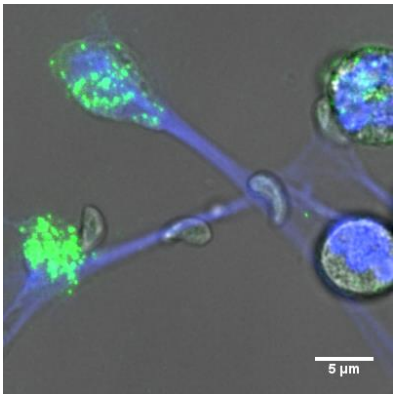
Ershun Zhou

Characterization of NETosis-derived effector mechanisms against *Besnoitia besnoiti* in cattle



INAGURAL DISSERTATION
zur Erlangung des Grades eines
Dr. med. vet.
beim Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

Aus dem Institut für Parasitologie, Fachbereich Veterinärmedizin,
Justus-Liebig-Universität, Giessen
Prof. Dr. Carlos Hermosilla

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Eingereicht von
Ershun Zhou
Tierarzt aus China

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LIST OF PUBLICATIONS AND CONFERNCE CONTRIBUTIONS

Original papers

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Conejeros, I., Velásquez, Z. D., Grob, D., **Zhou, E.**, Salecker, H., Hermosilla, C., Taubert, A. (2019). Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation. (submitted manuscript).

Zhou, E., Conejeros, I., Gärtner, U., Mazurek, S., Hermosilla, C., Taubert, A. (2019). Metabolic requirements of *Besnoitia besnoiti* tachyzoite-triggered NETosis. (submitted manuscript).

Zhou, E., Silva, L.M.R., Conejeros, I., Velásquez, Z., Hirz, M., Gärtner, U., Jacquiet, P., Taubert, A., Hermosilla, C. (2019). *Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis and seeming to correlate with autophagy. (submitted manuscript).

Zhou, E., Conejeros, I., Zhang, X., Yang, Z., Taubert, A., Hermosilla, C. (2019). Investigations of molecular mechanism on *Neospora caninum* tachyzoites-induced NETosis. (manuscript in submission process).

Muñoz-Caro, T., Conejeros, I., **Zhou, E.**, Pikhovych, A., Gärtner, U., Hermosilla, C., & Taubert, A. (2018). *Dirofilaria immitis* Microfilariae and Third-stage larvae induce canine NETosis resulting in Different Types of neutrophil extracellular Traps. *Front Immunol*, 9.

Conference contributions

1. **Ershun Zhou**, Ivan Conejeros, Anja Taubert, Carlos Hermosilla. (2017) Investigations on molecular mechanisms involved in *Besnoitia besnoiti*-mediated NETosis. 10th Conference on Life Sciences of the International Giessen Graduate Center for Life Sciences (GGL), 27-28 September 2017, Giessen, Germany

2. **Ershun Zhou**, Ivan Conejeros, Anja Taubert, Carlos Hermosilla. (2018) Analysis of molecular mechanisms involved in *Besnoitia besnoiti*-mediated NETosis. 28th Annual Meeting of the German Society for Parasitology (DGP), 21–24 March 2018, Berlin, Germany. VPA-P-06

3. **Ershun Zhou**, Ivan Conejeros, Zahady Velásquez, Anja Taubert, Carlos Hermosilla. (2018) Investigations on the role of autophagy in *Besnoitia besnoiti*-mediated bovine NETosis. Meeting of the Parasitology Section of the German Veterinary Medical Society (DVG), 02-04 July 2018, Giessen, Germany

4. **Ershun Zhou**, Iván Conejeros, Zahady Velásquez, Anja Taubert, Carlos Hermosilla. (2018) Investigations on the role of autophagy in *Besnoitia besnoiti*-mediated bovine NETosis. 11th Conference on Life Sciences of the International Giessen Graduate Center for Life Sciences (GGL), 19-20 September 2018, Giessen, Germany

5. **Ershun Zhou**, Iván Conejeros, Sybille Mazurek, Anja Taubert, Carlos Hermosilla. (2019) Metabolic requirements of *Besnoitia besnoiti* tachyzoite-triggered NETosis. Meeting of the Parasitology Section of the German Veterinary Medical Society (DVG), 17-19 June 2019, Leipzig, Germany

ABBREVIATIONS

EFSA - European Food Safety Authority

PVM - Parasitophorous vacuole membrane

PCR - Polymerase chain reaction

PAMPs - Pathogen associated molecular patterns

DAMPs - Danger associated molecular patterns

PMN - Polymorphonuclear neutrophils

NETs - Neutrophil extracellular traps

MPO - Myeloperoxidase

NE - Neutrophil elastase

DCs - Dendritic cells

MHC - Major histocompatiblity complex

NK cells - Natural killer cells

NOX - NADPH oxidase

ATP - Adenosine triphosphate

TCA - Tricarboxylic acid

TLRs - Toll-like receptors

CLRs - C-type lectin receptors

NLRs - NOD-like receptors

RLRs - RIG-I-like receptors

mTOR - Mechanistic target of rapamycin

SEM - Scanning electron microscopy

MCT1 - Monocarboxylate transporter 1

BUVEC - Bovine umbilical vein endothelial cells

FDG - 2-Fluoro-2-deoxy-D-glucose

DON - 6-Diazo-5-oxo-L-norleucine

DCA - Dichloroacetic acid

OT - Oxythiamine

OXA - Oxamate

2-DOG - 2-Deoxy-D-glucose

NAD^+ - Nicotinamide adenine dinucleotide

TABLE OF CONTENTS

1. INTRODUCTION

A parasite is an organism living on and/or within another organism for gaining access to nutrients and energy from parasitized hosts in order to fulfill its lifecycle. Impacts of ecto- and endoparasites on their hosts range from miniscule to lethal effects, and known to be an important driving force in shaping host populations in evolution (Minchella and Scott, 1991). Farther, parasite species which can cause disease in humans, domestic animals, wildlife and invertebrates, are mainly classified into three systematic groups [i. e. protozoa, helminths (nematodes, cestodes, trematodes, acanthocephalans, pentastomids) and arthropods (mites, crustaceans, and insects)] and worldwide distributed in terrestrial as well as marine ecosystems (Hermosilla et al., 2015; Kleinertz et al., 2014).

Many protozoan parasites are of veterinary importance, and protozoan infections in terrestrial and marine mammals are responsible not only for causing significant losses of animal production due to severe morbidity and mortality but also for having impact on wildlife animal health population (Hermosilla et al., 2018; Patra et al., 2017; Villagra-Blanco et al., 2019). Parasitic protozoa are unicellular, eukaryotic, microorganisms which possess typical cellular structures (e. g. nucleus, mitochondrium, Golgi) and some other specialized subcellular organelles such as a flagellum and/or cilia which can lead to independent motility (Chávez-Munguía et al., 2007; Kaur; Peck, 1977; Santoro et al., 2007; Weise et al., 2000). Protozoa are among the most common parasitic organisms distributed worldwide present in terrestrial as well as aquatic environments, and are getting increasing attention as human and animal pathogens, or potential vehicles (Shanan et al., 2015).

Based on the mode of locomotion, protozoa are mainly divided into four subphyla: Sarcodina (amoebae), Ciliophora (ciliates), Zoomastigophora (flagellates) and the Apicomplexa (sporozoa) (Imam, 2009). The Apicomplexa are spore-forming unicellular parasites, and are characterized by lacking obvious locomotory structures and presence of an apical complex in their infective life cycle stages (Siński and Behnke, 2004). The apicomplexan subphylum contains a large group of obligate intracellular protozoan organisms with more than 6000 named and probably more than one million unnamed species (Seeber and Steinfelder, 2016), many of which are of significant medical and economic importance. There are numerous apicomplexan genera highly pathogenic to their hosts causing severe diseases, such as *Plasmodium*, *Babesia*, *Cryptosporidium*, *Cystoisospora*, *Sarcocystis, Neospora, Frenkiella, Eimeria*, *Besnoitia* and *Toxoplasma* (Votýpka et al., 2017). Besides, recently the genus

Besnoitia which causes besnoitiosis in different animal species are drawing more and more attention from researchers in the field of parasitology due to increased number of clinical cases of besnoitiosis and rapid geographic expansion into non-endemic geographic areas (Álvarez-García et al., 2013a; Basso et al., 2013; Jacquiet et al., 2010; Ryan et al., 2016).

Although clinical besnoitiosis is frequently reported in intermediate hosts (e. g. cattle) in literature, very little knowledge is available on final host spectrum and complete life cycle, and thus still being a great challenge for scientific community. Nonetheless, advances in molecular biology, immunology, pathogenesis, and genetics will most likely provide promising breakthroughs in the field of *Besnoitia*-related research.

1.1 Genus *Besnoitia*

Besnoitia spp. are cyst-forming apicomplexan protozoan parasites, and besnoitiosis caused by these species is distributed worldwide in both domestic and wild animals, such as cattle, donkeys, horses, goats, sheep, reindeer, caribou, zebras, rodents, rabbits, and lizards (Dubey and Yabsley, 2010). Until now 10 species of *Besnoitia* have been discovered including *B. akadoni* (Dubey et al., 2003a), *B. bennetti* (Bennett, 1927), *B. besnoiti* (Franco and Borges, 1916), *B. caprae* (Bwangamoi, 1967), *B. darlingi* (SCHNEIDER, 1967), *B. jellisoni* (Ernst et al., 1968), *B. neotomofelis* (Frenkel and Lunde, 1953), *B. oryctofelisi* (Dubey and Lindsay, 2003), *B. tarandi* (Hadwen, 1922), and *B. wallacei* (Ng'ang'a et al., 1994). It is suspected that all *Besnoitia* species have a heteroxenous (two-host) life cycle, in which there is a predator acting as final host and a prey species acting as intermediate host species (Olias et al., 2011). However, limited knowledge is known on their complete life cycle except for *B. darlingi* [cats (final hosts); opossums and lizards (intermediate hosts)], *B. wallacei* [cats (final hosts); rodents (intermediate host)] and *B. oryctofelisi* [cats (final hosts); rabbits (intermediate hosts)] (Dubey and Lindsay, 2003).

As already stated above, the intermediate host spectrum contains diverse animals, such as horses, donkeys (Els et al., 1993), cattle (Njagi et al., 1998), goats (Cheema and Toofanian, 1979), reindeer (Ayroud et al., 1995), rabbits (Basson et al., 1970), opossums and mice (Shkap et al., 1987a). Initially both domestic and wild cats were thought to be definitive hosts for all *Besnoitia* spp. (Dubey, 1977; Rommel, 1978; Wallace and Frenkel, 1975), but this conclusion was doubted by other researchers due to irreproducible observations (Basso et al., 2011; Diesing et al., 1988).

1.2 *Besnoitia besnoiti*

The species *Besnoitia besnoiti* (phylum Alveolata, subphylum Apicomlexa, family Sarcocystidae) is the causative agent of the disease known as bovine besnoitiosis (Álvarez-García et al., 2013a; Cortes et al., 2003; Cortes et al., 2014; Ryan et al., 2016). *B. besnoiti* infections can lead to reduced fertility and productivity in cattle thereby causing high economic losses, not only in Europe, but also in Asia, Africa and South America (Bigalke et al., 2004; Cortes et al., 2014; Vogelsang and Gallo, 1941). Mild to severe clinical signs, such as anasarca, oedema, orchitis, vulvovaginitis, hyperkeratosis, characteristic skin, and mucosal cysts, are due to *B. besnoiti* tachyzoite and bradyzoite replication in diverse intermediate host

vessels, tissues and organs. Until now, there are neither commercially available effective drugs nor vaccines against this parasite species (Cervantes-Valencia et al., 2019).

1.2.1 Background

In the past, the occurrence of bovine besnoitiosis was studied in Portugal between 1885 and 1915 (Franco and Borges, 1915), but then little attention was paid on this disease. Consistently, until the 1990s, bovine besnoitiosis was reported exclusively from Mediterranean European countries such as Portugal (Cortes et al., 2003), Spain (Juste et al., 1990), and France (Bourdeau et al., 2004). Continuous successive case reports in literature on bovine besnoitiosis from Portugal (Cortes et al., 2006), Spain (Fernández-García et al., 2009), France (Jacquiet et al., 2010), Germany (Schares et al., 2009), Italy (Gollnick et al., 2010; Rinaldi et al., 2013, 201), Switzerland (Basso et al., 2013), and Hungary (Hornok et al., 2014) clearly indicated a re-emergence and widespread of this disease in middle Europe (Álvarez-García et al., 2013b). Based on the increased number of cases and geographic expansion of bovine besnoitiosis in Europe, the European Food Safety Authority (EFSA) classified this disease as an emerging disease within EU in 2010 (European Food Safety Authority, 2010).

1.2.2 Morphology

B. besnoiti is a tissue cyst-forming apicomplexan protozoan parasite which has two stages in bovine intermediate host: tachyzoites and bradyzoites. The morphology of both *B. besnoiti* stages can be described as crescent-shaped- or 'banana'-shaped-forms and containing common apicomplexan organelles such as the apical complex composed of a polar ring and a conoid, apicoplast, rhoptries, micronemes, exonemes and dense granules (please refer to Fig. 1).

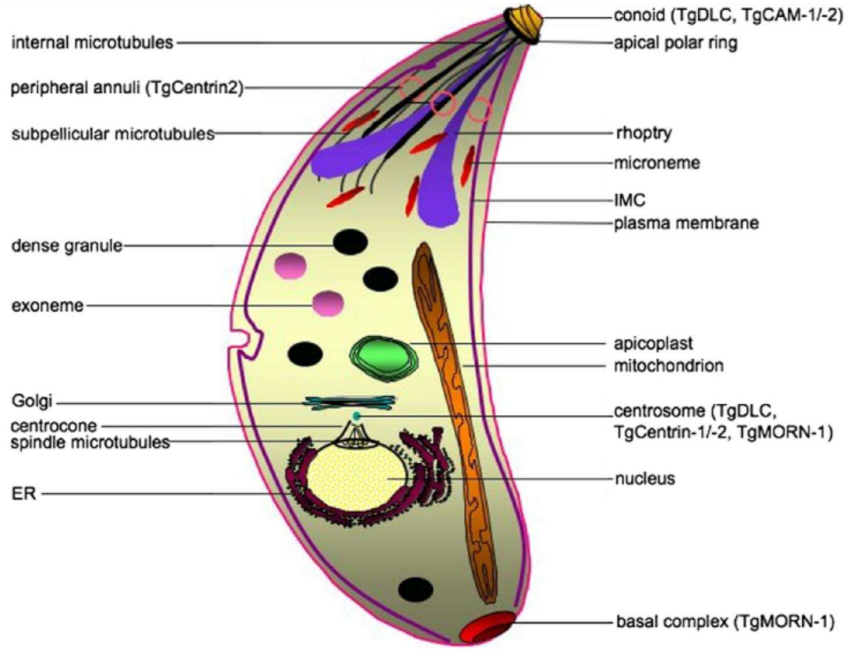

Fig. 1. Scheme of a *Besnoitia besnoiti*-tachyzoite/bradyzoite stage. Shown are the cytoskeleton elements (microtubules and centrocone), the apical complex (micronemes, rhoptries, conoid and apical polar ring), the pellicle (inner membrane complex and plasma membrane), the secretory organelles (exonemes, dense granules, micronemes and rhoptries), the non-secretory intracellular organelles (mitochondrion, apicoplast, nucleus, endoplasmic reticulum and Golgi) and the basal complex. Note that not all members of the phylum contain the full repertoire shown in the scheme (adapted from Santos JM et al., 2009).

In vivo large-sized tissue cysts of *B. besnoiti* clearly differ morphological/morphometric from other cyst-forming coccidian parasites, i. e. *T. gondii*, *N. caninum, Hammondia* spp. and *Sarcocystis* spp. (Dubey et al., 2013), thereby consisting of a thick three-layered cyst wall (Basso et al., 2013; Cortes et al., 2006; Fernández-García et al., 2009; Gollnick et al., 2010; Schares et al., 2009). This complex three-layered *B. besnoiti*-cyst wall consists of the following layers: *i)* outermost layer with connective tissue, *ii)* middle layer containing host cell nuclei, and *iii)* internal parasitophorous vacuole membrane (PVM) containing thousands of bradyzoites (please refer to Fig. 2). The host cell nuclei enclosed in the tissue cyst is an additional common feature of *Besnoitia*-cysts, which is distinct from other tissue cyst-forming coccidia (Dubey et al., 2013; Fernández-García et al., 2009).

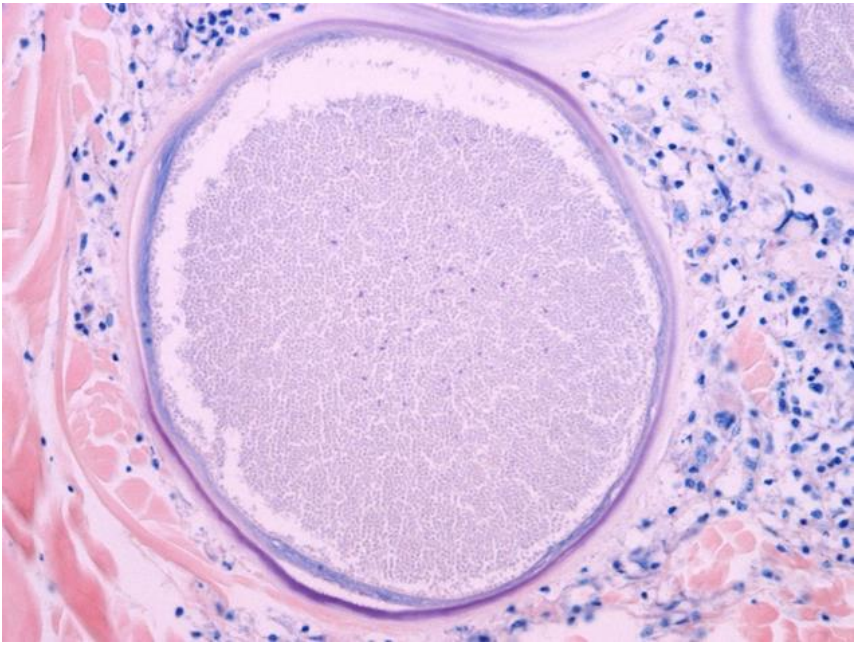

Fig. 2. Visualization of a subcutaneous connective large-sized tissue cyst of *Besnoitia besnoiti* with its characteristic thick three-layered wall in the subdermis of a naturally infected heifer from France (Giemsa staining 20 ×).

1.2.3 Life cycle

Currently only four species of *Besnoitia* (i. e. *B. darlingi*, *B. wallacei*, *B. oryctofelisi* and *B. neotomofelis*) are completely known regarding their heteroxenous life cycles, and for all these species domestic cats (*Felis silvestris catus*) acting as definitive hosts, are known (Dubey and Yabsley, 2010; Dubey et al., 2003b; Frenkel, 1977; Smith and Frenkel, 1977). Thus, it has been suspected that *B. besnoiti* has also a two-host life cycle like other cyst-forming coccidian parasites of the Sarcocystidae family in which cattle and wild cervids (i. e. roe deers, deers) act as intermediate hosts, and a carnivore host being the definitive host (Basso et al., 2011). However, the complete life cycle of *B. besnoiti* still remains obscure (Fig. 3). Early studies suggested that domestic- and wild cats were acting as definitive hosts for *B. besnoiti* since they shed oocysts after oral ingestion of cyst-containing tissues (Peteshev et al., 1974). Nonetheless, former study have not been reproduced by others, and thus biology

remaining unsolved until now (Basso et al., 2011; Diesing et al., 1988). In the intermediate host, this parasite develops two infective asexual stages: fast replicating tachyzoite stages and slower replicating bradyzoite stages (Fig. 3).

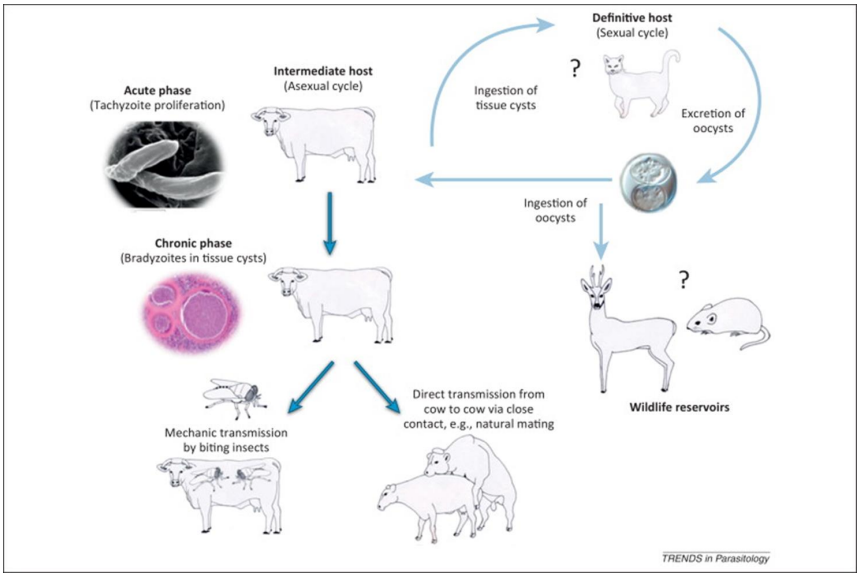

Fig. 3. Life cycle and transmission routes of *Besnoitia besnoiti* in cattle and cervids. It is suspected that *B. besnoiti* has a heteroxenous life cycle. Two infective asexual stages of *B. besnoiti* develop within intermediate hosts (bovids, cervids): fast-replicating tachyzoites and slower-dividing bradyzoites, which gather into macroscopic cysts located inside cells of the subcutaneous connective tissue. The complete life cycle of the parasite remains unknown, although epidemiological data suggest an important role for horizontal transmission by either direct contact, through natural mating, or mechanical transmission by blood-sucking arthropods. Domestic cats (*F. silvestris catus*) have been suggested as final host and to shed environmentally resistant oocysts in their faeces after ingestion of meat containing tissue cysts, as it occurs for other *Besnoitia* species. Sporulated *B. besnoiti*-oocysts are suspected to be formed after sexual replication of the parasite in the intestine of final host and to be similar to oocysts described for other members of subfamily Toxoplasmatinae (e. g., *Toxoplasma gondii* and *Neospora caninum*). The role of wild ruminants and rodents as putative hosts of the parasite remains to be elucidated (adapted from Álvarez-García et al., 2013).

1.2.4 Transmission of *Besnoitia besnoiti*

So far, mechanical indirect transmission (see Fig. 3) among cattle is the only experimentally confirmed mode of transmission of *B. besnoiti* via hypodermic needles or blood-sucking insects such as tsetse flies and tabanids (Bigalke, 1968; Pols, 1960). Since *B. besnoiti* survives only for a short time in insects and whose activities are dependent on seasonal fluctuations (Álvarez-García et al., 2013a; Liénard et al., 2011), bovine besnoitiosis is endemic where these blood-sucking insects have the chance to be constantly exposed to cattle with epidermal *B. besnoiti* cyst formation (Gentile et al., 2012).

In addition, a direct transmission route (see Fig. 3) from cattle to cattle (e. g. through mating, wounds or lacerations) was speculated to be possible since bradyzoites might be able to cross mucous membranes after mechanical tissue cyst rupture and infect other host cells (Cortes et al., 2014). However, this mode of transmission has not been confirmed yet. Moreover, ingestion of oocysts shed in faeces of definitive hosts and most probably found on oocyst-contaminated pastures or premises, was also proposed to be a way of *B. besnoiti* infection for cattle (Peteshev et al., 1974).

Besides these transmission routes, national as well as international cattle trade might in addition contribute to the introducing *B. besnoiti*-infected animals into a naïve herd or a previously non-endemic area throughout European countries (Alvarez-Garcia et al., 2013, 2014). Some reports in literature agree with this hypothesis in which subclinical and undiagnosed cases of bovine besnoitiosis may act as reservoirs for infection and spread of the disease within a herd (Alvarez-Garcia et al., 2014).

1.2.5 Geographical distribution of bovine besnoitiosis

Bovine besnoitiosis occurs in many European countries, such as Spain, Portugal, France, Italy, Germany, Switzerland, and Hungary (Hornok et al., 2014), Africa (Bigalke et al., 2004), Asia (Cortes et al., 2014) and South America (Vogelsang and Gallo, 1941). It has been considered as an emerging disease in Europe by EFSA since 2010, and clinical cases of besnoitiosis are still increasing and its expansion has reached new European countries such as Ireland, Belgium, and Croatia (Beck et al., 2013; Ryan et al., 2016; Vanhoudt et al., 2015).

The first case in Germany was observed in a cattle farm allocated in Bavaria and was confirmed by morphological studies of *B. besnoiti*-infected skin samples (Mehlhorn et al.,

2009). Shortly after, another study showed natural occurring cases of bovine besnoitiosis in a herd closely located to the previous Bavarian farm (Mehlhorn et al., 2009), and thereafter confirmed by *B. besnoiti*-specific serology and by polymerase chain reaction (PCR) (Rostaher et al., 2010). It was suggested that this outbreak of bovine besnoitiosis in Germany occurred due to the importation of cattle from enzootic regions of France (Gollnick et al., 2009; Majzoub et al., 2010).

In Africa, this disease has been widely distributed in many tropical/subtropical countries such as South Africa, Swaziland, Mozambique, Zimbabwe, Angola, Congo, Kenya, Cameroon and Nigeria (Shanan et al., 2015), and Asia (Lee et al., 1970; Peteshev et al., 1974; Wang and Liu, 1987). In America, it has also been reported in Venezuela and Colombia (Trujillo and Benavides, 2011; Vogelsang and Gallo, 1941) but no data exist in literature on the occurrence of cattle besnoitiosis neither from Central American- nor from North American countries.

Several reports suggest that there is an enzootic occurrence in Nigeria, Uzbekistan, Kazakhstan, China and South Korea, but no information is currently available from these countries (Olias et al., 2011). Alongside, a more recent seroprevalence survey showed no presence of antibodies against *B. besnoiti* in South Australian cattle (Nasir et al., 2012) but additional large-scale epidemiological studies are urgently needed to confirm/assess the free-status of bovine besnoitiosis for Australia (Nasir et al., 2012).

1.2.6 Clinical signs of bovine besnoitiosis

Typically, cattle affected by bovine besnoitiosis have an acute and a chronic phase of disease resulting in different clinical signs. In the acute phase of disease, *B. besnoiti*-tachyzoites replicate rapidly in monocytes, polymorphonuclear neutrophils (PMN), macrophages, fibroblasts and host endothelial cells of vessels and infected animals show pyrexia, intensive respiratory disorder, increased heart rates, subcutaneous oedema, swollen joints, conjunctivitis, nasal discharge, photophobia, reduced milk yield and orchitis in bulls (Álvarez-García et al., 2013b; Bigalke, 1981; Cortes et al., 2014). Abortion can also occur in this stage of diseases in pregnant cattle (Cortes et al., 2014). Besides, *B. besnoiti*-infected animals with nephrotic syndrome may die due to severe hypoalbuminaemia, hyperproteinuria and mild leukocytosis (Dubey et al., 2013).

One to 2 weeks later, *B. besnoiti*-bradyzoites proliferate slowly to form cysts in subcutaneous connective tissue, and infected animals show dramatic thickening, hardening and folding or wrinkling of affected skin, hair loss, dermatitis and the presence of characteristic macroscopic subcutaneous thick-walled tissue cysts, a gradual deterioration of body condition, and weight loss (Pols, 1960). Macroscopic *B. besnoiti*-cysts in scleral conjunctiva, vestibule and vulvae are usually visible via close visual inspection (Cortes et al., 2014). Affected bulls show irreversible testicular lesions (atrophy, sclerosis and necrosis) leading to chronic epididymitis and orchitis which result in infertility or even sterility (Kumi-Diaka et al., 1981).

1.2.7 Diagnosis

Only based on the clinical signs of infected animal, bovine besnoitiosis may frequently be misdiagnosed from other diseases, such as fungal dermal infections, bacterial dermal infections, parasitic mange (i. e. *Sarcoptes*, *Chorioptes*, *Psoroptes*, *Psorergates*, *Demodex*), pediculosis (e. g. *Bovicola, Haematopinus, Lignognathus, Solenopotes*), mineral deficiency, photosensitivity or even blue tongue virus infection (Cortes et al., 2014). When *B. besnoiti*-infected animals develop the chronic phase of disease, bovine besnoitiosis can be diagnosed by a combination of apparent clinical manifestations and histological examination of skin samples. Manifested alopecia, hard, thick, and wrinkled skin, and macroscopically visible cysts in sclera conjunctiva and vulvae (Fernandez-Garcia et al., 2009) can be included to diagnose this parasitic disease. Moreover, the detection of *B. besnoiti*-DNA in tissue samples by parasite-specific PCR (Schares et al., 2011) and detection of antibodies against *B. besnoiti* using an avidity enzyme-linked immunosorbent assay (ELISA) (Schares et al., 2013) have been regarded as appropriate diagnostic techniques in the acute phase of clinical bovine besnoitiosis.

However, only few infected animals develop clinical signs, whereas most of infected animals remain seropositive but asymptomatic (Bigalke, 1981; Goldman and Pipano, 1983; Janitschke et al., 1984; Neuman, 1972). Therefore, highly sensitive and specific diagnostic methods (i. e. PCR and serology tests) are needed not only for diagnosis of bovine besnoitiosis in subclinical cattle herds but also for large-scale epidemiological studies on disease spreading. Consistently, a conventional and a real-time fluorescent ITS1 rDNA PCR has been developed (Cortes et al., 2007), and successfully used to detect *B. besnoiti* in skin biopsies of chronic infected animals, but has not been adapted to detect tachyzoites in the

blood circulation during the early phase of infection (Jacquiet et al., 2010). Other serological techniques, including IFAT and Western blots, have also been reported for the detection of *B. besnoiti* infections (Cortes et al., 2006; Fernandez-Garcia et al., 2009; García-Lunar et al., 2013, 2). Specificity and sensitivity have been shown to be high in Western blot (96.4% and 91%) and in ELISA (96% and 87%), respectively. As such, ELISA tests complemented with parasite-specific Western blots have been suggested to reliable methods to diagnose both disease forms, namely the symptomatic- as well as the asymptomatic bovine besnoitiosis (Cortes et al., 2006).

1.2.8 Treatment and control

Currently there is no effective treatment for animals suffering bovine besnoitiosis (Cervantes-Valencia et al., 2019). Treatments with the administration of pentamidine, aureomycin, formalin, sodium iodide, sulfamerazine, mycostatin and terramycin failed to cure besnoitiosis in rabbits (Pols, 1960). Drugs including oxytetracycline, sulfonamides, trimethoprim, halofuginone, diminazene aceturate, and pentamidine were also tested to treat experimentally *Besnoitia* spp.-infected gerbils or rabbits, but oxytetracycline showed some effect in gerbils only when it was administered concurrently with the parasite (Shkap et al., 1987b). Moreover, the nitro-thiazolide nitazoxanide, a range of bromo-derivatives and a new-generation of pentamidine derivatives were found to exert significant activity against *B. besnoiti* tachyzoites, but studies *in vivo* have not been performed yet (Cortes et al., 2011). More recently, also antiparasitic efficacy of curcumin against *B. besnoiti* tachyzoites *in vitro* was demonstrated (Cervantes-Valencia et al., 2019). Curcumin, a polyphenolic compound derived from *Curcuma longa* rhizomes is well-known for its anti-parasitic protozoan effects and functional inhibition assays revealed that curcumin treatments reduced tachyzoite viability and inducing lethal effects in up to 57% of exposed tachyzoites with an IC_{50} in 5.93 µM *in vitro* (Cervantes-Valencia et al., 2019).

In South Africa, live-attenuated vaccines based on *B. besnoiti*-tachyzoites isolated from blue wildebeest (*Connochaetes taurinus*) were developed *in vitro* and used to control bovine besnoitiosis (Bigalke et al., 1974). Although sub-clinical infection could not completely be prevented, cattle were protected from clinical infection for up to four years (Bigalke et al., 2004), and these live-attenuated vaccines were recommended for use in weaners and older animals. However, their use is geographically limited, as live-attenuated vaccines pose the risk of introducing the parasite into non-endemic areas and inducing carriers among

vaccinated cattle, which is of particular concern due to very limited knowledge on pathogenesis, immunity, transmission routes and life cycle (Cortes et al., 2014).

As already stated, chemotherapy is unsatisfactory and attenuated live vaccines are not authorized in most of European countries (Jacquiet et al., 2010). Therefore, the control of bovine besnoitiosis mainly relies on management measures coupled with diagnosis. These approaches can be carried out by two steps: *i)* avoiding introduction of parasites into a naïve herd by rigorous testing of all new animal entering the herd, *ii)* and/or avoiding the spread of disease by gradually decreasing its prevalence within herds, which is a long-term step-by-step, selective culling strategy together with biosecurity measurements as postulated elsewhere (Álvarez-García et al., 2013a). Besides, associated risk factors with this disease including sharing pastures and natural mating with shared bulls should be avoided in endemic areas of bovine besnoitiosis (Álvarez-García et al., 2013b, Cortes et al., 2014).

1.3 Innate immune system and its effector cells

The host immune system (please refer to Fig. 4) against any invading pathogen has been traditionally divided into innate and adaptive immunity. The first level of protection is provided by anatomic and physiologic barriers, such as intact skin, mucous membranes, low stomach pH, and bacteriolytic lysozyme in tears, saliva, milk and are all components of the innate immune system (Turvey and Broide, 2010; Villagra-Blanco et al., 2019). Once invasive pathogens pass through these anatomic/physiologic barriers, innate immune effector cells, commonly known as leukocytes are recruited to the site of infection, become immediately activated and provide a rapid non-specific host innate immune response which acts efficiently against a broad range of different pathogens such as virus, bacteria, fungi and protozoan/metazoan parasites (Hermosilla et al., 2014; Villagra-Blanco et al., 2019). Moreover, leukocytes of innate immunity play a crucial role in initiating the subsequent adaptive cellular immune response, and are also associated with the removal of invading pathogens in adaptive immunity (Janeway et al., 2001).

