAH). For inhibition and maximum DNA release controls, DNase I and Triton X treatments were used, respectively. Stimulation with zymosan served as a positive control (1 mg/mL) and PMN in plain medium as a negative control.

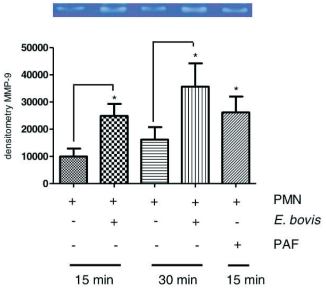

Figure 5 Release of MMP-9 from PMN granule contents after sporozoite-exposure. Bovine PMN were exposed to *E. bovis* sporozoites and the supernatants were analyzed for MMP-9 activities via zymography in gelatin-containing polyacrylamide gels and consecutive densitometric estimations. Recombinant MMP-9 was used as an internal standard. Stimulation of PMN with PAF (100 nM) served as a positive control. Each bar represents arithmetic means ± SEM of x PMN donors.

Sporozoite-triggered NETosis is neither stage- nor parasite species- nor host-specific

To account for stage-specificity we tested *E. bovis* merozoites I for the capability to induce NET. As depicted in Figure 7C, merozoite I stages significantly triggered NET when compared to non-exposed controls ($p \le 0.01$). These data were confirmed by SEM analyses illustrating the formation of a delicate, NET-like network of thicker and thinner strands of fibres originating from dead PMN and being firmly attached to the merozoites I (Figure 7A). In addition, SEM analyses indicated NET-like structures also to be induced by oocyst stages (Figure 7B). These data clearly argue against a stage-specificity of *E. bovis*-triggered NETosis highlighting the capability of PMN to equally respond to different parasitic stages of this parasite.

In order to analyze whether *E. bovis*-triggered NETosis is a parasite-specific event or rather reflects a general mechanism that commonly accounts for most coccidian parasites, we analyzed NET formation of bovine PMN after exposure to the sporozoite stage of a non-bovine *Eimeria* spp. (the caprine-specific species *E. arloingi*) and of two non-*Eimeria* coccidian species (*T. gondii* and *I. suis*). The data clearly show that the sporozoites of all three parasite species equally induced significant NET formation (*E. arloingi* and *T. gondii*: $p \le 0.01$, *I. suis*: $p \le 0.05$, Figure 8B). Overall, these findings rather argue against a parasite-specific mechanism and propose NET formation as a generally valid effector mechanism against the sporozoite stage of different coccidian species.

Given that *E. bovis* is a strict host-specific parasite and, in consequence, exclusively infects bovines, we then analyzed whether *E. bovis*-mediated NET formation is a host-specific event. Therefore, PMN of different host origins (horse, goat, dog and cattle) were controlled for NET formation after exposure to *E. bovis* sporozoites. The data clearly revealed that PMN of all host types reacted by significant NET formation ($p \le 0.01$, Figure 8A) which argues against a host-specific reaction.

NET structures significantly entrap *Eimeria bovis* sporozoites

In order to analyze the capacity of NET to trap viable *E. bovis* sporozoites, we established a quantitative parasite-entrapment-assay by using FITC-stained parasites and zymosan pre-activated bovine PMN as described elsewhere [17]. The experiments revealed a proportion of 43.4% of sporozoites to be immobilized in NET structures when compared to non-exposed sporozoites (data not shown). These results indicate a rather high efficacy of NET as an effector mechanism considering that almost every second parasite was entrapped and most probably hampered from host cell invasion as reported elsewhere [5].

Discussion

NET formation depends on the assembly/activation of the NADPH oxidase complex and the resulting production of ROS [7]. Since these mechanisms were reported as Ca^{2+}-mediated in the bovine system [31], it appears likely that NETosis is a Ca^{2+}-dependent process, as recently demonstrated by Gupta et al. [20]. The fact that NET formation is induced by thapsigargin [33] which mobilizes Ca^{2+} from intracellular pools, also indicates a key role of Ca^{2+} in NETosis. Since SOCE was proven to play a crucial role in Ca^{2+}-dependent ROS production [34] we here used 2-APB a well-known inhibitor of SOCE for functional assays. The pre-incubation of PMN with 2-APB prior to sporozoite exposure resulted in a significant diminishment of NETosis and therefore confirmed the assumption that parasite-triggered NET formation is a Ca^{2+}-(SOCE)-dependent process.

Besides being SOCE-dependent, ROS production is up-regulated in a CD11b (CR3)-dependent manner in bovine PMN [31]. In turn, up-regulation of CD11b proves as SOCE-dependent since it is inhibited by 2-APB [32] leading us to the assumption that the neutrophil integrin receptor CD11b may also be involved in sporozoite-mediated NETosis. Indeed, we found significantly enhanced levels of surface CD11b expression on parasite-exposed bovine PMN. The fact that antibody-mediated blockage of CD11b additionally resulted in NET diminishment suggests CD11b to be involved in sporozoite-triggered NETosis and to function as a PMN-derived receptor in this effector mechanism. These findings were in accordance

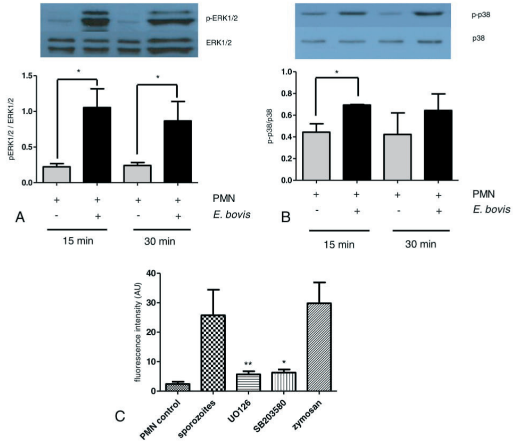

Figure 6 Expression of phosphorylated ERK1/2- and p38 MAPK in sporozoite-exposed PMN and influence of their inhibition on parasite-triggered NETosis. Total proteins of sporozoite-exposed PMN and non-exposed controls were analyzed by SDS/PAGE and immunoblot technique using specific antibodies against non-phosphorylated and phosphorylated ERK1/2 **(A)** and p38 MAPK **(B)**. Respective bands were densitometrically analyzed. Each bar represents the arithmetic means ± SEM of three PMN donors. Differences were regarded as significant at a level of $p \leq 0.05$ compared to negative controls. **(C)** NET formation in presence of ERK 1/2 (UO126) and p38 (SB 203580) MAPK inhibitors. Stimulation with zymosan served as a positive control; PMN in plain medium as a negative control.

with Byrd et al. [21] who reported on CD11b as a potential ligand in NETosis since antibody-mediated blockage of this integrin inhibits fibronectin-dependent NET formation to *C. albicans* hyphae.

Moreover, SOCE is tightly regulated via ERK1/2 MAPK phophorylation, i.e., by a signaling pathway that was recently described to be involved in NETosis [35]. In addition, ROS-dependent activation of ERK and p38 MAPK was demonstrated to mediate PMA-induced NET release from human neutrophils [35]. In accordance to the latter reports and to data on other pathogens [12,21] we here show a sporozoite-triggered up-regulation of ERK1/2 and p38 MAPK phosphorylation in parasite-exposed bovine PMN. The key role of the ERK1/2- and p38 MAPK-dependent signaling pathway in NETosis was confirmed in functional inhibition experiments showing that parasite-triggered NETosis was significantly diminished in the presence of ERK-and p38 inhibitors.

We furthermore focused on the relevance of NE and MPO and confirmed the key role of these two molecules in *E. bovis*-triggered NETosis. As with other bovine pathogens [18], exposure to sporozoites led to an increase of both, NE and MPO enzymatic activities in bovine PMN. Functional inhibition assays confirmed the relevance of these enzymes since parasite-mediated NETosis was significantly blocked when NE and MPO inhibitors were applied. Finally, the co-localization of DNA-rich filaments with NE- or MPO-derived signals in sporozoite-induced NET structures emphasized the classical characteristics of NET. These results were in line with recent findings on other coccidian parasites, such as *E. arloingi* [16] or *B. besnoiti* [17].

Whilst NE and MPO originate from azurophilic PMN granules, other molecules such as metalloproteinases (MMP), are contained in tertiary (gelatinase) granules. We here analyzed whether MMP-9 is released from PMN upon sporozoite exposure. Zymographic analyses of PMN supernatants confirmed a parasite-mediated MMP-9-release into PMN supernatants. So far, the functional role of MMP-9 in NETosis is unclear. However, it is worth noting that the release of MMP-9 is regulated by the ERK 1/2 and p38 MAPK signaling pathway [36]. Interestingly, Carmona Rivera et al. [37] showed an impaired endothelial function induced by NET-externalized

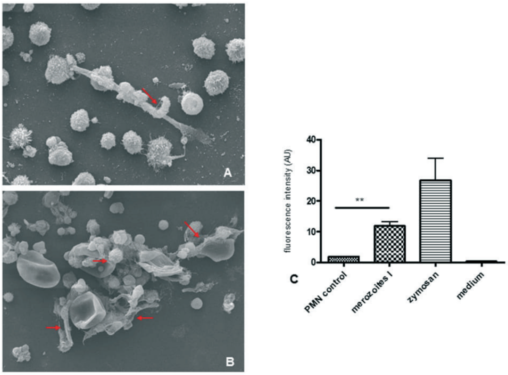

Figure 7 NET formation triggered by *E. bovis* merozoites I and oocyst stages. (A) Scanning electron microscopy (SEM) analysis of *E. bovis* merozoite I-exposed PMN. The arrow indicates a merozoite I being trapped in NET structures**. (B)** SEM analysis of *E. bovis* oocyst-exposed PMN revealing NET-like, delicate PMN-derived filaroid structures being attached to oocysts as indicated by arrows. **(C)** Quantification of *E. bovis* merozoite I-induced NET formation. PMN were exposed to *E. bovis* merozoites I, zymosan (positive control) or plain medium (negative control). NET formation was estimated by Pico Green-derived fluorescence intensities. Arithmetic means of three PMN donors, minimum and maximum.

MMP. Given that *E. bovis* sporozoites infect and mature within endothelial cells, a yet unclear interrelation between NETosis and parasite development within endothelium may exist.

Overall, since the first description of NET as an innate effector mechanism [6], most studies have focused on NETosis driven by bacterial, viral and fungal pathogens and, less frequently, on parasitic pathogens [13]. Nonetheless, there is increasing evidence on the relevance of NET as a defense mechanism against protozoan infections in vitro and in vivo [12,13]. Thus, NET-related data are available on some protozoan pathogens, such as *E. bovis, Toxoplasma gondii, Plasmodium falciparum, E. arloingi,* and *B. besnoiti* [5,12,15-17] and *Leishmania spp.* [14].

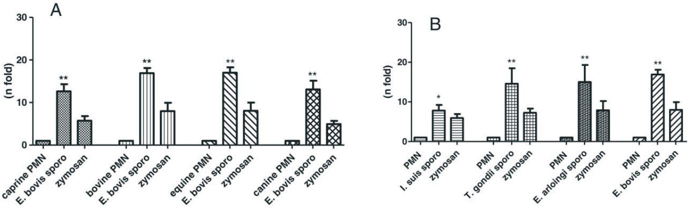

Figure 8 *E. bovis*-sporozoite triggered NETosis of PMN of different host origins and NET formation induced by different coccidian sporozoites. (A) PMN of caprine, bovine, equine and canine origin were exposed to *E. bovis* sporozoites and respective NET formation was measured according to Pico Green-derived fluorescence intensities. **(B)** NET formation of bovine PMN in response to sporozoites of different coccidian species (*I. suis*, *T. gondii*, *E. arloingi* and *E. bovis*). In both experiments, stimulation with zymosan served as positive controls and PMN in plain medium were used for negative controls. Arithmetic means of three PMN donors, minimum and maximum.

The current data suggest NETosis as a generally valid effector mechanism against coccidian parasites. Thus, NET release occurred irrespective of the parasite stage (*E. bovis* sporozoites, merozoites I, oocysts), and PMN origin (bovine, caprine, equine, canine). In agreement, data on different *Leishmania* stages [14] also indicated NETosis as a stage-independent defense mechanism. In addition, *T. gondii*-triggered NETosis has been reported for both human and murine PMN [12] indicating host-independent reactions. Overall, it is noteworthy that the degree of entrapment clearly differed amongst different parasite species and stages. Thus, we here report on 43% of *E. bovis* sporozoites to be immobilized within bovine NET structures, whilst in the caprine system even 72% of *E. arloingi* sporozoites were trapped [16] but only 34% of *B. besnoiti* tachyzoites were found ensnared in NET being expelled from bovine PMN [17]. Since all these parasites exhibit an obligatory intracellular replication, extracellular immobilization via NET will have a tremendous implication on the outcome of the disease as previously postulated [16,17].

Furthermore, NETosis was not parasite species-specific since sporozoites of different coccidian parasites (*E. bovis*, *E. arloingi*, *I. suis*, *T. gondii*) equally triggered NET formation. To our best knowledge this is the first report on *T. gondii* and *I. suis* sporozoite-induced NET extrusion whilst tachyzoite stages of *T. gondii* [12] and sporozoites of *E. arloingi* [16] have already been reported before. Especially in the case of *T. gondii*, which exhibits an enormous proliferation at the tachyzoite stage, the intervention of the immune system at a very early time point, i.e. when sporozoites invade the host system and before non-sexual parasite replication occurs, will have a higher impact on the outcome of disease than tachyzoite-triggered reactions. Taking into account that PMN have been demonstrated to actively transmigrate into the intestinal lumen and were found alive in the gut mucus [38], it seems feasible to assume that they may interact with luminal pathogen stages, such as ingested *Eimeria* oocysts, in vivo. Consistently, we here show that oocysts of *E. bovis* are trapped and sometimes almost encaged by NET structures in vitro, thereby most probably preventing proper excystation of sporozoites. This novel anti-excystation mechanism of NET has recently been hypothesized for *E. arloingi* oocysts, in which sporozoites were impeded to escape from NET-covered preformed excystation sites (micropyles) of sporulated oocysts [16]. Thus, in the in vivo situation, luminal occurring NETosis might intervene very early after oral infection with the parasite and abrogate infection even before infectious stages (sporozoites) are able to evade immune reactions via intracellular positioning.

In summary, NET formation seems to be an ancient and highly conserved host effector mechanism of PMN acting against several pathogens as an early host immune reaction. In the present work, we added new data on the molecular mechanisms involved in parasite-triggered NETosis and call for more investigations on receptor-ligand-interactions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: TMC, JMH, IC, PA, RAB, MAH, CH, AT. Performed the experiments: TMC, JMH, IC, PA. Analyzed the data: TMC, JMH, IC, PA, RAB, CH, AT. Contributed reagents/materials/analysis tools: TMC, JMH, IC, PA, RAB, MAH, CH, AT. Wrote the paper: TMC, CH, AT. All authors read and approved the final manuscript.

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NADPH oxidase, MPO, NE, ERK1/2, p38 MAPK and Ca^{2+} influx are essential for *Cryptosporidium parvum*-induced NET formation

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ABSTRACT

Cryptosporidium parvum causes a zoonotic infection with worldwide distribution. Besides humans, cryptosporidiosis affects a wide range of animals leading to significant economic losses due to severe enteritis in neonatal livestock. Neutrophil extracellular trap (NET) formation has been demonstrated as an important host effector mechanism of PMN acting against several invading pathogens. In the present study, *C. parvum*-mediated NET formation was investigated in human and bovine PMN *in vitro*. We here demonstrate that *C. parvum* sporozoites indeed trigger NET formation in a time-dependent manner. Thereby, the classical characteristics of NETs were demonstrated by co-localization of extracellular DNA with histones, neutrophil elastase (NE) and myeloperoxidase (MPO). A significant reduction of NET formation was measured following treatments of PMN with NADPH oxidase-, NE- and MPO-inhibitors, confirming the key role of these enzymes in *C. parvum*-induced NETs. Additionally, sporozoite-triggered NETosis revealed as dependent on intracellular Ca^{++} concentration and the ERK 1/2 and p38 MAPK-mediated signaling pathway. Moreover, sporozoite-triggered NET formation led to significant parasite entrapment since 15% of the parasites were immobilized in NET structures. Consequently, PMN-pre-exposed sporozoites showed significantly reduced infectivity for epithelial host cells confirming the capability of NETs to prevent active parasite invasion. Besides NETs, we here show that *C. parvum* significantly up-regulated CXCL8, IL6, TNF-α and of GM-CSF gene transcription upon sporozoite confrontation, indicating a pivotal role of PMN not only in the bovine and human system but most probably in other final hosts for *C. parvum*.

1. Introduction

Cryptosporidium parvum is a protozoan parasite belonging to the Cryptosporiidae family within the phylum Alveolata. Parasites of the genus *Cryptosporidium* are euryxenous apicomplexan parasites with a zoonotic potential, thus putting immunocompromised humans as well as livestock animals at risk (Urquhart et al., 1996). Cryptosporidiosis is considered as a typical 'water- and food-borne disease' with a worldwide distribution that is characterized by enteritis accompanied by diarrhea, dehydration, weight loss and abdominal pain. A recent global enteric multi-center study (GEMS) revealed that *Cryptosporidum* spp. is the second leading cause of infant diarrhea associated with toddler mortality in developing countries (Kotloff et al., 2013). However, in immunocompromised hosts chronic cryptosporidiosis becomes a life-threatening parasitosis (McDonald et al., 2013; Blackwelder et al., 2012).

