CARACTERIZAÇÃO DA INFECÇÃO POR *EIMERIA* SPP. EM CAPRINOS NO ALENTEJO

CHARACTERIZATION OF *EIMERIA* SPP INFECTIONS IN GOATS IN ALENTEJO

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Aos meus Avós Ivete e Ircílio Machado por todo o apoio e amor que sempre me deram.

“O sucesso é ter quem fique feliz com o meu sucesso,
tão simples, e é mesmo assim,
vale-me quem me abrace quando estou feliz,
e no final das contas é mais por isso que estou feliz...
todas as vitórias são colectivas, sobretudo as individuais.“

Pedro Chagas Freitas

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ABBREVIATIONS

AMPs – antimicrobial peptides
BPI – bacterial permeability increasing protein
BUVEC – bovine umbilical vein endothelial cells
CCL2 – CC-chemokine ligand 2
CCL5 – CC-chemokine ligand 5
CFSE – carboxyfluorescein succinimidyl ester
COX-2 – prostaglandin-endoperoxide synthase 2
CXCL1 – CXC-chemokine ligand 1
CXCL8 – CXC-chemokine interleukine-8
CXCL10 – CXC-chemokine 10
DNA – deoxyribonucleic acid
DNase – deoxyribonuclease
DPI – diphenylene iodondium
ECs – endothelial cells
ETs – extracellular traps
GM-CSF – granulocyte-macrophage colony-stimulating factor
H3 – histone H3
ICAM-1 – intracellular adhesion molecule 1
IFN-γ – interferon gamma
IgA – immunoglobuline A
IgG2 – immunoglobuline G2
IgM – immunoglobuline M
IMC – inner membrane complex
MARC-145 – Rhesus monkey fetal kidney cells
MDBK – Madin-Darby bovine kidney cells
MHC – Major histocompatibility complex
MPO – myeloperoxidase
NADPH – nicotinamide adenine dinucleotide phosphate-oxidase
NE – neutrophil elastase
NET – neutrophil extracellular trap
OPG – oocysts per gram of faeces
PGRPs – peptido-glycan recognition proteins
PMN – polymorphonuclear neutrophils
PV – parasitophorous vacuole
PVM – PV membrane
RANTES – regulation upon activation normal T cell expressed and secreted
RONs – rhoptry neck proteins
ROS – reactive oxygen species
SCID – severe combined immunodeficiency
SEM – scanning electron microscopy
VCAM-1 – vascular cellular adhesion molecule 1
WFB1 – wall forming bodies 1
WFB2 – wall forming bodies 2

ABSTRACT

Coccidiosis is a major concern in goat industry leading to high economic losses. In Alentejo, the pathogenic *Eimeria arloingi* was one of most prevalent species identified. A new *E. arloingi* European strain was isolated and a suitable *in vitro* culture system for the first merogony in primary endothelial cells was established. Moreover, parasite-host endothelial cells interactions were investigated during macromeront formation.

Polymorphonuclear neutrophils (PMN) release neutrophil extracellular traps (NETs). We have demonstrated for the first time NET formation performed by caprine PMN exposed to different stages of *E. arloingi* (sporozoites and oocysts), suggesting NETosis as an important effector mechanism in the early innate immune response to this infection in goats.

The new *E. arloingi* (strain A) will be useful for better comprehension of early host innate immune reactions against this parasite *in vitro/in vivo* as well as to further our investigations in the complex *Eimeria*-host endothelial cell interactions.

Key words: Coccidiosis; *Eimeria arloingi*; Endothelial cell modulation; Innate immune reactions; Neutrophil extracellular traps

RESUMO

A coccidiose constitui um enorme problema na indústria caprina, causando graves perdas económicas. No Alentejo, a espécie patogénica *Eimeria arloingi* foi uma das mais frequentemente identificadas. Uma nova estirpe europeia de *E. arloingi* foi isolada e um sistema de cultura *in vitro* foi estabelecido para o desenvolvimento da primeira merogonia em células endoteliais primárias e as interações parasita-células endoteliais do hospedeiro foram investigadas durante a formação dos macromerontes.

Os neutrófilos (PMN) extrudem redes extracelulares (Nets). Pela primeira vez demonstramos a formação de NETs por PMN caprinos expostos a diferentes estádios de *E. arloingi* (esporozoitos e oocistos), sugerindo que a NETosis é um importante mecanismo da resposta imune inata nesta infecção em cabras.

A nova *E. arloingi* (estirpe A) será útil para uma melhor compreensão das reações imunes inatas contra este parasita *in vitro*/*in vivo*, bem como para progredir nas nossas investigações sobre as complexas interações entre *Eimeria*-células endoteliais do hospedeiro.

Palavras-chave: Coccidiose; *Eimeria arloingi*; Modulação das células endoteliais; Reações imunes inatas; Redes extracelulares de neutrófilos

1. INTRODUCTION

1.1. Social and economic importance of goat industry

According to FAOSTAT (2014), about one billion goats were reared for meat and milk production worldwide in 2012 (Fig. 1). In addition, to assure nutritional demands of small farmers in deprived regions and nourishment of growing ethnic populations, faith-based consumers and health-conscious people, goat production also guarantees working opportunities for a considerable number of people worldwide, apart from contributing to the cleaning of forest areas and fire prevention (Silva et al. 2014b).

Portugal represents the eighth largest national goat industry within the European Union (Fig. 1), rearing about 400.000 goats (FAOSTAT 2014). Most of them (21.6 %) are allocated in Alentejo (GPP 2009), where goats support the stability and maintenance of rural communities, thereby avoiding the abandonment of this underprivileged region. Especially in Alentejo, a semi-arid region with poor soil conditions and lack of vegetation, goats are raised in extensive or semi-extensive systems. These environments are not suitable for rearing other domestic animals that need feed supplementation, but goats are undemanding animals that require low quality feed for the production of valuable meat and milk products (Oltjen and

Beckett 1996). Despite their resistance, goats are still affected by a variety of diseases and parasites, which cause an array of health problems in goat production (Ruiz et al. 2014).

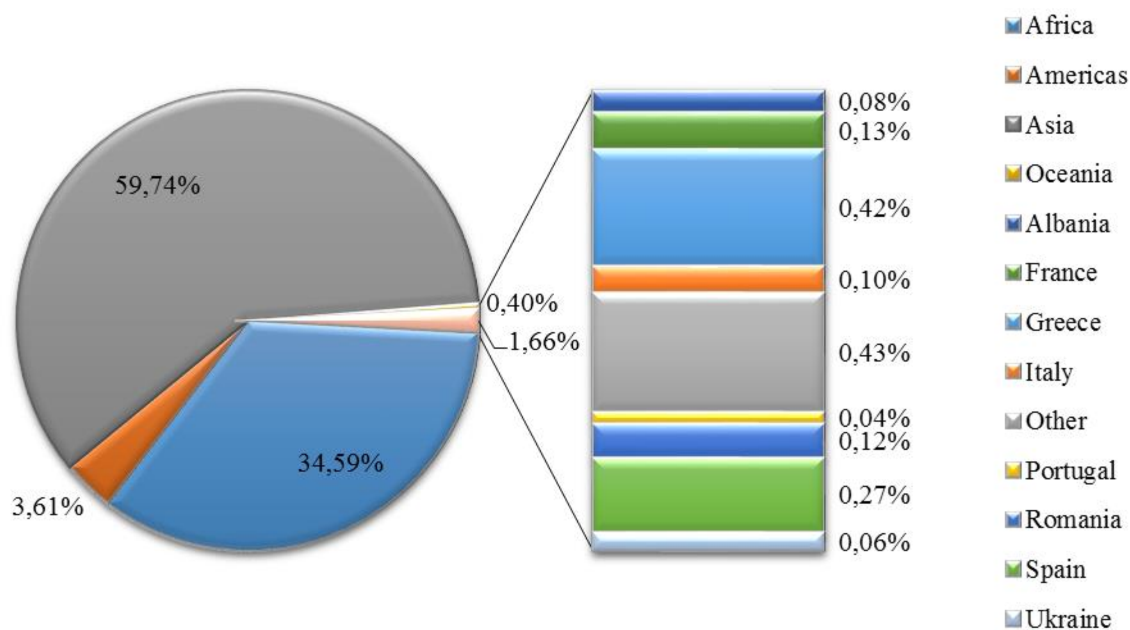

FIG. 1. World population of goats distributed by continents (2012)
Most important European countries in goat industry are listed in the bar. Portugal has 0.04% of the global goat population.

Overall and compared with other livestock animals, goats represent still a neglected species regarding basic, clinical and epidemiological research. Nevertheless, under the framework of Food and Agriculture of the European Community, the CAPARA COST Action (FAO805) – Caprine Parasitology – Goat-parasite interactions: from knowledge to control – brought researchers from more than 28 countries together to address this farm animal species. CAPARA supported the establishment of an efficient network of European researchers working on different aspects of goats parasitology and health management to improve the understanding of the different components explaining the specificities of goat-parasite interactions as well as to develop suitable strategies to control parasitic diseases in goats (CAPARA 2012).

1.2. Apicomplexan parasites

Apicomplexa is the subphylum of single-celled eukaryotic protozoa responsible for diseases of recognizable medical and economic importance both in humans and in farm animals (Muller and Hemphill 2013; Francia and Striepen 2014). Apicomplexan parasites infect different hosts and specific host cell types, in which they hide from the host immune response, while depleting their nutrients (Striepen et al. 2007). Amongst the most relevant apicomplexan genera in farm animals are *Babesia* spp. (Gohil et al. 2013), *Besnoitia* spp. (Cortes et al. 2014), *Cryptosporidium* spp. (Slapeta 2013), *Eimeria* spp. (Chartier and Paraud 2012), *Cystoisospora* spp. (Schwarz et al. 2014), *Neospora* spp. (Reichel et al. 2014), *Sarcocystis* spp. (Bahari et al. 2014), *Theileria* spp. (Li et al. 2014) and *Toxoplasma gondii* (Abi Abdallah et al. 2012; Coppens 2013). Despite the enormous socio-economic impact of these parasites in farm animal production worldwide, until now, *Toxoplasma* and *Plasmodium* have been the best studied parasites (Chapman 2014), mainly because of their antropozoonotic potential and high impact on human health worldwide.

All apicomplexan parasites are obligate intracellular protozoa and are characterized by the unique apical complex of parasite different stages, such as sporozoites, tachyzoites, bradyzoites and merozoites. The apical complex is formed of structural and secretory elements and it is crucial in the active host cell invasion process, parasitophorous vacuole (PV) formation and parasite division within infected host cells (Morrissette and Sibley 2002; Striepen et al. 2007; Katris et al. 2014). The apical complex contains a polar-ring complex, a conoid, subpellicular microtubules, rhoptries, micronemes and dense granules (Chobotar and Scholtyseck 1982). The polar ring is located at the anterior part of apicomplexan invasive stages. It consists of a ring of microtubules, which operate as one of the microtubule-organizing centers essential for the arrangement of host cell structures during intracellular

growth and replication of the parasite (Morrissette and Sibley 2002; Striepen et al. 2007). The hollow-shaped conoid is located in the middle of the apical complex and plays a mechanical role in active invasion process of host cells (Morrissette and Sibley 2002). Additionally, longitudinal subpellicular microtubules arise and anchor to the polar-ring and are associated with the inner membrane complex (IMC), which is important for the apicomplexan shape and physical stability (Morrissette and Sibley 2002). Rhoptries, micronemes and dense granules are highly specialized secretory apicomplexan organelles that secret important molecules for the parasite intracellular survival (Morrissette and Sibley 2002; Striepen et al. 2007). Therefore, their secreted products are required for three essential apicomplexan actions: (*i*) gliding motility, (*ii*) host cell invasion and (*iii*) early intracellular life establishment by PV formation (Dubremetz et al. 1998; Blackman and Bannister 2001; Morrissette and Sibley 2002; Souza 2006; Striepen et al. 2007; Ravindran and Boothroyd 2008). Rhoptries are tear drop-shaped organelles that are connected by a thin duct to the apical part of the parasite. The number of rhoptries can vary from two to more than six elements depending on the apicomplexan genus, species and stage [e. g. sporozoites, merozoites, bradyzoites, tachyzoites, metrozoites, dormozoites (hypnozoites)] (Blackman and Bannister 2001). Shortly after parasite adhesion to the host cell membrane, rhoptric molecules are secreted. Rhoptry neck proteins (RONs) have been described to participate in the parasite-cell membrane tight junction formation during active host cell invasion process (Alexander et al. 2005; Alexander et al. 2006). Furthermore, rhoptry proteins, such as the merozoite-specific 22-kDa rhoptry protein of *E. nieschulzi,* have been described as integral molecule of the PV membrane (PVM) (Rick et al. 1998). Micronemes are small elliptic-shaped organelles important for specific host cell recognition, binding and gliding motility (Dubremetz et al. 1998). Dense granules represent cytoplasmic spherical-shaped organelles containing high concentration of proteins that are secreted after internalization of the parasite and incorporated

into both, the PVM and the intravacuolar membranous network (Mercier et al. 2005). Structural elements of the apical complex mediate orientation of the parasite and are considered as the focal point for secretory organelle discharge (Katris et al. 2014). Moreover, after contact of the apical complex with the host cell membrane, host cell invasion is accomplished in a host cell-independent process, based on the active gliding motility of the parasite being regulated by specialized actin- and myosin-elements within the pellicula (parasite membrane) (Striepen et al. 2007).

Irrespectively of the genus, apicomplexan parasites share the same phases of the life cycle, with endogenous (parasitic) and exogenous (environment) phase, comprising three generations: sporogony, merogony, and gamogony. Sporogony represents an asexual replication period resulting in the formation of infective stages, the sporozoites. Sporozoites invade specific host cells and thereafter undergo a second step of asexual replication, named merogony, in which merozoites are formed. Merogony occurs in various host cell locations and for genetically defined numbers of generations, which are characteristic for each species. Certain merozoites differentiate into haploid macro- (female) and micro- (male) gametes which than have to fuse (known as syngamy), becoming a diploid zygote – known as the sexual gamogony. Immediately after syngamy, the zygote undergoes meiosis to re-establish haploid forms – sporogony (Striepen et al. 2007). The repetition of cycles of host cell invasion, parasite replication, host cell lysis and parasite invasion of new cells results in the vast tissue damage which is a typical characteristic of apicomplexan infections (Morrissette <and Sibley 2002). Free stages, such as sporozoites and merozoites, must promptly invade new specific host cells after being released from ruptured host cells in order to continue their life cycle and to avoid extracellular attacks of host leukocytes, such as neutrophils and monocytes (Morrissette and Sibley 2002; Abi Abdallah and Denkers 2012).

1.3. *Eimeria* species

More than one thousand species are known within the *Eimeria* genus belonging to the subphylum Apicomplexa (order Eimeriida, family Eimeriidae). Almost all species of *Eimeria* are strictly host specific (monoxenous) enteropathogens of vertebrates (Witcombe and Smith 2014), which develop within specific host cells at specific sites of the intestinal mucosa. The disease caused by the genus *Eimeria* is known either as coccidiosis or as eimeriosis and has been reported worldwide as a major livestock health problem in different production systems (Foreyt 1990; Lima 2004; Daugschies and Najdrowski 2005; Witcombe and Smith 2014). Particularly in poultry (Chapman 2014), rabbits (Nosal et al. 2014) and ruminants (Daugschies and Najdrowski 2005) *Eimeria* infections are well-known to cause severe intestinal lesions, and consequently, considerable economic losses worldwide. In addition, extra-intestinal stages of some *Eimeria* species have been reported in different host locations, causing hepatic-, renal-, and, less commonly, splenic- or pulmonary-coccidiosis (Collins et al. 1988; Dai et al. 1991; Morgan et al. 2013). In the last years, numerous studies have been conducted to improve the knowledge on this disease, e. g. in poultry (Blake and Tomley 2014; Chapman 2014), in rodents (Shi et al. 2000), in rabbits (Akpo et al. 2012) and in cattle (Hammond et al. 1966; Hermosilla et al. 1999; Hermosilla et al. 2012). Despite the negative impact of coccidiosis on goat industry, caprine *Eimeria* infections have been neglected for several decades. Nonetheless, few studies on caprine coccidiosis have recently been published (Ruiz et al. 2006; Hashemnia et al. 2011; Hashemnia et al. 2012; Ruiz et al. 2014), but most of them were related to clinical and epidemiological topics (Rakhshandehroo et al. 2013; Ruiz et al. 2014).

Alike other Apicomplexa the life cycle of *Eimeria* spp. is complex and contains three different obligatory steps: sporogony, merogony and gamogony. The sporulation of the

oocysts takes place in the environment under appropriate humidity, temperature and oxygen supply conditions, resulting in the infective form of the parasite, the sporulated oocyst, in which four sporocysts develop, each containing two sporozoites. After oral ingestion by the final host the sporulated oocyst undergoes excystation in the gut lumen and releases sporozoites that must infect specific intestinal cells. Most of *Eimeria* species develop within intestinal epithelial cells while some highly pathogenic species (e. g. *E. bovis*, *E. ninakohlyakimovae*, *E. arloingi*) infect endothelial host cells. When first merogony is completed merozoites I are released from mature meronts. Thereafter, merozoites I migrate to the ileum, caecum or colon epithelium and undergo the second merogony, followed by the sexual phase of gamogony. The gamogony also occurs in host epithelial cells of the gut and results, after syngamy, in the formation of new oocysts. After the rupture of oocyst-carrying epithelial cells, unsporulated oocysts are shed into the environment and the life cycle of *Eimeria* is completed (Taylor and Catchpole 1994).

Considering the pathogenesis of ruminant eimeriosis, the damage in the intestinal mucosa during development and proliferation of these intracellular parasites significantly interferes with the digestive process and with homeostasis. In consequence, adverse effects on animal welfare and production performance leading to significant economic losses, even in the absence of visible clinical signs (Daugschies and Najdrowski 2005), are a common feature in coccidiosis. Diarrhoea leads to plasmatic potassium concentration increases, hypoproteinaemia, hyponatraemia and hypochloraemia in biochemical blood analysis of *Eimeria*-infected animals (Lima 2004). Most *Eimeria* species are considered as low pathogenic since they induce mild pathology and mild or non-clinical disease. Additionally, in non-intensive farming conditions and after low dose *Eimeria* infections, a rather beneficial balance between the parasite and the host immune system can be established. In this case, either no clinical signs are detected (subclinical coccidiosis) or rather moderate diarrhoea

occurs (Daugschies and Najdrowski 2005; Witcombe and Smith 2014). On the other hand, certain species such as e. g. *E. tenella* and *E. necatrix* (chicken), *E. intestinalis* and *E. stiedae* (rabbit), *E. bovis, E. alabamensis* and *E. zuernii* (cattle), *E. bakuensis* and *E. ovinoidalis* (sheep), or *E. ninakohlyakimovae* and *E. arloingi* (goat) are considered as highly pathogenic species. Apart from common clinical signs observed in clinical coccidiosis, such as anaemia, weakness, lethargy, anorexia, poor weight gain, dehydration, growth delay, low feed conversion and catharrhalic diarrhoea, severe haemorrhagic diarrhoea often is observed in the presence of highly pathogenic species. In contrast to non-pathogenic ruminant *Eimeria* spp., highly pathogenic ones (e. g. *E. arloingi*) possess a massive replication capacity during their first merogony within host endothelial cells of the lacteals, producing macromeronts of up to 400 µm size each containing > 120.000 merozoites I and occasionally causing even sudden death of infected animals (Koudela and Bokova 1998; Daugschies and Najdrowski 2005; Chapman 2014; Silva et al. 2014b).

1.3.1. Epidemiology of *Eimeria* infections (coccidiosis)

Coccidiosis severity results from complex interactions between the parasite and the host, being additionally influenced by many environmental circumstances (Hashemnia et al. 2014). In principle, naïve animals of all ages are susceptible to eimeriosis. However, younger animals represent the most susceptible group and almost inevitably develop the disease. Especially under intensive rearing conditions young animals are unavoidably exposed to *Eimeria* spp. infection owing to the ubiquity of infectious stages (Soe and Pomroy 1992; Taylor and Catchpole 1994; Daugschies and Najdrowski 2005). Still, the severity of coccidiosis is not only influenced by the age, but also by other intrinsic factors. One of the most import risk factors being associated with clinical disease in ruminants is stress,

especially when acting on young animals. As such, the weaning period, the transport, re-grouping, inadequate feeding, the parturition period or concomitant infections with other pathogens result in a higher risk of coccidiosis and contribute to the complication of the condition (Faber et al. 2002; Lima 2004; Daugschies and Najdrowski 2005). Moreover, apart from characteristics of the parasite itself (species and even strains), the genetical background and related host immune system efficacy most probably contributes to the individual clinical outcome of the disease (Daugschies and Najdrowski 2005; Witcombe and Smith 2014).

In Serpentina goats, a rustic breed from Alentejo, Portugal, infections with the most pathogenic species of *Eimeria* resulted in the absence of classical clinical signs of coccidiosis, indicating an effective innate and adaptive immune response of this breed (see Chapter 2).

The pathogenicity and the site of infection of different *Eimeria* species (or even strains) influence the outcome of clinical coccidiosis (Rose 1987). The most pathogenic species in ruminants [*E. bovis*, *E. zuernii* (cattle), *E. ninakohlyakimovae*, *E. arloingi*, *E. christenseni* (goat), and *E. bakuensis* (sheep)] do not infect epithelial host cells as most other *Eimeria* spp. But have to transverse the intestinal epithelium and to invade endothelial cells of the central lymph capillaries of the intestinal villi (Hermosilla et al. 2012) where they form macromeronts, a process that requires prolonged replication time and vast modulation of the host cell (Taubert et al. 2010; Lutz et al. 2011; Hermosilla et al. 2012). *Eimeria bovis* as well as other apicomplexan- (*T. gondii, N. caninum*) induced modulation of the host endothelial cell was clearly demonstrated by Hermosilla et al. (2008) and Taubert et al. (2006). In *E. bovis in vitro* infections of primary bovine umbilical vein endothelial cells (BUVEC), the parasite induced a reorganization of several elements of the host cell cytoskeleton in order to support the enlargement of host cells by macromeront formation (>250 µm) within the PV. Additionally, the parasite recruits host cell-organelles, such as mitochondria and ER, close to the PVM to gain access to host cell energy and nutrients (C. Hermosilla, personal

observation). Other host cell functional categories are significantly modulated by apicomplexan parasites, and will be addressed in more detail in Chapter 1.4.

The severity of coccidiosis is also determined by the proliferation capacity of the infective *Eimeria* species as defined by the number of merogonies and number of merozoites being produced per merogony, since these will later determine the number of destroyed cells per ingested sporulated oocysts. Thus, the dose of infection and the magnitude of the reinfection can considerably influence the extend and the outcome of coccidiosis (Lima 2004).

Once in contact with coccidian stages, a host protective species-specific immunity develops (Rose 1987; Daugschies and Najdrowski 2005). During a primary ruminant *Eimeria* infection, an expansion of both $CD4^{+}$- and $CD8^{+}$-T cells subsets generally occurs (Hermosilla et al. 1999; Suhwold et al. 2010). In prepatency a rather Th1-dominated cellular immune responses was observed being characterized by an enhanced antigen-specific IFN-γ production (Taubert et al. 2008). As shown previously by Faber et al. (2002), an active host immune response was generally related with the synthesis of *Eimeria*-specific IgM-, IgG2- and IgA-antibodies in cattle, being combined with decreased oocyst shedding. However, Faber et al. (2002) could not significantly prove antibody-based immunoprotection of calves against *E. bovis* infection which is in contrast to findings of Catchpole and Devonshire in 1989 (reviewed by Yvoré 1989) showing that maternal antibodies are protective for new-born lambs in *E. crandallis* infections. However, host humoral immune responses are not fully protective since reinfections represent a common feature in coccidiosis, but do generally not result in clinical disease, confirming the importance of primary infections for the outcome of protective immunity (Daugschies and Najdrowski 2005).

Infected animals are the natural source for environmental contamination and subsequent infection of naïve and young animals (Lima 2004). The degree of environmental

contamination varies largely with the type of herd management. The risk of coccidiosis outbreaks is greater in intensive husbandries, where environmental contamination is high due to excessive population densities. High infection pressure is thereby increasing the individual risk to acquire clinical coccidiosis. Generally, in this type of intensive managements, humidity and temperature conditions empower the sporogony of freshly shed oocysts thereby increasing the disposability of infectious exogenous stages (Lima 2004; Daugschies and Najdrowski 2005; Ruiz et al. 2006). Furthermore, climate conditions play an important role in the epidemiology of coccidiosis since the development of exogenous infectious stages depends on adequate temperatures (Ruiz et al. 2006). Thus, under propitious conditions of humidity, aeration and temperature oocyst sporulation occurs in several days (Christensen 1939; Daugschies and Najdrowski 2005; Ruiz et al. 2006).

1.3.2. Control of *Eimeria* spp. coccidiosis

The prevalence of *Eimeria* spp. in ruminants is generally high, entailing significant economic losses caused mainly by decreased meat or milk production, costs of treatments, prophylaxis and metaphylaxis, and even death of heavily infected animals. Thus, an effective control of the disease is essential to avoid clinical coccidiosis in the farms, especially in those with histories of high morbidity and mortality in young animals (Ruiz et al. 2006). Nowadays, control of coccidiosis is based on the improvement of management practices combined with treatments of infected animals and chemoprophylaxis/metaphylaxis applying specific anticoccidial drugs (Ruiz et al. 2014).

In order to reduce the risk of eimeriosis outbreaks, adequate management measures should be considered. Husbandries with insufficient hygiene bear a higher risk of disease due to high environmental contamination with oocysts. Secondly, high values of humidity and high

concentrations of ammonia and carbon dioxide, lead to a higher rate of sporulation of shed oocysts, increasing the availability of infective stages. Consequently, only mild subclinical coccidiosis was observed in ruminant herds with good hygienic status (Lima 2004; Daugschies and Najdrowski 2005; Smith and Sherman 2009). Improved hygiene practices in turn will decrease the ingestion of sporulated oocysts. Therefore, reducing the number of animals per group and keeping feeders and water sources free of faeces contamination will increase the health status of the herd (Lima 2004; Smith and Sherman 2009). Furthermore, oocysts are quite resistant to environmental degradation and are even more resistant when being sporulated (Smith and Sherman 2009), but can be degraded by desiccation, sunlight, heat and some disinfectants (e. g. disinfectants with high concentrations of sodium hypochlorite or cresol) (Lima 2004). However, given that oocysts are generally covered by faecal material, they may not generally be killed by these compounds, and persist infective for long periods (up to 4.5 years at 4°C; C. Hermosilla, personal communication), even on pastures, in stables and during the winter season.

Commonly, kids are always kept on the same pastures year after year, which may increase the risk of caprine coccidiosis due to high infection pressure of naïve animals. Hence, pastures should be kept drained to avoid accumulation of water, which provides humidity conditions for oocyst sporulation and thus perpetuation of infective sporulated oocysts where animals graze (Daugschies and Najdrowski 2005).

The treatment of infected animals is inevitable in coccidiosis outbreaks and the first control measure to be implemented should be the isolation of diarrhoeic animals from the group to stop environmental contamination. Supportive care during coccidiosis therapy should be taken into account in order to re-establish homeostasis, and to correct dehydration and electrolyte imbalances. Additionally, broad-spectrum antibiotics are indicated to prevent secondary bacterial infections and septicaemia owing to parasite-induced disruption of the

intestinal mucosal barrier (Smith and Sherman 2009). For the last years, specific anticoccidial compounds have been used in coccidiosis outbreaks and most of them delivered satisfactory results when administrated in the early phase of the disease. Many anticoccidial drugs mainly target gamonts (Daugschies and Najdrowski 2005). However, in active clinical coccidiosis, application of these drugs are of limited value since they are coccidiostats which slow down but do not entirely interrupt coccidial replication (Smith and Sherman 2009). Still, administration of these compounds hampers gamogony and formation of new oocysts and is therefore of benefit for heavily infected animals.

Chemo-prophylactic and -metaphylactic programs are more effective tools to prevent coccidiosis outbreaks than therapeutic approaches (Daugschies and Najdrowski 2005; Iqbal et al. 2013). Sulphonamides, which interfere with asexual replication, are frequently applied (Daugschies and Najdrowski 2005) and represent the oldest coccidiostatic drugs. However, parasite resistance to these compounds have been reported worldwide (Smith and Sherman 2009). In contrast to sulphonamides, benzene acetonitrile compounds (e. g. toltrazuril and diclazuril) act against all endogenous parasitic generations (merogonies and gamogony) and therefore became particularly useful in the metaphylaxis of ruminant coccidiosis when all animals of a group were treated (Daugschies and Najdrowski 2005; Ruiz et al. 2012). Single doses of toltrazuril and diclazuril have been described effective in the control of clinical disease and oocysts excretion (Daugschies and Najdrowski 2005; Ruiz et al. 2012). In contrast, decoquinate, which acts against sporozoites and trophozoites, must be administrated continuously as in-feed medication for effective treatments (Daugschies and Najdrowski 2005). The coccidiostatic compound amprolium, a thiamine antagonist acting upon the first generation meronts, inhibits merozoites differentiation (Daugschies and Najdrowski 2005; Smith and Sherman 2009) and may also suppress the sexual stages and sporulation of the oocysts. Young et al. (2011) recently confirmed the efficacy of amprolium

for treatment of pathogenic *Eimeria* species in goat kids by using high doses. Additional drugs with known efficacies against coccidia are monensin, lasalocid, salinomycin and other ionophores, which are all widely used in poultry, cattle, sheep and goat industries. They affect the cation transport through the parasite cell membrane, but they are potentially toxic to the ruminant host if being overdosed (Smith and Sherman 2009; Witcombe and Smith 2014).

Many anticoccidial drugs are no longer as effective as they were when were first introduced into the market owing to drug resistance (Chapman et al. 2013). Currently, the use of anticoccidial drugs as feed additives is widely used for coccidiosis control. Extensive use of these drugs has caused the development of drug-resistant *Eimeria* species worldwide. Due to the genomic plasticity of coccidia, resistant species are able to multiply and survive in the presence of a drug that would normally abolish or prevent replication of the same non-resistant species (McDonald and Shirley 2009; Coppens 2013; Ruiz et al. 2014; Witcombe and Smith 2014). Moreover, some Apicomplexa-specific drug treatments present poor overall potency, activity which is restrict to particular parasitic stages and unwanted side effects (Coppens 2013). Lately, recent and growing public demand for residue-free livestock products has encouraged new research lines. Owing to drug resistance phenomena and the lack of new drugs on the market, research activities focused on the development of vaccines and plant-derived, ecologically friendly compounds acting against coccidia (Chapman et al. 2013; Coppens 2013; Iqbal et al. 2013; Witcombe and Smith 2014). However, the search for new drug targets is an ongoing process and mainly concerns parasite auxotrophic metabolic pathways. Coppens (2013) described apicomplexan parasites to contain unique, non-mammalian enzymatic systems for the biosynthesis of specific lipids, which become valid drug targets in the future.

Different vaccines against *Eimeria* spp. have been used in the past, such as live attenuated vaccines, parasites lysates, total antigens or antigen fractions from killed parasites or subunit

vaccines (Jenkins 2001; Monney and Hemphill 2014; Ruiz et al. 2014; Witcombe and Smith 2014). Immunoprophylaxis mainly has been applied in poultry industry and to date commercial *Eimeria* vaccines are only available for poultry coccidiosis (McDonald and Shirley 2009). Concerning goat eimeriosis, Ruiz et al. (2014) have recently shown protective effects when using live attenuated oocysts of *E. ninakohlyakimovae* for challenge infections of goat kids, thus preventing clinical coccidiosis manifestations. In this trial Ruiz et al. (2014) have clearly demonstrated the usefulness of gamma-irradiated oocysts, which conferred cellular immune protection whilst inducing low pathogenicity. In general, *Eimeria* spp. infections induce strong species-specific protective immune responses, which reduce the clinical signs of coccidiosis. Hence, the isolation of a new pathogenic caprine *Eimeria* species would add to the currently available vaccine portfolio against caprine coccidiosis. In this context, with the present work we were able to isolate for the first time in Europe a new *E. arloingi* (strain A) from Alentejo, Portugal (see Chapter 3). Investigations on the efficacy of this new isolate in immunization protocols for goat kids, both as single species and a multi-species approach (in combination with *E. ninakohlyakimovae*), will constitute a future project.

The public interest in the development of alternative and environmentally-friendly anticoccidial agents for coccidia control has been growing in the last years. The efficacy of different plant compounds against coccidia have been investigated by Kommuru et al. (2014), who observed less signs of coccidiosis in infected goats fed with pelleted sericea lespedeza (*Lespedeza cuneata*) when compared to control groups. Thus, this treatment led to a more than 90 % reduction of oocyst shedding. Given that goats preferably engorge this kind of plants while grazing may deliver a self-medication on the level of prophylaxis or even therapeutic or prophylactic. The same behaviour has been shown in primates parasitized with gastro-intestinal nematodes (Hoste et al. 2008; Amit et al. 2013).

1.4. Caprine coccidiosis

Caprine coccidiosis is a ubiquitous and frequent clinical and subclinical disease affecting the profitability of goat industry worldwide (Norton 1986; O'Callaghan 1989; Agyei et al. 2004; Hashemnia et al. 2014). Coccidiosis might affect up to 100% of 4-10 weeks old goat kids (Mehlhorn and Armstrong 2001), depending on the type of management, immune status of animals, and climate conditions (Ruiz et al. 2006). Therefore, significant economic losses are implied, mostly due to high mortality rates that may concern > 50% of the kids (Jalila et al. 1998; Smith and Sherman 2009). This condition could be prevented with a correct diagnosis of involved Eimeria species and prophylactic control measures. Diagnosis should not only rely on epidemiological and clinical factors, since moderate infections or infections with non-pathogenic species may induce subclinical coccidiosis or transient non-haemorrhagic diarrhoea usually being attributed to other pathogens, but should be proven by laboratory investigations (Daugschies and Najdrowski 2005; Silva et al. 2013). Between one week and one month of age, other pathogens than Eimeria spp. can cause abdominal pain and acute diarrhoea episodes in kids such as cryptosporidiosis, colibacillosis, enterotoxaemia, salmonellosis, viral enteritis and dietary diarrhoea, and consequently, they must also be considered as potential ethiologic agents (Smith and Sherman 2009). In cases of massive Eimeria infections, clinical signs occur in the prepatent period hampering oocyst detection in faecal samples. Consequently, diagnosis would only be possible by detection of *Eimeria* endogenous stages in intestinal tissues of necropsied recently deceased animals or in fragments of intestinal mucosa free of faeces (Lima 2004). Nowadays, the classical copromicroscopy is still the most widely employed technique for the detection of parasitic stages, regardless of the availability of other molecular, biochemical or immunological diagnostic approaches which usually bear rather high costs which still make them unavailable

in routine practice (Cringoli et al. 2010; Vadlejch et al. 2011; Silva et al. 2013). Besides clinical observations and the analysis of the epidemiologic status of the goat herds, the quantification of oocyst shedding is helpful for proper treatments, especially in young kids (Ruiz et al. 2012; Iqbal et al. 2013). However, the number of oocysts shed by animal is not strictly related to the severity of clinical disease since in cases of infections with pathogenic species many oocysts may be trapped within tissue and fibrin thereby reducing oocyst shedding (Daugschies and Najdrowski 2005). Thus, the number of shed oocysts by animal is not strictly related to the severity of clinical disease. Overall, routine diagnostics on faecal samples is recommended including the determination of the species being involved in respective samples. Given that oocyst shedding may be discontinuous, e. g. peaking in stressful conditions, the continuous control of oocyst shedding within the herd is recommended The morphologic differentiation of sporulated caprine Eimeria spp. oocysts is based on their shape and size, the presence or absence of a micropyle and polar cap, and the colour and thickness of the oocyst walls (Levine 1985; Eckert 1995).

Due to the lack of available data concerning caprine coccidiosis in Portugal, the first aim of this work was to epidemiologically characterize caprine Eimeria spp. infections in the province of Alentejo (see Chapter 2).

At least 18 different Eimeria species have been described to infect goats worldwide (Soe and Pomroy 1992; Smith and Sherman 2009). *Eimeria arloingi* (Marotel 1905), *E. ninakohlyakimovae* (Yakimoff and Rastegaieff 1930) and also *E. christenseni* (Lima 1980) are considered as the most pathogenic ones (Sayin et al. 1980; Levine 1985; Yvoré et al. 1985) due to their massive replication capacity within the first merogony in host endothelial cells and to the large-scaled erosion of the affected intestinal mucosa (Soe and Pomroy 1992; Ruiz et al. 2006; Taylor et al. 2007). *Eimeria arloingi* and *E. ninakohlyakimovae* were reported as the most frequent species present in caprine flocks from several countries

(Balicka-Ramisz 1999; Ruiz et al. 2006; Balicka-Ramisz et al. 2012; Silva et al. 2013; Kheirandish et al. 2014), which might indicate a well-adapted host endothelial cell-parasite interaction within their definitive hosts. Common clinical manifestations of infections with these species include severe haemorrhagic diarrhoea, weight losses, dehydration and poor growth rates (Koudela and Bokova 1998; Ruiz et al. 2013). Even sudden death in heavily infected goat kids has been reported (Balicka-Ramisz et al. 2012; Rakhshandehroo et al. 2013; Silva et al. 2014b). Other commonly detected caprine species are *E. alijevi* (Musaev 1970), *E. hirci* (Lima 1980), *E. caprina* (Lima 1979), *E. caprovina* (Lima 1980) and *E. jolchijevi* (Lima 1980). All these species develop in epithelial cells of the small and large intestine (Taylor et al. 2007). In general these species are considered non or mild pathogenic; however, they are still reported as responsible for subclinical caprine coccidiosis (Taylor et al. 2007).

1.4.1. *Eimeria arloingi*: peculiarities of its life cycle and pathogenesis

Considering all *Eimeria* species identified in goats so far, *E. arloingi* is reported as one of the most pathogenic species. Given that this species is regularly identified as the most frequent one in previous caprine coccidiosis reports, it is of major concern in many countries (Chartier and Paraud 2012; Hashemnia et al. 2012; Silva et al. 2013; Silva et al. 2014b). Marotel originally described *E. arloingi* in 1905 as *Coccidium arloingi* and for many years it was accepted that *E. arloingi* infected both, sheep and goats (Hashemnia et al. 2012). Nonetheless, attempts to transfer the morphologically indistinguishable *Eimeria* species, *E. arloingi* and *E. ovina*, to different hosts revealed that they were two different species and as other *Eimeria* spp. behaved strictly species-specific (Sayin et al. 1980).