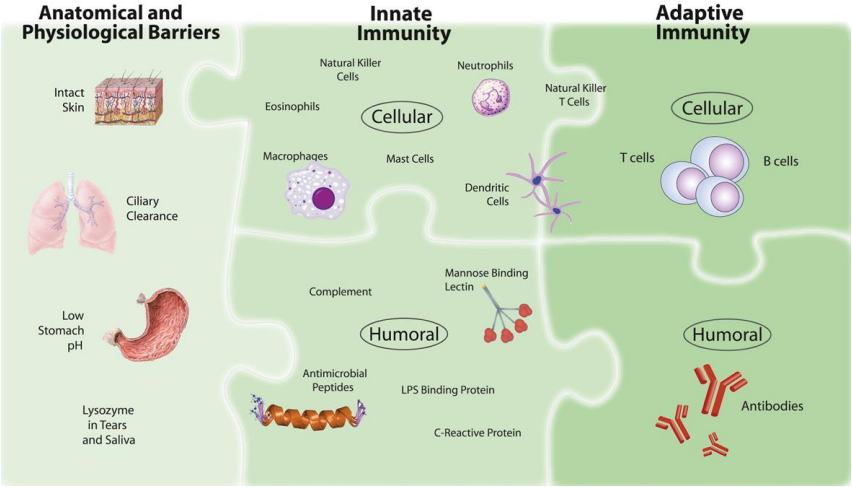

Fig. 4. Integrated mammalian immune system. The mammalian microbial defense system can be simplistically viewed as consisting of three levels: *i)* anatomic and physiologic barriers; *ii)* innate immunity system; and *iii)* adaptive immunity system. In common with many classification systems, some elements are difficult to categorize. For example, NK T cells and dendritic cells (DCs) can be classified as being on the cusp of innate and adaptive immunity rather than being firmly in one camp (adapted from Turvey et al., 2010)

There are three recognition strategies used by the host innate immune system to fight against invading pathogens. Firstly, a number of receptors on the surface of phagocytic leukocytes are able to recognize so-called pathogen associated molecular patterns (PAMPs) such as bacterial components, which are highly conserved structures, expressed by numerous microorganisms. Moreover, the host innate immune system detects the danger in form of damage associated molecular patterns (DAMPs) such as high mobility group box 1 (HMGB 1) protein and/or other endogenous molecules such as alarmins, heat shock proteins (HSPs), ATP and uric acid which are released during tissue damage in inflammation processes (Foell et al., 2007; Krysko et al., 2011; Srikrishna and Freeze, 2009; Turvey and Broide, 2010; Zheng et al., 2011). Furthermore, innate immune recognition can also be undertaken by detecting molecules which are expressed by infected host cells (Turvey and Broide, 2010).

The host innate immune system contains a number of professional phagocytes with specialized functions, such as basophils, eosinophils, polymorphonuclear neutrophils (PMN),

DCs, Langerhans cells, mast cells, NK cells, monocytes and macrophages (Silva et al., 2016; Villagra-Blanco et al., 2019).

1.3.1 Characteristics of mammalian PMN

PMN are the most abundant leukocytes in the blood of terrestrial and marine mammalians and being produced daily by bone marrow in large numbers, and which are known to possess a short life nonetheless playing an essential role in host innate immunity (Hermosilla et al., 2014; Selders et al., 2017; Silva et al., 2016; Villagra-Blanco et al., 2019). Three main antimicrobial functions (Fig. 5) of PMN are recognized: phagocytosis, degranulation, and the release of neutrophil extracellular traps (NETs).

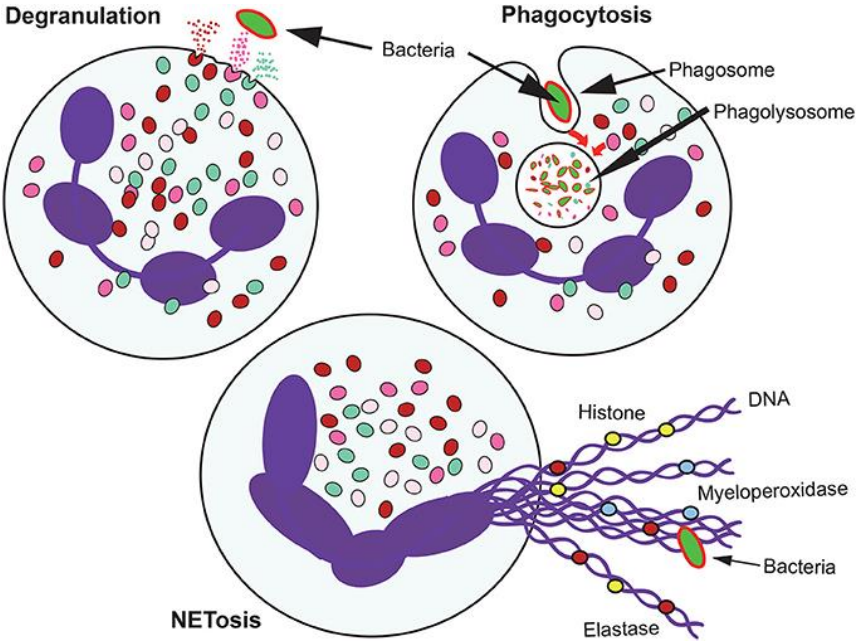

Fig. 5. Antimicrobial defense mechanisms of activated mammalian PMN. When PMN recognize microbial pathogens through pathogen recognition receptors (PRRs), they deploy different functions to destroy invasive microbial agents. Phagocytosis involves the ingestion of the microorganism into a phagocytic vacuole which upon maturation becomes a phagolysosome. In this new organelle, the microorganism is destroyed by the action of low pH, and degradation through enzymes contained in PMN granules. Alternatively, PMN also degranulate to their exogenous environment the content of

their granules containing important antimicrobial peptides and proteases (neutrophil elastase, myeloperoxidase). PMN can also cast neutrophil extracellular traps (NETs) formed by DNA fibers decorated with nuclear histones and proteins from the granules (adapted from Rosales, 2018).

Phagocytosis is a process in which these professional phagocytes including PMN engulf and destroy invading microorganisms. This process can be divided into four main steps: *(i)* recognition of the target particles including microorganisms and apoptotic cells, *(ii)* particle internalization, *(iii)* phagosome formation, and *(iv)* phagolysosome maturation (Rosales and Uribe-Querol, 2017). Briefly, the target particles are recognized by the PRRs on the surface of cell membrane, surrounded by pseudopodia in a zipper-like mechanism (Griffin et al., 1975; Griffin et al., 1976), and then a vesicle called phagosome including the particles is formed by the fusion of cell membrane. Phagosome matures via a series of membrane fusion and fission events to become a phagolysosome that is an acidic, hydrolytic compartment to degrade the ingested particles (Aderem, 2003). Microorganisms within a phagolysosome are destroyed by many factors including oxygen radicals, nitric oxide, anti-microbial proteins, anti-microbial peptides, binding proteins and hydrogen ion transport (Uribe-Querol and Rosales, 2017).

Moreover, PMN contain a large number of granules full of various compounds and exert their antimicrobial properties through degranulation (Segal, 2005). There are three types of granules present in mammalian PMN: azurophilic granules, specific granules and gelatinase granules. Azurophilic granules also named primary granules are the first formed particles during PMN maturation. These granules may contain many antimicrobial molecules, such as bactericidal/permeability-increasing protein, phospholipase A2, myeloperoxidase (MPO), defensins, cathepsin G, lysozyme, acid hydrolases, proteoglycans and some serine proteases including neutrophil elastase (NE), proteinase 3 (Faurschou and Borregaard, 2003; Korkmaz et al., 2010; Villagra-Blanco et al., 2019), and have been regarded as the microbicidal vesicles mobilized during phagocytosis (Mollinedo, 2003). In addition, azurophil granule degranulation is confined mainly to internalized phagocytic vacuoles (Leffell and Spitznagel, 1975). Specific granules named secondary granules are the second type of PMN granules produced during PMN development, and they also contain some antimicrobial molecules such as unsaturated lactoferrin, calprotectin, lysozyme, human cathelicidin, and neutrophil gelatinase-associated lipocalin (Bullen and Armstrong, 1979; Teng et al., 2017). Gelatinase granules known as tertiary granules have few antimicrobial compounds, but are rich in matrix metalloproteases (MMPs), such as gelatinase and leukolysin (Teng et al., 2017). PMN

granules are mobilized to the cell membrane and degranulate for secretion of their contents once the receptors [e. g. Toll-like receptors (TLRs)] expressed on the PMN plasma membrane or phagosomal membrane signal to the cytoplasm (Lacy, 2006; Villagra-Blanco et al., 2019).

In addition, NETosis is a late discovered cell death process of activated PMN (Brinkmann and Zychlinsky, 2012a; Fuchs et al., 2007), resulting in the release of a meshwork of sticky extracellular DNA fibers decorated with antimicrobial peptides and enzymes such as histones (H1, H2A/H2B, H3, H4), NE, lactoferrin, pentraxin and MPO among others (Brinkmann and Zychlinski, 2012; Silva et al., 2016; Villagra-Blanco et al., 2019). Over the past fifteen years, NETosis has become the most studied areas of PMN function, and it has been nowadays considered as a novel, ancient and conserved effector mechanism of mammalian PMN to fight against invading pathogens (Brinkmann et al., 2004; Papayannopoulos and Zychlinsky, 2009; Pilsczek et al., 2010). More details about NETosis are discussed in **Chapter 1.4.**

1.3.2 Other innate immune cells

Besides PMN, there are also some other professional phagocytes such as monocytes, macrophages, DCs and mast cells that ingest and degrade foreign pathogens and/or dead cells by phagocytosis (Hermosilla et al., 2014).

Macrophages literally 'big eaters' derived from circulating monocytes and are a type of large white blood cells that are found in both the bloodstream and in tissues. Macrophages live for months patrolling our body and keeping it clean from invasive pathogens (Taubert et al., 2009). They are able to locate and destroy particles such as bacteria, viruses, fungi, and parasites (Lauvau and Hohl, 2015). Moreover, monocytes and macrophages produce pro-inflammatory molecules and can activate other immune responses thereby offering a bridge between the innate and adaptive immune systems (Hirayama et al., 2017; Taubert and Zahner, 2001; Taubert et al., 2009).

DCs are the most potent professional antigen-presenting cells, which are responsible for capture, processing, and presentation of antigens on their surface to T helper (h) cells linking innate and adaptive immune responses (Chistiakov et al., 2015). DCs must be present with the appropriate major histocompatiblity complex (MHC) expressed on the cell membrane surface since antigens alone cannot activate Th cells. During the development of an adaptive immune response, the phenotype and function of DCs play an extremely crucial role in the

initiation of tolerance, memory, and some polarised Th cell differentiation (Gaurav and Agrawal, 2013).

Mast cells are found in most tissues close to the external environment such as skin, airways, and intestine (Marshall and Bienenstock, 1994), and can actively participate in the early recognition of pathogens and mediate allergic responses by releasing pro-inflammatory chemicals such as cytokines and chemokines (Marquardt and Wasserman, 1982; Urb and Sheppard, 2012). Moreover, mast cells can not only kill invading pathogens by phagocytosis and ROS production, but also send signals to other tissues to modulate both innate immune responses via releasing a variety of mediators (Abraham and John, 2010; Dawicki and Marshall, 2007). In addition, mast cells also play a key role on host defense against parasitic protozoan infections (Lu and Huang, 2017).

NK cells are a type of cytotoxic lymphocytes of the host innate immune system, and have many biological functions such as tumor cell surveillance and killing pathogen-infected host cells. NK cells contain granules filled with perforin and granzymes. When NK cells meet its target they bind to it and release perforin and granzymes to puncture holes into the membrane of the target resulting in cell rupture or apoptosis induction (Paust and Von Andrian, 2011). Unlike other lymphocytes (for example, Th cells) that are limited to recognize only targets which conjugate with their surface receptors, NK cells use the CD16 receptor on the surface of cell membrane to recognize and bind to any tumor cells or virally-infected host cells (Paust and Von Andrian, 2011). Recently, NK cells have been shown to possess the characteristics of adaptive immunity and can acquire immunological memory similar to that of Th and B cells (O'Sullivan et al., 2015; Paust and Von Andrian, 2011).

Besides, eosinophils are involved in allergic responses and host defense against parasitic infections (Klion and Nutman, 2004; Weller, 1997). Basophils are closely related to mast cells that are found in tissues only (Galli et al., 1991), and basophils are also involved in allergic reactions and capable of releasing histamine which helps to trigger inflammation, and heparin which prevents blood from clotting in some innate immune reactions (Crivellato, 2013).

1.4 Neutrophil extracellular traps (NETs)

NETs were first described to kill bacteria in 2004, and they are large, extracellular, web-like structures composed of mainly DNA, histones, various cytoplasmic proteins, and many

granule proteins such as NE, cathepsin G, MPO, lactoferrin, and gelatinase (Brinkmann et al., 2004). Over fifteen years, NETs have been found to have a potent antimicrobial activity and also involved in many inflammatory and autoimmune disorders (Brinkmann and Zychlinsky, 2012b; Vorobjeva and Pinegin, 2014). NETs can be released by activated PMN mainly through two pathways: suicidal NETosis and vital NETosis (Fig. 6).

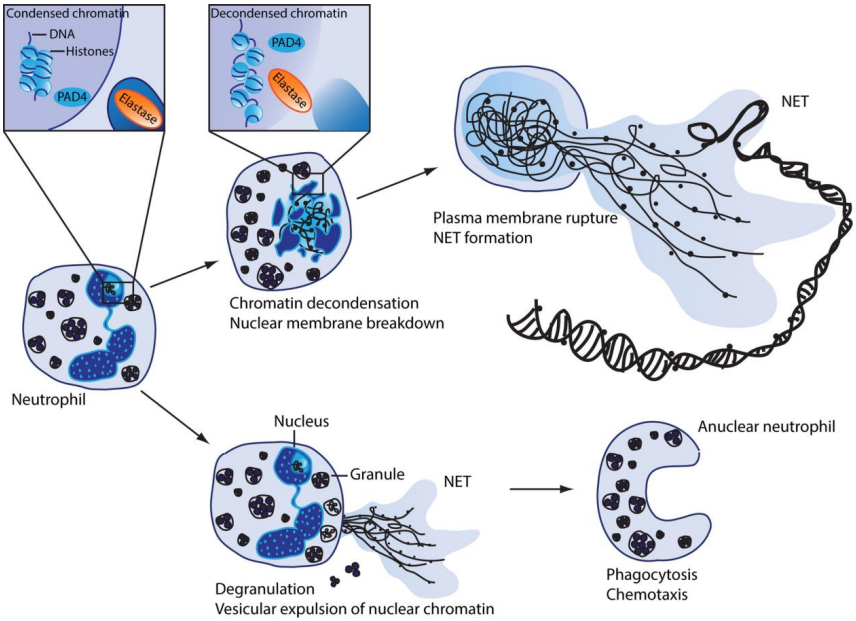

Fig. 6. Suicidal- and vital NETosis. The nuclear enzyme PAD4 citrullinates histones in activated PMN, and thereby promotes weakening of the electrostatic binding between histones and DNA within nucleosomes. After breakdown of the nuclear and granule membrane, NE translocates into the nucleus to drive further unfolding of chromatin by processing histones. Decondensed DNA with citrullinated histones and granule proteins meet within the PMN cytosol. NETosis ends when NETs-chromatin with associated proteins are expelled from disrupted PMN. The outcome of this PMN cell death is known as suicidal NETosis. In contrast, without compromising cell integrity, vital NETosis involves the secreted expulsion of mitochondrial or nuclear chromatin via vesicles. The PMN surface membrane reseals and results in a viable anuclear/amitochondrial PMN which continues performing other functions such as phagocytosis, crawling activities, degranulation and chemotaxis (adapted from van Avondt and Hartl, 2018).

Suicidal NETosis was initially described as a NADPH oxidase (NOX)-dependent cellular effector mechanism, which induces the extrusion of nuclear-derived DNA and cytoplasmic granule enzymes leading to formation of DNA-rich networks being decorated with histones and various potent antimicrobial granular effector molecules, such as NE, MPO, lactoferrin, pentraxin, peptidoglycan recognition proteins or calprotectin (Brinkmann and Zychlinsky, 2012a; Fuchs et al., 2007; Hahn et al., 2013; Parker and Winterbourn, 2013). A variety of invasive pathogens such as bacteria, virus, fungi, protozoan and metazoan parasites, might either be immobilized within released sticky NET structures or being killed via local high concentration of antimicrobial histones, peptides, and proteases (Behrendt et al., 2010; Muñoz-Caro et al., 2018; Nathan, 2006; Silva et al., 2016). Classical suicidal NETosis is signaled via Raf-MEK-ERK-dependent pathways (Fuchs et al., 2007; Hakkim et al., 2011; Villagra-Blanco et al., 2017b; Wartha and Henriques-Normark, 2008). Alongside NOX-dependent NETosis, also NOX-independent NETosis has been reported to exist and seem to be linked to a substantial reduction of ERK1/2 activation and weak Akt activation, whilst p38 MAPK activation appears similar in both types of NETosis (Douda et al., 2015; Khan and Palaniyar, 2017; Villagra-Blanco et al., 2017a, b).

In addition to suicidal NETosis, PMN have also been shown to undergo vital NETosis without cell lysis, thus remaining viable and functional including migration, degranulation and the capability of active bacteria phagocytosis (Yipp et al., 2012). Vital NETosis was demonstrated to rapidly occur via nuclear envelope blebbing and vesicular exportation in an oxidant-independent manner (Pilsczek et al., 2010). This vital rapid NETosis (within 30 min after PMN activation) is induced by Gram-positive bacteria mediated by TLR2 and complement (Yipp et al., 2012), and by *Candida albicans* dependent on fibronectin and complement factors (Byrd et al., 2013). So far, vital NETosis has not been described in response parasites but some hints of vital NETosis against apicomplexan parasites have been documented. As such, isolated harbour seal (*Phoca vitulina*)-derived PMN exposed to *T. gondii* tachyzoites rapidly (within 10 min) released a “chameleon tongue-like” structure towards tachyzoites thereby maintaining PMN cellular integrity (Reichel et al., 2015). The same is true for recent live cell imaging analysis of bovine PMN acting against highly motile trypomastigote stages of *Trypanosoma brucei* where a similar “chameleon tongue-like” phenomenon was reported (Daniela Grob, unpublished data). Furthermore, PMN seem able to release small-sized mitochondria-derived NETs without suffering cell death (Yousefi et al., 2009).

Suicidal NETosis was reported to be triggered by different protozoan parasites *in vitro* and *in vivo*, including *Plasmodium falciparum* (Baker et al., 2008), *Leishmania* spp. (Denkers and Abi Abdallah, 2012; Guimarães-Costa et al., 2009; Guimarães-Costa et al., 2014), *Eimeria bovis* (Behrendt et al., 2010; Muñoz-Caro et al., 2015a), *Eimeria arloingi* (Silva et al., 2014a), *T. gondii* (Abdallah et al., 2012; Reichel et al., 2015; Yildiz et al., 2017), *Cryptosporidium parvum* (Muñoz-Caro et al., 2015b), *N. caninum* (Villagra-Blanco et al., 2017a; Villagra-Blanco et al., 2017b; Wei et al., 2016), *Trypanosoma cruzi* (de Buhr et al., 2018; Sousa-Rocha et al., 2015), *Entamoeba histolytica* (Ventura-Juarez J. et al., 2016) and *B. besnoiti* (Muñoz-Caro et al., 2014; Maksimov et al., 2016).

Moreover, a vast number of molecules have been identified as NETosis stimuli such as ionomycin (Francis et al., 2014), nicotine (Hosseinzadeh et al., 2016), PMA (Brinkmann et al., 2004; Brinkmann et al., 2010; Desai et al., 2016), LPS (Yousefi et al., 2009), IL-8 (Gupta et al., 2005), TNF-α (Keshari et al., 2012), zymosan (Caro et al., 2014; Silva et al., 2014a), hydrogen peroxide (Fuchs et al., 2007), platelet activating factor (Caudrillier et al., 2012; Clark et al., 2007), thapsigargin (Gupta et al., 2010), chemotactic complement-derived peptide complement factor 5 (C5a) (Di Martinelly and Artiba, 2004), Fc receptors (Urban et al., 2006), IFN-γ (Yousefi et al., 2008), lipophosphoglycans (LPG) of *Leishmania* spp. promastigote stages (Guimarães-Costa et al., 2009), *Staphylococcus epidermidis* δ-toxin (Cogen et al., 2010), and antimicrobial peptide LL-37 (Neumann et al., 2014a; Neumann et al., 2014b).

Besides PMN, extracellular traps (ETs) are also released by other leukocytes against invasive pathogens such as macrophages (Braian et al., 2013), monocytes (Chow et al., 2010), mast cells (Köckritz-Blickwede et al., 2008), eosinophils (Yousefi et al., 2008; Muñoz-Caro et al., 2016), and basophils (von Köckritz-Blickwede and Nizet, 2009) of mammals including human (Wong and Jacobs Jr, 2013), cattle (Muñoz-Caro et al., 2014; Wei et al., 2018), goats (Yang et al., 2018), dogs (de Buhr et al., 2018), rats (Delbosc et al., 2011), mice (Boe et al., 2015) and harbour seals (Reichel et al., 2015).

1.5 Metabolism during NETosis

1.5.1 Metabolism

Metabolism is a general cellular biochemical process that is defined to describe all chemical reactions occurring in living cells and organisms, and which are usually divided into two

metabolic pathways: *i)* catabolism which consumes energy to assemble small molecules (blocking units) into larger ones, and *ii)* anabolism which disassembles large molecules into smaller ones to produce energy.

All living organisms and cells covert their food (nutrient) energy supply to a form that organisms or cells can use them via diverse metabolic pathways (Fig. 7). Firstly, the major constituents of foods, i. e. carbohydrates, lipids, and proteins, are broken down into smaller subunits such as amino acids, monosaccharides, and fatty acids via digestion (El Bacha et al., 2010). Molecules such as glucose, fatty acids, glycerols, and amino acids, followed by being transported throughout the body via the circulating system or within cells are essential and being dependent (aerobic/oxidative metabolism) or independent of oxygen (anaerobic metabolism). Secondly, these subunits are converted into a few metabolites via several chemical reactions included in differing metabolic pathways such as glycolysis. In the end, the metabolites are converted into carbon dioxide (CO_2) and water, and a large number of energy in the form of adenosine triphosphate (ATP) are produced via the oxygen-dependent reactions of the tricarboxylic acid (TCA) cycle and electron transport chain (El Bacha et al., 2010).

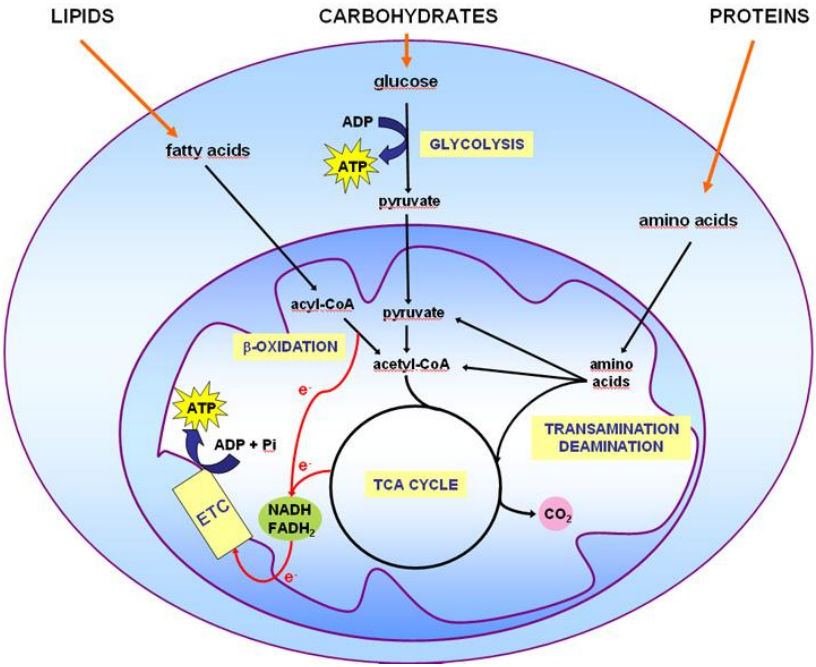

Fig. 7. Schematic representation of energy extraction in oxidative metabolism. The body breaks down food into amino acids, monosaccharides, and fatty acids, and then cells degrade these molecules to a few simple metabolites. At the end, the oxygen-dependent reactions of the tricarboxylic acid (TCA) cycle and electron transport chain liberate large amounts of energy in the form of ATP (adapted from El Bacha et al., 2010)

Carbohydrates including both simple and complex sugars are the major source of energy for living cells (Moore and Hatfield, 1994), and monosaccharide glucose is the pivotal molecule in carbohydrate metabolism. Energy from carbohydrates is extracted by living cells mainly via four pathways: glycolysis, conversion of pyruvate to acetyl CoA, the TCA cycle, and the electron transport chain. Glycolysis is a series of ten enzyme-catalyzed reactions occurring in the cytosol, at the end of which two ATP, two NADH, and two pyruvate molecules are finally produced (Moore and Hatfield, 1994). When oxygen is absent or mitochondria are limited, pyruvate is converted into lactate along with the regeneration of NAD+ that is needed by glycolysis. If oxygen is readily available, each pyruvate molecule yields one acetyl CoA, one CO_2, and one NADH in the mitochondria. Subsequently, acetyl CoA is converted to oxaloacetate, which enters the TCA cycle. TCA cycle is a circular pathway composed of a

sequence of reactions in the mitochondria, through which each acetyl CoA generates one GTP, three NADH, and one $FADH_2$. In the end, NADH and FADH deliver pairs of high-energy electrons to the electron transport chain located in the inner mitochondrial membrane, and a large number of ATP are released via oxidative phosphorylation. Totally, 36 net ATP are produced from one glucose molecule (Lopaschuk and Saddik, 1992).

Fats and oils are both triglyceride molecules, which play an important role in storing energy. To extract energy from triglycerides, they are firstly degraded into glycerol and fatty acids. Fatty acids contain almost all the energy in triglycerides, and can be transferred by carnitine into mitochondria where they are disassembled via beta-oxidation and a group of acetyl CoA are produced. As mentioned above, one acetyl CoA can generate numerous ATP via TCA cycle and electron transport. Therefore, a triglyceride molecule containing three fatty acids produces more than ten times ATP than a glucose molecule after a complete metabolic breakdown process (Vander Heiden et al., 2009).

Proteins are not used as primary sources of energy (Hoffman and Falvo, 2004), since they have a variety of vital structural and functional roles in different forms of blocking units such as enzymes, signaling receptors, structural members, intracellular trafficking components, ion pumps, and ion channels (Alberts et al., 2002). However, if carbohydrates and fats are not available amino acids degraded from proteins can be used as an alternative energy source. Firstly, amino acids are converted into carbon skeletons via deamination. These carbon skeletons from different types of amino acids form different products such as pyruvate, acetyl-CoA, acetoacetyl-CoA, α-ketoglutarate, suc-CoA, fumarate, and oxaloacetate (Berg et al., 2002), and enter different catabolic pathways. Therefore, the amount of ATP produced from one amino acid depends upon which catabolic pathway it enters (Alberts et al., 2002; Berg et al., 2002).

1.5.2 Metabolism of PMN

All cells in mammalian species need energy to maintain their basic functions. Unlike other immune cells such as lymphocytes and macrophages, PMN have relatively few mitochondria and which are less active (Fossati et al., 2003; Maianski et al., 2004), and thus PMN obtain their energy mainly from the glycolysis pathway (Borregaard and Herlin, 1982; Van Raam et al., 2008). As for other host cells, ATP is the fundamental energy carrier to fuel cellular functions of PMN such as phagocytosis and degranulation. Thus, it was reported that ATP

required for diverse critical PMN-derived NOX activities resulting in reaction oxygen species (ROS) production (Zhang et al., 1996), degranulation (Kannan, 2001), and phagocytosis (Borregaard and Herlin, 1982), are mainly produced through glucose catabolism resulting in lactate production (Beck and Valentine, 1952). Only 2-3 % glucose is involved in the oxidation via the TCA cycle in PMN (Beck, 1958). In the absence of glucose, the rate of ATP generation in PMN is enhanced via glycogenolysis during phagocytosis, and the content of intracellular ATP is significantly decreased (Borregaard and Herlin, 1982). Conversely, in presence of glucose, ATP concentration has a quick reduction in order to perform phagocytosis, but the rate of ATP generation from glucose to lactate is not increased (Borregaard and Herlin, 1982).

In contrast to PMN-derived metabolic requirements for oxidative burst-, degranulation- and phagocytic activities, only two reports exist on PMN metabolic pathways being associated with NETosis. Recently, Menegazzo et al. (2015) reported that NETosis was dependent on glucose molecules. As such, high glucose concentrations increased PMN undergoing NETosis as well as their NETs release (Menegazzo et al., 2015; Rodríguez-Espinosa et al., 2015). Moreover, inhibition assays of glycolysis and ATP synthase revealed that glycolysis played a crucial role during NETosis, and seeming that oxidative phosphorylation was not so important as expected (Rodríguez-Espinosa et al., 2015).

In addition, the amino acid glutamine has been reported to be helpful for the increasing ability of PMN to display proper antimicrobial defense mechanisms. Studies showed that glutamine enhanced the bactericidal ability (Ogle et al., 1994) and improved phagocytosis of PMN (Furukawa et al., 2000), indicating that glutaminolysis also plays an important role in PMN metabolism.

1.6 Autophagy during NETosis

Autophagy is an intracellular programmed degradation system, which recycles unnecessary or damaged components including proteins and organelles, and is essential in cellular response to different stress factors such as hypoxia, inflammation and oxidative burst (Skendros et al., 2018). Autophagy (or macroautophagy) is a dynamic multi-step process which is mainly divided into four stages controlled by autophagy-related (Atg) proteins (please refer to Fig. 8): *i)* induction and initiation of autophagy, *ii)* expansion and completion of phagophore, *iii)* autophagosome maturation and fusion with lysosome, *iv)* and degradation

of engulfed components (You et al., 2016). Autophagosome as a landmark of autophagy is a double-membraned organelle, and formed by the elongation of a unique isolation membrane (phagophore), which sequesters unnecessary cytoplasmic contents. At the end, autophagosomes fuse with lysosomes to degrade and recycle the cargo inside (Bernard and Klionsky, 2013). LC3 is a small soluble protein that is distributed ubiquitously in mammalian tissues and cultured cells which is a well-known protein to form a stable association with the autophagosome membrane (Tanida et al., 2008; Bernard and Kionsky, 2013). Once activation, LC3-I (a cytosolic form of LC3) is conjugated to phosphatidylethanolamine to form LC3-II, which is then recruited to autophagosomal membranes (Tanida et al., 2008). Thus, LC3 is widely used as a marker for microscopic detection of autophagosomes.

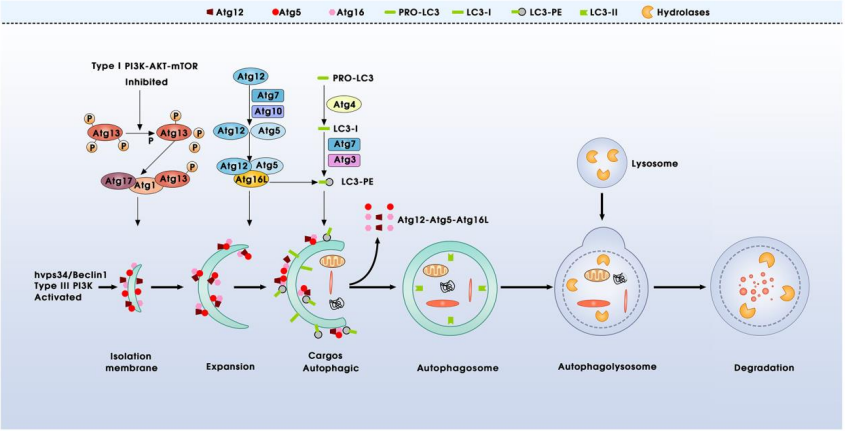

Fig. 8. Schematic model of autophagy process. Autophagy is a multi-step process involving at least four main phases, which is controlled by more than 30 autophagy-related (Atg) proteins and mediated by two ubiquitin-like conjugation systems, Atg12-Atg5 and Atg8/LC3: including the initiation, vesicle expansion and completion, maturation and fusion, and ultimate degradation of the membrane and its contents within the lysosomes (according to You et al., 2017).

The intracellular process of autophagy has been shown to be involved in various cellular functions including proliferation, differentiation, homeostasis and cell survival (Levine and Kroemer, 2008; Mizushima, 2007), and playing a crucial role during diverse diseases such as neurodegenerative disorders [e. g. Alzheimer's syndrome, transmissible spongiform encephalopathies (TSE), Parkinson- and Huntington syndrome] (Rubinsztein et al., 2007; Williams et al., 2006), liver diseases (Rautou et al., 2010), muscle diseases (Sandri, 2010),

heart diseases (Gustafsson and Gottlieb, 2009; Kirshenbaum, 2012; Wang et al., 2015) and even in cancer progression (Mathew et al., 2007). Besides, autophagy is also implicated in a variety of inflammatory and autoimmune diseases such as systemic lupus erythematosus (Zhang et al., 2015), Zika virus infection (Chiramel and Best, 2018) and Crohn's disease (Hooper et al., 2016).

Accumulating studies have also demonstrated the role of autophagy in host innate immune defence against different kinds of pathogens including bacteria, protozoa, and viruses (Deretic, 2005; Levine and Deretic, 2007; Schmid and Münz, 2007). Autophagy contributes in the elimination of invading microorganisms such as *Mycobacterium tuberculosis* (Gutierrez et al., 2004; Watson et al., 2015), *Shigella* spp. (Krokowski et al., 2018; Ogawa et al., 2005), and *Streptococcus* spp. (Nakagawa et al., 2004; Ogawa et al., 2018). In the early recognition of invading microorganisms, host innate immune system senses the presence of microbes via PRRs, which contain Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (Takeuchi and Akira, 2010). It has been revealed that autophagy is activated by the stimulation of a group of TLRs and thereafter able to eliminate intracellular pathogens (Delgado et al., 2008; Franco et al., 2017; Sanjuan et al., 2007; Vural et al., 2015; Xu et al., 2007). Lipopolysaccharide (LPS) known as a TLR4 ligand is capable of inducing autophagosome formation in murine RAW264.7 macrophage-like cells (RAW), which is dependent on TLR4, TRIF, RIP, and p38 MAPK (Xu et al., 2007). Two TLR7 ligands, i. e. single-stranded RNA and imiquimod, induced LC3 puncta formation in RAW and J774 macrophage-like cells, and the formation of LC3 puncta depended on TLR7, Beclin 1, and MyD88 (Delgado et al., 2008).