In young calves, *C. parvum* is one of the most important causes of heavy watery diarrhea leading to high economic losses in the cattle industry (Lendner and Daugschies, 2014; Hoffman and Sandoval, 1989; Moon et al., 1982; Tzipori et al., 1988; Shahiduzzaman and Daugschies, 2012). Hosts are orally infected by the uptake of sporulated *C. parvum* oocysts that were previously shed in the feces of infected animals. After ingestion, sporozoites are released upon stimulatory factors in the intestinal environment (Fayer, 1997) and infect the brush border of epithelial cells of the upper intestine (Fayer et al., 2004). Here they are localized intracellularly but extracytoplasmatic in the epithelial host cell and induce the formation of a parasitophorous vacuole (PV) (Karanis et al., 2007).

Concerning host immune reactions against *C. parvum*, most of the studies have focused on the cellular adaptive immunity of the hosts mainly by applying *C. parvum*-infected mice models (Aguirre et al., 1994; Blanshard et al., 1992; Chen et al., 2003; McDonald, 2008; Schmidt et al., 2001). In contrast, little is known on early innate immune reactions, especially of PMN and other professional

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phagocytes against *C. parvum in vivo*. Some reports on *C. parvum*-related early host innate reactions indicate a significant contribution of PMN and macrophages to the protective inflammatory response in cryptosporidiosis *in vivo* (Codices et al., 2013; Lacroix-Lamande et al., 2002; Takeuchi et al., 2008; Tzipori, 1988; Zadrozny et al., 2006). PMN are the most abundant leukocyte population in the blood of mammalians and often are reported as the earliest leukocytes to arrive at the site of infections (Stvrtinova et al., 2001). Besides phagocytosis and the production of molecules with antimicrobial properties, such as reactive oxygen species (ROS), PMN utilize the formation of neutrophil extracellular traps (NETs) as effector mechanism. This antimicrobial tool of PMN was firstly described in a landmark study by Brinkmann et al. (2004) showing that PMN are capable to release granular proteins and chromatin forming thin extracellular fibers which bind and kill Gram-positive and -negative bacteria (Fuchs et al., 2007). DNA is the main structural component of NETs and is decorated with antimicrobial proteins composed mainly of nuclear histones, granula-derived neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin, pentraxin and gelatinase among others (Brinkmann et al., 2004; Hermosilla et al., 2014; Papayannopoulos and Zychlinsky, 2009; Wartha and Henriques Normark, 2008). This novel form of cell death, now termed ETosis, depends on PMN-derived ROS production via intrinsic NADPH oxidase activities (Fuchs et al., 2007; Wartha and Henriques Normark, 2008).

While most studies have focused on bacterial, viral and fungal pathogens, little attention has been paid to the role of NETs in the early host innate immune responses against parasites (for reviews see Abi Abdallah and Denkers, 2012; Hermosilla et al., 2014). Thus, the first report on parasite-triggered NETosis to occur *in vivo* and *in vitro* was published in 2008 on *Plasmodium falciparum* (Baker et al., 2008). So far, parasite-triggered NET formation was mainly described for protozoan parasites, such as *Leishmania* spp. (Gabriel et al., 2010; Guimarães-Costa et al., 2009, 2010, 2011; Wang et al., 2011), *Eimeria bovis* (Behrendt et al., 2010; Muñoz-Caro et al., 2015), *Toxoplasma gondii* (Abi Abdallah et al., 2012; Reichel et al., 2015), *Besnoitia besnoiti* (Muñoz-Caro et al., 2014) and *E. arloingi* (Silva et al., 2014). However, only two reports on ETosis in response to helminth parasites exist so far and refer to the trematode *Schistosoma japonicum* (Chuah et al., 2013) and the nematode *Strongyloides stercoralis* (Bonne-Année et al., 2014). Furthermore, bovine monocyte-released extracellular traps (ETs) acting against tachyzoites of *B. besnoiti* (Muñoz-Caro et al., 2014) and harbor seal (*Phoca vitulina*) monocyte-triggered ETs against tachyzoites of *T. gondii* (Reichel et al., 2015) have recently been described adding a new leukocyte type to the portfolio of parasite-induced ET forming cells.

We here aimed to investigate in depth *C. parvum*-triggered ETosis in human and bovine PMN and to characterize molecules being involved in this innate effector mechanism. Furthermore, we analyzed chemokine (CXCL1, CXCL8, CXCL10, CCL2, CCL5), cytokine (IL6, TNF-α) as well as iNOS and GM-CSF gene transcription in *C. parvum*-exposed PMN as additional immune reaction of PMN. These first *C. parvum*-induced NET findings could be in the near future extended to other mammalian host PMN in order to better understand this complex molecular orchestra of the intestinal innate immunity.

2. Materials and methods

2.1. Parasites

Cryptosporidium parvum (in-house strain) was maintained by serial passages of oocysts in 1-day-old calves at the Institute of Parasitology, University of Leipzig, Germany. Oocysts were purified by a combined sedimentation–flotation technique according to Najdrowski et al. (2007) and stored in phosphate-buffered saline (PBS, pH 7.4), supplemented with penicillin/streptomycin (200 µg/ml each) and amphotericin B (5 µg/ml) at 4 °C until further use for a maximum of 6 months. The medium for *C. parvum* oocyst storage was changed at monthly intervals.

For sporozoite excystation, oocysts were incubated 10 min in 2% hypochlorite solution (final concentration) and washed two times with cold PBS (10 min, 1500 × *g*, 4 °C). Thereafter, the oocysts pellet was re-suspended in excystation medium (0.75% sodium taurocholat in PBS for the NET experiments; 0.4% sodium taurocholat for the infection assay) and incubated for 2 (NET experiments) and 3 (infection assay) h at 37 °C. For the NET experiments sporozoites were counted using a Neubauer hemocytometer chamber (Brand W., Germany).

2.2. Isolation of bovine and human PMN

Healthy adult dairy cows ($n = 3$) were bled by puncture of the jugular vein and blood was collected in 50 ml plastic tubes (Nunc), containing 0.1 ml heparin (Sigma-Aldrich) as anticoagulant. Heparinized blood was diluted in an equal volume of PBS containing 0.02% EDTA (Sigma-Aldrich), layered on Biocoll Separating Solution® (Biochrom AG) and centrifuged (800 × *g*, 45 min). After the removal of plasma, lymphocytes and monocytes, the pellet was suspended in 25 ml distilled water and shaken for 40 s to lyse erythrocytes. Osmolarity was immediately re-adjusted by adding the appropriate amount of Hanks salt solution (4 ml, HBSS 10×, Biochrom AG). PMN were washed twice, re-suspended in RPMI 1640 medium (Gibco), counted, and assessed for cell viability by using trypan blue (Sigma) exclusion tests. Finally, the cells were incubated at 37 °C and 5% CO_2 for at least 30 min before use. All animal procedures were performed according to the Justus Liebig University Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the State of Hesse (Regierungspräsidium Giessen) and in accordance to the current German Animal Protection Laws. Identification number of animal care and project license: GI 18/10-Nr.A51/2012 544_AZ952 (*Eimeria bovis*-oocyst production); GI 18/10-Nr.A9/2012 521_AZ877 (bovine blood samples). European Animal Welfare Legislation: ART13TFEU.

Isolation of human PMN was performed by using healthy male voluntary blood donors ($n = 3$). Heparinized blood was transferred into 55% and 70% Easycoll® (Biochrom) gradients in 50 ml plastic tubes (Falcon). Then, tubes were centrifuged (400 × *g*, 20 min, without break). After discarding plasma and PBMC layer, red blood lysis was performed by using the same procedure as for bovine PMN. Finally, PMN were re-suspended in RPMI 1640 medium (Gibco), counted and assessed for cell viability by using trypan blue exclusion test (Sigma-Aldrich).

2.3. Scanning electron microscopy (SEM)

Bovine PMN were incubated with either freshly isolated *C. parvum* sporozoites or oocysts at a ratio of 2:1 for 60 min on poly-L-lysine (Sigma-Aldrich) pre-coated coverslips. After incubation, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 15 min and washed with 0.1 M cacodylate buffer (all Merck). The cells were then post-fixed in 1% osmium tetroxide (Merck) in 0.1 M cacodylate buffer, washed three times in distilled water, dehydrated in ascending ethanol concentrations, critical point dried by CO_2-treatment and sputtered with gold. Specimens were examined using a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

2.4. Quantification of NET

NET formation was quantified using Pico Green®, a DNA-binding dye (Invitrogen). Therefore, bovine PMN ($n = 3$) were re-suspended in serum-free medium RPMI 1640 without phenol red and incubated in duplicates for 30 and 60 min with *C. parvum* sporozoites (37 °C, 2:1 ratio). In a parallel setting, the following inhibitors were applied: the NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 μM, Sigma-Aldrich), the NE inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone (CMK, 1 mM, Sigma-Aldrich, according to Scapinello et al., 2011) and the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH, 100 μM, Calbiochem, according to Parker et al., 2012), the store-operated calcium entry (SOCE) inhibitor 2-aminoethoxydiphenyl borate (2-APB, 100 μM), UO126 as inhibitor of ERK 1/2 (50 μM; Sigma-Aldrich, according to Muñoz-Caro et al., 2015) and SB 202190 (10 μM; Sigma-Aldrich) as inhibitor of p38 MAPK signaling pathway (Muñoz-Caro et al., 2015). For inhibition experiments, PMN were pre-incubated in the respective inhibitor in serum-free medium RPMI 1640 without phenol red for 30 min prior to exposure to *C. parvum* sporozoites. For NET quantification 50 μl of micrococcal nuclease buffer (New England Biolabs) containing 0.1 U/μl micrococcal nuclease (New England Biolabs) was added to each well and incubated (15 min, 37 °C). Afterwards each sample was centrifuged at $300 \times g$ for 5 min. The supernatant was transferred into a 96-well flat-bottom plate (100 μl per well in duplicates). Fifty microliters of Pico Green® (1:200 in 10 mM Tris base buffered with 1 mM EDTA) was added to each sample and the samples were incubated (4 min, in the dark). NET-formation was determined by spectrofluorometric analysis at an excitation wavelength of 484 nm and an emission wavelength of 520 nm using an automated plate monochrome reader (Varioskan Flash®; Thermo Scientific). NET formation was quantified by fluorescence intensity analyses. For negative controls, PMN in plain medium were used. For positive controls, stimulation of PMN with zymosan (1 mg/ml; Sigma-Aldrich) was used. Furthermore, in order to evaluate parasite dose-dependent effects on NET induction, different PMN:sporozoites ratios were applied (1:1, 1:2, 1:3) and processed as described earlier. Moreover, for the quantification of NETs in human PMN ($n = 3$) cells were re-suspended in serum-free medium RPMI 1640 without phenol red and incubated in duplicates for 60 and 120 min with *C. parvum* sporozoites (37 °C, 1:1 ratio, 4×10^5 cells/96-well, duplicates). For NADPH oxidase enzyme inhibition, DPI (10 μM, Sigma-Aldrich) was used as described earlier. In addition, to prove the DNA nature of human NETs, co-culture of cells and *C. parvum* sporozoites was exposed to DNase I treatment (90U; Sigma-Aldrich) 15 min before the incubation period. Human NET formation was quantified by fluorescence intensity analyses as described earlier. As negative control, human PMN in plain medium were used and for positive controls, human PMN stimulated with zymosan (1 mg/ml; Sigma-Aldrich) were used. NET formation was quantified by fluorescence intensity analyses as described earlier.

2.5. Visualization of NETs and detection of histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in Cryptosporidium parvum *sporozoites-induced NET structures*

Bovine PMN were co-cultured with *C. parvum* sporozoites (ratio 1:1, 60 min) on poly-L-lysine-treated coverslips and fixed with 4% (w/v) paraformaldehyde (20 min, in the dark, Merck). NET structures were visualized by staining extracellular DNA with Sytox Orange (Invitrogen) according to Martinelli et al. (2004). For the detection of histones and MPO within NET structures the following specific antibodies were used: anti-histone antibodies (clone H11-4 monoclonal antibody, 1:1000; Merck Millipore and rabbit anti-bovine histone H3, phospho S10 DyLight® 488, 1:100; ab139848, Abcam), anti-NE antibodies (rabbit polyclonal anti-human neutrophil elastase, 1:200, AB68672, Abcam) and anti-MPO antibodies (rabbit polyclonal anti-bovine MPO, Alexa Fluor 488, 1:200; ABIN906866, Antibodies-online.com). Fixed samples were washed three times with PBS, blocked with BSA [1% (w/v) in PBS, 30 min, RT, Sigma-Aldrich] and incubated in histone, -NE (for 24 h) or anti-MPO (for 24 h) antibody solution (RT, in the dark). The samples were then gently washed with PBS and mounted in anti-fading buffer (Moviol®, Sigma-Aldrich) according to Muñoz-Caro et al. (2014). Visualization was achieved by using an inverted Olympus® IX81 fluorescence microscope.

2.6. Quantification of PMN-derived reactive oxygen species (ROS) production

ROS production was measured by oxidation of DCFH-DA (2′, 7′-dichlorofluorescein–diacetate; Sigma-Aldrich) to fluorescent DCF according to Conejeros et al. (2011) with some modifications. Briefly, PMN ($n = 3$) were suspended in HBSS containing Ca^{2+} (0.9 mM $CaCl_2$) and incubated with *C. parvum* sporozoites (37 °C, 1:1 ratio, 2.5×10^5 cells/96-well, duplicates, 30 min). For positive controls, stimulation of PMN with zymosan (1 mg/ml) was used. For negative controls, PMN in plain medium were used. Following incubation, DCFH-DA (10 μg/ml) was added to each well. The fluorescence intensity of each sample was measured at 485 nm excitation and 530 nm emission wavelengths. The relative fluorescence units (RFU) were recorded after 30 min of incubation.

2.7. Sporozoite entrapment assay

For the quantification of sporozoite entrapment we followed the method described by von Köckritz-Blickwede et al. (2010) and Muñoz-Caro et al. (2014), with some slight modifications. Briefly, triplicates of bovine PMN ($n = 3$, 2×10^5/96-well) were pre-activated by zymosan treatment (1 mg/ml, 30 min, 37 °C). Meanwhile, *C. parvum* sporozoites were stained with CFSE dye (2.5 μM, Invitrogen, 10 min, 37 °C as described by Hermosilla et al. (2008)) and washed twice in PBS ($3000 \times g$, 10 min). Thereafter, zymosan-stimulated PMN were exposed to CFSE-labeled *C. parvum* sporozoites ($512 \times g$, 10 min; subsequent incubation for 30 min at 37 °C; 1:1 ratio). Equal numbers of non-exposed CFSE-stained sporozoites were used as reference and the values were assigned to 100%. The samples were thoroughly washed twice in RPMI 1640 medium and measured for fluorescence intensities at 485/538 nm wavelength. The percentage of entrapment was calculated as follows: [(A485/538 nm tachyzoites exposed to PMN)/(A485/538 nm non-exposed tachyzoites)] × 100%.

2.8. Host cell invasion assay

A total of 3×10^5 human ileocecal colorectal adenocarcinoma (HCT-8) cells were seeded per well in a 24 well plate and cultured in growth medium [RPMI, 10% FCS, 1% sodium pyruvate (NaPy), streptomycin/penicillin (200 μg/ml, 200 U/ml), 5 μg/ml amphotericin B] until confluency and infected with 5×10^5 *C. parvum* sporozoites/well. *Cryptosporidium parvum* sporozoites were either pre-exposed to PMN (3:1, 30 min), pre-exposed to PMN and additionally treated by DNase I (1.3 Units/μl) or left untreated and then used for host cell infection. After 3 h of incubation (37 °C, 5% CO_2, 0.4% sodium taurocholat) remaining oocyst shells and extracellular sporozoites were washed of and cells were cultured for a period of 48 h. The infected HTC-8 cells were detached 48 h p.i. by trypsinization (10 min, 37 °C), pelleted ($300 \times g$ for 5 min), re-suspended in 200 μl PBS and stored at −20 °C. Thereafter, DNA was extracted using the Qiagen QIAamp DNA Mini Kit according to the manufacturer's instructions for cultured cells and eluted in 50 μl distilled water. After determining the DNA concentration using a spectrophotometer (Eppendorf, Hamburg, Germany) DNA concentration was adjusted in all samples and stored at −20 °C until use. The parasite load per well was quantified by determining the *C. parvum* hsp70 gene copy numbers using a TaqMan based qPCR according to Shahiduzzaman et al. (2009). The PCR reactions were prepared in a total volume of 25 µl consisting of 1 × PCR buffer (Maxima Probe/ROX qPCR Master Mix (2X), Life Technologies), 300 nM CP_hsp_70_fwd and 900 nM CP_hsp_70_rvs, 200 nM of HSP_70_SNA. A plasmid standard equalling 10^2–10^6 gene copies or target sample template DNA with the same concentration in each experiment (500–800 ng/rxn) was added to the respective wells. Negative controls containing DNA of non-infected HTC-8 cells and no template controls were included in each assay. The qPCR reactions were run on an Mx3000P QPCR system (Stratagene, La Jolla, USA) with an initial polymerase activation step at 95 °C for 15 min followed by 40 cycles at 94 °C for 15 s and at 60 °C for 1 min. The infectivity of PMN pre-exposed *C. parvum* sporozoites was calculated relative to non-treated ones.