Life cycle of *E. arloingi* comprises the formation of two generations of meronts (Fig. 2). After ingestion of sporulated oocyst, excystation of sporozoites in gut lumen and migration of sporozoites through epithelial cells, first merogony occurs in the host endothelial cells of the lacteals of the villi of duodenum, jejunum and ileum, but also in the endothelial cells of the sinuses of mesenteric lymph nodes draining these regions. First-generation macromeronts can reach sizes of up to 240 µm, develop within 12-14 days and may release > 120.000 merozoites I, each (Taylor et al. 2007; Hashemnia et al. 2012). Smaller second-generation meronts develop in epithelial cells of the villi and the crypts of the lower jejunum (Taylor et al. 2007), within 12 days and release 8-24 merozoites II (Sayin et al. 1980; Hashemnia et al. 2012). Merozoites II differentiate into sexual macro- (female) and micro- (male) gametocytes within host epithelial cells and, after gamogony, new oocysts are shed into the environment (for illustration see Fig. 2).

Hashemnia et al. (2012) recently reported on morphopathological aspects of *E. arloingi* experimental infections. The authors denoted a correlation between infection dose and the onset and severity of clinical signs being presented by mild to severe degrees of depression, dehydration, paleness of conjunctiva and semi-liquid to liquid diarrhoea. Following experimental caprine *E. arloingi* infections, a thickening of the mucosa and the presence of large non-pedunculated to polyp-like whitish nodules reaching the size of 3-5 mm within the jejunum and ileum were reported at necropsy (Hashemnia et al. 2012; Silva et al. 2014b). Moreover, mucosal leukocyte infiltration mainly by lymphocytes, eosinophils, polymorphonuclear neutrophils (PMN) and macrophages has been described (see Chapter 2).

Since first merogony of *E. arloingi* occurs within highly immunoreactive host endothelial cells (Ruiz et al. 2010), the aim of this work was to establish a suitable *in vitro* system by using primary endothelial host cells to be as close as possible to the *in vivo* situation. Further on, we aimed to study in detail the development of pathogenic stages and their host cell

modulation (see Chapter 3). Additionally, the species-specificity of the host immune response against *Eimeria* and even species variation factors (e. g. strains) (Rose 1987) justify the isolation of a new pathogenic species occurring endemically in Portuguese goats and in other European countries (see Chapter 2) (Silva et al. 2013; Silva et al. 2014b).

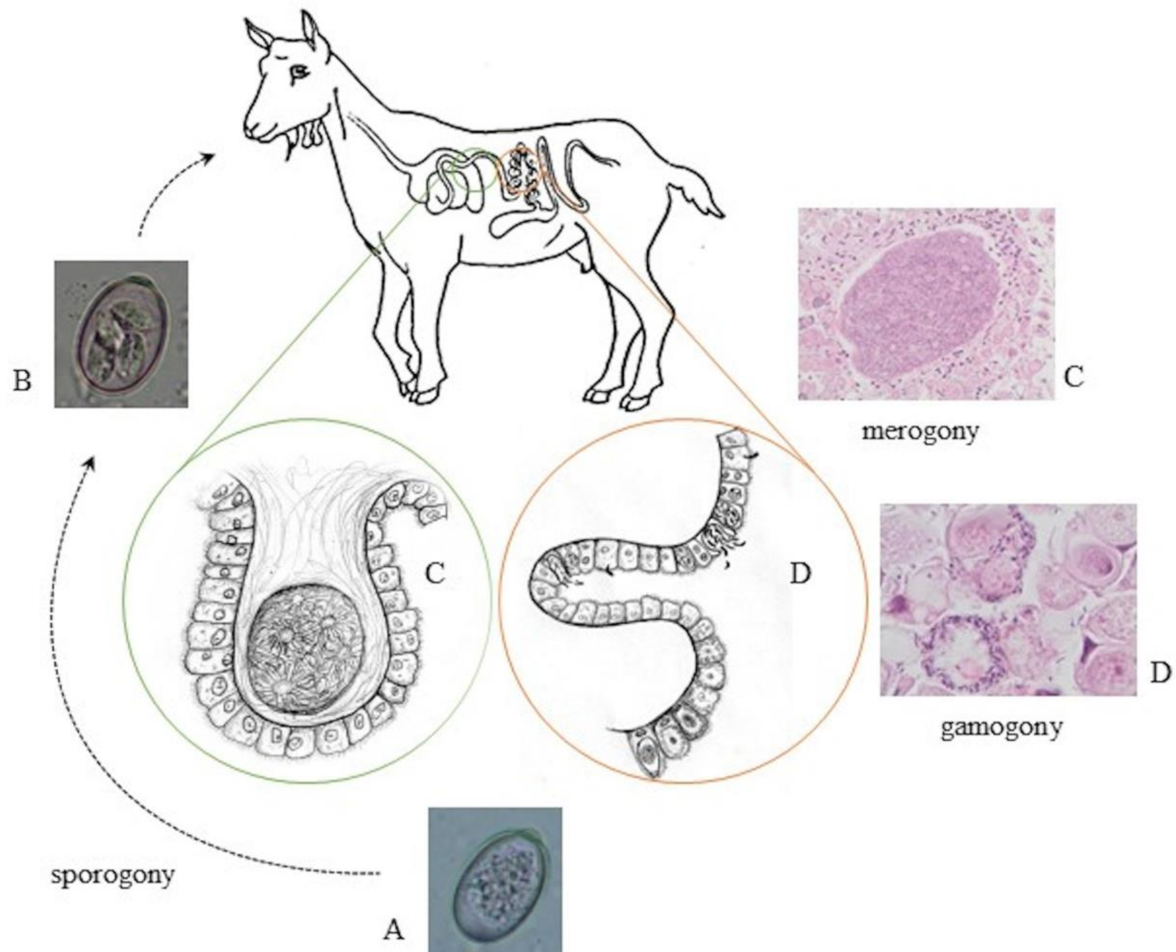

FIG. 2. Life cycle of *Eimeria arloingi*

Infected animals shed unsporulated oocysts (A), which undergo sporogony in the environment. Infective stages (B, sporulated oocysts) remain on the pasture until ingestion by goats (primary or challenge infection). During the excystation process in the gut lumen, oocysts release eight sporozoites, each. Freshly released sporozoites must traverse the epithelium and invade endothelial cells of the lacteals of the villi of duodenum, jejunum and ileum, or, less commonly, endothelial cells of the sinuses of mesenteric lymph nodes draining these regions. First merogony results in macromeronts (C), which may release more than 120.000 merozoites I. Free-released merozoites I then must invade epithelial cells of the duodenum, jejunum or ileum and develop into small second-

generation meronts (second merogony). Differentiation of second-generation merozoites into macro- or microgametocytes is designated gamogony (D) and takes place in epithelial cells (duodenum, jejunum, ileum). The life cycle is completed when unsporulated oocysts are shed into the environment.

1.5. Host cell-parasite interactions

In biology, parasitism is defined as relation between an organism (parasite) that grows, multiplies, feeds and shelters on or in a different organism (host) while contributing minimal to nothing to the survival of its host, thereby causing morbidity or mortality (Todar 2006). As obligatory intracellular organisms, intracellular stages of all apicomplexan parasites cause the pathogenicity of the infection. Therefore, *in vitro* systems have been used for many years in order to investigate in detail complex host-parasite interactions and in many cases have entirely replaced the requirement of experimental animals (Muller and Hemphill 2013). Especially concerns on animal welfare, animal protection laws and the public´s interest in science impelled the development of *in vitro* culture systems, which were recognized as powerful and useful tools in a large number of research fields. Some examples of what have been accomplished with suitable *in vitro* culture systems have been reviewed by Muller and Hemphill (2013) and are here enumerated: knowledge of basic biology of parasites – *T. annulata* (Li et al. 2014); host-parasite interactions – *E. tenella* (Tierney et al. 2007); development of tools for diagnostic proposes – *B. besnoiti* (Cortes et al. 2006); factors involved in innate resistance – *T. gondii* (Cavailles et al. 2006); vaccines candidates and drug effectiveness – *B. bovis* (Salama et al. 2013), and identification of chemotherapic compounds – *B. besnoiti* (Cortes et al. 2011).

The host immune system governs host-parasite interactions to a great extent. Mainly, it is responsible for protection of the organism against infectious agents, including bacteria, viruses, fungi and parasites. However, pathogens possess a great variety of host evasion

mechanisms that facilitate their development by directly interact with host defence tools. Some parasites are capable of producing endonucleases and DNases which allow them to free themselves from extracellular traps (ETs) produced by activated polymorphonuclear neutrophils (PMN) during host innate immune responses (Riyapa et al. 2012; Seper et al. 2013; Guimaraes-Costa et al. 2014), or, like in the case of *E. papillata* infections in mice, parasites can induce clear goblet cell hypoplasia and depletion of mucus secretion to gain better access to epithelial cells (Dkhil et al. 2013). Furthermore, *Eimeria* spp. modulate a series of host cell-derived processes in order to complete their intracellular life cycle, such as the host cell cycle (Taubert et al. 2010), host cell structure – e. g. by the modulation of the cytoskeleton (Hermosilla et al. 2008), inhibition of apoptosis (Lang et al. 2009), immune responses or even host cell metabolism (Hermosilla et al. 2008; Taubert et al. 2010), all to achieve completion of their own life cycle. Particularly in the case of early host innate immune reactions against apicomplexan, the development of a suitable *in vitro* culture system allows detailed analyses of complex host endothelial cell-*Eimeria* interactions as previously reported for *E. bovis* (Hermosilla et al. 2006; Taubert et al. 2008; Taubert et al. 2010; Hermosilla et al. 2012).

1.5.1. Host immune reactions against *Eimeria*

The immune system can be academically divided in two main sections: the innate or non-specific immune system and the adaptive or specific immune system. The adaptive immune system is highly specific for particular pathogens and it is either triggered when innate immune responses fail to eliminate the pathogen or when reactions of the innate immune system initiate the transition to adaptive immune response. The adaptive immune response can confer long-lasting protection, in contrast to the innate immune system which is known to

act immediately upon exposure and thereby represents the first line of defence (Alberts et al. 2002). In coccidiosis, respective parasite species reveal a high host- and site-specificity, which is assumed to result from genetically determined parasite factors in combination with specific host immune responses (Rose 1987; Witcombe and Smith 2014). Induction of strong species-specific protective immune response, which prevents clinical disease, is generally induced by homologous challenge infections (Ruiz et al. 2014) and relies on acquired immunological memory actions provided by the cellular adaptive immune system. This process is the basis of successful vaccination used for coccidiosis control in chickens (Long et al. 1982; Zhang et al. 2014). Nonetheless, further research focused on other host species to be protected from coccidiosis, such as turkeys (Poplstein and Vrba 2011), bovines (Svensson et al. 1996) and goats (Ruiz et al. 2014).

a) Host innate immune responses

In contrast to the cellular adaptive immune response (Rose and Hesketh 1979; Rose et al. 1992; Hermosilla et al. 1999; Shi et al. 2001), relatively little is known on host innate immune reactions against *Eimeria* infections in ruminants, even though the host innate immune system is considered to be an evolutionarily older system than the adaptive one and acts as the first-line of defence (Alberts et al. 2002; Tschopp et al. 2003). Physical barriers of mucosa, epithelial cells, endothelial cells and leukocytes form part of the host innate immune system. Skin and other epithelial surfaces, such as the lining of lungs and gut, establish the most external physical and natural barriers between the organism and invasive pathogens. Additionally, specialized epithelial cells (goblet cells) secrete antimicrobial products and gel-forming mucins, which are the major component of mucus and contribute to the maintenance of the mucosal barrier (Roxstrom-Lindquist et al. 2006). Mucus interfaces act as

local defence mechanisms, protect epithelial cells from host digestive enzymes and constitute the first barrier between the intestinal epithelium and pathogens (Roxstrom-Lindquist et al. 2006; Tierney et al. 2007). For instance, the antimicrobial peptides defensin and lactoferrin, which cover the epithelial surfaces, inhibit the adhesion of pathogens to epithelium by hampering their invasion or by even killing them (Alberts et al. 2002; Roxstrom-Lindquist et al. 2006). Likewise, it has been shown that mucin exerts a direct negative effect on the attachment of *E. tenella* to epithelial cells *in vitro* thereby reducing the number of intracellular sporozoites (Tierney et al. 2007).

Furthermore, endothelium, lining blood and lymphatic vessels, establishes a physical barrier between blood and lymph and the adjacent tissues. Besides trophic (modulation of metabolic homeostasis), tonic (vascular hemodynamics) and trafficking functions (cell extravasation), endothelial cells exhibit important immunoregulatory functions, rendering them the first cells to interact with foreign pathogens (Mai et al. 2013). Furthermore, endothelial cells are highly immunoreactive cells since they actively participate in both innate and adaptive immune responses, demonstrating the crucial interplay between both systems (i. e., components of the innate immune system influence the adaptive immune system and vice versa). For instance, they synthesize a broad spectrum of immunoregulatory molecules such as chemokines which are involved in the recruitment of immune cells by chemotaxis, in leukocyte activation, endothelium transmigration and in the regulation of inflammatory processes by interactions with growth factors, cytokines, and adhesion molecules (Taubert et al. 2006).

According to the relative position of their cysteine residues, chemokines are classified in four different families (CXC, CC, C, and CX_3C chemokines). CXC chemokines such as chemokine (CXC motif) ligand 1 [(CXCL1, previously growth-related oncogene protein α (GRO-α], the CXC-chemokine interleukin-8 (CXCL8, previously IL-8) and CXC motif

chemokine 10 [CXCL10, previously interferon gamma-induced protein 10 (IP-10)] predominantly attract PMN and lymphocytes, whilst CC chemokines [CC motif ligand 2 (CCL2), previously monocyte chemotactic protein-1 (MCP-1); CCL5, previously regulation upon activation normal T cell expressed and secreted (RANTES)] are more effective in the recruitment of other leukocytes and T helper cells rather than PMN (Taubert et al. 2006). Additionally, endothelial cells even serve as antigen presenting cells by expressing major histocompatibility complex (MHC) I and II molecules, which facilitate the recognition of pathogen epitopes by T-cells and expedites their infiltration in tissues (Taubert et al. 2010; Mai et al. 2013).

Endothelial cells are the specific host cells of a number of coccidian parasites *in vivo*, particularly for pathogenic *Eimeria* species forming macromeronts [e. g. *E. bovis* (cattle), *E. arloingi*, *E. ninakohlyakimovae* (goat), *E. bakuensis* (sheep), *E. rajhastani* (camels)], and as well as for other coccidian species [e. g. *T. gondii*, *N. caninum* (Taubert et al. 2006) and *B. besnoiti* (Alvarez-Garcia et al. 2014b)].

The development of macromeront by certain *Eimeria* species is related to their rather longer persistence in their host endothelial cell, when compared to faster replicating parasites, such as *T. gondii, N. canimum* or *B. besnoiti,* and hence, to avoid the stimulation of host endothelial cells may, in turn, result in pro-inflammatory host cell reactions. In this work, interactions between *E. arloingi* sporozoites and primary endothelial host cells were studied (see Chapter 3). Furthermore, early host innate immune reactions, i. e. parasite-triggered neutrophil extracellular trap (NET) formation was investigated and compared to reactions being induced by a closely related, but fast replicating apicomplexan parasite, *B. besnoiti* (see Chapter 5)(Muñoz-Caro et al. 2014).

The collection and isolation of primary endothelial host cells is a rather difficult, time consuming, and expensive process. Therefore, different cell lines have also been tested with the purpose of identifying permanent cell lines capable of supporting *E. arloingi* replication (Hermosilla et al. 2002; Tierney et al. 2007; Ruiz et al. 2010). However, the current data show that the *in vitro* infection of endothelial host cells represents a superior model to investigate host-parasite interactions (see Chapter 3).

b) Host innate leukocytes

Host innate leukocytes are a major component of the innate immune system comprising PMN, monocytes, macrophages, dendritic cells, natural killer cells, basophils, mast cells and eosinophils. Until recently, the most acknowledged function of PMN, monocytes and macrophages was the phagocytosis promoting the internalization of pathogens in cytosolic phagosomes and their fusion with intracellular granules forming phagolysosomes. Within these intracellular structures, a combination of non-oxidative [antimicrobial peptides (AMPs) – e. g. defensins, proteases] and oxidative mechanisms [antimicrobial reactive oxygen species (ROS)] act to eliminate pathogens (Hermosilla et al. 2014). Especially, PMN as the most abundant leukocyte cell type play an important role in innate immunity and predominantly act via the production of immunomodulatory molecules, such as cytokines [e.g. IL-6, IL-12, tumour necrosis factor-α (TNF-α)] and chemokines (e. g. CCL2, CXCL1, CXCL8, CXCL10), which attract other immunocompetent cells to the site of infection in order to start acquired cellular immune responses (Behrendt et al. 2010).

1.6. Neutrophil extracellular traps

PMN are fascinating cells able to kill viable pathogens far beyond their lifespan. Ten years ago, Brinkmann et al. (2004) revealed a novel effector mechanism, which represents a general and ancient mechanism to eliminate invasive pathogens, known as neutrophil extracellular trap (NET) formation. NETs are classically composed of extracellular protein-studded DNA matrices capable of extracellular trapping and killing of pathogens (Brinkmann et al. 2004). The protein mass of NETs accounts for approximately 70% and is mainly composed of core histones (H2A, H2B, H3, H4), microbicidal granular enzymes and peptides/proteins (Brinkmann et al. 2012) such as bacterial permeability-increasing protein (BPI), myeloperoxidase, cathepsin G, lactoferrin, gelatinase, peptido-glycan recognition proteins (PGRPs), calprotectin and elastase (Abi Abdallah and Denkers 2012; Hermosilla et al. 2014).

NET formation (Fig. 3) occurs when (*i*) PMN become activated by contact with pathogenic stages inducing the activation of the NADPH oxidase cascade and the intracellular production of ROS. Additionally, PAD4-dependent NET formation has been reported (von Kockritz-Blickwede and Nizet 2009). Subsequently, (*ii*) the nuclear envelope degrades and chromatin decondensates. Then, (*iii*) nuclear contents mix with cytoplasmatic granular contents. Finally, (*iv*) the DNA-protein mixture is expelled from the cells by a cytoskeleton-dependent shrinkage of the dead PMN (Brinkmann et al. 2004; Hermosilla et al. 2014).

This novel cell death process, which is distinct from apoptosis and necrosis, is nowadays well-known as NETosis. NETosis has been widely associated with extracellular bacteria and has been shown to occur in numerous vertebrates and invertebrates including humans, mice, cows, horses, cats, dogs, chickens, goats, fish, insects and even crustaceans (see Chapters 4 and 6).

Previous studies report on NET formation in the presence of other protozoan parasites within the subphylum Apicomplexa: *P. falciparum* (Baker et al. 2008), *E. bovis* (Behrendt et al. 2010) and *T. gondii* (Abi Abdallah et al. 2012). Since there was clear evidence of *E. bovis*-triggered NETosis (Behrendt et al. 2010), the aim of this work was to investigate whether the interaction of caprine PMN with *E. arloingi* stages would also result in caprine NET formation (see Chapter 4).

Eimeria arloingi sporozoites – after having excystated in the intestinal lumen – traverse the intestinal mucosa of the small intestine in order to infect lymphatic endothelial cells to fulfil their first merogony. During parasite migration, *E. arloingi* sporozoites are exposed to leukocytes, such as PMN, mainly when entering the lymphatic capillaries of the ileum. Given that host cell invasion is an indispensable requirement of *E. arloingi* for successful *in vivo* survival and replication (Silva et al. 2014a), leukocyte-parasite-encounter may represent a good opportunity for the host innate immune system to either eliminate or at least reduce the degree of infection (Abi Abdallah and Denkers 2012).

The key role of PMN and other leukocytes, such as monocytes and macrophages, in ruminant *Eimeria* infections has been proven *in vitro, ex vivo* and *in vivo* experiments (Behrendt et al. 2008; Suhwold et al. 2010; Taubert et al. 2010). In line with these findings, other authors have described the crucial role of PMN during rodent coccidiosis. Thus, PMN-depleted SCID mice showed severe coccidiosis due to primary *E. papillata* infections when compared to wild type mice. (Schito and Barta 1997). Others have described a significant reduction of the *in vitro* infection rate of PMN-exposed *E. bovis* sporozoites, which may have a tremendous impact on subsequent apicomplexan parasite proliferation (see Chapter 5)(Muñoz-Caro et al. 2014).

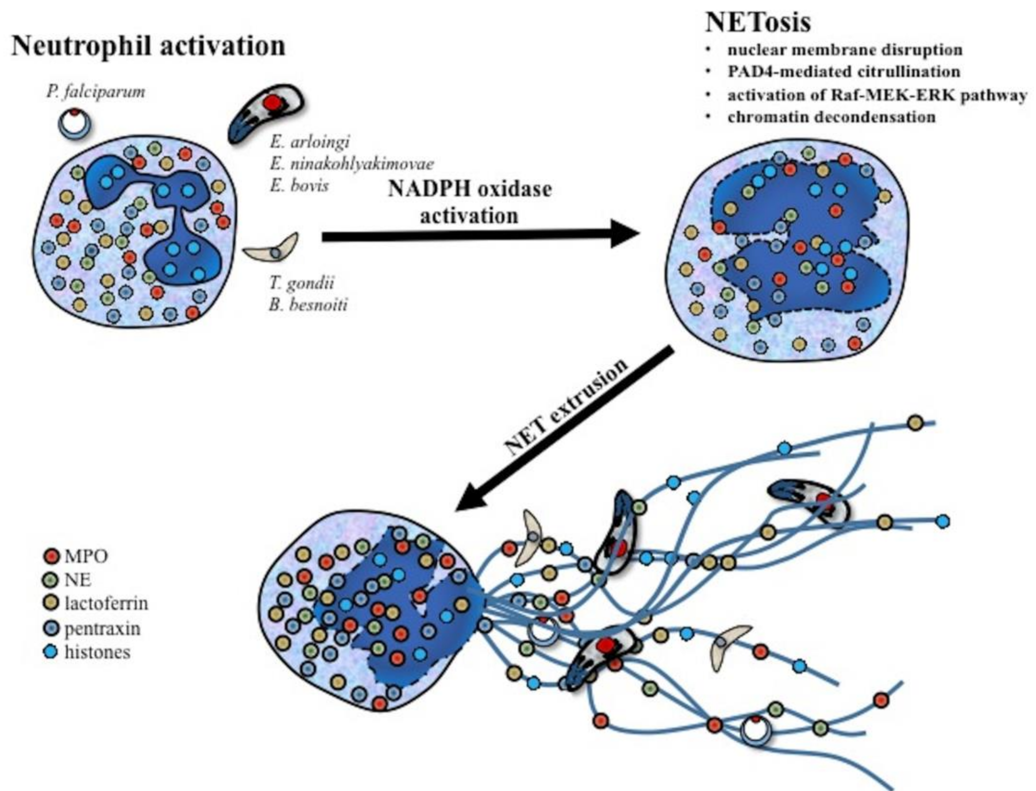

FIG. 3. Mechanisms of parasite-triggered neutrophil extracellular traps (NETs) release

PMN become activated by the contact with different apicomplexan parasite stages, such as trophozoites of *Plasmodium falciparum*, sporozoites of *Eimeria bovis*, *E. arloingi* or *E. ninakohlyakimovae*, tachyzoites of *Toxoplasma gondii* or *Besnoitia besnoiti*. Stimulation of PMN results in the activation of NADPH oxidase, the intracellular production of reactive oxygen species (ROS) and 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 degradation 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 extruded by a cytoskeleton-dependent shrinkage of the dead PMN. Released NET structures being decorated 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 (adapted from Hermosilla et al. 2014).

Recently, other innate immune leukocytes have also been described to release extracellular traps (ETs) and, consequently, this process was re-named as ETosis (Muniz et al. 2013). ETs are released by basophils containing mitochondrial DNA instead of nuclear DNA (Morshed et al. 2014), by macrophages (Bonne-Annee et al. 2014), mast cells (von Kockritz-Blickwede and Nizet 2009) and eosinophils (Yousefi et al. 2008). Moreover, monocytes can also strongly extrude ETs as shown for the exposure to *B. besnoiti* tachyzoites and *E. bovis* sporozoites (Muñoz Caro et al., accepted manuscript) and *E. arloingi* and *E. ninakohlyakimovae* sporozoites (L. Silva, personal observation).

2. *EIMERIA* INFECTION IN GOATS IN SOUTHERN PORTUGAL

This chapter is based on the following published paper:

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Eimeria infections in goats in Southern Portugal

Infeções por *Eimeria* em caprinos do Sul de Portugal

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Abstract

Coccidiosis caused by *Eimeria* species is a major form of intestinal infection affecting intensively and semi-intensively reared goats. The province of Alentejo is the main goat-producing area in Portugal. Therefore, all 15 Serpentina goat farms in Alentejo were analyzed regarding the occurrence and diversity of *Eimeria* species. Fecal samples obtained from 144 animals (52.1% dairy goats, 47.9% pre-pubertal goats) were examined using the modified McMaster technique to determine the number of oocysts per gram of feces. *Eimeria* spp. oocysts were present in 98.61% of the fecal samples and, overall, nine different *Eimeria* species were identified. The most prevalent species were *E. ninakohlyakimovae* (88%) and *E. arloingi* (85%), followed by *E. alijevi* (63%) and *E. caprovina* (63%). The average number of oocysts shed was significantly lower in dairy goats than in pre-adult animals. Astonishingly, no clinical signs of coccidiosis were observed in any of the animals examined, even though they were shedding high numbers of oocysts and were infected with highly pathogenic species. Thus, implementation of routine diagnostic investigation of the occurrence and diversity of caprine *Eimeria* species may be a useful tool for determination and better understanding of their potential economic impact on goat herds in southern Portugal.

Keywords: *Eimeria,* coccidiosis, goats, infection, Portugal.

Resumo

A coccidiose causada por espécies de *Eimeria* é a maior infecção intestinal que afeta regimes intensivos e semi-intensivos de caprinos. A região do Alentejo é a mais importante na indústria caprina em Portugal. Assim, todas as 15 explorações de caprinos da raça Serpentina do Alentejo foram analisadas para determinar a frequência e diversidade de espécies de *Eimeria* presentes. Amostras fecais de 144 animais (52,1% adultas, 47,9% jovens) foram examinadas com a técnica de McMaster modificada para determinar o número de oocistos por grama de fezes. Oocistos de *Eimeria* spp. estavam presentes em 98,61% das amostras fecais e nove espécies distintas foram identificadas. As espécies mais frequentes foram *E. ninakohlyakimovae* (88%) e *E. arloingi* (85%), seguidas por *E. alijevi* (63%) e *E. caprovina* (63%). A média do número de oocistos excretados foi significativamente menor em adultas do que em jovens. Surpreendentemente, não foram observados quaisquer sinais clínicos em nenhum dos animais examinados, apesar de eliminarem elevados números de oocistos e de estarem infectados com espécies altamente patogénicas. A prática de diagnósticos de rotina para identificação de espécies de *Eimeria* caprinas pode ser um importante instrumento para o melhor entendimento do nefasto impacto da doença em explorações de caprinos no Sul de Portugal.

Palavras-chave: *Eimeria*, coccidiosis, caprinos, infecção, Portugal.

Introduction

Caprine coccidiosis caused by the apicomplexan protozoan genus *Eimeria* is a worldwide and frequent intestinal parasitosis of goats (NORTON, 1986; O'CALLAGHAN, 1989; AGYEI et al., 2004). It affects the profitability of the industry, particularly in rural, semi-arid geographic regions that are economically dependent on goat rearing, such as the Mediterranean basin (RUIZ et al., 2006), Africa (KANYARI, 1993), Asia (FAIZAL; RAJAPAKSE, 2001) and Latin America (CAVALCANTE et al., 2012). Economic losses result from the high mortality rate among goat kids, reduction

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of productivity and delayed weight gains of parasitized animals (LIMA, 2004; RUIZ et al., 2012).

Goats of all ages are in principle susceptible to *Eimeria* spp. infections, but younger animals are more likely to develop the disease (TAYLOR; CATCHPOLE, 1994). Depending on the type of management, caprine coccidiosis might affect 100% of goat kids within the age range of 4-10 weeks, thus directly affecting animal health and the profitability of the goat industry (KOUDELA; BOKOVÁ, 1998; RUIZ et al., 2010). *Eimeria*-infected goat kids show clinical signs particularly during the weaning period, ranging from non-hemorrhagic to severe hemorrhagic diarrhea, with accompanying weight loss, dehydration and growth delay (KOUDELA; BOKOVÁ, 1998; RUIZ et al., 2006, 2012).

Although goats can be parasitized by 16 different *Eimeria* species, most of them do not cause visible clinical coccidiosis. Therefore, diagnosis must be combined with species identification in order to avoid economic losses. *Eimeria ninakohlyakimovae* and *E. arloingi* are considered to be the most pathogenic species (KOUDELA; BOKOVÁ, 1998; CHARTIER; PARAUD, 2012).

During the last two decades, there has been remarkable growth in the goat industry and currently about one billion goats are being reared for meat and milk production worldwide. It is evident that the goat industry is not only playing a big role in the nutritional security of small farmers in many poor countries but also assures working opportunities for a sizeable population around the world. This particular feature of the goat industry also includes Portugal. Approximately half a million goats are kept in this country, which represents the sixth largest national goat industry within the European Union (GPP, 2009). In Portugal, goats are usually raised in extensive or semi-extensive systems, mostly in semi-arid geographic regions with poor soil conditions and a lack of vegetation apart from some bushes. These environments are not appropriate for rearing other domestic animals. Thus, goats play an important role in stabilizing and maintaining rural communities, thereby avoiding the abandonment of these poor regions by the human population. The most important Portuguese province for the goat population is Alentejo, where 21.6% of all Portuguese goats are kept (GPP, 2009). Of the six native Portuguese goat breeds, the Serpentina breed is the one at risk of extinction, according to the IUCN Red List threatened categories (IUCN, 2013), since there are fewer than 5,000 pure mature individuals (APCRS, 2014) in existence. Several efforts have been made in the past years to maintain this breed, including financial support from national and European programs like the PRODER program (PRODER, 2011). Serpentina goats are well adapted to semi-arid climate conditions and are used for both milk and meat production. Nevertheless, semi-intensive and intensive systems of goat rearing provide excellent environmental conditions for accumulation and transmission of *Eimeria* oocysts and, in the absence of effective control measures, clinical coccidiosis is the inevitable outcome (RUIZ et al., 2006). There are, however, records in some herds of deaths among kids around the weaning period, which have reached 20% of newly born animals and have been correlated with coccidiosis (APCRS, personal communication). Therefore, in the present study, we concentrated on this special goat breed and examined 15 Serpentina goat herds for the presence, diversity and parasite load of *Eimeria* species, considering both dairy goats (> 1 year) and pre-pubertal goats (5-12 months of age). The overall aim of this survey was to characterize *Eimeria* spp. infections and identify species occurring in the southern part of Portugal using classical coprological methods of diagnosis in dairy goat herds, so as to gain better understanding of caprine coccidiosis epidemiology in semi-arid areas.

Materials and Methods

This study was carried out in the province of Alentejo, which comprises one third of the total area of Portugal, covering 31,604.9 km^2 area, with an average altitude of 200 m (INE, 2010). The Serpentina goat farms were distributed throughout Alentejo. The climate is semi-arid with hot and dry summers but damp and cold winters; the average rainfall is less than 700 mm per year. The rainy season lasts from late autumn to early spring. There are currently 15 Serpentina goat herds in this region; all of them present semi-extensive production systems. More than 70% are medium-sized farms keeping more than 200 dairy goats. There are two breeding seasons: one in autumn (September-October) and another in winter (January-February). Male goat kids are mainly sold for meat consumption during the Christmas and Easter periods, while some female goat kids are kept for replacement, when presenting exceptional phenotypic characteristics.

Fecal samples were collected rectally from 144 goats. All fecal samples were placed in plastic bags and kept at 4 °C until coprological analyses were performed, which was done at the Victor Caeiro Parasitology Laboratory, University of Évora, Portugal. The fecal samples were classified into two categories: dairy goats (n = 75) and pre-pubertal goats (n = 69).

For each farm, the total number of animals, distribution according to age, number and schedule of anthelminthic or anti-coccidial treatments and place of birth were surveyed. The two categories of animals were grouped as follows: pre-pubertal goats (group 1: 5-12 months of age) and dairy goats (group 2, split into three age groups: 2-4 (2a), 5-7 (2b) and 8-11 (2c) year old animals). Individual fecal samples from five animals per group from each herd were taken from the rectum and each animal was checked for common clinical signs relating to coccidiosis (diarrhea, dirty posteriors and dehydration). The numbers of oocysts per gram of feces (OPG) were determined by means of the modified McMaster technique (THIENPONT et al., 1979).

Morphological identification of *Eimeria* oocysts was carried out based on shape and size of the oocysts, presence or absence of the micropyle and polar cap and color and thickness of oocyst walls (LEVINE, 1985; ECKERT et al., 1995). Oocysts and oocyst sporogony were collected as previously described (HERMOSILLA et al., 2002). Oocysts were isolated from the feces by means of saturated sugar solution (1.3 g/l density) and the suspension was transferred into Petri dishes covered with small glass panels. Oocysts that bonded to the glass panels were washed off with water and were added to 2% (w/v) potassium dichromate solution to allow complete sporulation, at room temperature and with regular aeration for 2-5 days. After concentration, simple flotation using saturated saline solution (1.20 g/l) was performed

and the oocysts were observed and measured under a microscope to determine the relative frequency of each species.

Fecal oocyst counts were transformed into the logarithm of OPG plus one [log (OPG + 1)] to obtain normal distribution. Levene's test, Student's t test, and one-way ANOVA were used to analyze the data (R® software, version 2.13.1) and determine whether any differences were significant; $p < 0.05$ was accepted as a statistically significant difference.

Additionally, due to an unexpected event, histopathological sections were produced from intestinal samples from a kid that died due to coccidiosis in one of the farms. During the necropsy, intestinal tissue samples from the duodenum, jejunum and ileum were collected, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin-eosin for observation under an optical microscopic.

Results

As the survey revealed, most of the Serpentina goat farms (80%) subjected all animals to anthelminthic treatments every six months, medicating with a combination of either benzimidazoles and macrocyclic lactones or benzimidazoles and salicylanilides. Only two goat farms had a metaphylactic program for coccidiosis control, in which kids were treated orally with a single dose of diclazuril (Vecoxan®, Virbac, Portugal) at two weeks of age. Generally, parturition occurred on the pasture and kids were fed by natural lactation and were kept together with the dairy goats until weaning and slaughtering time (about two month of age).

Eimeria spp. oocysts were detected in 98.6% of the fecal samples: 74 samples originating from adult dairy goats and 68 from pre-pubertal animals. In total, nine different *Eimeria* species were identified in the present study. The most prevalent species were *E. ninakohlyakimovae* (88%), followed by *E. arloingi* (85%), *E. alijevi* (63%) and *E. caprovina* (63%). Other species observed less frequently were *E. hirci*, *E. caprina*, *E. jolchijevi*, *E. christenseni* and *E. apsheronica*, which were present in 52, 52, 49, 39 and 26% of the samples, respectively. The most prevalent species in dairy goats was *E. ninakohlyakimovae*, while in pre-adults *E. arloingi* was found most frequently. Two to five different *Eimeria* species were observed simultaneously in the feces, thus indicating concomitant infections in more than 50% of the samples (Figure 1). Furthermore, 6-9 different species were present in more than 40% of the samples.

Overall, OPG counts varied greatly, ranging from 3.3×10 to 3.4×10^5. Within the same goat herd and at the same sampling time, pre-pubertal OPGs ranged from 2.6×10^3 to 3.4×10^5. The mean number of oocysts shed was significantly higher ($p < 0.001$) in pre-pubertal goats (11,358 ± 49,754) than in adult dairy goats (996 ± 1,066). In contrast, no significant differences were detected between the different dairy goat age ranges (groups 2a-c). Despite high OPG counts, no diarrhea, dirty posteriors or dehydration, which are commonly associated with caprine coccidiosis, were detected in any animal included in the current study.

The mean OPG counts for each farm sampled in the two goat classes are represented in Figure 2. Pre-pubertal goats had significantly higher OPG counts, except for herd number 10, in which one of the pre-pubertal animals showed no oocysts in the feces. Differences in OPG values, in comparing dairy goats between herds, were highly significant ($p < 0.001$). This also held true for pre-pubertal groups ($P < 0.001$).

Additionally, in one of the farms, a two-month-old goat kid suffering from severe coccidiosis presented yellowish diarrhea and

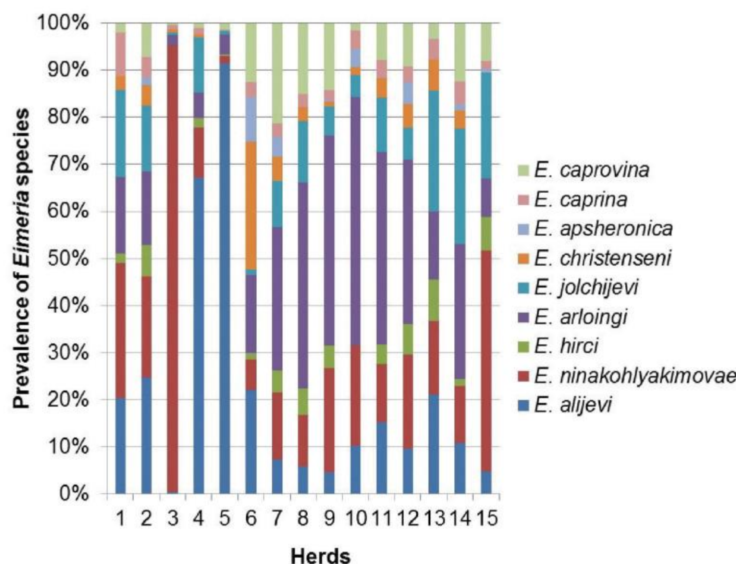

Figure 1. Prevalence of *Eimeria* species found in each Serpentina goat herd in Alentejo, Portugal.