Consistently to these findings, autophagy has been described to occur in PMN derived from diverse mammalian species, including mice and humans (Mitroulis et al., 2010), and further occurring in a phagocytosis-independent and phagocytosis-dependent manner (Huang et al., 2009; Mitroulis et al., 2010). These autophagy findings in PMN are similar to data demonstrated in the past for macrophage active phagocytosis (Deretic, 2006; Shi and Kehrl, 2008, 88; Xu et al., 2007). Recently, first evidence suggested that in addition to PMN phagocytosis, autophagy was also necessary to adequately prime PMN to undergo NETosis (Park et al., 2017; Remijsen et al., 2011; Skendros et al., 2018). Besides Atg proteins, autophagy is regulated by the metabolic sensor molecule AMP-activated kinase α (AMPKα) and by the mechanistic target of rapamycin (mTOR) (Laplante and Sabatini, 2013). Processes of PMN-autophagy resulting in NETosis appear to be mainly linked to PMA-activated PMN

and furthermore in sterile inflammation (Mitroulis et al., 2010) by a mechanism which seems dependent on mTOR activation (Itakura and McCarty, 2013). A recent study showed that mTOR signaling regulated extrusion of NETosis via induction of hypoxia-inducible factor 1 α (HIF-1α) protein expression. In this context, it was demonstrated that PMN expression of HIF-1α and NETosis were significantly reduced via the blockage of mTOR activity with rapamycin (McInturff et al., 2012), which is contrary to another study which revealed enhancement of NETosis by inhibition of mTOR signaling (Itakura and McCarty, 2013). In this doctoral thesis, we intend to investigate for the first time the role of autophagy in *B. besnoiti* induced NETosis. Furthermore, different signaling pathways of NETosis, NETosis phenotypes [i. e. spread-, diffuse-, aggregated-NETs (Muñoz-Caro et al., 2018); 'cell free'- and 'anchored'-NETs (Tanaka et al., 2014)], and metabolic requirements in *B. besnoiti*-triggered suicidal NETosis will be here investigated. Last but not least, both parasitic stages to be found during bovine besnoitiosis *in vivo*, namely tachyzoites and bradyzoites, will be used and compared concerning their capabilities in NETosis induction and impact on primary bovine endothelium.

2. PUBLICATIONS

2.1 Simultaneous and positively correlated NET formation and autophagy in *Besnoitia besnoiti* tachyzoite-exposed bovine polymorphonuclear neutrophils

Zhou, E., Conejeros, I., Velásquez, Z., Muñoz-Caro, T., Gärtner, U., Hermosilla, C., Taubert, A. (2019). **Simultaneous and positively correlated NET formation and autophagy in *Besnoitia besnoiti* tachyzoite-exposed bovine polymorphonuclear neutrophils**. *Front Immunol*, 10, 1131. doi: 10.3389/fimmu.2019.01131

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Initiative	Weitestgehend eingeständig
Projektplannung	Weitestgehend eingeständig
Durchführung des Versuches	Wesentlich
Auswertung der Experimente	Wesentlich
Erstellung der Publikation	Weitestgehend eingeständig

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Simultaneous and Positively Correlated NET Formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-Exposed Bovine Polymorphonuclear Neutrophils

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Given that *B. besnoiti* tachyzoites infect host endothelial cells of vessels *in vivo*, they become potential targets for professional phagocytes [e.g., polymorphonuclear neutrophils (PMN)] when in search for adequate host cells or in case of host cell lysis. It was recently reported that *B. besnoiti*-tachyzoites can efficiently be trapped by neutrophil extracellular traps (NETs) released by bovine PMN. So far, the potential role of autophagy in parasite-triggered NET formation is unclear. Thus, we here analyzed autophagosome formation and activation of AMP-activated protein kinase α (AMPKα) in potentially NET-forming innate leukocytes being exposed to *B. besnoiti* tachyzoites. Blood was collected from healthy adult dairy cows, and bovine PMN were isolated via density gradient centrifugation. Scanning electron microscopy confirmed PMN to undergo NET formation upon contact with *B. besnoiti* tachyzoites. Nuclear area expansion (NAE) analysis and cell-free and anchored NETs quantification were performed in *B. besnoiti-induced* NET formation. Interestingly, tachyzoites of *B. besnoiti* additionally induced LC3B-related autophagosome formation in parallel to NET formation in bovine PMN. Notably, both rapamycin- and wortmannin-treatments failed to influence *B. besnoiti*-triggered NET formation and autophagosome formation. Also, isolated NETs fail to induce autophagy suggesting independence between both cellular processes. Finally, enhanced phosphorylation of AMP activated kinase α (AMPKα), a key regulator molecule of autophagy, was observed within the first minutes of interaction in tachyzoite-exposed PMN thereby emphasizing that *B. besnoiti*-triggered NET formation indeed occurs in parallel to autophagy.

Keywords: *Besnoitia besnoiti*, PMN, NET formation, autophagy, cattle, AMPKα

INTRODUCTION

Besnoitia besnoiti is a cyst-forming apicomplexan protozoan parasite that causes bovine besnoitiosis which is traditionally endemic in Africa and Asia. Recent continuous reports on bovine besnoitiosis outbreaks in several European countries (1–9) indicated a re-emergence and spread of this disease in Europe (10) and led to the classification as emerging disease by the European Food Safety

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

Authority (EFSA) in 2010. Overall, bovine besnoitiosis has a detrimental impact on both, individual animal welfare (e.g., pain, oedemas, fever, abortion, orchitis, male infertility, severe skin lesions) and cattle industry (losses).

So far, very little data is available on early host innate immune reactions during primary acute *B. besnoiti* infections *in vivo* (11) and *in vitro* (12, 13) despite the fact that early host innate defense reactions should be critical for the outcome of the disease. In this sense, PMN play a pivotal role since they are the most abundant leukocyte population in blood and the first ones to be recruited to sites of infection. As reported for other mammalian species, bovine PMN own several efficient effector mechanisms to combat apicomplexan stages, such as phagocytosis (14), production of reactive oxygen species (ROS) (15) and *in vitro* excretion of antimicrobial peptides. Additionally, the release of neutrophil extracellular traps (NETs) in response to coccidian protozoa was reported (13, 16–18). NETs are commonly released via a PMN-derived cell death process known as NET formation (19). Suicidal NET formation was described as a NADPH oxidase (NOX)-dependent cellular mechanism which induces the extrusion of DNA and nuclear and cytoplasmic granule enzymes leading to the formation of DNA-rich networks being decorated with histones and various potent antimicrobial granular effector molecules, such as neutrophil elastase (NE), myeloperoxidase (MPO), lactoferrin, pentraxin, peptidoglycan recognition proteins, or calprotectin (19–22). A variety of invasive pathogens such as bacteria, virus, fungi, protozoan, and metazoan parasites, might either be immobilized within released sticky NET structures or be killed via local high concentration of antimicrobial histones, peptides, and proteases (16, 20, 23–25).

Classical suicidal NET formation is signaled via Raf-MEK-ERK-dependent pathways (18, 19, 26, 27). Besides NOX-dependent NET formation, NOX-independent NET formation also exists and seems to be linked to a substantial reduction of ERK1/2 activation and weak Akt activation, whilst p38 MAPK activation appears similar in both types of NET formation (28, 29). In addition to suicidal NET formation, PMN have also been shown to undergo vital NET formation without cell lysis, thus remaining viable and retaining the capability of active phagocytosis of bacteria (30). Furthermore, PMN seem able to release small-sized mitochondria-derived NETs without suffering cell death (31). So far, vital NET formation has not been described in response parasites. Suicidal NET formation was reported to be triggered by different protozoan parasites *in vitro* and *in vivo*, including *Plasmodium falciparum* (32), *Leishmania* spp., *Eimeria bovis* (16, 33), *Eimeria arloingi* (17), *Toxoplasma gondii* (34–36), *Cryptosporidium parvum* (37), *Neospora caninum* (18, 38, 39), *Trypanosoma cruzi* (40), *Entamoeba histolytica* (41), and *B. besnoiti* (12, 13).

Autophagy is an essential intracellular degradation system, that recycles cell components as proteins and organelles and it is essential in the cellular response to stress (42). In neutrophils, autophagy has been described in PMN derived from mouse and human (43, 44). Interestingly, first evidences suggest that autophagy is necessary and can prime PMN to undergo NET formation (42, 45, 46). Besides other molecules, autophagy is regulated by the metabolic sensor molecule AMP activated kinase α (AMPKα) and by the mechanistic target of rapamycin (mTOR) (47). The processes of autophagy and NET formation appear to be linked in PMA-activated PMN and in sterile inflammation (44) by a mechanism which seems dependent on mTOR activation (48).

So far, *B. besnoiti*-mediated NET formation seems to be NOX-, NE- MPO-dependent and capable to efficiently hamper tachyzoites from active host cell invasion (13). On this regard, AMPK is been described as critical molecule of the autophagic process and governing critical functions in PMN as ROS production, chemotaxis and phagocytosis (49, 50). Despite this, nothing is known on the role of autophagy or autophagy-related molecules such as AMPKα in *B. besnoiti*-triggered NET formation.

Aim of the current study was to analyze the presence of autophagy during *B. besnoiti*-triggered suicidal NET formation. Therefore, we first confirmed NET formation induction by *B. besnoiti* tachyzoites and then showed that both, NET formation and autophagy are performed independent of rapamycin (stimulator of autophagy via mTOR binding), wortmannin (inhibitor of PI3K-mediated autophagy), treatments. In addition, we studied the release of extracellular DNA induced by *B. besnoitia* tachyzoites in presence of the autophagy-related molecules: LY294002 (inhibitor of PI3K-mediated autophagy) parthenolide (NF-κB inhibitor) or WP1130 (ubiquitinase inhibitor). Interestingly, NET formation and autophagosome formation occur simultaneously in tachyzoite-exposed PMN and is accompanied by a rapid phosphorylation of AMPKα.

MATERIALS AND METHODS

Ethics Statement

This study was conducted in accordance to Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols were approved by Ethic Commission for Experimental Animal Studies of Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No.521_AZ) and in accordance to European Animal Welfare Legislation: ART13TFEU and current applicable German Animal Protection Laws.

Parasites

All NET formation -related experiments were performed with tachyzoite stage of the apicomplexan parasite *B. besnoiti* (strain Bb Evora04) which was initially isolated from the field in Portugal as previously reported (13).

Host Cell Culture and *B. besnoiti* Tachyzoite Maintenance

Permanent Madin-Darby bovine kidney cells (MDBK) were used as host cells for *B. besnoiti* tachyzoite production *in vitro*. MDBK monolayers were cultured in 75 cm^2 plastic tissue culture flasks (Greiner) at 37°C and 5% CO_2 atmosphere until confluency using RPMI 1640 (Sigma) cell culture medium supplemented with 2% fetal bovine serum (FBS, Merck), 1% penicillin (500 U/ml) and streptomycin (500 mg/ml) (both Sigma-Aldrich).

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

Confluent MDBK layers were infected with 2×10^6 vital tachyzoites of *B. besnoiti*.

For experiments under physiological flow conditions, primary bovine umbilical vein endothelial cells (BUVEC) were isolated according to the method described by Taubert et al. (51). Briefly, umbilical cords retrieved from newborn calves via *Sectio caesarea* were enriched with 0.9% Hanks balanced salt solution (HBSS)-HEPES buffer (pH 7.4; Gibco) supplemented with 1% penicillin (500 U/ml; Sigma-Aldrich) and streptomycin (500 mg/ml; Sigma-Aldrich) and kept at 4°C until use. For isolation of host endothelial cells, the lumen of umbilical veins were infused with 0.025% collagenase type II solution (Worthington Biochemical Corporation). Veins were ligated and incubated for 20 min at 37°C and 5% CO_2 atmosphere. Then, veins were gently massaged and collagenase-cell suspensions were harvested in 50-ml plastic tubes (Nunc) containing 1 ml FCS (Gibco) to inactivate collagenase type II. After two centrifugations (400 × *g*, 10 min, 4°C), endothelial cells were resuspended in complete ECGM (endothelial cell growth medium; PromoCell), plated in 25 or 75 cm^2 plastic culture flasks (Greiner) and cultured at 37°C and 5% CO_2 atmosphere until confluency. For flow assays, BUVEC were grown on Thermanox® coverslips (Nunc) until confluency.

Isolation of Bovine PMN

Healthy adult dairy cows ($n = 9$) served as blood donors. Animals were bled by puncture of jugular vein and 30 ml blood was collected in 12 ml heparinized sterile plastic tubes (Kabe Labortechnik). Approximately 20 ml of heparinized blood were diluted in 20 ml sterile PBS with 0.02% EDTA (Sigma-Aldrich), layered on top of 12 ml Biocoll® separating solution (density = 1.077 g/l; Biochrom AG) and centrifuged (800 × *g*, 45 min). After removal of plasma and mononuclear cells, the cell pellet was suspended in 25 ml bi-distilled water and gently mixed during 40 s to lyse erythrocytes. Osmolarity was rapidly restored by adding 4 ml of 10 × Hanks balanced salt solution (Biochrom AG). For complete erythrocyte lysis, this step was repeated twice and PMN were later suspended in sterile RPMI 1640 medium (Sigma-Aldrich). PMN counts were analyzed in a Neubauer haemocytometer. Finally, freshly isolated bovine PMN were allowed to rest at 37°C and 5% CO_2 atmosphere for 30 min until further use.

Scanning Electron Microscopy (SEM)

Bovine PMN were co-cultured with vital *B. besnoiti* tachyzoites (ratio 1:4) for 60 min on coverslips (10 mm diameter; Thermo Fisher Scientific) pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich) at 37°C and 5% CO_2. After incubation, 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. Finally, all samples were visualized via a Philips® XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

Immunofluorescence Microscopy Analyses for Visualization of *B. besnoiti*-Triggered NETosis

Bovine PMN were co-cultured with *B. besnoiti* tachyzoites (ratio 1:4) for 3 h (37°C and 5% CO_2 atmosphere) on 0.01% poly-L-lysine pretreated coverslips (15 mm diameter, Thermo Fisher Scientific), fixed by adding 4% paraformaldehyde (Merck) and stored at 4°C until further experiments.

For NET visualizing, Sytox Orange (Life Technologies) was used to stain DNA and anti-histone (clone H11-4, 1:1,000; Merck Millipore #MAB3422), anti-NE (AB68672, 1:1,000, Abcam), or anti-MPO (orb11073, 1:1,000, Byorbit) antibodies were used to stain specific proteins on ETs structures. Therefore, fixed samples were washed three times with PBS, blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min at RT and incubated with corresponding primary antibody solutions for 1 h at RT. Thereafter, samples were washed thrice with PBS and incubated in secondary antibody solutions (Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG, both Life Technologies, 60 min, 1:1,000, RT). Finally, samples were washed thrice in PBS and mounted in anti-fading buffer (ProLong Gold Antifade Mountant; Thermo Fisher Scientific). Visualization was achieved using an inverted IX81 fluorescence microscope equipped with an XM 10 digital camera (Olympus).

Extracellular DNA-Based Quantification of NETs

Bovine PMN were suspended in medium RPMI 1640 lacking phenol red and serum, confronted with vital *B. besnoiti* tachyzoites (96-well plates, duplicates) at a final PMN:tachyzoites ratio of 1:4 (2×10^5 PMN + 8×10^5 *B. besnoiti* tachyzoites). Samples were incubated at 37°C and 5% CO_2. For the autophagy-related experiments, bovine PMN were pretreated with different concentrations of rapamycin (10, 50, 100, 200 nM), wortmannin (10, 50, 100, 200 nM), LY294002 100 µM, parthenolide 90 µM, or WP1130 5 µM for 30 min, then stimulated by *B. besnoiti* tachyzoites at a 1:4 PMN:tachyzoites ratio for 3 h. After incubation, samples were treated with 0.5 U/ml micrococcal nuclease (New England Biolabs) for 15 min and pelleted (300 × *g*, 5 min). Supernatants were collected for NET quantification which was performed by Picogreen®-based fluorometric measurements (19).

NETs are divided into two distinct forms: one is released away from neutrophils named cell-free NETs, and the other is those that are anchored to neutrophils (namely anchored NETs). For "cell free"- and "anchored"-NETs determination according to Tanaka et al. (52), the plate was directly centrifuged at 300 × *g* for 5 min after incubation. The supernatants were transferred into a new 96-well plate to measure "cell-free"-NETs and the pellets were used for "anchored"-NETs estimation. For both sampling methods, 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), and then extracellular DNA was detected and quantified by PicoGreen®-derived fluorescence intensities using an automated multiplate reader (Varioskan, Thermo Scientific) at 484 nm excitation/520 nm emission.

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

Estimation of "Anchored" NETs on *B. besnoiti*-Infected BUVEC Under Physiological Flow Conditions

BUVEC ($n = 3$) were cultured on Thermanox® (Nunc) coverslips pre-coated with bovine fibronectin (10 μg/ml, 2 h RT; Sigma-Aldrich) until confluency and infected with 2.5×10^5 freshly isolated *B. besnoiti* tachyzoites. Twenty-four h. p. i., coverslips were washed to remove residual tachyzoites and placed into a parallel flow chamber (53). Bovine PMN (2.5×10^6 PMN in 500 μl medium) were perfused into the system at a constant wall shear stress of 1.0 dyn/cm^2 (syringe pump sp100i®; World Precision Instruments). For "anchored"-NET formation visualization, coverslips were fixed, washed, and stained for DNA and histones as described above. Images were taken under an inverted fluorescence microscope (DM IRB; Leica) equipped with a digital camera (Olympus).

Nuclear Decondensation-Based Quantification of NETs

Nuclear expansion-based quantification of NETs was performed according to the method described by Papayannopoulos et al. (54). Briefly, bovine PMN ($n = 3$) were pretreated with rapamycin (50 nM), wortmannin (50 nM) or plain medium (RPMI 1640, Sigma-Aldrich) for 30 min and then exposed to *B. besnoiti* tachyzoites for 3 h at a 1:4 PMN:tachyzoites ratio. After incubation, PMN were fixed by 4% paraformaldehyde (Merck) and stained with 5 μM Sytox Orange® (Life Technologies) for 30 min at RT. Five images were captured randomly for each condition using an inverted fluorescence microscope (Olympus IX 81) and nuclear area size of single cells was analyzed using ImageJ® software as described by Gonzalez et al. (55). Cells that presented decondensed nucleus and exceeded the threshold of 50 μm^2 were considered as PMN undergoing NET formation. Overall, 1,200–1,700 PMN were analyzed for each experimental condition in samples from three different donors.

Autophagosome Detection by Immunofluorescence Analysis

LC3 protein is a marker for autophagosomes (56) with LC3-I being cytosolic and LC3-II being membrane-bound and enriched in the autophagic vacuole. Analysis of autophagosome formation in PMN was performed according to Itakura and McCarty (48). In brief, bovine PMN ($n = 3$) were deposited on poly-L-lysine (0.01%) pre-treated coverslips (15 mm diameter, Thermo-Fisher scientific), pretreated with rapamycin (50 nM) or wortmannin (50 nM) for 30 min before being exposed to *B. besnoiti* tachyzoites at a 1:4 PMN:tachyzoite ratio for 3 h. After incubation, cells were fixed with 4% paraformaldehyde (10 min), permeabilized by ice cold methanol treatment (3 min at 4°C) and blocked with blocking buffer (5% BSA, 0.1% Triton X-100 in sterile PBS; all Sigma-Aldrich) for 60 min at RT. Thereafter, cells were incubated overnight at 4°C in anti-LC3B antibody solution (cat#2775 Cell Signaling Technology) diluted 1:200 in blocking buffer. After incubation, samples were washed thrice with PBS and incubated 30 min in the dark and RT in a 1:500 dilution of goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen). After three washes in PBS, samples were mounted in Prolong Anti-fading reagent with DAPI® (Invitrogen) on glass slides and images were taken applying confocal microscopy (Zeiss LSM 710). To estimate LC3B-positive cells, the background fluorescence signal was determined in control conditions for FITC (green) and DAPI (blue) channels. Image processing was carried out with Fiji ImageJ® using Z-project and merged channel plugins and restricted to overall adjustment of brightness and contrast.

Immunoblotting-Based Analysis of LC3B- and AMPK-Expression in Bovine PMN

Proteins from tachyzoite-exposed and non-exposed bovine PMN were extracted by lysing 5×10^6 PMN using a ultrasound sonicator (20 s, 5 times) in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.5% Na-deoxycholate; 0.1% SDS; 150 mM NaCl; 2 mM EDTA; 50 mM NaF, all Roth) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged (10,000 × g, 10 min, 4°C) to sediment intact cells and nuclei, the supernatant was transferred to new tubes and the protein content was quantified via Coomassie Plus Assay Kit (Thermo Scientific) according to the manufacturer's instructions. For immunoblotting, samples were supplemented with 6 M urea. After boiling (95°C, 5 min), 60 μg of total protein/slot were electrophoresed in 12 or 15% polyacrylamide gels (100 V, 90 min) using a Mini-PROTEAN Tetra Cell system (Biorad). Proteins were then transferred (300 mA, 2h) to polyvinylidene difluoride (PVDF) membranes (Millipore) using a semidry blotting instrument (Mini-transfer blot, Biorad). Samples were first incubated in blocking solution (3% BSA in TBS containing 0.1% Tween, all Sigma-Aldrich) (1 h, RT) and then overnight at 4°C in anti-LC3B (Cat#2775, 1:1,000, Cell Signaling), anti-Atg5 (Cat#ab108327, 1:1,000 abcam), and anti-AMPKα T172 (Cat#5832, 1:1,000 Cell Signaling) antibody solution diluted in blocking solution. Detection of vinculin (Cat#sc-73614, 1:1,000, Santa Cruz) was used for the normalization of the sample. Signal detection was accomplished by applying solutions of corresponding secondary antibodies conjugated with peroxidase (Cat#31430, 1:40,000 and Cat#31460, 1:40,000; both Pierce) and enhanced chemiluminescence detection system (ECL® plus kit, GE Healthcare). Protein signals were recorded in a ChemoCam Imager® (Intas Science Imaging). Protein sizes were controlled by a protein ladder (PageRuler® plus prestained protein ladder ~10–250 kDa; Thermo Fisher Scientific). Quantification of protein band intensities was performed by the use of Image J® software (Fiji version using gel analyzer plugin).

Statistical Analysis

Statistical significance was defined by a p value <0.05. p value were determined by applying non-parametric analyses: Mann-Whitney test when two experimental conditions were compared and Kruskal-Wallis test followed by Dunn's *post-hoc* test for multiple comparisons. Correlation between LC3B-positive and NETotic PMN was determined by Spearman correlation test. All graphs (mean ± SD) and statistical analyses were generated by the use of Graph Pad software (v. 7.03).

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

RESULTS

Visualization of *B. besnoiti*-Triggered NETs in Bovine PMN

SEM analysis showed that bovine PMN (**Figure 1**) exposed to vital *B. besnoiti* tachyzoites released NET-like structures and many *B. besnoiti* tachyzoites were firmly trapped by these filaments (**Figures 1A–C**). To verify that bovine PMN were indeed undergoing NET formation, the main components of NETs [i.e., histones (H11-4) and NE] were visualized by immunostaining. Co-localization analyses of extracellular DNA being adorned with H11-4, NE (**Figures 1D–I**) in parasite-entrapping structures confirmed classical characteristics of NETs. Furthermore, tachyzoites were entangled in these NETs structures confirming the observations in SEM analysis (**Figures 1D,I**; Control condition is shown in **Figures S6** and **S7**).

Given that *B. besnoitia* tachyzoites develop within endothelial host cells, we wondered whether infected endothelium would also contribute to NET formation. Therefore, we chose an experimental approach which mimicked the *in vivo* situation in a small vessel: controlled physiological flow condition of 1.0 dyn/cm^2 shear stress was applied on endothelial cell layers and a fixed number of PMN were floating over *B. besnoiti*-infected endothelial cells in parallel plate chamber assay (53). Under these flow conditions, bovine PMN also underwent "anchored"-NET formation on *B. besnoiti*-infected BUVEC which was also corroborated by co-localization of extracellular DNA decorated with histones (**Figure 2**).

Effects of Autophagy on *B. besnoiti*-Stimulated NET Formation in Bovine PMN

To investigate effects of autophagy on *B. besnoiti*-triggered bovine NET formation, initially we used the mTOR-mediated autophagy inducer rapamycin (48) and the PIK3-mediated autophagy inhibitor wortmannin (57).

In a first experimental series, NET formation was measured based on PicoGreen®-derived fluorescence intensities as previously described (18, 19) thereby rather targeting late phase of NETosis. Overall, confrontation of bovine PMN with *B. besnoiti* tachyzoites resulted in a significant increase of NET formation when compared to control groups ($p = 0.02$–0.03; **Figures 3A–C**). Given that we always experience high individual variations in NET-related assays, the reactions induced in PMN derived from each animal ($n = 9$) are also depicted (**Figure 3A**). However, parasite-mediated NET formation was neither affected by rapamycin (tested in a range from 10 to 200 nM, **Figure 3B**) nor by wortmannin treatments (tested in the same range of concentration, **Figure 3C**). This lack of effect was also observed when non-stimulated PMN were treated with these compounds (100 nM) for control purposes (**Figure 3D**).

In a second series of experiments, an alternative method of NET quantification was applied which allowed for "cell free"- and "anchored"-NETs distinction by following the methodology described by Tanaka et al. (52). Overall, zymosan treatment which was used for positive control resulted in a highly significant increase of both types of NETs, i.e., "cell free"- and "anchored"-NETs in bovine PMN ($p < 0.0001$; **Figures 3E–H**). In addition, confrontation of PMN with *B. besnoiti* tachzoites in principle also triggered both kinds of NETs as seen for "anchored"-NETs in the rapamycin-related ($p = 0.008$; **Figure 3E**) and the wortmannin-related data set ($p = 0.007$; **Figure 3G**) and for "cell free"-NETs in the wortmannin-related dataset ($p = 0.02$; **Figure 3H**). Interestingly, the induction of "anchored"-NETs by parasite stages was more evident than the induction of "cell free"-NETs. In agreement with the data mentioned above, neither rapamycin nor wortmannin treatments led to altered parasite-triggered "cell free"- nor "anchored" NET formation (**Figures 3E–H**). Since autophagy is a very complex process, and various signaling pathways are involved in autophagosome formation, we used more pharmacological regulatory factors to check if autophagy affects NET formation via appropriate pathways. In our experimental setting, LY294002 (PI3K inhibitor) and pathenolide (NF-κB inhibitor) nor WP1130 (deubiquitinase inhibitor) treatments did not alter anchored and cell free NET formation (**Figure 3I,J**).

In a third series of experiments, we chose to analyze parasite-triggered NET formation based on nuclear area expansion (NAE). In general, PMN undergo several stages of NET formation including NE- and MPO-dependent chromatin decondensation (54). Decondensed PMN nuclei are considered as a marker for early "NETotic" processes (55). In order to determine if *B. besnoiti* induces NAE in bovine PMN, as well as to estimate if rapamycin or wortmannin influences this parameter of NET formation, 1,200–1,700 cells were individually analyzed per condition (**Figures 4C–H**) and data illustrated via frequency histograms (**Figure 4I**) and percentage of cells undergoing early NET formation (**Figure 4J**). In agreement to data on the later phase of NET formation (**Figure 3**), confrontation with *B. besnoiti* tachyzoites significantly induced nuclear area expansion in a higher percentage of bovine PMN thereby indicating early NET formation processes ($p = 0.03$); illustrated in **Figures 4B,F**, data sets in **Figures 4I,J**, control condition is shown in **Figures 4A,C**. Interestingly, we also observed an increase of PMN populations showing NAE in case of rapamycin treatments (inducer of autophagy) of tachyzoite-exposed PMN, however, these reactions showed no significance in relation to untreated controls (PMN + *B. besnoiti*) due to the high individual variation of the donors already mentioned above (**Figure 4J**). Given that significant differences were indeed detected referring to rapamycin-treated tachyzoite-exposed groups and to parasite-free rapamycin controls ($p = 0.04$), an influence of rapamycin and therefore of autophagy on parasite-triggered NET formation may be stated in that sense that induction of autophagy leads to enhanced early NET formation. The fact that this effect could not be detected by the other methods of NET quantification used before (see **Figure 3**) may be due to the targeted early/late phase of NET formation.

Effects of *B. besnoiti* Tachyzoite Exposure on Autophagosome Formation in PMN

Given that data on NAE indicated a link between autophagy and parasite-triggered early NET formation, we additionally analyzed the effect of tachyzoite exposure on PMN-derived

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

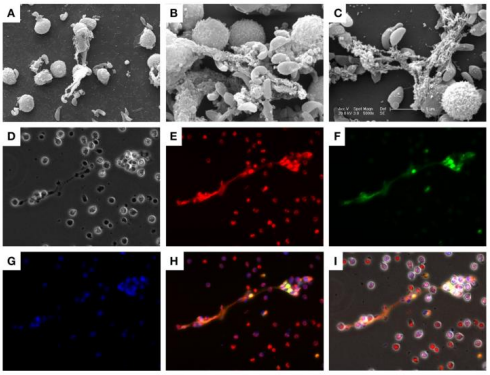

FIGURE 1 | *Besnoitia beonoiti* tachyzoite-induced NET formation in bovine PMN. Co-cultures of bovine PMN and *B. besnoiti* tachyzoites were fixed and analyzed by scanning electron microscopy (SEM) analysis. **(A–C)** NETs, defined as chromatin extracellular structures forming a meshwork in contact with the tachyzoites, were confirmed and visualized via immunostaining. **(D)** Phase contrast image; **(E)** DNA staining; Sytox Orange; **(F)** histone (H11-4) staining; **(G)** neutrophil elastase (NE) staining; **(H)** Merged image of **(E–G)**; **(I)** Merged image of all channels.

autophagosome formation. During autophagy, the cytosolic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II which allows LC3 to become associated with autophagic vesicles (58). We therefore used an antibody directed against the splice variant LC3B as a marker to investigate the effect of tachyzoite exposure to PMN-derived autophagy and of autophagy on *B. besnoiti*-induced bovine NET formation. Overall, exposure to *B. besnoiti* tachyzoites led to significant autophagosome formation in exposed bovine PMN ($p = 0.01$); for illustration, see **Figures 5A,B**; triangles, for data see **Figure 5D**. Notably, cells undergoing autophagy also showed NET formation against *B. besnoiti* tachyzoites, which were firmly entrapped in DAPI-labeled chromatin structures (illustrated in **Figure 5B**, arrows, more images are shown in **Figure S3**). Thus, we analyzed LC3B expression in tachyzoites-exposed PMN and in rapamycin and wortmannin pretreated PMN (**Figure 5C**). The percentage of LC3B positive cells was increased in the *B. besnoitia* exposed condition but was not affected by rapamycin or wortmannin (**Figure 5D**). However, an increase of 8% was observed in PMA-treated PMN that were incubated previously with rapamycin **Figure S1**. Finally we analyzed the data by Spearman correlation test indicating a positive correlation of NET formation and autophagy in tachyzoite-exposed PMN (**Figure 5D**). In a different approach we evaluated autophagosome formation in PMN confronted with isolated NETs obtained as described (59). Isolated NETs failed to induce autophagosome formation. Figures and analysis are shown in **Figure S2**.

B. besnoiti Tachyzoite Exposure Induces LC3B and p-AMPKα Protein Expression in Bovine PMN

Given that exposure to *B. besnoiti* tachyzoites induces autophagy in bovine PMN, we here analyzed the protein expression of LC3B and of phosphorylated AMPKα as a key regulator molecule of autophagy in a kinetic experiment (protein extracts of tachyzoite-exposed PMN isolated after 5, 30, 60, and 180 min of co-culture). For control reasons, protein extracts from pure *B. besnoiti* tachyzoites (three different isolates, 60 min of incubation) were included in the assays (**Figure 6**). Immunoblotting analyses revealed a non-statistically significant increase in LC3BII expression in *B. besnoiti* tachyzoite-confronted PMN when compared non-exposed PMN (**Figure 6**). Atg 5, which plays a role in the formation and elongation of autophagosomes does not show a detectable difference in protein expression when PMN were confronted with *B. besnoiti* tachyzoites (**Figure S4**). In addition, immunoblotting experiments revealed a very distinct expression profile on p-AMPKα. Here we used an antibody that is specific for AMPK that showed phosphorylation in the alpha subunit and thereby reflected AMPK activation. Whilst neither pure tachyzoites nor PMN alone showed any signal of this molecule, AMPKα expression was clearly induced by tachyzoite exposure of PMN thereby showing a fast response

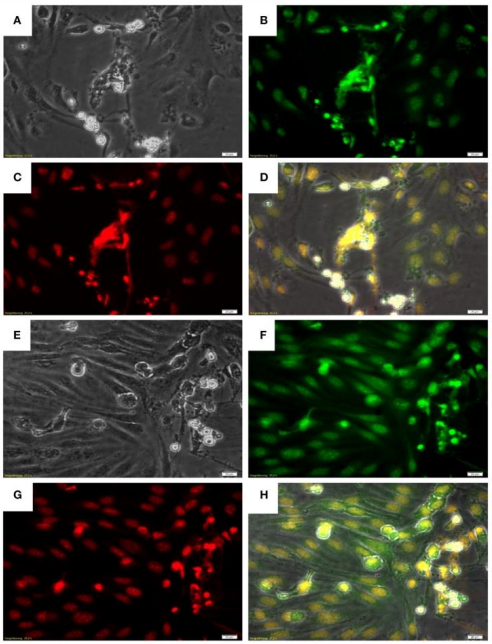

FIGURE 2 | NET formation by PMN on *Besnoitia besnoiti*-infected BUVEC under physiological flow conditions. *B. besnoiti*-infected BUVEC on Thermanox coverslips were placed into the parallel plate flow chamber and bovine PMN were perfused into the system at a constant wall shear stress of 1.0 dyn/cm^2. For NET visualization, the coverslips were fixed and stained with Sytox Orange jointly with an anti-histone antibody. **(A,E)** Phase contrast images; **(B,F)** histone staining with anti-histone antibody; **(C,G)** DNA staining using Sytox Orange; **(D,H)** Merged images: DNA (red), histones (green) and phase contrast.

pattern with enhanced expression only within the first 30 min of contact (**Figure 7**, lower panel). In all samples, two distinct protein bands at the level of the expected size (~62 and 50 kDa) were observed in AMPKα-positive Immunoblots, which most probably represents parts of a cleaved form of AMPK, a common process occurring in leukocytes (60). For AMPK, a transient pattern was observed in both, *B. besnoiti* confronted and non-confronted PMN (**Figure 7**, middle panel) showing peaks of expression at 30 and 180 min. For comparison, p-AMPKα band densitometry was obtained and normalized by vinculin signal, reinforcing the clear effect over AMPK phosphorylation showed in immunoblots. Due to the lack of signal of AMPK in animal 3, the normally used ratio of pAMPK/AMPK was not possible to apply for all the donors; however the graph corresponding to this ratio is shown in the **Figure S5**.