2.9. *RT-PCR for the quantification of CXCL1, CXCL8, CXCL10, CCL2, CCL5, IL6, TNF alpha, GM-CSF and iNOS gene transcripts in PMN*

Bovine PMN ($n = 4$; 1×10^6) were co-cultured with *C. parvum* sporozoites (1×10^6, 1 h, 37 °C). PMN alone were equally processed for negative controls and PMN stimulated with zymosan (1 mg/ml) were used as positive controls. After incubation, co-cultures were centrifuged (400 × *g*, 10 min) and each pellet was directly lysed for total RNA isolation by addition of 594 µl buffer RLT lysis buffer, (RNeasy Mini Kit, Qiagen), and 6 µl 2-mercaptoethanol, (Serva) per sample. Thereafter, total RNA isolation was performed using the RNeasy kit according to manufacturer's protocol. In order to guarantee absolute genomic DNA digestion, a second genomic DNA digestion step was performed. Therefore, 1 µg of total RNA was treated with 1 U DNase I (1 U/µl, New England Biolabs) in 10 × DNase reaction buffer (37 °C, 30 min, New England Biolabs) and DNase I was inactivated by heating the sample (65 °C, 10 min). Total RNA samples were stored at −80 °C until further use. cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis System (Invitrogen) according to manufacturer's protocol with slight modifications. For first-strand cDNA synthesis, the following constituents were mixed: 1 µg of total RNA, 0.5 µl oligo d(T) (50 ng/µl), 1 µl hexamer primer (50 ng/µl), 1 µl dNTP mix (10 mM) and the volume was adjusted with DEPC-treated water to 13.5 µl total volume. The samples were incubated at 65 °C for 5 min and then immediately cooled on ice. For the second strand synthesis, the following ingredients were added: 2.5 µl of 10 × RT buffer, 5 µl 25 mM $MgCl_2$, 2.5 µl 0.1 M DTT, 1 µl RNase OUT (40 U/µl, Invitrogen) and 0.5 µl Super Script® III enzyme (200 U/µl). The samples were incubated at 25 °C for 10 min followed by incubation steps at 50 °C for 50 min and 5 min at 85 °C. Finally, samples were adjusted to 200 µl total volume with 1 × TE buffer and the samples were stored at −20 °C. Realtime PCRs for the quantification of the gene transcripts of the chemokines CXCL1, CXCL8, CXCL10, CCL2 and CCL5, the cytokines IL6 and TNFα s well as GM-CSF and iNOS (for primers and probes see Supplementary Table S1) were performed in a 10 µl total volume containing 400 nM forward and reverse primers, 200 nM probe, 2 µl cDNA and 8 µl PCR master mix (Quanta Perfecta qPCR FastMix®). The reaction conditions for all systems were as follows: hold at 95 °C for 5 min, 45 cycles at 94 °C for 15 s, and 60 °C for 60 s. PCRs were performed utilizing an automated real-time PCR fluorometer (Rotor-Gene Q®, Qiagen). Non-template controls (NTCs) were included in each experiment. Cycle threshold (Ct) values ≥40 were considered as non-significant amplification.

2.10. *Statistical analyses*

Co-culture/stimulation conditions were compared by one- or two-factorial analyses of variance (ANOVA) with repeated measures. Differences were regarded as significant at a level of $p \le 0.05$.

3. Results

3.1. Cryptosporidium parvum *stages induce NET formation in bovine and human PMN*

SEM analyses revealed NET formation to be induced by both *C. parvum* parasitic stages, oocysts and sporozoites. Thus, NET structures released from bovine PMN appeared as fine and thick strands contacting sporozoites and oocysts, thereby occasionally entirely covering the oocysts (Fig. 1A, B) indicating that *C. parvum* mediated-NET formation is not a stage-specific event and suggesting that NET formation may lead to the mechanic impairment of oocyst excystation.

The parasite-triggered induction of NETs was confirmed via fluorescence microscopy analyses applying specific antibodies against H3, NE and MPO alongside with DNA-staining thereby proving the classical characteristics of NETs. Thus, the DNA-positive (estimated via extracellular DNA-positive fibers) backbone of NETs co-localized with histone H3-, NE- and MPO-positive signals indicating a pivotal role of these molecules in *C. parvum*-triggered NETosis (Fig. 1C and D, for NE-positive reactions and further illustrations see Supplementary Fig. S1).

Quantification of NETs revealed that *C. parvum* sporozoites indeed triggered NETosis in bovine PMN ($p \le 0.05$, Fig. 3) and human PMN ($p \le 0.01$, Fig. 5B). As expected, the stimulation with zymosan serving as positive control significantly enhanced NET formation of bovine PMN ($p \le 0.01$, Fig. 3) and of human PMN ($p \le 0.01$, Fig. 5B). *Cryptosporidium parvum*-mediated NET formation also appeared as dose-dependent and significant differences were observed in ratios 1:2 and 1:3 when compared to negative controls (both $p \le 0.05$, Fig. 3). Given that NETosis is reported as obligatory linked to neutrophil ROS production, we furthermore analyzed ROS production of bovine PMN in response to *C. parvum* sporozoites. As expected, exposure of bovine PMN to sporozoites resulted in a significant upregulation of neutrophil ROS production ($p \le 0.01$, Fig. 2). The stimulation of PMN with zymosan, which was used as positive control, induced an even stronger, significant reaction compared to that of sporozoites ($p \le 0.0001$, Fig. 2).

Applying a quantitative parasite-entrapment assay using CFSE-stained parasites, 15 ± 4% of *C. parvum*-sporozoites were shown to be significantly immobilized within NET structures compared to non-exposed parasites ($p \le 0.01$, Fig. 4). Trypan blue exclusion tests revealed that NET formation did not interfere with the sporozoite viability since 97% of PMN-exposed *C. parvum* sporozoites remained vital after PMN-exposure. The same rate of sporozoite mortality was detected in non-exposed sporozoites (data not illustrated).

3.2. Cryptosporidium parvum *sporozoite-induced NETosis is a NADPH oxidase-, NE-, MPO-, ERK1/2-, p38 MAPK- and SOCE-dependent process*

The actual role of the NET-associated enzymes NADPH oxidase, MPO and NE was analyzed via functional inhibition experiments using DPI, ABAH and CMK, respectively. All inhibitor treatments resulted in a significant decrease of parasite-triggered NET formation in the bovine system ($p \le 0.01$ for all treatments; Fig. 5A), thus demonstrating the key role of these enzymes in *C. parvum*-induced NET formation. Moreover, in human PMN a NADPH oxidase-dependent *C. parvum*-NET induction was observed resulting in a significant diminishment of NET formation ($p \le 0.01$, Fig. 5B). Furthermore, *C. parvum* induced NETs in human PMN were efficiently abolished by DNase I treatment ($p \le 0.01$; Fig. 5B) proving the typical characteristic of NETs.

In addition, we looked for the precise molecular signaling pathway being involved in *C. parvum*-triggered NETosis. The use of

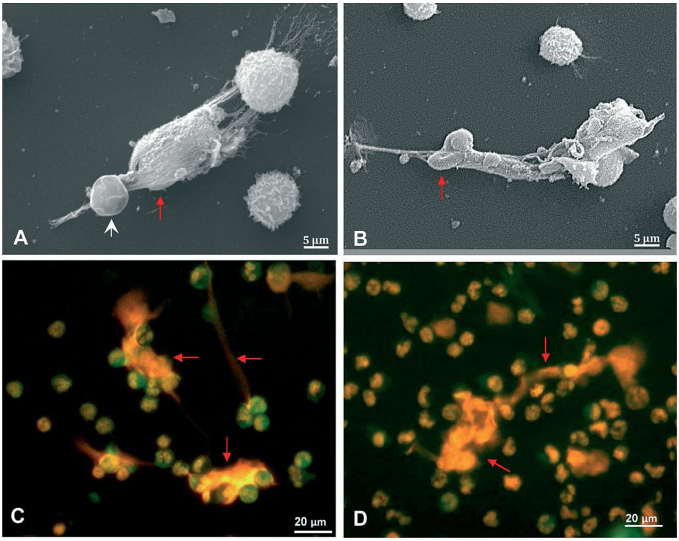

Fig. 1. *Cryptosporidium parvum*-triggered NET formation. A,B: Following co-cultures, scanning electron microscopy analysis revealed the presence of thicker and thinner filaments originating from PMN exposed to *C. parvum* oocysts and sporozoite stages. (A) *C. parvum* oocyst loosely immobilized in NETs (arrowhead) and one specimen almost completely covered by PMN-released NET structures (red arrow). (B) *C. parvum* sporozoite (red arrow) trapped in PMN derived-extracellular fibers. C,D: Co-localization of NET-derived DNA with histones (C) and MPO (D). Co-cultures of bovine PMN and *C. parvum* sporozoites were fixed, permeabilized, stained for DNA using Sytox Orange and probed for histones and MPO using anti-MPO and anti-histone (H3) along with adequate conjugate systems. (C) NET-DNA stained with Sytox orange (red) and anti-MPO (green). (D) NET-DNA stained with Sytox orange (red) and anti-histone (green). Photomicrographs represent exemplary images of 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inhibitors interfering with ERK1/2- (UO126) and p38 MAPK- (SB 202190) signaling pathways in functional NET assays resulted in a significant reduction of parasite-induced NET extrusion ($p \leq 0.01$ for both inhibitors; Fig. 5A) proving a significant involvement of the ERK1/2 and p38 MAPK-mediated signal pathway in parasite-triggered NETosis.

Given that ROS production of bovine PMN is Ca^{2+}-dependent (Conejeros et al., 2011; Muñoz-Caro et al., 2015), we here furthermore tested whether *C. parvum*-induced NET formation is dependent on store-operated Ca^{2+} entry (SOCE). The use of 2-APB, known as potent SOCE-inhibitor, resulted in a significant reduction of parasite-triggered NET release ($p \leq 0.01$; Fig. 5A), emphasizing the importance of SOCE for functional parasite-induced NETosis.

3.3. *Cryptosporidium parvum-induced NETs hamper active host cell invasion*

To successfully complete their life cycle, *C. parvum* sporozoites must invade host epithelial cells of the gut to fulfill first merogony. In order to assess the impact of *C. parvum*-induced NETs on active sporozoite host cell invasion, oocysts of *C. parvum* were pre-incubated with bovine PMN before being used for host cell infection. To prove NETs as underlying mechanism we additionally treated samples with DNase I following an appropriate reaction time of 30 min to dissolve potential NET structures. The pre-exposure of parasites with PMN led to sporozoite immobilization and reduced the sporozoite invasion capacity for host cells since significantly decreased infection rates were found (un-exposed *vs.* PMN-exposed oocysts: $p \leq 0.001$, Fig. 6). Thus, the numbers of intracellular parasites were reduced by $36 \pm 1.5\%$ which might have an important impact on subsequent *C. parvum* proliferation. The fact that this reaction was reversible and could be entirely restored by DNase I treatment proved the impairment to be owned by NET extrusion (Fig. 6) and furthermore evidenced a lack of NET-mediated killing of *C. parvum* sporozoites.

3.4. *Exposure of bovine PMN to* C. parvum *sporozoites induces CXCL8, IL6, TNFα and GM-CSF gene transcription*

Real-time PCR analyses of PMN exposed to *C. parvum* sporozoites revealed an up-regulation of several immunoregulatory molecules as summarized in Fig. 7. Relative to medium controls, co-cultures of PMN with *C. parvum* sporozoites induced a gene transcriptional up-regulation of the PMN-derived chemokine CXCL8

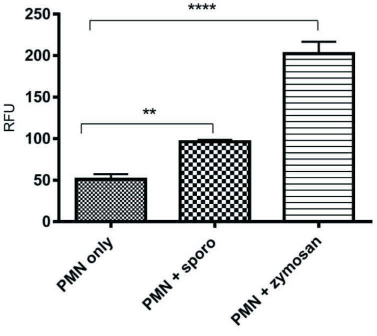

Fig. 2. ROS production in *Cryptosporidium parvum* sporozoite-exposed bovine PMN. Bovine PMN were exposed to *C. parvum* sporozoites and ROS production was measured via the oxidation of DCFH-DA to fluorescent DCF. Zymosan (1 mg/ml) stimulation of PMN served as positive control and PMN in plain medium as negative control. Arithmetic means of three PMN donors, minimum and maximum.

(2.16-fold, $p \le 0.05$), the cytokines IL-6 (7.40-fold, $p \le 0.01$) and TNF-α (2.587-fold, $p \le 0.05$), as well as GM-CSF (2.41-fold, $p \le 0.05$), while the transcriptional levels of the CXCL1, CXCL10, CCL2, CCL5 and iNOS genes were not significantly affected. The overall strongest reactions were found in the case of IL-6.

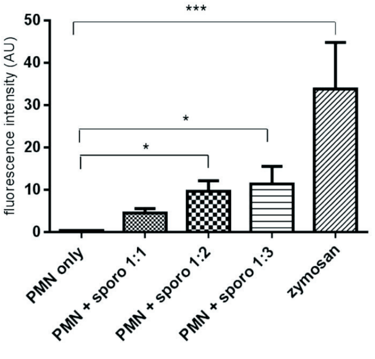

Fig. 3. Dose-dependency of *Cryptosporidium parvum* sporozoite-triggered NET formation. PMN and *C. parvum* sporozoites were co-cultured at different ratios (PMN : sporozoites = 1:1, 1:2, 1:3). After incubation, samples were analyzed for extracellular DNA by quantifying Pico Green-derived fluorescence intensities. Each condition was performed in duplicates. Arithmetic means of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \le 0.05$.

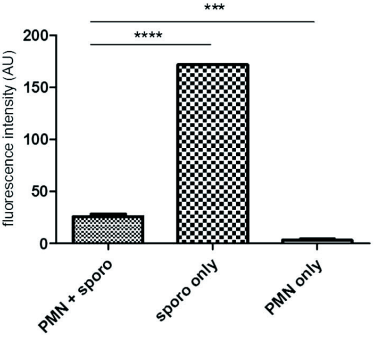

Fig. 4. Quantification of *Cryptosporidium parvum* sporozoite entrapment in NET structures. Prior to NET experiments, *C. parvum* sporozoites were stained with CFSE and PMN ($n = 3$) were activated by zymosan treatment. Pre-activated PMN were then exposed to CFSE-stained sporozoites (30 min at 1:1 ratio). Thereafter, non-trapped sporozoites were thoroughly washed off and the fluorescence intensities of trapped CFSE-stained sporozoites were calculated in relation to identical numbers of non-exposed CFSE-stained sporozoites. Differences were regarded as significant at a level of $p \le 0.05$.

4. Discussion

PMN are considered as a fundamental component of the host innate immune system representing the first line of defense against pathogens and the first ones to be recruited to the site of infection (Brinkmann et al., 2004; Hahn et al., 2013; Von Köckritz-Blickwede and Nizet, 2009). Besides phagocytosis and oxidative burst-related defense actions, the extrusion of NETs is nowadays considered as one of the main effector mechanisms of PMN against infectious agents (Brinkmann and Zychlinsky, 2012; Hahn et al., 2013). Most studies on pathogen-triggered NETs focused on bacterial, viral and fungal infections (Aulik et al., 2010; Fuchs et al., 2007; Jenne et al., 2013; Urban et al., 2009). So far, some protozoan parasites have been identified as potent NET inducers (Abi Abdallah et al., 2012; Baker et al., 2008; Behrendt et al., 2010; Guimarães-Costa et al., 2009; Muñoz-Caro et al., 2014, 2015; Reichel et al., 2015; Silva et al., 2014). To the best of our knowledge, we here describe for the first time the release of NETs from bovine and human PMN in response to the apicomplexan parasite *C. parvum*, which is known as an important anthropozoonotic agent affecting children and immunocompromised humans as well as neonates of domestic livestock animals worldwide.