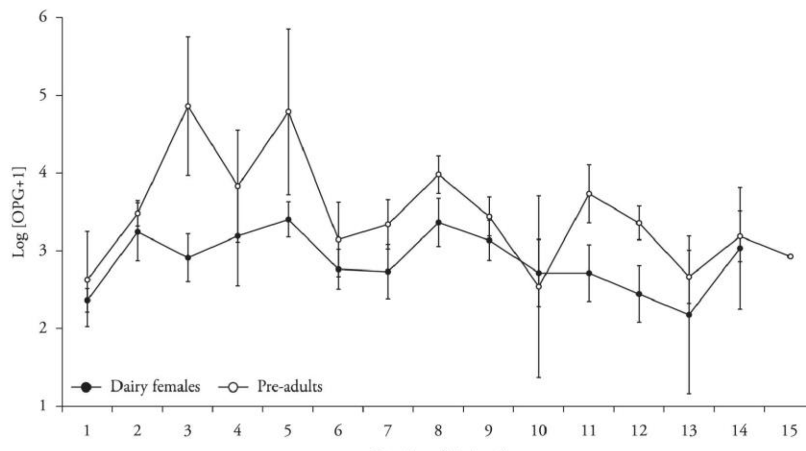

Figure 2. *Eimeria* spp. oocyst counts (oocysts per gram of feces, OPG) among dairy females and pre-adults in Serpentina goat herds in Alentejo, Portugal. Data are expressed as transformed values of log (OPG + 1).

low body weight and was found dead on the pasture. Although this animal was not part of the investigation owing to its young age, a necropsy was performed and contributed to this investigation, since it indicated that severe cases of caprine coccidiosis definitely occur in young goat kids within the herds examined. The OPG count of this affected animal was greater than 1×10^6 and the most frequent species found were *E. arloingi* (96.9%), *E. ninakohlyakimovae* and *E. caprovina* (1.4% each) and *E. christenseni* (0.3%). At necropsy, pathological lesions similar to those reported for *E. arloingi* mono-infections in goats were found with large non-pedunculated to polyp-like whitish nodules, reaching the size of 3-5 mm within the jejunum and ileum (Figure 3A). Tissue alterations were characterized by hyperplasia of the intestinal villi and mild inflammation of the mucosa, on hematoxylin-eosin stained gut sections. Mucosal leukocyte infiltration mainly consisted of lymphocytes, eosinophils, polymorphonuclear neutrophils and macrophages, as well as plasma cells to a lesser extent. Different developmental stages of *E. arloingi* were found, including trophozoites, immature and mature first-generation macroschizonts, second-generation schizonts, macrogamonts, microgamonts, free microgametocytes and immature and mature oocysts (Figure 3B, C). Autolysis and necrosis of the affected villi were also observed, and the presence of necrotic epithelial cells and scattered developmental stages of the parasite were also seen within the lumen of the intestinal mucosa, as previously reported (HASHEMNIA et al., 2012). Bacterial infection was also observed in the affected gut lumen section.

Discussion

This study demonstrates that *Eimeria* species are widely distributed in the Serpentina goat herds of Alentejo, Portugal. The high prevalence of *Eimeria* infections found is consistent with previous reports from other regions experiencing comparable semi-arid climate conditions (DE LA FUENTE; ALUNDA, 1992; RUIZ et al., 2006). Furthermore, the high OPG counts observed in pre-pubertal goats was consistent with previous age-related studies on caprine coccidiosis (PENZHORN et al., 1994; KOUDELA; BOKOVÁ, 1998). The infection intensity and frequency were lower in adult dairy goats, probably owing to previous exposure to *Eimeria* spp., thus resulting in partial protective immunity, in comparison with pre-pubertal goats. The two herds in which metaphylactic programs for caprine coccidiosis control were being conducted had OPG counts within the range of the non-treated animals. This showed that the treatment selected had failed and indicated that there was a need to improve the metaphylaxis method in order to achieve a reduction in oocyst shedding and thereby decrease the environmental contamination. As proposed by Ruiz et al. (2012), on goat farms with a history of serious clinical caprine coccidiosis, precise timing of treatments using the anti-coccidial drug diclazuril improves the control over coccidiosis in the farm and prevents severe clinical status (RUIZ et al., 2012).

In general, adult animals are protected by cellular immune responses induced by primary *Eimeria* infections, in terms of clinical coccidiosis. However, this is not linked to complete interruption of internal parasite development, since these animals continue to shed *Eimeria* oocysts throughout their lives (TAYLOR; CATCHPOLE, 1994). This can be explained by the enzootic stability achieved between host and parasite, thus leading to non-clinical status among adult goats and even among pre-pubertal goats. In the current study, no significant differences in the OPGs between adult age ranges were found. In contrast to these findings, other studies have reported a slight increase in the excretion of oocysts among goats older than seven years of age, which has been interpreted as relative weakness of the host immune system (CHARTIER; PARAUD, 2012). Occasionally, host protective immune reactions against *Eimeria* infections fail due to adverse conditions, involving stress, dietary changes, prolonged travel, extremes of temperatures, weather conditions, environment

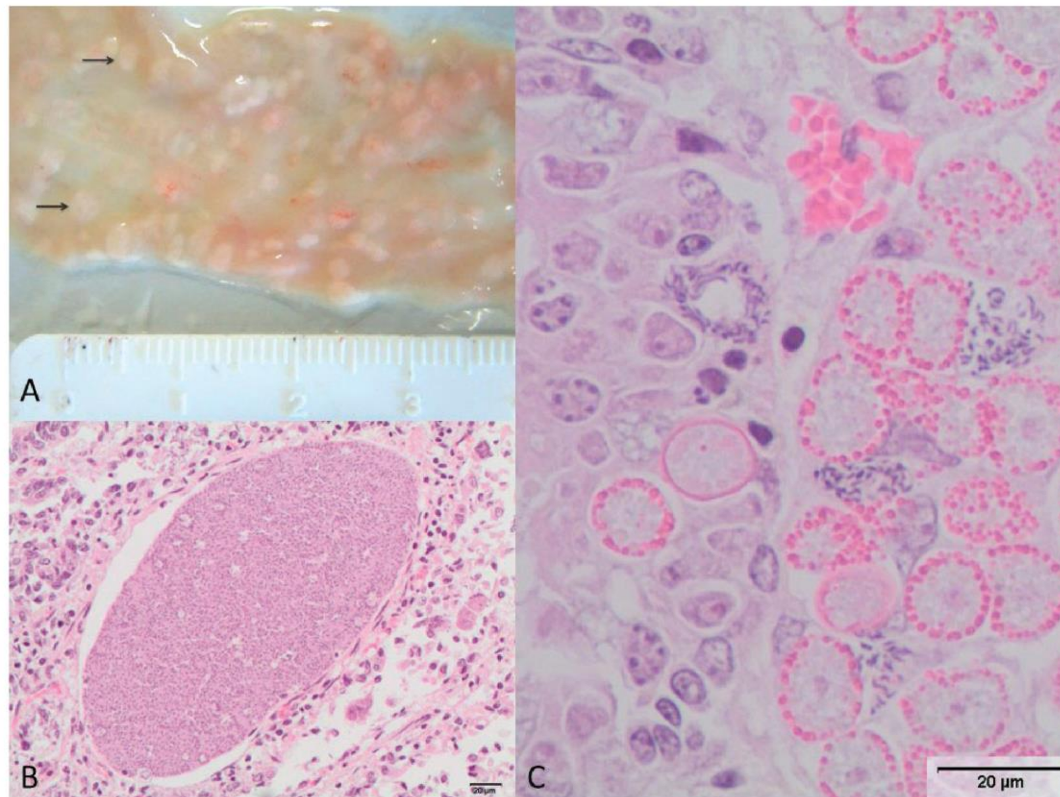

Figure 3. A. Jejunum. Natural infection of a two-month-old kid. Presence of scattered whitish pin-head size nodules (3-5 mm in diameter) (arrows). B. Multinucleated first-generation macroschizont. C. Cluster of macro and microgamonts and primitive oocyst.

changes, severe concomitant infections, nutritional status and other factors (TAYLOR; CATCHPOLE, 1994; DAUGSCHIES; NAJDROWSKI, 2005; RUIZ et al., 2006).

Eimeria ninakohlyakimovae and *E. arloingi* were the most predominant of the nine *Eimeria* species identified in the current study (Figure 2), which is in accordance to previous reports relating to other geographical areas (PENZHORN et al., 1994; AGYEI et al., 2004; RUIZ et al., 2006). These two species are well known as the most pathogenic species in cases of caprine coccidiosis (LEVINE, 1985; YVORÉ et al., 1985; KOUDELA; BOKOVÁ, 1998; CHARTIER; PARAUD, 2012). The general occurrence rate of *E. christenseni* was in agreement with previous studies, which also revealed higher prevalences among young goats than among adult dairy goats (LIMA, 1980; JALILA et al., 1998; HARPER; PENZHORN, 1999; HASSUM; MENEZES, 2005; RUIZ et al., 2006). *Eimeria christenseni* is considered to be one of the most pathogenic species in animals less than six months of age (AUMONT et al., 1984; LIMA, 2004). Nonetheless, other field surveys have shown very low prevalences of *E. christenseni*, not only in adults but also in young animals (KUSILUKA et al., 1996; AGYEI et al., 2004; FREITAS et al., 2005; CAVALCANTE et al., 2012). These differences in prevalence may be attributed to varying geographical conditions (CHARTIER; PARAUD, 2012), e. g. < 7% prevalence of *E. christenseni* was found in Ghana (AGYEI et al., 2004), Spain (RUIZ et al., 2006) and Brazil (CAVALCANTE et al., 2012), whilst higher prevalence was observed in the United States (LIMA, 1980).

The pathological findings among the samples from the deceased two-month-old goat kid clearly indicated that *E. arloingi* was the prime cause of death and confirmed the high pathogenicity of this *Eimeria* species in young animals, as previously described (YVORÉ et al., 1985; KOUDELA; BOKOVÁ, 1998). Since this goat kid almost exclusively shed *E. arloingi* oocysts, this *Eimeria* species could even be considered to be a monocausal parasitic agent that led to the animal's death. However, because of the delayed discovery of this animal, it cannot be ruled out that the bacteria found in the histological analysis might have been a concomitant infection that was proven to aggravate the clinical outcome of coccidiosis in goat and poultry (NORTON, 1986; PRUKNER-RADOVCIC et al., 1995), or that invaded the mucosa after the animal's death.

In conclusion, this survey clearly indicates that *Eimeria* infections are highly prevalent in Portugal, especially in Serpentina goats in the province of Alentejo. Although goats were affected by the most pathogenic species (e.g. *E. ninakohlyakimovae*, *E. arloingi*) and although pre-pubertal goats in particular shed high numbers of oocysts, no diarrhea, dirty posteriors or dehydration (symptoms commonly associated with caprine coccidiosis) were observed in any of the animals examined, which might indicate that there had been adequate development of protective cellular adaptive immunity against *Eimeria* spp.

Overall and compared with other livestock animals, goats are still a neglected species with regard to basic, clinical and epidemiological research. Nevertheless, these animals are the only reliable and economically viable source of meat and milk in certain climate areas. Foundations supporting research on caprine infections (e.g. CAPARA: COST action FA0805 of the European Community) currently pay tribute to the important value of this particular farm animal. Consequently, generation of epidemiological data on caprine *Eimeria* spp. will provide valuable data for future research on caprine coccidiosis.

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References

Agyei AD, Odonkor M, Osei-Somuah A. Concurrence of *Eimeria* and helminth parasitic infections in West African Dwarf kids in Ghana. *Small Rumin Res* 2004; 51(1): 29-35. http://dx.doi.org/10.1016/S0921-4488(03)00184-6

Aumont G, Yvoré P, Esnault A. Experimental coccidiosis in goats. 1. Experimental model. Effects of parasitism on the feeding behaviour and the growth of animals; intestinal lesions. *Ann Rech Vet* 1984; 15(4): 467-73. PMid:6529115.

Associação Portuguesa de Caprinicultores da Raça Serpentina - APCRS. *Livro genealógico da raça* [online]. Évora: APCRS; 2014 [cited 2014 Apr 17]. Available from: http://www.cabraserpentina.pt.

Cavalcante AC, Teixeira M, Monteiro JP, Lopes CW. *Eimeria* species in dairy goats in Brazil. *Vet Parasitol* 2012; 183(3-4): 356-8. PMid:21852038. http://dx.doi.org/10.1016/j.vetpar.2011.07.043

Chartier C, Paraud C. Coccidiosis due to *Eimeria* in sheep and goats, a review. *Small Rumin Res* 2012; 103(1): 84-92. http://dx.doi.org/10.1016/j.smallrumres.2011.10.022

Daugschies A, Najdrowski M. Eimeriosis in cattle: current understanding. *J Vet Med B Infect Dis Vet Public Health* 2005; 52(10): 417-27. PMid:16364016. http://dx.doi.org/10.1111/j.1439-0450.2005.00894.x

Eckert J, Braun R, Shirley MW, Coudert P. *Biotechnology*: guidelines on techniques in coccidiosis research. COST 89/820. Luxembourg: Office for Official Publications of the European Communities; 1995.

Faizal AC, Rajapakse RP. Prevalence of coccidia and gastrointestinal nematode infections in cross bred goats in the dry areas of Sri Lanka. *Small Rumin Res* 2001; 40(3): 233-8. http://dx.doi.org/10.1016/S0921-4488(01)00179-1

Freitas FLC, Almeida KS, Nascimento AA, Machado CR, Veschi JLA, Machado RZ. Species of the genus *Eimeria* Schneider, 1875 (Apicomplexa: Eimeriidae) in dairy goats maintained in intensive system in the São José do Rio Preto region, São Paulo State, Brazil. *Rev Bras Parasitol Vet* 2005; 14(1): 7-10. PMid:16153337.

de la Fuente C, Alunda JM. A quantitative study of *Eimeria* infections of goats from central Spain. *Vet Parasitol* 1992; 41(1-2): 7-15. http://dx.doi.org/10.1016/0304-4017(92)90003-R

Gabinete de Planeamento e Políticas - GPP. *Animal production yearbook 2008/2009*. Lisboa: GPP; 2009.

Harper CK, Penzhorn BL. Occurrence and diversity of coccidia in indigenous, Saanen and crossbred goats in South Africa. *Vet Parasitol* 1999; 82(1): 1-9. http://dx.doi.org/10.1016/S0304-4017(98)00266-0

Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi S. Experimental caprine coccidiosis caused by *Eimeria arloingi*: morphopathologic and electron microscopic studies. *Vet Res Commun* 2012; 36(1): 47-55. PMid:22127426. http://dx.doi.org/10.1007/s11259-011-9511-9

Hassum IC, Menezes RC. Natural infection with species of the genus *Eimeria* in small ruminant raised in two municipalities of the State Rio de Janeiro. *Rev Bras Parasitol Vet* 2005; 14(3): 95-100. PMid:16229752.

Hermosilla C, Barbisch B, Heise A, Kowalik S, Zahner H. Development of *Eimeria bovis* in vitro: suitability of several bovine, human and porcine endothelial cell lines, bovine fetal gastrointestinal, Madin-Darby Bovine Kidney (MDBK) and African green monkey kidney (VERO) cells. *Parasitol Res* 2002; 88(4): 301-7. PMid:11999015. http://dx.doi.org/10.1007/s00436-001-0531-1

Instituto Nacional de Estatística - INE. *Statistical Yearbook of Alentejo Region 2010*. Lisboa: INE; 2010.

International Union for Conservation of Nature - IUCN. *The IUCN Red List of Threatened Species*. Version 2013.2 [online]. Switzerland: IUCN, 2013 [cited 2014 Apr 17]. Available from: http://www.iucnredlist.org.

Jalila A, Dorny P, Sani R, Salim NB, Vercruysse J. Coccidial infections of goats in Selangor, peninsular Malaysia. *Vet Parasitol* 1998; 74(2-4): 165-72. http://dx.doi.org/10.1016/S0304-4017(97)00133-7

Kanyari PW. The relationship between coccidial and helminth infections in sheep and goats in Kenya. *Vet Parasitol* 1993; 51(1-2): 137-41. http://dx.doi.org/10.1016/0304-4017(93)90204-Z

Koudela B, Boková A. Coccidiosis in goats in the Czech Republic. *Vet Parasitol* 1998; 76(4): 261-7. http://dx.doi.org/10.1016/S0304-4017(97)00147-7

Kusiluka LJM, Kambarage DM, Matthewman RW, Harrison LJS, Daborn CJ. Coccidiosis of small ruminants in Tanzania. *Small Rumin Res* 1996; 21(2): 127-31. http://dx.doi.org/10.1016/0921-4488(96)00860-7

Levine JD. *Veterinary protozoology*. Ames: ISU Press; 1985.

Lima JD. Prevalence of coccidian in domestic goats from Illinois, Indiana, Missouri and Wisconsin. *Int. Goat Sheep Res* 1980; 1: 234-41.

Lima JD. Coccidiose dos ruminantes domésticos. *Rev Bras Parasitol Vet* 2004; 13(S1): 9-13.

Norton CC. Coccidia of the domestic goat *Capra hircus*, with notes on *Eimeria ovinoidalis* and *E. bakuensis* (syn. *E. ovina*) from the sheep *Ovis aries*. *Parasitology* 1986; 92(Pt 2): 279-89. PMid:3714300. http://dx.doi.org/10.1017/S0031182000064052

O'Callaghan MG. Coccidia of domestic and feral goats in South Australia. *Vet Parasitol* 1989; 30(4): 267-72. http://dx.doi.org/10.1016/0304-4017(89)90095-2

Penzhorn BL, Rognlie MC, Hall LL, Knapp SE. Enteric coccidia of Cashmere goats in southwestern Montana, USA. *Vet Parasitol* 1994; 55(1-2): 137-42. http://dx.doi.org/10.1016/0304-4017(94)90064-7

Programa de Desenvolvimento Rural - PRODER. *Protecção da biodiversidade doméstica*. Nota explicativa. Version V12.01.2011 [online]. Portugal: PRODER; 2011 [cited 2014 Apr 17]. Available from: http://www.proder.pt/conteudo.aspx?menuid=494.

Prukner-Radovcic E, Milakovic-Novak L, Ivesa-Petricevic S, Grgic N. *Clostridium chauvoei* in hens. *Avian Pathol* 1995; 24(1): 201-6. PMid:18645777. http://dx.doi.org/10.1080/03079459508419060

Ruiz A, Gonzalez JF, Rodriguez E, Martín S, Hernández YI, Almeida R, et al. Influence of climatic and management factors on Eimeria infections in goats from semi-arid zones. *J Vet Med B Infect Dis Vet Public Health* 2006; 53(8): 399-402. PMid:17010046. http://dx.doi.org/10.1111/j.1439-0450.2006.00985.x

Ruiz A, Behrendt JH, Zahner H, Hermosilla C, Pérez D, Matos L, et al. Development of *Eimeria ninakohlyakimovae* in vitro in primary and permanent cell lines. *Vet Parasitol* 2010; 173(1-2): 2-10. PMid:20594648. http://dx.doi.org/10.1016/j.vetpar.2010.05.023

Ruiz A, Guedes AC, Munoz MC, Molina JM, Hermosilla C, Martín S, et al. Control strategies using diclazuril against coccidiosis in goat kids. *Parasitol Res* 2012; 110(6): 2131-6. PMid:22193521. http://dx.doi.org/10.1007/s00436-011-2746-0

Taylor MA, Catchpole J. Review article: coccidiosis of domestic ruminants. *Appl Parasitol* 1994; 35(2): 73-86. PMid:8087156.

Thienpont D, Rochette F, Van Parijs OFJ. *Diagnosing helminthiasis through coprological examination*. Beerse: Janssen Research Foundation; 1979.

Yvoré P, Esnault A, Naciri M. La coccidiose caprine: effets de contaminations mono ou multispécifiques. *Rec Med Vet* 1985; 161: 347-1.

3. SUITABLE *IN VITRO EIMERIA ARLOINGI* MACROMERONT FORMATION IN HOST ENDOTHELIAL CELLS AND MODULATION OF ADHESION MOLECULE, CYTOKINE AND CHEMOKINE GENE TRANSCRIPTION

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ORIGINAL PAPER

Suitable in vitro *Eimeria arloingi* macromeront formation in host endothelial cells and modulation of adhesion molecule, cytokine and chemokine gene transcription

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Abstract *Eimeria arloingi* infections can cause severe haemorrhagic enteritis in young goat kids, thereby leading to high economic losses in goat industry worldwide. We aimed to isolate a new *E. arloingi* strain and establish a suitable in vitro culture system for the first merogony. *E. arloingi* oocysts were collected from naturally infected goat kids in the province of Alentejo, Portugal. For the maintenance of *E. arloingi* (strain A), kids kept under strict parasite-free conditions were orally infected with 10^3 sporulated oocysts each. Further, a new excystation protocol was successfully established to obtain viable sporozoites for further in vitro development in primary bovine umbilical vein endothelial cells (BUVEC). Overall, *E. arloingi* first merogony was successfully accomplished in BUVEC leading to macromeront formation (up to 150 μm) and the release of fully developed merozoites I stages. Moreover, host endothelial cell-parasite interactions were investigated in order to determine the extent of modulation carried out by *E. arloingi* in BUVEC during the first merogony. Gene transcription of adhesion molecules (E-selectin, P-selectin, VCAM-1, ICAM-1) was enhanced in the first hours *post-infection* (p.i.) in *E. arloingi*-infected BUVEC. BUVEC activation due to invasion was also shown by increased chemokine (CXCL8, CCL2, CCL5), cytokine (GM-CSF) and COX-2 gene transcription. The new *E. arloingi* (strain A) will be useful for better comprehension of early host innate immune reactions against this parasite in vitro/in vivo as well as to further our investigations in the complex *Eimeria*-host endothelial cell interactions.

Keywords *Eimeria arloingi* · Coccidiosis · Endothelial host cells · In vitro · Pro-inflammatory molecules

Introduction

The apicomplexan protozoa *Eimeria arloingi* is a monoxenous intracellular goat parasite, causing caprine coccidiosis worldwide. Caprine coccidiosis has been associated with considerable impact on animal health, as well as significant economic losses in goat industry (Soe and Pomroy 1992; Silva et al. 2014b). Caprine coccidiosis might affect up to 100 % of 4–10-week-old goat kids (Mehlhorn and Armstrong 2001), depending on the type of management and geographic area in which they preside (Ruiz et al. 2006). Alongside *Eimeria ninakohlyakimovae*, *E. arloingi* is considered one of the most pathogenic species in caprine coccidiosis, causing a severe haemorrhagic enteritis which results in clinical manifestations such as diarrhoea, weight loss, dehydration and poor growth (Koudela and Boková 1998; Ruiz et al. 2013; Silva et al. 2014b). Even sudden death in heavily *E. arloingi*-infected goat kids has been reported (Balicka-Ramisz et al. 2012; Rakhshandehroo et al. 2013; Silva et al. 2014b).

Currently, approximately 400,000 goats are reared in Portugal, with 21.6 % of them being allocated in the province of Alentejo (GPP 2009), thereby representing the most relevant region of the Portuguese goat industry. Due to high prevalence of *E. arloingi* infections worldwide, and due to its high pathogenicity in caprine coccidiosis, we aimed to establish a pure *E. arloingi* strain for further detailed

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investigations. Particularly, investigations related to early host innate immune reactions (e.g. polymorphonuclear neutrophils (PMNs)) against this parasite and complex host endothelial cell-parasite interactions as previously reported from other *Eimeria* species in cattle (Hermosilla et al. 2006, 2012; Taubert et al. 2006a, b, 2010).

The endogenous development of *E. arloingi* involves two generations of meronts and a gamogony. First-generation macromeronts (up to 240 μm) develop in host endothelial cells (ECs) of the lacteals of the villi of duodenum, jejunum and ileum, and also in ECs of the sinuses of mesenteric lymph nodes draining these regions within 9–12 days *post-infection* (p.i.) and releasing >120,000 merozoites I (Taylor et al. 2007; Hashemnia et al. 2012). Smaller second-generation meronts containing 8 to 24 merozoites II (Sayin et al. 1980; Hashemnia et al. 2012) develop within 12 days p.i. in epithelial cells of the villi and the cripts of lower jejunum (Taylor et al. 2007).

Since the first merogony occurs within highly immunoreactive host ECs (Ruiz et al. 2010; Hermosilla et al. 2012), the aims of this work were also to establish a suitable in vitro system by using primary ECs to be as close as possible to the in vivo situation, and to determine the extent of *E. arloingi*-induced modulation of infected ECs. To achieve this, a new excystation protocol was established for *E. arloingi* oocysts in order to isolate viable sporozoites for further intracellular development and production of viable *E. arloingi*-merozoites I in vitro. As suitable ECs, we used primary bovine umbilical vein endothelial cells (BUVEC) as previously described by Hermosilla et al. (2002), which allowed *E. arloingi* macromeront formation and consequent production of viable merozoites I.

Additionally, *E. arloingi*-induced modulation of the gene transcription of adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1), cytokines (GM-CSF) and chemokines (CXCL8, CCL2, CCL5) was investigated.

To our best knowledge, this is the first description of *E. arloingi* in vitro culture and may be useful for further detailed analyses on the modulation of these highly immunoreactive ECs by *E. arloingi*.

Materials and methods

Animals

Three male White German goat kids were purchased from a local goat milk farmer at the age of 3 days and each treated with a single dose of 20 mg/kg bodyweight (bw) toltrazuril (Baycox® 5 %, Bayer) and 0.1 mg/kg bw halofuginone (Halocur®, Intervet) for 7 consecutive days. Faecal samples were weekly collected and assessed for parasitic infections. When deemed parasite free, they were maintained under parasite-free conditions in autoclaved stainless steel metabolic cages (Woetho) within a large animal stable of the Institute of Parasitology, Justus Liebig University (JLU), Giessen, with laminar flow lock entrance until experimental infection. Animals were fed with milk substitute (CombiMilk® Lämmermilch, Agravis) and commercial starter pellet concentrates (Lämmerpellets®, Deuka). Water and sterilized hay were given ad libitum. All animal 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 with the current German Animal Protection Laws.

Parasites

E. arloingi (strain A) was initially isolated in 2012 from naturally infected goat kids, in the province of Alentejo, Portugal. Faecal samples collected directly from the rectum of kids and dairy goats were assessed with a modified McMaster technique (Whitlock 1948). *Eimeria* species identification (Levine 1985) was based on morphometric characteristics after sporulation at room temperature (RT), with aeration every 2 to 4 h by stirring the oocysts mixture. Respective proportions present in samples were determined, and samples containing more than 80 % of *E. arloingi* oocysts were selected and conserved at 4 °C. A simple flotation process was performed on these samples (10 min, RT) with saturated sodium chloride solution with a final density of 1.20 g/l. Each cover slide was then washed with distilled water in a Petri dish and observed with an invert microscope (CKX41, Olympus®). Oocysts with morphometric characteristics consistent to the ones of *E. arloingi* (Levine 1985) were isolated from the suspension by using a glass Pasteur pipette and added to a 2 % (*w*/*v*) potassium dichromate solution to prevent bacterial/fungal contamination according to Hermosilla et al. (2002). A total of 3×10^3 oocysts collected by this technique were then stored at 4 °C in 2 % (*w*/*v*) potassium dichromate solution until further experimental animal infection took place in Giessen, Germany.

In order to obtain a pure *E. arloingi* (strain A), three parasite-free male White German goat kids were orally infected with 1×10^3 sporulated *E. arloingi* oocysts, at 6 weeks of age. Collection of excreted *E. arloingi* oocysts and sporogony of oocysts were performed as previously described (Hermosilla et al. 2002). Briefly, excreted *E. arloingi* oocysts were isolated from the faeces, beginning at 18 days p.i., for 5 consecutive days (Jackson 1964). For elimination of debris, daily collected faeces were washed with tap water under pressure through three different sized sieves (250, 150 and 100 μm). After the last rinse, oocyst solution was pelleted for 24 h, and supernatant was afterwards discharged. Pellets were mixed (1:1) with saturated sugar solution (1.3 g/l density) and the suspension was transferred into sedimentation trays covered with glass panels. Every 2 h, oocysts bound to glass

panels were withdrawn with water and treated as mentioned before to allow complete sporulation. Sporulated *E. arloingi* oocysts were stored at 4 °C until further usage.

Excystation of *E. arloingi*

For the isolation of viable *E. arloingi* sporozoites, the following modified excystation protocol was used (Fayer and Hammond 1967). Sporulated *E. arloingi* oocysts stock solution was added to 4 % (*v*/*v*) sodium hypochlorite solution and magnetically stirred on ice for 20 min. After mixing in the vortex for 20 s, oocyst solution was centrifuged (300×*g*, 5 min) and supernatant was mixed with bi-distilled water (1:1). Washed oocyst solution was then layered in Percoll™ (GE Healthcare, UK) 60 % gradients and centrifuged for 20 min at 400×*g* to remove remaining debris. After centrifugation, oocyst bands were 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 (37 °C, 20 h). Afterwards, oocysts were re-suspended in the following 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, due to the lack of caprine bile availability). Afterwards, oocysts were incubated up to 4 h (37 °C, 5 % CO_2 atmosphere). Every hour, excystation progress was checked under an inverted microscope (IX81, Olympus®) to estimate the number of free-released sporozoites. Freshly released sporozoites of *E. arloingi* were washed two times (600×*g*, 15 min) with cell culture medium 199 (M199, Gibco) supplemented with 2 % (*v*/*v*) foetal calf serum (FCS, Gibco) and 1 % penicillin (*v*/*v*, 500 U/ml; Sigma-Aldrich) and streptomycin (*v*/*v*, 500 μg/ml; PS; Sigma-Aldrich) and finally suspended in tissue culture medium (2×10^6 sporozoites/ml).

First merogony of *E. arloingi* in vitro

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated as previously described (Taubert et al. 2006a) and cultured in EC growth medium (ECGM, PromoCell). BUVEC were seeded into 25-cm^2 tissue culture flasks (Greiner) and incubated at 37 °C and 5 % CO_2 atmosphere. Medium was changed with modified ECGM (ModECGM, ECGM mixed with 70 % (*v*/*v*) M199 (Gibco), 2 % FCS and 1 % PS) every 2–3 days until confluent BUVEC monolayers (n=3) were infected with 2.5×10^5 freshly excysted *E. arloingi* sporozoites (strain A) per 25-cm^2 flask. Culture medium was changed at 24 h p.i. and then successively every 2 days p.i. In order to keep up with *E. arloingi* macromeront development and to observe merozoites I release, infected cells were daily checked under an inverted microscope (IX81, Olympus®) for a period of 22 days p.i.

Initial infection rate was determined in 20 power vision fields randomly selected at ×630 magnification. First merogony was closely followed and arbitrarily selected meronts (n=20/BUVEC) were measured at 7, 10, 13, 17, 20 and 22 days p.i. using the software CellSens® Dimension 1.7® (Olympus).

For comparative reasons, two permanent host cell lines were also infected with 7.5×10^5 freshly excysted *E. arloingi* sporozoites (strain A) per 75-cm^2 culture flask. Madin-Darby bovine kidney (MDBK) cells were maintained in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 5 % FCS and 1 % PS. MARC-145 (Rhesus monkey foetal kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % FCS and 1 % PS. Medium was changed every 2–3 days until confluent MDBK and MARC-145 monolayers were infected. Culture medium was changed at 24 h p.i. and then successively every 2 days. Further *E. arloingi* macromeront development was closely observed in these additional permanent host cell types until 22 days p.i.

Improvement of the *E. arloingi* first merogony development in vitro

To improve the early in vitro development of *E. arloingi*, we studied different protein, carbohydrate and lipid supplementations of culture media: ModECGM, already containing 2 % FCS (control); ModECGM+10 mM glucose (D(+)-glucose anhydrous; Roth); ModECGM+10 % FCS; and ModECGM+2.5 μM oleic acid (Cayman Chemicals). After establishment of BUVEC infection (n=3), culture mediums were changed every 2 days. BUVEC cultures with oleic acid supplementation were in contact with ModECGM+50 μM oleic acid for 1 h prior to medium change in order to induce production of lipid droplets as lipid intracellular storage according to Martin and Parton (2006). At 22 days p.i., merozoites I were harvested and counted with a Neubauer chamber.

BUVEC infection and RNA isolation

Confluent BUVEC monolayers (n=3) maintained in 25-cm^2 plastic tissue culture flasks were infected with 2.5×10^5 freshly excysted *E. arloingi* sporozoites. Respective non-infected BUVEC were analysed in parallel as negative controls. Infected BUVEC were harvested for RNA isolation at 3, 6, 12 and 24 h p.i. by direct lysis (per flask: 594 μl buffer RLT lysis buffer, RNeasy Mini Kit, Qiagen, and 6 μl 2-mercaptoethanol, Serva).

Isolation of total RNA and DNase I treatment

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) in 10× DNase reaction buffer (37 °C, 30 min). DNase with 1 μl EDTA (50 mM, Thermo Scientific) was inactivated by heating the sample (65 °C, 10 min). Total RNA probes were stored at −80 °C until further use.

Reverse transcription of total RNA

The complementary DNA (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 DNase-treated total RNA, 0.5 μl oligo d(T) (50 ng/μl), 1 μl of 50 ng/μl hexamer primer, 1 μl of 10 mM dNTP mix and DEPC-treated water was adjusted 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) and 0.5 μl Super Script III enzyme (200 U/μl). The samples were incubated at 25 °C for 10 min followed by a 50 °C incubation for 50 min and 5 min at 85 °C. Finally, nuclease-free dH_2O with 1× TE buffer was adjusted to 200-μl total volume and stored at −20 °C.

Real-time qPCR for the relative quantification of chemokine (CXCL8, CCL2, CCL5), cytokine (GM-CSF), COX-2 and adhesion molecules (E-selectin, P-selectin, VCAM-1, ICAM-1)

Real-time qPCR was 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. The real-time qPCR primers here used were all previously published by Taubert et al. (2006b) and Hermosilla et al. (2006).

Statistical analysis

For statistical analysis of variance, ANOVA with repeated measures was performed to compare co-culture/stimulation conditions. The Bonferroni method was used as a follow-up test to ANOVA. The qPCR data analysis was based on the ΔΔCt method and data was normalized to GAPDH results as housekeeping gene. Fold change expression compared to control=$2^{-\Delta\Delta Ct}$, with the following: ΔCt control (control samples)=Ct control housekeeping gene − Ct control target gene; ΔCt treated (test samples)=Ct treated houskeeping gene − Ct treated target gene; and ΔΔCt=ΔCt control (control samples) − ΔCt treated (test samples). Data analyses were performed with GraphPad PRISM® 6 (GraphPad Software, Inc., USA). An unpaired *t* test was used to compare gene transcription levels between infected and non-infected BUVEC. *P* values <0.05 were here considered as a significant difference.

Results

Morphological characteristics of *E. arloingi* (strain A) and sporogony

Eimeria arloingi (strain A) sporulated oocysts presented an ellipsoidal shape and were slightly flattened at the micropylar end. The outer oocyst wall was smooth and colourless and the inner layer was yellowish. A prominent micropyle was always present at the pole of the oocyst and was covered by a prominent colourless polar cap (Fig. 1a, red arrow). Measurements of oocysts revealed a medium length of 26 to 34 μm (30.10±1.92) and a medium width of 17 to 24 μm (21.10±1.64).

The *E. arloingi* sporogony at RT (18–19 °C) varied from 5 to 12 days. Within sporulated oocysts, sporocysts were rather elongated ovoid in morphology and presented an average length of 13.76±1.76 μm and an average width of 7.53± 0.64 μm. Both Stiedae body and residual bodies were present within *E. arloingi* sporocysts. There was no oocyst residuum although polar granules were not always present (in 32 % were not observed). When they were within the same oocyst, one to several oocyst residuums were observed.

The three experimentally *E. arloingi*-infected goat kids (10^3 oocysts) shed in total 580×10^6 oocysts, showing tremendous biologic potential of this new field isolated species. Newly shed *E. arloingi* (strain A) oocysts were then collected, counted and transferred into 75-cm^2 tissue culture flasks (Greiner) suspended in 2 % potassium dichromate at 4 °C until further use.

Moreover, still ongoing molecular characterization of *E. arloingi* (strain A) as well as first phylogeny studies on other related ruminant *Eimeria* species (e.g. *Eimeria bovis*, *E. ninakohlyakimovae*, *Eimeria ovinoidales*) was additionally conducted at the Royal Veterinary College (Damer Blake; data not shown) in the UK.

Excystation and motility of *E. arloingi* sporozoites

Viable sporozoites were successfully obtained from sporulated *E. arloingi* (A) oocysts. After 30 min of incubation in

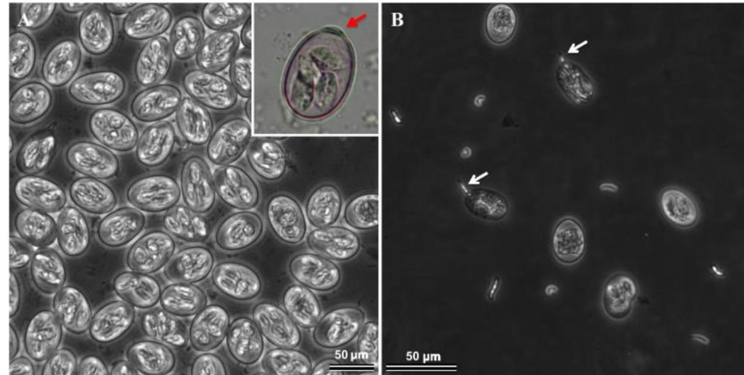

Fig. 1 Excystation of *Eimeria arloingi* sporozoites. **a** *E. arloingi* oocysts during excystation process and detailed visualization of the polar cap (*red arrow*). **b** Sporozoites (*white arrow*) egress through opened oocyst micropyle (Color figure online)

excystation medium, first free-released sporozoites were visible. Sporozoites of *E. arloingi* showed dynamic movement while inside sporocysts, and later on when the Stiedae body was absent, sporozoites rapidly moved into the cavity of the oocysts. Within the oocyst cavity, sporozoites moved very actively until they succeeded to get in contact with the micropyle/polar cap and finally egressed through the micropyle (Fig. 1b, arrows, and Supplementary Data Movie 1). The excystation process continued up to 4 h, as approximately 90 % of the sporozoites were released from sporulated oocysts. Furthermore, freshly released sporozoites showed typical movements of gliding motility and contractility on the surface of ECs.