DISCUSSION

The initial description of NET formation was released in 2004 and was followed by the discovery of a novel programmed cell death pathway nowadays known as ETosis (19, 20, 61). ETosis comprises a unique series of cellular events by which

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

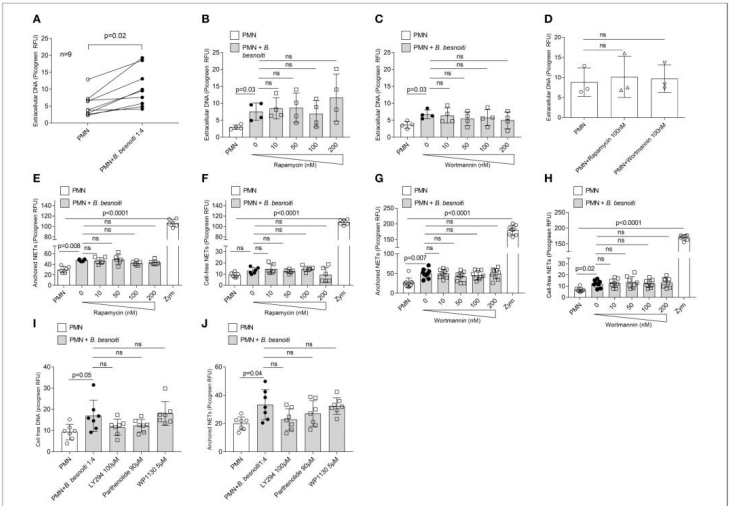

FIGURE 3 | Effects of rapamycin and wortmannin treatments on *Besnoitia besnoitia*-triggered NET formation as detected by PicoGreen-based quantification. Bovine PMN were pre-treated with increasing concentrations of rapamycin and wortmannin for 30 min and then exposed to *B. besnoiti* tachyzoites for 3 h. After incubation, the samples were analyzed as follows. **(A–D)** samples were treated with micrococcal nuclease for 15 min, thereafter centrifuged and extracellular DNA present in the supernatants were analyzed using PicoGreen-based quantification. **(E–H)** samples were centrifuged immediately and the supernatants were analyzed for "cell free" NETs. Moreover, remaining pellets located at the bottom were investigated for "anchored" NETs. For both approaches, extracellular DNA was detected and quantified via PicoGreen-derived fluorescence intensities. In all assays, zymosan treatments (1 mg/ml) were used for positive controls. Statistical analyses were performed by Kruskal-Wallis test followed by Dunn's test for multiple comparisons. *p*-values are indicated in the graphs (Mean ± SD).

nuclear contents, including chromatin and histones, mix with granular/cytoplasmic components and are released from the cell to form sticky extracellular structures capable of trapping and killing microorganisms (20, 62). Meanwhile, ETosis has been implicated in diverse diseases ranging from conditions of sterile inflammation, i.e., human gout (63, 64) and bovine synovitis (65), reproduction disorders (66, 67), cancer (68, 69), and autoimmune diseases (70, 71). Since the initial report on PMN, other leukocyte types, such as monocytes, macrophages, eosinophils, basophils, and mast cells were identified to extrude ETs (72, 73).

Concerning stimuli, both chemicals and pathogens can trigger ETosis (74, 75). Several reports have demonstrated that ETosis is efficiently induced by protozoan and metazoan parasites, such as *T. gondii, E. bovis*, *Cryptosporidium parvum, N. caninum, Dirofilaria immitis, Haemonchus contortus,* and *Schistosoma japonicum* (18, 23, 34, 37, 76, 77). Recently, Muñoz-Caro et al. (13) confirmed *B. besnoiti* tachyzoites as potent NET inducers by quantification of extracellular DNA using Picogreen®. In the current study, we aimed to take a more detailed look on *B. besnoiti*-triggered NET formation and to dissect these immune reactions into different NET types ("anchored"/"cell-free" NETs) and time-dependent reactions (early phase of NET formation vs. finalized NETs) by using different methodical approaches. Nevertheless, as proof of principle, ETosis events were first confirmed by SEM analysis in *B. besnoiti* tachyzoites-exposed bovine PMN. Immunostaining analyses revealed that these extracellular structures were mainly composed of DNA being decorated with histones, MPO and NE, thereby confirming classical components of ETs.

As also performed in the current study and leading to the confirmation of tachyzoites as NET inducers, NET formation can be estimated by quantification of extracellular DNA using specific probes as Picogreen® (19) following DNA digestion in cell culture microplates. The advantage of this approach is

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

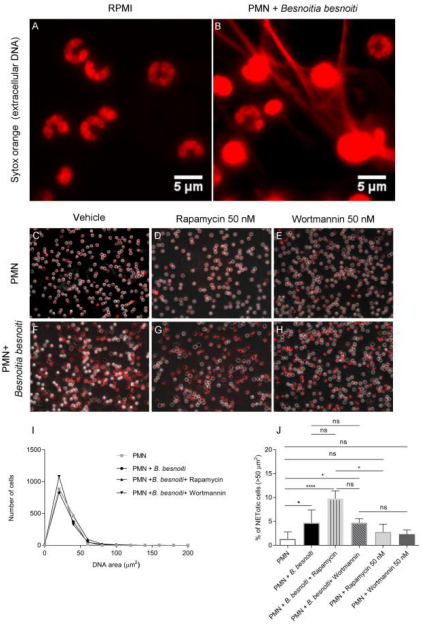

FIGURE 4 | Effects of rapamycin and wortmannin treatments on *Besnoitia besnoiti*-triggered NET formation as detected by nuclear area expansion (NAE)-based quatification. Bovine PMN were incubated in media or exposed to *B. besnoiti* tachyzoites on coverslips under different experimental conditions. **(A)** PMN alone (zoom); **(B)** PMN+*B. besnoiti* (zoom) showing PMN doing NETs and considered as positive for the cell analysis software; **(C)** PMN + rapamycin 50 nM; **(D)** PMN+*B. besnoiti* + rapamycin 50 nM; **(E)** PMN + wortmannin 50 nM; **(F)** PMN+*B. besnoiti* **(G)** PMN+*B. besnoiti* + rapamycin 50 nM; **(H)** PMN+*B. besnoiti* + wortmannin 50 nM. After fixation, NAE of 1,200–1,500 cells per condition was analyzed by ImageJ **(I)** and the percentage of NETotic cells (NAE > 50 μm^2) was calculated **(J)**. Statistical significance was defined by a *p*-value <0.05 (*$p < 0.05$, ****$p < 0.0001$) in Mann-Whitney test followed by a Dunn's multiple comparisons test.

obvious since several compounds can be tested in the same experiment, however it is advised by several authors that NET quantification should always be confirmed by microscopy (55, 78). In the current study we broadened the methodical panel to dissect between early events during NET formation by analyzing nuclear decondensation of PMN and late events by estimating the formation of "cell free" and "anchored" NETs. Overall, exposure of PMN to *B. besnoiti* tachyzoites led to a significant induction of nuclear decondensation and of "anchored"-NETs. Interestingly, the data were more consistent in terms of magnitude of the response in the formation of "anchored" (1.7-fold increase) than "cell free" NETs (1.4-fold increase). Thus, it appears that tachyzoites mainly induce "anchored" NETs. Of note was the inter-donor variation concerning NET formation quantification using this technique. However, this observation is in line with high inter-donor variations in terms of quantity and quality of NETs induced by soluble mediators such as PMA and A23187 (59, 79). In a further experimental approach using *B. besnoiti*-infected primary endothelium in a parallel plate flow chamber,

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

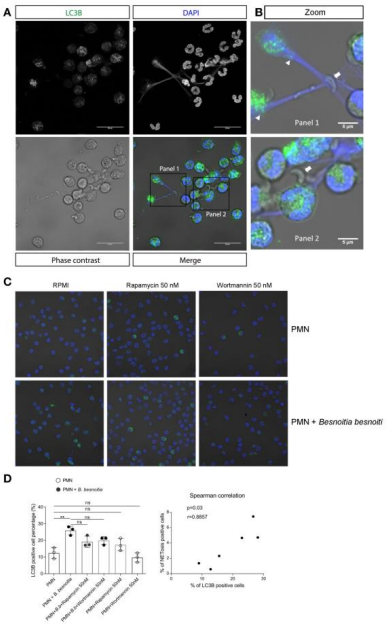

FIGURE 5 | Autophagy and NET formation occurs simultaneously in *B. besnoiti*-exposed PMN. Bovine PMN were exposed to *B. besnoiti* tachyzoites for 3 h. Samples were fixed and permeabilized for LC3B-based immunostaining to determine autophagosome formation by confocal microscopy. **(A)** Panel showing the staining for LC3B (green), DAPI (blue) phase contrast (gray scale) and merge. **(B)** Zoom-in showing autophagosome formation (triangles) and NETs entrapping tachyzoites (arrows). **(C,D)** PMN were pretreated with rapamycin or wortmannin (50 nM for 30 min) and then exposed to *B. besnoitia* tachyzoites. After 3 h of incubation, the samples were stained for LC3B **(C)** and the number of autophagosome-positive cells was determined (**D**, left graph). Finally, Spearman test (**D**, graph on the right side) revealed a positive correlation ($r = 0.89$) between LC3B expression and NETotic cells. (**$p < 0.01$, Kruskal Wallis test, followed by Dunn's *post-hoc* test for multiple comparisons).

we could confirm the formation of "anchored"-NETs under physiological flow conditions by co-localization of extracellular DNA with histones. This confirms a potential cross-talk between activated endothelium and leukocytes to be recruited to site of infection. Whether these extruded "anchored"-NETs might impact on intracellular located *B. besnoiti* tachyzoites needs further investigation. However, this observation might have implications on the outcome of cattle besnoitiosis since the tachyzoite stages are indeed infecting endothelium of vessels *in vivo* (10, 12).

Autophagy is a physiological process within the body which maintains homeostasis or normal function of cells by protein degradation and turnover of destroyed cell organelles for new cell formation after cellular stress (80). Furthermore, autophagy has been shown to play a pivotal role in regulating early innate leukocyte-associated effector mechanisms against pathogens, such as phagocytosis (81), cytokine secretion (82), and NET formation (46). In this regards, mTOR pathway plays a key role in NET formation via regulation of autophagy pathways (48). Thus, Park et al. (50) showed using a Sytox Green-related assay

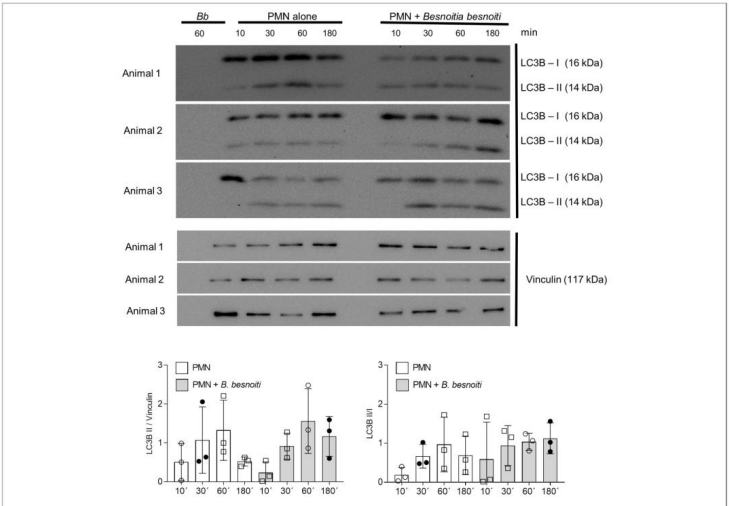

FIGURE 6 | LC3B expression in *B. besnoiti* tachyzoite-exposed PMN. Protein extracts of *B. besnoiti* tachyzoite-exposed PMN ($n = 3$) were performed at different time points after exposure and analyzed for LC3B-I and LC3B-II expression by immunoblotting. The expression of vinculin was controlled as an internal reference protein.

for NET quantification, that rapamycin pretreatments primed human PMN enhancing NET formation in response to PMA. This reaction was reversed by a panel of different autophagy inhibitors. In this set-up, rapamycin treatments alone did not influence NET formation. In our experimental set-up using *B. besnoiti* tachyzoites instead of PMA, rapamycin treatments did not influence the degree of parasite-triggered bovine NET formation when using PicoGreen-based analyses on total NETs and "anchored"/"cell-free" NETs. In addition, treatments with the autophagy-inhibitor wortmannin failed to affect parasite-triggered NET formation. This observation was complemented with the use of the PI3K inhibitor LY294002, observing a non-significant decrease of "anchored" NETs formation. Same result was observed by the use of NF-κB inhibitor. However, when estimating early NET formation via nuclear area expansion, (NAE) analysis, we found that rapamycin pretreatments indeed primed bovine PMN for enhanced NET formation in response to tachyzoites. The discrepancy between the different methods of NET detection may be explained by two factors: first, autophagy appears to precede NET formation and may therefore rather be linked to early NET formation that to late NET-related effects, and secondly, the NAE-based assay appeared more sensitive for the detection of tachyzoite-triggered NET formation and may therefore have produced an improved resolution of the data. We therefore assume that early tachyzoite-triggered NET formation is indeed linked to autophagy in bovine PMN. Furthermore, the fact that formation of LC3B-positive autophagosomes was observed in bovine PMN while casting NETs supported the potential role of autophagy in PMN-derived responses against tachyzoite stages.

Autophagosomes are double-membraned vesicles formed during autophagy, which represent characteristic markers of autophagy. LC3 is a small, soluble protein, which is distributed ubiquitously in mammalian tissues and in cultured cells. During autophagy, LC3-I (a cytosolic form of LC3) is conjugated to phosphatidylethanolamine to form LC3-II, which is then recruited to autophagosomal membranes (58). Therefore, LC3-II is widely used as a marker for the microscopic detection of intracellular autophagosomes. The LC3 gene family comprises three members, LC3A, LC3B and LC3C, and LC3B represents the most used endogenous autophagic marker (58). Certain studies have revealed that autophagy is required for NET formation

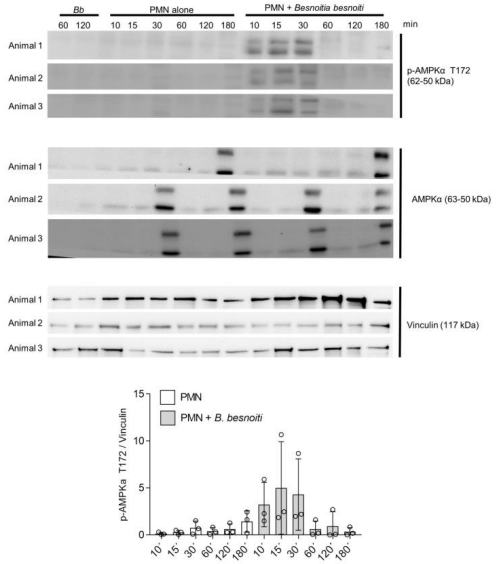

FIGURE 7 | *B. besnoiti* induces the phosphorylation of AMPK in bovine PMN. Protein extracts of 5×10^6 PMN confronted with 20×10^6 *B. besnoiti* tachyzoites from three different donors were obtained at different time points and the kinetics were analyzed for LC3B-I and LC3B-II (upper panel) and AMPKα T172 phosphorylation (middle panel) through western blot. For AMPK, the activation of the enzyme was detected in the first 30 min of interaction (middle panel). Vinculin detection via western blot was performed for the three donors as a protein quantity loading controls.

(46, 83) and that autophagy induction triggers NET formation (45, 46, 83). To detect autophagy in *B. besnoiti* tachyzoite-exposed PMN as a matter of principle, autophagosome formation was visualized by LC3B-based immunostaining. Confocal microscopy clearly showed that confrontation of PMN with *B. besnoiti* tachyzoites indeed caused a significant increase of autophagosome formation. As a highly interesting finding, we additionally observed that autophagic PMN also performed NET formation. However, neither rapamycin nor wortmannin pre-treatments had any influence on PMN-derived autophagosome formation, reinforcing the observation that these processes were mTOR-independent. Our results are in line with those obtained in human PMN, where rapamycin by its own is not able to induce autophagy but increases the autophagosome formation induced by PMA (48). In line, mTOR-independent induction of autophagy was also reported in a distinct population of PMN from sepsis patients which showed increased PMA-triggered NET formation activity (45).

AMPKα is a key metabolic master regulator in eukaryotes with high impact on several important cellular mechanisms. AMPKα activation is initiated by changes in the metabolic status which result from inhibition of ATP generation during hypoxia, glucose deprivation and increased ATP consumption (84). Previous observations in PMN showed that AMPK activation decreased PMA-induced ROS production in human PMN (49), but enhanced PMN chemotaxis, bacterial killing, and phagocytosis (50). Moreover, AMPK promotes autophagy by directly activating Ulk1 which is a mTOR downstream enzyme during autophagosome formation (85). On the other hand, inhibition of AMPK in mice model induced histone 3 secretion, suggesting that AMPK activation contributed to

Simultaneous and Positively Correlated NET formation and Autophagy in *Besnoitia besnoiti* Tachyzoite-exposed Bovine Polymorphonuclear Neutrophils

murine NET formation (86). Since autophagy is a complex process and could be initiated via various signaling pathways, we tried to check if AMPK pathway is involved in *B. besnoiti* tachyzoite-induced autophagy. Our current data show that confrontation of PMN with *B. besnoiti* tachyzoites clearly induced AMPKα activation in a time-dependent manner. Thus, AMPKα phosphorylation was immediately induced from the very beginning of parasite-PMN interactions until 30 min of co-culture. So far, it is unclear if enhanced AMPKα activation is linked to autophagy or NET formation or both in tachyzoite-exposed neutrophils, but this will be a matter for further research.

ETHICS STATEMENT

This study was conducted in accordance to Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols were approved by Ethic Commission for Experimental Animal Studies of Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No.521_AZ) and in accordance to European Animal Welfare Legislation: ART13TFEU and current applicable German Animal Protection Laws.

AUTHOR CONTRIBUTIONS

CH, AT, and IC: designed the project and experiments. EZ: carried out most of the experiments. TM-C: under flow experiments, SEM. UG: SEM and confocal microscope. ZV: LC3B confocal microscopy and Western blots. IC, CH, and AT: prepared the manuscript. IC, ZV, and EZ: prepared the figures. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01131/full#supplementary-material

Figure S1 | Autophagy induction in bovine PMN. Bovine PMN were treated with PMA (20 nM) and rapamycin (100 nM) for 1 h, and bovine PMN treated with media was used as control. Samples were fixed and permeabilized with pure cold methanol for LC3B-based immunostaining to determine autophagosome formation by confocal microscopy. **(A,B)** showing merged images with the staining for LC3B (green), DAPI (blue) in the control group. **(C,D)** showing merged images with the staining for LC3B (green), DAPI (blue) in the treated group. The right graph shows the percentage of autophagosome-positive cells **(E)**.

Figure S2 | Isolated NETs fails to induce autophagosome formation in bovine PMN. Isolated NETs from *B. besnoiti*-confronted PMN were isolated as described by Barrientos (59). Bovine PMN were treated with isolated NETs for 1 h, and bovine PMN treated with media was used as control. Samples were fixed and permeabilized with pure cold methanol for LC3B-based immunostaining to determine autophagosome formation by confocal microscopy. **(A,B)** showing merged images with the staining for LC3B (green), DAPI (blue) in the control group. **(C,D)** showing merged images with the staining for LC3B (green), DAPI (blue) in the treated group. The right graph shows the percentage of autophagosome-positive cells **(E)**.

Figure S3 | Autophagy and NET formation occurs simultaneously in *B. besnoitia*-exposed PMN. Bovine PMN were exposed to *B. besnoitia* tachyzoites for 3 h. Samples were fixed and permeabilized for LC3B-based immunostaining to determine autophagosome formation by confocal microscopy. **(A–F)** control group: **(A,C,E)** phase contrast, **(B,D,F)** merged images; **(G–L)** PMN+B. besnoiti group: **(G,I,K)** phase contrast **(H,J,L)** merged images. Blue: DNA staining with DAPI, green: autophagosomes staining with L3CB antibody.

Figure S4 | Atg5 protein expression in *B. besnoitia*-confronted PMN.

Figure S5 | Densitometry quantification of p-AMPKa T127/AMPK.

Figure S6 | NET formation in control bovine PMN (1/2). Analysis at the same time-point of the experiments performed with *B. besnoitia* tachyzoites. **(A)** Phase contrast image; **(B)** DNA staining: Sytox Orange; **(C)** histone (H11-4) staining; **(D)** neutrophil elastase (NE) staining; **(E)** Merged image of **B–D** and **(F)** Merged image of all channels **(A–D)**.

Figure S7 | NET formation in control bovine PMN (2/2). Analysis at the same time-point of the experiments performed with *B. besnoitia* tachyzoites. **(A)** Phase contrast image; **(B)** DNA staining: Sytox Orange; **(C)** histone (H11-4) staining; **(D)** neutrophil elastase (NE) staining; **(E)** Merged image of **B–D** and **(F)** Merged image of all channels **(A–D)**.

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2.2 Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

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Abstract: *Besnoitia besnoiti* tachyzoites infect and develop in bovine endothelial cells *in vivo* and trigger the release of neutrophil extracellular traps (NETs) from bovine polymorphonuclear neutrophils (PMN). The purpose of this study was to analyze if pure *B. besnoiti* tachyzoite- or calcium ionophore (A23187)-triggered NETs would damage endothelial host cells and subsequently influence intracellular development and proliferation of *B. besnoiti* tachyzoites in primary bovine endothelial cells. Thus, we here evaluated endothelial host cell damage triggered by histone 2A (H2A) and *B. besnoiti* tachyzoite-induced NET preparations and furthermore estimated the effects of PMN floating over *B. besnoiti*-infected endothelium under physiological flow conditions on endothelial host cell viability. Overall, all treatments (H2A, *B. besnoiti*-triggered NETs and floating PMN) significantly induced endothelial cell death of *B. besnoiti*-infected host cells. However, thought host cell damage led to significantly altered intracellular parasite development with respect to parasitophorous vacuole diameter and numbers, the total proliferation of the parasite over time was not significantly affected by these treatments thereby denying any direct effect of NETs on intracellular *B. besnoiti* replication.

Keywords: NETs; BUVEC; PMN; endothelium; innate; DNA; histones; *Besnoitia*

1. Introduction

The obligate intracellular parasite *Besnoitia besnoiti* (*B. besnoiti*) replicates *in vivo* in endothelium and represents the causal agent of besnoitiosis in cattle. Bovine besnoitiosis has a high impact on animal welfare and cattle production. Since 2010, bovine besnoitiosis has been classified as an emerging disease by the European Food Safety Authority (EFSA). Within the life cycle of *B. besnoiti*, cattle act as intermediate hosts whilst the final host, shedding oocysts, is still unknown [1,2].

Polymorphonuclear neutrophils (PMN) and endothelium are key players of host innate immune responses both interacting with *B. besnoiti* stages during acute infection [3]. Besides classical effector mechanisms, such as reactive oxygen species (ROS) production, phagocytosis and degranulation, PMN are able to extrude chromatin structures decorated with granular proteins that are able to ensnare and eventually kill pathogens. These structures were first reported by Brinkmann et al. [4] and named neutrophil extracellular traps (NETs). They are involved in several

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

physiopathological processes and are described as a defense mechanism that is directed against pathogens (bacteria, fungi, parasites) but also induced by soluble mediators (for review on NET inducers and respective mechanisms please refer to [5,6]). In case of apicomplexan parasites, NETs were reported to be released by PMN of different donor origin in response to stages of *B. besnoiti* [3], *Neospora caninum*, [7,8], *Toxoplasma gondii* [9,10], *Eimeria bovis* [11,12], *Plasmodium falciparum* [13] and *Cryptosporidium parvum* [14], thereby highlighting the conserved nature of NETosis throughout parasite and host species.

Direct interactions of PMN or NETs with endothelium at physiological conditions have already been reported [15–17] and indicated a critical role of PMN in the pathophysiology of endothelium impairment. Nonetheless, little data is still available with respect to NET-derived effects on parasite-infected endothelium *in vitro* and *in vivo*. In general, endothelial cells react upon parasite infections by a broad spectrum of immune-related reactions, such as upregulation of adhesion molecules (e. g. ICAM-1, VCAM-1, P-selectin, E-selectin), chemokines and PMN adhesion onto activated endothelium [11,18–22]. With this regard, our previous data showed that *B. besnoiti* infections induced the following early innate immune reactions in primary bovine umbilical endothelial cells (BUVEC): *i)* increased gene transcription of adhesion and inflammatory molecules (ICAM-1, CXCL1, CXCL8, CCL5 and COX-2), *ii)* augmented PMN adhesion to BUVEC layers and *iii)* release of NETs under physiological flow conditions [18].

PMN-derived NETs affect endothelium by increasing endothelial cell (EC) layer permeability and directly damaging single endothelial cells [23,24]. Additionally, NETs induce the expression of leukocyte adhesion molecules in activated ECs and, consequently, enhance local inflammatory responses[25]. EC damage is mainly explained by enhanced PMN-derived ROS production [26] and by transiently increased abundance of proteases/proteins in the microenvironment of vessels. Major NET components that were already proven as inducers of EC damage are histone 2A (H2A) [24], citrullinated histone 3 (H_3Cit) [27] and MMP-9 (through activation of endothelial MMP-2) [28]. Refering to DNA-induced EC damage, it was reported that both DNA-dependent [23] and DNA-independent EC impairment might occur [24], however, this is still a matter of debate.

The aim of the current study was to determine whether bovine PMN and especially *B. besnoiti* tachyzoite-triggered NETs in addition to major single NET components, such as DNA and H2A [27], induce cytotoxicity and damage in ECs and further alter intracellular *B. besnoiti* tachyzoite development in endothelial host cells. The current methods included fluorescence- and 3D live cell microscopy applying static or physiological flow conditions on *B. besnoiti*-infected and non-infected primary bovine umbilical vein endothelial cells (BUVEC). Respective analyses were performed on BUVEC treated with H2A and NET preparations triggered by *B. besnoiti* tachyzoites or the calcium ionophore A23187, a well known inducer of PMN activation and inducer of NETs.

Current data revealed that *B. besnoiti*-triggered NETs, A23187-induced NETs and H2A all induced cytotoxicity and damage in *B. besnoiti*-infected bovine endothelial cells. With respect to parasite intracellular development, *B. besnoiti* parasitophorous vacuole (PV) diameter and number per host cell were found diminished in treated BUVEC. However, the total tachyzoite proliferation over time was not significantly affected by NET-derived treatments, thereby denying a direct effect of NETs on intracellular *B. besnoiti* replication.

2. Materials and Methods

2.1. Ethic statement

This study was conducted in accordance to Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols were approved by the Ethic Commission for Experimental Animal Studies of the Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No.521_AZ), and in accordance to European Animal Welfare Legislation (ART13TFEU) and current applicable German Animal Protection Laws.

2.2. Primary host endothelial cell isolation and maintenance

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

Primary bovine umbilical vein endothelial cells (BUVEC) were isolated from umbilical cords obtained from calves born by *sectio caesarea* at the Clinic of Obstetrics, Gynecology and Andrology of Small and Large Animals, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Germany. Umbilical cords 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-Aldrich) for a maximum of 16 h before use. For the isolation of endothelial cells (EC), 0.025% collagenase type II (Worthington Biochemical Corporation) suspended in Pucks solution (Gibco) was infused into the lumen of ligated umbilical veins and incubated for 20 min at 37 °C in 5% CO_2 atmosphere. After gently massaging umbilical veins, the cell suspension was collected in RPMI-1640 medium and supplemented with 1 ml fetal calf serum (FCS, Gibco) in order to inactivate collagenase type II. After two washes (350 x *g*, 12 min, 20 °C), cells were resuspended in complete endothelial cell growth medium (ECGM, PromoCell, supplemented with 10% FCS), plated in 25 cm^2 tissue plastic culture flasks (Greiner) and kept at 37 °C in 5% CO_2 atmosphere until confluency. BUVEC were cultured in modified ECGM medium [EGCM, diluted at 30% in M199 medium, supplemented with 5% FCS (Greiner) and 1% penicillin and streptomycin (Sigma-Aldrich)] with medium changes every 2-3 days. BUVEC cell layers were used for *B. besnoiti* infections after three passages *in vitro*.

2.3. Isolation of bovine PMN

Healthy adult dairy cows served as blood/PMN donors. Animals were bled by puncture of jugular vein and peripheral blood was collected in heparinized sterile plastic tubes (Kabe Labortechnik). 20 ml of heparinized blood was mixed with 20 ml sterile PBS supplemented with 0.02% EDTA (Sigma-Aldrich), the mixture was carefully layered on top of 12 ml Biocoll® separating solution (density = 1.077 g/l; Biochrom AG) and centrifuged (800 × *g*, 45 min). After removal of plasma and peripheral blood mononuclear cells (PBMC), cell pellet was suspended in 25 ml bi-distilled water and gently mixed for 40 s to lyse erythrocytes. Osmolarity was rapidly restored by supplementing appropriate volumes of Hanks balanced salt solution (4 ml, HBSS 10x; Biochrom AG). For entire erythrocyte lysis, this step was repeated twice and PMN were later suspended in sterile RPMI 1640 medium supplemented with 1% penicillin and 1% streptomycin (Sigma-Aldrich). Counting and PMN were performed in a Neubauer haemocytometer chamber. Finally, freshly isolated bovine PMN were allowed to rest at 37 °C and 5% CO2 atmosphere for 30 min until further use.

2.4. In vitro maintenance and harvesting of B. besnoiti (strain Bb1Evora04) tachyzoites

Permanent Madin-Darby bovine kidney (MDBK) cells were used as host cells to maintain *B. besnoiti* tachyzoites (strain Bb1Evora04) *in vitro*. MDBK cell layers were cultured in 75 cm^2 plastic tissue culture flasks (Greiner) containing RPMI 1640 (Sigma-Aldrich) cell culture medium supplemented with 2% FBS (Merck), 1% penicillin (500 U/ml) and streptomycin (500 mg/ml) (both Sigma-Aldrich) at 37 °C and 5% CO_2 atmosphere until confluency. MDBK cell layers were then infected with 2 × 10^6 vital tachyzoites of *B. besnoiti*. Harvesting of *B. besnoiti* tachyzoites was performed as described previously [3].

2.5. Detection of extracellular DNA and protein markers of NETs by confocal and fluorescence microscopy.

Bovine PMN were co-cultured with *B. besnoiti* tachyzoites (ratio 1:4) for 3 h (37 °C and 5% CO_2 atmosphere) on 0.01% poly-ʟ-lysine pretreated coverslips (15 mm diameter, Thermo Fisher Scientific), fixed by adding 4% paraformaldehyde (Merck) and stored at 4 °C until further staining.

For ET visualization, Sytox Orange 5 µM (Life Technologies) was used to stain DNA and anti-histone (clone H11-4, 1:500; Merck Millipore #MAB3422) and anti-NE (AB68672, 1:500, Abcam) antibodies were used to stain specific proteins on ETs structures. Therefore, fixed samples were washed three times with PBS, blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min at RT and

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

incubated with corresponding primary antibody solutions for 1 h at RT. Thereafter, samples were washed thrice with PBS and incubated in secondary antibody solutions (Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 405 goat anti-rabbit IgG, both Life Technologies, 60 min, 1:500, RT). Finally, samples were washed thrice in PBS and mounted in anti-fading buffer (ProLong Gold Antifade Mountant; Thermo Fisher Scientific). Visualization was achieved applying confocal microscopy (Zeiss LSM 710) and using an inverted IX81 fluorescence microscope equipped with an XM 10 digital camera (Olympus). Image processing was carried out with Fiji ImageJ software using Z-project and merged channel plugins restricted to overall adjustment of brightness and contrast.

2.6. Preparation of B. besnoiti tachyzoite- or A23187-induced NETs

Isolation of NETs was performed as previously described by Barrientos et al. [29] with some modifications. Briefly, 1.5 x 10^6 bovine PMN/well were seeded in 12-well culture plates and stimulated either with A23187 (5 µM) or 6x10^6 *B. besnoiti* tachyzoites (= 1:4 PMN:tachyzoites ratio) for 3 h (37 °C, 5% CO_2). After incubation, the medium was carefully aspirated and wells were washed twice with 1 ml of PBS. Then, 400 µl of *Alu*I (4 U/ml, New England Biolabs) was added and plates were incubated for 20 min at 37 °C and 5% CO_2. Thereafter, samples were recovered and centrifuged for 5 min at 300 x *g* to remove cells and debris. NET preparations were immediately stored at -80 °C until further quantification and use.

DNA content of NET preparations was estimated by Quant-iT PicoGreen (Thermo Scientific). Briefly, 2 µL of each NET sample was mixed with 98 µL of TE buffer (1 M Tris pH 7.4; 0.5 M EDTA pH=8.0) and incubated for 5 min at room temperature (RT), protected from light. Afterwards, DNA content was quantified in a Varioskan fluorescence automated multiplate reader (Thermo Scientific) applying exposition/emission wavelengths of 480/520 nm, respectively. All DNA measurements were performed in duplicates. A standard λ-DNA curve was used to interpolate the DNA concentration of the samples.

2.7. Estimation of NET-, DNA- and histone 2A (H2A)-induced endothelial cell death

Three different BUVEC isolates were cultured to 100% confluency on 96- or 24-well plates (Greiner Bio-One) depending on the experiment setting. For H2A-related experiments, cells were treated with 10 or 100 µg H2A/mL for 4 or 12 h. For DNA and NET preparations experiments cells were treated for 12 h. To control the influence of components of NETs, *B. besnoiti* secreted and excreted substances and to test if a protein alone as BSA can influence the observed results we tested incubating for 4h and 12 h also BSA at the same concentrations used for histone H2A (10 and 200 µg/ml), viable and heat-inactivated *B. besnoiti* tachyzoites and supernatant recovered from *B. besnoiti* cell culture after 3h of infection, named excretory secretory (E/S) components at two different dilutions. At each time point, medium was removed and cells were analyzed for cytotoxic effects via live/dead staining (5 µM Sytox Orange® diluted in modified ECGM medium, Thermo Scientific, 10 min, RT, in the dark). Fluorescence intensity was estimated at 574/570 nm excitation and emission wavelengths, respectively.