In agreement with observations in other coccidian genera (Abi Abdallah et al., 2012; Behrendt et al., 2010; Muñoz-Caro et al., 2014, 2015; Reichel et al., 2015; Silva et al., 2014), we here document NET-like structures being attached to sporozoites and oocysts of *C. parvum*. DNA staining of *C. parvum*-induced NETs proved the DNA nature of these PMN-derived extracellular structures. Additionally, the resolution of these structures by DNase I treatments confirmed the DNA backbone nature of *C. parvum*-triggered NETs. Alongside to DNA, other classical NET components, such as nuclear histones and antimicrobial enzymes were detected in *C. parvum*-induced NETs. Thus, co-localization experiments showed the simultaneous presence of H3, NE and MPO in *C. parvum*-triggered NETs corroborating typical

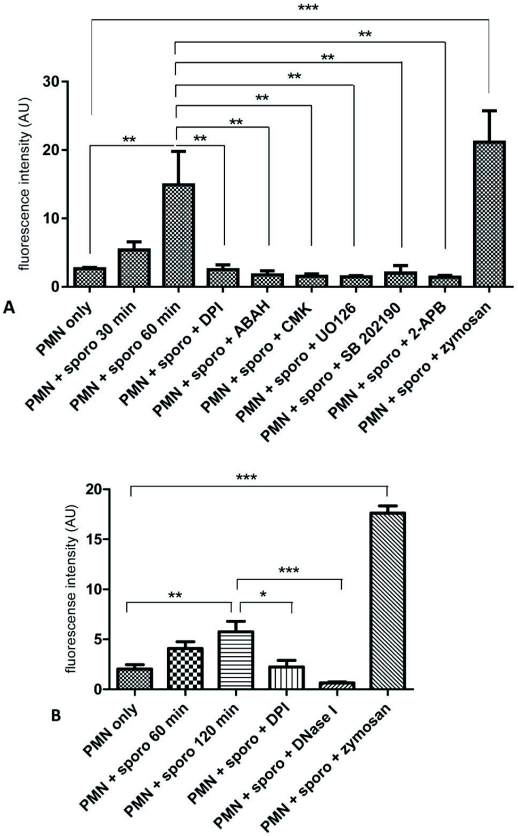

Fig. 5. Blockage of *Cryptosporidium parvum*-triggered NETosis via inhibitors of NADPH oxidase, MPO, NE, ERK 1/2, p38 and SOCE. A: NET inhibition assays were performed by pre-incubating PMN with inhibitors of NADPH oxidase (DPI, 10 μM), NE (CMK, 1 mM), MPO (ABAH, 100 μM), ERK 1/2 (UO126, 50 μM), p38 (SB, 10 μM) and SOCE (2-APB, 100 μM) prior to *C. parvum* sporozoite exposure and NET measurement. B: *C. parvum*-induced NET formation was demonstrated in human PMN ($n = 3$, 60 and 120 min). NADPH oxidase inhibition was performed by using DPI, 10 μM. For the resolution of NETs, DNase I control was included. In both experiments, for positive controls zymosan treatment of PMN was used. PMN in plain medium served as negative control. Differences were regarded as significant at a level of $p \leq 0.05$ and $p \leq 0.01$.

characteristics of NETs. Accordingly, the key role of MPO and NE in *C. parvum*-induced NETosis was emphasized by the use of their inhibitors ABAH and CMK, which both resulted in a significant reduction of parasite-triggered NET formation.

A classical characteristic of NETosis is its obligate linkage to NADPH oxidase-dependent ROS production. We here show that ROS are indeed induced in *C. parvum* sporozoite-exposed PMN. Interestingly, *C. parvum*-infection in neonatal piglets resulted not only

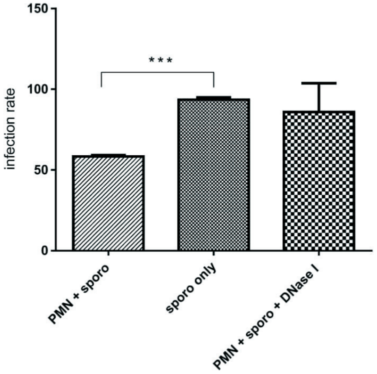

Fig. 6. *Cryptosporidium parvum* induced-NET formation prevents active host cell invasion. To assess the capacity of NETs to prevent active *C. parvum* invasion into HCT-8 as host cells, HCT-8 cells were infected with *C. parvum* sporozoites (5×10^5 sporozoites/well) that were either used untreated for infection (positive control), pre-exposed to bovine PMN ($n = 3$, 60 min) and then used for host cell infection or pre-exposed to PMN and treated with DNase I (for 15 min at the end of incubation period) before host cell infection.

in mucosal PMN infiltration but also in increased MPO activities *in vivo* (Zadrozny et al., 2006) underlining the overall importance of ROS-dependent MPO release. In addition and in agreement with other reports on parasite-triggered NETosis (Baker et al., 2008; Behrendt et al., 2010; Muñoz-Caro et al., 2014; Reichel et al., 2015; Silva et al., 2014), *C. parvum*-triggered NET extrusion also proved to be NADPH oxidase-dependent. Thus, NADPH oxidase blockage via DPI treatment resulted in a significant diminishment of *C. parvum*-induced NET formation both in bovine and human PMN.

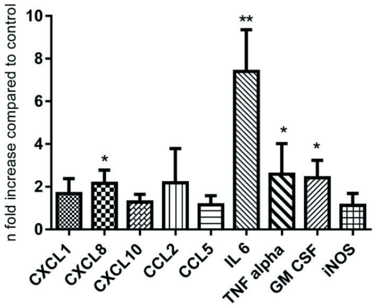

Fig. 7. Effects of *Cryptosporidium parvum* sporozoite exposure to PMN on chemokine (CXCL1, CXCL8, CXCL10, CCL2, CCL5), cytokine (IL6, TNF-α), GM-CSF and iNOS gene transcription. Bovine PMN were exposed to *C. parvum* sporozoites at a ratio of 1:1 or to plain medium as negative control (1 h, 37 °C). Thereafter total RNA was isolated from PMN samples, reverse transcribed and assayed with real-time PCR systems for the detection of bovine CXCL1, CXCL8, CXCL10, CCL2, CCL5, IL6, TNF-α, GM-CSF and iNOS mRNA equivalents. The graphic is representative for 4 independent experiments Arithmetic means of 4 PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p \leq 0.05$.

In accordance to *E. bovis*-based NET data (Behrendt et al., 2010) but in contrast to *B. besnoiti*-induced ETs (Muñoz-Caro et al., 2014), *C. parvum* sporozoite-triggered NETosis was time-dependent. Furthermore, NET structures were observed being firmly attached to *C. parvum* sporozoites and oocysts thus supporting the quantitative data of sporozoite entrapment showing that a considerable proportion of these parasites were captured and immobilized by NETs. Moreover, *in vitro* infection trials involving PMN-pre-exposed *C. parvum* oocysts revealed a significant reduction of their infectivity (36%) for host cells owing to NET formation. The pivotal role of NETs in parasite immobilization was proven by the fact that NET-induced invasion inhibition was reversed by DNase I treatment. Furthermore, this result proved that the parasites were indeed not killed by NETs as was postulated for several bacterial pathogens (Parseghian and Luhrs, 2006) and protozoan parasites (Behrendt et al., 2010; Muñoz-Caro et al., 2014; Reichel et al., 2015; Silva et al., 2014). Overall, these data confirm the capability of NET to hamper *C. parvum* sporozoites from active host cell *in vivo* infection by immobilizing them. Given that *C. parvum* obligatory needs to invade host cells for further development and proliferation it appears feasible to suggest NETosis as effective defense mechanism against *C. parvum*.

Given that PMN-derived ROS production is known to be dependent on store-operated calcium entry (SOCE) (Hallett et al., 1990; Muñoz-Caro et al., 2015; Parekh and Putney, 2005; Sandoval et al., 2007), we applied the SOCE inhibitor 2-APB in NET-related functional assays, as reported elsewhere (Conejeros et al., 2011; Muñoz-Caro et al., 2014). In agreement with recent data on another apicomplexan parasite (Muñoz-Caro et al., 2015), *C. parvum*-triggered NETosis also revealed as Ca^{2+}-dependent since 2-APB treatments resulted in a significant reduction of parasite-triggered NET formation. A Ca^{2+}-dependency of NET induction was also recently reported for NET extrusion by human PMN in response to non-parasitic stimulators (Gupta et al., 2014).

Moreover, inhibitor analyses revealed a pivotal role of ERK1/2- and p38 MAPK-signaling pathways in *C. parvum*-induced NETosis. Thus, functional inhibition of these kinases resulted in a significant diminishment of parasite-triggered NET formation. Similar findings have recently been published on *T. gondii*- (Abi Abdallah et al., 2012) and *E. bovis*-triggered NETosis (Muñoz-Caro et al., 2015) evidencing a general role of these signaling pathways in apicomplexan mediated-NETosis.

While apicomplexan sporozoite stages have recently been demonstrated as potent NET inducers (Behrendt et al., 2010; Muñoz-Caro et al., 2015; Silva et al., 2014), we here demonstrate that also the oocyst stage of *C. parvum* is capable to induce NETosis. These findings correspond well to those observed in *E. arloingi*-triggered NET formation, where oocysts were shown to induce NETs. Given that *E. arloingi* oocysts were occasionally entirely covered by NETs (Silva et al., 2014), the authors speculated that NET-mediated blockage of the oocyst micropyle might hamper sporozoite excystation and, consequently, abrogate the parasite life cycle at a very early stage of infection. Since mammalian PMN have been demonstrated to actively transmigrate into the intestinal lumen (Brazil et al., 2013; Seper et al., 2013; Sumagin et al., 2013; Szabady and McCormick, 2013) to fulfill their antimicrobial activities, they should also be able to interact with different luminal parasite stages, such as freshly ingested *C. parvum* oocysts as postulated elsewhere (Hermosilla et al., 2014; Silva et al., 2014).

Moreover, we here show that *C. parvum*-driven innate immune reactions of PMN are not restricted to NETosis. Thus, exposure to *C. parvum* sporozoites induced the gene transcription of several pro-inflammatory molecules in bovine PMN. Consequently, a significant up-regulation of the gene transcription of the chemokine CXCL8, of the cytokines TNF-α and IL6 and of the growth factor GM-CSF was detected in sporozoite-exposed PMN providing evidence for their modulatory role in the transition of ongoing immune responses (Leutenegger et al., 2000; Taubert et al., 2006). In agreement, mRNAs of IL6 and TNF-α were also induced in bovine PMN that had been exposed to sporozoites of *E. bovis* (Behrendt et al., 2008). Furthermore, other studies demonstrated a rapid mobilization of TNF-α to the surface of mouse neutrophils in response to microbial antigens and protozoan parasites such as *T. gondii* (Bennouna and Denkers, 2005; Bennouna et al., 2006). While CXCL8 and GM-CSF primarily act on innate immune cells and propagate further PMN recruitment which has already been demonstrated for *C. parvum*-infected pigs (Argenzio et al., 1990), TNF-α and IL6 production may additionally cause lymphocyte chemotaxis thereby contributing to the transition to adaptive immune responses (Boss and Neeck, 2000; Cronstein, 2007).

The results of this study demonstrate for the first time *C. parvum* sporozoites and oocysts as significant inducers of NETosis. Considering the life cycle of *C. parvum* which includes active proliferation in gut epithelial cells during acute phase of the disease and the recruitment of PMN during cryptosporidiosis *in vivo* (Codices et al., 2013; Takeuchi et al., 2008; Zadrozny et al., 2006), parasite entrapment via NET formation may be of particular importance since the lysis of infected epithelial cells will lead to direct exposure of parasitic stages to luminal PMN. Additionally, also ingested *C. parvum* oocysts as well as free-released sporozoites will be exposed to luminal PMN of the intestine. However, the actual role of NETs in *C. parvum*-infected hosts *in vivo* is not clear yet and should be addressed in the near future.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2015.05.007.

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RESEARCH Open Access

Leucocyte-derived extracellular trap formation significantly contributes to *Haemonchus contortus* larval entrapment

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Abstract

Background: Polymorphonuclear neutrophil (PMN) and eosinophil extracellular trap (ETs) formation has recently been described as an important host effector mechanism against invading pathogens. So far, scarce evidence on metazoan-triggered ET formation has been published. We here describe for the first time *Haemonchus contortus*-triggered ETs being released by bovine PMN and ovine eosinophils in response to ensheathed and exsheathed third stage larvae (L_3).

Methods: The visualization of ETs was achieved by SEM analysis. The identification of classical ETs components was performed via fluorescence microscopy analysis. The effect of larval exsheathment and parasite integrity on ET formation was evaluated via Pico Green®- fluorescence intensities. ETs formation under acidic conditions was assessed by using media of different pH ranges. Parasite entrapment was evaluated microscopically after co-culture of PMN and L_3. ET inhibition experiments were performed using inhibitors against NADPH oxidase, NE and MPO. Eosinophil-derived ETs were estimated via fluorescence microscopy analysis.

Results: L_3 significantly induced PMN-mediated ETs and significant parasite entrapment through ETs structures was rapidly observed after 60 min of PMN and L_3 co-culture. Co-localization studies of PMN-derived extracellular DNA with histones (H3), neutrophil elastase (NE) and myeloperoxidase (MPO) in parasite-entrapping structures confirmed the classical characteristics of ETs. *Haemonchus contortus*-triggered ETs were significantly diminished by NADPH oxidase-, NE- and MPO-inhibition. Interestingly, different forms of ETs, i.e. aggregated (*agg*ETs), spread (*spr*ETs) and diffused (*diff*ETs) ETs, were induced by L_3. *Agg*ETs and *spr*ETs firmly ensnared larvae in a time dependent manner. Significantly stronger *agg*ETs reactions were detected upon exposure of PMN to ensheathed larvae than to exsheathed ones. Low pH conditions as are present in the abomasum did not block ETosis and led to a moderate decrease of ETs. Eosinophil-ETs were identified extruding DNA via fluorescence staining.

Conclusion: We postulate that ETs may limit the establishment of *H. contortus* within the definitive host by immobilizing the larvae and hampering them from migrating into the site of infection. Consequently, *H. contortus*-mediated ET formation might have an impact on the outcome of the disease. Finally, besides PMN-triggered ETs, we here present first indications of ETs being released by eosinophils upon *H. contortus* L_3 exposure.

Keywords: *Haemonchus contortus*, Neutrophil extracellular traps, Innate immunity, ETosis

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Background

Haemonchus contortus is a gastrointestinal nematode with worldwide distribution. This abomasal parasite leads to significant economic losses particularly in small ruminant livestock [1]. Haemonchosis is acquired by ingesting infective third-stage larvae (L_3) from contaminated pasture. After exsheathment, L_3 penetrates the abomasal glands where it develops to fourth-stage larvae (L_4) and thereafter to dioecious haematophagous pre-adult larvae (L_5) and adults. Adult *H. contortus* nematodes are found in the lumen of the abomasum in herbivores [2–5]; or the stomach of omnivores [6] consuming up to 0.05 ml host blood per worm daily [7], which results in hemorrhagic abomasitis (gastritis), anaemia, oedema and associated complications often leading to death of severely infected animals [8].

H. contortus infections in ruminants are known to elicit a Th2 type-dominated host immune response being characterized by the recruitment of large numbers of eosinophils, mast cells and globule leucocytes and to the production of locally active and circulating antibodies [9–11]. Nonetheless, little is known on the very early host innate immune responses against *H. contortus.* In this scenario, the relative inaccessibility of infective *H. contortus* L_3 within abomasal/gastric glands for host leucocytes poses unique challenges to the innate immune system, which has evolved several specialized strategies for parasite control [12]. Parasite colonization of the host abomasum initially depends on the motility of the larvae and the parasite load. Thus, some host individuals, after sensitization via previous infections, can modify the microenvironmental conditions of this niche to expel the parasite [13]. There is evidence showing that helminths activate the alternative complement pathway binding opsonins on their surface [14]. Moreover, within the innate immune response polymorphonuclear neutrophils (PMN; [15]) and eosinophils are considered as fundamental leucocytes forming the first line of defense against metazoan nematodes and the first leucocytes to be recruited to the site of infection [16–19]. Various authors have reported that eosinophils are capable of immobilizing infective larvae of diverse species of nematodes in vitro and in vivo [9, 10, 20, 21]. Furthermore, incubation of *H. contortus* L_3 with antibodies raised against $HcsL_3$ antigens in the presence of ovine eosinophils resulted in significant larval killing after 24 h [20]. In addition, it has been demonstrated that eosinophils are essentially involved in the expulsion of diverse nematodes in vivo, such as *Strongyloides stercoralis* [22], *Onchocerca lienalis* [23], *Trichinella spiralis* [24] and *Trichostrongylus colubriformis* [25]. Alongside phagocytosis and oxidative burst, leucocytes are capable of triggering extracellular traps (ETs) as a novel effector mechanism. This results in the cellular release of granule proteins and chromatin upon activation that together form extracellular fibers capable of binding and killing Gram-positive and -negative bacteria and parasites [16, 26]. So far, the mechanism of ET formation has been attributed to PMN [16], mast cells [27], macrophages [28], eosinophils [29] and monocytes [30, 31] and thus appears to be a general effector mechanism of innate immune cells. Most studies on pathogen-triggered ETs have been focused on bacterial, viral and fungal infections [17, 32–34]. However, little attention has been paid to parasites as ET-inducers [26] and studies of ET induction by parasites have mainly focused on protozoans [35–40]. So far, only two helminth species, i.e. *Schistosoma japonicum* [41] and *Strongyloides stercolaris* [15], have been proven to induce NETs.

With the present work we add a new species to the group of metazoan-ET-inducers and highlight the capability of ETs to entrap this large parasite. The current data suggest *H. contortus*-mediated NET formation may influence the outcome of the infection in affected animals in vivo. Furthermore, we here provide first evidence on eosinophil-derived ET formation in response to a helminth parasite.