In vitro development of *E. arloingi*

Host cell invasion (BUVEC, MDBK, MARC-145)

BUVEC confluent monolayers were exposed to freshly isolated *E. arloingi* sporozoites to allow parasite invasion and further intracellular development. The majority of the infective sporozoites had completed the invasion phase within 60 min, with an initial infection rate of 8.03 % (Fig. 2). Intracellular sporozoites were found to be close to the host cell nucleus (Fig. 3a) and also to move from cell to cell, without visible damages. Occasional sporozoite egression was observed from day 2 onwards until 22 days p.i. At 24 h p.i., extracellular sporozoites (Fig. 3a, white arrow) were clearly different in size and shape than intracellular sporozoites, being longer and thinner than intracellular sporozoites (Table 1). MDBK confluent monolayers were infected by freshly excysted sporozoites in as little as 60 min. After 2 h p.i., sporozoites were already found intracellularly. Despite rapid invasion of sporozoites, infection rates (4.05 %) were low (Fig. 2). No further development was observed until 22 days p.i. Sporozoites were observed inside a small parasitophorous vacuole (PV) as in BUVEC, and occasional egression was observed after 2 days p i. until 18 days p.i. Regarding MARC-145 confluent monolayers, *E. arloingi* sporozoites infected 1.81 % of available host cells (Fig. 2). Once again, egression was observed after 2 days p.i. until 7 days p.i. No further development was detected, other than a small PV surrounding intracellular sporozoites. In neither MDBK nor MARC-145 cell *E. arloingi* cultures was merozoites I production achieved.

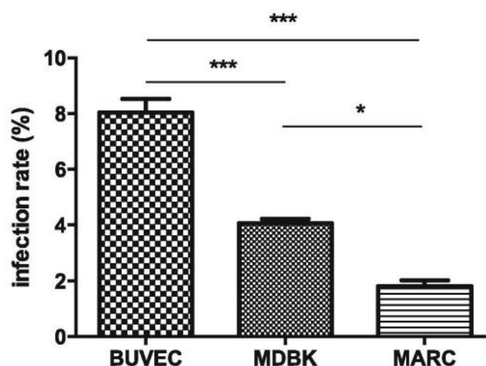

Fig. 2 Infection rates (%) of *Eimeria arloingi* sporozoites in BUVEC, MDBK, and MARC-145 cells. *E. arloingi* sporozoite invasion was determined 24 h p.i. in three different host cell lines, one primary host cell line (BUVEC) and two permanent cell lines (MDBK, MARC-145). Invasion of host cells of bovine origin (BUVEC, MDBK) was greater than that of non-bovine host cells (MARC-145). BUVEC were the most suitable cells tested presenting by showing higher infection rates and allowing further development of *E. arloingi* macromeronts

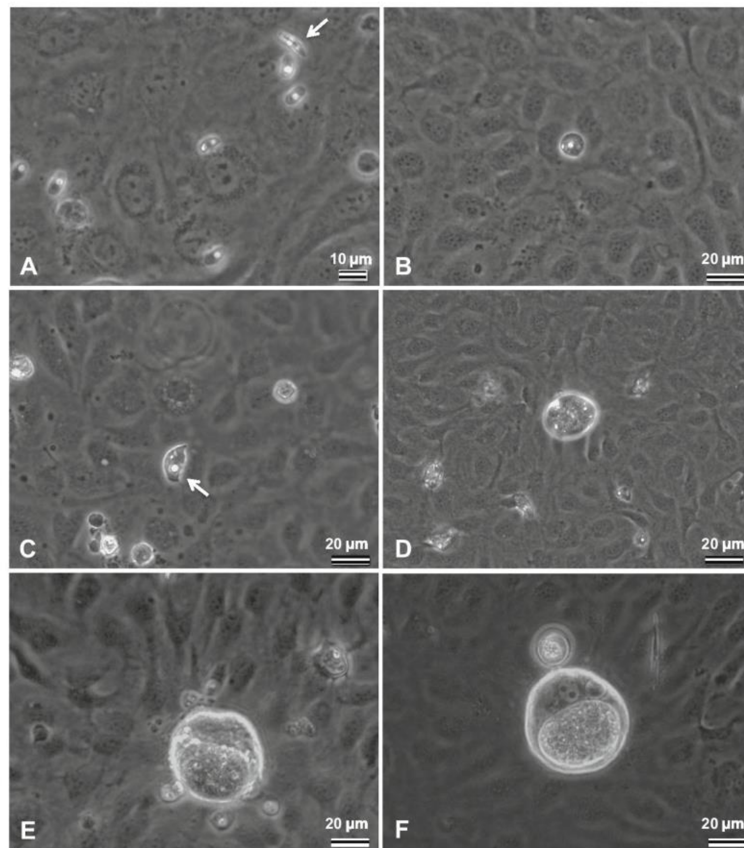

Fig. 3 Infection and in vitro development of *Eimeria arloingi* in BUVEC. Confluent BUVEC were infected with freshly excysted *E. arloingi* sporozoites, and macromeront development was monitored until 22 days p.i. **a** One day p.i., extracellular sporozoites (*arrow*) showed different morphology than intracellular sporozoites; **b** 4 days p.i., trophozoites. Immature macromeronts are observed in **c** 7 days p.i. (*arrow*), **d** 13 days p.i., **e** 17 days p.i. and **f** 20 days p.i

Development of E. arloingi intracellular stages in BUVEC

On day 4 p.i., intracellular sporozoites continued their development into rounded up trophozoite stages (Fig. 3b). Nevertheless, some sporozoites and/or trophozoites persisted unaltered until the end of the experiment whereas others started their development later in time, resulting in unsynchronous macromeront formation. Further development of *E. arloingi* trophozoites resulted in immature meronts (Fig. 3c–f) observed from 5 to 20 days p.i., but not all immature meronts developed into mature macromeronts. Macromeront enlargement throughout in vitro development is registered in Table 1. Mature macromeronts of *E. arloingi* were observed at 22 days p.i. showing typical rosette-like structures (Fig. 4a), clearly corresponding to morphological features of in vivo occurring macromeront stages (Silva et al. 2014b). Additionally, small immature macromeronts were released from BUVEC monolayer and later on re-integrating into BUVEC cell layer as previously described in other apicomplexan parasite species (Hermosilla 2009; van de Sand et al. 2005). At 22 days p.i., rupture of mature macromeronts and release of viable merozoites I were observed (Fig. 4b and Supplementary Data Movie 2)

E. arloingi merozoites I production in BUVEC

With the purpose of improving the in vitro development of *E. arloingi* macromeront formation and the resulting mass production of viable merozoites I, different protein, carbohydrate and lipid supplement treatments were additionally tested in infected BUVEC cultures. As shown in Fig. 5, the supplementation of 10 mM glucose resulted in significantly higher production of fully developed merozoites I than untreated controls and other supplementations, i.e. carbohydrates and lipids ($P<0.0001$). The supplementation of EC medium with proteins (10 % FCS) and lipids (2.5 µM oleic acid), contrary

Table 1 Morphological changes during in vitro development of *Eimeria arloingi* first merogony

		Infection with 2.5×10^5 sporozoites
Development stage n=20	Days post-infection	BUVEC confluent monolayer (25 cm^2)
Sporozoites (μm)	0	8×2[a]
	1	5×3[b]
Immature meronts (μm)	7	9×7
	10	14×12
	13	54×35
Mature macromeronts (μm)	17	74×52
	20	82×71
	22	96×84

BUVEC confluent monolayers were cultivated and infected with 2.5×10^5 *E. arloingi* sporozoites. Different stages were measured (n=20) throughout the development of the parasite. Extracellular and intracellular sporozoites, as well immature meronts and mature macromeronts, were measured in order to register enlargement throughout development

[a] Extracellular

[b] Intracellular

to expectations, did not significantly improve in vitro *E. arloingi* macromeront formation.

E. arloingi infection of BUVEC triggers temporary upregulation of adhesion molecule gene transcription

E. arloingi infection significantly induced (P<0.05) upregulation of adhesion molecules such as E-selectin, P-selectin, ICAM-1 and VCAM-1 gene transcription in BUVEC (Fig. 6a). E-selectin showed an eightfold increase when compared to controls (P<0.001) at 6 h p.i. followed by a rapid decrease at 12 h p.i. Regarding P-selectin, a rapid increase of gene transcription was detected at 3 h p.i. (P<0.001) which continued until 12 h p.i. (P<0.05). At 24 h p.i. the levels of transcription decreased to almost control levels in both genes. ICAM-1 gene transcription revealed an increase of up to threefold at 6 h p.i. and a marked decrease at 12 h p.i. The same time courses were shown in case of VCAM-1 gene transcription. However, in this case, a 16-fold increase (P<0.05) was detected throughout the investigated time course.

Effects of *E. arloingi* infection on chemokine (CXCL8, CCL2, CCL5), cytokine (GM-CSF) and COX-2 gene transcription

Eimeria arloingi triggered effects on the EC gene transcription of chemokines, cytokine and COX-2 (Fig. 6b). Upregulation of CXCL8 resulted in a highly significant difference detected at 3 h p.i. (P<0.001) and remaining at high levels until 12 h p.i. At 24 h p.i., a marked decrease was observed. Considering CC chemokines (CCL2 and CCL5), *E. arloingi* infection induced analogous upregulation of these molecules showing similar time courses. Both CCL2 and CCL5 upregulations were detected at 3 h p.i. and increasing until 12 h p.i. (>16-fold). Comparing to CXC chemokine, the CC chemokine levels of transcription increase were gradual, while the CXCL8 chemokine expression was high throughout the time course. *E. arloingi* infection induced significant differences in CCL5 gene transcription when compared to non-infected cell levels at 3 and 6 h p.i. (P<0.05).

Eimeria arloingi triggered moderated GM-CSF transcription levels, increasing from 3 h p.i. (twofold) to 12 h p.i. (fourfold), but no significant differences were detected when comparing infected to non-infected cells. As in all the other cases, at 24 h p.i., a decrease of gene transcription to control levels was observed.

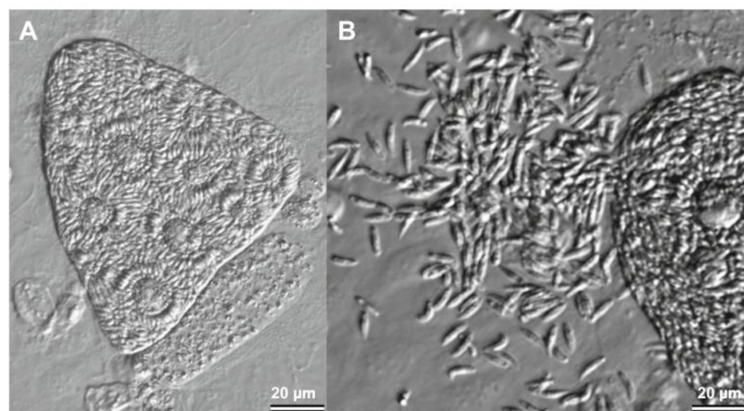

Fig. 4 Mature *Eimeria arloingi* macromeront in BUVEC. **a** At 22 days p.i., macromeront with rosette structure containing fully developed merozoites I. **b** Rupture of mature macromeront and release of viable merozoites I (see Supplementary Data Movie 2)

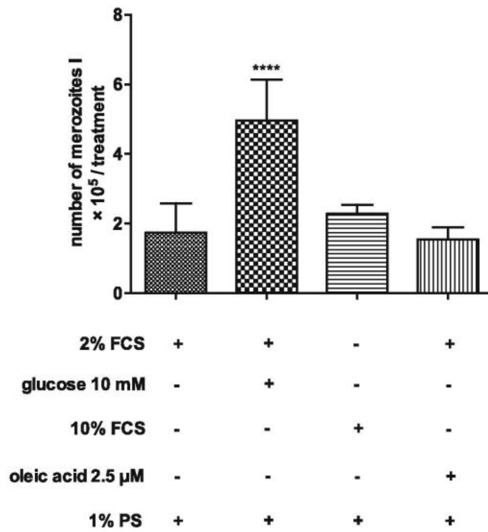

Fig. 5 Total merozoites I production under different culture conditions. To estimate the influence of different supplements on merozoite production, *E. arloingi*-infected BUVEC were treated with different medium supplements. Thereafter, released merozoites I were harvested and counted. The supplementation consisted of 10 mM glucose, 10 % FCS and 2.5 μM oleic acids which were added to endothelial cell culture medium—control (2 % FCS and 1 % PS)—until the release of merozoites I. Glucose supplementation resulted in significant increment of total number of merozoites I ($P<0.0001$)

A mean of fourfold change in COX-2 transcription induced by *E. arloingi* infection was observed throughout the time course, and no significant differences in gene transcription were registered when comparing infected to non-infected cells.

Discussion

Eimeria arloingi is one of the most pathogenic species in caprine coccidiosis, causing severe haemorrhagic enteritis due to its massive replication capacity within the first merogony in host ECs (Soe and Pomroy 1992; Ruiz et al. 2006). In this work, we accomplished the isolation of a pure Portuguese field *E. arloingi* (strain A), which presents the same morphometric characteristics previously described for this caprine species (Pellérdy 1974; Sayin et al. 1980; Levine 1985). Worldwide and particularly in Portugal, caprine coccidiosis is largely caused by *E. arloingi* infections (Silva et al. 2013, 2014b), mainly in kids (Sayin et al. 1980; Agyei et al. 2004), and sometimes even causing death to a significant percentage of animals (Silva et al. 2014b). This *E. arloingi* (strain A) will allow further investigations, particularly in characterizing early host innate immune reactions against this apicomplexan parasite (Silva et al. 2014a), and allow detailed analyses of complex host EC-parasite interactions as reported for related ruminant *Eimeria* species (Hermosilla et al. 2006, 2012; Taubert et al. 2008, 2010).

During the excystation process, *E. arloingi* sporozoites demonstrated to undergo a different oocyst egress mechanism than observed for the closely related *E. bovis* or *E. ninakohlyakimovae*, both presenting two thin oocyst walls and inconspicuous micropyle (Eckert 1995). In vitro excystation of these two former species involves, firstly, the

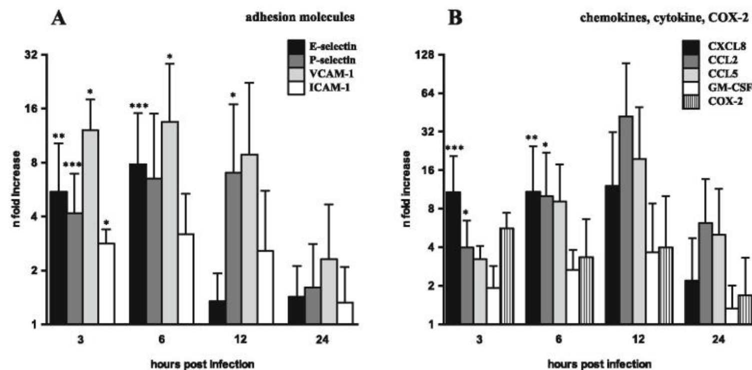

Fig. 6 Transcription of **a** E-selectin (*black*), P-selectin (*grey*), VCAM-1 (*light grey*) and ICAM-1 (*white*), and **b** CXCL8 (*black*), CCL2 (*grey*), CCL5 (*light grey*), GM-CSF (*white*) and COX-2 (*pattern*) genes in BUVEC during *Eimeria arloingi* infections in vitro. BUVEC were grown to confluence and infected with 2.5×10^5 sporozoites. Non-infected controls were cultured in parallel for each time point. Results are expressed as *n*-fold increase in comparison to controls. Total RNA was isolated at 3, 6, 12 and 24 h p.i. One microgram total RNA was reversely transcribed into cDNA and probed with real-time qPCR systems for the detection of the mRNA equivalents. Arithmetical means of three different BUVEC isolates are shown and standard deviations.*$P<0.05$, **$P<0.01$, ***$P<0.001$

rupture of oocyst walls, releasing sporocysts containing sporozoites. Thereafter, activated sporozoites within sporocysts set themselves free from single sporocysts. In case of *E. arloingi*, a well-developed micropyle and polar cap are present. After the removal of oocyst polar cap, sporozoites become extremely active inside oocysts, but as oocysts walls are thicker (Eckert 1995) and do not rupture, sporozoites must rapidly move into the cavity of the oocysts. While sporozoites get in contact with the opened micropyle, they egress from oocysts as previously reported by Jackson (1964) and as observed (Fig. 1 and Supplementary Data Movie 1). Successful excystation process resulted in viable sporozoites which presented adequate gliding motility, infectivity and further in vitro intracellular development, even when using sterile filtered non-specific bile (bovine) as previously demonstrated for other caprine *Eimeria* species (Ruiz et al. 2010).

In vivo, *E. arloingi* sporozoites obligatory invade host ECs of the central lacteals of the intestinal villi and develop into first-generation macromeronts as integrant part of their endogenous life cycle. Nevertheless, in in vitro conditions, *E. arloingi* sporozoites were able to invade BUVEC and to undergo successful intracellular macromeront development, thus culminating with the release of fully developed merozoites I at 22 days p.i. (Fig. 4b and Supplementary Data Movie 2). It was expected that *E. arloingi* sporozoites successfully developed in other host species cell types as reported for other ruminant *Eimeria* species which revealed no host cell type specificity (Hermosilla et al. 2002; Ruiz et al. 2010). According to Ruiz et al. (2010), BUVEC are suitable cells for in vitro development of *E. ninakohlyakimovae* and resulted as well in the production of viable merozoites I. Since 2 days p.i. until the end of the experiment, sporozoite egress was observed as parasites left initial infected host cells and invaded new BUVEC. This feature was also mentioned to occur in vitro in other apicomplexan parasites (Mota and Rodriguez 2001; Behrendt et al. 2008; Ruiz et al. 2010). Sporozoite egress is commonly associated with the need of sporozoites to reach their final specific host cells, as seen for *Plasmodium falciparum*, *E. bovis* and *E. ninakohlyakimovae*, thereby transmigrating through cells in a unique, alternative invasion process by breaching the plasma membrane without forming a parasitophorous vacuole (Mota et al. 2001; Behrendt et al. 2008). This alternative invasion process of apicomplexan parasites has been reported exclusively for sporozoite stages. It seems feasible to assume that the search of a suitable host cell, which has appropriated metabolic resources for further macromeront development, may occur by this alternative invasion process, as postulated by Behrendt et al. (2008), or in order to escape intracellular defence mechanisms such as host cell apoptosis (Mota and Rodriguez 2001).

Changes in the morphology and size of *E. arloingi* intracellular stages (Table 1) were accompanied with a morphological change of the host nucleus in infected host ECs. In the course of time, host cell nucleus changed from usual dark spotted to “fried egg” shape (heterochromatin), where the light-coloured euchromatine spreads over almost the whole area of the nucleus and the nucleoli coalesce to form single or multiple nucleoli, as previously reported for other ruminant *Eimeria* species (Ruiz et al. 2010; Taubert et al. 2010). Mature *E. arloingi* macromeronts at the same day p.i. exhibited different sizes and morphologies. Despite the fact that *E. arloingi* macromeronts presented non-structured type with merozoites I diffused in the macromeront or rosette structure type with merozoites I clustered in several rosettes within the macromeront (Fig. 4a), merozoites I production was not influenced by the macromeront structure in vitro.

In order to further investigate the effects of different supplements on *E. arloingi* macromeront formation in vitro, we tested glucose, protein or lipid (oleic acids) supplementation. As infection rates and macromeront development were quite similar to those of non-treated controls, total merozoites I production was used as a measurement for successful replication in BUVEC monolayers. The role of carbohydrates in many biological processes has been described before: Carbohydrates participate in cell adhesion, cell-cell communication and host-pathogen interactions (Sanz et al. 2013). Additionally, for the in vitro apicomplexan parasite growth, carbohydrates seem to be fundamental (van Schalkwyk et al. 2008; Preuss et al. 2012). In line with these findings, we also witnessed the importance of glucose in the increment of merozoites I production (Fig. 5). In contrast, the addition of greater amounts of FCS to cell cultures did not have the estimated effect. As BUVEC multiply faster with incremented dosages of FCS (10 % FCS), we expected that the extra protein content in medium would also enhance parasite replication. Besides the slight increment of total merozoites I production, differences were not significant when compared with supplemented 2 % FCS medium. Regarding lipid supplementation, it has already been described as the key role of oleic acid in the intraerythrocytic proliferation of *P. falciparum* (Mi-Ichi et al. 2007; Gratraud et al. 2009). Furthermore, in the *E. bovis* in vitro system, supplementation of oleic acid significantly enhanced merozoites I production (Hamid et al., submitted manuscript). Unfortunately, in our experiment, the supplementation with the same amount of oleic acid did not result in greater production of *E. arloingi* merozoites I.

Overall, in vitro *E. arloingi* macromeront development was in accordance with previously described data for other related *Eimeria* spp. (Hermosilla et al. 2002; Ruiz et al. 2010). Thus, mature macromeront I cultures and availability of merozoites I

represent useful tools for the replacement of animal experiments concerning pharmaceutical screenings against these particular parasitic stages, as well as for basic research applications.

One of the first EC reactions against pathogens is their activation. This state of intensified responsiveness can be induced by various factors and stimuli, including cytokines and active parasite invasion (Taubert et al. 2006b; Mai et al. 2013). Upon activation, EC can produce a broad range of adhesion molecules, cytokines and proinflammatory chemokines.

E- and P-selectin are both relevant adhesion molecules, which mediate initial contact, known as tethering, between ECs and leukocytes, while ICAM-1 and VCAM-1 adhesion molecules regulate leukocyte rolling and leukocyte firm adhesion on ECs (Hermosilla et al. 2006). In *E. arloingi*-infected BUVEC, E-selectin transcription increased from 3 to 6 h p.i., whereas P-selectin showed the highest transcription values at 12 h p.i. Conversely, *E. arloingi* infections resulted in a different pattern of E- and P-selectin gene transcription with lower levels, when compared with *E. bovis*-induced upregulation of these molecules (Hermosilla et al. 2006). It has been discussed that even contact of ECs with secreted proteins by apicomplexan parasites may already activate ECs without parasite host cell invasion (Hermosilla et al. 2006). However, secretion of parasite-specific proteins during host cell invasion constitutes a well-known aspect of apicomplexan invasion process. Thus, *E. arloingi* infection enhanced adhesion molecule gene transcription shows the parasite's ability to activate BUVEC. Moreover, increasing transcription of ICAM-1 and VCAM-1 was also observed from 3 to 6 h p.i., contrary to *E. bovis* infection where the peak was detected at 4 and 1 h, respectively. In agreement with a previous report by Hermosilla et al. (2006), a decrease in adhesion molecule gene transcription was observed from 6 h p.i. onwards. Especially, sporozoites of *Eimeria* spp. may display counteracting mechanisms to avoid, or downregulate, intense host cellular immune reactions (Hermosilla et al. 2012), thus achieving survival.

Comparative studies investigating relevant immunoregulatory molecules such as CXC and CC chemokines, cytokines (GM-CSF) and enzymes responsible for the synthesis of prostaglandins revealed a relative weak impact of *E. bovis* infections when compared to *Toxoplasma gondii* and *Neospora caninum* infections (Taubert et al. 2006b), thereby showing that *E. bovis* rendered less intense ECs.

Due to phylogenetic similarity with *E. bovis*, we expected *E. arloingi* to be able to avoid or downregulate EC activation. However, since our in vitro system is constituted with BUVEC of bovine origin, and *E. arloingi* is a caprine-specific parasite, this might explain the early stronger reaction induced by *E. arloingi* sporozoites, as seen with other apicomplexan (Taubert et al. 2006b). In the first 12 h p.i., we observed stronger enhanced transcription of studied genes (CXC and CC chemokines) confirming that the invasion process of sporozoites represents a triggering event in BUVEC activation (Taubert et al. 2006b). CXCL8 gene transcription levels presented a significant increase at 3 h p.i. and a peak at 12 h p.i., contrary to *T. gondii* and *N. caninum* which revealed a peak of expression at 4 h p.i. In case of *E. bovis* infection, triggered gene transcription was moderated and revealed a peak at the same time point as *E. arloingi* infections. Thus, the response of BUVEC to *E. arloingi* infection was similar to *T. gondii* and *N. caninum* infections in the first hours, but then induced same reactions as *E. bovis* infections. Considering CC chemokine (e.g. CCL2 and CCL5) expression, analogous time courses were observed for these molecules. In both cases at 3 h p.i., a 4-fold increase was detected and it continued to increase until 12 h p.i., reaching >16-fold enhancement. Comparing to the CC chemokine data published by Taubert et al. (2006b), all three species, namely *E. bovis*, *T. gondii* and *N. caninum*, presented a CC chemokine gene transcription peak at 12 h p.i. Besides the fact that these are different parasites and even different stages (sporozoites for *E. arloingi*/*E. bovis*, tachyzoites for *T. gondii*/*N. caninum*), the reactivity of BUVEC should also be taken into account. With their own variability, maybe BUVEC are responsible for small differences in gene transcription.

Transcription of the GM-CSF gene was moderate, increasing from 3 to 12 h p.i., similar to the pattern observed in *E. bovis* infection. As referred for other encoding genes in case of *T. gondii* and *N. caninum* infections, an earlier increase of GM-CSF was detected (Taubert et al. 2006a; 2006b). As in all the other cases, at 24 h p.i., a decrease of GM-CSF gene transcription to control levels was observed. In contrast, COX-2 gene transcription remained stable and no significant differences were registered when compared to controls. COX-2 expression is usually associated with cell damage, which in our in vitro *E. arloingi* study was seldomly observed.

So far, we could recently confirm the key role of PMN via neutrophil extracellular traps (NETs) formation in early host innate immune reactions against different *E. arloingi* stages (Silva et al. 2014a), and resulting ECs activation might attract PMN to the site of macromeront formation. Nevertheless, more detailed investigations on host-parasite interactions as well as innate immune reactions should be carried out to fully understand how this parasite manages to guarantee its massive replication in such a hostile and immune-reactive host cell.

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References

Agyei AD, Odonkor M, Osei-Somuah A (2004) Concurrence of *Eimeria* and helminth parasitic infections in West African Dwarf kids in Ghana. Small Rumin Res 51:29–35, doi:16/S0921-4488(03)00184-6

Balicka-Ramisz A, Ramisz A, Vovk S, Snitynskyj V (2012) Prevalence of coccidia infection in goats in Western Pomerania (Poland) and West Ukraine region. Ann Parasitol 58:167–171

Behrendt JH, Taubert A, Zahner H, Hermosilla C (2008) Studies on synchronous egress of coccidian parasites (*Neospora caninum*, *Toxoplasma gondii*, *Eimeria bovis*) from bovine endothelial host cells mediated by calcium ionophore A23187. Vet Res Commun 32: 325–332. doi:10.1007/s11259-007-9033-7

Eckert J (1995) COST 89/820: biotechnology. Guidelines on techniques in coccidiosis research. Office for Official Publications of the European Communities

Fayer R, Hammond DM (1967) Development of first-generation schizonts of *Eimeria bovis* in cultured bovine cells. J Protozool 14:764–772

GPP (2009) Animal Production Yearbook 2008/2009. Lisboa-Portugal

Gratraud P, Huws E, Falkard B, Adjalley S, Fidock DA, Berry L, Jacobs WR Jr, Baird MS, Vial H, Kremer L (2009) Oleic acid biosynthesis in *Plasmodium falciparum*: characterization of the stearoyl-CoA desaturase and investigation as a potential therapeutic target. PLoS One 4:e6889. doi:10.1371/journal.pone.0006889

Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi S (2012) Experimental caprine coccidiosis caused by *Eimeria arloingi*: morphopathologic and electron microscopic studies. Vet Res Commun 36:47–55. doi:10.1007/s11259-011-9511-9

Hermosilla C (2009) Studien zur Interaktion des Rinderkokzids *Eimeria bovis* mit seiner Wirtszelle. Verlag, VVB Laufersweiler

Hermosilla C, Barbisch B, Heise A, Kowalik S, Zahner H (2002) Development of *Eimeria bovis* in vitro: suitability of several bovine, human and porcine endothelial cell lines, bovine fetal gastrointestinal, madin-darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells. Parasitol Res 88:301–307

Hermosilla C, Zahner H, Taubert A (2006) Eimeria bovis modulates adhesion molecule gene transcription in and PMN adhesion to infected bovine endothelial cells. Int J Parasitol 36:423–431. doi: 10.1016/j.ijpara.2006.01.001

Hermosilla C, Ruiz A, Taubert A (2012) *Eimeria bovis*: an update on parasite-host cell interactions. Int J Med Microbiol 302:210–215. doi:10.1016/j.ijmm.2012.07.002

Jackson AR (1964) The isolation of viable coccidial sporozoites. Parasitology 54:87–93

Koudela B, Boková A (1998) Coccidiosis in goats in the Czech Republic. Vet Parasitol 76:261–267

Levine ND (1985) Veterinary Protozoology. Iowa State University Press, Ames, IA

Mai J, Virtue A, Shen J, Wang H, Yang XF (2013) An evolving new paradigm: endothelial cells-conditional innate immune cells. J Hematol Oncol 6:61. doi:10.1186/1756-8722-6-61

Martin S, Parton RG (2006) Lipid droplets: a unified view of a dynamic organelle. Nat Rev Mol Cell Biol 7:373–378. doi:10.1038/nrm1912

Mehlhorn H, Armstrong PM (2001) Encyclopedic Reference of Parasitology: Biology, Structure, Function. vol Bd. 1. Springer

Mi-Ichi F, Kano S, Mitamura T (2007) Oleic acid is indispensable for intraerythrocytic proliferation of *Plasmodium falciparum*. Parasitology 134:1671–1677. doi:10.1017/S0031182007003137

Mota MM, Rodriguez A (2001) Migration through host cells by apicomplexan parasites. Microbes Infect 3:1123–1128

Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V, Rodríguez A (2001) Migration of Plasmodium sporozoites through cells before infection. Science 291(5501):141–144

Pellérdy LP (1974) Coccidia and coccidiosis, 2nd edn. Verlag Paul Parey, Berlin Hamburg

Preuss J, Maloney P, Peddibhotla S, Hedrick MP, Hershberger P, Gosalia P, Milewski M, Li YL, Sugarman E, Hood B, Suyama E, Nguyen K, Vasile S, Sergienko E, Mangravita-Novo A, Vicchiarelli M, McAnally D, Smith LH, Roth GP, Diwan J, ChungTD JE, Rahlfs S, Becker K, Pinkerton AB, Bode L (2012) Discovery of a plasmodium falciparum glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase inhibitor (R, Z)-N-((1-ethylpyrrolidin-2-yl)methyl)-2-(2-fluorobenzylidene)-3-oxo-3,4-dihydr o-2H-benzo[b][1,4]thiazine-6-carboxamide (ML276) that reduces parasite growth in vitro. J Med Chem 55:7262–7272. doi:10.1021/jm300833h

Rakhshandehroo E, Razavi SM, Nazifi S, Farzaneh M, Mobarraei N (2013) Dynamics of the enzymatic antioxidants during experimental caprine coccidiosis. Parasitol Res 112:1437–1441. doi:10.1007/s00436-013-3273-y

Ruiz A, Gonzalez JF, Rodriguez E, Martin S, Hernandez YI, Almeida R, Molina JM (2006) Influence of climatic and management factors on *Eimeria* infections in goats from semi-arid zones. J Vet Med B Infect Dis Vet Public Health 53:399–402. doi:10.1111/j.1439-0450.2006.00985.x

Ruiz A et al (2010) Development of *Eimeria ninakohlyakimovae* in vitro in primary and permanent cell lines. Vet Parasitol 173:2–10. doi:10.1016/j.vetpar.2010.05.023

Ruiz A, Matos L, Muñoz MC, Hermosilla C, Molina JM, Andrada M, Rodríguez F, Pérez D, López A, Guedes A, Taubert A (2013) Isolation of an *Eimeria ninakohlyakimovae* field strain (Canary Islands) and analysis of its infection characteristics in goat kids. Res Vet Sci 94:277–284. doi:10.1016/j.rvsc.2012.08.003

Sanz S, Bandini G, Ospina D, Bernabeu M, Marino K, Fernandez-Becerra C, Izquierdo L (2013) Biosynthesis of GDP-fucose and other sugar nucleotides in the blood stages of *Plasmodium falciparum*. J Biol Chem 288:16506–16517. doi:10.1074/jbc.M112.439828

Sayin F, Dincer S, Milli U (1980) The life cycle and pathogenicity of *Eimeria arloingi* (Marotel, 1905) Martin, 1909, in Angora kids and an attempt at its transmission to lambs. Zentralbl Veterinarmed B 27: 382–397

Silva LMR, Vila-Vicosa MJM, Maurelli MP, Morgoglione ME, Cortes HCE, Cringoli G, Rinaldi L (2013) Mini-FLOTAC for the diagnosis of *Eimeria* infection in goats: an alternative to McMaster. Small Rumin Res 114:280–283. doi:10.1016/j.smallrumres.2013.06.017

Silva LM, Muñoz-Caro T, Gerstberger R, Vila-Vicosa MJ, Cortes HC, Hermosilla C, Taubert A (2014a) The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps. Parasitol Res 113(8):2797–807. doi:10.1007/s00436-014-3939-0

Silva LMR, Vila-Viçosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE (2014b) *Eimeria* infections in goats in Southern Portugal. Rev Bras Parasitol Vet 23:7. doi:10.1590/S1984-29612014051

Soe AK, Pomroy WE (1992) New species of *Eimeria* (Apicomplexa: Eimeriidae) from the domesticated goat *Capra hircus* in New Zealand. Syst Parasitol 23:195–202

Taubert A, Hermosilla C, Behrendt J, Zahner H (2006a) Reaction of bovine endothelial cells in vitro to coccidia (*Eimeria bovis*, *Toxoplasma gondii*, *Neospora caninum*) infections as the expression of a non-adaptive immune response. Berl Munch Tierarztl Wochenschr 119:274–281

Taubert A, Zahner H, Hermosilla C (2006b) Dynamics of transcription of immunomodulatory genes in endothelial cells infected with different coccidian parasites. Vet Parasitol 142:214–222. doi:10.1016/j.vetpar.2006.07.021

Taubert A, Hermosilla C, Suhwold A, Zahner H (2008) Antigen-induced cytokine production in lymphocytes of *Eimeria bovis* primary and challenge infected calves. Vet Immunol Immunopathol 126:309–320. doi:10.1016/j.vetimm.2008.09.003

Taubert A, Wimmers K, Ponsuksili S, Jimenez CA, Zahner H, Hermosilla C (2010) Microarray-based transcriptional profiling of *Eimeria bovis*-infected bovine endothelial host cells. Vet Res 41:70. doi:10.1051/vetres/2010041

Taylor MA, Coop RL, Wall R (2007) Veterinary parasitology, 3rd edn. Blackwell, Oxford, Ames, Iowa

van de Sand C, Horstmann S, Schmidt A, Sturm A, Bolte S, Krueger A, Lütgehetmann M, Pollok JM, Libert C, Heussler VT (2005) The liver stage of *Plasmodium berghei* inhibits host cell apoptosis. Mol Microbiol 58:731–742. doi:10.1111/j.1365-2958.2005.04888.x

van Schalkwyk DA, Priebe W, Saliba KJ (2008) The inhibitory effect of 2-halo derivatives of D-glucose on glycolysis and on the proliferation of the human malaria parasite *Plasmodium falciparum*. J Pharmacol Exp Ther 327:511–517. doi:10.1124/jpet.108.141929

Whitlock HV (1948) Some modifications of the McMaster helminth egg-counting technique and apparatus. J Counc Sci Ind Res 21:177–180

4. THE APICOMPLEXAN PARASITE *EIMERIA ARLOINGI* INDUCES CAPRINE NEUTROPHIL EXTRACELLULAR TRAPS

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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

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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

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

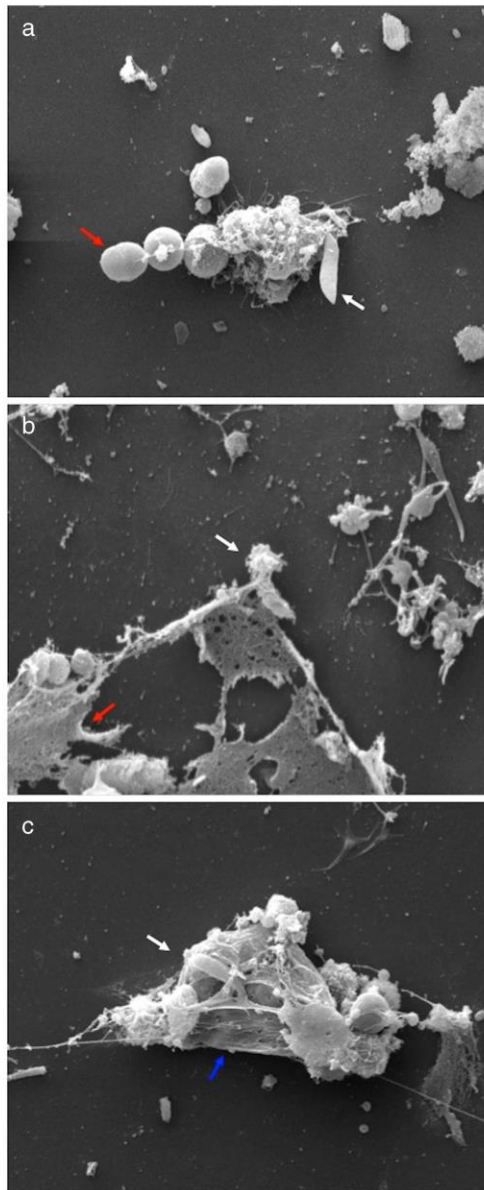
Illustrations of NETs being induced by *E. arloingi* stages were obtained with Sytox Orange® (S-11368, Invitrogen) nucleic acid staining. In brief, 10^5 PMN in serum-free RPMI 1640 medium (Gibco) were seeded on poly-L-lysine-treated glass coverslips in a six-well plate (Nunc) and exposed to sporozoites 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

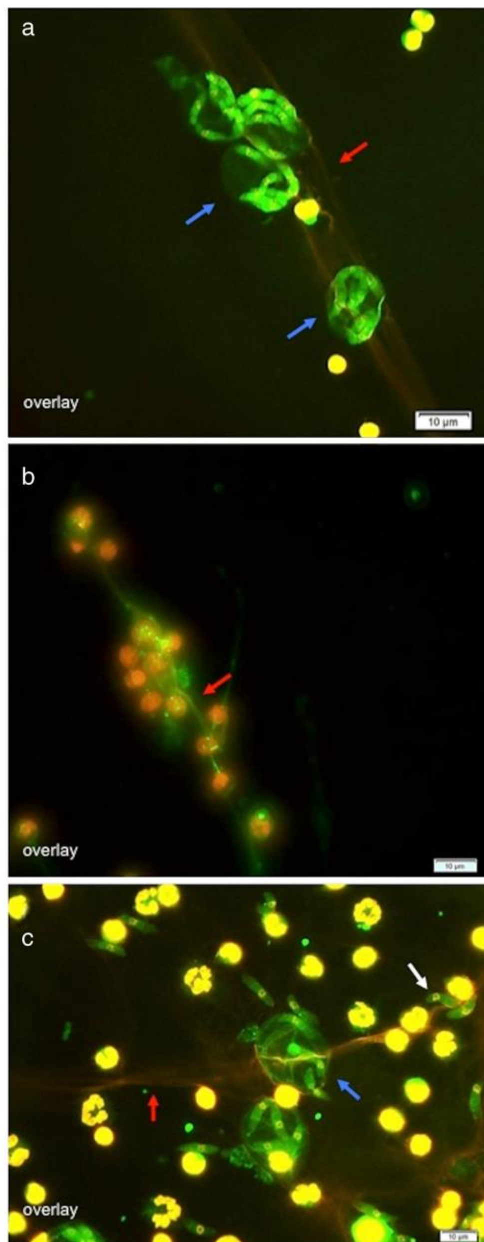
NET formation trigged by *Eimeria arloingi* stages—visualization and characterization

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).