2.8. Physiological flow condition experiments

Three different BUVEC isolates were cultured in µ-slide-0.4 Luer chambers (IBIDI®, Martinsried, Germany) until confluency. BUVEC layers were infected with *B. besnoiti* tachyzoites 12 h before performing flow condition experiments or left uninfected for controls. Culture plates were mounted on the stage of a motorized inverted microscope (Olympus Microscope IX81) using a top-stage incubator (IBIDI®, Martinsried, Germany) with a controlled atmosphere of 5%CO_2 and 37 °C. The chambers containing non-infected or *B. besnoiti*-infected BUVEC were connected to a pump flow system using Luer adapters and a constant physiological wall shear stress of 1.0 dyn/cm^2 was applied (syringe pump sp100i; World Precision Instruments) for 5 min of perfusion of either pure medium or a solution 5x10^6 PMN/ml. After perfusion, plates were carefully removed from IBIDI®

chambers, cells were fixed in 4% paraformaldehyde (PFA) at RT for 10 min and finally washed thrice with sterile PBS for further staining.

2.9. Determination of endothelial cell damage using isolectin GS-IB4

PFA-fixed BUVEC layers were treated with blocking/permeabilization solution (PBS with 3% BSA, 0.1% saponin; 1 h, RT). Thereafter, the samples were incubated in isolectin GS-IB4 who binds predominantly to the cell membrane of blood vessel endothelia [30]. Isolectin GS-IB4 was used at a concentration of 20 µg/ml, diluted in blocking/permeabilization solution, conjugated with Alexa Fluor 594 (Molecular Probes) incubating the samples for 20 min at RT, in a humidified chamber according to Tanaka et al. [31]. Then, samples were washed thrice in PBS and mounted using DAPI-containing mounting medium Fluoromount G® (ThermoFisher). Of each sample, five random microscopic images were taken applying identical conditions of exposition, light intensity and compensation (Olympus Microscope IX81). Image processing was carried out by Fiji ImageJ® using merged-channel-plugins restricted to overall adjustment of brightness and contrast. In brief, images were converted to 8 bit and greyscale color. A sharpen filter was applied followed by the color threshold selection used as background the isolectin-negative regions. The lack of isolectin-derived signals in BUVEC layers was defined as EC damage as described elsewhere [29]. For calculation, data from isolectin-negative area were divided by those of the total area to obtain the percentage of EC damage in each analyzed image. Overall, data from a total of 15 images (resulting from three different BUVEC isolates) were included in the calculation.

2.10. Determination of B. besnoiti rosettes number and parasitophorous vacuoles (PV) diameter

Three different BUVEC isolates were included in all experiments. For each experimental condition, 5 microscopic images of *B. besnoiti*-infected BUVEC and non-treated controls were randomly taken via phase contrast microscopy (Olympus Microscope IX81®). The number of *B. besnoiti* rosettes present in the PV of infected host cells were counted and PV sizes reflecting differential stages of tachyzoite replication were measured manually by three independent observers using Image J® software (NIH).

2.11. Quantification of B. besnoiti tachyzoites by qPCR

The number of free-released *B. besnoiti* tachyzoites (extracellular tachyzoites in cell culture supernatants) was determined by qPCR using primers described previously by Cortes et al. [32].

2.12. Graphical representation of results and statistical analyses

Statistical significance was defined by a p-value < 0.05. If not otherwise stated, p-value was determined using ANOVA followed by Dunnett's multiple comparisons test. For analysis on EC damage under physiological flow conditions, comparison of experimental conditions was performed using Sidak post-test. Data are presented as bar graphs (showing mean ± SD) or box-whiskers graphs (showing middle line at median and lines at maximum and minimum values). Capital letter N is used to denote the number of biological replicates (BUVEC isolates) and minuscule letter n is used when the analysis referred to *B. besnoiti* rosettes numbers. All statistical analyses and graphs were performed via Graph Pad® v. 7.03 software.

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

3. Results

3.1 B. besnoiti tachyzoites induce bovine NETs

Classical markers of NETs were detected when isolated bovine PMN were confonted with isolated *B. besnoiti* tachyzoites at 1:4 ratio and observed under confocal microscopy. Nuclear decondensation and extracellular DNA being extruded without compromise of the cell membrane of the PMN was detectable by means of Sytox Orange dye after 3h of co-incubation (Fig 1A). Tachyzoites being trapped in NETs was also observed (Fig 1B), Images are representative of the experiments conducted with PMN isolated from three different animals. Also, main components of formed NETs as Histones and neutrophil elastase (NE) was also observed by immunostaining. Colocalization of extracellular DNA with these proteins confirmed classical characteristics of NETs (Fig 1C) Additional figures showing co-localization of characteritic markers of NETs is showed in supplementary material (Fig S1).

3.2. H2A, B. besnoiti tachyzoite- and A23187-derived NET preparations are all cytotoxic for BUVEC

Cell death was determined by a live/dead-staining with Sytox Orange® which only enters into cells with compromised membranes (= dead cells). Overall, treatments of BUVEC with H2A at concentrations ≥ 10 μg/ml resulted in significantly enhanced cell death when applied for 4 h (H2A 10 μg/ml vs non-treated control: $p = 0.030$, H2A 200 μg/ml vs nontreated control: $p = 0.001$; Fig 2A) or 12 h (H2A 10 μg/ml vs non-treated control: $p = 0.002$, H2A 200 μg/ml vs nontreated control: $p = 0.0001$ Fig 2B). In addition, we tested if pure NET preparations obtained from bovine PMN being stimulated either with *B. besnoiti* tachyzoites (Bb-NETs) or the calcium ionophore A23187 (A23187-NETs) also induced BUVEC death in a static system. Preparation of pure NETs resulted in concentrations of 161.5±35 ng DNA/ml for A23187-NETs and 169±17 ng DNA/ml for Bb-NETs. Overall, an average of 40 ng DNA per 10^6 PMN was obtained. For the estimation of cytotoxic effects on BUVEC, NETs were used at two different concentrations defined as 1X and 2X corresponding to a final concentration of 3.3 and 6.6 ng DNA/ml, respectively. Respective data showed that both, A23187-NET (2X vs non-treated control: $p = 0.016$) and Bb-NET (1X vs non-treated control: $p = 0.001$; 2X vs non-treated control: $p = 0.014$) treatments induced significant cytotoxic effects in when exposure lasted for 12 h (Fig. 2C). For control, pure λDNA was applied to BUVEC at 10 μg/ml and also significantly induced cytotoxic effects in BUVEC (λDNA vs non-treated control: $p = 0.0004$; Fig 2C). When the influence of protein alone (BSA), *B. besnoiti* tachyzoites (live and heat-killed), and excretory/secretory molecules (E/S) (Fig 2D, E) was found that only viable *B. besnoiti* tachyzoites after 12 h of incubation induce cytotoxicity in BUVEC (Fig 2E)

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

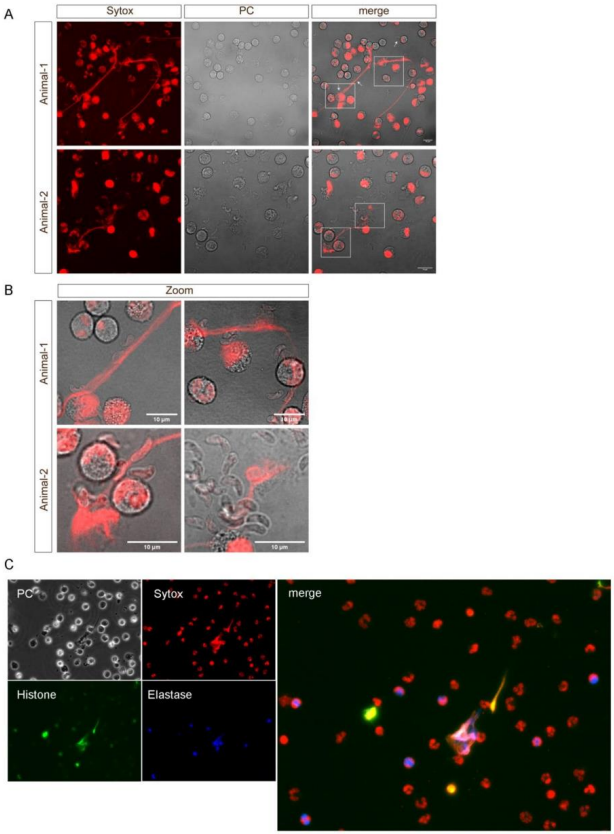

Figure 1. *B. besnoiti* tachyzoites induce NETs. 2 x 10^5 PMN were confronted with 8 x 10^5 *B. besnoiti* tachyzoites (1:4 ratio) for 3h at 37°C and 5% CO_2. Samples were fixed and stained for DNA (Sytox Orange). Nuclear dedondensation and extracellular DNA being extruded without compromising the cell membrane was observed under confocal microscopy (A) being *B. besnoiti* tachyzoites are entangled in these structures (B) Confirmation of characteristic protein markers for NETs co-localizing with DNA (red) was performed by immunofluorescence microscopy detecting Histones (green) and neutrophil elastase (blue) (C)

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

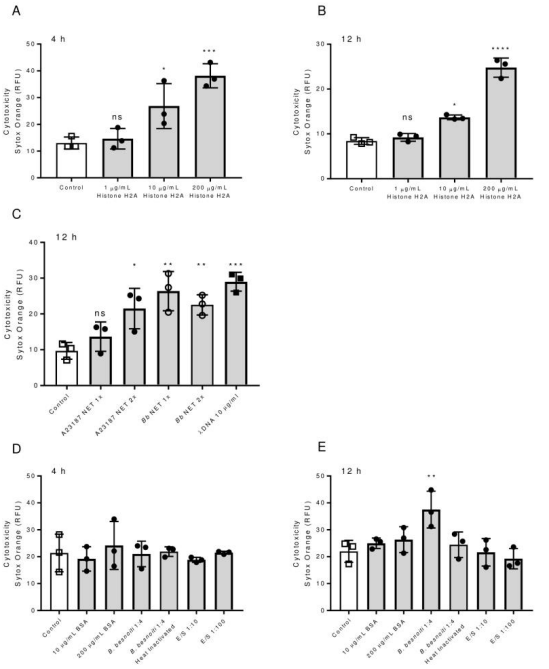

Figure 2. Histone 2A (H2A) and isolated NETs induces cell death in BUVEC. BUVEC were treated with H2A at concentrations of 1-200 µg/ml for 4 (A) and 12 (B) h and with isolated NET derived from bovine PMN confronted with *B. besnoiti* tachyzoites (*Bb* NET) or stimulated with A23187 (A23187 NET) for 12 h (C). For control purposes BSA, heat killed and viable *B. besnoiti* tachyzoites and excretory/secretory (E/S) molecules was used. Cell death was evaluated using Sytox Orange® staining. Bars represent mean ± SD. Statistical significance ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$; $p < 0.0001$ ****) was determined by ANOVA followed of a Dunnet post-test comparing experimental vs control conditions (N= 3).

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

3.3. PMN induce endothelial cell damage in B. besnoiti-infected BUVEC under flow conditions

We recently showed that PMN being perfused over *B. besnoiti*-infected BUVEC show increased endothelial adhesion and additionally perform NETosis [2]. Here, we applied a constant physiological shear stress of 1 dyn/cm^2 onto BUVEC and additionally conducted the experiments under controlled temperature (37 °C) and atmosphere (5% CO_2) conditions. Medium was perfused over BUVEC layers infected with *B. besnoiti* tachyzoites for 12 and 18 hours for a time period of 5 min under presence or absence of floating bovine PMN. Non-infected BUVEC were used as controls. Following perfusion, BUVEC layers were assayed for the EC damage marker isolectin-IB4 coupled with Alexa Fluor 594 and stained with DAPI as nuclear marker (exemplary images are illustrated in Fig 3A). Unmerged and phase contrast images confirming PMN adhesion on BUVEC layers are presented in supplementary material (Fig S2). Overall, perfusion of PMN over non-infected BUVEC induced minor EC damage (17.48 ± 10.58 %), whilst perfusion of medium alone hardly affected BUVEC layers (2.83 ± 0.47 %) (Fig. 3B). Interestingly, when PMN were perfused over *B. besnoiti*-infected cell layers, EC damage significantly increased to 35.47 ± 9.20 % (*B. besnoiti* infection vs non-infected controls: p = 0.028) at 12 hours of infection (Fig. 3B). Given that perfusion of medium alone did not induce considerable EC damage in *B. besnoiti*-infected BUVEC (3.43 ± 0.57 % Fig 3B), the former effect could not be due to an enhanced sensitivity of infected BUVEC towards shear stress conditions. Consequently, medium only-related data on non-infected and *B. besnoiti*-infected cells did not differ significantly.

3.4. H2A treatments decrease PV diameter and number of rosettes in B. besnoiti-infected host cells but does not affect total tachyzoite production over time

Since we observed that H2A and NETs and PMN induced cytotoxicity and damage on infected BUVEC under static and physiological flow conditions, we next analyzed whether treatments with H2A as a major component of NETs may also influence the intracellular development of *B. besnoiti* (for experimental set-up see Fig. 4A). Therefore, we determined *B. besnoiti* PV diameters which reflect the typical division stages of *B. besnoiti* tachyzoites (Fig. 4A). In addition, we quantified the number of rosettes present in each host cell. Overall, H2A treatments induced a decrease of the PV diameter independent of the time point of H2A supplementation (Fig 4C). As such, this effect was observed at time points, 4 and 12 h p.i. and at both H2A concentrations (10 and 100 μg/ml) (H2A-treated vs untreated: 10 μg-4 h: p = 0.014, 100 μg-4 h: p = <0.0001; 10 μg-4 h: p = <0.0001; 100 μg-12 h: p = <0.0001). Refering to mean rosette numbers/host cell, no significant difference was observed between non-treated and H2A-treated samples when varying PV loads per host cell were statistically analyzed within one condition (Fig S3). Given that up to 15 rosettes were detected in non-treated cells, PV number-derived categories were formed and compared to each other. Following this strategy, no changes in mean PV numbers in H2A-treated BUVEC were observed (Fig 5A) whilst the general infection rate remained unchanged (Fig 5B). Accordingly, when estimating tachyzoite production over time (30 hours), no significant differences were observed between H2A-treated cells and untreated controls (Fig 5C).

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

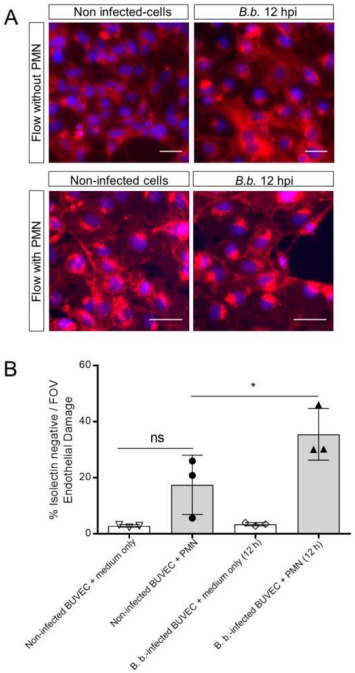

Figure 3. PMN induce damage on *B. besnoiti*-infected BUVEC under physiological flow conditions. PMN or medium alone were perfused at a constant shear stress of 1 dyn/cm² over *B. besnoiti*-infected and non-infected BUVEC. After 5 min of perfusion, cell layers were fixed and stained with DAPI for nuclei and with Alexa Fluor 594-conjugated isolectin-IB4 that binds endothelium. Continuity of membrane by means of the covered surface was evaluated for each experimental condition and the percentage of endothelial damage was determined. Five random pictures from three different BUVEC isolates were included in each experimental condition. Cell layers were analyzed using an epifluorescence microscope (**A**, representative images). FOV= Field of view. Bars represent mean ± SD. Statistical significance ($p < 0.05$) was determined by ANOVA followed by a Sidak post-test for multiple comparisons.

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

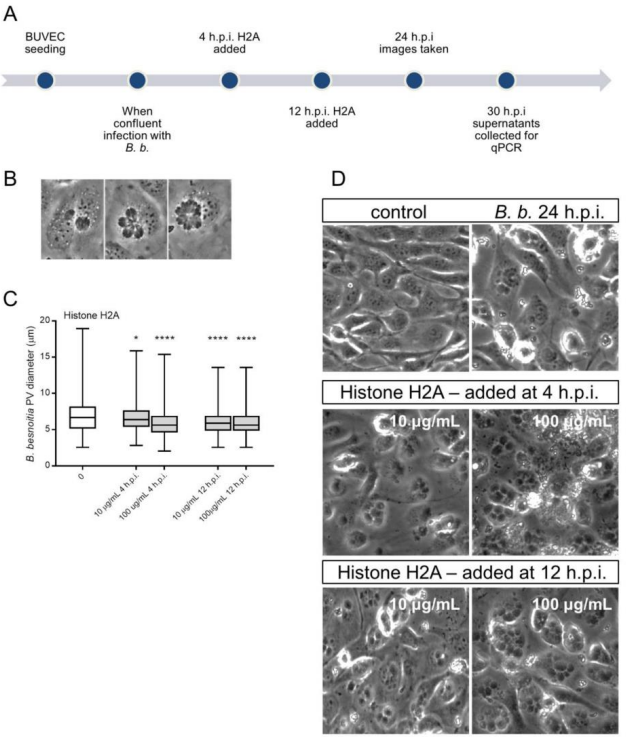

Figure 4. Histone 2A (H2A) treatment of *B. besnoiti*-infected BUVEC reduces *B. besnoiti* parasitophorous vacuole (PV) diameter. BUVEC (three different isolates, $N = 3$) were treated with H2A at 10 or 100 µg/ml at 4 and 12 h. p. i. (for experimental procedure refer to Fig. 4**A**). At 24 h. p. i. experimental conditions were documented by 5 randomly taken images using a phase contrast microscope (**B**, **D**). (**B**) Shows the typical development of *B. besnoiti* rosettes within 24 hours of infection (non-synchronous tachyzoite division leads to the formation of 2-mers to 16-mers). Here, the diameter of each *B. besnoiti* PV was measured ($n = 825$) and plotted as box and whiskers plot (**C**), line at median, bars indicating maximum and minimum values. Statistical significance ($p < 0.05$ *; $p < 0.0001$ ****) was determined by ANOVA followed of a Dunnet post-tests comparing experimental vs control condition at 4 and 12 h. p. i. In (**D**) a representative image of each experimental condition is shown.

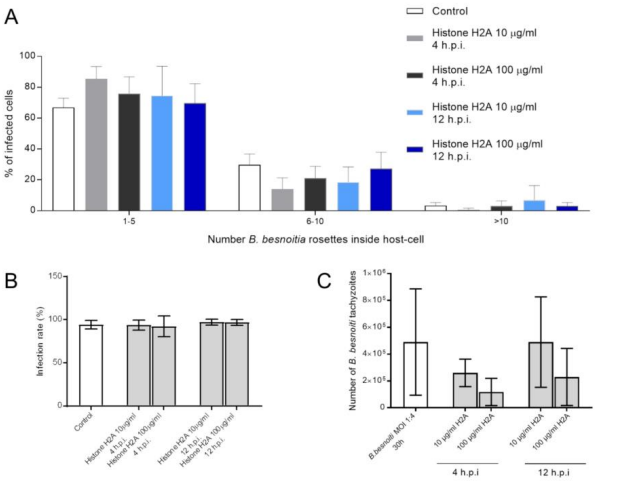

Figure 5. Histone 2A (H2A) did not affect the number of intracellular *B. besnoiti* rosettes. *B. besnoiti*-infected BUVEC (three different isolates, $N = 3$) were treated with H2A (10 or 100 µg/ml) at 4 and 12 h. p. i. At 24 h. p. i. cell layers were analyzed by randomly taking 5 microscopic images using phase contrast microscopy. The total number of *B. besnoiti* rosettes per host cell was determined and the percentage of host cells carrying different numbers of PV was estimated and sub-grouped in different categories (with 1-5, 6-10 and >10 rosettes/cell, respectively; **A**). Furthermore, the infection rate **(B)** and the total number of tachyzoites being released into cell supernatant within 30 hours p. i. was estimated via qPCR (**C).** Box and whiskers plot, line at median, bars indicating maximum and minimum values. Bar graph shows mean ± SD. Statistical significance was determined by ANOVA followed of a Dunnet post-tests comparing experimental vs control condition at 4 and 12 h. p. i. (n = 238).

3.5. Treatments of B. besnoiti-infected BUVEC with Bb-NETs and A23187-NETs affect PV development and total tachyzoite production

Overall, treatments of *B. besnoiti*-infected BUVEC layers with Bb-NETs or A23187-NETs at 4 and 12 hours p. i. differentially affected *B. besnoiti* PV diameter. Whilst A23187-NETs induced a significant diminishment of PV size at both time points of treatment [A23187-NETs-1X (4h) vs untreated control: $p < 0.0001$; A23187-NETs-1X (12h) vs untreated control:$p = 0.050$; A23187-NETs-2X (12h) vs untreated control: $p < 0.0001$], treatments with Bb-NETs had no effects when performed at 4 hours p. i. (Fig. 6A). However, in case of later Bb-NET treatments (12 h p. i.), a significant decrease of PV diameters was estimated for 1X concentration (Bb-NETs-1X (12 h): $p = 0.001$) (Fig. 6A, lower panel). When rosette numbers/host cell were analyzed and normalized as percentage of the infected host cells which contained 1 to 15 rosettes, we observed a significant decrease in the number of host cells which contained only one *B. besnoiti* rosette per cell in case of Bb-NET and A23187-NET treatments [A23187-NETs-1X (4h) vs untreated control: $p = 0.0001$; A23187-NETs-2X (4h) vs untreated control: $p = 0.0001$; Bb-NETs-1X (4h) vs untreated control: $p = 0.0002$; Bb-NETs-2X (4h) vs

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

untreated control: p = 0.0001; Bb-NETs-1X (12h) vs untreated control: p = 0.015] (Fig. 7A). When estimating total tachyzoite production and release over 30 hours, a striking difference was observed when comparing Bb-NET- and A23187-NET-related treatments: whilst total tachyzoite proliferation was not altered by Bb-NETs (Fig. 7B), treatments with A23187-NETs led to a significant and dramatic (almost 10-fold) increase of tachyzoite numbers present in cell culture supernatants [A23187-NETs-1X (4h) vs untreated control: p = 0.0001; A23187-NETs-2X (4h) vs untreated control: p = 0.0001; A23187-NETs-1X (12h) vs untreated control: p = 0.0001; A23187-NETs-2X (12h) vs untreated control: p = 0.0001] (Fig. 7B). This effect was independent of both, A23187-NET concentration and the time point of supplementation.

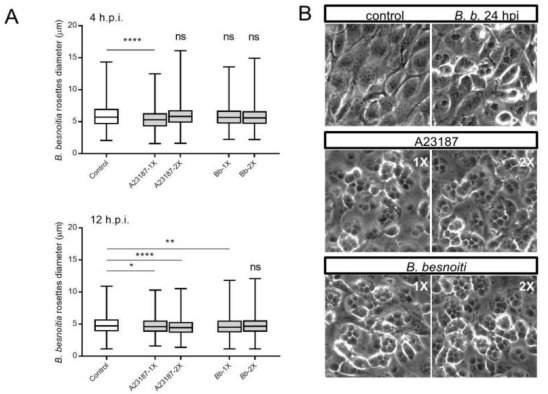

Figure 6. *Bb*-NETs and A231887-NETs induced a decrease in rosette diameter in *B. besnoiti*-infected BUVEC. *Bb*-NETs or A231887-NETs were added at two different concentrations (1X and 2X) to infected BUVEC cells (three different isolates, $N = 3$) at 4 (A, upper panel) and 12 (A, lower panel) h. p. i. At 24 h. p. i. experimental conditions were documented within 5 random pictures using a phase contrast microscope and the diameter of *B. besnoiti* rosettes was determined ($n = 1115$). Box and whiskers plot, line at median, bars indicating maximum and minimum values. Statistical significance was determined by ANOVA followed of a Dunnet post-tests comparing experimental vs control condition at 4 and 12 h. p. i. (**A**). Representative images infected BUVEC cells to which *Bb* and A23187 NETs were added at 12 h. p. i. are shown (**B**).

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

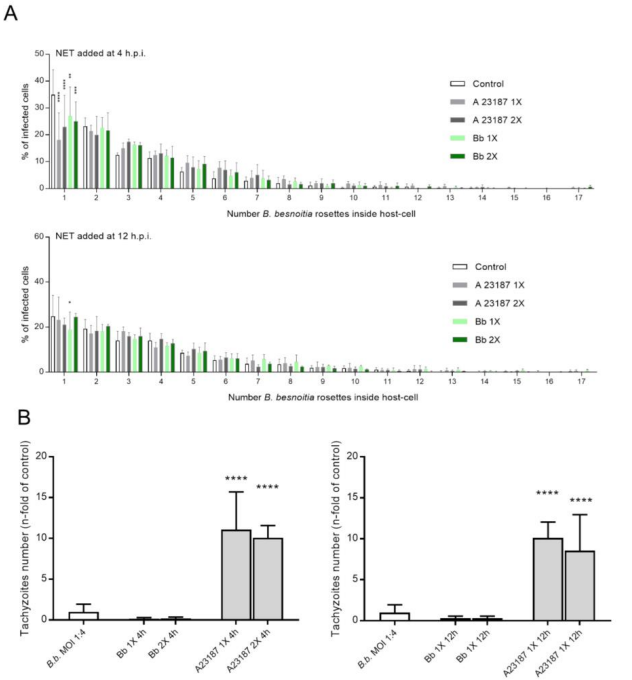

Figure 7. Effects of *Bb*-NET and A23187-NET treatments on intracellular *B. besnoiti* rosettes formation. *B. besnoiti*-infected BUVEC (three different isolates, $N = 3$), were treated with *Bb*-NETs or A231887-NETs at 1X and 2X concentrations at 4 and 12 h. p. i. At 24 h. p. i. cells were analyzed by randomly taking 5 random images for each experimental condition using phase contrast microscopy. The total number of *B. besnoiti* rosettes per host cell was determined and the percentage of host cells carrying different numbers of rosettes was calculated (**A**) In addition, the total number of tachyzoites being released into cell supernatant within 30 hours p. i. was estimated via qPCR (**B**).Bars represents mean ± SD. Statistical significance was determined by ANOVA followed of a Dunnet post-tests comparing experimental vs control condition at 4 ($n = 1115$) and 12 h. p. i ($n = 970$) $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***; $p < 0.001$ ****.

4. Discussion

B. besnoiti tachyzoites in acute phase infects predominantly host endothelial cells from different organs and vessels in vivo [18]. In the acute stage, the toxic effect has been related to increased vascular permeability. It is described that this lesions are mainly located in small and medium size vessels, but also in arteries [2]. In this report we used BUVEC primary endothelial cells isolated from three different animals in order to be closest possible to the *in vivo* situation.

We have recently demonstrated that *B. besnoiti* tachyzoites are a potent NET inducers [3] and that perfusion of bovine PMN over *B. besnoiti*-infected bovine endothelial cells leads to enhanced PMN adhesion and NET deposition on endothelium [18]. In this report, we present for the first time evidence that pure NET preparations as well as PMN perfusion under physiological share stress conditions lead to damage and cell death of parasite-infected endothelial host cells. In addition, we demonstrate that, though PV sizes appear to be affected by NET treatments, NET-related endothelial cell damage fails to significantly influence total parasite proliferation. The current finding emphasizes the hypothesis that excess NET formation may contribute to pathogenesis driven by cell-toxic side-effects and that these immune defense-related structures fail to exert lethal effects on tachyzoite stages.

In the current study, we worked with pure NET preparations that were released from bovine PMN either in response to *B. besnoiti* tachyzoites or after stimulation with the Ca^{++} ionophore A23187. In this regard, isolation of NETs was achieved by partial digestion of the DNA backbone by the enzyme nuclease Alu*I* as demonstrated by Barrientos et al. [28]. We here obtained comparable amounts of DNA for both inducers (161.5±35 ng/ml for A23187-NETs and 169.17±17 ng/ml for *Bb*-NETs, respectively). These DNA values are one order of magnitude below the ones described for human PMN stimulated with A23187 [29] when normalized as µg of DNA per 1 x 10^6 PMN. This difference might rely on species-specific differences in the activity of human and bovine PMN [33] and on peculiarities of PMN activation induced by calcium influxes in cattle [34]. No other data exist so far on the recovery or isolation of parasite-induced NETs to conduct a reliable comparison. However it is expected that NETs derived from *B. besnoiti* tachyzoites and A23187-stimulated PMN contains neutrophil elastase, histones and MPO considering the detection of these proteins in immunofluorescence of NETs induced by *B. besnoiti* in in-vitro static and under flow conditions.

H2A is a key component of NETs and NET-derived H2A was recently reported as a potent inducer of epithelial- and endothelial cell death in both, primary and permanent cell lines [24]. In line, the current data confirmed this effect for primary bovine endothelial cells and additionally showed that cytotoxicity for BUVEC was also observed when cell layers were treated with A23187-NETs and *Bb*-NETs. Considering that histones present in NETs show a lower molecular mass compared to chromatin-derived histones (which may be due to post-translational modifications [35] and are not as concentrated as when pure H2A is applied, different molecules present in NETs will also have driven these effects. In addition, given that *B. besnoiti*-infected BUVEC generally showed a high infection rate (at least 95 % of BUVEC were infected) it additionally appears unlikely that exclusively non-infected cells died within the infected cell layer.

The current study evaluated furthermore endothelial damage under controlled atmosphere and physiological flow conditions by means of the specific marker isolectin IB-4, a lectin derived from *Griffonia simplicifolia* that binds preferentially endothelium in blood vessels. [29], and used before in NETs-endothelium damage related studies [31]. Our estimation of the endothelial damage showed that *B. besnoiti*-infected endothelial cells were indeed significantly affected by perfused bovine PMN. Endothelium-PMN interactions, mainly referring to leukocyte adhesion cascades, have extensively been studied comprising a series of steps and signaling pathways [15]. In case of *B. besnoiti*-infected endothelium, it is known that changes in BUVEC host cell metabolism [36]. Also, gene expression of cytokines CXCL1, CXCL8, CCL2 and CCL5 and the adhesion molecules VCAM-1, P-selectin, ICAM-1 and E-selectin was increased at 12 h.p.i and in this context, induction of PMN adhesion and NETosis under flow occurs [18]. In this study we add evidence that bovine PMN can also induce damage on activated endothelium at the same time point through PMN adhesion and NET release.

Bovine neutrophil extracellular traps induce damage of *Besnoitia besnoiti*-infected host endothelial cells but fail to affect total parasite proliferation

These lethal effects most probably are due to a transient higher concentration of proteases and ROS as hypothesized by others [26].

As an interesting finding, we here showed that presence of H2A but also of A23187-NETs and *Bb*-NETs led to a decrease of intracellular PV diameter thereby reflecting earlier developmental stages with less numbers of tachyzoites (two- and four-mers of tachyzoites have a smaller diameter than 8- or 16-mers). Noteworthy, this phenomenon occurred irrespective of the time point of *B. besnoiti* infection. The two time points here used, i. e. 4 and 12 h. p. i., reflect *B. besnoiti* replication at the beginning (4 h p.i.: before first division) and in the middle (12 h p. i.: after two divisions) of first merogony, but before lysis of infected BUVEC occurs (from 20 hours ongoing). It must be noted that different strains *B. besnoiti* have different lytic cycle characteristics in vitro when a permanent cell line (MARC-145) is used as a host [37] Our results cannot be compared in terms of the lytic cycle since we use the Bb1Evora04 *B. besnoiti* strain, that was not included in this study and as a host cell we used isolated primary cells (BUVEC). Thus the timepoint to measure endothelial damage were rationally selected based on the gene expression profile of BUVEC cells infected with *B. besnoiti* tachyzoites and the formation of NETs over infected endothelium [18]. At a first glance, the effects on PV diameter could indicate a direct detrimental effect of H2A or NET preparations on tachyzoite development. However, when host cells were analyzed for rosettes numbers, less cells carrying only one rosette were detected in the case of A23187- and *Bb*-NET treatments. This effect can be linked to selective endothelial cell death, which leads to the release of (obviously) vital tachyzoites which then "rescue" themselves by invading neighboring cells. Since they first have to establish within these new host cells, proliferation onset is delayed and smaller PV is found. We consistently observed similar effects in other adverse cell culture conditions, e. g. when cells died out of nutritional deficits. As s general finding, this effect only occurs when single BUVEC die within a total cell layer, not when a cell layer entirely detaches. In agreements with this hypothesis, total *B. besnoiti* tachyzoite production was not significantly affected in NET-treated BUVEC. The latter data clearly indicated that direct lethal effects of NET preparations on intracellular tachyzoites did not occur.

In contrast to H2A or *Bb*-NETs, treatments of *B. besnoiti*-infected BUVEC with A23187-NETs led to a striking and significant increase of *B. besnoiti* tachyzoite production and release into cell culture supernatants within 30 hours of infection. The basis of these effects remains unclear although it is known that NET-derived protein composition varies according to the stimuli and may therefore exert different reactions. However, it must be taken into account that existing data mainly refer to PMA-stimulated PMN [35,38], and that also a dependence on the PMN donor species as well as on the quantity of key components present in NETs has been reported [29]. Nevertheless, since Behrendt et al. [39] reported on A23187-induced tachyzoite host cell egress in case of the closely related coccidian parasites *Toxoplasma gondii* and *Neospora caninum*, the increase of free tachyzoites may also have been induced by residues of A23187 compound present in NET preparations (even though these preparations were thoroughly washed before use). However, Behrendt et al. [39] reported that A23187-induced tachyzoite egress largely depends on PV maturity and on A23187 concentration. Thus, only 10 μM A23187 treatment led to egress of tachyzoites from immature PV. Interestingly, reactions were species-dependent since already 1 μM of this compound caused egress of 86% of *T. gondii* tachyzoites but had no effect on *N. caninum* tachyzoites. Given that we here used 5 μM A23197 for NET induction but removed the compound by consecutive washings rather argues against A23187-induced egress as cause for enhanced tachyzoite numbers in cell culture supernatants.