Methods

Ethic statement

All animal procedures were performed according to the Moredun Research Institute regulations and to the Justus Liebig University Animal Care Committee guidelines, approved by the Ethic Commission for Experimental Animal Studies of the State of Hesse and in accordance to the current European Animal Welfare Legislation: ART13TFEU.

Animals and parasites

Three male Merino sheep were purchased from a local farmer at the age of five months, treated with a single dose of toltrazuril [20 mg/kg body weight (bw), Baycox® 5 %; Bayer Animal Health] and benzimidazole (10 mg/kg bw, Panacur; Bayer Animal Health). The sheep were controlled for parasitic infections via weekly coprological analyses and, when deemed parasite free, maintained under parasite-free conditions within a large animal stable equipped with a laminar flow lock entrance until experimental infection (Institute of Parasitology, Justus Liebig University, Giessen, Germany). Animals were fed with commercial starter pellet concentrates (Lämmerpellets®, Deuka). Water and sterilized hay were given *ad libitum*.

Animals were infected *per os* with 8×10^3 viable ensheathed *H. contortus* L_3 (in house strain) suspended in tap water. Following prepatency of approximately three weeks, cotton faecal collection bags were fixed to the anuses of sheep to collect faeces and were emptied each day.

The isolation of excreted *H. contortus* eggs and exogenous in vitro culture into third stage larvae were performed as previously described elsewhere [42]. Faecal samples (10–50 g) were transferred to a jar and mixed with commercially purchased sawdust until a crumbly consistency was obtained, and, if necessary, dampened with tap water. Thereafter, the jars were capped and incubated at 27–28 °C for 7–8 days. After incubation, tap water was added to the culture until the jar was filled up to the brim, the jar was turned upside down on a petri dish. Then, 10–20 ml of tap water was added into the petri dish and the jars were incubated overnight at room temperature (RT). Thereafter, the fluid containing *H. contortus* ensheathed L_3 was collected, transferred to a conical tube (Greiner) and the L_3 were sedimented at unit gravity (at least 30 min, RT). Afterwards, the supernatant was discarded; the L_3 of *H. contortus* were counted, suspended in sterile PBS and stored at 4 °C until further use. L_3-related ET experiments were performed within 4 weeks after parasite collection in order to prevent morphological alterations or death of the larvae.

Exsheathment of *Haemonchus contortus* L_3

For exsheathment, vital ensheathed L_3 (1500 larvae in 5 ml tap water) were exposed to sodium hypochlorite solution (0.3 % *v*/*v*; 5 min, RT, Merck). Active larval exsheathment was observed under an inverted DMIRB® microscope (Leica). When at least 80 % of the *H. contortus* L_3 had exsheathed, the larvae were washed in sterile PBS (400 × *g*, 3 × 5 min) and kept at 4 °C for further use within the same day.

Isolation of bovine PMN and ovine eosinophils

For PMN isolation, healthy adult female Holstein-Frisian cows ($n = 3$) were bled by puncture of the jugular vein. Heparinized blood was diluted under sterile conditions in an equal volume of sterile phosphate buffered saline (PBS) containing 0.02 % EDTA (Sigma-Aldrich). The mixture was layered on Biocoll® separating solution (Biochrom AG) and centrifuged at 800 × *g* for 45 min. After removal of plasma, lymphocytes and monocytes in the upper layers of the gradient, the cells were re-suspended in 25 ml sterile distilled water to lyse erythrocytes. Osmolarity was immediately readjusted by adding 10x sterile Hank's Balanced Salt Solution (HBSS, Biochrom AG) and the cells were pelleted (400 × *g*, 10 min). Thereafter, the cells were washed twice (400 × *g*, 10 min, 4 °C) in complete RPMI 1640 medium without phenol red (Sigma-Aldrich). The cells were counted in a Neubauer haemocytometer chamber and incubated at 37 °C and 5 % CO_2 atmosphere for at least 30 min before use. The PMN-enriched samples had > 90 % purity.

For ovine eosinophil isolation, heparinized blood (25 ml) was collected from Texel-cross lambs ($n = 3$), with patent *H. contortus* infection, diluted 1:2 with sterile PBS- containing 0.05 % EDTA (Sigma-Aldrich) and layered on top of a 70 % Percoll gradient (25 ml, Sigma-Aldrich). The sample was centrifuged (20 min, 400 × *g*, 4 °C). Within the gradient, the eosinophil-enriched layer appeared in between the peripheral blood mononuclear cell- (PBMC) and the PMN-rich layer. Eosinophils were withdrawn by aspiration in a sterile Pasteur pipette, re-suspended in 25 ml PBS-EDTA and washed in PBS (400 × *g*, 2 × 5 min, 4 °C). The cells were re-suspended in culture medium [RPMI 1640, 10 % heat inactivated foetal calf serum (hiFCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol, all Sigma-Aldrich]. Eosinophil purity was determined by light microscopy of cells stained with Diff-Quick and haematoxylin-eosin staining after cytocentrifugation onto glass slides. Eosinophil counting was performed in a Neubauer haemocytometer chamber. Eosinophils were incubated at 37 °C and 5 % CO_2 atmosphere for at least 30 min before use. The viability of eosinophils was tested by using the trypan blue exclusion test (Sigma-Aldrich). Eosinophil-enriched samples had 30 % eosinophils.

Scanning electron microscopy (SEM)

Bovine PMN ($n = 3$, 5×10^5) were co-cultured with vital ensheathed L_3 of *H. contortus* (100 larvae/sample) on poly-$_L$-lysine (Sigma-Aldrich) pre-coated coverslips (60 min, 37 °C). After incubation, the samples were fixed in 2.5 % glutaraldehyde (60 min, RT, Merck), post-fixed in 1 % osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and spayed with gold. Thereafter, samples were examined with a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

Visualization of NETs and detection of histones, neutrophil elastase (NE) and myeloperoxidase (MPO) in *Haemonchus contortus*-induced NETs

Bovine PMN ($n = 3$, 5×10^5) were co-cultured with vital ensheathed *H. contortus* L_3 (100 larvae/sample) on poly-$_L$-lysine-treated coverslips (60 min, 37 °C). Thereafter, the samples were fixed (overnight, 4 % paraformaldehyde, on ice, Merck) and stored at 4 °C until further use. The experiments were performed in duplicates. In each set of data, 2 coverslips were used per condition and the entire coverslip was further analysed. NET structures were visualized by staining extracellular nucleic acid with Sytox Orange® as previously recorded [43–45]. For the detection of histones, MPO and NE within NET structures the following specific antibodies were used: anti-histone H1, H2A/H2B, H3, H4 monoclonal antibody [mouse clone H11-4, 1:1000, 1 h, RT, Merck Millipore], anti-MPO antibodies (rabbit polyclonal anti-MPO, Alexa Fluor 488, 1:200, 24 h, RT, ABIN906866, antibodies-online.com) and anti-NE antibodies (rabbit polyclonal anti human NE, 1:200, 24 h, RT, AB68672, Abcam). Prior to antibody exposure, the samples were blocked with bovine serum albumin (30 min, BSA 2 %, Sigma-Aldrich). Following antibody exposure, the samples were washed twice with PBS and incubated in respective secondary antibody solutions [Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (both from Life Technologies) 30 min, 1:500, RT]. Finally, the samples were stained with Sytox Orange® (S-11368, Invitrogen, 1:1000, 15 min in the dark), washed with PBS and mounted in anti-fading buffer (Mowiol®; Sigma-Aldrich). The visualization of NETs based on co-localized extracellular DNA staining and histone-, MPO- and NE-derived signals was achieved by using an inverted Olympus IX81® fluorescence microscope.

Visualization of eosinophil-derived *Haemonchus contortus*-induced ETs

Co-culture of ovine eosinophils ($n = 3$, 1×10^6) with either ensheathed or exsheathed *H. contortus* L_3 (150 larvae/sample each) was carried out on poly-$_L$-lysine-treated coverslips (60 min, 37 °C). After the incubation period, the samples were fixed (overnight, 4 % paraformaldehyde, on ice). Eosinophilic ET structures were visualized by staining extracellular DNA with Pico Green® (Life Technologies, 1:200 diluted in 10 mM Tris/1 mM EDTA) according to [45]. Moreover, eosinophilic granules were labelled with alcoholic Eosin Y® solution (10 s, RT, Sigma-Aldrich) and thereafter washed twice with sterile PBS. The cells were then mounted in ProLong® anti-fading reagents (Life Technologies). The visualization of eosinophil-derived ETs was achieved using an inverted Olympus IX8® phase contrast/fluorescence microscope.

Quantification of *Haemonchus contortus*-induced NETs

Bovine PMN ($n = 3$; 5×10^5 cells/200 μl) were exposed either to vital ensheathed larvae (40 larvae/sample) or heat-inactivated (60 °C, 60 min) larvae (40 larvae/sample) in RPMI 1640 medium (1 % PS, without phenol red) for 60 min at 37 °C in 96-well flat-bottom plates (Nunc). Thereafter, micrococcal nuclease was added (5 U/well, New England Biolabs) and incubated for 15 min at 37 °C. Then the samples were centrifuged ($250 \times g$, 5 min) and the supernatants (100 μl/well) were transferred to a 96-well flat-bottom plate (Nunc). The samples were stained by Pico Green® (50 μl/well, 1:200 in 10 mM Tris/1 mM EDTA) and NET formation was quantified by spectrofluorometric analysis at an excitation wavelength of 484 nm and emission wavelength 520 nm using an automated plate monochrome reader (Varioskan Flash®; Thermo Scientific). For negative controls, PMN in plain medium were used. Stimulation of PMN with zymosan (1 mg/ml, Invitrogen) served as positive control. For the quantification of the different types of NETs, bovine PMN ($n = 3$, 5×10^5) were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to *H. contortus* L_3 (20 larvae/sample) in 300 μl RPMI medium (1 % PS, without phenol red) for 30 min and 1 h at 37 °C. Thereafter NET formation was detected via fluorescence microscopy analysis using an anti-histone H1, H2A/H2B, H3, H4 monoclonal antibody [mouse clone H11-4, 1:1000, 1 h, RT] jointly with a secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG). Moreover, for staining extracellular DNA, Sytox Orange® (S-11368, Invitrogen, 1:1000, 15 min in the dark) was used.

Entrapment assay of *Haemoncus contortus* L_3

Bovine PMN ($n = 3$, 5×10^5) were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to ensheathed *H. contortus* L_3 (100 larvae/sample) in 300 μl RPMI medium (1 % PS, without phenol red, Sigma-Aldrich) for 30 and 60 min at 37 °C. Thereafter, the coverslips were fixed (4 % paraformaldehyde, overnight on ice) and PMN-entrapped larvae were counted by using an inverted DMIRB® phase-contrast microscope (Leica). Larvae were considered as entrapped when *agg*NET or *spr*NET were in contact with the larvae. The data are expressed as percentage of entrapped L_3 relative to the total amount of *H. contortus* L_3.

NET-associated larvicidal assay

Bovine PMN ($n = 3$, 5×10^5) were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to ensheathed *H. contortus* L_3 (50 larvae/sample) in 300 μl RPMI medium (1 % PS, without phenol red) for 24, 36 and 48 h at 37 °C. Thereafter, DNase I (Sigma Aldrich) was added to the coverslips to resolve NET structures and larval survival was determined microscopically based on the presence or absence of larval motility. In addition, parasite viability was evaluated by using the trypan blue exclusion test (1:10 dilution, Sigma-Aldrich). For reference samples, the equal number of non-PMN-exposed larvae was used.

Identification and quantification of aggregated NETs (*agg*NETs) induced by *Haemonchus contortus* L_3

Bovine PMN ($n = 3$, 5×10^5) were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to both, ensheathed and exsheathed *H. contortus* L_3 (50 larvae/sample) in 300 μl RPMI medium (1 % PS, without phenol red) for 1 h at 37 °C. Thereafter, the samples were fixed (4 % paraformaldehyde, overnight, on ice). Each sample was analysed microscopically using an inverted phase-contrast microscope (DMIRB®, Leica). *Agg*NETs were defined as clusters of NET-like structures with a "ball of yarn" morphology (see Fig. 2f red arrow) being larger than 50 μm in diameter. Within each sample, all structures with the described characteristics were counted. For negative controls, PMN in plain medium was used. Stimulation of PMN with zymosan (1 mg/ml) served as positive controls as described elsewhere [45].

NET inhibition assays

For NET inhibition assays the following inhibitors were used according to [46] and [39]: the NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 μM, Sigma-Aldrich), the MPO inhibitor 4-Aminobenzoic acid hydrazide (ABAH, 100 μM, Calbiochem) and the NE inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone (CMK, 1 mM, Sigma-Aldrich). PMN ($n = 3$, 5×10^5) were pre-incubated with respective inhibitors (30 min, 37 °C, 5 % CO_2) prior to exposure to vital ensheathed L_3 (80 larvae/sample, 60 min). For no-inhibitor controls, non-treated PMN were exposed to larvae. For negative controls, PMN in plain medium were used. Stimulation of PMN with zymosan (1 mg/ml) served as positive control. NET formation was quantified as described above.

Haemonchus contortus-induced NETs under acidic conditions

In order to verify whether NET formation occurs in abomasal acidic pH conditions, the cell culture medium (RPMI 1640) was adjusted to different acidic pH conditions: 2.0, 2.5, 3.0 and 3.5 prior to NET assays. For controls a pH of 7.0 was applied. Bovine PMN ($n = 3$, 5×10^5) were seeded on poly-$_L$-lysine pre-coated coverslips and exposed to *H. contortus* L_3 (20 larvae/sample) in 300 μl RPMI medium (1 % PS, without phenol red) for 1 h at 37 °C. Thereafter NET formation was detected using a fluorescence microscope as previously described using an anti-histone H1, H2A/H2B, H3, H4 monoclonal antibody [mouse clone H11-4, 1:1000, 1 h, RT] jointly with a secondary antibody (Alexa Fluor® 488 goat anti-mouse IgG). Moreover, for staining extracellular DNA, Sytox Orange® (S-11368, Invitrogen, 1:1000, 15 min in the dark) was used.

Statistical analysis

By using normal distribution of the data, co-culture/stimulation conditions were compared by one- or two-factorial analyses of variance (ANOVA) with repeated measures. Differences were regarded as significant at a level of $p \leq 0.05$ and analysed by GraphPad Prism® programme.

Results

Haemonchus contortus L_3 induce different types of NETs

Scanning electron microscopy (SEM) analyses revealed that exposure of bovine PMN to ensheathed third-stage larvae of *H. contortus* triggers the formation of morphologically different NETs. Typically "diffuse" NETs (*diff*NETs) are composed of a complex of extracellular decondensed chromatin decorated with antimicrobial proteins with globular and compact form with a size of 25–28 nm diameter. Moreover, "spread" NETs (*spr*NETs) were observed consisting of smooth and elongated web-like structures of decondensed chromatin and antimicrobial proteins composed exclusively by thin fibers with a diameter of 15–17 nm (Fig. 1a, b). Furthermore, a marked presence of so-called "aggregated" NETs (*agg*NETs), according to [47] were displayed as large clusters of NET-like structures with a "ball of yarn"-like clumpy and massive appearance involving a high number of PMN. These conglomerates of NETs were composed by extracellular chromatin studded with phagocyte-granular proteins with sizes larger than 50 μm in diameter. *Spr*NETs and *agg*NET formation led to larval entrapment and, in some cases of *agg*NETs, to an almost entire coverage of the larvae (Fig. 1c, d). Overall, both forms of NETs, i.e. *spr*NET and *agg*NET, appeared capable of firmly ensnaring the larvae (see Additional files 1, 2: movies 1 and 2). Furthermore, we also observed a significant reduction in larval forward-motility due to an anchor-like effect of fine NET structures (*spr*NETs), which are connected to the *agg*NETs entrapping larvae and clearly hampering larval motility (see Additional files 1, 2: movies 1 and 2).

Fluorescence-based analyses confirmed the classical components of NET formation. As such, staining by Sytox Orange® (Fig. 2a, d, g) proved the DNA nature of extracellular NET-like structures being formed by PMN after *H. contortus* L3 exposure. In addition, MPO-, NE- and histone- (H1, H2A/H2B, H3 and H4) positive signals were detected in co-localization with DNA-positive NET structures (Fig. 2c, f, i). Interestingly, we also observed different forms of NETs, i.e., *spr*NETs (Fig. 2c, f, white arrows), *agg*NETs (Fig. 2f, red arrows), diffused NETs (*diff*NET, Fig. 2f, yellow arrow) and clasp-like NET formation (Fig. 2 i, red arrow) sticking mainly to the anterior part of the larvae, consistent with observations made by SEM analyses (see Fig. 1).