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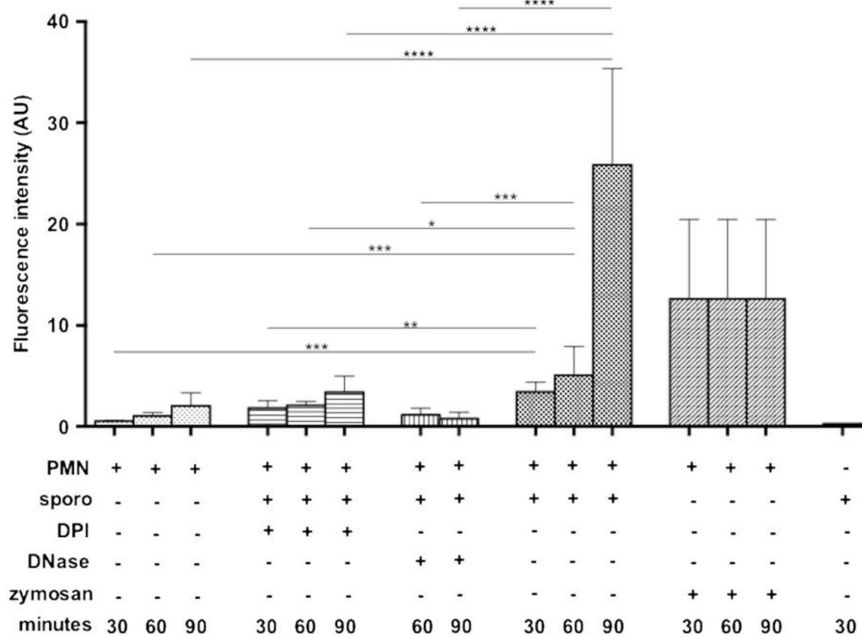

Fig. 3 Kinetic and inhibition assays on *E. arloingi* sporozoite-induced NETosis. PMN and sporozoites were incubated in a 1:1 ratio for 30, 60, and 90 min. Stimulation with zymosan (1 mg/ml) was used as positive control and sporozoites only as negative control. For NET inhibition, DNase- and DPI-treatments 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 ($p<0.001$).

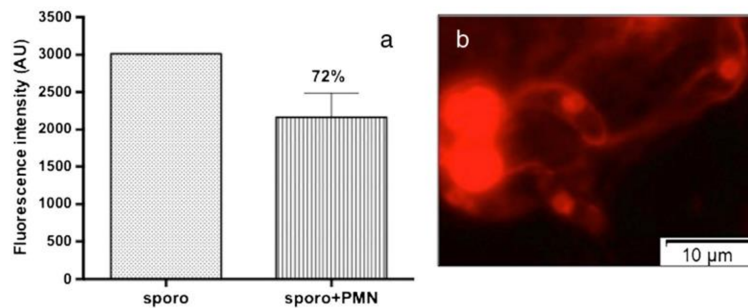

Fig. 4 Quantification of sporozoite entrapment in NET structures. Quantification of *E. arloingi* sporozoite entrapment in NETs (**a**) was performed after incubation of zymosan-stimulated PMN ($n=3$) with sporozoites^{CFSE}. Non-trapped sporozoites^{CFSE} were washed off, and the resulting fluorescence 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

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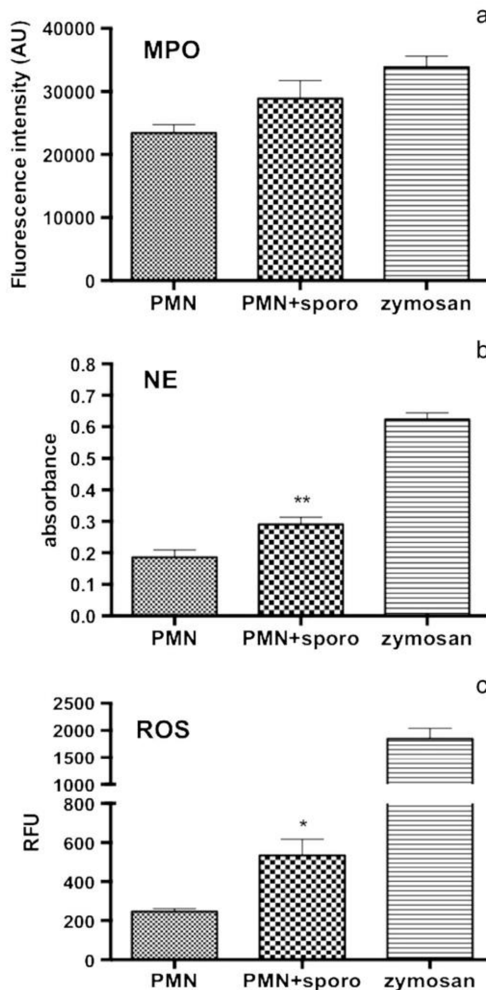

Fig. 5 Enzymatic activities of MPO, NE, and NADPH oxidase in caprine PMN after exposure to *E. arloingi* sporozoites. Caprine PMN were exposed to *E. arloingi* sporozoites, zymosan (positive control), or plain medium (negative control) for 60 min. Thereafter, enzymatic activities of NE and MPO as well 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).

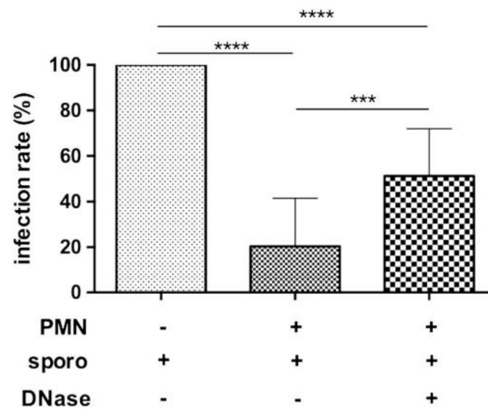

Fig. 6 Infectivity of *E. arloingi* sporozoites after exposure to caprine PMN. *Eimeria arloingi* sporozoites were co-cultured with caprine PMN for 90 min in the presence or absence of DNase. Incubation in plain medium served as PMN-free, positive control (the respective infection rate was set as 100 %). 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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References

Abi Abdallah DS, Denkers EY (2012) Neutrophils cast extracellular traps in response to protozoan parasites. Front Immunol 3:382. doi:10.3389/fimmu.2012.00382

Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE, Denkers EY (2012) Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. Infect Immun 80(2):768–777. doi:10.1128/IAI.05730-11

Adler H et al (1996) Differential regulation of inducible nitric oxide synthase production in bovine and caprine macrophages. J Infect Dis 173(4):971–978

Alghamdi AS, Foster DN (2005) Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. Biol Reprod 73(6):1174–1181. doi:10.1095/biolreprod.105.045666

Altincicek B, Stotzel S, Wygrecka M, Preissner KT, Vilcinskas A (2008) Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J Immunol 181(4):2705–2712

Aulik NA, Hellenbrand KM, Klos H, Czuprynski CJ (2010) Mannheimia haemolytica and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect Immun 78(11):4454–4466. doi:10.1128/IAI.00840-10

Baker VS et al (2008) Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in Plasmodium falciparum infected children under six years of age. Malar J 7:41. doi:10.1186/1475-2875-7-41

Behrendt JH, Ruiz A, Zahner H, Taubert A, Hermosilla C (2010) Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite Eimeria bovis. Vet Immunol Immunopathol 133(1):1–8. doi:10.1016/j.vetimm.2009.06.012

Bianchi M et al (2009) Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood 114(13):2619–2622. doi:10.1182/blood-2009-05-221606

Bonne-Annee S et al (2014) Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval Strongyloides stercoralis. Microbes Infect. doi:10.1016/j.micinf.2014.02.012

Brazil JC et al (2013) alpha3/4 Fucosyltransferase 3-dependent synthesis of Sialyl Lewis A on CD44 variant containing exon 6 mediates polymorphonuclear leukocyte detachment from intestinal epithelium during transepithelial migration. J Immunol 191(9):4804–4817. doi:10.4049/jimmunol.1301307

Brinkmann V, Zychlinsky A (2007) Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol 5(8):577–582. doi:10.1038/nrmicro1710

Brinkmann V, Zychlinsky A (2012) Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol 198(5):773–783. doi:10.1083/jcb.201203170

Brinkmann V et al (2004) Neutrophil extracellular traps kill bacteria. Science 303(5663):1532–1535. doi:10.1126/science.1092385

Brown GB, Roth JA (1991) Comparison of the response of bovine and human neutrophils to various stimuli. Vet Immunol Immunopathol 28(3–4):201–218

Bruns S et al (2010) Production of extracellular traps against Aspergillus fumigatus in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. PLoS Pathog 6(4):e1000873. doi:10.1371/journal.ppat.1000873

Chow OA et al (2010) Statins enhance formation of phagocyte extracellular traps. Cell Host Microbe 8(5):445–454. doi:10.1016/j.chom.2010.10.005

Chuah C et al (2013) Spatial and temporal transcriptomics of Schistosoma japonicum-induced hepatic granuloma formation reveals novel roles for neutrophils. J Leukoc Biol 94(2):353–365. doi:10.1189/jlb.1212653

Chuammitri P, Ostojic J, Andreasen CB, Redmond SB, Lamont SJ, Palic D (2009) Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. Vet Immunol Immunopathol 129(1–2):126–131. doi:10.1016/j.vetimm.2008.12.013

Conejeros I, Patterson R, Burgos RA, Hermosilla C, Werling D (2011) Induction of reactive oxygen species in bovine neutrophils is CD11b, but not dectin-1-dependent. Vet Immunol Immunopathol 139(2–4):308–312. doi:10.1016/j.vetimm.2010.10.021

Conejeros I, Velasquez ZD, Carretta MD, Alarcon P, Hidalgo MA, Burgos RA (2012) 2-Aminoethoxydiphenyl borate (2-APB) reduces alkaline phosphatase release, CD63 expression, F-actin polymerization and chemotaxis without affecting the phagocytosis activity in bovine neutrophils. Vet Immunol Immunopathol 145(1–2):540–545. doi:10.1016/j.vetimm.2011.12.006

Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V (2009) Mouse neutrophil extracellular traps in microbial infections. J Innate Immun 1(3):181–193. doi:10.1159/000205281

Fuchs TA et al (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 176(2):231–241. doi:10.1083/jcb.200606027

Gabriel C, McMaster WR, Girard D, Descoteaux A (2010) Leishmania donovani promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol 185(7):4319–4327. doi:10.4049/jimmunol.1000893

Grinberg N, Elazar S, Rosenshine I, Shpigel NY (2008) Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic Escherichia coli. Infect Immun 76(6):2802–2807. doi:10.1128/IAI.00051-08

Guimarães-Costa AB et al (2009) Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A 106(16):6748–6753. doi:10.1073/pnas.0900226106

Guimarães-Costa AB, Nascimento MT, Wardini AB, Pinto-da-Silva LH, Saraiva EM (2012) ETosis: a microbicidal mechanism beyond cell death. J Parasitol Res 2012:929743. doi:10.1155/2012/929743

Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol 66(11):1146–1154. doi:10.1016/j.humimm.2005.11.003

Hahn S, Giaglis S, Chowdhury CS, Hosli I, Hasler P (2013) Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. Semin Immunopathol 35(4):439–453. doi:10.1007/s00281-013-0380-x

Hellenbrand KM, Forsythe KM, Rivera-Rivas JJ, Czuprynski CJ, Aulik NA (2013) Histophilus somni causes extracellular trap formation by bovine neutrophils and macrophages. Microb Pathog 54:67–75. doi:10.1016/j.micpath.2012.09.007

Hermosilla C, Barbisch B, Heise A, Kowalik S, Zahner H (2002) Development of Eimeria bovis in vitro: suitability of several bovine, human and porcine endothelial cell lines, bovine fetal gastrointestinal, Madin-Darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells. Parasitol Res 88(4):301–307

Hermosilla C, Stamm I, Taubert A, Lutz K, Zahner H, Menge C (2008) Fluorescent Eimeria bovis sporozoites and meront stages in vitro: a helpful tool to study parasite-host cell interactions. Parasitol Res 102(4):777–786. doi:10.1007/s00436-007-0849-4

Hermosilla C, Caro TM, Silva LM, Ruiz A, Taubert A (2014) The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitology 1–10 doi:10.1017/S0031182014000316

Hosseinzadeh A, Messer PK, Urban CF (2012) Stable redox-cycling nitroxide tempol inhibits NET formation. Front Immunol 3:391. doi:10.3389/fimmu.2012.00391

Jackson AR (1964) The isolation of viable coccidial sporozoites. Parasitology 54:87–93

Jenne CN et al (2013) Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. Cell Host Microbe 13(2):169–180. doi:10.1016/j.chom.2013.01.005

Martinelli S et al (2004) Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem 279(42):44123–44132. doi:10.1074/jbc.M405883200

Mehlhorn H, Armstrong PM (2001) Encyclopedic reference of parasitology: biology, structure, function. Springer

Muñoz Caro T, Hermosilla C, Silva LM, Cortes H, Taubert A (2014) Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite Besnoitia besnoiti. PLoS One 9(3):e91415. doi:10.1371/journal.pone.0091415

Ng TH, Chang SH, Wu MH, Wang HC (2013) Shrimp hemocytes release extracellular traps that kill bacteria. Dev Comp Immunol 41(4):644–651. doi:10.1016/j.dci.2013.06.014

Palic D, Ostojic J, Andreasen CB, Roth JA (2007) Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol 31(8):805–816. doi:10.1016/j.dci.2006.11.010

Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191(3):677–691. doi:10.1083/jcb.201006052

Ramos-Kichik V et al (2009) Neutrophil extracellular traps are induced by Mycobacterium tuberculosis. Tuberculosis 89(1):29–37. doi:10.1016/j.tube.2008.09.009

Ruiz A et al (2006) Influence of climatic and management factors on Eimeria infections in goats from semi-arid zones. J Vet Med B Infect Dis Vet Public Health 53(8):399–402. doi:10.1111/j.1439-0450.2006.00985.x

Sahoo G, More T, Singh VK (2000) Evaluation of oxygen-dependent immunodefences of the polymorphonuclear cells of some tropical ruminants. Vet Res Commun 24(8):505–515

Seper A et al (2013) Vibrio cholerae evades neutrophil extracellular traps by the activity of two extracellular nucleases. PLoS Pathog 9(9):e1003614. doi:10.1371/journal.ppat.1003614

Soe AK, Pomroy WE (1992) New species of Eimeria (Apicomplexa: Eimeriidae) from the domesticated goat Capra hircus in New Zealand. Syst Parasitol 23:195–202

Sumagin R, Robin AZ, Nusrat A, Parkos CA (2013) Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment. Mucosal Immunol. doi:10.1038/mi.2013.106

Szabady RL, McCormick BA (2013) Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products. Front Immunol 4:220. doi:10.3389/fimmu.2013.00220

Tschopp J, Martinon F, Burns K (2003) NALPs: a novel protein family involved in inflammation. Nat Rev Mol Cell Biol 4(2):95–104. doi:10.1038/nrm1019

Urban CF, Reichard U, Brinkmann V, Zychlinsky A (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol 8(4):668–676. doi:10.1111/j.1462-5822.2005.00659.x

Wardini AB et al (2010) Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. J Gen Virol 91(Pt 1):259–264. doi:10.1099/vir.0.014613-0

5. NEUTROPHIL EXTRACELLULAR TRAPS AS INNATE IMMUNE REACTION AGAINST THE EMERGING APICOMPLEXAN PARASITE *BESNOITIA BESNOITI*

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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-Aldrich) 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-

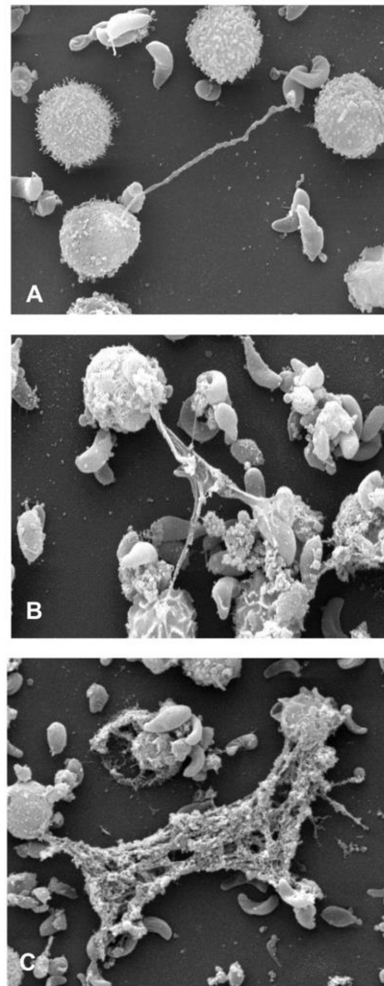

Figure 1. NETs formed by bovine PMN after confrontation with *B. besnoiti* tachyzoites. Scanning electron microscopy analysis revealed NETs being formed by bovine PMN co-cultured with *B. besnoiti* tachyzoites for different time periods [(A) 10 min (B) 30 min, (C) 60 min] in the absence of serum. (A) 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 $76.4\pm2.03\%$ DNA release of the PMN, respectively. In contrast, in parasite-free negative controls $4.03\pm0.33\%$ 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

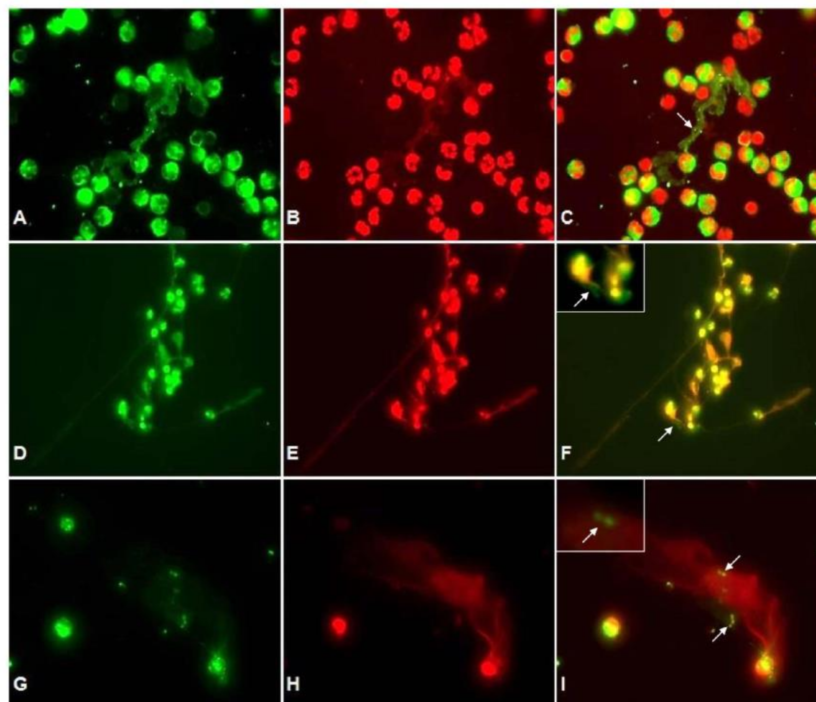

Figure 2. Co-localization of DNA with histones (H3), NE and MPO in tachyzoite-induced NET structures. Co-cultures of bovine PMN and *B. besnoiti* tachyzoites were fixed, permeabilized, stained for DNA using Sytox Orange (red: B, E, H) and probed for MPO (green: A), histones (green: D) and NE (green: 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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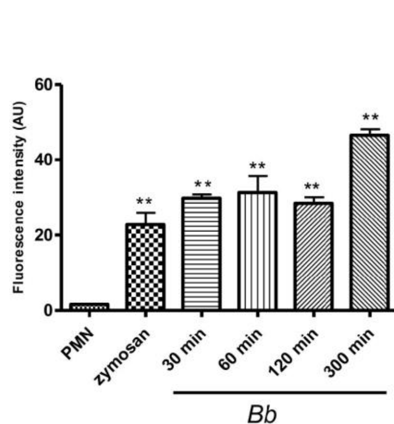

Figure 3. Kinetics of tachyzoite-triggered NET formation. PMN were incubated with *B. besnoiti* tachyzoites (ratio 2:1; 4×10^5 PMN: 2×10^5 tachyzoites), zymosan (1 mg/ml, positive control) or plain medium (negative control) for different time periods. After incubation, samples were analysed for extracellular 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 $p\leq0.05$.
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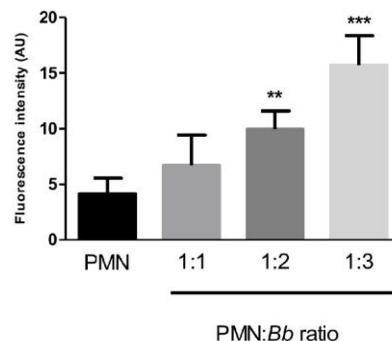

Figure 4. Dose-dependency of tachyzoite-triggered NET formation. PMN and *B. besnoiti* tachyzoites were incubated at different ratios (PMN:tachyzoites = 1:1, 1:2, 1:3). After incubation, samples were analysed for extracellular DNA by quantifying PicoGreen-derived fluorescence intensities. Each condition was performed in duplicates. Arithmetic means of three PMN donors, minimum and maximum. Differences were regarded as significant at a level of $p\leq0.05$.
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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.

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

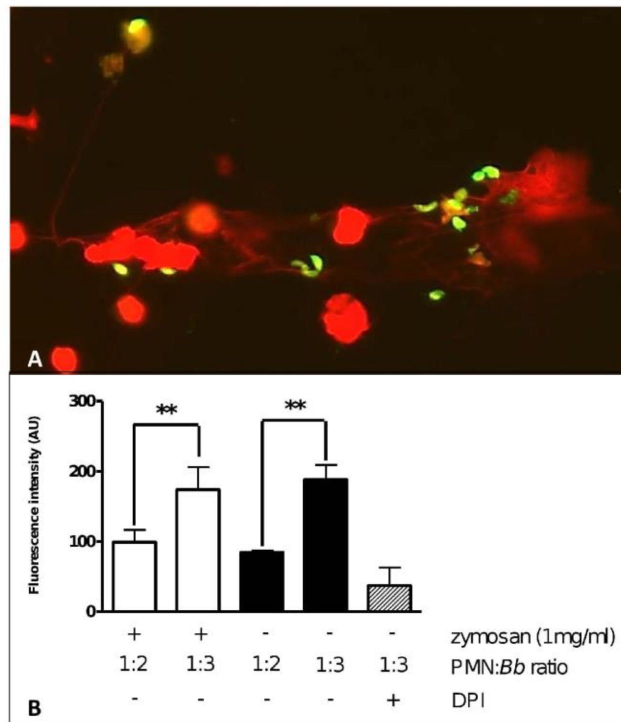

Figure 5. Quantification of *B. besnoiti* tachyzoite entrapment in NET structures. Entrapment of *B. besnoiti* tachyzoites within NET structures was illustrated after the exposure of CFSE- (7.5 μM, 37°C, 30 min) stained tachyzoites to bovine PMN and a subsequent DNA-staining by Sytox Orange (A). Quantification 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 $p\leq0.05$.
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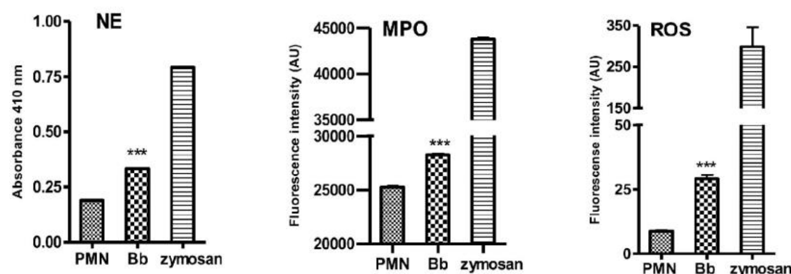

Figure 6. ROS production and enzymatic activities of NE and MPO in tachyzoite-exposed bovine PMN. Bovine PMN were exposed to *B. besnoiti* tachyzoites, zymosan (positive control) or plain medium (negative control) for 30 min. Thereafter, enzymatic activities of NE and MPO as well as ROS production were 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 $p\leq0.05$.
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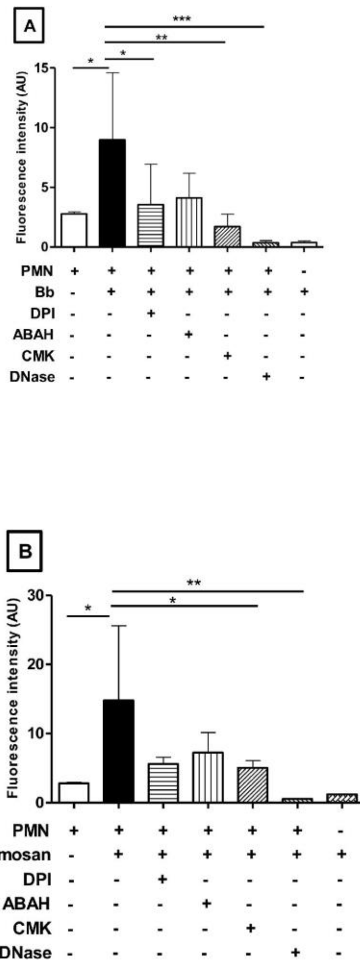

Figure 7. Inhibition of *B. besnoiti* tachyzoite-triggered NET formation. Fig. 7A: PMN were exposed to *B. besnoiti* tachyzoites in the presence or absence of inhibitors and DNase I (90 U). Cells were pre-incubated with DPI (10 μM), NE inhibitor (CMK, 1 mM) and the MPO inhibitor ABAH (100 μM) prior to 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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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.

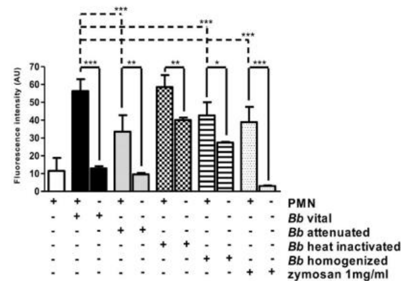

Figure 8. NET formation induced by differentially treated tachyzoites. Bovine PMN were exposed to vital, UV- attenuated, homogenized, and heat-inactivated *B. besnoiti* tachyzoites for 90 min. Stimulation with zymosan (1 mg/ml) served as positive control, plain medium was used as negative control. Samples 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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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

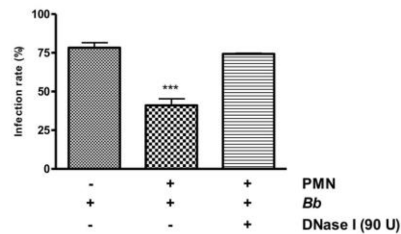

Figure 9. Infectivity of *B. besnoiti* tachyzoites after exposure to bovine PMN. Vital *B. besnoiti* tachyzoites were co-cultured for 3 h with bovine PMN (= PMN + *B.b.*) allowing for effective NET formation. To dissolve potential NET structures, DNase was supplemented 15 min before the end of the incubation 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.

References

1. Cortes H, Leitao A, Vidal R, Vila-Vicosa MJ, Ferreira ML, et al. (2005) Besnoitiosis in bulls in Portugal. Vet Rec 157: 262–264.
2. Fernandez García A, Risco Castillo V, Pedraza Diaz S, Aguado Martínez A, Alvarez García G, et al. (2009) First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J Parasitol 95(2): 474–476.
3. Jacquiet P, Boucher C, Liénard E, Grisez C, Prévot F, et al. (2009) Besnoitiose bovine: enquêtes épidémiologiques dans le sud de la France. Journées Nationales GTV: 1213–1217.
4. Schares G, Basso W, Majzoub M, Cortes HC, Rostaher A, et al. (2009) First in vitro isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet Parasitol 163: 315–322.
5. Gollnick NS, Gentile A, Schares G (2010) Diagnosis of bovine besnoitiosis in a bull born in Italy. Vet Rec 8:166(19):599.
6. Gentile A, Militerno G, Schares G, Nanni A, Testoni S, et al. (2012) Evidence for bovine besnoitiosis being endemic in Italy-first in vitro isolation of *Besnoitia besnoiti* from cattle born in Italy. Vet Parasitol 184: 108–115.
7. Rinaldi L, Maurelli MP, Musella V, Bosco A, Cortes H, et al. (2013) First cross-sectional serological survey on *Besnoitia besnoiti* in cattle in Italy. Parasitology Research112: 1805–1807.
8. Basso W, Braun U, Deplazes P, Gottstein B, Hilbe M, et al. (2012) [First cases of besnoitiosis in cattle in Switzerland]. Schweiz Arch Tierheilkd 154(11): 469–74.
9. Bovine Besnoitiosis: An emerging disease in Europe (2010) EFSA Journal 8(2): 1499.
10. Behrendt JH, Hermosilla C, Hardt M, Failing K, Zahner H, et al. (2008) PMN-mediated immune reactions against *Eimeria bovis*. Vet Parasitol 151(2–4): 97–109.
11. Wilson CB, Remington JS (1979) Activity of human blood leukocytes against *Toxoplasma gondii*. J Infect Dis 140: 890–895.
12. MacLaren A, De Souza W (2002) Further studies on the interaction of *Toxoplasma gondii* with neutrophils and eosinophils. J. Submicrosc. Cytol Pathol 34: 99–104.
13. MacLaren A, Attias M, de Souza W (2004) Aspects of the early moments of interaction between tachyzoites of *Toxoplasma gondii* with neutrophils. Vet Parasitol 125: 301–312.
14. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, et al. (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532–1535.
15. Urban CF, Reichard U, Brinkmann V, Zychlinsky A (2006) Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell Microbiol 8(4): 668–76.
16. Aulik NA, Hellenbrand KM, Klos H, Czuprynski CJ (2010) *Mannheimia haemolytica* and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect Immun 78(11): 4454–66.
17. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, et al. (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 176(2): 231–41.
18. Wartha F, Beiter K, Normark S, Henriques-Normark B (2007) Neutrophil extracellular traps: casting the NET over pathogenesis. Curr Opin Microbiol 10: 52–56.
19. Papayannopoulos V, Zychlinsky A (2009) NETs: a new strategy for using old weapons. Trends Immunol 30(11): 513–21.
20. Wartha F, Henriques Normark B (2008) ETosis: a novel cell death pathway. Sci Signal 1(21): pe25.
21. Abi Abdallah DS, Denkers EY (2012) Neutrophils cast extracellular traps in response to protozoan parasites. Front Immunol 3:382.
22. Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE, et al. (2012) *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. Infect Immun 80(2): 768–77.
23. Baker VS, Imade GE, Molta NB, Tawde P, Pam SD, et al. (2008) Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in *Plasmodium falciparum* infected children under six years of age. Malaria Journal 29: 7:41.
24. Behrendt JH, Ruiz A, Zahner H, Taubert A, Hermosilla C (2010) Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. Vet Immunol Immunopathol 133(1): 1–8.
25. Guimarães-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, et al. (2009) *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A 106(16): 6748–53.
26. Gabriel C, McMaster WR, Girard D, Descoteaux A (2010) *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. J Immunol 185(7): 4319–27.
27. Taubert A, Hermosilla C, Behrendt J, Zahner H (2006) [Reaction of bovine endothelial cells in vitro to coccidia (*Eimeria bovis*, *Toxoplasma gondii*, *Neospora caninum*) infections as the expression of a non-adaptive immune response]. Berl Munch Tierarztl Wochenschr 119(7–8): 274–81. [Article in German]
28. Y Zhao, B Huang, S Huang, H Zheng, YQ Li, et al. (2013) Evaluation of the adjuvant effect of pidotimod on the immune protection induced by UV-attenuated *Toxoplasma gondii* in mouse models. Parasitol Res 2(9): 3151–60.
29. Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, et al. (2004) Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J BiolChem 279: 44123–44132.
30. Lippolis JD, Reinhardt TA, Goff JP, Horst RL (2006) Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. Vet Immunol Immunopathol 15:113(1–2): 248–55.
31. Hermosilla C, Stamm I, Taubert A, Lutz K, Zahner H, et al. (2008) Fluorescent *Eimeria bovis* sporozoites and meront stages in vitro: a helpful tool to study parasite-host cell interactions. Parasitol Res 102(4): 777–86.
32. Conejeros I, Jara E, Carretta MD, Alarcón P, Hidalgo MA, et al. (2012) 2-Aminoethoxydiphenyl borate (2-APB) reduces respiratory burst, MMP-9 release and CD11b expression, and increases l-selectin shedding in bovine neutrophils. Res Vet Sci 92(1): 103–10.
33. Conejeros I, Patterson R, Burgos RA, Hermosilla C, Werling D (2011) Induction of reactive oxygen species in bovine neutrophils is CD11b, but not dectin-1-dependent. Vet Immunol Immunopathol 139 (2–4): 308–12.
34. Parker H, Albrett AM, Kettle AJ, Winterbourn CC (2012) Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. J Leukoc Biol 91(3): 369–76.
35. Köckritz-Blickwede M, Chow O, Ghochani M, Nizet V (2010) Visualization and Functional Evaluation of Phagocyte Extracellular Traps. Methods in Microbiology 37: 139–160.
36. Brown GB, Roth JA (1991) Comparison of the response of bovine and human neutrophils to various stimuli. Vet Immunol Immunopathol 28(3–4): 201–18.
37. von Köckritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, et al. (2008) Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood 111: 3070–3080.
38. Linch SN, Kelly AM, Danielson ET, Pero R, Lee JJ, et al. (2009) Mouse eosinophils possess potent antibacterial properties in vivo. Infect Immun 77: 4976–4982.
39. Saitoh T, Komano J, Saitoh Y, Misawa T, Takahama M, et al. (2012) Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe 12(1): 109–16.
40. Brinkmann V, Zychlinsky A (2007) Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol 5(8):577–82 Review .
41. Álvarez-García G, Frey CF, Mora LMO, Schares G (2013) A century of bovine besnoitiosis: an unknown disease re-emerging in Europe. Trends in Parasitology 29: 407–415.

6. THE INTRIGUING HOST INNATE RESPONSE: NOVEL ANTI-PARASITIC DEFENCE BY NEUTROPHIL EXTRACELLULAR TRAPS

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SPECIAL ISSUE ARTICLE 1489

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, *Staphylococcus 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

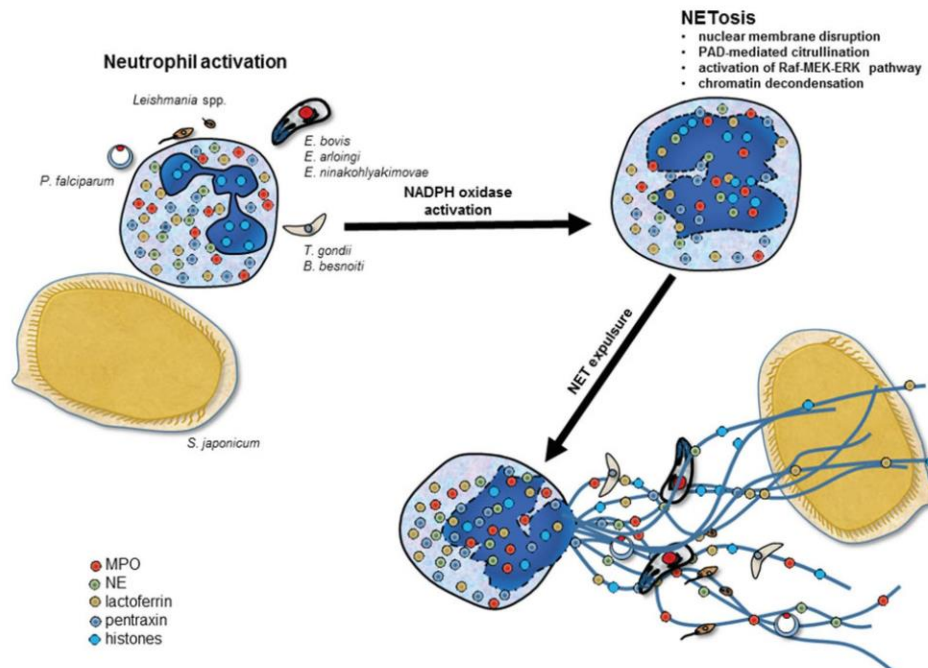

Fig. 1. Mechanisms of parasite-triggered neutrophil extracellular traps (NETs) release. PMN become activated by the contact with different protozoan parasite stages, such as trophozoites of *Plasmodium falciparum*, sporozoites of *Eimeria bovis*, *E. arloingi* or *E. ninakohlyakimovae*, tachyzoites of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

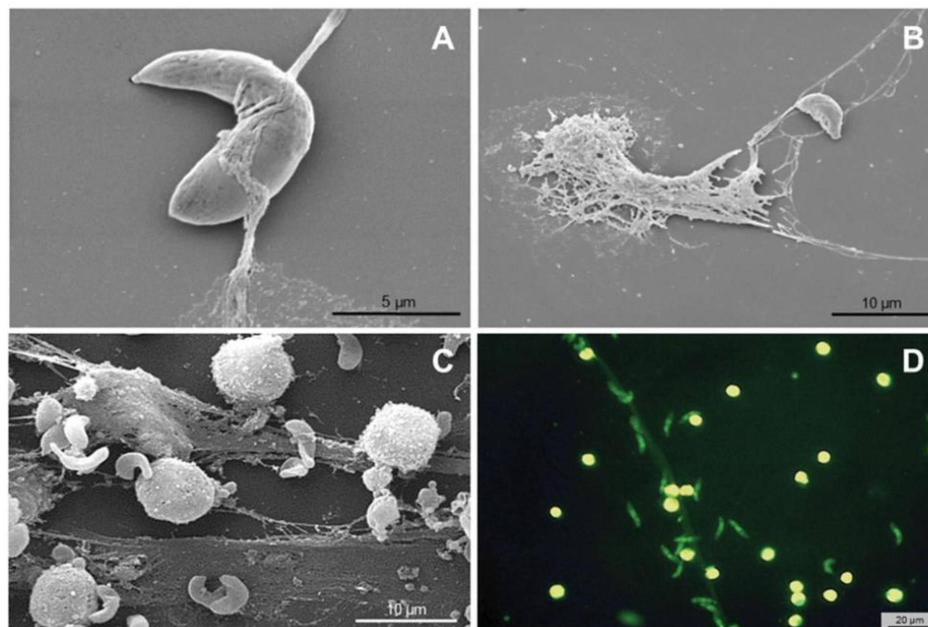

Fig. 2. NET formation triggered by different coccidian (*Eimeria bovis*, *E. arloingi*, *Toxoplasma gondii*, *Besnoitia besnoiti*) species. Bovine PMN were co-incubated with sporozoites of *E. bovis*/*E. arloingi* and tachyzoites of *T. gondii*/*B. besnoiti*, respectively, and thereafter analysed by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

Abi Abdallah, D. S. and Denkers, E. Y. (2012). Neutrophils cast extracellular traps in response to protozoan parasites. *Frontiers in Immunology* **3**, 382. doi: 10.3389/fimmu.2012.00382.