Overall, we here present new data on the damaging capacity of *B. besnoiti* tachyzoite- and A23197-triggered NETs and H2A and showed that PV diameter and number of rosettes/host cell may rather indirectly than directly have been affected by NET-driven host cell death. Our data did not include parasite viability as a parameter and thus is not possible to give a conclusion on this regard.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: PMN release NETs in response to *B. besnoiti* tachyzoites. Figure S3. Additional Images of DAPI/Isolectin IB4 staining

of experimental conditions. Figure S3: Distribution of the parameter rosette number per cell in H2A-treated BUVEC

Author Contributions: Conceptualization, A.T., C.H., I.C., and Z.V; Investigation, Z.V., D.G., E.Z., H.S.; Validation, I.C. and Z.V.; Formal Analysis, I.C., Z.V. and A.T..; Resources, A.T., C.H.; Writing-Original Draft Preparation, I.C.; Writing-Review & Editing, Z.V., A.T., C.H.; Supervision, A.T., C.H.; Project Administration, C.H., A.T.; Funding Acquisition, A.T.

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2.3. Metabolic requirements of *Besnoitia besnoiti* tachyzoite-triggered NETosis

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Eigener Anteil inder Publikation:

Initiative	Weitestgehend eingeständig
Projektplannung	Weitestgehend eingeständig
Durchführung des Versuches	Wesentlich
Auswertung der Experimente	Wesentlich
Erstellung der Publikation	Weitestgehend eingeständig

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Metabolic requirements of *Besnoitia besnoiti* tachyzoite-triggered NETosis

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Abstract

Besnoitia besnoiti is the causative agent of bovine besnoitiosis, a disease affecting both, animal welfare and cattle productivity. NETosis represents an important and early host innate effector mechanism of polymorphonuclear neutrophils (PMN) that also acts against *B. besnoiti* tachyzoites. So far, no data are available on metabolic requirements of *B. besnoiti* tachyzoite-triggered NETosis. Therefore, here we analyzed metabolic signatures of tachyzoite-exposed PMN and determined the relevance of distinct PMN-derived metabolic pathways via pharmacological inhibition experiments. Overall, tachyzoite exposure induced a significant increase in glucose and serine consumption as well as glutamate production in PMN. Moreover, tachyzoite-induced cell-free NETs were significantly diminished via PMN pretreatments with oxamate and dichloroacetate which both induce inhibition of lactate release as well as oxythiamine, which inhibits pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and transketolase, thereby indicating a key role of pyruvate- and lactate-mediated metabolic pathways for proper tachyzoite-mediated NETosis. Furthermore, NETosis was increased by enhanced pH conditions, however, inhibitors of MCT-lactate transporters (AR-C141900, AR-C151858) failed to influence NET formation. Moreover, a significant reduction of tachyzoite-induced NET formation was also achieved by treatments with oligomycin A (inhibitor of ATP synthase) and NF449 (purinergic receptor P2X1 antagonist) thereby suggesting a pivotal role of ATP availability for tachyzoite-mediated NETosis. In summary, the current data provide first evidence on carbohydrate-related metabolic pathways and energy supply to be involved in *B. besnoiti* tachyzoites-induced NETosis.

Keywords: *Besnotia besnoiti*, PMN, NETosis, metabolic signatures, glycolysis, ATP

1. Introduction

Besnoitia besnoiti is an obligate intracellular apicomplexan protozoan which was first described in 1912 (Besnoit and Robin, 1912). Bovine besnoitiosis is an endemic disease in several countries of Europe as well as in the Middle East, Asia, South America and Africa (Bigalke et al., 2004; Cortes et al., 2014) causing significant economic losses in cattle industry (Jacquiet et al., 2010; Maqbool et al., 2012). Based on increased cases and geographic expansion, the European Food Safety Authority (EFSA) graded bovine besnoitiosis as an emerging disease in Europe (European Food Safety Authority, 2010). Meanwhile, numerous reports elaborated clinical signs and histopathological alterations in infected cattle, but limited data are available on early interactions of innate immune cells with *B. besnoiti* stages, despite the fact that these leukocytes are the first line of defence and also the first ones to be recruited to infection sites (Maksimov et al., 2016; Muñoz Caro et al., 2014). Among these leukocytes, polymorphonuclear neutrophils (PMN) are the most abundant granulocytes in blood acting rapidly against invading pathogens, including apicomplexan parasites (Baker et al., 2008; Behrendt et al., 2010; Villagra-Blanco et al., 2017, 2019). Besides phagocytosis and degranulation (Behrendt et al., 2008; Lacy, 2006), PMN also efficiently combat and eventually kill invading pathogens by undergoing NETosis (Brinkmann, 2018; Fuchs et al., 2007). NETosis comprises the release of nuclear or mitochondrial DNA being adorned with histones and various antimicrobial molecules, such as neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, lactoferrin, pentraxin, and gelatinase (Brinkmann et al., 2004; Delgado-Rizo et al., 2017; Hahn et al., 2013; Parker and Winterbourn, 2013). A variety of pathogens including bacteria, virus, fungi and parasites might either be immobilized within released sticky NET structures or be killed via a high local concentration of antimicrobial histones, peptides and proteases (Behrendt et al., 2010; Muñoz-Caro et al., 2018; Nathan, 2006; Silva et al., 2016; Villagra-Blanco et al., 2019).

So far, few reports are available on *B. besnoiti*-triggered ETosis which include two types of leukocytes, PMN, and monocytes (Muñoz Caro et al., 2014; Muñoz-Caro et al., 2014). Both leukocyte populations were shown to rapidly release ETs in response to *B. besnoiti* tachyzoites (Maksimov et al., 2016; Muñoz Caro et al., 2014) thereby hampering these stages from host cell invasion (Muñoz-Caro et al., 2014). *B. besnoiti* tachyzoite-induced ETosis was

shown to be reduced by NADPH oxidase (NOX)-, NE- and MPO inhibitors (Muñoz Caro et al., 2014) thereby indicating the pivotal role of these enzymes for parasite-induced ETosis, which is consistent to other ETosis-related investigations (Metzler et al., 2011; Papayannopoulos et al., 2010; Rada et al., 2013). Parasite entrapment assays revealed that one third of *B. besnoiti* tachyzoites were efficiently immobilized by NETosis (Muñoz Caro et al., 2014), implying that this effector mechanism may play a crucial role during acute phase of cattle besnoitiosis (Muñoz-Caro et al., 2014). Interestingly, in a physiological flow condition-related experimental setting, Maksimov et al. (2016) showed that bovine PMN not only adhere to *B. besnoiti*-infected primary host endothelium but also undergo NETosis at this interface.

Basic information on metabolic requirements of PMN or other immune cells during parasite-triggered ETosis is scarce. In general, extracellular ATP disposability and activation of P2 receptor-mediated purinergic signaling pathways seem essential for early host innate immune responses of PMN (Wang and Chen, 2018). As such, P2-mediated purinergic signaling is involved in regulating other essential PMN functions, such as chemotaxis, phagocytosis, oxidative burst and degranulation (Wang and Chen, 2018). This pathway also seems essential for NET formation since *Neospora caninum*-mediated NETosis was significantly blocked by a P2Y2 receptor inhibitor (Villagra-Blanco et al., 2017). Thus, we here aimed to explore the impact of key metabolic pathways as well as the role of the purinergic receptor P2X1 and of monocarboxylate transporter 1 (MCT1) in *B. besnoiti*-triggered bovine NETosis. In this connection, the metabolic signatures of *B. besnoiti* tachyzoite-exposed PMN were determined by assessing the metabolic conversion rates of glycolysis (glucose, pyruvate, and lactate) in addition to the amino acids glutamine, glutamate, alanine, serine and aspartate in cell culture supernatants (Mazurek et al., 1997; Taubert et al., 2016). Furthermore, the relevance of the corresponding pathways as well as the role of the purinergic receptor P2X1 in *B. besnoiti*-induced NETosis was analyzed via functional inhibition assays. Given that enhanced lactate and glutamate production may lead to PMN acidification, we also studied the role of pH in parasite-induced NETosis via pH-adjusted media and pharmacological inhibition of MCT1 which transports lactic acid across biological membranes.

2. Materials and Methods

2.1 Ethic statement

This study was performed in accordance to the Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols were approved by the Ethic Commission for Experimental Animal Studies of the Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No. 521_AZ), and in accordance to prevalent European Animal Welfare Legislation: ART13TFEU and current applicable German Animal Protection Laws.

2.2 *Besnoitia besnoiti* tachyzoite maintenance and collection

All NETosis-related experiments were performed with *B. besnoiti* tachyzoites of strain Bb Evora04 which was initially isolated from the field in Portugal. The permanent Madin-Darby bovine kidney (MDBK) cell line was used for *B. besnoiti* tachyzoite production *in vitro*. Briefly, MDBK were cultured in 75 cm^2 plastic tissue culture flasks (Greiner, Frickenhausen, Germany) at 37 °C and 5% CO_2 atmosphere until confluency using RPMI 1640 (Sigma-Aldrich, R0883, Steinheim, Germany) cell culture medium supplemented with 2% fetal bovine serum (FBS, Biochrom AG, S0115, Berlin, Germany), 1% penicillin (500 U/ml) and streptomycin (500 mg/ml) (Sigma-Aldrich, P4333, Steinheim, Germany). Confluent MDBK layers were infected with 2×10^6 vital *B. besnoiti* tachyzoites. Free-liberated viable tachyzoites were collected from cell culture supernatants, filtered through a 5-µm syringe filter (Merck Millipore, Darmstadt, Germany), washed in sterile PBS and pelleted (400 × *g*, 12 min). Tachyzoites were re-suspended in cell culture medium RPMI 1640 (Sigma-Aldrich, R7509, Steinheim, Germany) supplemented with 1% penicillin (500 U/ml) and streptomycin (500 mg/ml), counted in a Neubauer chamber and placed at 37 °C and 5% CO_2 atmosphere for further experimental use.

2.3 Bovine PMN isolation

Healthy adult dairy cows (n = 4) served as blood donors. Animals were bled by puncture of the jugular vein and 30 ml peripheral blood was collected in 12 ml heparinized sterile plastic tubes (Kabe Labortechnik, Nümbrecht-Elsenroth, Germany). Approximately 20 ml of heparinized blood was re-suspended in 20 ml sterile PBS with 0.02% EDTA (Roth, Karlsruhe, Germany), slowly layered on top of 12 ml Biocoll® separating solution (density = 1.077 g/l; Biochrom AG, Berlin, Germany) and centrifuged (800 × *g*, 45 min).

After extraction of plasma and peripheral mononuclear blood cells, the cell pellet was re-suspended in 25 ml distilled water and gently shaken during 40 s to lyse erythrocytes. Osmolarity was rapidly restored by Hank's balanced salt solution (4 ml, HBSS 10 ×; Sigma-Aldrich, Steinheim, Germany). To complete erythrocyte lysis, this step was repeated twice and PMN were re-suspended in sterile RPMI 1640 medium (Sigma-Aldrich, R7509, Steinheim, Germany). Finally, freshly isolated bovine PMN were allowed to rest at 37 °C and 5% CO_2 atmosphere for 30 min until further use (Behrendt et al., 2010; Muñoz-Caro et al., 2014).

2.4 Scanning electron microscopy (SEM)

Bovine PMN were co-cultured with *B. besnoiti* tachyzoites (ratio 1:4) for 60 min (37 °C, 5% CO_2) on coverslips (15 mm diameter, Thermo Fisher Scientific, Braunschweig, Germany) pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich). After incubation, cells were fixed in 2.5% glutaraldehyde (Merck, Darmstadt, Germany), post-fixed in 1% osmium tetroxide (Merck, Darmstadt, Germany), washed in distilled water, dehydrated, critical point dried by CO_2-treatment and sputtered with gold. Finally, all samples were visualized via a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

2.5 Visualization of *B. besnoiti*-triggered NETosis by immunofluorescence microscopy

Bovine PMN were co-cultured with *B. besnoiti* tachyzoites (ratio 1:4) for 3 h (37 °C, 5% CO_2 atmosphere) on 0.01% poly-L-lysine pre-treated coverslips (15 mm diameter, Thermo Fisher Scientific, Braunschweig, Germany), fixed in paraformaldehyde (4%, Merck, Darmstadt, Germany) and stored at 4 °C until further use.

For NET visualization, Sytox Orange (Life Technologies, S11368, Eugene, USA) was used to stain DNA and anti-histone (clone H11-4, 1:1,000; Merck Millipore #MAB3422, Darmstadt, Germany), anti-NE (AB68672, 1:1,000, Abcam, Cambridge, UK) or anti-MPO (orb11073, 1:1,000, Byorbit, Cambridge, UK) antibodies were used to stain respective proteins on ET structures. Therefore, fixed samples were washed thrice with PBS, blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany, 30 min, RT) and incubated with corresponding primary antibody solutions (1 h, RT). After three times of washing in PBS, samples were incubated in secondary antibody solutions (Alexa Fluor 488 goat anti-mouse

IgG or Alexa Fluor 488 goat anti-rabbit IgG, both Life Technologies, Eugene, USA, 60 min, 1:1,000, RT). Finally, samples were washed thrice in PBS and mounted in anti-fading buffer (ProLong Gold Antifade Mountant; Thermo Fisher Scientific, Carlsbad, USA). Visualization was achieved using an inverted IX81 fluorescence microscope equipped with an XM 10 digital camera (Olympus).

2.6 Quantification of 'cell-free' and 'anchored' NETs

Bovine PMN (in cell culture medium RPMI 1640 lacking phenol red and serum) were confronted with *B. besnoiti* tachyzoites at a PMN:tachyzoites ratio of 1:4 (2×10^5 PMN : 8×10^5 tachyzoites, 96-well format) for 3 hours. After incubation, 'cell-free NET' quantification was performed according to Tanaka et al. (2014). Briefly, after centrifugation (300 × *g*, 5 min) resulting supernatants were transferred into a new 96-well plate to measure 'cell free NETs' and remaining cell pellet samples were used for 'anchored NETs' estimation (Tanaka et al., 2014). Therefore, Pico Green® (Invitrogen, Eugene, USA, 1:200 dilution in 10 mM Tris base buffered with 1 mM EDTA, 50 µl/well) was added to each supernatant and pellet sample. Extracellular DNA was detected and quantified by PicoGreen®-derived fluorescence intensities using an automated multiplate reader (Varioskan, Thermo Scientific, Vantaa, Finland) at 484 nm excitation/520 nm emission as described elsewhere (Muñoz Caro et al., 2014; Villagra-Blanco et al., 2017).

2.7 Estimation of metabolic conversion rates in cell culture supernatants

For metabolic conversion rate experiments, bovine PMN were pre-treated with cytochalasin D to block active tachyzoite phagocytosis (10 µg/ml, 10 min, Sigma, C8273, 15 min), then confronted with heat-inactivated *B. besnoiti* tachyzoites (60 °C, 30 min) or plain medium (RPMI 1640 with 1.5 mM glucose) at MOIs of 3:1 and 6:1 (9×10^6 or 18×10^6 tachyzoites/3× 10^6 PMN) for 6 hours (37 °C, 5% CO_2 atmosphere). Thereafter, the cells were pelleted (400 × g, 10 min, 4 °C) and supernatants were collected, immediately frozen in liquid nitrogen and stored at -80 °C for further analysis. For measuring metabolite concentrations, the samples were heated for 15 min at 95 °C and centrifuged (8000 × *g* for 10 min). In the supernatants, glucose, pyruvate, lactate, glutamine, glutamate, alanine, serine, and aspartate were measured using a bench top random access clinical chemistry analyzer as described previously (Mazurek et al., 1997; Taubert et al., 2016). The conversion rates of the

corresponding metabolites were calculated in nanomoles per (h × 10^6 cells) in relation to plain medium samples which were incubated in parallel at identical conditions for reference purposes.

2.8 Pharmacological inhibition assays and pH-related experiments

For inhibition assays, bovine PMN were pretreated with inhibitors for 30 min and then co-cultured with *B. besnoiti* tachyzoites (1:4 PMN:tachyzoites ratio, 3 h, 37 °C, 5% CO_2). The following inhibitors were here used: 2-fluor-2-deoxy-D-glucose (FDG, 2 mM, Sigma-Aldrich; glucose analogue which cannot be further degraded after phosphorylation by glycolytic hexokinase and therefore inhibits glycolysis), sodium dichloroacetate (DCA, 8 mM, Sigma-Aldrich; inhibitor of pyruvate dehydrogenase kinase), oxythiamine (OT, 50 µM, Sigma-Aldrich; inhibitor of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase), sodium oxamate (OXA, 50 mM, Sigma-Aldrich; structural analog of pyruvate, inhibitor of lactate dehydrogenase), oligomycin A (5 µM, Sigma-Aldrich; inhibitor of ATP-Synthase in mitochondrial respiration), theobromine (100 µM, Sigma-Aldrich; inhibitor of P1A1-mediated purinergic signaling), NF449 (100 µM, Tocris; purinergic receptor antagonist with high specificity for $P2X_1$), AR-C 141990 (1 µM, Tocris; MCT1 inhibitor) and AR-C 155858 (1 µM, Tocris; inhibitor of MCT1 and MCT2). All inhibitor concentrations were selected based on previous studies (Aronsen et al., 2014; Rodríguez-Espinosa et al., 2015; Seliger et al., 2013; Taubert et al., 2016; Villagra-Blanco et al., 2017; Wang et al., 2013).

For pH-related experiments, RPMI 1640 medium was adjusted to different pH values of 6.6, 7.0, 7.4, and 7.8 by HCL or NaOH (both Merck, Darmstadt, Germany) supplementation as previously described (Naffah de Souza et al., 2018). Bovine PMN were suspended in RPMI 1640 medium at diverse pH values and exposed to tachyzoites. Experiments were performed as follows: 2 × 10^5 PMN were seeded in duplicates into 96-well plates and co-cultured with 8 × 10^5 *B. besnoiti* tachyzoites or incubated in plain pH-adjusted medium (controls) for 3 h (37 °C, 5% CO_2 atmosphere).

2.9 Statistical analysis

Data were illustrated as means ± SEM of at least three biological replicates and two technical replicates. One-way analysis of variance and Dunnett's multiple comparison tests were here

performed by using GraphPad Prism 7®. Differences were considered as significant at a level of $p \leq 0.05$.

3. Results

3.1 *B. besnoiti* tachyzoites triggered NET formation

To confirm the NET-related functional capability of *B. besnoiti* tachyzoites in the current experimental setting, NET formation in response to these parasitic stages were here demonstrated by SEM and immunofluorescence analysis (Fig. 1). As illustrated by SEM, bovine PMN indeed released NET-like structures when co-cultured with *B. besnoiti* tachyzoites and numerous tachyzoites were found trapped by these filaments (Fig. 1A-C). To verify classical characteristics of NETosis, co-localization of extracellular DNA with H1-H4, and NE (Fig. 1D-I) in parasite-entrapping structures was here confirmed.

3.2 *B. besnoiti* tachyzoite exposure altered metabolic conversion rates in PMN

Metabolic signatures of tachyzoite-exposed PMN were analysed by the estimation of metabolite conversion rates in the cell cultivation supernatants of the cells. It has to be noted that the sometimes strong individual variance of non-syngene blood donors (cows) often hampers a significant outcome of PMN-related experiments. To exclusively monitor NETosis-driven responses and to block reactions driven by phagocytosis or active parasite infection, PMN were pre-treated with cytochalasin D and exposed to heat-inactivated parasite stages. Biochemical analyses of supernatants from PMN and tachyzoite co-cultures revealed a significant [PMN + tachyzoites (1:6) *vs* medium control: $p < 0.05$] increase in PMN-derived glucose consumption (Fig. 2). Since tachyzoites had been heat-inactivated prior to PMN exposure, these reactions could only be attributed to PMN. The glucose carbons can either be channeled into synthetic pathways debranching from glycolytic intermediates or can be degraded to pyruvate with regeneration of energy. Pyruvate can be released into the medium or it can be introduced in various metabolic pathways: reduction to lactate by lactate dehydrogenase and release into the medium, amination to alanine by transaminases and release into the medium, oxidative decarboxylation to acetyl-CoA by pyruvate dehydrogenase and channeling into the citric acid, respectively. In line, pyruvate, lactate and alanine release increased in PMN:tachyzoite co-cultures. However, none of the three metabolites reached

significant levels (Fig. 2). Co-cultivation with tachyzoites was also accompanied by a significant decrease in glutamine production [PMN + tachyzoites (1:3) *vs* medium control: $p < 0.05$] (Fig. 2) Like glutamine, glutamate was released into the medium by plain PMN. Tachyzoite exposure induced a significant increase in glutamate release [PMN + tachyzoites (1:6) *vs* medium control: $p < 0.001$] (Fig. 2). Taken together, the glutamine and glutamate data argue against glutaminolysis (= energy regenerating glutamine degradation) as an important metabolic pathway for parasite-driven NETosis. The decrease in glutamine release may be caused by an inhibition of ATP-dependent glutamine synthetase. A source for glutamate production is aspartate which provides the amino group for amination of α-ketoglutarate to glutamate catalyzed by aspartate aminotransferase. Measurement of aspartate conversion rates revealed a trend towards increase in aspartate consumption in PMN tachyzoite co-cultures which, however, this increase was not significant. A part of the pyruvate and lactate released from tachyzoite exposed PMN may also derive from serine, whose consumption rate significantly increased [PMN + tachyzoites (1:3) *vs* medium control: $p < 0.01$] (Fig. 2). Overall, the current data indicate that differential metabolic pathway activation is indeed required for tachyzoite-driven NETosis.

3.3 *B. besnoiti*-induced 'cell-free' NETosis was blocked via inhibition of specific metabolic pathways

We next investigated the relevance of PMN metabolism during NETosis via the presence of selected metabolic inhibitors which interfere with glycolysis and ATP regeneration (see also metabolic scheme in Fig. 3). Concretely, we studied the impact of PMN pre-treatments with chemical compounds acting on the glucose-lactate and citric acid cycle-axis (FDG: blocks glycolysis at hexokinase level; oxamate: inhibits lactate formation by blocking lactate dehydrogenase; oxythiamine: blocks pyruvate conversion to acetyl-CoA via pyruvate dehydrogenase inhibition, succinyl-Co-A production by inhibition of α-ketoglutarate dehydrogenase as well as the non-oxidative pentose phosphate pathway by inhibiting transketolase; DCA: activates the conversion of pyruvate to acetyl-CoA by inhibiting pyruvate dehydrogenase kinase) and on mitochondrial ATP regeneration by inhibiting ATP-synthase within the mitochondrial respiration chain (oligomycin).

Overall, quantification of 'anchored' and 'cell-free' NETs confirmed that tachyzoites trigger

bovine PMN to release either form of NETs (Fig. 4). Functional inhibition experiments revealed that this process was independent of glucose consumption since 2 mM FDG did not influence NET formation (Fig. 4). In contrast, a significant decrease of 'cell-free' NET formation was observed in case of DCA, oxythiamine, oxamate and oligomycin A treatments (treated PMN + tachyzoites *vs* non-treated PMN + tachyzoites: DCA: $p < 0.05$, oxythiamine: $p < 0.01$, oxamate: $p < 0.01$, oligomycin A: $p < 0.001$) (Fig. 4), implying that efficient *B. besnoiti* tachyzoite-induced 'cell free' NET formation depended in some way on the products and pathways of the corresponding enzymes (pyruvate dehydrogenase kinase-, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, transketolas-, lactate dehydrogenase and mitochondrial ATP synthase). Interestingly, these findings only applied to 'cell-free' NETs whilst the formation of 'anchored' NETs was not affected by these inhibitors.

3.4 *B. besnoiti*-induced NETosis depended on P2X1-mediated ATP binding

Here we investigated the relevance of purinergic signaling pathways in *B. besnoiti* tachyzoite-induced NETosis. Therefore, PMN were pre-treated with two specific inhibitors: theobromine which inhibits P1A1-mediated purinergic signaling and NF449 which blocks P2X1-mediated purinergic signaling. As an interesting finding we here show that NF449 pretreatment almost entirely abolished parasite-triggered NET formation when compared to non-treated controls (treated PMN + tachyzoites *vs* non-treated PMN + tachyzoites: $p < 0.0001$) whilst theobromine treatments had no effect (Fig. 5). These data indicated that tachyzoite-induced NETosis selectively depends on P2X1-mediated ATP binding but is independent of P1A1-mediated purinergic signaling.

3.5 *B. besnoiti*-induced NETosis increased with raising pH values but is not influenced by MCT inhibition

Several studies demonstrated that PMN show reduced chemotaxis, respiratory activity and bactericidal capacity at acidic pH (Cao et al., 2015; Lardner, 2001) which is consistently reported in terms of inflammation (Lardner, 2001; Riemann et al., 2015). Here we investigated the effects of varying extracellular pH conditions (pH of 6.6, 7.0, 7.4. and 7.8) on parasite triggered NETosis. Interestingly, NETosis was already triggered in plain PMN when the pH was raised to 7.8 thus indicating that alkalization of the extracellular compartment led to PMN activation and subsequent NET formation (Fig. 6). However, this

only applied for 'anchored' NETs and not for 'cell-free' NETs. In tachyzoite-exposed PMN the same tendency was observed for both NET forms since elevated pH conditions led to enhanced NETosis (Fig. 6). Thus, significantly more NETs were formed in response to parasite stages when pH was raised to pH 7.8 thereby indicating a dependence of NET formation on alkaline pH. Interestingly, a lowering of pH below neutral led to diminished 'anchored' NET formation ('anchored' NETs: pH 7.4 vs. pH 6.6: $p < 0.05$ and pH 7.0 vs. pH 6.6: $p < 0.05$) (Fig. 6) thereby indicating that NET formation might be impaired in acidic conditions of inflammation.

Monocarboxylate transporters (MCTs) are important cellular pH regulators (Pinheiro et al., 2010), which control proton-linked transport of monocarboxylates, such as L-lactate, pyruvate, and ketone bodies, across the plasma membrane (Halestrap, 2012). As visualized in Fig. 7, *B. besnoiti* tachyzoite-induced NETosis was neither significantly altered via pretreatments with an inhibitor of MCT1 (AR-C 155858) nor by a blocker of MCT1 and MCT2 (AR-C 141990) thereby implying that these transporters were irrelevant for parasite-triggered NET formation.

4. Discussion

In the present study, we aimed to investigate the relevance of selective metabolic pathways in bovine PMN for *B. besnoiti*-induced NETosis. Metabolic signatures of PMN that were blocked in their phagocytotic activity and then exposed to invasion-defective (heat-inactivated) tachyzoites revealed a significant increase in glucose and serine consumption, an increase in glutamate release as well as a decrease in glutamine release during NETosis. Lactate, pyruvate and alanine release as well as aspartate consumption also indicated an increase during NETosis. However, the latter increase did not reach a significance level for any of the metabolites. Taken together, these data point at an increased energy regenerating conversion of glucose and serine to pyruvate and lactate. Besides reduction to lactate, pyruvate itself may be released into the medium. In addition, intracellular pyruvate may be converted into alanine or infiltrated into the citric acid cycle. The current experimental setting did not indicate glutamine as an energy source in the cells since it was produced in untreated PMN. However, the decrease in glutamine release during NETosis may be due to the enhanced glutamate release (Figure 3).

Even though an increase in glucose consumption was observed, pharmacological inhibition of glycolysis by 2-Fluor-2-desoxy-D-Glucose did not result in a significant reduction of parasite-triggered NETosis. This result is in contrast to Rodríguez-Espinosa et al. (2015) who showed that treatments with the closely related compound 2-desoxy-glucose led to a significant reduction of PMA-induced NETosis. Whether these differences rely on the mode of NET stimulation or host-derived differences (Rodríguez-Espinosa et al. stimulated human PMN with the potent inducer PMA, which does hardly function in the bovine system) remains unclear. Nevertheless, pharmacological interference with the glucose-pyruvate-lactate-axis indeed confirmed the importance of lactate and pyruvate generation during NETosis since treatments with both, oxamate and dichloroacetate efficiently blocked *B. besnoiti*-induced formation of 'cell-free' NETs and induced NET diminishment to control level (plain PMN). Oxamate inhibits lactate dehydrogenase thereby reducing lactate release and regeneration of NAD^+ (Ratter et al., 2018) which both may have an impact on NETosis. An inhibition of NAD^+ recycling leads to a blockage of glycolytic glyceraldehyde 3-P dehydrogenase thereby inhibiting glycolysis. Dichloroacetate (DCA) inhibits pyruvate dehydrogenase kinase thereby activating the conversion of pyruvate to acetyl CoA by pyruvate dehydrogenase and inhibiting the conversion of pyruvate to lactate by lactate dehydrogenase (Nayak et al., 2018; Wu et al., 2018). Interestingly, on the other hand, treatments with oxythiamine which is an antagonist of thiamin-PP and inhibits pyruvate dehydrogenase (Tylicki et al., 2005) did also induce a significant reduction of parasite-triggered NET formation. However, besides pyruvate dehydrogenase, oxythiamine is also a cosubstrate of α-ketoglutarate dehydrogenase within the citric acid cycle and transketolase within the non-oxidative pentose phosphate pathway. Further metabolic characterization has to show which enzymes are mainly responsible for the inhibition of NETosis by oxythiamine.

The current data additionally point at an important role of ATP regeneration during NETosis. Thus, treatments with oligomycin, an inhibitor of mitochondrial ATP synthase, entirely abolished tachyzoite-induced 'cell-free' NET formation and led to NET values even lower than those in plain PMN controls, which is in line to findings on oligomycin treatments in PMA-induced NETosis (Rodríguez-Espinosa et al., 2015). ATP as an energy source is produced either by glycolysis or by mitochondrial respiration. However, PMN comprise only few mitochondria (Fossati et al., 2003; Maianski et al., 2004) and, in contrast to current

findings, older reports suggested that these organelles do not play a key role in PMN-related energy metabolism (Borregaard and Herlin, 1982). Consequently, glycolysis should represent the main pathway for energy disposal. In line, PMA stimulation led to increased glucose uptake in human PMN and PMA-induced NET formation revealed as glucose-dependent (Rodríguez-Espinosa et al., 2015). A dependency of NETosis on glycolytic ATP production was also described in a mouse model by Amini et al., (2018). Interestingly, in mice glycolytic ATP production was shown to depend upon optic atrophy 1 molecules (Amini et al., 2018), which are of mitochondrial origin and reduce the activity of mitochondrial electron transport complex I in neutrophils thereby linking both metabolic pathways to another. Accordingly, blockage of mitochondrial ATP synthesis via oligomycin in the current study showed effective inhibition effects on parasite-triggered NET formation thereby confirming the relevance of mitochondrial ATP production for proper NETosis function. Notably, oligomycin treatments also impaired chemotaxis and respiratory burst in human PMN (Fossati et al., 2003), the latter of which is linked to NETosis process. Interestingly, a recent study reported that mitochondrial ATP is required for human PMN activation and that inhibition of mitochondrial ATP synthesis had minor effects on intracellular ATP levels, but inhibited the release of ATP into the extracellular space in human PMN (Bao et al., 2014). In turn, extracellular ATP acts as a messenger molecule and promotes communication between adjacent cells. Furthermore, it drives purinergic signaling-dependent mechanisms via various purinergic receptors. Consistently, hydrolysis of extracellular ATP inhibited the ATP-dependent process of PMN migration, and inhibition of purinergic signaling blocked PMN activation and impaired innate host responses to bacterial infection (Chen et al., 2010). Furthermore, purinergic receptors are involved in PMN chemotaxis (Tweedy et al., 2016), phagocytosis (Wang et al., 2017), oxidative burst (Chen et al., 2010), apoptosis (Vaughan et al., 2007) and degranulation (Grassi, 2010). There are two main families of purine receptors: P1 and P2 receptors. So far, nineteen different purinergic receptor subtypes for extracellular ATP and adenosine were recorded, including eight P2Y, seven P2X and four P1 (adenosine) receptor subtypes (Burnstock, 2007; Ralevic and Burnstock). It has been reported that extracellular ATP regulates PMN chemotaxis via P2Y2 receptors (Chen et al., 2006) and that P2Y receptors are involved in PMN adhesion to endothelium (DiStasi and Ley, 2009; Jacobson et al., 2009). Interestingly, PMN-derived P2X and P2X7R surface receptors are required for NLRP3-mediated inflammasome activation and bacterial killing (Karmakar et al.,

2016). In the current study, we found that P2X1 receptors play a crucial role in *B. besnoiti* tachyzoite-induced NETosis since pharmacological inhibition of this receptor by NF449 treatments totally abolished the formation of both, 'anchored' and 'cell-free' NETs upon parasite exposure. In line, receptors of the P2 family were recently recorded as important players in NET formation. Thus, P2Y2 receptor blockage significantly diminished *N. caninum* tachyzoite-triggered NETosis (Villagra-Blanco et al., 2017) and inhibition of P2Y6 receptor subtype led to blockage of neutrophil activation and aggregated NET formation induced by gout-associated monosodium urate crystals (Sil et al., 2017).

Distinct cell types, such as leukocytes, skeletal muscle cells, and most tumor cells, mainly produce ATP via glycolysis and need to export lactic acid to avoid cytoplasmic acidosis (Merezhinskaya et al., 2004). Lactic acid transport across cell membranes is mediated by MCTs. The MCT family contains 14 members, amongst which MCT1-4 represent the predominant transporters for lactate uptake and efflux (Halestrap and Meredith, 2004, 16, Dhup et al., 2012; Halestrap, 2013). However, the current data on MCT1/2 inhibition denied any influence of these receptors on *B. besnoiti*-induced NETosis. MCT1 typically imports lactate in oxidative cells, whilst MCT4 rather exports lactate derived from glycolysis. As such, other transporters than MCT1 may be involved in parasite-driven NETosis. As mentioned before, energy for neutrophil functions is mainly derived from glycolysis and therefore, MCT4-related lactate transport may be more important in bovine NETosis. Nevertheless, since NETosis was associated with increased lactate and glutamate release of the PMN which both induce an acidification of the medium we here also studied the effect of extracellular pH on *B. besnoiti* tachyzoite-triggered NETosis. It is well-known that extracellular pH modulates the functions of immune cells (Kellum et al., 2004; Lardner, 2001), including PMN (Trevani et al., 1999), macrophages (Malayev and Nelson, 1995) and lymphocytes (Nakagawa et al., 2015). Extracellular acidosis is the most common condition related to various pathological situations and most studies rather focused on acidification than alkalinization effects. Referring to PMN, older studies documented that acidic extracellular pH-induced PMN migration (Nahas et al., 1971; Rabinovich et al., 1980; Zigmond and Hargrove, 1981) and respiratory burst (Ahlin et al., 1995; Gabig et al., 1979; Leblebicioglu et al., 1996; Simchowitz, 1985). A more recent study revealed that extracellular acidification caused delayed human PMN-derived apoptosis, enhanced endocytosis and inhibited bacterial

killing (Cao et al., 2015). Likewise, intracellular killing mechanisms of bovine PMN were also inhibited by extracellular acidification (Craven et al., 1986). Recently, it was reported that extracellular acidification inhibited ROS-dependent NET formation (Behnen et al., 2017). In line, we here documented hat acidification (pH 6.6) led to a decrease of NET formation when compared to neutral pH (pH 7.4.). Thus, acidification commonly found in conditions of inflammation may impair proper NETosis in the in vivo situation. Additionally, the current data indicated that extracellular alkalization led to enhanced *B. besnoiti* tachyzoite-induced NETosis, which is in accordance to NET-related reports in the human system (Maueröder et al., 2016; Naffah de Souza et al., 2018). Given that intracellular alkalinization induces cytosolic calcium flux (Li et al., 2012) and elevated levels of calcium are required for PAD4-mediated citrullination of histone H3 and subsequent NETosis (Naffah de Souza et al., 2018; Villagra-Blanco et al., 2017), enhancement of *B. besnoiti*-induced NETosis with raising extracellular pH might be based on altered calcium fluxes.