Quantitative analyses on total NET formation showed that ensheathed and exsheathed L_3 significantly induced NETosis in bovine PMN ($p \leq 0.05$, Fig. 3a). As expected, this reaction was significantly and almost entirely resolved by DNase I treatment in both, exsheathed and ensheathed larvae (larvae vs. DNase I: $p \leq 0.05$, Fig. 3a). Furthermore, quantitative assessment of *agg*NETs revealed that exposure of PMN to ensheathed L_3 significantly induced *agg*NET in bovine PMN when compared to negative controls ($p \leq 0.01$, Fig. 3b). Comparative approaches revealed that ensheathed larvae triggered NETosis significantly more than exsheathed ones ($p \leq 0.05$, Fig 3b), indicating that surface cuticle components are able to effectively trigger NETosis. Furthermore, analysis of the frequency of different NET types induced by *H. contortus* was performed for different time spans (30 and 60 min). As outcomes of this experiment, at both time points the three different types of NETs were observed to be significantly

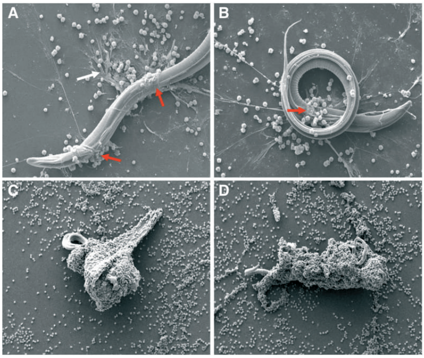

Fig. 1 Co-culture of *Haemonchus contortus* larvae and bovine PMN triggers NET formation. Scanning electron microscopy (SEM) analysis revealed different forms of NET formation induced by *H. contortus* L_3 in bovine PMN contributing to larval immobilization. **a**, **b**: *H. contortus* trigger spread NET formation (*spr*NET) **c**, **d**: *H. contortus* trigger aggregated NET formation (*agg*NET)

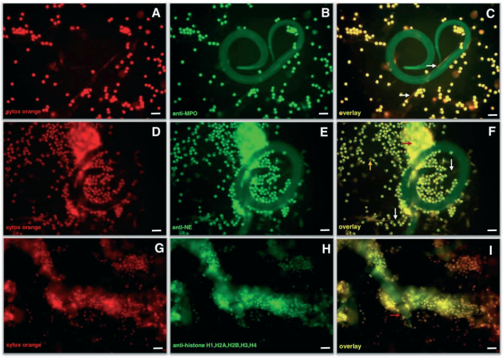

Fig. 2 Co-localization of exracellular DNA with histones, MPO and NE in *H. contortus*-triggered NET structures. Co-cultures of bovine PMN with *H. contortus* L_3 were fixed, permeabilized, stained for DNA using Sytox Orange and probed for histones using anti-histone H1, H2A/H2B, H3, H4 monoclonal antibodies, for MPO using anti-MPO and for NE using anti-NE antibodies. **a**, **d**, **g**: Extracellular DNA stained with Sytox Orange (red). **b**, **e**, **h**: MPO, NE and histones staining within NET structures (green), respectively. **c**, **f**, **i**: Overlay of NET-DNA with MPO, NE, and histones respectively. Note that distinct forms of NETs were observed in fluorescence microscopy: *spr*NET (**c**, **f** white arrows), *agg*NET (**f** red arrow) *diff*NET (**f** yellow arrow) and *clasp-like* NET (**i** red arrow). Photomicrographs represent exemplary images of 3 independent experiments

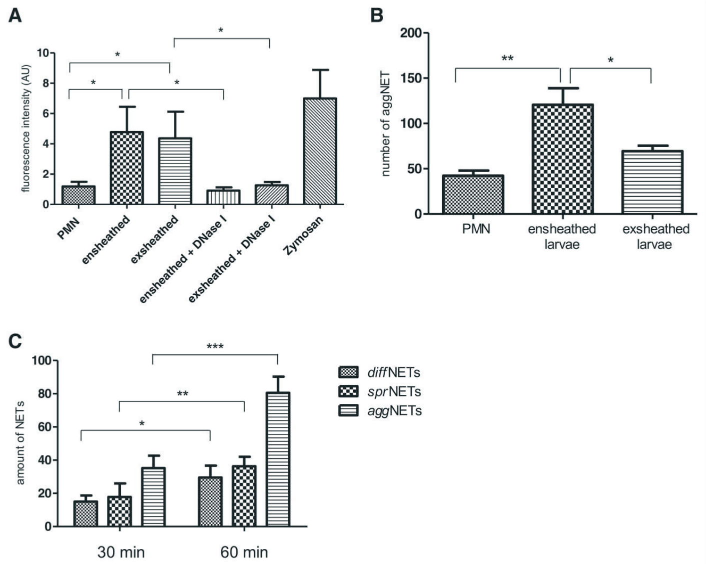

Fig. 3 *Haemonchus contortus* L_3 induce the formation of *agg*NET upon exposure to bovine PMN. **a**: Bovine PMN were confronted with ensheathed and exsheathed *H. contortus* L_3. NET measurement was performed using PicoGreen-derived fluorescence intensities and DNase I was applied 15 min before the end of incubation to the co-culture of PMN with both ensheathed and exsheathed larvae. **b**: Bovine PMN were confronted with ensheathed and exsheathed *H. contortus* L_3 and *agg*NET were counted using phase-contrast microscopy based on a size larger than 50 mm diameter. As negative control, PMN in plain medium were used. PMN exposed to zymosan (1 mg/ml) served as positive control. **c**: Bovine PMN ($n = 3$, 5×10^5) were exposed to *H. contortus* L_3 (20 larvae/sample) for 30 and 60 min at 37 °C. For the quantification of different types of NETs, fluorescence microscopy analysis was performed using an anti-histone H1, H2A/H2B, H3, H4 monoclonal antibody, [mouse clone H11-4, 1:1000, 1 h, RT], jointly with a secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse IgG). Extracellular DNA was analysed via Sytox Orange® staining (S-11368, Invitrogen, 1:1000).

increased ($p \leq 0.001$, Fig. 3c). Interestingly, at both time points predominantly *agg*NET were detected more frequently. Most probably, sprNET and diffNET are stages of the same process being *agg*NETs the latest stage. The difference of *diff*NET and *spr*NET formation along the two time points exhibited a significant increased ($p \leq 0.05$ and $p \leq 0.01$, respectively; Fig. 3c) showing overall a clear time-dependent pattern.

Haemonchus contortus L_3-triggered NETosis occurs irrespective of parasite viability or pH environmental conditions

In order to analyse the role of *H. contortus* L_3 viability and integrity in parasite-induced NETosis, either viable or heat-inactivated larvae were used for NET induction. The quantitative assessment of NETs revealed that both preparations equally induced NETosis (viable and heat-inactivated larvae vs. control: both $p \leq 0.05$; Fig. 4a), i.e. the viability or integrity of larvae is irrelevant for NET induction.

Given that, following oral infection, ensheathed L_3 of *H. contortus* reach the acidic environment of the abomasum and, after exsheathment, infect abomasal glands, we additionally tested whether NETosis also occurs in acidic conditions by testing different pH ranges (2.0, 2.5, 3.0 and 3.5) of the culture medium. Overall, even under low acidic conditions NETs were significantly formed in response to *H. contortus* L_3 (pH 2.0 vs. control: $p \leq 0.05$; pH 2.5 vs. control: $p \leq 0.05$; pH 3 vs. control: $p \leq 0.05$;

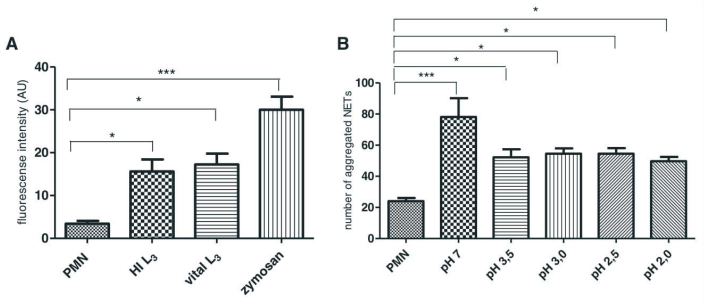

Fig. 4 *H. contortus*-triggered NETosis is independent on the parasite integrity and low pH conditions. **a**: Bovine PMN were exposed to vital ensheathed larvae, heat-inactivated larvae or parasite-free controls. NET measurement was performed using PicoGreen-derived fluorescence intensities. **b**: Bovine PMN were exposed to *H. contortus* L_3 at different pH conditions (pH 2.0–3.5) and NET measurement was performed using Sytox Orange®-derived fluorescence intensities with an anti-histone H1, H2A/H2B, H3, H4 monoclonal antibody, [mouse clone H11-4, 1:1000, 1 h, RT], jointly with a secondary antibody (Alexa Fluor® 488 Goat Anti-Mouse IgG). As positive control, stimulation of PMN by zymosan (1 mg/ml) was used. For negative control, PMN in plain medium were used

pH 3.5 vs. control: $p \le 0.05$, Fig. 4b). However, at pH 7 a significantly stronger reaction was observed compared to the negative control ($p \le 0.001$) and lowering of the pH to 2.0 led to a reduction of NET formation of approximately 30 % when compared to pH 7 (Fig. 4b).

NETs entrap *Haemonchus contortus* L_3 in a time-dependent and non-lethal manner

In order to evaluate time dependency of *H. contortus*-mediated NET entrapment, bovine PMN were incubated with *H. contortus* for different time spans. As a result, significant differences were observed between 30 and 60 min of exposure ($p \le 0.01$, Fig. 5) leading to 46.9 % and 75.8 % entrapment of larvae, respectively, indicating that this process is time-dependent.

To elucidate whether *H. contortus* L_3 may be killed by NETs, parasite survival was evaluated by microscopic measures (motility + trypan blue exclusion test) following long-term PMN exposure. Overall, *H. contortus* larvae appeared reduced in their motility since only 80 % of the larvae displayed active motility compared to 100 % in non-exposed control larvae (data not shown). However, trypan blue exclusion test neither revealed damage of the larval cuticle nor trypan blue up-take indicating that the larvae were still alive after exposure to NETs.

Haemonchus contortus-triggered NET formation is NADPH oxidase and MPO-dependent

To elucidate the role of different NET-associated molecules in *H. contortus* L_3-triggered NETosis, inhibition experiments were performed using blockers of NADPH oxidase (DPI), MPO (ABAH) and NE (CMK). Pretreatments of PMN with DPI, CMK and ABAH resulted in a significant reduction of parasite-induced NET formation when compared with controls lacking inhibitors ($p \le 0.05$; Fig. 6), indicating a key role of NADPH oxidase, NE and MPO in *H. contortus* L_3-mediated NETosis.

Haemonchus contortus L_3 induce eosinophil-derived ETs

Ovine eosinophils were isolated at a purity of 30 % as demonstrated in Diff-Quick-stained cytospins (Fig. 7a). Given that the proportion of eosinophils in these preparations was not high enough to perform reliable quantitative assays on ETosis and further enrichment attempts failed, we made attempts to detect ET formation microscopically. Following exposure to *H. contortus* L_3, several eosinophils (indicated by their red granules) were found in direct contact to the larvae (Fig. 7b). In addition, some of these clearly extruded PicoGreen®-positive DNA onto the larval surface indicating that these cells performed ETosis (Fig. 7b).

Discussion

The present study delivers first evidence on the release of ETs as part of the early innate immune response of PMN and eosinophils to third stage larvae of the nematode *H. contortus*, an important and pathogenic species in the global veterinarian sector [6, 48–50]. NETs mainly consist of chromatin, nuclear histones (H1, H2A/H2B, H3, H4) and granular components, such as NE, MPO,

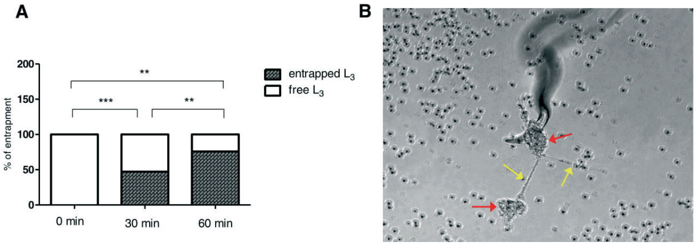

Fig. 5 Entrapment of *H. contortus* L_3 within NET structures is time-dependent. **a**: Bovine PMN were co-cultured with *H. contortus* L_3. Entrapped larvae were counted microscopically under different time points. **b**: Phase contrast image showing the anterior part of a L_3 being entrapped in NET structures. Red arrows: *agg*NET. Yellow arrows: anchor-like effect of fine NET structures mainly originating from *agg*NETs

lactoferrin and gelatinase [16, 51, 52]. We here confirmed the typical characteristics of NETs by co-localization experiments on extracellular DNA and histones, NE and MPO. Furthermore, functional inhibition experiments proved the relevance of NADPH oxidase, NE and MPO in *H. contortus*-induced NETosis since treatments with specific inhibitors significantly reduced parasite-triggered NET formation. This is in accordance to other reports on parasite-induced NET formation [30, 31, 39, 40, 45, 53].

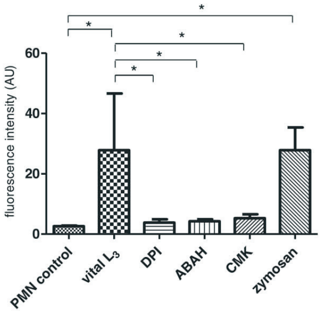

Fig. 6 Blockage of *Haemonchus contortus* L_3-triggered NETosis via inhibitors of NADPH oxidase, MPO, NE. NET inhibition assays were performed by pre-incubating PMN with inhibitors of NADPH oxidase (DPI, 10 μM), NE (CMK, 1 mM), MPO (ABAH, 100 μM) prior to *H. contortus* exposure. NET measurement was performed using PicoGreen-derived fluorescence intensities. For positive controls, zymosan treatment of PMN was used (1 mg/ml). PMN in plain medium served as negative control

During haemonchosis, infective *H. contortus* L_3 must actively migrate through the lumen of the reticulum, the rumen and the abomasum in order to infect gastric glands of the latter organ. By performing this migration, *H. contortus* larvae become potential targets of leucocytes of the innate immune system, which are known to actively migrate into the intestinal lumen [54–57]. This encounter drives the initiation of innate immune reactions as previously demonstrated by O'Connell et al. [58] showing a CXCR2/IL-17 dependent neutrophil recruitment in response to nematode infections. Referring to the infection biology of *H. contortus*, it has to be considered that exsheathed larvae will reside within the abomasum under low pH conditions. However, the current data on pH-dependent parasite-triggered NET formation clearly indicate that NETosis also occurs in these acidic conditions.

So far, several reports exist on protozoan-induced NET formation [31, 35–40, 45, 53, 59] whilst only few data are available on metazoan-triggered NETosis (for overview see Hermosilla et al., [26]). As such, *S. japonicum* was recently identified as NET-inducer in vitro and in vivo [41]. Another report demonstrated the capability of the nematode *Strongyloides stercoralis* to trigger NETosis [15]. In agreement with the current study, a NET-based ensnarement of *S. stercoralis* larvae was also demonstrated [15].

As an interesting feature, observed in this study was the release of different types of NETs, i.e. of *diff*NETs, *spr*NETs and *agg*NETs upon contact with *H. contortus* larvae, with all of them promoting a time-dependent ensnarement of the larvae. Given that no parasite killing was observed even within a prolonged period of 24 h, the tight immobilization of larvae appears as the key mechanism of *H. contortus*-triggered NET formation.

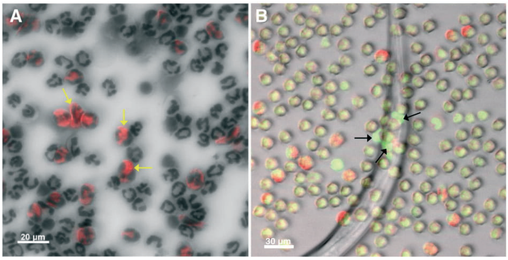

Fig. 7 *Haemonchus contortus* L_3 induce ETs in ovine eosinophils. **a**: Eosinophil population from ovine blood showing 30 % of enrichment as detected in Diff-Quick-stained cytospin preparations. **b**: Eosinophil-derived ETs being released towards an *H. contortus* L_3 [see co-localization of eosin granules (red) with eosinophil-derived extracellular DNA (green)]

Therefore, we hypothesize that even though NETs themselves do not appear capable of killing *H. contortus* larvae, they may entrap larvae and prevent the active migration into the gastric glands of the abomasum and expose them to other leucocytes.