Abi Abdallah, D. S., Lin, C., Ball, C. J., King, M. R., Duhamel, G. E. and Denkers, E. Y. (2012). *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. *Infection and Immunity* **80**, 768–777. doi: 10.1128/IAI.05730-11.

Alghamdi, A. S. and Foster, D. N. (2005). Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. *Biology of Reproduction* **73**, 1174–1181. doi: 10.1095/biolreprod.105.045666.

Altincicek, B., Stotzel, S., Wygrecka, M., Preissner, K. T. and Vilcinskas, A. (2008). Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. *Journal of Immunology* **181**, 2705–2712.

Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J. and den Boer, M. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* **7**, e35671.

Ashford, R. W. (2000). The leishmaniases as emerging and reemerging zoonoses. *International Journal for Parasitology* **30**, 1269–1281.

Aulik, N. A., Hellenbrand, K. M., Klos, H. and Czuprynski, C. J. (2010). *Mannheimia haemolytica* and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. *Infection and Immunity* **78**, 4454–4466. doi: 10.1128/IAI.00840-10.

Aulik, N. A., Hellenbrand, K. M. and Czuprynski, C. J. (2012). *Mannheimia haemolytica* and its leukotoxin cause macrophage extracellular trap formation by bovine macrophages. *Infection and Immunity* **80**, 1923–1933. doi: 10.1128/IAI.06120-11.

Bainton, D. F., Ullyot, J. L. and Farquhar, M. G. (1971). The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *Journal of Experimental Medicine* **134**, 907–934.

Baker, V. S., Imade, G. E., Molta, N. B., Tawde, P., Pam, S. D., Obadofin, M. O., Sagay, S. A., Egah, D. Z., Iya, D., Afolabi, B. B., Baker, M., Ford, K., Ford, R., Roux, K. H. and Keller, T. C., III (2008). Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in *Plasmodium falciparum* infected children under six years of age. *Malaria Journal* **7**, 41. doi: 10.1186/1475-2875-7-41.

Behrendt, J. H., Clauss, W., Zahner, H. and Hermosilla, C. (2004). Alternative mechanism of *Eimeria bovis* sporozoites to invade cells *in vitro* by breaching the plasma membrane. *Journal of Parasitology* **90**, 1163–1165. doi: 10.1645/GE-3285RN.

Behrendt, J. H., Hermosilla, C., Hardt, M., Failing, K., Zahner, H. and Taubert, A. (2008). PMN-mediated immune reactions against *Eimeria bovis*. *Veterinary Parasitology* **151**, 97–109. doi: 10.1016/j.vetpar.2007.11.013.

Behrendt, J. H., Ruiz, A., Zahner, H., Taubert, A. and Hermosilla, C. (2010). Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. *Veterinary Immunology and Immunopathology* **133**, 1–8. doi: 10.1016/j.vetimm.2009.06.012.

Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A. and Henriques-Normark, B. (2006). An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Current Biology* **16**, 401–407. doi: 10.1016/j.cub.2006.01.056.

Bianchi, M., Hakkim, A., Brinkmann, V., Siler, U., Seger, R. A., Zychlinsky, A. and Reichenbach, J. (2009). Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* **114**, 2619–2622. doi: 10.1182/blood-2009-05-221606.

Bliss, S. K., Marshall, A. J., Zhang, Y. and Denkers, E. Y. (1999). Human polymorphonuclear leukocytes produce IL-12, TNF-alpha, and the chemokines macrophage-inflammatory protein-1 alpha and -1 beta in response to *Toxoplasma gondii* antigens. *Journal of Immunology* **162**, 7369–7375.

Bliss, S. K., Butcher, B. A. and Denkers, E. Y. (2000). Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. *Journal of Immunology* **165**, 4515–4521.

Borregaard, N. and Cowland, J. B. (1997). Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**, 3503–3521.

Breman, J. G. and Brandling-Bennett, A. D. (2011). The challenge of malaria eradication in the twenty-first century: research linked to operations is the key. *Vaccine* **29** (Suppl. 4), D97–D103. doi: 10.1016/j.vaccine.2011.12.003.

Brinkmann, V. and Zychlinsky, A. (2007). Beneficial suicide: why neutrophils die to make NETs. *Nature Reviews Microbiology* **5**, 577–582. doi: 10.1038/nrmicro1710.

Brinkmann, V. and Zychlinsky, A. (2012). Neutrophil extracellular traps: is immunity the second function of chromatin? *Journal of Cell Biology* **198**, 773–783. doi: 10.1083/jcb.201203170.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. and Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535. doi: 10.1126/science.1092385.

Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S., Thywissen, A., Jeron, A., Latge, J. P., Brakhage, A. A. and Gunzer, M. (2010). Production of extracellular traps against *Aspergillus fumigatus in vitro* and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathogen* **6**, e1000873. doi: 10.1371/journal.ppat.1000873.

Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Kotb, M., Feramisco, J. and Nizet, V. (2006). DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Current Biology* **16**, 396–400. doi: 10.1016/j.cub.2005.12.039.

Burke, M. L., McManus, D. P., Ramm, G. A., Duke, M., Li, Y., Jones, M. K. and Gobert, G. N. (2010). Temporal expression of chemokines dictates the hepatic inflammatory infiltrate in a murine model of schistosomiasis. *PLoS Neglected Tropical Diseases* **4**, e598. doi: 10.1371/journal.pntd.0000598.

Chuah, C., Jones, M. K., Burke, M. L., Owen, H. C., Anthony, B. J., Mcmanus, D. P., Ramm, G. A. and Gobert, G. N. (2013). Spatial and temporal transcriptomics of *Schistosoma japonicum*-induced hepatic granuloma formation reveals novel roles for neutrophils. *Journal of Leukocyte Biology* **94**, 353–365. doi: 10.1189/jlb.1212653.

Chuammitri, P., Ostojic, J., Andreasen, C. B., Redmond, S. B., Lamont, S. J. and Palic, D. (2009). Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Veterinary Immunology and Immunopathology* **129**, 126–131. doi: 10.1016/j.vetimm.2008.12.013.

Cogen, A. L., Yamasaki, J., Muto, J., Sanchez, K. M., Alexander, L. C., Tanios, J., Lai, Y., Kim, J. E., Nizet, V. and Gallo, R. (2010). *Staphylococcus epidermidis* antimicrobial δ-toxin

(phenol-soluble modulin-γ) cooperates with host antimicrobial peptides to kill group A S*treptococcus*. *PLoS ONE* **5**, e8557.

Dabritz, H. A. and Conrad, P. A. (2010). Cats and *Toxoplasma*: implications for public health. *Zoonoses and Public Health* **57**, 34–52. doi: 10.1111/j.1863-2378.2009.01273.x.

Daugschies, A. and Najdrowski, M. (2005). Eimeriosis in cattle: current understanding. *Journal of Veterinary Medicine – Series B – Infectious Diseases and Veterinary Public Health* **52**, 417–427. doi: 10.1111/j.1439-0450.2005.00894.x.

Dubey, J. P. (2009). History of the discovery of the life cycle of *Toxoplasma gondii*. *International Journal for Parasitology* **39**, 877–882.

Ermert, D., Urban, C. F., Laube, B., Goosmann, C., Zychlinsky, A. and Brinkmann, V. (2009). Mouse neutrophil extracellular traps in microbial infections. *Journal of Innate Immunity* **1**, 181–193. doi: 10.1159/000205281.

Faber, J. E., Kollmann, D., Heise, A., Bauer, C., Failing, K., Burger, H. J. and Zahner, H. (2002). Eimeria infections in cows in the periparturient phase and their calves: oocyst excretion and levels of specific serum and colostrum antibodies. *Veterinary Parasitology* **104**, 1–17.

Friend, S. C. and Stockdale, P. H. (1980). Experimental *Eimeria bovis* infection in calves: a histopathological study. *Canadian Journal of Comparative Medicine* **44**, 129–140.

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V. and Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. *Journal of Cell Biology* **176**, 231–241. doi: 10.1083/jcb.200606027.

Gabriel, C., Mcmaster, W. R., Girard, D. and Descoteaux, A. (2010). *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. *Journal of Immunology* **185**, 4319–4327. doi: 10.4049/jimmunol.1000893.

Grinberg, N., Elazar, S., Rosenshine, I. and Shpigel, N. Y. (2008). Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic *Escherichia coli*. *Infection and Immunity* **76**, 2802–2807. doi: 10.1128/IAI.00051-08.

Guimarães-Costa, A. B., Nascimento, M. T., Froment, G. S., Soares, R. P., Morgado, F. N., Conceicao-Silva, F. and Saraiva, E. M. (2009). *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proceedings of the National Academy of Sciences USA* **106**, 6748–6753. doi: 10.1073/pnas.0900226106.

Guimarães-Costa, A. B., Nascimento, M. T. C., Wardini, A. B., Pinto-da-Silva, L. H. and Saraiva, E. M. (2011). ETosis: a microbicidal mechanism beyond cell death. *Journal of Parasitology Research* **2012**, 1–11. doi: 10.1155/2012/929743.

Gupta, A. K., Hasler, P., Holzgreve, W., Gebhardt, S. and Hahn, S. (2005). Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Human Immunology* **66**, 1146–1154. doi: 10.1016/j.humimm.2005.11.003.

Hahn, S., Giaglis, S., Chowdhury, C. S., Hosli, I. and Hasler, P. (2013). Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. *Seminars in Immunopathology* **35**, 439–453. doi: 10.1007/s00281-013-0380-x.

Hakkim, A., Fuchs, T. A., Martinez, N. E., Hess, S., Prinz, H., Zychlinsky, A. and Waldmann, H. (2011). Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Natural Chemical Biology* **7**, 75–77. doi: 10.1038/nchembio.496.

Hellenbrand, K. M., Forsythe, K. M., Rivera-Rivas, J. J., Czuprynski, C. J. and Aulik, N. A. (2013). *Histophilus somni* causes extracellular trap formation by bovine neutrophils and macrophages. *Microbial Pathogenesis* **54**, 67–75. doi: 10.1016/j.micpath.2012.09.007.

Hermosilla, C., Zahner, H. and Taubert, A. (2006). *Eimeria bovis* modulates adhesion molecule gene transcription in and PMN adhesion to infected bovine endothelial cells. *International Journal for Parasitology* **36**, 423–431. doi: 10.1016/j.ijpara.2006.01.001.

Hermosilla, C., Ruiz, A. and Taubert, A. (2012). *Eimeria bovis*: an update on parasite-host cell interactions. *International Journal of Medical Microbiology* **302**, 210–215. doi: 10.1016/j.ijmm.2012.07.002.

Hsu, S. Y., Hsu, H. F., Davis, J. R. and Lust, G. L. (1972). Comparative studies on the lesions caused by eggs of *Schistosoma japonicum* and *Schistosoma mansoni* in livers of albino mice and rhesus monkeys. *Annals of Tropical Medicine and Parasitology* **66**, 89–97.

Lippolis, J. D., Reinhardt, T. A., Goff, J. P. and Horst, R. L. (2006). Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. *Veterinary Immunology and Immunopathology* **113**, 248–255. doi: 10.1016/j.vetimm.2006.05.004.

Logters, T., Margraf, S., Altrichter, J., Cinatl, J., Mitzner, S., Windolf, J. and Scholz, M. (2009). The clinical value of neutrophil extracellular traps. *Medical Microbiology and Immunology* **198**, 211–219. doi: 10.1007/s00430-009-0121-x.

MacLaren, A. and De Souza, W. (2002). Further studies on the interaction of *Toxoplasma gondii* with neutrophils and eosinophils. *Journal of Submicroscopic Cytology and Pathology* **34**, 99–104.

Maclaren, A., Attias, M. and De Souza, W. (2004). Aspects of the early moments of interaction between tachyzoites of *Toxoplasma gondii* with neutrophils. *Veterinary Parasitology* **125**, 301–312. doi: 10.1016/j.vetpar.2004.07.006.

Muñoz Caro, T., Hermosilla, C., Silva, L. M. R., Cortes, H. and Taubert, A. (2014). Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite *Besnoitia besnoiti*. *PLoS One* **9**, e91415. doi: 10.1371/journal.pone.0091415.

Mota, M. M., Pradel, G., Vanderberg, J. P., Hafalla, J. C., Frevert, U., Nussenzweig, R. S., Nussenzweig, V. and Rodriguez, A. (2001). Migration of *Plasmodium* sporozoites through cells before infection. *Science* **291**, 141–144. doi: 10.1126/science.291.5501.141.

Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. *Nature Reviews Immunology* **6**, 173–182. doi: 10.1038/nri1785.

Palic, D., Ostojic, J., Andreasen, C. B. and Roth, J. A. (2007). Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. *Development and Comparative Immunology* **31**, 805–816. doi: 10.1016/j.dci.2006.11.010.

Ramos-Kichik, V., Mondragon-Flores, R., Mondragon-Castelan, M., Gonzalez-Pozos, S., Muniz-Hernandez, S., Rojas-Espinosa, O., Chacon-Salinas, R., Estrada-Parra, S. and Estrada-Garcia, I. (2009). Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis*. Tuberculosis (Edinburgh) **89**, 29–37. doi: 10.1016/j.tube.2008.09.009.

Ross, A. G., Bartley, P. B., Sleigh, A. C., Olds, G. R., Li, Y., Williams, G. M. and McManus, D. P. (2002). Schistosomiasis. *New England Journal of Medicine* **346**, 1212–1220. doi: 10.1056/NEJMra012396.

Szabady, R. L. and McCormick, B. A. (2013). Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products. *Frontiers in Immunology* **4**, 220. doi: 10.3389/fimmu.2013.00220.

Taubert, A. (2011). *Untersuchungen zur zellulären Immunantwort sowie zu wirtszellgebundenen Reaktionen gegen Eimeria bovis-infektionen*. VVB Laufersweiler Verlag, 40.

Tenter, A. M., Heckeroth, A. R. and Weiss, L. M. (2000). *Toxoplasma gondii*: from animals to humans. *International Journal for Parasitology* **30**, 1217–1258.

Urban, C. F., Reichard, U., Brinkmann, V. and Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiology* **8**, 668–676. doi: 10.1111/j.1462-5822.2005.00659.x.

Urban, C. F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C., Nacken, W., Brinkmann, V., Jungblut, P. R. and Zychlinsky, A. (2009). Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathogens* **5**, Article ID, e1000693.

Von Kockritz-Blickwede, M. and Nizet, V. (2009). Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. *Journal of Molecular Medicine* (*Berlin, Germany*) **87**, 775–783. doi: 10.1007/s00109-009-0481-0.

Von Kockritz-Blickwede, M., Goldmann, O., Thulin, P., Heinemann, K., Norrby-Teglund, A., Rohde, M. and Medina, E. (2008). Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* **111**, 3070–3080. doi: 10.1182/blood-2007-07-104018.

Von Lichtenberg, F., Erickson, D. G. and Sadun, E. H. (1973). Comparative histopathology of schistosome granulomas in the hamster. *American Journal of Pathology* **72**, 149–178.

Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., Hayama, R., Leonelli, L., Han, H., Grigoryev, S. A., Allis, C. D. and Coonrod, S. A. (2009). Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *Journal of Cell Biology* **184**, 205–213. doi: 10.1083/jcb.200806072.

Wang, Y., Chen, Y., Xin, L., Beverley, S. M., Carlsen, E. D., Popov, V., Chang, K. P., Wang, M. and Soong, L. (2011). Differential microbicidal effects of human histone proteins H2A and H2B on *Leishmania* promastigotes and amastigotes. *Infection and Immunity* **79**, 1124–1133. doi: 10.1128/IAI.00658-10.

Wardini, A. B., Guimaraes-Costa, A. B., Nascimento, M. T., Nadaes, N. R., Danelli, M. G., Mazur, C., Benjamim, C. F., Saraiva, E. M. and Pinto-Da-Silva, L. H. (2010). Characterization of

neutrophil extracellular traps in cats naturally infected with feline leukemia virus. *Journal of General Virology* **91**, 259–264. doi: 10.1099/vir.0.014613-0.

Wartha, F., Beiter, K., Albiger, B., Fernebro, J., Zychlinsky, A., Normark, S. and Henriques-Normark, B. (2007). Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiology* **9**, 1162–1171. doi: 10.1111/j.1462-5822.2006.00857.x.

Wilson, C. B. and Remington, J. S. (1979). Activity of human blood leukocytes against *Toxoplasma gondii*. *Journal of Infectious Diseases* **140**, 890–895.

Yipp, B. G., Petri, B., Salina, D., Jenne, C. N., Scott, B. N., Zbytnuik, L. D., Pittman, K., Asaduzzaman, M., Wu, K., Meijndert, H. C., Malawista, S. E., De Boisfleury Chevance, A., Zhang, K., Conly, J. and Kubes, P. (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. *Nature Medicine* **18**, 1386–1393. doi: 10.1038/nm.2847.

Yousefi, S., Gold, J. A., Andina, N., Lee, J. J., Kelly, A. M., Kozlowski, E., Schmid, I., Straumann, A., Reichenbach, J., Gleich, G. J. and Simon, H. U. (2008). Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nature Medicine* **14**, 949–953. doi: 10.1038/nm.1855.

Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I. and Simon, H. U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death and Differentiation* **16**, 1438–1444. doi: 10.1038/cdd.2009.96.

7. Discussion and Outlook

The vast biodiversity of apicomplexan parasites and their impact in human and livestock health provide enough incentive motives for a long-term investment in parasitology research, as nowadays we do not have appropriate solutions for all of these parasitic diseases. As such, coccidiosis caused by parasites of the genus *Eimeria* is still one of the most widespread and ubiquitous infections of livestock reared under different production systems, especially in ruminants, poultry, and rabbits (Foreyt 1990; Lima 2004; Daugschies and Najdrowski 2005; Chapman 2014; Nosal et al. 2014; Witcombe and Smith 2014).

Particularly in goats, high prevalences of *Eimeria* spp.-infections have been reported worldwide. Regardless of climatic conditions or type of management, coccidiosis is a considerable problem, inclusively in semi-arid regions such as the ones observed in Alentejo, Portugal (de la Fuente and Alunda 1992; Ruiz et al. 2006; Silva et al. 2013; Silva et al. 2014b). In principle, even under adverse climatic conditions, such as desiccation, direct sunlight or extreme high temperatures, certain *Eimeria* oocysts (e. g. *E. cameli, E. dromedarii, E. bactriani*) remain infectious for long periods of time showing their exceptional resistant properties (Faizal and Rajapakse 2001; Ruiz et al. 2006; Sazmand et al. 2012). The *Eimeria*-oocyst wall, a robust structure resistant to a variety of environmental and chemical agents, grants this property (Mai et al. 2009). Oocyst wall formation occurs within *Eimeria*-infected host cells, by conjugating a variable number of membranes depending on *Eimeria* species to be considered. Thus, two (*E. arloingi*), three (*E. acervulina*), four

(*E. maxima*), or even up to five membranes (*E. perforans*) have been described to form oocyst walls (Mai et al. 2009). In the case of *E. arloingi*, the oocyst wall has two distinct membrane layers, the outer electron-dense layer [formed by fusion of wall forming bodies 1 (WFB1)] and the inner electron-lucent layer (formed by the fusion of WFB2), thus providing a protective survival barrier for adverse climatic condition or chemical agents (Razavi et al. 2014). Consequently, resilience of caprine *Eimeria*-oocysts facilitates continuous infection of hosts and perpetuation of coccidiosis in goat herds allocated in semi-arid regions, as seen in Alentejo, Portugal.

In total, nine different caprine *Eimeria* species were identified in Alentejo (Silva et al. 2014b), in agreement to other reports with similar geographical areas (Penzhorn et al. 1994; Agyei et al. 2004; Ruiz et al. 2006). Our survey constitutes the first published epidemiological study in this region and, evidently, contributes to clarify the presence of pathogenic and non-pathogenic species responsible for caprine coccidiosis. The pathogenic species *E. arloingi* and *E. ninakohlyakimovae* (Levine 1985; Chartier and Paraud 2012) were the most predominant species identified (Silva et al. 2014b). These results were expected due to the severity of clinical signs and death of young animals witnessed by veterinarians and goat producers in Alentejo. Additionally, *E. christenseni*, also a pathogenic species, was more predominant among young animals than dairy goats, as previously confirmed by high prevalences in kids with less than six months of age (Jalila et al. 1998; Lima 2004; Hassum and Menezes Rde 2005). Nevertheless, variations in prevalence of *E. christenseni* registered in adults and young animals, previously reported in diverse epidemiological studies, have been attributed to differences in geographical conditions (Chartier and Paraud 2012). Non-pathogenic species were also identified in this epidemiological survey such as *E. alijevi*, *E. hirci*, *E. jolchijevi*, *E. aspheronica*, *E. caprina* and *E. caprovina* (Silva et al. 2014b). Even if these species are considered non-pathogenic, their multiplication in epithelial host cells causes disturbances in

homeostasis, and consequent economic losses due to low performance of *Eimeria*-infected animals.

Young animals are more susceptible than adults during coccidiosis outbreaks, and the impact on young animals' health is usually higher than in older animals. Higher OPG counts observed in our survey corroborated findings of previous age-related studies on caprine coccidiosis (Penzhorn et al. 1994; Koudela and Bokova 1998), where mainly goat kids were more frequently affected by *Eimeria*-infections. Adult goats exposed to primary *Eimeria*-infections develop a species-specific protective cellular immunity, resulting in lower intensity of infection and lower OPG during homologous challenge infections, when comparing to naïve kids (Taylor and Catchpole 1994). At this point, an enzootic stability between host and parasite can be achieved, leading to non-clinical manifestations among adult goats and even among some previously exposed young animals to low *Eimeria*-infection doses. In the case of Serpentina goats, which were the only Portuguese breed to be included in our survey, *E. arloingi* and *E. ninakohlyakimovae*-infected animals, although showing particularly high OPG values, displayed no clinical signs of coccidiosis, such as severe diarrhoea, dirty posteriors or dehydration. Probably the well-developed Serpentina cellular adaptive immune response against these two caprine *Eimeria* spp. could be the plausible reason for this phenomenon. Nonetheless, the natural innate immune system of this Portuguese goat breed compared to others should also be taken into account; but obviously further research on early host innate immune reactions against these species should be conducted to confirm this hypothesis (Silva et al. 2014b).

Clinical coccidiosis outcome is mostly determined by the pathogenicity of the species, even sometimes by the strain of *Eimeria* spp., the infection dose, and the site of replication of the parasite (Rose 1987). Consistently, most pathogenic species in goats, namely *E. arloingi* and *E. ninakohlyakimovae*, must traverse the intestinal epithelium to invade deeper located

host endothelial cells of the central lymph capillaries of the intestinal villi. Moreover, the first merogony of ruminant pathogenic *Eimeria* species results in huge macromeronts (up to 240 µm in size) and the release of > 120.000 merozoites I (Ruiz et al., 2010; Hermosilla et al., 2012). This intracellular massive replication requires not only a prolonged replication time but also an efficient host cell modulation [e. g. modulation of host cell cytoskeleton (Hermosilla et al. 2008), modulation of host cell apoptosis (Lang et al. 2009), modulation of host endothelial cell immune reactions (See Chapter 3), and also host cell metabolism to gain access to nutrients (Hermosilla et al. 2008; Taubert et al. 2010)].

In mono *E. arloingi*-infections, the presence of large non-pedunculated to polyp-like whitish nodules, reaching the size of 3-5 mm, within the jejunum and ileum mucosa are related to the site of the parasite's massive replication (Hashemnia et al. 2012; Silva et al. 2014b). Accordingly, the pathological findings in intestinal samples from a deceased two-month-old goat kid clearly indicated a natural occurring *E. arloingi* mono-infection as prime cause of death (Silva et al. 2014b), and thus confirming its high pathogenicity especially in young animals (Yvoré et al. 1985; Koudela and Bokova 1998).

Caprine coccidiosis results from complex host-parasite interactions, influenced by a variety of factors which can later on determine the severity of the disease (Hashemnia et al. 2014) including age, parasite species, stress, genetic susceptibility, physical condition and immunologic state of the host (Ruiz et al. 2006). Stress caused to animals during their lives is an important risk factor associated with coccidiosis outbreaks in ruminants. The weaning period, transport, frequent re-grouping, inadequate feeding, peripartum period, or even concomitant infections might result in higher risk of coccidiosis (Faber et al. 2002; Lima 2004; Daugschies and Najdrowski 2005). Consequently, early recognition of risk factors, identification of *Eimeria* species to be found in goat herds as well as continuous assessment of

OPG throughout the animal production life are crucial in the efficient control of caprine coccidiosis.

Regarding the impact of peripartum stress on OPG counts, a follow up of *Eimeria*-oocysts excretions was conducted in two goat farms in Alentejo, one week before and until three months after kidding, on a weekly base (Silva et al. 2012). Examined kids (*n* = 14) and dairy goats (*n* = 12) presented concomitant infections with 2 to 7 different species. Again *Eimeria arloingi*, *E. ninakohlyakimovae* and *E. alijevi* were the most frequently identified species in both groups. Interestingly, the goat herd set in a more humid environment presented always higher OPG counts in all investigated animals when compared to the herd with more insolation and drier premises, thus confirming the importance of adequate humidity levels for oocyst sporulation and further maintenance of infective stages (sporulated oocysts) in the environment. After parturition, there was a weekly decrease in the number of shed oocysts in dairy goats, but no significant differences were registered when comparing consecutive weeks, revealing a rather slow decrease of oocysts shedding (Silva et al. 2012). Meanwhile, kids started to shed oocysts from the second or third week of age onwards, mainly due to acquired infection in their first days of life. Commonly, ingestion of sporulated oocysts occurs while suckling on contaminated udders of their mothers or by ingestion of contaminated soil and other food materials (Silva et al. 2011). Kids OPG counts revealed an interesting pattern with a peak of oocysts shedding every 2 to 3 weeks. Silva et al. (2011) reported a peak of OPG in lambs at 6 weeks of age, probably associated with the introduction of lambs into pasture and the beginning of grazing season. In our survey, goat kids from both herds remained in isolated premises, where gazing was not possible. However, kids became more active and curious, so accidental ingestion of sporulated oocysts could have easily occurred while exploring surroundings. The biphasic pattern of oocysts excretion can also be related to different pre-patent periods of *Eimeria* spp. found in this study or even related to the presence of so called ‘hypnozoites’/’dormozoites’, previously described for some ruminant *Eimeria*

(Speer et al. 1985). *In vivo*, these 'hynozoites/dormozoites' might remain intracellularly inactive until more favourable conditions are found for further macromeront development and production of merozoites I (Markus 2011).

In both caprine coccidiosis epidemiology studies, kids were the most important source of environmental contamination since they always showed higher OPG values when compared to older animals (Silva et al. 2012; Silva et al. 2014b). Therefore, goat kids born later on during the kidding season may present higher risk of clinical coccidiosis, due to a more contaminated environment, if no appropriate management is considered. Only two goat herds in this survey had running metaphylactic programs against caprine coccidiosis as control strategy (Silva et al. 2014b); nonetheless, both farms presented OPG counts within the ranges of non-treated animals. This fact clearly indicates the requirement of improved metaphylaxis programs in order to achieve real reduction of oocysts shedding and, thereby, decreasing environmental contamination. Ruiz et al. (2012) stated that in goat farms with previous history of serious clinical caprine coccidiosis, an efficient metaphylactic control program should be included by taking into account, firstly, the precise time point of treatments by using diclazuril, and secondly, higher treatment doses than the ones recommended in order to prevent severe clinical coccidiosis outbreaks.

Currently, we have evidence that caprine *Eimeria*-infections are endemic and highly prevalent in Portugal. The economic impact of caprine coccidiosis is enormous, yet neglected in the goat industry. In contrast to this situation, in the poultry industry in which costs represent a constant concern, it was estimated that more than $3 billion are spent annually worldwide exclusively for coccidiosis prevention (ThePoultrySite 2013). Also in ruminants, coccidiosis has a great economic impact (Foreyt 1990; Daugschies and Najdrowski 2005; Chartier and Paraud 2012). Daugschies and Najdrowski (2005) estimated costs due to coccidiosis in cattle and buffalo of US$731 million/year in 1980 and lost profit amounts of

US$400 million/year in 2002. However, the current impact of coccidiosis on the small ruminants industry worldwide is still not well documented (Chartier and Paraud 2012). More than three decades ago, Fitzgerald (1980) estimated US$140 million/year of economic losses caused by ovine- and caprine-coccidiosis. Nowadays, we truly believe this value is considerably higher due to the fact that the numbers of reared goats have dramatically increased since 1980, reaching one billion goats raised worldwide (FAOSTAT 2014). Thus, the search for new coccidiosis control measurements, such as the development of suitable immunoprophylaxis in caprine eimeriosis (e. g. attenuated vaccines) is hereby justified (Ruiz et al. 2014).

Despite academic recognition on the importance of caprine coccidiosis and its financial impact in goat production systems, there is still unawareness within breeders. The absence of common visible clinical signs, such as diarrhoea, does not indicate the absence of the disease in their flocks. Indeed, the perpetuation of uncontrolled subclinical caprine coccidiosis will almost inevitably result in coccidiosis outbreaks with devastating consequences in the goat production, often associated with mortality of young animals. Therefore, early interventions of veterinarians through reliable diagnostic tools, as well as effective control programs, are of great importance in integrated control strategies of caprine coccidiosis.

As mentioned before, *E.* arloingi infections are widely distributed (Chartier and Paraud 2012; Hashemnia et al. 2012), particularly in Southern Portugal (Silva et al. 2013; Silva et al. 2014b) and mainly in kids (Sayin et al. 1980; Agyei et al. 2004), thereby justifying the isolation of a new *E. arloingi* (strain A) which will allow further detailed caprine studies. Investigations related to early host innate immune reactions [e. g. caprine NETosis induced by *E. arloingi* (Silva et al. 2014a)] and others linked to complex molecular host endothelial cell-parasite interactions (see Chapter 3) can also be performed, as previously reported for the closely related *Eimeria* species in cattle (Hermosilla et al., 2006; Taubert et al., 2006; Taubert

et al., 2010; Hermosilla et al., 2012). To our best knowledge, there was only one isolated pure *E. arloingi* strain so far (Parasitology Department of Shiraz University, Iran), which has been recently characterized in *in vivo* infections (Hashemnia et al. 2011; Hashemnia et al. 2012; Hashemnia et al. 2014; Razavi et al. 2014) and also genetically and phylogenetically (Khodakaram-Tafti et al. 2013). Isolation of this new European *E. arloingi* (strain A) represents an important tool for investigations related to antigenic differences between strains (Kawazoe et al. 2005), which have been reported to exist in chicken *Eimeria* spp. (Tomley 1994a; Tomley 1994b). Furthermore, the isolation of a highly prolific *E. arloingi* (strain A) (see Chapter 3, isolation of more than 580×10^6 oocysts out of 3×10^3 initial sporulated oocysts) will allow promising future investigations on immunoprophylaxis against *E. arloingi*-infections as recently demonstrated for *E. ninakohlyakimovae* (Ruiz et al. 2014). Therefore, further investigations on the applicability of this new caprine *E. arloingi*-isolate for immunization trails, both alone and combined with *E. ninakohlyakimovae* will be carried out in the near future. Our main goal will be to develop a commercial suitable vaccine against these two highly pathogenic species, and thus actively contribute to the control of caprine coccidiosis worldwide.

So far, no *in vitro* system was ever established for *E. arloingi*, although similar *in vitro* systems have been reported for other ruminant *Eimeria* (Hammond et al. 1966; Hammond and Fayer 1968; Hermosilla et al. 2002; Ruiz et al. 2010). Consequently, the development of a suitable *in vitro* system for *E. arloingi* sporozoites by using the same *in vivo* specific host cells – primary endothelial host cells – allows advanced and detailed molecular parasite-host cell interaction studies. Excystation of *Eimeria* oocysts is a key event for any suitable *in vitro* system. *In vivo*, two types of stimuli are necessary during excystation process: the first one provided by the rumen environment and the second one by the trypsin-bile solution of the small intestine (Jackson 1962). Here, a successful *E. arloingi in vitro* excystation protocol was established, adapted from excystation protocols previously described for *E. bovis*

(Hermosilla et al. 2002). Thus, using bovine bile and swine trypsin we were able to obtain viable *E. arloingi* sporozoites *in vitro* (see Chapter 3), showing that bile salts and trypsin are mandatory components for proper excystation even if coming from other host species (bovine, swine). However, *E. arloingi* sporozoites experienced a different sporozoites egress mechanism than the one observed in closely related *E. bovis* (Hermosilla et al. 2002) and *E. ninakohlyakimovae* (Ruiz et al. 2010), both presenting two thin oocysts walls and inconspicuous micropyle (Eckert 1995). *In vitro* excystation of these two former species involves the rupture of oocyst walls degraded by trypsin releasing sporocysts, which contain two sporozoites each. Subsequently, activated sporozoites enclosed within sporocysts set themselves free from single sporocysts. In case of *E. arloingi*, a well-developed micropyle and polar cap are present (Levine 1985). After few minutes of incubation in trypsin-bile excystation solution, *E. arloingi*-oocyst polar caps detach and sporozoites become extremely active and mobile inside oocysts. *Eimeria arloingi* oocyst walls do not rupture due to their thickness, and do not release sporocysts (Eckert 1995). Afterwards, *E. arloingi* sporozoites leave sporocysts into the cavity of the oocysts. Meanwhile, sporozoites get in contact with the opened micropyle, and actively egress from oocysts by gliding motility, as previously reported by Jackson (1964) (see supplementary data, Movie 1). We can only speculate about the reasons for this peculiar excystation process, since we do not know the real biological functions of the micropyle and the polar cap. Neither the molecules nor enzymes involved in *E. arloingi* excystation process are known. Eventually, micropyle and polar cap are features of *Eimeria* species with more resistant oocysts. Having their sporozoites protected by sporocysts surrounded by two thick oocyst walls, allows *E. arloingi* to master and to overcome adverse climatic environmental conditions, such as desiccation and high temperatures. Therefore, the presence of a micropyle might be an alternative way to regulate water within exogenous *E. arloingi*-oocysts. Unfortunately, no data is available concerning the role of oocyst micropyle and its implications during excystation *in vivo*, but further

investigations of micropyle- and polar cap-related molecules, as recently reported for *E. nieschulzi* (Wiedmer et al. 2011), are expected to take place.