In summary, this study provides a better understanding on the relevance of metabolic pathways, purinergic signaling and pH conditions involved in *B. besnoiti* tachyzoite-induced NETosis.

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Conflict of interest

There is no conflict of interest.

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Fig. 1 *Besnoitia besnoiti* tachyzoite-induced NETosis in bovine PMN. Co-cultures of bovine PMN and *B. besnoiti* tachyzoites were fixed and analyzed by scanning electron microscopy analysis (A-C). NETs, defined as chromatin extracellular structures forming a meshwork in contact with tachyzoites, were confirmed and visualized via immunostaining. (D) Phase contrast image; (E) DNA staining; Sytox Orange; (F) histone (H1-H4) staining; (G) neutrophil elastase staining; (H) Merged image of E, F, and G; (I) Merged image of all channels.

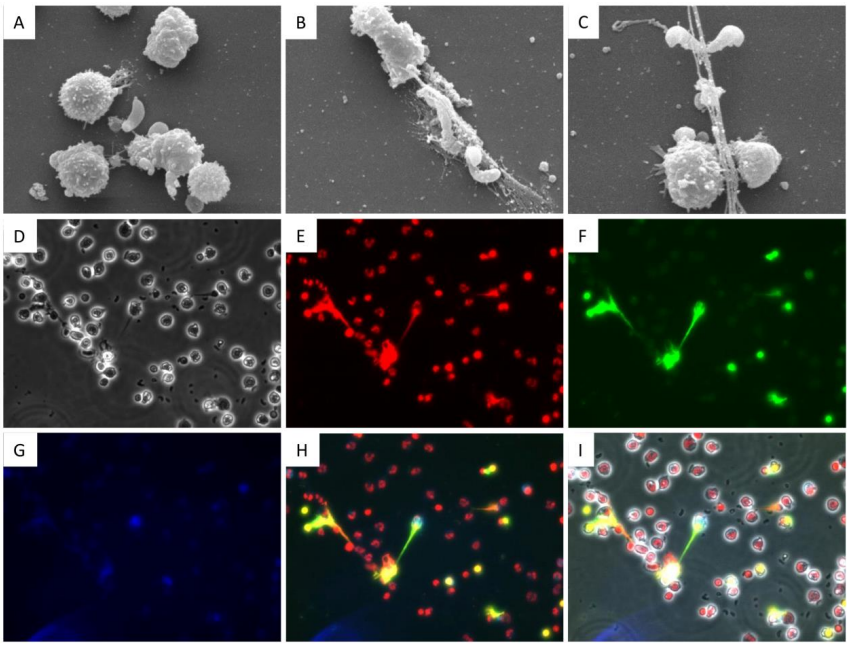
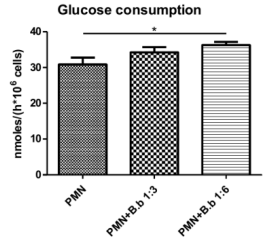
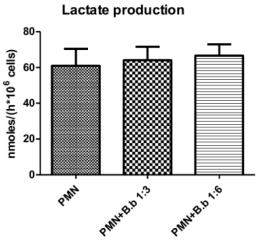
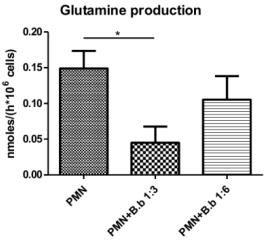


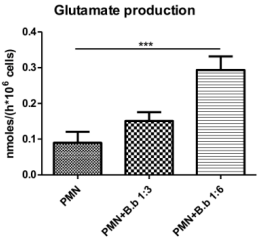
Fig. 2 Metabolic conversion rates in bovine PMN exposed to *B. besnoiti* tachyzoites. Bovine PMN were pre-treated with cytochalasin D for 15 min, then confronted for 6 h with heat-inactivated *B. besnoiti* tachyzoites or plain medium at MOIs of 3:1 and 6:1. Metabolites present in cell culture supernatants were measured using a bench top random access clinical chemistry analyzer and the conversion rates of these metabolites were calculated in nanomoles (nM) per (hour × 10^6 cells) in relation to plain medium samples which were

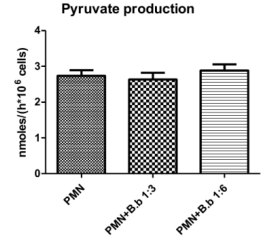
incubated in parallel for reference purpose. Values are presented as mean ± SEM (n = 8) in the graphs and *p* values of <0.05 were considered statistically significant.

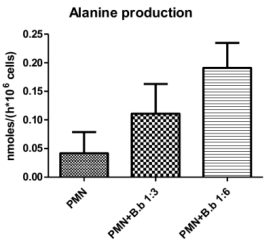












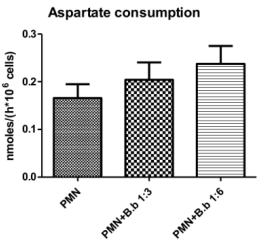


Fig. 3 Metabolic scheme and overview on molecules analyzed in the current study. Molecules in blue letters were analyzed in PMN supernatants, molecules in red letters represent the inhibitors used in the current study. FDG = fluoro 2-deoxy-D glucose, DCA = dichloroacetate, OT = oxythiamine, OXA = oxamate

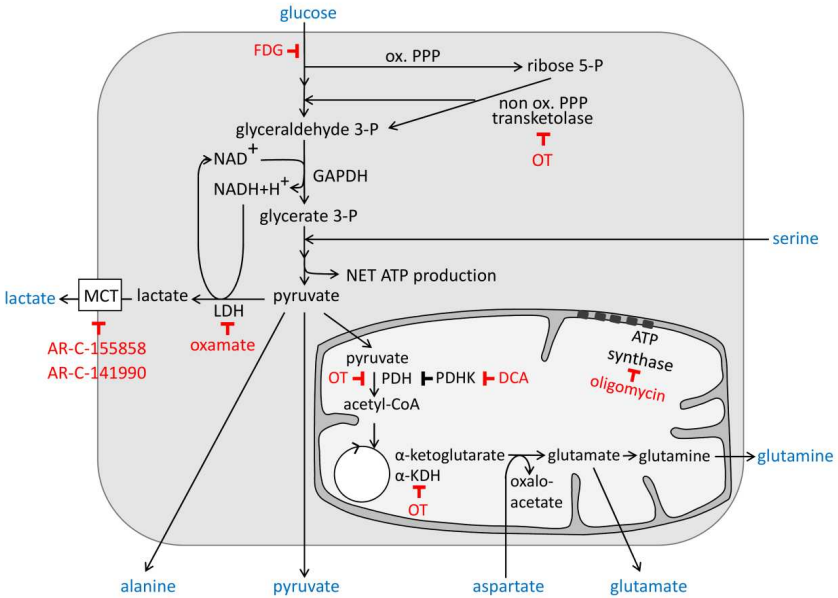


Fig. 4 Effects of FDG, DCA, OT, OXA and oligomycin D treatments on parasite-triggered NETosis. Bovine PMN (n = 3) were pre-treated for 30 min with FDG (2 mM), DON (4 µM), DCA (8 mM), OT (50 µM), OXA (50 mM) and oligomycin (5 µM) and then exposed to *B. besnoiti* tachyzoites for 3 h. Cell culture supernatants were collected for 'cell-free' NET measurement, and the pellets were used for 'anchored' NET estimation. Extracellular DNA was quantified by PicoGreen®-derived fluorescence intensities using an automated multi-plate reader (Varioskan, Thermo Scientific). Values are presented as mean ± SEM in the graphs and p values of <0.05 were considered statistically significant.

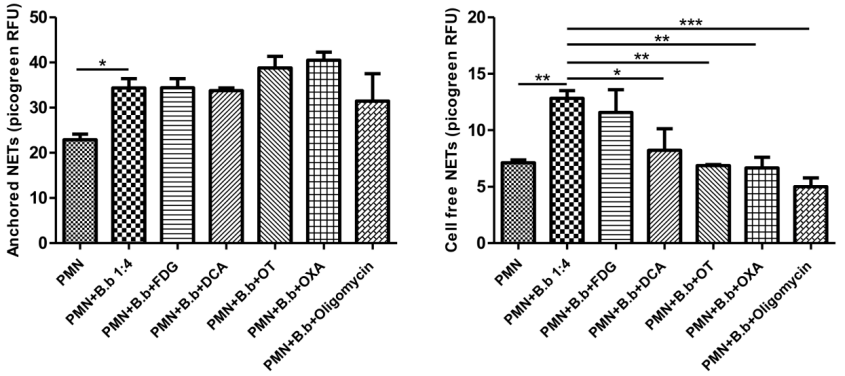


Fig. 5 Effects of purinergic signaling inhibition on *B. besnoiti*-induced bovine NETosis. Bovine PMN (n = 3) were pre-treated for 30 min with NF449 (100 μM, inhibitor of $P2X_1$ receptor) or theobromine (100 μM, inhibitor of $P1A_1$ receptor), followed by the exposure to *B. besnoiti* (ratio 1:4) for 3 h. Cell supernatants were collected for 'cell-free' NET measurement, and the pellets were used for 'anchored' NET estimation. Extracellular DNA was detected and quantified by PicoGreen®-derived fluorescence intensities using an automated multi-plate reader (Varioskan, Thermo Scientific). Values are presented as mean ± SEM in the graphs and p values of <0.05 were considered statistically significant.

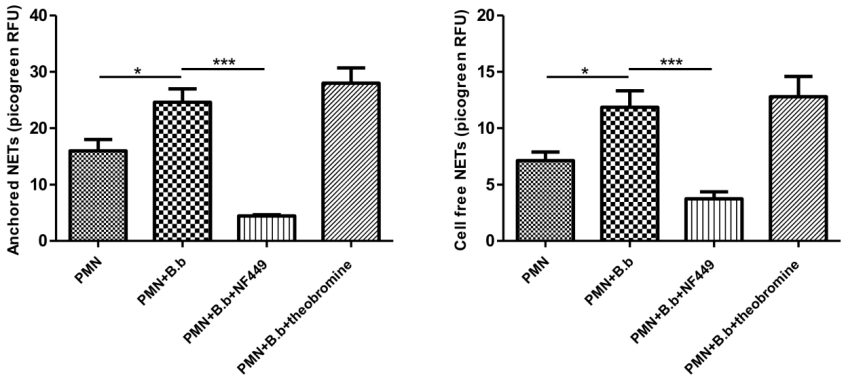


Fig. 6 Effects of extracellular pH on *B. besnoiti*-mediated bovine NETosis. Bovine PMN (n = 3) were suspended in RPMI 1640 media adjusted to different pH values (6.6, 7.0, 7.4 and 7.8), and then exposed to tachyzoites (ratio 1:4) for 3 h. Cell supernatants were collected for 'cell-free' NET measurement, and the pellets were used for 'anchored' NET estimation. Extracellular DNA was detected and quantified by PicoGreen®-derived fluorescence intensities using an automated multi-plate reader (Varioskan, Thermo Scientific). Values are presented as mean ± SEM in the graphs and p values of <0.05 were considered statistically significant.

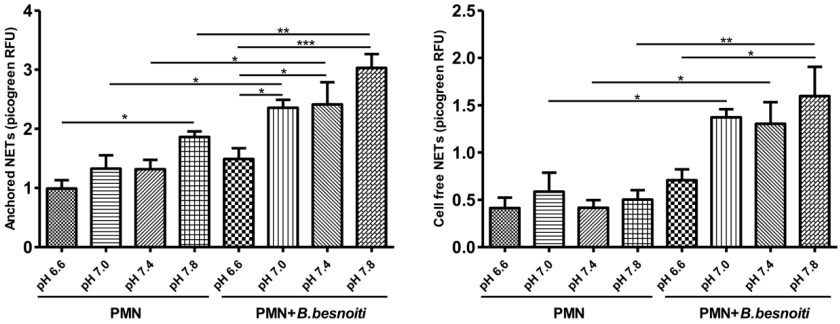
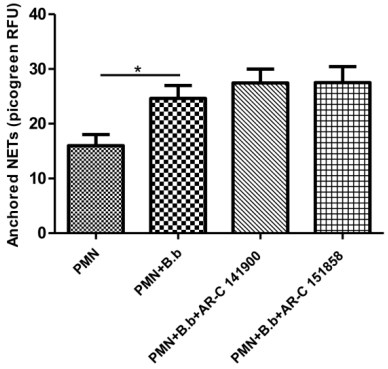
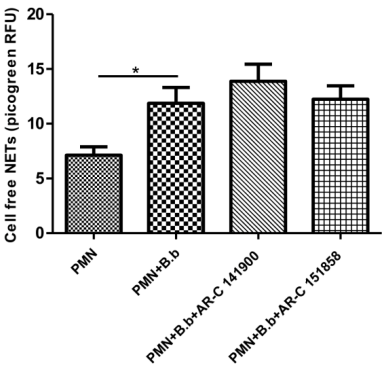


Fig. 7 Effects of monocarboxylate transporter (MCT) inhibition on *B. besnoiti* tachyzoite- induced bovine NETs. Bovine PMN (n=3) were pre-treated for 30 min with AR-C141990 (1 µM, MCT1 inhibitor) or AR-C155858 (1 µM, inhibitor of MCT1 and MCT2), followed by the exposure to *B. besnoiti* tachyzoites (ratio 1:4). Cell supernatants were collected for 'cell-free' NET measurement, and the pellets were used for 'anchored' NET estimation. Extracellular DNA was detected and quantified by PicoGreen®-derived fluorescence intensities using an automated multi-plate reader (Varioskan, Thermo Scientific). Values are presented as mean ± SEM in the graphs and p values of <0.05 were considered statistically significant.


Anchored NETs (picogreen RFU)
40
30
20
10
0
*
PMN
PMN+B.b
PMN+B.b+AR-C 141900
PMN+B.b+AR-C 151858


Cell free NETs (picogreen RFU)
20
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PMN
PMN+B.b
PMN+B.b+AR-C 141900
PMN+B.b+AR-C 151858

2.4. *Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy

Zhou, E., Silva, L.M.R., Conejeros, I., Velásquez, Z., Hirz, M., Gärtner, U., Jacquiet, P., Taubert, A., Hermosilla, C. (2019). *Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy. (submitted manuscript)

Eigener Anteil inder Publikation:

Initiative	Weitestgehend eingeständig
Projektplannung	Weitestgehend eingeständig
Durchführung des Versuches	Wesentlich
Auswertung der Experimente	Wesentlich
Erstellung der Publikation	Weitestgehend eingeständig

***Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy**

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***Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy**

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***Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy**

Abstract

Besnoitia besnoiti is an obligate intracellular apicomplexan protozoan parasite, which causes bovine besnoitiosis. Recent increased emergence within Europe was responsible for significant economic losses in cattle industry due to significant reduction of productivity. However, still limited knowledge exists on interactions between *B. besnoiti* and host innate immune system. Here, *B. besnoiti* bradyzoites were successfully isolated from tissue cysts located in skin biopsies of a naturally infected animal, and we aimed to investigate for the first time reactions of polymorphonuclear neutrophils (PMN) exposed to these vital bradyzoites. Freshly isolated bovine PMN were confronted to *B. besnoiti* bradyzoites. Scanning electron microscopy (SEM)- and immunofluorescence microscopy-analyses demonstrated fine extracellular networks released by exposed bovine PMN resembling suicidal NETosis. Classical NETosis components were confirmed via co-localization of extracellular DNA decorated with histone 3 (H3) and neutrophil elastase (NE). Live cell imaging by 3D holotomographic microscopy (Nanolive®) unveiled rapid vital NETosis against this parasite. A significant increase of autophagosomes visualized by specific-LC3B antibodies and confocal microscopy was observed in *B. besnoiti*-stimulated bovine PMN when compared to non-stimulated group. As such, a significant positive correlation (p = 0.042) was found between *B. besnoiti*-triggered suicidal NETosis and autophagy. These findings suggest that vital- as well as suicidal-NETosis might play a role in early innate host defense mechanisms against released *B. besnoiti* bradyzoites from tissue cysts, and possibly hampering further parasitic replication. Our data generate first hints on autophagy being associated with *B. besnoiti* bradyzoite-induced suicidal NETosis and highlighting for first time occurrence of parasite-mediated vital NETosis.

Keywords: *Besnoitia besnoiti*, bradyzoites, bovine PMN, autophagy, vital NETosis

***Besnoitia besnoiti* bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy**

1. Introduction

Besnoitia besnoiti, an obligate intracellular apicomplexan parasite, was firstly described in 1912 (Besnoit and Robin, 1912). Several reports on bovine besnoitiosis [i. e. Portugal (Cortes et al., 2006), Spain (Fernández-García et al., 2009), France (Jacquiet et al., 2010a), Germany (Schares et al., 2009), Italy (Gollnick et al., 2010; Rinaldi et al., 2013), Switzerland (Basso et al., 2013) and Hungary (Hornok et al., 2014)] clearly indicate the spread of this disease within Europe (Álvarez-García et al., 2013). Based on increased number of cattle besnoitiosis cases and its geographic expansion into previous non-endemic countries, the European Food Safety Authority (EFSA) classified bovine besnoitiosis as an emerging disease within EU in 2010 (EFSA, 2010). Besides Europe, bovine besnoitiosis is also a vastly endemic disease in the Middle East, Asia, South America (Trujillo and Benavides; Vogelsang and Gallo, 1941) and Africa (Bigalke et al., 2004; Cortes et al., 2014) causing significant economic losses in cattle industry due to significant reduction of productivity (Jacquiet et al., 2010a; Maqbool et al., 2012).

Typically, bovine besnoitiosis is characterized by an acute and a chronic phase with different clinical signs. In the acute phase, *B. besnoiti*-infected cattle present pyrexia, intensive respiratory disorders, increased heart rates, subcutaneous oedema, anasarca, swollen joints, conjunctivitis, nasal discharge, photophobia, reduced milk yield and orchitis associated with permanent infertility in bulls (Álvarez-García et al., 2013; Bigalke, 1981; Cortes et al., 2014). During the chronic phase of disease, *B. besnoiti* bradyzoites proliferate slowly within the epidermis, subcutaneous tissues, mucous membranes and/or sclera, and form characteristic cysts within mesenchymal host cells, related to dramatic thickening, hardening, folding, wrinkling of the skin (also termed “elephant skin”), alopecia, and gradual deterioration of body condition and weight loss (Pols, 1960). Until now, the complete life cycle of *B. besnoiti* is not entirely known and final host species are unidentified carnivores. Nevertheless, direct contact between infected and non-infected animals (e. g. natural mating, nasopharyngeal route) and insect-mediated transmission through biting flies [i. e. tabanids (*Tabanus* spp.), stable flies (*Stomoxys calcitrans*)] have been suggested as suitable transmission routes (Gollnick et al., 2015; Gutiérrez-Expósito et al., 2017; Tainchum et al., 2018) and of epidemiological relevance (Sharif et al., 2019).

Besnoitia besnoiti bradyzoite stages induce suicidal- and rapid vital-NETosis being correlated with autophagy

So far, very limited knowledge exists on early interactions between circulating polymorphonuclear neutrophils (PMN) of host innate immune system with *B. besnoiti*, although these cells are the first ones to be recruited to infection sites. As such, PMN are the most abundant granulocytes in the blood and being the first line of defence against invading pathogens including parasites (Weissmann et al., 1980; Behrendt et al., 2010, Villagra-Blanco et al., 2017). Upon activation, and in addition to phagocytosis (Behrendt et al., 2008) and degranulation (Lacy, 2006), PMN also combat efficiently invading pathogens by releasing NETs (Brinkmann, 2018; Brinkmann and Zychlinsky, 2012; Fuchs et al., 2007). These NETs are composed of nuclear DNA decorated with different histones (H1, H2A/H2B, H3, H4) and various antimicrobial granular effector molecules and commonly released via a novel cell death process known as suicidal NETosis. Suicidal NETosis is characterized by nuclear and cell membrane rupture and the loss of main PMN functions such as chemotaxis, degranulation, and phagocytosis (Fuchs et al., 2007; Remijsen et al., 2011b; Yipp and Kubes, 2013). In contrast, vital NETosis can also occur by not affecting the continuation of mentioned PMN functions (Yipp and Kubes, 2013). Vital NETosis has been demonstrated in response to bacteria (Pilsczek et al., 2010), fungi (Byrd et al., 2013) and LPS-activated platelets (Clark et al., 2007). A landmark of vital NETosis is its rapid induction, normally within 30 min after PMN stimulation (Yipp and Kubes, 2013). In previous studies, it was shown that suicidal NETosis was able to efficiently trap *B. besnoiti* tachyzoites *in vitro* and that released suicidal NETosis was capable of hampering tachyzoites from active host cell invasion (Muñoz Caro et al., 2014). Furthermore, also bovine monocyte-derived extracellular traps (METosis) occurred when these phagocytes have been exposed to vital and motile *B. besnoiti* tachyzoites (Muñoz-Caro et al., 2014a), thereby expanding the spectrum of leukocytes undergoing ETosis (Villagra-Blanco et al., 2019).

Conversely, no data are available so far neither on interactions of bovine PMN with *B. besnoiti* bradyzoites nor the role of autophagy in parasite-induced NETosis. Autophagy has recently been indicated to play a crucial role not only influencing classical PMN-mediated effector mechanisms (e. g. phagocytosis) (Mitroulis et al., 2010; Skendros et al., 2018) but also actively regulating NETosis (Skendros et al., 2018). Thus, in the present study we intended firstly to investigate rapid vital- as well as suicidal-NETosis in bovine PMN

exposed to freshly isolated bradyzoites of *B. besnoiti* from subdermal tissue cysts and further to analyse the possible correlation of autophagy in *B. besnoiti* bradyzoite-mediated NETosis.

2. Materials and methods

2.1 Ethic statement

This work was performed in accordance to the Justus Liebig University Giessen Animal Care Committee Guidelines. Protocols were approved by the Ethic Commission for Experimental Animal Studies of the Federal State of Hesse (Regierungspräsidium Giessen; A9/2012; JLU-No.521_AZ), and in accordance to prevalent European Animal Welfare Legislation (ART13TFEU) and current applicable German Animal Protection Laws.

2.2 Animal data

In early 2018, a 4-year-old Limousine heifer (502 kg BW) from South France presented inappetence, limb oedema with desquamation and emaciation. Natural *B. besnoiti* infection was confirmed by PCR investigation. For animal treatment, flunixine meglumine (2.2 mg/kg; Finadyne®) and sulfamethoxine (40 mg/kg; Sulfaron®) were given. Three months later, the same animal was admitted to the Ecole Nationale Veterinaire Toulouse (ENVT) because of weakness, hyperkeratosis, and multifocal alopecia, and presence of multiple visible cysts within the sclera. One week later, the animal was euthanized due to severe emaciation. At necropsy, bovine besnoitiosis in the scleroderma phase was here diagnosed: multiple whitish punctuated cysts were observed in sclera and in mucous-cutaneous junctions of mouth and anus. Moreover, the skin of neck, shoulders, base of tail, hocks, and pasterns presented marked hyperkeratosis with crusty appearance. No other relevant clinical alterations were further noticed. Skin samples of affected areas have been collected, stored in sterile saline solution (4 ºC) and later on processed for histopathological evaluation as well as for parasite isolation.

2.3 Isolation of vital *Besnoitia besnoiti* bradyzoites

Skin biopsies were placed in a sterile Petri dish (Nunc) containing a small volume of sterile RPMI 1640 cell culture medium without phenol red (Sigma-Aldrich) supplemented with 2%

penicillin-streptomycin (Sigma-Aldrich). A sterile tweezer was used to hold the skin and with a sterile scalpel the skin surface was carefully scraped in order to release vital bradyzoites from these cysts. As soon as the RPMI 1640 cell culture medium became turbid, it was collected and filtered through a sterile gauze swab in a sieve into a 50-mL Falcon tube followed by centrifugation at 200 × *g* for 1 min at room temperature (RT). Then the supernatant was collected and transferred into a new 50-mL Falcon tube, centrifuged (400 × *g*, 12 min), and the pellet was washed again with RPMI 1640 medium to collect released bradyzoites. All supernatants were collected and centrifuged at 1500 × *g* for 10 min, and then the supernatant was discarded and pellet containing bradyzoites was resuspended in sterile RPMI 1640 cell medium. Vital and extremely motile *B. besnoiti* bradyzoites were isolated and afterwards counted in a Neubauer haemocytometer chamber (Supplementary data video 1). Isolated *B. besnoiti* bradyzoites were firstly stored for 30 min at 4 ºC and afterwards at -80 ºC in RPMI 1640 cell medium supplemented with 10% DMSO (Merck).

2.4 Histopathological examination

After successful bradyzoites isolation, parts of skin samples (5 × 5 mm) were stored in 10% phosphate-buffered formalin for histopathological examinations. Shortly, formalin-fixed samples were dehydrated using an ascending ethanol series, embedded in paraffin wax at 56 °C and finally sectioned at 3 µm tissue samples at the Institute of Veterinary Pathology, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Germany. Histological tissue samples have been stained using haematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Giemsa staining according to routine protocols and pathological findings/changes of *B. besnoiti*-infected skin samples were then evaluated under a light microscope (Nikon Eclipse 80i) equipped with a DS-Fi1 digital camera (Nikon).

2.5 Isolation of bovine PMN

Healthy adult dairy cows (n = 3) served as blood donors. Animals were bled by puncture of jugular vein and 30 ml peripheral blood was collected in 12 ml heparinized sterile plastic tubes (Kabe Labortechnik). Approximately 20 ml of heparinized blood was re-suspended in 20 ml sterile PBS with 0.02% EDTA (Sigma-Aldrich), slowly layered on top of 12 ml Biocoll® separating solution (density = 1.077 g/L; Biochrom AG), and centrifuged (800 × *g*,

45 min). After extraction of plasma and peripheral mononuclear blood cells (PBMC), the pellet was washed in 25 ml distilled water and gently shaken during 40 s in order to lyse erythrocytes. Osmolarity was rapidly restored by Hank's balanced salt solution (4 ml, HBSS 10x; Biochrom AG). To complete erythrocyte lysis, this step was repeated twice and PMN were later re-suspended in sterile RPMI 1640 medium (Gibco). Finally, freshly isolated bovine PMN were allowed to rest at 37 °C and 5% CO_2 atmosphere for 30 min until further use (Behrendt et al., 2010).

2.6 Scanning electron microscopy (SEM) analysis

Bovine PMN were co-cultured with vital *B. besnoiti* bradyzoites (ratio 1:4) for 3 h on coverslips (10 mm diameter; Nunc) pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich) in an incubator at 37 °C and 5% CO_2 atmosphere. After incubation, 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. Finally, all samples were visualized via a Philips® XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

2.7 Immunofluorescence microscopy analyses for visualization of *B. besnoiti* bradyzoite-triggered NETosis

Freshly isolated bovine PMN were co-cultured on 0.01% poly-L-lysine pre-treated coverslips (15 mm diameter) with *B. besnoiti* bradyzoites (ratio 1:4) for 3 h (37 °C and 5% CO_2 atmosphere), then fixed by adding 4% paraformaldehyde (Merck) for 15 min and stored at 4 °C until further epifluorescence microscopy experiments.

For visualization of suicidal NETosis-related structures, Sytox Orange® (Life Technologies) was used to stain extracellular DNA, anti-histone 3 (H3; clone H11-4, 1:1,000; Merck Millipore) and anti-neutrophil elastase (NE) antibodies (AB68672, 1:1,000, Abcam) were used to label H3 and NE on NETosis structures. In brief, fixed samples were washed thrice with sterile PBS, then blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) at RT for 15 min, incubated with corresponding primary antibodies (1 h; RT), and then incubated with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 488 goat

anti-rabbit IgG, both Life Technologies, 60 min, 1:1,000, RT), and finally incubated for 15 min with Sytox Orange® (Life Technologies). After incubation, samples were carefully mounted with anti-fading solution (ProLong Gold® anti-fading buffer; Thermo Fisher Scientific), and thereafter visualized using an inverted IX81® epifluorescence microscope (Olympus) equipped with a digital camera XM10® (Olympus).

2.8 Live cell interactions between bovine PMN and *B. besnoiti* bradyzoites investigated by live cell 3D holotomographic microscopy

Isolated PMN (1×10^6) were centrifuged at $300 \times g$ for 10 min at RT, supernatant was carefully discarded and cells were suspended in 2 ml of pre-warmed RPMI 1640 cell medium. One ml of this PMN solution was placed in an Ibidi® cell plate 35 mm low profile, and the plate was incubated in an Ibidi® chamber at 5% CO_2 and 37 °C. PMN were allowed to settle down (30 min) to bottom of a plate and then 2×10^6 *B. besnoiti* bradyzoites were added to the center of the plate. Acquisition was set for refractive index (RI; 3D tomography) for a time lapse of 155 min every 30 s in a Nanolive Fluo-3D Cell Explorer® (Nanolive) microscope. At the end of the experiment, images were exported using Steve software v.2.6® (Nanolive). Using Image J software (Fiji version 1.7, NIH), every frame was exported using z-projection, maximum intensity algorithm and the video movie was constructed using 1 frame per 5 s of speed. For zoomed video, the region of interest was cropped and the same procedure described above was applied. Digital staining and 3D rendering of activated PMN was performed by using Steve software v.2.6® (Nanolive).

2.9 Autophagosome detection by immunofluorescence analysis

LC3 has been used as a classical marker for autophagosomes (Karim et al., 2007), being LC3-I cytosolic and LC3-II membrane-bound and enriched in the autophagic vacuole. Therefore, we tested whether LC3 expression might be present during *B. besnoiti* bradyzoite-induced NETosis as described elsewhere (Zhou et al., 2019). Briefly, bovine PMN ($n = 3$) were added on 0.01% poly-L-lysine pre-coated coverslips (15 mm diameter; Nunc), then stimulated by *B. besnoiti* bradyzoites for 1 h at RT. After incubation, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with cold methanol (Merck) for 3 min, and blocked by using the following blocking buffer [5% BSA (Sigma-Aldrich), 0.1% Triton X-

100 (Sigma-Aldrich) in sterile PBS] for 60 min at RT. After removing blocking buffer, cells were incubated overnight at 4 °C with rabbit anti-LC3B antibodies (Cat#2775, 1:200, Cell Signaling Technology) diluted in blocking buffer, washed three times with PBS, incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Invitrogen) for 1h in the dark at RT. After being washed three times with PBS, coverslips were mounted by prolonged anti-fading reagent with DAPI (Invitrogen) on glass slides (Nunc), and images were taken using an inverted epifluorescence microscope IX 81® (Olympus) and/or by using confocal microscopy analysis (LSM 710®; Zeiss).

2.10 Statistical analysis

Results are illustrated as means ± SEM of at least three independent experimental settings. One-way analysis of variance and Dunnett's multiple comparison test and Spearman correlation test were here performed and by using GraphPad Prism 7®. Differences were considered as significant at a level of $p \leq 0.05$.

3. Results

3.1 Histopathological examination of *B. besnoiti*-infected skin

Histopathological examination revealed multifocal large-sized round to ovoid *B. besnoiti*-cysts present in the dermis, panniculus and underlying muscle layer (Fig. 1A). Early tissue cysts were small, approximately 10-20 µm and contained a parasitophorous vacuole (PV) with few banana-shaped 3-5 µm structures (bradyzoites). Mature *B. besnoti*-cysts were huge (up to 400 µm) and the parasitophorous vacuole contained thousands of bradyzoite stages (please see Fig. 1B and C). Mature cysts containing numerous typical banana-shaped *B. besnoiti* bradyzoites had a three-layered cyst wall (10-30 µm of thickness): *i)* the outer wall composed of compressed collagen type I fibers, *ii)* the middle wall layer representing a thick hyaline capsule composed of extracellular matrix, and *iii)* the inner wall layer composed of a small rim of host cell cytoplasm with often multiple flattened nuclei containing the PV (Fig. 1C). The outer wall of compressed collagen as well as the bradyzoites stained mildly and middle hyaline layer was brightly stained with PAS. Using Giemsa staining, the inner rim of the middle hyaline layer of mature cysts stained purple, while the outer rim of the hyaline layer was translucent and the inner layer containing the host cell cytoplasm as well as the

bradyzoites stained blue (Fig. 1C). Surrounding *B. besnoiti*-tissue cysts there was a mild to moderate multifocal to coalescing infiltrate composed of macrophages, PMN, fewer lymphocytes, plasma cells, eosinophils, and rare multinucleated giant cells. Few tissue cysts were ruptured and surrounded by abundant macrophages including multinucleated giant cells, numerous PMN, and eosinophils which were sometimes arranged in clusters as well as fewer lymphocytes and plasma cells. There was mild to moderate diffuse epithelial hyperplasia and moderate orthokeratotic hyperkeratosis.

3.2 *Besnoitia besnoiti* bradyzoite-triggered suicidal NETosis was unveiled via SEM- and immunofluorescence microscopy-analyses

To investigate whether *B. besnoiti* bradyzoites were capable to induce suicidal NETosis, bovine PMN exposed to bradyzoites were analysed by SEM. Fine network structures were observed in *B. besnoiti* bradyzoites-stimulated bovine PMN (Fig. 2), and many bradyzoites were trapped by those structures (Fig. 2) visualized in SEM analysis. Alongside, different morphologies of bovine PMN were observed around these fine networks. Whilst typical smooth rounded PMN have been found in close proximity to bradyzoites indicating a rather inactivation status, other exposed PMN showed disrupted cell membrane surfaces and thereby releasing extracellular filaments entrapping firmly bradyzoites by cell death. Former PMN status corresponded well to previously described suicidal (lytic) NETosis and METosis against this apicomplexan protozoan where extracellular fibers mainly derived from dead PMN and monocytes (Muñoz Caro et al., 2014a; Muñoz-Caro et al., 2014).