It is intriguing that parasite-mediated NET induction proved to be independent of the ensheathment status of the larvae, since both, ensheathed and exsheathed larvae equally triggered NET formation. So far, it is unclear how PMN recognize the larvae in terms of NETosis and which parasite-derived molecules are involved this process. However, the data on ex- and ensheathed larvae suggest that the molecules responsible are present on the cuticle and the surface of the larval body. In contrast, the fact that ensheathed larvae induce *agg*NETs more prominently than exsheathed ones also indicates the involvement of different parasite-derived triggers. However, PMN-derived sensing of the larvae may also be a matter of size since Branzk et al. [60] reported on the ability of PMN to distinguish between small and large sized pathogens and to selectively release NETs in response to large pathogens [60]. In addition, other physical properties of particles such as shape and rigidity can influence the response of phagocytes [61]. This would explain the induction of NET formation by large metazoan parasites such as *H. contortus* larvae, and makes biological sense as phagocytosis is probably ineffective against large multicellular pathogens. We hypothesize that a function of PMN-released *agg*NETs may be to prevent proper larval exsheathment in vivo. A blockage of larval exsheathment would abrogate parasite infections and thereby tremendously affect the outcome of disease. We also consider that ETs might serve to localize anthelmintic products of neutrophils and eosinophils in the near vicinity of parasites. As such, neurotoxin (eosinophil-derived neurotoxin or EDN) released by eosinophils would be more effective if localized near the parasite. The release of EDN may restrict motility of the larvae thereby preventing the shedding process and allowing adhering eosinophils to discharge their toxic granule content directly on the larval surface [20, 62]. This might serve to enhance the effects of these molecules impacting on larval motility. Moreover, parasite killing may be more likely to succeed if these anthelmintic molecules remain concentrated in the near vicinity of the parasite. Furthermore, ETosis is not a unique feature of PMN [63] but is also attributed to other immune cells, such as macrophages [64], mast cells [27, 65], monocytes [30, 31] and eosinophils [29, 66]. In accordance, we provide evidence on *H. contortus-induced eosinophil-derived ETs (EETs). Meanwhile, in vivo studies with the nematode Nippostrongylus brasiliensis* [62] demonstrate the formation of similar 'ET like entities' in presence of eosinophil-rich leucocytes and complement. In this model, larvae recovered from skin air pouches were aggregated into large clumps that included numerous leucocytes. Whilst many larvae eventually escaped from these aggregations, in mice with pre-existing eosinophilia, subsequent larval migration to the lungs was impaired [62]. This suggests that ET, leucocytes, and especially eosinophils, may be involved in damaging larvae in the very earliest stages of infection, such that the parasites are then more susceptible to entrapment and killing elsewhere in the pre-lung phases of migration through the host [62]. This may be of high relevance since eosinophils have been shown to adhere to *H. contortus* larvae both in vitro and in vivo and to efficiently kill these larvae [20]. Interestingly, Yousefi et al. [29] demonstrated that eosinophils release EETs of mitochondrial origin without undergoing cell death. However, whether *H. contortus*-triggered EETs also originating from mitochondria remains to be elucidated in the future. Nonetheless, our data strongly suggest EETs as an additional effector mechanism of eosinophils against *H. contortus*.

Conclusion

Overall, we postulate that NETs and EETs may limit the establishment of *H. contortus* within the definitive host by immobilizing the larvae and hampering them from migration to the site of infection. NETs and EETs may further facilitate the exposure of entrapped large sized parasites to other immunocompetent cells, such as monocytes and macrophages exhibiting larvicidal effects. In consequence, *H. contortus*-mediated ET formation will also have an impact on the in vivo situation and influence the outcome of the disease.

Additional file

Additional file 1: Parasite entrapment by *agg*NETs and the anchor-like effect of fine NET structures (*spr*NETs) derived from *agg*NETs. (AVI 474248 kb)

Additional file 2: Significant reduction in larval motility due to an anchor-like effect of fine NETs originated from *agg*NETs. (AVI 398927 kb)

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TMC and MCRR performed the NET quantification and in vitro experiments including *Haemonchus contortus* exsheathment and visualization of NETs. TMC and MCRR also participated in data analysis, design and coordination of the study and preparation of the manuscript. LMRS performed in vitro experiments including aggregated NET quantification and contributed in design and data analysis. UG and GM participated in preparation of samples and performance of scanning electron microscopy analysis. TMC, LMRS and TNM contributed in the isolation of eosinophils and ETs visualization in fluorescence microscopy. TNM also contributed in the correction of the manuscript. CH and AT participated in study design and coordination, data analysis and revision of the manuscript. In addition they contributed to blood collection for isolation of bovine PMN. All authors read and approved the final manuscript.

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Neutrophil extracellular traps in the intestinal mucosa of *Eimeria*-infected animals

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ABSTRACT

Objective: To investigate the presence of neutrophil extracellular traps (NETs) *in vivo* by analysing intestinal sections from experimentally *Eimeria bovis*- and naturally *Eimeria arloingi*-infected animals.

Methods: Intestinal samples of *Eimeria arloingi*- and *Eimeria bovis*-infected animals were analysed by using immunohistochemical and fluorescence approach by using monoclonal antibodies.

Results: Classical NET components were confirmed by co-localization of extracellular DNA being decorated with neutrophil elastase and histones in *Eimeria*-infected tissue samples. Here, extrusion of NETs was exclusively detected in intestinal polymorphonuclear neutrophils infiltrating *Eimeria*-infected sites. *In vivo* NETs were either found in close proximity or in direct contact to different *Eimeria* stages suggesting a stage-independent process. NETs were also found within the gut lumen driven by polymorphonuclear neutrophils that were contacting released oocysts.

Conclusions: We postulate that NETs might play an important role in innate defence reactions in coccidiosis therefore significantly altering the outcome of infection.

1. Introduction

Coccidiosis is a protozoan disease caused by different species of the genus *Eimeria* which causes considerable animal health problems and economic losses in the ruminant industry worldwide due to severe clinical enteritis and/or typhlocolitis [1–7]. Ruminant *Eimeria* infections with pathogenic species, such as *Eimeria bovis* (*E. bovis*) in cattle or *Eimeria arloingi* (*E. arloingi*) in goats, commonly induce clinical disease only in young animals, since homologous reinfections generally are under immunological control [8]. However, relatively little is known on early host innate immune reactions against *Eimeria* infections contributing to protection of animals through the interaction with cells of the cellular adaptive immune response [9–11]. In this context, polymorphonuclear neutrophils (PMN) play a key role since they are the most abundant cells in the blood and the first ones to be recruited to the site of infection [12–14]. PMN own several effector mechanisms to combat and eventually kill pathogens, such as phagocytosis, reactive oxygen species production, the release of antimicrobial peptides/proteins and the formation of neutrophil extracellular traps (NETs) [12,14,15]. NETs are generally released after PMN cell death and are primarily situated in the extracellular space [16]. The formation of NETs (NETosis) is a NADPH oxidase (NOX)-dependent mechanism [15,17–22], which leads to the extrusion of a mixture of nuclear and cytoplasmic granule contents leading to the formation of DNA-rich web-like structures being decorated with histones (H1, H2A/H2B, H3, H4) and granular effector molecules, such as neutrophil elastase (NE), lactoferrin, pentraxin, myeloperoxidase (MPO) and others [14,16,19]. Unlike NOX-dependent NETosis, NOX-independent NETosis is accompanied by a substantially lower level of ERK activation and rather moderate level of Akt activation,

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whereas the activation of p38 is similar in both pathways [23]. Irrespective of NOX-dependency, pathogens may either be immobilized within sticky DNA fibres or be killed via the local high concentration of effector molecules. Interestingly, Yipp *et al.* recently demonstrated that PMN, which undergo NETosis without cell lysis are still viable and retain their ability to phagocytise bacteria [24]. In agreement with these findings, PMN also seem to be able to release NETs of mitochondrial origin which are of smaller size than the ones originating from classical NETosis [25]. So far, NET formation was described to be induced by different protozoan parasites *in vitro*, such as *Plasmodium falciparum* [26], *Leishmania* spp. [27], *E. bovis* [22,28], *Toxoplasma gondii* (*T. gondii*) [29–31], *E. arloingi* [5], *Besnoitia besnoiti* (*B. besnoiti*) [20], and *Cryptosporidium parvum* [32]. In addition, monocyte-derived extracellular traps (ETs) have recently been reported to be formed in response to tachyzoites of *B. besnoiti* and *T. gondii in vitro* [29–31]. Recent analyses on *Eimeria*-induced NETosis confirmed its dependency on NOX, NE and MPO activities [5,22,28]. More detailed investigations on molecular mechanisms of *E. bovis*-triggered NETosis have demonstrated that this cell death pathway is CD11b-, ERK1/2-, p38-, mitogen-activated protein kinase- and Ca^{++}-dependent [22].

There is a vast amount of data on the *in vivo* role of NETs in various bacterial infections [12,33], in metabolic [34,35], reproductive [36,37] and autoimmune disorders [38–40], and in cancer progression [41,42]. However, *in vivo* data on NETs regarding parasitic diseases are scarce. The first evidence of parasite-induced NETs *in vivo* came from *Plasmodium falciparum*-infected children [26]. Detailed analyses of cutaneous *Leishmania* lesions from human patients in Brazil also proved the *in vivo* existence of *Leishmania*-triggered NETs as demonstrated by the simultaneous presence of extracellular DNA and histones [27]. Abi Abdallah *et al.* provided first indications on the *in vivo* relevance of NETs against *T. gondii* in a murine model of infection [30].

The aim of the current study was to show *in vivo* evidence on NETosis in response to *Eimeria* infections. Typical NET structures were found in gut tissue sections of both *E. bovis*- and *E. arloingi*-infected animals indicating that this effector mechanism naturally occurs during primary *Eimeria* infections. However, the actual efficacy of this effector mechanism *in vivo* remains to be elucidated in *Eimeria*-infected animals.

2. Materials and methods

2.1. Intestinal samples of E. arloingi- and E. bovis-infected animals

A two-month-old Serpentina goat kid of the province of Alentejo, Portugal, which died due to a severe natural *E. arloingi* infection served as donor for intestinal samples [5]. In the case of *E. bovis*, intestinal gut samples originating from experimentally *E. bovis*- (strain H) infected calves, which were published before [8], were used. Caprine and bovine intestinal gut samples (jejunum, ileum, caecum, colon) were withdrawn for immediate fixation [4% formaldehyde in phosphate-buffered saline (PBS), 24 h] and embedded in paraffin according to procedures described by Sühwold *et al.* [8]. Then 3–5 μm cross-sections of formalin-fixed tissues were deparaffinized according to standard histological procedures. Thereafter, the samples were exposed to descendant concentrations of isopropanol (90%, 80%, 70%, and 50%, 3 min each) and re-hydrated in distilled water (3 min). The samples were incubated in haematoxylin solution (Sigma–Aldrich) for 90 s, then washed 5 times in bi-distilled water and placed for 5 min in tap water. Afterwards, the samples were washed in bi-distilled water again, stained with eosin staining solution (Sigma–Aldrich, 30 s) and washed again twice in bi-distilled water. Finally, the samples were dehydrated in ascending isopropanol concentrations (70%, 80% and 90%, 30 s each), incubated twice in isopropanol (100%, 2 min) and twice in xylol (100%, 2 min). Finally, all samples were mounted with Pertex™ (Leica Biosystems) for further investigations.

2.2. Immunohistochemical detection of NETs

For the immunohistochemical detection of NETs, paraffin-fixed sections were deparaffinized as previously described. For antigen-demasking, a heating treatment was performed. Therefore, slides were cooked in a steamer in 10 mmol/L Tris base (Sigma–Aldrich) and 1 mmol/L ethylene diamine tetraacetic acid solution (pH 9.0) (Sigma–Aldrich), for 15 min for caprine samples and 30 min for bovine samples. Thereafter, the samples were allowed to cool down for 20 min at room temperature and then washed thrice in PBS for 2 min. To inhibit endogenous peroxidase activity the sections were exposed to 1% H_2O_2 (Sigma–Aldrich, 30 min, room temperature), then washed thrice in PBS (2 min). Unspecific protein binding was excluded by treatment with 1% bovine serum albumin (BSA) (Sigma–Aldrich) and 0.1% sodium azide in PBS (Sigma–Aldrich) for 30 min at room temperature. Afterwards, the samples were incubated in primary antibody solution [anti-histone H3 (D1H2) XP® rabbit monoclonal antibody, No. 4499 (Cell Signaling); overnight, 4 °C, 1:100 dilution in blocking solution]. The samples were washed thrice in PBS and exposed to the secondary antibody [goat anti-rabbit immunoglobulin G (H + L) secondary antibody, horseradish peroxidase conjugate (Life Technologies); 1:50 in PBS, 1 h, room temperature]. For signal development the samples were exposed to 3.3′-diaminobenzidine (Sigma–Aldrich, 125 μg/mL, 10 min, room temperature) and then washed thrice with PBS. Counterstaining was performed in haematoxylin staining solution (Sigma–Aldrich, 1:5 in distilled water, 90 s). Thereafter the samples were washed (5 min, distilled water) and dehydrated in ascending isopropanol concentrations (50%, 70%, 80% and 90%, 30 min each), isopropanol 100% (2 × 2 min) and xylene (100%, 2 × 2 min). The samples were mounted in Pertex™ (Leica Biosystems). In order to test for unspecific NET formation a rat ileum tissue section was equally processed in parallel. Visualization was achieved and documented by using an inverted Olympus BX51® microscope equipped with a digital camera and an analySIS® software (Olympus).

2.3. Fluorescence-based detection of NETs

Fluorescence-based detection of NETs was performed according to von Köckritz-Blickwede *et al.* with some slight modifications [17]. Briefly, the samples were deparaffinized in xylene (Fisher Scientific, 3 × 10 min), 100% alcohol (Fisher Scientific, 2 × 5 min), 95% alcohol (2 × 5 min) and 70% alcohol (2 × 5 min). Thereafter, the samples were washed with

PBS (3 × 10 dips) and heated in a microwave (2 × 5 min in citrate buffer, pH 6.0, Dako, S2369). Afterwards, the samples were cooled for 20 min at room temperature, washed thrice with PBS and blocked with 2% BSA–PBS + foetal calf serum (Sigma–Aldrich, 45 min, room temperature). The samples were then exposed to primary antibody solution (rabbit anti-human NE, 1:500; AB68672, Abcam, 3 h, 4 °C, humidity chamber, 2% BSA–PBS). To avoid drying-out, the cross sections were covered with parafilm. Then the samples were washed four times with PBS and incubated in secondary antibody solution (Invitrogen, Alexa Fluor® 488 conjugated goat anti-rabbit antibodies, 1:500, 30 min, room temperature, humidity chamber, covered with parafilm). After four washings with PBS, the samples were mounted either in ProlongGold® with 4′,6-diamidino-2-phenylindole (DAPI) staining or in ProlongGold® after staining with Sytox Orange® (Invitrogen, 1:1 000, 5 min, room temperature, in the dark). The visualization of extracellular DNA and NE-positive signals was achieved using an inverted Olympus IX81® fluorescence microscope.

3. Results

Haematoxylin-eosin-stained sections of *E. bovis*- and *E. arloingi*-infected intestinal tissue samples showed a strong leukocytic mucosal infiltration, mainly composed of PMN, monocytes and eosinophils, into parasitised areas of the jejunum, ileum and caecum/colon. Some mucosal leucocytes were found in direct contact with the surface of infected host cells carrying different *Eimeria* stages such as oocysts (Figures 1A and 2C), macrogamonts (Figures 1B,C and 2A,B) and also at the periphery of developing macromeronts (Figure 2D). These features demonstrate that these immune cells are capable to effectively transmigrate into affected intestinal mucosa *in vivo*. Accordingly, the histopathology of both *Eimeria* infections exhibited a dramatic damage due to a high parasitic load alongside with a striking epithelial destruction and detachment (dysentery). PMN were even found within the intestinal lumen in close contact with extracellular *E. bovis* oocysts (Figure 1D).

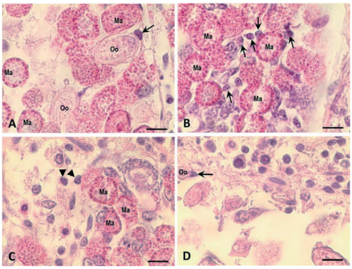

Figure 1. Haematoxylin-eosin staining of E. *bovis*-infected intestinal tissue.

A: Intestinal leucocyte contacting *E. bovis* oocysts (arrow). B and C: Intestinal leucocyte contacting *E. bovis* macrogamonts (arrows). D: Intestinal leucocyte contacting an *E. bovis* oocyst in lumen (arrow). Oo: Oocysts; Ma: Macrogamonts. Scale bars = 20 µm.

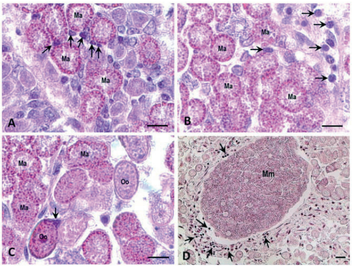

Figure 2. Haematoxylin-eosin staining of *E. arloingi*-infected intestinal tissue.

A and B: Leucocyte infiltration contacting *E. arloingi* macrogamont stages (arrows); C: Leucocyte infiltration contacting *E. arloingi* oocysts stages (arrows); D: *E. arloingi* macromeront being surrounded by leucocyte infiltration (arrows). Mm: Macromeront; Oo: Oocysts; Ma: Macrogamonts. Scale bars = 20 µm.