Freshly *in vitro* released *E. arloingi* sporozoites showed adequate gliding motility, infectivity, and further intracellular development. *In vivo*, *E. arloingi* is a highly host-specific parasite of goats in which sporozoites have to invade host ECs of the central lacteals of the intestinal villi. Nevertheless, under *in vitro* conditions, *E. arloingi* sporozoites were able to invade different host-cell types such as BUVEC, MDBK and MARC-145. Contrary to observations in BUVEC, after MDBK- and MARC-145-sporozoite invasion, no further development was witnessed (see Chapter 3). However, after infecting BUVEC, sporozoites were able to undergo successful intracellular macromeront development, ending with the release of fully developed merozoites I, at 22 days p. i. (see Chapter 3). According to Ruiz et al. (2010), BUVEC were also suitable host cells for *in vitro* first merogony development of *E. ninakohlyakimovae*. Thus, we were expecting a similar *E. arloingi* infection development. *In vitro* use of primary caprine umbilical vein endothelial cells (CUVEC) as host cells was not considered because of the widespread contamination of these endothelial cells with *Mycoplasma* spp., which clearly hampered *in vitro* development of *Eimeria* (Ruiz et al. 2010). Since 2 days p. i. until 22 days p. i., *E. arloingi* sporozoites egress was observed, as previously reported for other apicomplexan parasites (Mota and Rodriguez 2001; Behrendt et al. 2008; Ruiz et al. 2010). Sporozoite egress is commonly associated with the need of this parasite stage to reach its final specific host cells in deeper tissues, as reported for sporozoites of

P. falciparum, *E. bovis* and *E. ninakohlyakimovae*, thereby migrating through cells in a unique and alternative invasion mechanism by breaching the plasma membrane of the cell without forming a PV (Mota et al. 2001; Behrendt et al. 2004). Currently, it is completely unknown as to how sporozoites of *E. arloingi* and other related pathogenic ruminant *Eimeria* (e. g. *E. bovis*, *E. zuernii*, *E. christenseni*, *E. ninakohlyakimovae*, *E. bakuensis*, *E. cameli*)

cross *in vivo* the epithelium layer of the gut to invade the central lacteals endothelium. Thus, we speculate that the search for a suitable specific host cell is the main reason for this alternative sporozoite invasion process (Mota et al. 2001; Mota and Rodriguez 2002; Behrendt et al. 2004; Hermosilla et al. 2012). Macromeront development of *E. arloingi* requires the parasite's ability to modulate its highly immunoreactive host endothelial cell, and further to scavenge on host cell nutrients for offspring production (> 120.000 merozoites I). In this context, possible impact of cell culture medium supplementations (e. g. glucose, proteins, lipids) on *in vitro E. arloingi* merozoites I production were tested (see Chapter 3). Carbohydrates are well-known to mediate a variety of relevant biological processes such as cell adhesion, cell-cell communication and even host-pathogen interactions (Sanz et al. 2013). Additionally, for *in vitro* apicomplexan parasite growth they seem to be fundamental (van Schalkwyk et al. 2008; Preuss et al. 2012). We also proved the key role of glucose supplementation, which resulted in the increment of *E. arloingi* merozoites I production *in vitro* (see Chapter 3). In contrast, neither proteins- nor lipids-supplementation resulted in significant enhancement of *in vitro E. arloingi* merozoites I production. Regarding lipids supplementation, it has already been described a key role of oleic acid in intraerythrocytic proliferation of *P. falciparum in vitro* (Mi-Ichi et al. 2007; Gratraud et al. 2009). Conversely to our *E. arloingi* lipid-related assay, oleic acid supplementation of *E. bovis*-infected BUVEC resulted in significant increase of merozoites I production *in vitro* when compared to untreated cultures (Hamid et al., submitted manuscript).

Endogenous development of *in vitro E. arloingi* macromeronts was in accordance to previously described data for other related *Eimeria* spp. (Hermosilla et al. 2002; Ruiz et al. 2010). The production of *in vitro E. arloingi* macromeronts is of particular scientific interest for various reasons: (*i*) enduring *E. arloingi* macromeront maturation within highly immunoreactive host ECs strongly suggest that this parasite must rely on regulatory processes to guarantee its massive replication; (*ii*) there is evidence that first generation meronts

represent key targets for host protective immune responses (Rose et al. 1992; Shi et al. 2000; Shi et al. 2001; Taubert et al. 2008); (*iii*) mature macromeront I cultures should be useful tools for the replacement of animal experiments concerning pharmaceutical screenings; and (*iv*) they guarantee accessibility of other parasite stages (merozoites I), which might be needed for further basic molecular and immunological research.

One of the first reactions against pathogens is ECs activation, which represents a state of intensified responsiveness induced by different stimuli, including cytokines, chemokines and even active parasite invasion (Taubert et al. 2006; Mai et al. 2013). Upon activation, ECs can produce a broad range of adhesion molecules, cytokines and pro-inflammatory chemokines. Adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) regulate leukocyte adhesion to ECs and also monitor their movement (Hermosilla et al. 2006). E- and P-selectin mediate the reversible binding, known as tethering, between ECs and leukocytes, while ICAM-1 and VCAM-1 regulate leukocyte-rolling and firm adhesion onto ECs (for review see Wagner and Roth 2000). In *E. arloingi* infections, E-selectin transcription increased until 6 h p. i. while P-selectin transcription values peaked postponed at 12 h p. i.. Comparing to *E. bovis*-induced adhesion molecule up-regulation, *E. arloingi*-infection induced a different pattern with lower levels of E- and P-selectin up-regulation (Hermosilla et al. 2006). It was previously discussed that even contact of ECs with secreted molecules of apicomplexan parasites may already activate ECs without active parasite host cell invasion (Hermosilla et al. 2006). Secretion of parasite-specific proteins constitute a well-known feature in apicomplexan invasion process, and as sporozoites of *E. arloingi* induced up-regulation of adhesion molecules gene transcripts proved that the parasite has the capability to activate BUVEC. Moreover, ICAM-1 and VCAM-1 increasing transcription was also detected within the first 6 h p. i., contrary to *E. bovis*-infection where the peak for these molecules was earlier observed (4 h and 1h p. i., respectively). According with generated data of Hermosilla et al. (2006), a decrease of

adhesion molecules gene transcription was observed from 6 h p. i. onwards. Thus, sporozoites may display counteracting mechanisms to avoid, or at least down-regulate, ECs immune reactions, achieving their obligate intracellular replication (Hermosilla et al. 2012).

Relevant immunoregulatory molecules such as CXC- and CC-chemokines, cytokines (GM-CSF) and enzymes responsible for the synthesis of prostaglandins were also subject of comparative studies revealing a relative weak impact of *E. bovis*-infections when compared to *T. gondii*- and *N. caninum*-infections in BUVEC (Taubert et al. 2006).

As preliminary results of ongoing phylogenetic resemblance studies of *E. arloingi* and *E. bovis*, we expected also *E. arloingi* to be able to modulate ECs activation. Nevertheless, our *E. arloingi in vitro* system is established on BUVEC (bovine origin) and this might explain the stronger reaction induced by *E. arloingi*-sporozoites in these cells, similar to other apicomplexan findings (Taubert et al. 2006). In the first 12 h p. i., a stronger enhanced transcription of studied chemokine genes (CXC and CC) was observed, demonstrating again sporozoite active invasion as triggering event of BUVEC activation (Taubert et al. 2006). CXCL8 gene transcription peaked at 12 h p. i. resembling *E. bovis*-infection, contrary to *T. gondii*- and *N. caninum*-infected ECs which revealed higher transcription levels at 4 h p. i. (Taubert et al. 2006). Consequently, *E. arloingi* triggered similar BUVEC response to *T. gondii* and *N. caninum* within the first hours of infection, but later on induced similar reactions to *E. bovis*-infections. Concerning CCL2- and CCL5-chemokine gene transcription, analogous time courses were observed for these two pro-inflammatory molecules. At 3 h p. i., an increase in CCL2-gene transcription was detected and continued increasing until 12 h p. i.. Considering CC-chemokines, Taubert et al. (2006) reported a gene transcription peak at 12 h p. i., in all three investigated species, namely *E. bovis*, *T. gondii* and *N. caninum*. Besides the fact that different parasites and even different stages (sporozoites for *E. arloingi*/*E. bovis*, tachyzoites for *T. gondii*/*N. caninum*) were compared, the reactivity of single BUVEC isolates

should also be considered. With their own variability, maybe BUVEC isolates are responsible for small differences in gene transcription patterns.

Transcription of GM-CSF gene was rather moderate and similar to the pattern observed in *E. bovis*-infection, increasing from 3 h p. i. until 12 h p. i.. In contrast, *T. gondii*- and *N. caninum*-infections in BUVEC induced earlier GM-CSF reactions (Taubert et al. 2006). As for all the other studied molecules, at 24 h p. i., gene transcription levels decreased to controls levels. In contrast, COX-2 gene transcription remained stable throughout the time course and no significant differences were registered when compared to controls. COX-2 expression is usually associated with cell damage, which in our *E. arloingi in vitro* study was rarely observed.

The up-regulated gene transcription of pro-inflammatory chemokines and other immunomodulatory molecules by *E. arloingi* confirms the immune reactivity of ECs to foreign pathogen invasion. Additionally, endothelium-derived CC-chemokines and prostaglandins are known to be involved in the transition of innate into adaptive immune response (Zlotnik and Yoshie 2000; Zhang and Rivest 2001; Taubert et al. 2006).

Moreover, after *in vivo* excystation, *E. arloingi* sporozoites must confront directly all compartments of the host innate immune system, namely intestinal mucosa, epithelial cells, endothelial cells, and leukocytes (Tschopp et al. 2003). Therefore, basic research to better understand host innate immune reactions against parasites is urgently needed, since protective cellular adaptive immune responses are always dependent on early innate immune reactions. Particularly in the case of early host innate immune reactions against *Eimeria* spp., a small number of studies have been performed, a few of them showing the key role of PMN, monocytes and macrophages in ruminant coccidiosis (Behrendt et al. 2008; Behrendt et al. 2010; Silva et al. 2014a).

PMN are the most abundant leukocytes, representing approximately 60% of total white blood cells (Thrall et al. 2012). Being the first leukocytes to be recruited to the site of infection, PMN are the first line of defence against pathogens (Brinkmann et al. 2004; Nathan 2006; Ermert et al. 2009; Brinkmann and Zychlinsky 2012; Hahn et al. 2013). Therefore, PMN are considered of crucial value in host innate immune reactions, becoming extremely mobile and being able to respond immediately to pathogens after leaving the bone marrow (Hermosilla et al. 2014). Their main functions are phagocytosis- and oxidative burst-related defence actions, as well as NET formation (Brinkmann et al. 2004). During phagocytosis, pathogens are engulfed and internalized into phagosomes, which later on fuse with intracellular granules to form the phagolysosome, within which pathogens will be killed by a combination of non-oxidative (AMPs – cathelicidins, defensins, cathepsins and proteases) and oxidative mechanisms (ROS production via the NADPH oxidase complex) (Nathan 2006; von Kockritz-Blickwede and Nizet 2009; Hermosilla et al. 2014).

NETosis, the latest discovered effector mechanism of PMN, has been described in several species such as humans (Gupta et al. 2005), mice (Ermert et al. 2009), horses (Alghamdi and Foster 2005), cattle (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 demonstrated for the first time caprine NET formation, not only in response to recognized stimulus (zymosan) but also in response to the goat apicomplexan *E. arloingi* (Silva et al. 2014a).

In the last years, NET formation has been focused mainly on bacterial, viral or fungal infections (Urban et al. 2006; Fuchs et al. 2007; Aulik et al. 2010; Jenne et al. 2013; Hermosilla et al. 2014). Nonetheless, protozoan parasites have also been investigated as potent inducers of NETosis, e. g. *T. gondii* (Abi Abdallah et al. 2012), *P. falciparum* (Baker et

al. 2008), *E. bovis* (Behrendt et al. 2010), *Leishmania* spp. (Guimaraes-Costa et al. 2009), and, recently, *B. besnoiti* (Muñoz-Caro et al. 2014).

Bovine besnoitiosis is an important emerging disease in Europe (EFSA 2010), which has been already described in Portugal (Cortes et al. 2005; Waap et al. 2014), Spain (Fernandez-Garcia et al. 2009; Alvarez-Garcia et al. 2014a), France (Jacquiet et al. 2009), Germany (Mehlhorn et al. 2009; Schares et al. 2009), Italy (Gollnick et al. 2010; Rinaldi et al. 2013), Switzerland (Basso et al. 2013), and Hungary (Hornok et al. 2014). The first report of autochthonous bovine besnoitiosis in Central-Eastern Europe was closely related to human activities (e. g. cattle trading), resulting as the main factor in geographical spreading of this neglected parasitic disease (Hornok et al. 2014). *Besnoitia besnoiti* has a heteroxenous life cycle with intermediate and final hosts. Currently, the final host of cattle besnoitiosis is yet unknown, but ruminants (cattle, buffalo) are the most important intermediate hosts (Hornok et al. 2014). So far, little is known on adaptive and innate immune reactions against *B. besnoiti*. With the purpose to address early innate immune reactions against this parasite, we also have investigated the role of NETs in the course of the disease (Muñoz-Caro et al. 2014). During the acute phase of besnoitiosis, also known as anasarca phase, fast-replicating tachyzoites infect host ECs *in vivo* (Alvarez-Garcia et al. 2014b), as *E. arloingi* sporozoites. However, *E. arloingi* first merogony development requires a longer permanence in these host cells when compared to *B. besnoiti*-tachyzoites, which might implicate different modulation strategies of these parasites (see Chapter 3). Additionally, *B. besnoiti* invasion of BUVEC is faster and results in higher infection rates (see Chapter 5) when compared to *E. arloingi*-infections (see Chapter 3).

As suggested for *E. bovis* and *T. gondii* (Behrendt et al. 2010; Abi Abdallah et al. 2012), *E. arloingi*-induced NETosis counts on entrapment and immobilization of parasites, hampering sporozoite host cell invasion, rather than pathogen killing. Curiously, caprine

PMN entrapment capacity of *E. arloingi* sporozoites appears to be superior to the one observed in bovine PMN, since 70% of tested sporozoites were ensnared in caprine NETs (Silva et al. 2014a). Bovine NETs entrapped 30% of sporozoites, when challenging parasites to PMN at the same co-culture ratios (unpublished data). Furthermore, only 34% of CFSE-labelled *B. besnoiti* tachyzoites were trapped in bovine NET structures (Muñoz-Caro et al. 2014), confirming the greater capability of caprine NETs to entrap parasites.

Extracellular arrest of obligate intracellular replicating parasites will, obviously, prevent sporozoites/tachyzoites host cell invasion and, thus, abrogate life cycles of *E. arloingi* and *B. besnoiti* (Muñoz-Caro et al. 2014; Silva et al. 2014a). NET formation induced 40% reduction of *B. besnoiti* infectivity, after incubation with bovine PMN (180 min), while *E. arloingi* infectivity was reduced in 80%, after incubation with caprine PMN (90 min). In both cases, treatments with DNase I, which dissolve formed NET structures, revealed higher infectivity of sporozoites and tachyzoites, when compared to PMN-incubated parasites. Accordingly, trypan blue vital staining of the same parasites showed NETs did not vastly kill *E. arloingi* sporozoites (Silva et al. 2014a) and *B. besnoiti* tachyzoites (Muñoz-Caro et al. 2014), in agreement with reports on *E. bovis*-induced NET formation (Behrendt et al. 2010). In contrast to reports dealing with *Eimeria* sporozoites, NETs appeared to exhibit certain lethal effects on tachyzoites of *T. gondii* (Abi Abdallah et al. 2012) and *L. amazonensis* promastigotes (Guimaraes-Costa et al. 2009). Since the main replication step only follows after ECs 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.

Additionally, we have shown that caprine NETs is neither a stage- nor a parasite-specific effector mechanism of PMN. Caprine NETosis was observed in response to sporozoites and oocysts of *E. arloingi*, both by SEM and fluorescence analyses (Silva et al. 2014a). Whilst

sporozoite stages have been already demonstrated as potent NET-triggers (Behrendt et al. 2010), we here demonstrated for the first time that also oocysts of *E. arloingi* induced NETs. Similar findings were seen with *Leishmania*-triggered NETosis, since both promastigotes (*L. amazonensis*, *L. major*, *L. chagasi*) and amastigotes (*L. amazonensis*) promoted NETs, demonstrating non stage-specific NETs induction (Guimaraes-Costa et al. 2009; Guimaraes-Costa et al. 2012). As already mentioned, *E. arloingi* sporozoites must egress through opened micropyle (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, effective blockage of micropyle by NETs immediately hampers all sporozoites inside the oocyst from excystation. Recent investigations on PMN have demonstrated the capability of these leukocytes to actively transmigrate into the intestinal lumen (Brazil et al. 2013; Seper et al. 2013; Szabady and McCormick 2013; Sumagin et al. 2014), thereby also being able to interact with luminal pathogen stages, such as ingested oocysts, in the *in vivo* situation. It is therefore tempting to speculate that luminal NETs might effectively imped *E. arloingi* sporozoite excystation at the earliest time point after oral infection. SEM analyses performed in this study clearly showed oocysts and freshly excysted sporozoites being firmly entrapped by NET-like structures *in vitro*, showing once more the importance of NET formation in early innate immune response against caprine *E. arloingi* (Silva et al. 2014a).

Recent analyses doubt a strict species-specificity *Eimeria*-induced NETosis since caprine NETs was induced by (strictly host specific) *E. bovis* sporozoites, and *E. ninakohlyakimovae* sporozoites, and furthermore, bovine PMN also expelled NETs in response to non-bovine *E. arloingi* sporozoites (Muñoz-Caro et al., submitted manuscript). Likewise, future experiments with metazoan parasites (*Haemonchus contortus*) will be carried out with caprine PMN to identify possible NETs-dependent effector mechanisms against this parasite.

The DNA-nature of sporozoite-triggered NETs was demonstrated by staining of NET structures with the DNA-stain Sytox Orange® and the dissolution of parasite-induced filamentous structures by DNase I treatments. Besides chromatin, other components of NETs, such as nuclear histones (H3), NE, cathepsin G, MPO, lactoferrin and gelatinase, are described as pivotal for the microbiocidal effect of NETs (Brinkmann et al. 2004). Through co-localization experiments we were able to confirm these classical characteristics of NETs showing the simultaneous presence of H3, NE and MPO in caprine NETs. *Eimeria arloingi* sporozoite-triggered NETosis as well as *B. besnoiti* tachyzoite-triggered NET formation were time-dependent as also previously reported by Behrendt et al. (2010) for *E. bovis*. Caprine PMN reacted strongly upon zymosan by up regulating 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 *E.* arloingi-sporozoites (Silva et al. 2014a).

Moreover, enhanced NE enzymatic activity in sporozoite-exposed PMN confirmed the key role of this molecule in formation of NETs as suggested by others (Papayannopoulos et al. 2010). NADPH oxidase complex activation 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 diphenylene iodondium (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). Previously, a singular regulation process of inducible nitric oxide synthase production in bovine and caprine leukocytes was reported (Adler et al. 1996) as well as differential lower phagocytic activities in caprine PMN after being exposed to *Candida albicans* when compared to bovine- and buffalo-PMN responses (Sahoo et al. 2000). Thus,

findings of ROS-dependent NET induction in goats cannot be taken for granted (Silva et al. 2014a).

Even though NET formation has been studied *in vitro*, *in vivo* evidence of this innate immune effector mechanism is still scarce. Chuah et al. (2013) have shown the release of NETs *in vivo* in *Schistosoma japonicum*-induced hepatic granuloma formation and the role of PMN in the mediation of tissue damage and repair during *S. japonicum* egg-induced hepatic granulomatous lesions. In the case of *E. arloingi,* we could recently identify the presence of NET structures in histological intestinal tissue slides (supplementary data, Fig. 4) originated from a naturally *E. arloingi* infected goat kid, thereby clearly proving NETosis to occur also *in vivo* during coccidiosis. Interestingly in these tissue samples, a single *E. arloingi*-merozoite can be observed being firmly trapped by NET structures, confirming that *E. arloingi*-free stages are effectively trapped by caprine NETs (supplementary data, Fig. 4, arrow), as previously demonstrated *in vitro* (Silva et al. 2014a). Further analysis will be performed to co-localize other classical components of NETs in *E. arloingi*-infections *in vivo*, as well as in other apicomplexan infections of cattle.

Recently, other innate immune leukocytes have been described to release extracellular traps (ETs) (Muniz et al. 2013). ETs are released by basophils containing mitochondrial DNA instead of nuclear DNA (Morshed et al. 2014), macrophages (Bonne-Annee et al. 2014), mast cells (von Kockritz-Blickwede and Nizet 2009) and eosinophils (Yousefi et al. 2008). Moreover, we have recently shown monocyte-triggered ETs after the exposure to *B. besnoiti* tachyzoites and *E. bovis* sporozoites (Muñoz-Caro et al., accepted manuscript), as well as against *E. arloingi* and *E. ninakohlyakimovae* sporozoites (personal observation, data not published). Additionally, ETosis against metazoan parasites (e. g. *H. contortus*) and different leukocytes such as monocytes and eosinophils are being carried out in our lab in order to amplify our knowledge in innate immune responses.

Overall, we here presented specific data on the characterization of *Eimeria* infections in goats in Southern Portugal. Moreover, we provided for the first time epidemiological data concerning caprine coccidiosis in Portugal and we successfully isolated a caprine-specific *E. arloingi* (A). The advantages of keeping a brand new *Eimeria* species are countless. Without any doubts, the most tempting expectation of all these possibilities is the capability to develop a new protective vaccine against *E. arloingi*, as recently successfully achieved by the use of the attenuated caprine *E. ninakohlyakimovae* (GC) oocysts in Spain (Ruiz et al. 2014). Additionally, the development of a suitable *in vitro* culture system to gain access to additional parasite stages (merozoites I) proves the usefulness of this new *E. arloingi* (A) for future studies such as phylogenetic analysis (already in course with the kind collaboration of Dr. Damer Blake, Royal Veterinary College, London, UK), therapeutic target identification, endothelium-related immunological studies as well as basic research on the complex modulation of the host cell by *E. arloingi*. Other related data generated during the PhD scientific program is not yet available in this thesis, but will be published soon.

With this PhD work we were able to build up novel knowledge in very different fields of caprine coccidiosis, extending from epidemiology to complex innate immune reactions. Obviously, much more basic molecular research is needed to better understand this neglected enteric parasitosis of goats, but at least first insights into these exciting parasitosis were generated within this PhD thesis. The true journey starts now…

LIST OF OWN PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Original papers

Silva LMR, Vila-Vicosa MJ, Cortes HCE, Taubert A, Hermosilla C (2014) *In vitro Eimeria arloingi* macromeront formation in host endothelial cells and modulation of adhesion molecules-, cytokines- and chemokines-gene transcription. Parasitol Res. doi:10.1007/s00436-014-4166-4

Kleinertz S, Christmann S, **Silva LMR**, Hermosilla C, Taubert A (2014) Gastrointestinal parasite fauna of Emperor Penguins (*Aptenodytes forsteri*) at the Atka-Bay, Antarctis. Parasitol Res 113(11):4133-9. doi: 10.1007/s00436-014-4085-4

Munõz-Caro T, **Silva LMR**, Ritter C, Taubert A, Hermosilla C (2014) *Besnoitia besnoiti* tachyzoites induce monocyte extracellular trap formation. Parasitol Res 113(11):4189-97. doi: 10.1007/s00436-014-4094-3

Silva LMR, Vila-Vicosa MJ, Nunes T, Taubert A, Hermosilla C, Cortes HC (2014) *Eimeria* infections in goats in Southern Portugal. Rev Bras Parasitol Vet 23(2):280-6. doi:10.1590/S1984-29612014051

Silva LMR, Muñoz Caro T, Gerstberger R, Vila-Vicosa MJ, Cortes HC, Hermosilla C, Taubert A (2014) The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps. Parasitol Res 113(8):2797-807. doi:10.1007/s00436-014-3939-0

Muñoz-Caro T, Hermosilla C, **Silva LMR**, Cortes H, Taubert A (2014) Neutrophil Extracellular Traps as Innate Immune Reaction against the Emerging Apicomplexan Parasite *Besnoitia besnoiti*. PLoS One 9(3):e91415. doi:10.1371/journal.pone.0091415

Hermosilla C, Caro TM, **Silva LM**, Ruiz A, Taubert A (2014) The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitology 141(11):1489-98. doi:10.1017/S0031182014000316

Silva LMR, Vila-Viçosa MJM, Maurelli MP, Morgoglione ME, Cortes HCE, Cringoli C, Rinaldi, L (2013). Mini-FLOTAC for the diagnosis of *Eimeria* infection in goats: An alternative to McMaster. Small Rumin Res 114(2-3) 280-283.

Silva LMR, Magalhães FJR, Oliveira AMA, Coelho MCOC, Saldanha SV (2009). Reduction of cleft palate, secondary to transmissible venereal tumor, with palatal prosthesis. Revista Portuguesa de Ciências Veterinárias 104(569-572) 77-82.

Conference contributions

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

Silva LMR, Muñoz-Caro T, Vila-Viçosa MJM, Cortes H, Hermosilla C, Taubert A. Caprine neutrophil extracelular traps induced by *Eimeria arloingi*. PARATROP 2014 – Joint meeting of Parasitology and Tropical Medicine, 16th-19th July 2014, Zurich, Switzerland

Silva LMR, Muñoz-Caro T, Hermosilla C, Taubert A. Neutrophil extracelular traps as innate imune reactions against *Eimeria arloingi* stages. Jahrestagung der Deutschen Veterinärmedizinischen Gesellschaft, Fachgruppe Parasitologie und parasitäre Krankheiten, 30th June-2nd July 2014, Leipzig, Germany

Silva LMR, Muñoz-Caro T, Vila-Viçosa MJM, Cortes H, Hermosilla C, Taubert A. *Eimeria arloingi* stages trigger Neutrophil Extracellular Traps (NETs) as innate immune reaction. Annual Meeting Veterinärmedizinischer Arbeitskreis“ VIA 2014, 2nd-3rd May 2014, Jena, Germany

Silva LMR, Vila-Viçosa MJM, Cortes H, Hermosilla C, Taubert A. *Eimeria arloingi*: isolation of portuguese strain and *in vitro* culture trials. VI Congress of the Portuguese Society of Veterinary Sciences, 3rd-5th April 2014, Oeiras, Portugal

Silva LMR, Vila-Viçosa MJM, Cortes H, Hermosilla C, Taubert A. Caprine *Eimeria* infections: oocyst excretion in peri-parturient animals and goat kids. VI Congress of the Portuguese Society of Veterinary Sciences, 3rd-5th April 2014, Oeiras, Portugal

Silva LMR, Muñoz-Caro T., Vila-Viçosa MJM, Cortes H, Gerstberger R, Taubert A, Hermosilla C. Caprine neutrophil extracellular traps induced by *Eimeria arloingi*. 5th WGs CAPARA Meeting, 2nd-4th December 2013, Berlin, Germany

Silva, LMR, Muñoz-Caro, T, Vila-Viçosa, MJM, Cortes, H, Hermosilla, C, Taubert, A. *Eimeria arloingi* sporozoites induce neutrophil extracellular traps as innate immune reaction. 2nd International Meeting on Apicomplexan Parasites in Farm Animals, 31st October-2nd November 2013, Kusadasi, Turkey, Proceedings pp 84

Muñoz-Caro T, Hermosilla C, **Silva LMR**, Cortes H, Taubert A. *Besnoitia besnoiti* tachyzoites trigger the release of bovine Neutrophil Extracellular Traps. "ApiCowplexa": Apicomplexa in farm animals, 31st October-2nd November 2013, Kusadasi, Türkei. Proceedings pp 23

Silva LMR, Vila-Viçosa MJM, Cortes H, Hermosilla C, Taubert A. Isolation of a Portuguese *Eimeria arloingi* strain and first *in vitro* culture trials. Congress of the Spanish Society of Parasitology (SOCEPA), 17th-20th September 2013, Las Palmas, Great Canaria, Spain Proceedings pp 82

Hermosilla C, Muñoz-Caro T, **Silva LMR**, Cortes H, Taubert A. Neutrophil extracellular traps as innate immune reaction against the emerging apicomplexan parasite *Besnoitia besnoiti*. 24th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), 25th-29th August 2013, Perth, Australia

Silva LMR, Vila-Viçosa M, Nunes T, Taubert A, Hermosilla C, Cortes H. *Eimeria*-infections in goat kids and mothers in Southern Portugal. Spring Meeting of the Division Parasitology and Parasitic Diseases of the German Veterinary Society: diagnostic, epidemiology and control of parasitosis, 8th-10th July 2013, Giessen, Germany, Proceedings pp 152-153

Muñoz-Caro T., Hermosilla C, **Silva LMR**, Cortes H, Taubert A. Neutrophil extracellular traps as innate effector mechanism against *Besnoitia besnoiti* tachyzoites. Spring Meeting of the Division Parasitology and Parasitic Diseases of the German Veterinary Society: diagnostic, epidemiology and control of parasitosis, 8th-10th July 2013, Giessen, Germany, Proceedings pp 43-44

Silva LMR, Vila-Viçosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE. Follow-up of *Eimeria* excretion in goats before and after parturition in two farms in South Portugal. "Apicowplexa": Apicomplexa in farm animals. 25th-28th October 2012. Lisbon, Portugal

Silva LMR, Vila-Viçosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE. Follow up of *Eimeria* excretion in goats before and after parturition in two farms in South Portugal, 4th CAPARA Meeting (Caprine Parasitology, COST Action FA0805), 19th-21st September 2012, Las Palmas de Gran Canaria, Spain

Silva LMR, Vila-Viçosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE. Parasitic diversity in goats (Serpentina breed) with respect to *Eimeria* spp. in Alentejo, Portugal. Jahrestagung der Deutschen Veterinärmedizinischen Gesellschaft, Fachgruppe Parasitologie und parasitäre Krankheiten: 2nd-4th July 2012, Hannover, Germany

Silva LMR, Vila-Viçosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE. Coccidian diversity in the domestic goat breed (Serpentina) in Alentejo, Portugal. World Buiatric Congress, 2nd-8th June 2012, Lisbon, Portugal

Silva LMR, Vila-Viçosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE. Survey of *Eimeria* in goats (Serpentina) in Alentejo, Portugal. 3rd CAPARA Meeting, 10th-12th November 2011, Limassol, Cyprus.

REFERENCES

Abi Abdallah DS, Denkers EY (2012) Neutrophils cast extracellular traps in response to protozoan parasites. Front Immunol 3:382. doi:10.3389/fimmu.2012.00382

Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE, Denkers EY (2012) *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. Infect Immun 80(2):768-77. doi:10.1128/IAI.05730-11

Adler H, Adler B, Peveri P, Werner ER, Wachter H, Peterhans E, Jungi TW (1996) Differential regulation of inducible nitric oxide synthase production in bovine and caprine macrophages. J Infect Dis 173(4):971-8.

Agyei AD, Odonkor M, Osei-Somuah A (2004) Concurrence of *Eimeria* and helminth parasitic infections in West African Dwarf kids in Ghana. Small Rumin Res 51(1):29-35. doi:16/S0921-4488(03)00184-6

Akpo Y, Kpodekon MT, Djago Y, Licois D, Youssao IA (2012) Vaccination of rabbits against coccidiosis using precocious lines of *Eimeria magna* and *Eimeria media* in Benin. Vet Parasitol 184(1):73-6. doi:10.1016/j.vetpar.2011.08.012

Alberts B, Johnson A, Lewis J, Walter P, Raff M, Roberts K (2002) Molecular Biology of the Cell 4th Edition: International Student Edition. Routledge

Alexander DL, Arastu-Kapur S, Dubremetz JF, Boothroyd JC (2006) *Plasmodium falciparum* AMA1 binds a rhoptry neck protein homologous to TgRON4, a component of the moving junction in *Toxoplasma gondii*. Eukaryot Cell 5(7):1169-73. doi:10.1128/EC.00040-06

Alexander DL, Mital J, Ward GE, Bradley P, Boothroyd JC (2005) Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. PLoS Pathog 1(2):e17. doi:10.1371/journal.ppat.0010017

Alghamdi AS, Foster DN (2005) Seminal DNase frees spermatozoa entangled in neutrophil extracellular traps. Biol Reprod 73(6):1174-81. doi:10.1095/biolreprod.105.045666

Altincicek B, Stotzel S, Wygrecka M, Preissner KT, Vilcinskas A (2008) Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. J Immunol 181(4):2705-12.

Alvarez-Garcia G, Fernandez-Garcia A, Gutierrez-Exposito D, Quiteria JA, Aguado-Martinez A, Ortega-Mora LM (2014a) Seroprevalence of *Besnoitia besnoiti* infection and associated risk factors in cattle from an endemic region in Europe. Vet J 200(2):328-31. doi:10.1016/j.tvjl.2014.02.013

Alvarez-Garcia G, Garcia-Lunar P, Gutierrez-Exposito D, Shkap V, Ortega-Mora LM (2014b) Dynamics of *Besnoitia besnoiti* infection in cattle. Parasitology 141(11):1419-35. doi:10.1017/S0031182014000729

Amit M, Cohen I, Marcovics A, Muklada H, Glasser TA, Ungar ED, Landau SY (2013) Self-medication with tannin-rich browse in goats infected with gastro-intestinal nematodes. Vet Parasitol 198(3-4):305-11. doi:10.1016/j.vetpar.2013.09.019

Aulik NA, Hellenbrand KM, Klos H, Czuprynski CJ (2010) *Mannheimia haemolytica* and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect Immun 78(11):4454-66. doi:10.1128/IAI.00840-10

Bahari P, Salehi M, Seyedabadi M, Mohammadi A (2014) Molecular identification of macroscopic and microscopic cysts of sarcocystis in sheep in north khorasan province, iran. Int J Mol Cell Med 3(1):51-6.

Baker VS, Imade GE, Molta NB, Tawde P, Pam SD, Obadofin MO, Sagay SA, Egah DZ, Iya D, Afolabi BB, Baker M, Ford K, Ford R, Roux KH, Keller TC, 3rd (2008) Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in *Plasmodium falciparum* infected children under six years of age. Malar J 7:41. doi:10.1186/1475-2875-7-41

Balicka-Ramisz A (1999) Studies on coccidiosis in goats in Poland. Vet Parasitol 81(4):347-9.

Balicka-Ramisz A, Ramisz A, Vovk S, Snitynskyj V (2012) Prevalence of coccidia infection in goats in Western Pomerania (Poland) and West Ukraine region. Ann Parasitol 58(3):167-71.

Basso W, Lesser M, Grimm F, Hilbe M, Sydler T, Trosch L, Ochs H, Braun U, Deplazes P (2013) Bovine besnoitiosis in Switzerland: imported cases and local transmission. Vet Parasitol 198(3-4):265-73. doi:10.1016/j.vetpar.2013.09.013

Behrendt JH, Clauss W, Zahner H, Hermosilla C (2004) Alternative mechanism of *Eimeria bovis* sporozoites to invade cells in vitro by breaching the plasma membrane. J Parasitol 90(5):1163-5. doi:10.1645/GE-3285RN

Behrendt JH, Ruiz A, Zahner H, Taubert A, Hermosilla C (2010) Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*. Vet Immunol Immunopathol 133(1):1-8. doi:10.1016/j.vetimm.2009.06.012

Behrendt JH, Taubert A, Zahner H, Hermosilla C (2008) Studies on synchronous egress of coccidian parasites (*Neospora caninum*, *Toxoplasma gondii*, *Eimeria bovis*) from bovine endothelial host cells mediated by calcium ionophore A23187. Vet Res Commun 32(4):325-32. doi:10.1007/s11259-007-9033-7

Blackman MJ, Bannister LH (2001) Apical organelles of Apicomplexa: biology and isolation by subcellular fractionation. Mol Biochem Parasitol 117(1):11-25.

Blake DP, Tomley FM (2014) Securing poultry production from the ever-present *Eimeria* challenge. Trends Parasitol 30(1):12-9. doi:10.1016/j.pt.2013.10.003

Bonne-Annee S, Kerepesi LA, Hess JA, Wesolowski J, Paumet F, Lok JB, Nolan TJ, Abraham D (2014) Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval *Strongyloides stercoralis*. Microbes Infect 16(6):502-11. doi:10.1016/j.micinf.2014.02.012

Brazil JC, Liu R, Sumagin R, Kolegraff KN, Nusrat A, Cummings RD, Parkos CA, Louis NA (2013) alpha3/4 Fucosyltransferase 3-dependent synthesis of Sialyl Lewis A on CD44 variant containing exon 6 mediates polymorphonuclear leukocyte detachment from intestinal epithelium during transepithelial migration. J Immunol 191(9):4804-17. doi:10.4049/jimmunol.1301307

Brinkmann V, Goosmann C, Kuhn LI, Zychlinsky A (2012) Automatic quantification of *in vitro* NET formation. Front Immunol 3:413. doi:10.3389/fimmu.2012.00413

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. Science 303(5663):1532-5. doi:10.1126/science.1092385

Brinkmann V, Zychlinsky A (2007) Beneficial suicide: why neutrophils die to make NETs. Nat Rev Microbiol 5(8):577-82. doi:10.1038/nrmicro1710

Brinkmann V, Zychlinsky A (2012) Neutrophil extracellular traps: is immunity the second function of chromatin? J Cell Biol 198(5):773-83. doi:10.1083/jcb.201203170

CAPARA (2012) CAPARA (COST Action FA0805). http://www.capara.org/ Accessed 12.7.2014

Cavailles P, Sergent V, Bisanz C, Papapietro O, Colacios C, Mas M, Subra JF, Lagrange D, Calise M, Appolinaire S, Faraut T, Druet P, Saoudi A, Bessieres MH, Pipy B, Cesbron-Delauw MF, Fournie GJ (2006) The rat Toxo1 locus directs toxoplasmosis outcome and controls parasite proliferation and spreading by macrophage-dependent mechanisms. Proc Natl Acad Sci U S A 103(3):744-9. doi:10.1073/pnas.0506643103

Chapman HD (2014) Milestones in avian coccidiosis research: a review. Poult Sci 93(3):501-11. doi:10.3382/ps.2013-03634

Chapman HD, Barta JR, Blake D, Gruber A, Jenkins M, Smith NC, Suo X, Tomley FM (2013) A selective review of advances in coccidiosis research. Adv Parasitol 83:93-171. doi:10.1016/B978-0-12-407705-8.00002-1

Chartier C, Paraud C (2012) Coccidiosis due to *Eimeria* in sheep and goats, a review. Small Rumin Res 103(1):84-92. doi:10.1016/j.smallrumres.2011.10.022

Chobotar B, Scholtyseck O (1982) Ultrastructure. In: Long P (ed) The Biology of the Coccidia. University Park Press, Baltimore, Maryland

Christensen JF (1939) Sporulation and viability of oocysts of *Eimeria arloingi* from the domestic sheep. J Agric Res 59:0527-0534.

Chuah C, Jones MK, Burke ML, Owen HC, Anthony BJ, McManus DP, Ramm GA, Gobert GN (2013) Spatial and temporal transcriptomics of *Schistosoma japonicum*-induced hepatic granuloma formation reveals novel roles for neutrophils. J Leukoc Biol 94(2):353-65. doi:10.1189/jlb.1212653

Chuammitri P, Ostojic J, Andreasen CB, Redmond SB, Lamont SJ, Palic D (2009) Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. Vet Immunol Immunopathol 129(1-2):126-31. doi:10.1016/j.vetimm.2008.12.013

Collins JE, Dubey JP, Rossow KD (1988) Hepatic coccidiosis in a calf. Vet Pathol 25(1):98-100.

Coppens I (2013) Targeting lipid biosynthesis and salvage in apicomplexan parasites for improved chemotherapies. Nat Rev Microbiol 11(12):823-35. doi:10.1038/nrmicro3139

Cortes H, Leitao A, Gottstein B, Hemphill A (2014) A review on bovine besnoitiosis: a disease with economic impact in herd health management, caused by *Besnoitia besnoiti* (Franco and Borges, 1916). Parasitology 141(11):1406-17. doi:10.1017/S0031182014000262

Cortes H, Leitao A, Vidal R, Vila-Vicosa MJ, Ferreira ML, Caeiro V, Hjerpe CA (2005) Besnoitiosis in bulls in Portugal. Vet Rec 157(9):262-4.

Cortes HC, Muller N, Boykin D, Stephens CE, Hemphill A (2011) *In vitro* effects of arylimidamides against *Besnoitia besnoiti* infection in Vero cells. Parasitology 138(5):583-92. doi:10.1017/S0031182011000114

Cortes HC, Nunes S, Reis Y, Staubli D, Vidal R, Sager H, Leitao A, Gottstein B (2006) Immunodiagnosis of *Besnoitia besnoiti* infection by ELISA and Western blot. Vet Parasitol 141(3-4):216-25. doi:10.1016/j.vetpar.2006.05.023

Cringoli G, Rinaldi L, Maurelli MP, Utzinger J (2010) FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans. Nat Protoc 5(3):503-15. doi:10.1038/nprot.2009.235

Dai YB, Lin MC, Zhang SX, Fu AQ (1991) Hepatic coccidiosis in the goat. Int J Parasitol 21(3):381-2.

Daugschies A, Najdrowski M (2005) Eimeriosis in cattle: current understanding. J Vet Med B Infect Dis Vet Public Health 52(10):417-27. doi:10.1111/j.1439-0450.2005.00894.x

de la Fuente C, Alunda JM (1992) A quantitative study of *Eimeria* infections of goats from central Spain. Vet Parasitol 41(1-2):7-15.

Dkhil MA, Delic D, Al-Quraishy S (2013) Goblet cells and mucin related gene expression in mice infected with *Eimeria papillata*. ScientificWorldJournal 2013:439865. doi:10.1155/2013/439865

Dubremetz JF, Garcia-Reguet N, Conseil V, Fourmaux MN (1998) Apical organelles and host-cell invasion by Apicomplexa. Int J Parasitol 28(7):1007-13.

Eckert J (1995) COST 89/820: biotechnology. Guidelines on techniques in coccidiosis research. Office for Official Publications of the European Communities

EFSA (2010) Bovine Besnoitiosis: An emerging disease in Europe. EFSA Journal 8(2).

Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V (2009) Mouse neutrophil extracellular traps in microbial infections. J Innate Immun 1(3):181-93. doi:10.1159/000205281

Faber JE, Kollmann D, Heise A, Bauer C, Failing K, Burger HJ, Zahner H (2002) *Eimeria* infections in cows in the periparturient phase and their calves: oocyst excretion and levels of specific serum and colostrum antibodies. Vet Parasitol 104(1):1-17.

Faizal AC, Rajapakse RP (2001) Prevalence of coccidia and gastrointestinal nematode infections in cross bred goats in the dry areas of Sri Lanka. Small Rumin Res 40(3):233-238.

FAOSTAT (2014). FAO http://faostat.fao.org/default.aspx. Accessed 19.7.2014

Fernandez-Garcia A, Risco-Castillo V, Pedraza-Diaz S, Aguado-Martinez A, Alvarez-Garcia G, Gomez-Bautista M, Collantes-Fernandez E, Ortega-Mora LM (2009) First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J Parasitol 95(2):474-6. doi:10.1645/GE-1772.1

Fitzgerald PR (1980) The economic impact of coccidiosis in domestic animals. Adv Vet Sci Comp Med 24:121-43.

Foreyt WJ (1990) Coccidiosis and cryptosporidiosis in sheep and goats. Vet Clin North Am Food Anim Pract 6(3):655-70.

Francia ME, Striepen B (2014) Cell division in apicomplexan parasites. Nat Rev Microbiol 12(2):125-36. doi:10.1038/nrmicro3184

Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 176(2):231-41. doi:10.1083/jcb.200606027

Gohil S, Herrmann S, Gunther S, Cooke BM (2013) Bovine babesiosis in the 21st century: advances in biology and functional genomics. Int J Parasitol 43(2):125-32. doi:10.1016/j.ijpara.2012.09.008

Gollnick NS, Gentile A, Schares G (2010) Diagnosis of bovine besnoitiosis in a bull born in Italy. Vet Rec 166(19):599. doi:10.1136/vr.c2314

GPP (Gabinete de Planeamento e Políticas) (2009) Animal Production Yearbook 2008/2009, Lisboa, Portugal

Gratraud P, Huws E, Falkard B, Adjalley S, Fidock DA, Berry L, Jacobs WR, Jr., Baird MS, Vial H, Kremer L (2009) Oleic acid biosynthesis in *Plasmodium falciparum*: characterization of the stearoyl-CoA desaturase and investigation as a potential therapeutic target. PLoS One 4(9):e6889. doi:10.1371/journal.pone.0006889

Guimaraes-Costa AB, DeSouza-Vieira TS, Paletta-Silva R, Freitas-Mesquita AL, Meyer-Fernandes JR, Saraiva EM (2014) 3'-nucleotidase/nuclease activity allows *Leishmania* parasites to escape killing by neutrophil extracellular traps. Infect Immun 82(4):1732-40. doi:10.1128/IAI.01232-13

Guimaraes-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceicao-Silva F, Saraiva EM (2009) *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A 106(16):6748-53. doi:10.1073/pnas.0900226106

Guimaraes-Costa AB, Nascimento MT, Wardini AB, Pinto-da-Silva LH, Saraiva EM (2012) ETosis: A Microbicidal Mechanism beyond Cell Death. J Parasitol Res 2012:929743. doi:10.1155/2012/929743

Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol 66(11):1146-54. doi:10.1016/j.humimm.2005.11.003

Hahn S, Giaglis S, Chowdhury CS, Hosli I, Hasler P (2013) Modulation of neutrophil NETosis: interplay between infectious agents and underlying host physiology. Semin Immunopathol 35(4):439-53. doi:10.1007/s00281-013-0380-x

Hammond DM, Ernst JV, Miner ML (1966) The Development of First Generation Schizonts of *Eimeria bovis*. J Protozool 13(4):559-564. doi:10.1111/j.1550-7408.1966.tb01964.x

Hammond DM, Fayer R (1968) Cultivation of *Eimeria bovis* in Three Established Cell Lines and in Bovine Tracheal Cell Line Cultures. J Parasitol 54(3):559. doi:10.2307/3277083

Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi S (2011) Changing patterns of acute phase proteins and inflammatory mediators in experimental caprine coccidiosis. Korean J Parasitol 49(3):213-9. doi:10.3347/kjp.2011.49.3.213

Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi S (2012) Experimental caprine coccidiosis caused by *Eimeria arloingi*: morphopathologic and electron microscopic studies. Vet Res Commun 36(1):47-55. doi:10.1007/s11259-011-9511-9

Hashemnia M, Khodakaram-Tafti A, Razavi SM, Nazifi S (2014) Hematological and serum biochemical analyses in experimental caprine coccidiosis. J Parasit Dis 38(1):116-23. doi:10.1007/s12639-012-0205-1

Hassum IC, Menezes Rde C (2005) Natural infection with species of the genus *Eimeria* in small ruminant raised in two municipalities of the State Rio de Janeiro. Rev Bras Parasitol Vet 14(3):95-100.

Hermosilla C, Barbisch B, Heise A, Kowalik S, Zahner H (2002) Development of *Eimeria bovis in vitro*: suitability of several bovine, human and porcine endothelial cell lines, bovine fetal gastrointestinal, Madin-Darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells. Parasitol Res 88(4):301-7.

Hermosilla C, Burger HJ, Zahner H (1999) T cell responses in calves to a primary *Eimeria bovis* infection: phenotypical and functional changes. Vet Parasitol 84(1-2):49-64.

Hermosilla C, Caro TM, Silva LM, Ruiz A, Taubert A (2014) The intriguing host innate immune response: novel anti-parasitic defence by neutrophil extracellular traps. Parasitology 141(11):1489-98. doi:10.1017/S0031182014000316

Hermosilla C, Ruiz A, Taubert A (2012) *Eimeria bovis*: an update on parasite-host cell interactions. Int J Med Microbiol 302(4-5):210-5. doi:10.1016/j.ijmm.2012.07.002

Hermosilla C, Schropfer E, Stowasser M, Eckstein-Ludwig U, Behrendt JH, Zahner H (2008) Cytoskeletal changes in *Eimeria bovis*-infected host endothelial cells during first merogony. Vet Res Commun 32(7):521-31. doi:10.1007/s11259-008-9054-x

Hermosilla C, Zahner H, Taubert A (2006) *Eimeria bovis* modulates adhesion molecule gene transcription in and PMN adhesion to infected bovine endothelial cells. Int J Parasitol 36(4):423-31. doi:10.1016/j.ijpara.2006.01.001

Hornok S, Fedak A, Baska F, Hofmann-Lehmann R, Basso W (2014) Bovine besnoitiosis emerging in Central-Eastern Europe, Hungary. Parasit Vectors 7:20. doi:10.1186/1756-3305-7-20

Hosseinzadeh A, Messer PK, Urban CF (2012) Stable Redox-Cycling Nitroxide Tempol Inhibits NET Formation. Front Immunol 3:391. doi:10.3389/fimmu.2012.00391

Hoste H, Torres-Acosta JF, Aguilar-Caballero AJ (2008) Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? Parasite Immunol 30(2):79-88. doi:10.1111/j.1365-3024.2007.00987.x

Iqbal A, Tariq KA, Wazir VS, Singh R (2013) Antiparasitic efficacy of Artemisia absinthium, toltrazuril and amprolium against intestinal coccidiosis in goats. J Parasit Dis 37(1):88-93. doi:10.1007/s12639-012-0137-9

Jackson AR (1964) The Isolation of Viable Coccidial Sporozoites. Parasitology 54:87-93.

Jackson ARB (1962) Excystation of *Eimeria Arloingi* (Marotel, 1905): Stimuli from the Host Sheep. Nature 194(4831):847-849. doi:10.1038/194847a0

Jacquiet P, Boucher C, Liénard E, Grisez C, Prévot F, Salem A, Beuscart T, Bergeaud J-P, Alzieu J-P (2009) Besnoitiose bovine : enquêtes épidémiologiques dans le sud de la France. Journées Nationales G. T. V., Nantes, 1213-1217.

Jalila A, Dorny P, Sani R, Salim NB, Vercruysse J (1998) Coccidial infections of goats in Selangor, peninsular Malaysia. Vet Parasitol 74(2-4):165-72.

Jenkins MC (2001) Advances and prospects for subunit vaccines against protozoa of veterinary importance. Vet Parasitol 101(3-4):291-310.

Jenne CN, Wong CH, Zemp FJ, McDonald B, Rahman MM, Forsyth PA, McFadden G, Kubes P (2013) Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. Cell Host Microbe 13(2):169-80. doi:10.1016/j.chom.2013.01.005

Katris NJ, van Dooren GG, McMillan PJ, Hanssen E, Tilley L, Waller RF (2014) The apical complex provides a regulated gateway for secretion of invasion factors in *Toxoplasma*. PLoS Pathog 10(4):e1004074. doi:10.1371/journal.ppat.1004074

Kawazoe U, Bordin EL, de Lima CA, Dias LA (2005) Characterisation and histopathological observations of a selected Brazilian precocious line of *Eimeria acervulina*. Vet Parasitol 131(1-2):5-14. doi:10.1016/j.vetpar.2005.03.038

Kheirandish R, Nourollahi-Fard SR, Yadegari Z (2014) Prevalence and pathology of coccidiosis in goats in southeastern Iran. J Parasit Dis 38(1):27-31. doi:10.1007/s12639-012-0186-0

Khodakaram-Tafti A, Hashemnia M, Razavi SM, Sharifiyazdi H, Nazifi S (2013) Genetic characterization and phylogenetic analysis of *Eimeria arloingi* in Iranian native kids. Parasitol Res 112(9):3187-92. doi:10.1007/s00436-013-3494-0

Kommuru DS, Barker T, Desai S, Burke JM, Ramsay A, Mueller-Harvey I, Miller JE, Mosjidis JA, Kamisetti N, Terrill TH (2014) Use of pelleted sericea lespedeza (*Lespedeza cuneata*) for natural control of coccidia and gastrointestinal nematodes in weaned goats. Vet Parasitol. doi:10.1016/j.vetpar.2014.04.017

Koudela B, Bokova A (1998) Coccidiosis in goats in the Czech Republic. Vet Parasitol 76(4):261-7.

Lang M, Kann M, Zahner H, Taubert A, Hermosilla C (2009) Inhibition of host cell apoptosis by *Eimeria bovis* sporozoites. Vet Parasitol 160(1-2):25-33. doi:10.1016/j.vetpar.2008.10.100

Levine ND (1985) Veterinary Protozoology. Iowa State University Press, Ames, IA

Li Y, Liu Z, Yang J, Chen Z, Guan G, Niu Q, Zhang X, Luo J, Yin H (2014) Infection of small ruminants and their red blood cells with *Theileria annulata* schizonts. Exp Parasitol 137:21-4. doi:10.1016/j.exppara.2013.11.010

Lima JD (2004) Coccidiose dos ruminantes domésticos. Rev Bras ParasitolVet 13(suplemento 1):9-13.

Long PL, Millard BJ, Batty AF, da Vison C (1982) Immunisation against coccidiosis in chickens: tests under simulated field conditions. Avian Pathol 11(1):131-44. doi:10.1080/03079458208436088

Lutz K, Schmitt S, Linder M, Hermosilla C, Zahner H, Taubert A (2011) *Eimeria bovis*-induced modulation of the host cell proteome at the meront I stage. Mol Biochem Parasitol 175(1):1-9. doi:10.1016/j.molbiopara.2010.08.003

Mai J, Virtue A, Shen J, Wang H, Yang XF (2013) An evolving new paradigm: endothelial cells-conditional innate immune cells. J Hematol Oncol 6:61. doi:10.1186/1756-8722-6-61

Mai K, Sharman PA, Walker RA, Katrib M, De Souza D, McConville MJ, Wallach MG, Belli SI, Ferguson DJ, Smith NC (2009) Oocyst wall formation and composition in coccidian parasites. Mem Inst Oswaldo Cruz 104(2):281-9.

Markus MB (2011) The hypnozoite concept, with particular reference to malaria. Parasitol Res 108(1):247-52. doi:10.1007/s00436-010-2072-y

McDonald V, Shirley MW (2009) Past and future: vaccination against *Eimeria*. Parasitology 136(12):1477-89. doi:10.1017/S0031182009006349

Mehlhorn H, Armstrong PM (2001) Encyclopedic Reference of Parasitology: Biology, Structure, Function. Springer

Mehlhorn H, Klimpel S, Schein E, Heydorn AO, Al-Quraishy S, Selmair J (2009) Another African disease in Central Europa: Besnoitiosis of cattle. I. Light and electron microscopical study. Parasitol Res 104(4):861-8. doi:10.1007/s00436-008-1267-y

Mercier C, Adjogble KD, Daubener W, Delauw MF (2005) Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? Int J Parasitol 35(8):829-49. doi:10.1016/j.ijpara.2005.03.011

Mi-Ichi F, Kano S, Mitamura T (2007) Oleic acid is indispensable for intraerythrocytic proliferation of *Plasmodium falciparum*. Parasitology 134(Pt 12):1671-7. doi:10.1017/S0031182007003137

Monney T, Hemphill A (2014) Vaccines against neosporosis: what can we learn from the past studies? Exp Parasitol 140:52-70. doi:10.1016/j.exppara.2014.02.015

Morgan KJ, Alley MR, Pomroy WE, Gartrell BD, Castro I, Howe L (2013) Extra-intestinal coccidiosis in the kiwi (*Apteryx* spp.). Avian Pathol 42(2):137-46. doi:10.1080/03079457.2013.776665

Morrissette NS, Sibley LD (2002) Cytoskeleton of apicomplexan parasites. Microbiol Mol Biol Rev 66(1):21-38; table of contents.

Morshed M, Hlushchuk R, Simon D, Walls AF, Obata-Ninomiya K, Karasuyama H, Djonov V, Eggel A, Kaufmann T, Simon HU, Yousefi S (2014) NADPH oxidase-independent formation of extracellular DNA traps by basophils. J Immunol 192(11):5314-23. doi:10.4049/jimmunol.1303418

Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V, Rodriguez A (2001) Migration of *Plasmodium* sporozoites through cells before infection. Science 291(5501):141-4. doi:10.1126/science.291.5501.141

Mota MM, Rodriguez A (2001) Migration through host cells by apicomplexan parasites. Microbes Infect 3(13):1123-8.

Mota MM, Rodriguez A (2002) Invasion of mammalian host cells by *Plasmodium* sporozoites. Bioessays 24(2):149-56. doi:10.1002/bies.10050

Muller J, Hemphill A (2013) *In vitro* culture systems for the study of apicomplexan parasites in farm animals. Int J Parasitol 43(2):115-24. doi:10.1016/j.ijpara.2012.08.004

Muniz VS, Baptista-Dos-Reis R, Neves JS (2013) Functional extracellular eosinophil granules: a bomb caught in a trap. Int Arch Allergy Immunol 162(4):276-82. doi:10.1159/000354934

Muñoz-Caro T, Hermosilla C, Silva LMR, Cortes H, Taubert A (2014) Neutrophil Extracellular Traps as Innate Immune Reaction against the Emerging Apicomplexan Parasite *Besnoitia besnoiti*. PLoS One 9(3):e91415. doi:10.1371/journal.pone.0091415

Nathan C (2006) Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 6(3):173-82. doi:10.1038/nri1785

Ng TH, Chang SH, Wu MH, Wang HC (2013) Shrimp hemocytes release extracellular traps that kill bacteria. Dev Comp Immunol 41(4):644-51. doi:10.1016/j.dci.2013.06.014

Norton CC (1986) Coccidia of the domestic goat *Capra hircus*, with notes on *Eimeria ovinoidalis* and *E. bakuensis* (syn. *E. ovina*) from the sheep *Ovis aries*. Parasitology 92 (Pt 2):279-89.

Nosal P, Kowalska D, Bielanski P, Kowal J, Kornas S (2014) Herbal formulations as feed additives in the course of rabbit subclinical coccidiosis. Ann Parasitol 60(1):65-9.

O'Callaghan MG (1989) Coccidia of domestic and feral goats in South Australia. Vet Parasitol 30(4):267-72.

Oltjen JW, Beckett JL (1996) Role of ruminant livestock in sustainable agricultural systems. J Anim Sci 74(6):1406-9.

Palic D, Ostojic J, Andreasen CB, Roth JA (2007) Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol 31(8):805-16. doi:10.1016/j.dci.2006.11.010

Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191(3):677-91. doi:10.1083/jcb.201006052

Penzhorn BL, Rognlie MC, Hall LL, Knapp SE (1994) Enteric coccidia of Cashmere goats in southwestern Montana, USA. Vet Parasitol 55(1-2):137-42.

Poplstein M, Vrba V (2011) Description of the two strains of turkey coccidia *Eimeria adenoeides* with remarkable morphological variability. Parasitology 138(10):1211-6. doi:10.1017/S0031182011001090

Preuss J, Maloney P, Peddibhotla S, Hedrick MP, Hershberger P, Gosalia P, Milewski M, Li YL, Sugarman E, Hood B, Suyama E, Nguyen K, Vasile S, Sergienko E, Mangravita-Novo A, Vicchiarelli M, McAnally D, Smith LH, Roth GP, Diwan J, ChungTD, Jortzik E, Rahlfs S, Becker K, Pinkerton AB, Bode L. (2012) Discovery of a *Plasmodium falciparum* glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase inhibitor (R,Z)-N-((1-ethylpyrrolidin-2-yl)methyl)-2-(2-fluorobenzylidene)-3-oxo-3,4-dihydr o-2H-benzo[b][1,4]thiazine-6-carboxamide (ML276) that reduces parasite growth in vitro. J Med Chem 55:7262-7272. doi:10.1021/jm300833h

Rakhshandehroo E, Razavi SM, Nazifi S, Farzaneh M, Mobarraei N (2013) Dynamics of the enzymatic antioxidants during experimental caprine coccidiosis. Parasitol Res 112(4):1437-41. doi:10.1007/s00436-013-3273-y

Ravindran S, Boothroyd JC (2008) Secretion of proteins into host cells by Apicomplexan parasites. Traffic 9(5):647-56. doi:10.1111/j.1600-0854.2008.00723.x

Razavi SM, Hashemnia M, Khodakaram-Tafti A (2014) *Eimeria arloingi*: further studies on the development of some endogenous stages. Exp Parasitol 140:12-7. doi:10.1016/j.exppara.2014.03.011

Reichel MP, Mc AM, Pomroy WE, Campero C, Ortega-Mora LM, Ellis JT (2014) Control options for *Neospora caninum* - is there anything new or are we going backwards? Parasitology 141(11):1455-70. doi:10.1017/S0031182014000158

Rick B, Dubremetz JF, Entzeroth R (1998) A merozoite-specific 22-kDa rhoptry protein of the coccidium *Eimeria nieschulzi* (Sporozoa, Coccidia) is exocytosed in the parasitophorous vacuole upon host cell invasion. Parasitol Res 84(4):291-6.

Rinaldi L, Maurelli MP, Musella V, Bosco A, Cortes H, Cringoli G (2013) First cross-sectional serological survey on *Besnoitia besnoiti* in cattle in Italy. Parasitol Res 112(4):1805-7. doi:10.1007/s00436-012-3241-y

Riyapa D, Buddhisa S, Korbsrisate S, Cuccui J, Wren BW, Stevens MP, Ato M, Lertmemongkolchai G (2012) Neutrophil extracellular traps exhibit antibacterial activity against burkholderia pseudomallei and are influenced by bacterial and host factors. Infect Immun 80(11):3921-9. doi:10.1128/IAI.00806-12

Rose ME (1987) Immunity to *Eimeria* infections. Vet Immunol Immunopathol 17(1-4):333-43.

Rose ME, Hesketh P (1979) Immunity to coccidiosis: T-lymphocyte- or B-lymphocyte-deficient animals. Infect Immun 26(2):630-7.

Rose ME, Hesketh P, Wakelin D (1992) Immune Control of Murine Coccidiosis - Cd4+ and Cd8+ Lymphocytes-T Contribute Differentially in Resistance to Primary and Secondary Infections. Parasitology 105:349-354.

Roxstrom-Lindquist K, Palm D, Reiner D, Ringqvist E, Svard SG (2006) Giardia immunity-an update. Trends Parasitol 22(1):26-31. doi:10.1016/j.pt.2005.11.005

Ruiz A, Behrendt JH, Zahner H, Hermosilla C, Perez D, Matos L, Munoz Mdel C, Molina JM, Taubert A (2010) Development of *Eimeria ninakohlyakimovae* in vitro in primary and permanent cell lines. Vet Parasitol 173(1-2):2-10. doi:10.1016/j.vetpar.2010.05.023

Ruiz A, Gonzalez JF, Rodriguez E, Martin S, Hernandez YI, Almeida R, Molina JM (2006) Influence of climatic and management factors on *Eimeria* infections in goats from semi-arid zones. J Vet Med B Infect Dis Vet Public Health 53(8):399-402. doi:10.1111/j.1439-0450.2006.00985.x

Ruiz A, Guedes AC, Munoz MC, Molina JM, Hermosilla C, Martin S, Hernandez YI, Hernandez A, Perez D, Matos L, Lopez AM, Taubert A (2012) Control strategies using diclazuril against coccidiosis in goat kids. Parasitol Res 110(6):2131-6. doi:10.1007/s00436-011-2746-0

Ruiz A, Matos L, Munoz MC, Hermosilla C, Molina JM, Andrada M, Rodriguez F, Perez D, Lopez A, Guedes A, Taubert A (2013) Isolation of an *Eimeria ninakohlyakimovae* field strain (Canary Islands) and analysis of its infection characteristics in goat kids. Res Vet Sci 94(2):277-84. doi:10.1016/j.rvsc.2012.08.003

Ruiz A, Munoz MC, Molina JM, Hermosilla C, Andrada M, Lara P, Bordon E, Perez D, Lopez AM, Matos L, Guedes AC, Falcon S, Falcon Y, Martin S, Taubert A (2014) Immunization with *Eimeria ninakohlyakimovae*-live attenuated oocysts protect goat kids from clinical coccidiosis. Vet Parasitol 199(1-2):8-17. doi:10.1016/j.vetpar.2013.09.032

Sahoo G, More T, Singh VK (2000) Evaluation of oxygen-dependent immunodefences of the polymorphonuclear cells of some tropical ruminants. Vet Res Commun 24(8):505-15.

Salama AA, Terkawi MA, Kawai S, Aboulaila M, Nayel M, Mousa A, Zaghawa A, Yokoyama N, Igarashi I (2013) Specific antibody to a conserved region of *Babesia* apical membrane antigen-1 inhibited the invasion of *B. bovis* into the erythrocyte. Exp Parasitol 135(3):623-8. doi:10.1016/j.exppara.2013.09.017

Sanz S, Bandini G, Ospina D, Bernabeu M, Marino K, Fernandez-Becerra C, Izquierdo L (2013) Biosynthesis of GDP-fucose and other sugar nucleotides in the blood stages of *Plasmodium falciparum*. J Biol Chem 288(23):16506-17. doi:10.1074/jbc.M112.439828

Sayin F, Dincer S, Milli U (1980) The life cycle and pathogenicity of *Eimeria arloingi* (Marotel, 1905) Martin, 1909, in Angora kids and an attempt at its transmission to lambs. Zentralbl Veterinarmed B 27(5):382-397.

Sazmand A, Hamidinejat H, Hekmatimoghaddam S, Asadollahi Z, Mirabdollahi S (2012) *Eimeria* infection in camels (Camelus dromedarius) in Yazd province, central Iran. Trop Biomed 29(1):77-80.

Schares G, Basso W, Majzoub M, Cortes HC, Rostaher A, Selmair J, Hermanns W, Conraths FJ, Gollnick NS (2009) First in vitro isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet Parasitol 163(4):315-22. doi:10.1016/j.vetpar.2009.04.033

Schito ML, Barta JR (1997) Nonspecific immune responses and mechanisms of resistance to *Eimeria papillata* infections in mice. Infect Immun 65(8):3165-70.

Schwarz L, Worliczek HL, Winkler M, Joachim A (2014) Superinfection of sows with *Cystoisospora suis* ante partum leads to a milder course of cystoisosporosis in suckling piglets. Vet Parasitol. doi:10.1016/j.vetpar.2014.04.026

Seper A, Hosseinzadeh A, Gorkiewicz G, Lichtenegger S, Roier S, Leitner DR, Rohm M, Grutsch A, Reidl J, Urban CF, Schild S (2013) *Vibrio cholerae* evades neutrophil extracellular traps by the activity of two extracellular nucleases. PLoS Pathog 9(9):e1003614. doi:10.1371/journal.ppat.1003614

Shi MQ, Hirzmann J, Dafa'alla TH, Zahner H (2001) *In vivo* expression profiles of cytokine and iNOS mRNAs in rats infected with *Eimeria separata*. Vet Parasitol 97(2):131-40.

Shi MQ, Huther S, Burkhardt E, Zahner H (2000) Immunity in rats against *Eimeria separata*: oocyst excretion, effects on endogenous stages and local tissue response after primary and challenge infections. Parasitol Res 86(11):891-8.

Silva LMR, Muñoz Caro T, Gerstberger R, Vila-Vicosa MJ, Cortes HC, Hermosilla C, Taubert A (2014a) The apicomplexan parasite *Eimeria arloingi* induces caprine neutrophil extracellular traps. Parasitol Res 113(8):2797-807. doi:10.1007/s00436-014-3939-0

Silva LMR, Vila-Vicosa MJ, Nunes T, Taubert A, Hermosilla C, Cortes HC (2014b) *Eimeria* infections in goats in Southern Portugal. Rev Bras Parasitol Vet 23(2):280-6. doi:10.1590/S1984-29612014051

Silva LMR, Vila-Viçosa MJM, Maurelli MP, Morgoglione ME, Cortes HCE, Cringoli G, Rinaldi L (2013) Mini-FLOTAC for the diagnosis of *Eimeria* infection in goats: An alternative to McMaster. Small Rumin Res 114(2-3):280-283. doi:10.1016/j.smallrumres.2013.06.017

Silva LMR, Vila-Vicosa MJM, Nunes T, Taubert A, Hermosilla C, Cortes HCE (2012) *Eimeria* excretion in goats: peri-parturients and kids Apicomplexa in farm animals International meeting. Lisbon, p 82

Silva RM, Facury-Filho EJ, Souza MF, Ribeiro MF (2011) Natural infection by *Eimeria* spp. in a cohort of lambs raised extensively in Northeast Brazil. Rev Bras Parasitol Vet 20(2):134-9.

Slapeta J (2013) Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int J Parasitol 43(12-13):957-70. doi:10.1016/j.ijpara.2013.07.005

Smith MC, Sherman DM (2009) Goat Medicine. Wiley

Soe AK, Pomroy WE (1992) New species of *Eimeria* (Apicomplexa: Eimeriidae) from the domesticated goat *Capra hircus* in New Zealand. Syst Parasitol 23:195-202.

Souza W (2006) Secretory organelles of pathogenic protozoa. An Acad Bras Cienc 78(2):271-91. doi:/S0001-37652006000200008

Speer CA, Reduker DW, Burgess DE, Whitmire WM, Splitter GA (1985) Lymphokine-induced inhibition of growth of *Eimeria bovis* and *Eimeria papillata* (Apicomplexa) in cultured bovine monocytes. Infect Immun 50(2):566-71.

Striepen B, Jordan CN, Reiff S, van Dooren GG (2007) Building the perfect parasite: cell division in apicomplexa. PLoS Pathog 3(6):e78. doi:10.1371/journal.ppat.0030078

Suhwold A, Hermosilla C, Seeger T, Zahner H, Taubert A (2010) T cell reactions of *Eimeria bovis* primary and challenge-infected calves. Parasitol Res 106(3):595-605. doi:10.1007/s00436-009-1705-5

Sumagin R, Robin AZ, Nusrat A, Parkos CA (2014) Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment. Mucosal Immunol 7(4):905-15. doi:10.1038/mi.2013.106

Svensson C, Olofsson H, Uggla A (1996) Immunisation of calves against *Eimeria alabamensis* coccidiosis. Appl Parasitol 37(3):209-16.

Szabady RL, McCormick BA (2013) Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products. Front Immunol 4:220. doi:10.3389/fimmu.2013.00220

Taubert A, Hermosilla C, Suhwold A, Zahner H (2008) Antigen-induced cytokine production in lymphocytes of *Eimeria bovis* primary and challenge infected calves. Vet Immunol Immunopathol 126(3-4):309-20. doi:10.1016/j.vetimm.2008.09.003

Taubert A, Wimmers K, Ponsuksili S, Jimenez CA, Zahner H, Hermosilla C (2010) Microarray-based transcriptional profiling of *Eimeria bovis*-infected bovine endothelial host cells. Vet Res 41(5):70. doi:10.1051/vetres/2010041

Taubert A, Zahner H, Hermosilla C (2006) Dynamics of transcription of immunomodulatory genes in endothelial cells infected with different coccidian parasites. Vet Parasitol 142(3-4):214-22. doi:10.1016/j.vetpar.2006.07.021

Taylor MA, Catchpole J (1994) Review article: coccidiosis of domestic ruminants. Appl Parasitol 35(2):73-86.

Taylor MA, Coop RL, Wall R (2007) Veterinary parasitology, 3rd edn. Blackwell, Oxford, UK ; Ames, Iowa

ThePoultrySite (2013) The Poultry Site - High Cost of Coccidiosis in Broilers. http://www.thepoultrysite.com/poultrynews/28036/high-cost-of-coccidiosis-in-broilers Accessed 10.8.2014

Thrall MA, Weiser G, Allison R, Campbell TW (2012) Veterinary Hematology and Clinical Chemistry. Wiley

Tierney JB, Matthews E, Carrington SD, Mulcahy G (2007) Interaction of *Eimeria tenella* with intestinal mucin in vitro. J Parasitol 93(3):634-8. doi:10.1645/GE-1066R.1

Todar K (2006) Todar's Online Textbook of Bacteriology. University of Wisconsin-Madison Department of Bacteriology

Tomley F (1994a) Antigenic diversity of the asexual developmental stages of *Eimeria tenella*. Parasite Immunol 16(8):407-13.

Tomley FM (1994b) Characterization of rhoptry proteins of *Eimeria tenella* sporozoites: antigenic diversity of rhoptry epitopes within species of the genus *Eimeria* and among three asexual generations of a single species, *E. tenella*. Infect Immun 62(10):4656-8.

Tschopp J, Martinon F, Burns K (2003) NALPs: a novel protein family involved in inflammation. Nat Rev Mol Cell Biol 4(2):95-104. doi:10.1038/nrm1019

Urban CF, Reichard U, Brinkmann V, Zychlinsky A (2006) Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell Microbiol 8(4):668-76. doi:10.1111/j.1462-5822.2005.00659.x

Vadlejch J, Petrtyl M, Zaichenko I, Cadkova Z, Jankovska I, Langrova I, Moravec M (2011) Which McMaster egg counting technique is the most reliable? Parasitol Res 109(5):1387-94. doi:10.1007/s00436-011-2385-5

van Schalkwyk DA, Priebe W, Saliba KJ (2008) The inhibitory effect of 2-halo derivatives of D-glucose on glycolysis and on the proliferation of the human malaria parasite *Plasmodium falciparum*. J Pharmacol Exp Ther 327(2):511-7. doi:10.1124/jpet.108.141929

von Kockritz-Blickwede M, Nizet V (2009) Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. J Mol Med (Berl) 87(8):775-83. doi:10.1007/s00109-009-0481-0

Waap H, Nunes T, Cortes H, Leitao A, Vaz Y (2014) Prevalence and geographic distribution of *Besnoitia besnoiti* infection in cattle herds in Portugal. Parasitol Res. doi:10.1007/s00436-014-4035-1

Wagner JG, Roth RA (2000) Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. Pharmacol Rev 52(3):349-74.

Wardini AB, Guimaraes-Costa AB, Nascimento MT, Nadaes NR, Danelli MG, Mazur C, Benjamim CF, Saraiva EM, Pinto-da-Silva LH (2010) Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. J Gen Virol 91(Pt 1):259-64. doi:10.1099/vir.0.014613-0

Wiedmer S, Stange J, Kurth T, Bleiss W, Entzeroth R, Kurth M (2011) New insights into the excystation process and oocyst morphology of rodent *Eimeria* species. Protist 162(4):668-78. doi:10.1016/j.protis.2011.03.001

Witcombe DM, Smith NC (2014) Strategies for anti-coccidial prophylaxis. Parasitology 141(11):1379-89. doi:10.1017/S0031182014000195

Young G, Alley ML, Foster DM, Smith GW (2011) Efficacy of amprolium for the treatment of pathogenic *Eimeria* species in Boer goat kids. Vet Parasitol 178(3-4):346-9. doi:10.1016/j.vetpar.2011.01.028

Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E, Schmid I, Straumann A, Reichenbach J, Gleich GJ, Simon HU (2008) Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med 14(9):949-53. doi:10.1038/nm.1855

Yvoré P (1989) Coccidia and Intestinal Coccidiomorphs: Proceedings of the Vth International Coccidiosis Conference, Tours (France), 17-20 October 1989. INRA

Yvoré P, Esnault, A., Naciri, M. (1985) La coccidiose caprine. Effets de contaminations mono ou multispécifiques. Rec Méd Vét 161:347-351.

Zhang J, Rivest S (2001) Anti-inflammatory effects of prostaglandin E2 in the central nervous system in response to brain injury and circulating lipopolysaccharide. J Neurochem 76(3):855-64.

Zhang Y, Wang L, Ruan W, Zhang J, Yao P, Zhou S, An J (2014) Immunization with recombinant 3-1E protein in AbISCO(R)-300 adjuvant induced protective immunity against *Eimeria acervulina* infection in chickens. Exp Parasitol 141:75-81. doi:10.1016/j.exppara.2014.03.001

Zlotnik A, Yoshie O (2000) Chemokines: a new classification system and their role in immunity. Immunity 12(2):121-7.

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SUPPLEMENTARY DATA

Movie 1. *Eimeria arloingi* excystation. During excystation, *E. arloingi* sporozoites after activation become extremely active and egress through the micropyle.

Link:
https://www.youtube.com/watch?v=KLEP0-nKP2k&list=PLlxiM1Cz1_QkwjdudZWt_sf17IcC9b4R3&index=2

Movie 2. *Eimeria arloingi* merozoites I release. Rupture of mature macromeront and release of viable merozoites I at 22 days p. i..

Link: https://www.youtube.com/watch?v=9EHnqg1JWFg&list=PLlxiM1Cz1_QkwjdudZWt_sf17IcC9b4R3

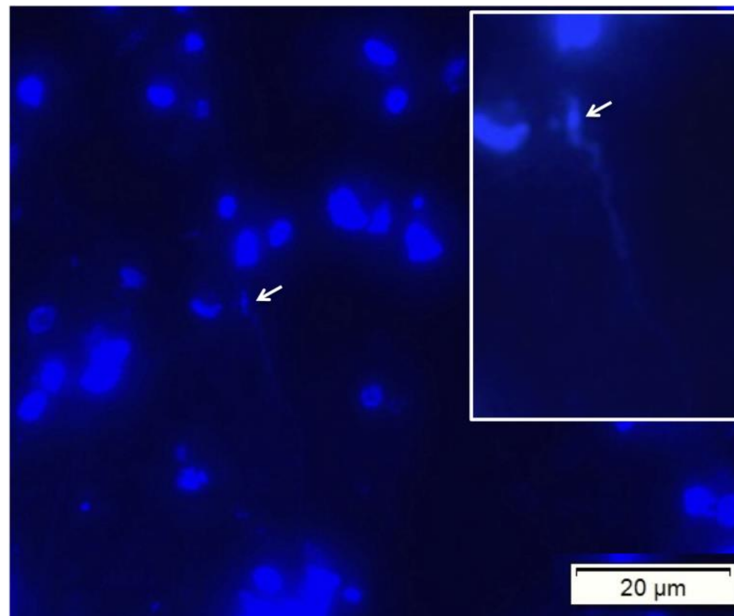

FIG. 4. First *in vivo* evidence of NETs in histological intestinal tissue slides of an *Eimeria arloingi*-infected goat kid

A single *Eimeria arloingi*-merozoite (arrow) is shown to be firmly entrapped within *in vivo* NETs in histological intestinal tissue slides. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye (blue).