In addition, the co-localization of mucosal extracellular DNA with histones (H1, H2A, H2B, H3, H4) (Figure 3) and NE (Figure 4) in *Eimeria*-induced NETs corroborated the classical characteristics of NETs *in vivo*. Furthermore, sections from the jejunum revealed a strong influx of PMN into *Eimeria*-infected areas with some of them releasing NETs as seen by the co-localization of H3 and extracellular nucleic acids derived from dead PMN (Figure 5), making this feature distinguishable from non-NET-releasing PMN which retain their typical cellular morphology. According to this, a recent study supports the use of immunostaining with citrullinated histone-3 antibodies to identify NETs in tissue sections showing that nuclear NETs extensions display orientations in different planes, in contrast to the ones observed in nuclear crush smears [43]. In both *Eimeria* species infections, single PMN were found releasing H3-positive NET structures in close proximity to *Eimeria* stages (Figure 6B). Diffused as well as small NET types were extruded by caprine PMN infiltrating mucosal areas of *E. arloingi* replication. Overall, *in vivo* NET-associated results clearly confirm previous

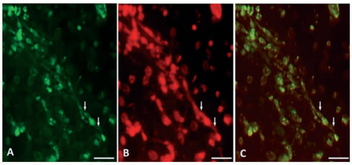

Figure 3. Co-localization of extracellular DNA and histones in E. arloingi-triggered NET structures in infected intestinal tissue.

Intestinal tissue (jejunum) sections from *E. arloingi*-infected animals were used for immunofluorescence analysis in order to identify NETs by (monoclonal) antibody-based detection of histones (H1, H2A, H2B, H3 and H4, in green). DNA was stained with Sytox orange (in red). A: Anti-histone staining of H1, H2A, H2B, H3 and H4; B: Sytox orange staining of DNA; C: Overlay of A and B. White arrows indicate NET structures being extruded from PMN. Scale bars = 20 µm.

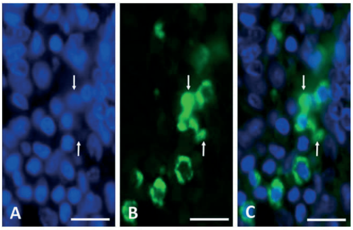

Figure 4. Co-localization of extracellular DNA and NE in *E. arloingi*-triggered NET structures in infected intestinal tissue.
Intestinal tissue (jejunum) sections from *E. arloingi* infected animals were used for immunofluorescence analysis in order to identify NETs by (polyclonal) antibody-based detection of NE (in green) in combination with DAPI staining (in blue) to identify nuclear and extracellular DNA. A: DAPI-stained DNA; B: NE staining; C: Overlay of A and B. White arrows indicate NET structures. Scale bars = 20 μm.

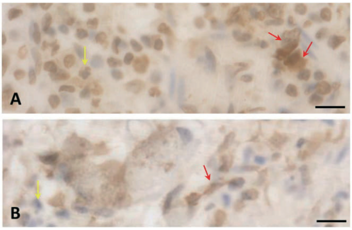

Figure 5. Histone detection in *E. bovis*- (A) and *E. arloingi*-infected (B) intestinal samples. NETs were identified by combining haematoxylin staining (in blue) with the (monoclonal) antibody-based detection of histone H3 [Cell Signaling, 1:100 (in brown)]. Red arrows indicate PMN releasing NET structures; yellow arrow shows inactive PMN. Scale bars = 20 μm.

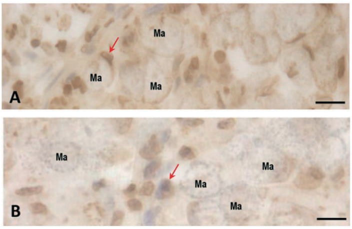

Figure 6. Histone detection in *E. arloingi*-infected intestinal samples.
A: PMN contacting *E. arloingi* macrogamont (arrow); B: PMN releasing NETs in close proximity to macrogamonts of *E. arloingi* (arrow). NETs were identified by combining haematoxylin staining (in blue) with the (monoclonal) antibody-based detection of histone H3 [Cell Signaling, 1:100 (in brown)]. Ma: Macromeront. Scale bars = 20 μm.

in vitro data on *E. bovis*- [10–22] and *E. arloingi*-triggered NET release [5], and their role as novel effector mechanism against these apicomplexan parasites.

4. Discussion

Early innate leucocyte-mediated reactions against bovine and caprine *Eimeria* parasites have scarcely been investigated in the past, although the first encounter between parasites and innate immune cells should be decisive for the subsequent outcome of infection [5,22]. PMN appear to play a pivotal role in ruminant *Eimeria*-triggered early host innate defence *in vivo* since this leucocyte population was identified in parasitized intestine of *E. bovis*- [44], *Eimeria ninakohlyakimovae*- [45] and *E. arloingi*-infected animals [5]. Detailed molecular investigations have revealed that PMN do not only interact directly with viable *E. bovis* stages and antigens, but also serve as an early source of immunomodulatory molecules, such as chemokine (C–C motif) ligand 3 and tumour necrosis factor α [10], which support monocyte/macrophage infiltration and activation [46]. PMN were also shown to adhere to *E. bovis*-infected endothelium under physiological flow conditions [47], and their phagocytic and oxidative burst activities were found enhanced in response to sporozoites of *E. bovis in vitro* and *ex vivo* [10]. Furthermore, *E. bovis*- and *E. arloingi*-triggered NETosis was reported as additional PMN effector mechanism *in vitro* [5,22,28].

The current *in vivo* data indicate NETosis as a generally occurring effector mechanism against *Eimeria* parasites. Co-localization studies on intestinal extracellular DNA being decorated with both histones and NE confirmed the presence of NETs in *Eimeria*-infected mucosa. Here, different patterns of NETs were observed as particularly seen in NE-positive staining which showed rather diffuse than spread form of NETs. Differential types of NETs have already been described in *Haemonchus contortus*-triggered NETosis [48]. Interestingly, *in vivo* NET release occurred irrespective of the *Eimeria* species and was also independent of the parasitic stages, *i.e.* merozoites I and oocysts, as previously demonstrated elsewhere [5,22]. In agreement, different *E. bovis* and *E. arloingi* stages (*i.e.* sporozoites, merozoites I and oocysts) were previously identified as potent NET inducers *in vitro* [5,22,28]. Moreover, it was demonstrated that *Eimeria*-induced NETosis is neither stage-, species- nor host-specific process [22]. The evidence of NET release *in vivo* in close proximity to parasitized areas containing intracellular *Eimeria* stages as well as NETs sticking to extracellular oocysts in the lumen of the intestine suggest NETosis as early host effector mechanism as previously postulated elsewhere [5,32].

Similar *in vivo* NET-related studies have been performed in other apicomplexan parasites such as *T. gondii* [49]. Here, *in vivo* NETs were confirmed by using immunohistochemistry analysis in *T. gondii*-infected mice lung tissue observing the extracellular DNA release co-localized with MPO molecules [30]. Nonetheless, in this former *in vivo* murine study neither direct contact of *T. gondii*-tachyzoites with NETs nor NETs-entrapped parasites were demonstrated [30]. These *in vivo* results coincide well with our findings where hardly any parasites were found entrapped by NETs. Taking into account that *in vivo* immunohistochemistry NET-related analyses of ruminant *Eimeria*-infected gut tissue sections might be a disadvantage due to the large size of the animals, it might be easier in the future to obtain evidence of parasites entrapped in NETs in *Eimeria*-infected rodent models, such as *Eimeria falciformis* or *Eimeria vermiformis* [50].

Referring to oocyst-induced NETosis, it appears noteworthy that in the case of *E. arloingi* oocysts (which are equipped with a micropyle), a blockage of sporozoite release by NETs was postulated [5]. In agreement, oocyst-induced NETosis was also reported for *Cryptosporidium parvum* where these stages were almost completely covered by NET structures [32]. Besides the interference with the *E. arloingi* excystation process, NETs were also released towards unsporulated oocysts in the gut lumen. However, so far it remains to be elucidated whether they are affected or even destroyed by the local high concentrations of antimicrobial peptides/proteases, such as NE, MPO, pentraxin, lactoferrin and gelatinase [14].

Not only PMN but also eosinophils and monocytes have been reported to play a crucial role in *E. bovis*-, *Eimeria ninakohlyakimovae*- and *E. arloingi*-induced coccidiosis [5,44–46]. Interestingly, ETs have recently also been reported to be released by other immune cells than PMN [29]. Thus, ETs can also be generated by macrophages [51,52], eosinophils [53,54], mast cells [55,56], basophils [57,58] and monocytes [21,31]. Independent of the leucocyte type, all ETs contain a vast amount of potent antimicrobial components and thus are able to interact with trapped pathogens [39]. Referring to parasite-driven formation of ETs, monocyte-derived ETs have recently been reported to be formed after exposure to *B. besnoiti* and *T. gondii* tachyzoites leading to parasite entrapment [21,31]. It is noteworthy that Taubert *et al.* also reported enhanced monocytic activities throughout experimental *E. bovis* infection although the detection of formation of ETs was not part of the study [46].

Regarding potential detrimental effects of NETs on *Eimeria* sp., extra- and intra-cellular stages have to be considered differently. Extracellular stages of *Eimeria* sp., such as sporozoites or merozoites in search of an adequate host cell, are unlikely to be killed by NETs, but were proven to be immobilized and hampered from host cell invasion [5,22,28]. However, intracellular stages can hardly be attacked by NET structures. Nevertheless, the function of NETs may here be attributed to other leucocyte recruitment (*e.g.* macrophages, cytotoxic $CD8^+$ cells) to the pathogen's site to deliver more effective parasitocidal actions. Alternatively, the local high concentration of NET-related antimicrobial molecules might additionally damage the cell membrane of infected cells, thereby exposing parasitic stages directly to NETs. Consistently to this assumption, in heavily *Eimeria*-infected mucosa, NETs were often observed sticking to epithelial host cells carrying intracellular stages. Actually the first ever published data on parasite-induced NETs also reported *in vivo* NETs entrapping *Plasmodium falciparum*-infected host cells (erythrocytes) within blood vessels [26]. Given that *E. bovis*-infected host cells express parasite-derived antigens (EbHSAg) on their surface membrane [59], these molecules might be recognized by PMN-derived pathogen recognition receptors, such as Toll-like receptors. In this context, we demonstrated the presence of mRNA transcripts of TLR1, TLR2, TLR4, TLR6, TLR7 and TLR10 genes in bovine PMN [60], and further characterized their pivotal role in the activation process of PMN after specific TLR-ligand binding [61]. In the human system there is some evidence on TLR4-dependent platelet–neutrophil interactions leading to the formation of NETs in plasma from severely septic patients [62]. Overall, future functional experiments have to clarify whether NETs may exhibit any detrimental effect on intracellular stages of *Eimeria* sp.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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8. CONTRIBUTION TO REVIEWS

SPECIAL ISSUE ARTICLE

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

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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

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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

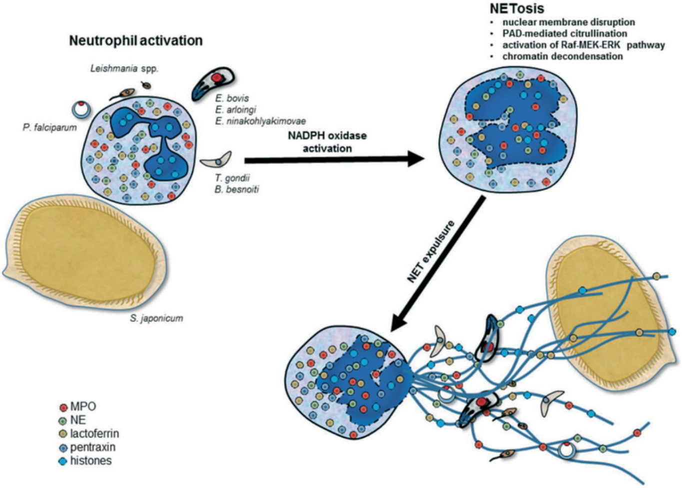

Fig. 1. Mechanisms of parasite-triggered neutrophil extracellular traps (NETs) release. PMN become activated by the contact with different protozoan parasite stages, such as trophozoites of *Plasmodium falciparum*, sporozoites of *Eimeria bovis*, *E. arloingi* or *E. ninakohlyakimovae*, tachyzoites of *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 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

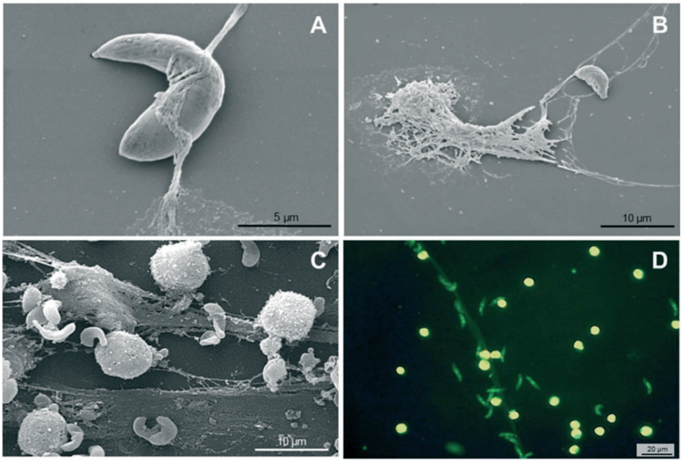

Fig. 2. NET formation triggered by different coccidian (*Eimeria bovis*, *E. arloingi*, *Toxoplasma gondii*, *Besnoitia besnoiti*) species. Bovine PMN were co-incubated with sporozoites of *E. bovis*/*E. arloingi* and tachyzoites of *T. gondii*/*B. besnoiti*, respectively, and thereafter analysed by 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 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).

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

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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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-1β, CC, and CXC chemokines (e.g., IL-8, IP-10, GRO-α, RANTES, and MIP-1α) [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, *spre*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.

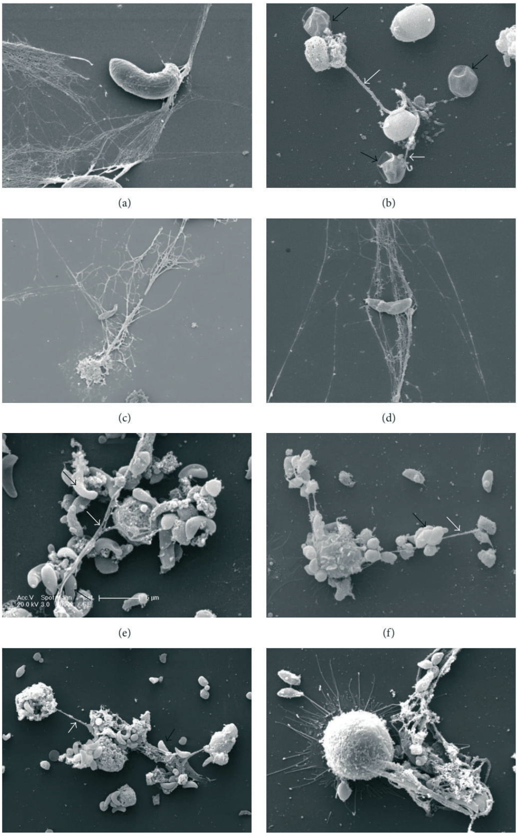
Plasmodium spp. parasites are mosquito-borne pathogens that cause malaria, a serious public health disease worldwide in the tropic and subtropics. Globally, an estimated 3.3 billion people are at risk of being infected with malaria of whom approximately 1.2 billion are at high risk (>1 in 1000 chance) 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].



(a) (b) (c) (d) (e) (f) (g) (h)

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.

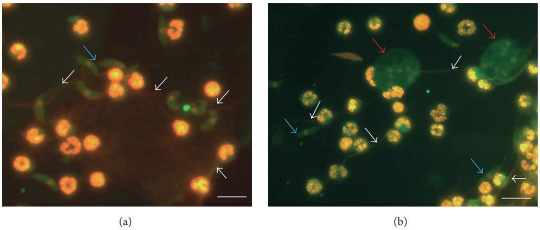


Figure 2: Colocalization of caprine NET-derived DNA and MPO. (a) Cocultures of caprine PMN and *Eimeria arloingi* sporozoites (blue arrows) were fixed, permeabilized, stained for DNA using Sytox Orange, and probed for MPO using anti-MPO along with adequate conjugate systems (white arrows); (b) cocultures 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 Ca^{2+}- (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
Eimeria bovis	Bovine	PMN	NOX NE MPO CD11b ERK 1/2 p38 MAPK SOCE	Muñoz-Caro et al. [23]
Eimeria arloingi	Caprine	PMN	NOX	Silva et al. [24]
Eimeria ninakohlyakimovae	Caprine	PMN	NOX	Pérez et al. (submitted manuscript)
		Monocytes	NOX	Pérez et al. (submitted manuscript)
Toxoplasma gondii	Mouse	PMN	ERK 1/2	Abi Abdallah et al. [25]
	Harbour seal	PMN	NOX NE MPO SOCE	Reichel et al. [26]
Besnoitia besnoiti	Bovine	PMN	NOX NE MPO	Muñoz-Caro et al. [27]
	Bovine	Monocytes	NOX MPO	Muñoz-Caro et al. [28]
Neospora caninum	Bovine	PMN	NOX NE MPO ERK 1/2 p38 MAPK SOCE P2Y2 PAD4	Villagra-Blanco et al. (submitted manuscript)
Cryptosporidium parvum	Bovine	PMN	NOX NE MPO ERK 1/2 p38 MAPK SOCE	Muñoz-Caro et al. [14]
Leishmania spp.	Human	PMN	NOX NE PAD4	Rochael et al. [29]
Trypanosoma cruzi	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 brasiliensis*) 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 ubiquitary 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.

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