In order to confirm whether bovine PMN were undergoing suicidal NETosis, main components of NETs formation [i. e. DNA, histones (H3) and NE] were visualized via immunostaining. In the control group (non-exposed PMN), no NETosis-like structures were observed by co-localization of H3 and NE (Fig. 3A-F). In contrast, the classical characteristics of suicidal NETosis were demonstrated in bovine PMN exposed to *B. besnoiti* bradyzoites by co-localization of extracellular DNA adorned with H3 and NE (Fig. 3G-L), and several bradyzoites being firmly trapped by NETosis as indicated by white arrows in Fig. 3L.

3.3 3D-holotomographic microscopy live cell imaging of *B. besnoiti* bradyzoite-triggered vital NETosis

Activation of bovine PMN and vital NETosis were additionally analysed by live cell 3D-holotomographic microscopy technology (Nanolive®). Activation of PMN occurred within the first 5 to 30 min of interaction with motile bradyzoites thereby showing pseudopod formation and rapid migration and crawling activities of PMN into the vision field showing bradyzoites. Noteworthy to mention was the observation of an elongated structure being rapidly tossed out from PMN after 30 min of parasite interaction. Due to the time point of occurrence and the non-lytic PMN phenotype of this 'chameleon tongue-like' reactions we interpreted this response as vital NETosis (Fig. 4A-B; please refer to Video S2). The digital staining and 3D reconstruction of vital NETosis showed clearly that neither the overall cell phenotype nor crawling activities were altered by the protrusion of this elongated structure (Video S3; Fig. 4C).

3.4 Autophagy occurred during *B. besnoiti*-triggered suicidal NETosis

Autophagy is a highly conserved intracellular degradation process not only to keep homeostasis or energy source of mammalian cells but also pivotal in several host innate immune functions (Germic et al., 2019). During autophagy, LC3 (microtubule-associated protein 1A/1B-light chain 3) is an important protein being involved in autophagosome formation, and it has been used as a classical marker of autophagosomes. As shown in Fig. 5, the majority of bovine PMN (see Fig. 5A, B, C, and D) were still round and inactive without stimulation of *B. besnoiti* bradyzoites. In contrast, most of bovine PMN exposed to *B. besnoiti* bradyzoites (see Fig. 5E, F, G, and H) were undergoing autophagy alongside with suicidal NETosis resulting in bradyzoite entrapment (Fig. 5F, G, and H), indicating a close association of these two cellular processes.

To investigate in more detail concomitant autophagy while *B. besnoiti*-induced suicidal NETosis, the percentages of 'NETotic cells' and LC3B-positive cells were calculated, and thereafter a Spearmann correlation test was performed. As seen in Fig. 6A, more cells were undergoing suicidal NETosis in *B. besnoiti* bradyzoite-stimulated bovine PMN when compared to non-stimulated PMN. Moreover, a significant positive correlation ($r = 0.3735$, p

= 0.042) was found between suicidal NETosis and autophagy in *B. besnoiti* bradyzoite-stimulated bovine PMN (Fig. 6B) compared to negative controls.

4. Discussion

Bovine besnoitiosis is caused by the parasite *B. besnoiti* which is a cyst forming apicomplexan parasite closely related to *Toxoplasma gondii* and *Neospora caninum* (Ellis et al., 2000). Chronic bovine besnoitiosis is characterized by tissue cyst formation, especially in the skin and in the mucosa of diverse organs (e. g. eyes, genitals) with associated progressive thickening, folding, hardening, wrinkling or lesions of affected skin or mucosa. Characteristic large-sized cysts of *B. besnoiti* containing thousands of bradyzoites were identified in skin samples of naturally infected heifer by histopathological examinations in accordance to previous reports (Cortes et al., 2005; Frey et al., 2013; Gentile et al., 2012; Jacquiet et al., 2010b; Rostaher Ana et al., 2010). Affected animal did not benefit from implemented treatments and its deteriorated clinical status determined euthanasia. However, even if the clinical status of this animal had remained unaltered, culling would have been the better control measurement for cattle besnoitiosis in the farm.

NETosis is an effective defence process of activated PMN to ensnare and eliminate invading pathogens by releasing web-like extracellular traps which consist of DNA as a backbone, histones (H1, H2A/H2B, H3, H4), and diverse anti-microbial peptides/proteases such as cathepsin G, α-defensin, pentraxin, cathelicidin (LL37), lactoferrin, calprotectin and others (Amulic and Hayes, 2011; Hermosilla et al., 2014). Recently, more attention has been paid on pivotal role of NETosis against protozoan- and metazoan-parasites in various terrestrial and marine mammalian species (Behrendt et al., 2010; Muñoz Caro et al., 2014; Reichel et al., 2015; Silva et al., 2014; Villagra-Blanco et al., 2017b) as well as gastropods (Lange et al., 2017). Consequently, PMN-derived NETosis and monocyte-derived METosis exerted potent entrapment capacities against *B. besnoiti* tachyzoites indicating that these two leukocyte populations might reduce parasite replication during the acute phase of infection as previously postulated (Muñoz Caro et al., 2014a, b). Nevertheless, no data in literature are still available on early NETosis against *B. besnoiti* bradyzoites although bradyzoites are released from tissue cysts *in vivo* (Langenmayer et al., 2015).

Here, for the first time, we demonstrated that bradyzoites of *B. besnoiti* were also able to induce NETosis in a similar manner as tachyzoites (Muñoz Caro et al., 2014), and thus proving that *B. besnoiti*-triggered NETosis is a rather parasite stage-independent effector mechanism. Bradyzoites and tachyzoites of *B. besnoiti* are known to exhibit different antigens (Fernandez-Garcia et al., 2009; Schares et al., 2010), and whether bradyzoite-specific antigens induced bovine suicidal NETosis could not be here answered (Muñoz-Caro et al., 2015; Muñoz-Caro et al., 2018; Silva et al., 2014). In line with these findings, other reports have also shown that different apicomplexan parasitic stages of the same species are able to induce NETosis (Muñoz-Caro et al., 2015; Silva et al., 2014; Villagra-Blanco et al., 2017a; Villagra-Blanco et al., 2017b). SEM analysis unveiled the presence of classical web-like structures released by bovine PMN exposed to *B. besnoiti* bradyzoites, as previously observed for tachyzoites (Maksimov et al., 2016; Muñoz Caro et al., 2014a). Additionally, the main components of NETosis [i. e. DNA, histones (H3), NE] were here identified and visualized via immunostaining and proving that these web-like structures were mainly suicidal NETosis. In addition, live cell imaging by 3D holotomographic microscopy showed rapid vital NETosis within the first 30 min without compromising the general structure of PMN cell membrane as well as crawling activity. In the past, it has been proposed that PMN subpopulations are able to elicit different types of NETosis and that only 20-30% undergo suicidal NETosis (Fuchs et al., 2007; Yipp and Kubes, 2013). Interestingly, suicidal NETosis seems to be more related to chemical stimuli as PMA, requiring hours to occur, meanwhile vital NETosis is more related to biological triggering agents of NETosis such as bacteria or fungi (Yipp and Kubes, 2013). To our knowledge, this is the first time that vital NETosis is evidenced by 3D live cell imaging as a PMN response to motile parasite stages.

As stated above, bradyzoites can be released from tissue cysts after either host induced- or after mechanical rupture (Langenmayer et al., 2015; Schulz, 1960). Farther, Langenmayer et al. (2015) suggested that during chronic bovine besnoitiosis intravascular circulation of ‘zoites’ might be possible after mechanical rupture of cysts located directly underneath vascular endothelium or after reactivation of tissue cysts and stage conversion into tachyzoite stages. Irrespective of these *in vivo* scenarios, released bradyzoites would be immediately in close contact to PMN and extruded NETosis might ultimately hamper bradyzoite

dissemination. Released bradyzoites might not be immediately identified in the bloodstream of infected animals due to pro-inflammatory host innate immune reactions as proposed elsewhere (Langenmayer et al., 2015) and *in vivo* PMN are among the first ones to be recruited to inflammation/infection sites (Fuchs et al., 2007; Villagra-Blanco et el., 2019; Zhou et al., 2019).

Our present results demonstrated also that autophagy was associated with bradyzoite-triggered NETosis. These findings corresponded well to recent data on *B. besnoiti* tachyzoite-mediated suicidal NETosis with concomitant autophagy (Zhou et al., 2019). While autophagy process has recently been tightly associated with NETosis the exact molecular mechanisms and autophagy pathways are still not completely clear (Remijsen et al., 2011a, b; Ullah et al., 2017; Skendros et al., 2018). Autophagy is an essential intracellular degradation mechanism to regulate protein and organelle turnover in many living cells thereby maintaining homeostasis and intracellular energy balance (Levine and Kroemer, 2008). During the process of autophagy, intracellular autophagosomes ultimately fuse with lysosomes to degrade and recycle the inside cargo (Bernard and Klionsky, 2013). LC3 is a small soluble protein which is distributed ubiquitously in mammalian tissues and known to form stable associations with the membrane of autophagosomes (Tanida et al., 2008). Thus, LC3 is widely used as a classical marker for microscopical detection of autophagosomes (Koukourakis et al., 2015; Park et al., 2017). Previous studies have revealed that autophagy is required for NETosis (Remijsen et al., 2011a, b; Ullah et al., 2017), and that autophagy induction significantly increased NETosis (Park et al., 2017). Accordingly, LC3B-stained autophagosomes were detected concomitant in PMN extruding suicidal NETosis towards *B. besnoiti* bradyzoites. These findings confirm that autophagy is required in bovine NETosis not only against *B. besnoiti* tachyzoite- (Zhou et al., 2019) but also against bradyzoite-stages as Spearmann test revealed a significant positive correlation between these two processes. In agreement to our findings, autophagy has also been reported to prime PMN not only for increased NETosis but also for increased phagocytosis during sepsis (Park et al., 2017). Whether PMN-derived phagocytosis through autophagy is expected to occur in the chronic phase of cattle besnoitiosis *in vivo* needs further investigations.

In summary, we describe for the first time the ability of bovine PMN to cast NETosis against motile *B. besnoiti* bradyzoites evidencing the importance of this ancient and well-conserved effector mechanism of early host innate immune system in cattle. Furthermore, LC3B-stained autophagosomes were detected in *B. besnoiti* bradyzoite-exposed PMN casting NETs resulting in a significant positive correlation of autophagy and parasite-induced suicidal NETosis. However, further autophagy-related investigations should elucidate other molecular mechanisms in this cell pathway. Finally, *B. besnoiti*-mediated vital NETosis resulted in a rapid extrusion and retraction of a 'chameleon tongue-like' structure, which is the first hint for this type of NETosis against apicomplexan parasites. Exact machinery, *B. besnoiti*-specific antigens and PMN receptors leading to fast parasite-triggered vital NETosis need further investigations.

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Author contributions

PJ provided the tissue samples. CH, AT, IC designed the project and experiments. LS isolated bradyzoites from tissue, and guided the manuscript. EZ carried out most of the experiments. MH Histopathological examination. UG: SEM. ZV: LC3B confocal microscopy. IC, CH and AT polished the manuscript. IC and EZ analyzed and prepared the figures. All authors reviewed the manuscript.

Conflict of interest

The authors declare no conflict of interest

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Fig. 1. Histopathological examination of skin biopsy. (A) Characteristic tissue cysts of *Besnoitia besnoiti* were observed by Periodic acid-Schiff (PAS) staining 4×; (B) a large-sized cyst of *B. besnoiti* is illustrated by haematoxylin and eosin (H&E) staining 20×; (C) a huge cyst of *B. besnoiti* with a typical three-layered wall is illustrated by Giemsa staining 20×.

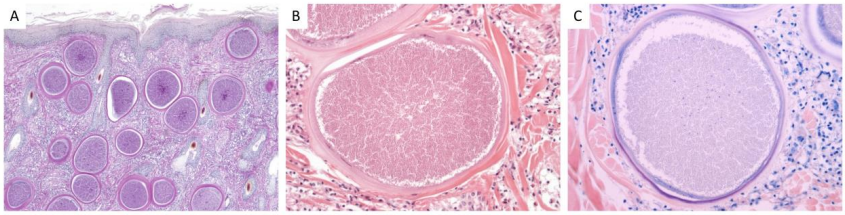


Fig. 2. Suicidal NETosis of bovine PMN after confrontation with *Besnoitia besnoiti* bradyzoites. Scanning electron microscopy (SEM) analysis revealed NETosis being formed by bovine PMN co-cultured with *B. besnoiti* bradyzoites, and these extracellular structures resulted in a fine meshwork containing bradyzoites as indicated by white arrows (A and B).

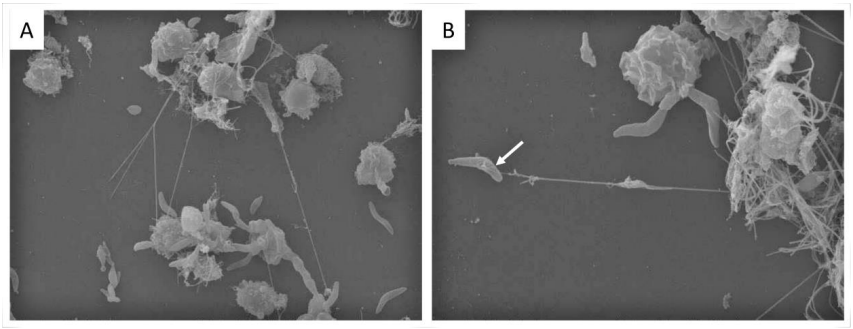


Fig. 3. Suicidal NETosis was visualized by co-localization of DNA with histones (H3) and neutrophil elastase (NE) in *B. besnoiti* bradyzoite-exposed bovine PMN. After 3 h of incubation, co-cultures of bovine PMN and *B. besnoiti* bradyzoites in a 1:4 ratio were fixed, permeabilized, and then suicidal NETosis was visualized via immunostaining. (A-F) PMN alone group: (A) phase contrast image (B) DNA staining; Sytox Orange; (C) H3 staining; (D) NE staining; (E) merged image without phase contrast image; (F) merged image with phase

contrast image. (G-L) PMN+bradyzoites group: (G) phase contrast image (H) DNA staining; Sytox Orange; (I) H3 staining; (J) NE staining; (K) merged image without phase contrast image; (L) merged image with phase contrast image.

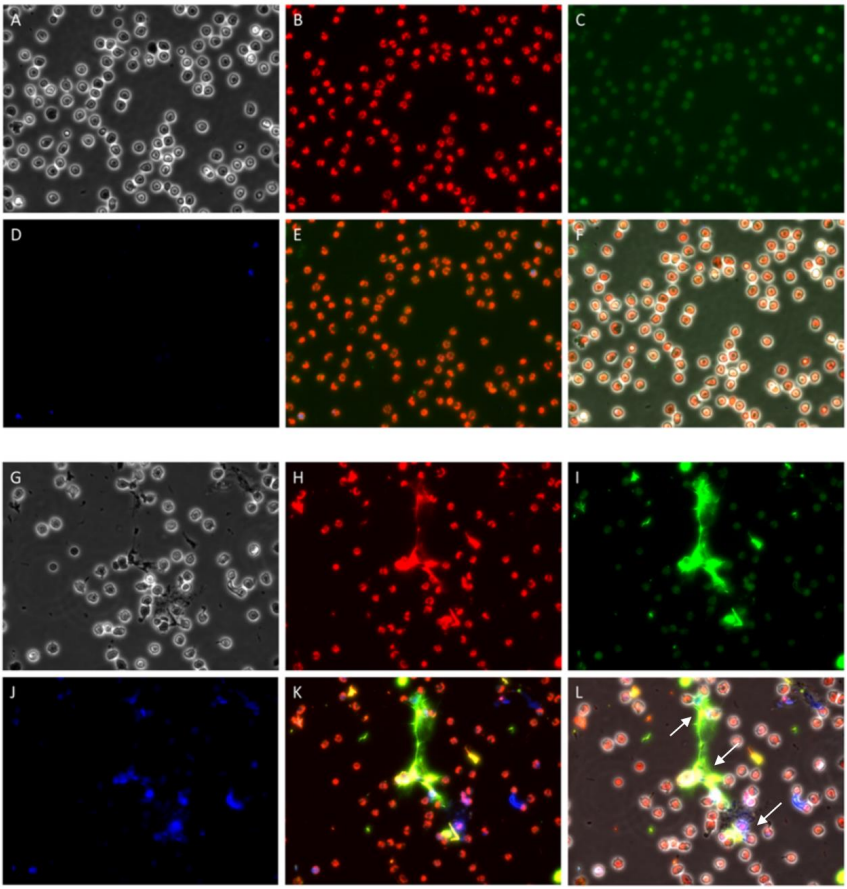


Fig. 4. *Besnoitia besnoiti* bradyzoites induced vital NETosis. Live cell 3D holotomographic microscopy (Nanolive®) analysis under controlled temperature and atmosphere conditions was performed for 1 h of interactions registering images every 30 s (A). At 31 min of incubation a tossing vital NETosis is observed without compromising the overall structure of PMN (B). Digital staining and 3D holotomographic reconstruction of tossed vital NETosis (C).

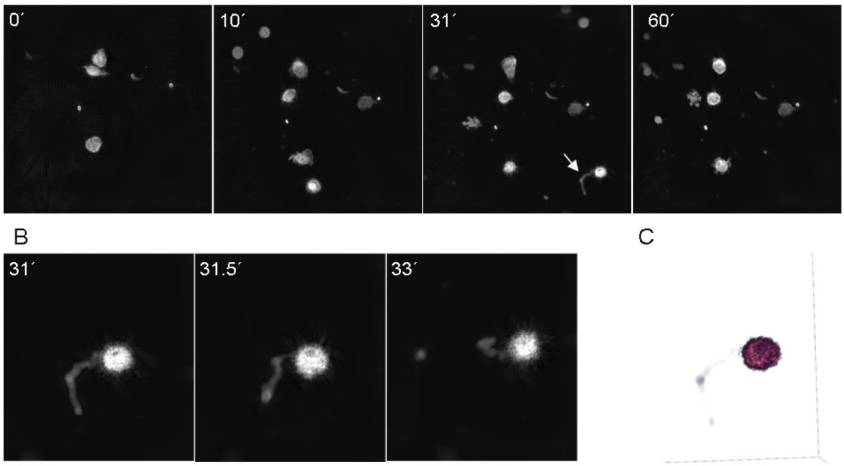


Fig. 5. Autophagy is occurring in *Besnoitia besnoiti*-triggered suicidal NETosis. Bovine PMN (n = 3) were exposed to *B. besnoiti* bradyzoites on coverslips for 1 h at 37 °C, 5% CO2. Samples were fixed and thereafter permeabilized for LC3B-based immunostaining in order to determine autophagosome formation by confocal microscopy analysis. All images are merges of LC3B (green), DAPI (blue), phase contrast (grey scale). Images A, B, C, and D are from PMN group, images E, F, G, and H are taken from PMN+bradyzoites group.

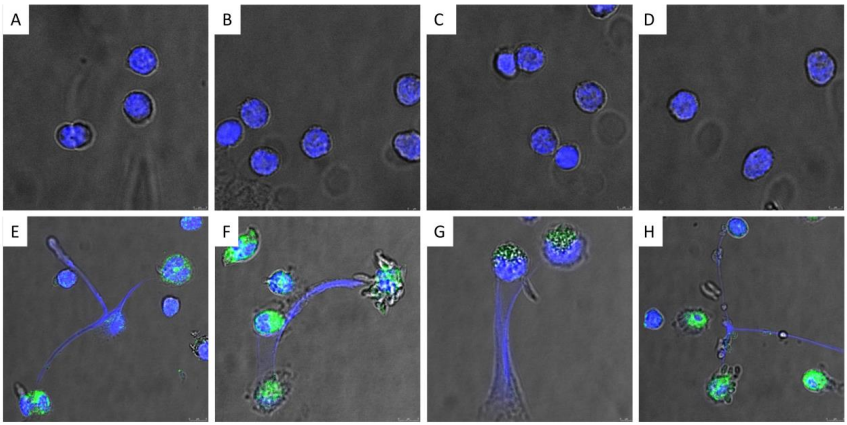
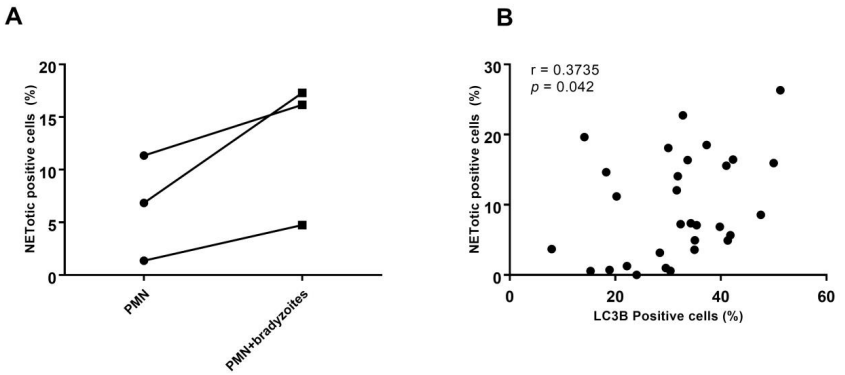


Fig. 6 Autophagy has a significant correlation with *Besnoitia besnoiti* bradyzoite-triggered suicidal NETosis. The number of NETotic (A) and LC3B-positive PMN was counted using ImageJ and the percentages over total cells were calculated. A positive correlation between *B. besnoiti*-induced LC3B expression and NETotic cells was analysed by Spearman test (B). Results are represented as before-after graph with data derived from 3 different animals ($n = 3$). p values of <0.05 were considered as statistically significant.



3. DISCUSSION AND OUTLOOK

Current work focused on bovine PMN-derived effector mechanism of NETosis in response to different parasitic stages of *B. besnoiti* (tachyzoites and bradyzoites) *in vitro*. Thus, this thesis mainly characterized the role of autophagy and glycolytic metabolism in *B. besnoiti* tachyzoite-induced bovine NETosis. Firstly, *B. besnoiti* tachyzoite-induced NETosis was confirmed by scanning electron microscopy (SEM) and immunofluorescence analyses. The metabolite conversion of PMN confronted with heat-inactivated *B. besnoiti* tachyzoites was measured by various functional enzymatic assays. Then, the role of diverse metabolic pathways in *B. besnoiti* tachyzoite-induced NETosis was determined via some specific metabolic inhibitors (i. e. FDG, DON, DCA, OT, OXA, and oligomycin), and the assessment of possible role of purinergic metabolism was tested via functional inhibition of purine receptors (i. e. P2X1 and P1A1) expressed on bovine PMN (Zhou et al., 2019b). Moreover, we investigated the role of pH in *B. besnoiti* tachyzoite-induced NETosis via modified media with different pH values and/or pharmacological inhibition of monocarboxylate transporter 1 (MCT1) which carries lactic acid across biological membranes (Zhou et al., 2019b). Furthermore, we detected concomitant autophagosome formation during *B. besnoiti* tachyzoite-induced NETosis and assessed the correlation of autophagy and NETosis in *B. besnoiti* stimulated bovine PMN (Zhou et al., 2019a).

In addition, we obtained *B. besnoiti* infected tissue samples from a naturally infected heifer from France and successfully isolated numerous *B. besnoiti* bradyzoites, which were here in addition investigated regarding their capacities to trigger bovine NETosis. Consequently, we showed for the first time, that *B. besnoiti* bradyzoites also induced NETosis in a similar manner to tachyzoites and analyses by SEM- and immunofluorescence microscopy corroborated bradyzoite-mediated NETosis (Zhou et al., 2019c). Farther, the correlation of autophagy and NETosis in *B. besnoiti* bradyzoite stimulated bovine PMN was evaluated in this thesis showing that both processes occur but independently from each other (Zhou et al., 2019c).

Originally most studies focused on associations of NETosis induction by bacteria and fungi, including *Staphylococcus aureus* (Pilsczek et al., 2010), *Streptococcus pneumonia* (Beiter et al., 2006), *Haemophilus influenzae* (Juneau et al., 2011), *Klebsiella pneumonia* (Papayannopoulos et al., 2010), *Listeria monocytogenes* (Munafo et al., 2009),

Mycobacterium tuberculosis (Ramos-Kichik et al., 2009), *Shigella flexneri* (Brinkmann et al., 2004), *Aspergillus nidulans* (Bianchi et al., 2011), *Aspergillus fumigatus* (McCormick et al., 2010), and *Candida albicans* (Urban et al., 2006), but less frequently by parasites. Nonetheless, increasing attention has been paid to adverse effects of NETosis against unicellular and metazoan parasites as part of early host innate immune reactions of various mammal species *in vitro* and *in vivo* (Hermosilla et al., 2014; Silva et al.,. 2016; Abdallah et al., 2012; Villagra-Blanco et al., 2019). So far, a vast number of protozoan species have been described as potent NETosis inducers, including apicomplexan parasites such as *P. falciparum* (Baker et al., 2008), *E. bovis* (Behrendt et al., 2010), *N. caninum* (Villagra-Blanco et al., 2017a), *T. gondii* (Abdallah et al., 2012), *B. besnoiti* (Caro et al., 2014), *C. parvum* (Muñoz-Caro et al., 2015b), *E. arloingi* (Silva et al., 2014a), and the euglenozoan parasites *Leishmania amazonensis* (Guimarães-Costa et al., 2009), *L. mexicana* (Hurrell et al., 2015), *L. major* , *L. infantum* (Guimarães-Costa et al., 2014), *L. braziliensis* (Morgado et al., 2015), *L. chagasi* and *L. donovani* (Gabriel et al., 2010). Alongside protozoans, NETosis was also induced by nematodes such as *S. stercoralis* (Bonne-Année et al., 2014), the filarial *B. malayi* (McCoy et al., 2017), *Dirofilaria immitis* (Muñoz-Caro et al., 2018), *L. sigmodontis* (Pionnier et al., 2016), the metastrongylid nematodes *A. vasorum* , *A. abstrusus* and *T. brevior* (Lange et al., 2017) and the trematodes *Schistosoma japonicum* (Chuah et al., 2013) as well as *Trichobilharzia regenti* (Skala et al., 2018).

Regarding *B. besnoiti*-induced extracellular traps (ETosis), scare knowledge is so far available, and only three reports are published in literature. Consequently, *B. besnoiti* tachyzoite-induced bovine ETosis released by PMN and monocytes were described, and classical ETosis structures were corroborated by co-localization of DNA with histones, NE and MPO (Muñoz-Caro et al., 2014a, b). Moreover, ETosis formation induced by *B. besnoiti* tachyzoites was reduced by pre-incubation with NOX-, NE- and MPO-inhibitors, indicating the important role of these enzymes in *B. besnoiti*-triggered ETosis extrusion and consistent with other publications in the field of ETosis (Metzler et al., 2011; Papayannopoulos et al., 2010; Rada et al., 2013). Parasite entrapment assays revealed that one third of *B. besnoiti* tachyzoites were efficiently immobilized by NETosis derived from PMN, thereby impeding host cell invasion and further replication (Muñoz-Caro et al., 2014a), implying that this effector mechanism might also occur during the acute phase of infection and could represent a relevant host innate immune reaction *in vivo* (Muñoz-Caro et al., 2014a).

In addition, bovine PMN adhered to *B. besnoiti* tachyzoite-infected primary bovine umbilical vein endothelial cell (BUVEC) layers, and releasing NETosis under physiological flow conditions, indicating a possible role of NETosis against parasites in the bloodstream (Maksimov et al., 2016). In order to verify this hypothesis posted by Maksimov et al. (2016), we additionally here analysed whether pure *B. besnoiti* tachyzoites- or calcium ionophore (A231187)-triggered NETosis would damage endothelial host cells and subsequently influence intracellular development and proliferation of *B. besnoiti* tachyzoites in primary bovine endothelium (Conejeros et al., 2019). Thus, endothelium injury triggered by H2A, floating PMN and *B. besnoiti*-induced NETosis preparations (i. e. 'cell free'- and 'anchored'-NETs), was evaluated under physiological flow conditions on endothelial host cell viability. Overall, all treatments (NETosis-derived H2A, *B. besnoiti*-triggered NETosis and floating PMN) significantly induced endothelium cell death of *B. besnoiti*-infected host cells (Conejeros et al., 2019). However, though host cell damage led to significantly altered intracellular parasite development with respect to parasitophorous vacuole diameter and numbers, the total proliferation of the parasite over time was not significantly affected by these treatments thereby denying any direct effect of NETosis on intracellular *B. besnoiti*-tachyzoite replication *in vitro* (Conejeros et al., 2019).

No data are available on metabolic requirements of bovine PMN during NETosis process neither for PMN stimulated with *B. besnoiti* tachyzoites nor bradyzoites. Farther there is no report on bovine PMN reactions exposed to vital and highly motile *B. besnoiti* bradyzoites. In this thesis, we reported on NETosis-like structures of bradyzoite-stimulated bovine PMN via SEM analysis (Zhou et al., 2019c). Bradyzoite-mediated NETosis was also confirmed by co-localization of main components: DNA, histones, and NE, which is in line with the other mentioned publications above (Zhou et al., 2019a, b; Conejeros et al., 2019).

To investigate in more depth the role of bovine PMN metabolism in *B. besnoiti* tachyzoite-induced NETosis, metabolic turnover rates of isolated PMN confronted with heat-inactivated *B. besnoiti* tachyzoites were measured by enzymatic assays using a bench top random access clinical chemistry analyzer as described by Mazurek et al. (1997). Glucose consumption, lactate production, glutamate production, serine consumption, aspartate, and alanine production was increased in by *B. besnoiti* tachyzoite-exposed bovine PMN compared to untreated bovine PMN, showing an enhancement of glycolysis, glutaminolysis, and serine metabolism in bovine PMN stimulated by dead tachyzoites (Zhou et al., 2019b). Furthermore, we investigated the effects of different metabolic pathways on *B. besnoiti* tachyzoite-induced

NETosis via appropriate pharmacological inhibition. As such, FDG- and DON-treatments resulted in a slight reduction of 'cell-free' NETs, and DCA-treatment caused a higher decrease in NETosis than FDG and DON, but all these treatments did not lead to a statistically significant difference (Zhou et al., 2019b). Interestingly, OT-treatment abolished *B. besnoiti* tachyzoite-induced 'cell-free' NETs. Similarly, treatment with OXA significantly blocked 'cell-free' NETs induced by *B. besnoiti* tachyzoites. In addition, oligomycin (an inhibitor of ATP synthase) treatment completely abolished 'cell-free' NETs formation in *B. besnoiti* tachyzoites-exposed bovine PMN, in which NETosis was even lower than of untreated PMN. As mentioned before, glycolysis is the first step to extract energy via breaking down glucose into pyruvate, and contains ten steps in the serial metabolic process. In mammalian PMN, there are very few mitochondria (Fossati et al., 2003; Maianski et al., 2004), and it has been reported that they do not play a critical role in energy metabolism (Borregaard and Herlin, 1982), thus glycolysis seems the main pathway to provide energy for PMN functions. PMA stimulation increased glucose uptake in stimulated PMN, and PMA-induced NETosis was dependent on glucose and glutamine to some extent. Similarly, the glycolytic rate in PMN was increased, and NETosis induced by PMA was inhibited by 2-DOG and oligomycin, indicating that glycolysis and ATP synthase play an important role during *B. besnoiti*-induced NETosis (Zhou et al., 2019b).

As already stated, energy required for PMN-derived effector mechanisms is mainly obtained from intracellular glycolysis pathway instead of mitochondria. However, our results revealed that NETosis was significantly decreased through inhibition of mitochondrial ATP synthesis. Furthermore, oligomycin treatment led to the inhibition of respiratory burst- and chemotaxis-activities of treated PMN which is in agreement with a previous report on the same metabolic topic (Fossati et al., 2003). These observations showed that mitochondrial ATP synthesis is required for some bovine PMN-derived effector mechanisms and demands further research approaches on species-specific differences of PMN activities (Zhou et al., 2019b). A recent study showed that inhibition of mitochondrial ATP synthesis had a minor effect on intracellular ATP levels, but inhibited release of ATP into the extracellular space of stimulated human PMN (Bao et al., 2014). Extracellular ATP can act as an extracellular messenger molecule for communications between adjacent cells, and driving purinergic-dependent signaling pathways via various specific receptors expressed on PMN surface. Consequently, hydrolysis of extracellular ATP inhibited PMN migration, and inhibition of purinergic signaling blocked effectively PMN activation and impaired innate host responses

to bacterial infection, implying that they are essential for PMN activation and innate immune defence (Chen et al., 2010).

Purinergic receptors are required for regulation of main displayed PMN functions, such as chemotaxis (Tweedy et al., 2016), phagocytosis (Wang et al., 2017), oxidative burst (Chen et al., 2010), apoptosis (Vaughan et al., 2007), and degranulation (Grassi, 2010). There are two main families of purine receptors: P1 receptors and P2 receptors. So far, nineteen different purinergic receptor subtypes of extracellular ATP and adenosine (ATP breakdown products) have been reported, including eight P2Y receptor subtypes, seven P2X receptor subtypes, and four P1 (adenosine) receptor subtypes (Burnstock, 2007; Ralevic and Burnstock, 2013). P2Y receptors are G protein-coupled receptors, and it has been reported that extracellular ATP regulates PMN chemotaxis via P2Y2 receptors (Chen et al., 2006). P2Y are farther involved in PMN adhesion onto endothelial cells via binding of ATP and UTP to PMN surface (DiStasi and Ley, 2009; Jacobson et al., 2009). P2X receptors are ligand-gated ion channel receptors, and P2X7R receptor expression on activated PMN is required for NLRP3-dependent inflammasome activation and bacterial killing (Karmakar et al., 2016).

In the present thesis, we found that P2X1 receptors played a crucial role during *B. besnoiti* tachyzoite-induced NETosis in stimulated bovine PMN since pharmacological inhibition of P2X1 receptors clearly abolished NETosis (Zhou et al., 2019b). Interestingly, coagulopathies have been linked to PMN activation and released intravascular NETosis (Kimball et al., 2016; Qi et al., 2017), and in pathogenesis P2X1 receptors expressed on PMN and platelets have been found to be involved in the pathogenesis of thrombosis (Darbousset et al., 2014). All these results together indicate that P2X1 play a crucial role in NETosis-mediated thrombosis (Darbousset et al., 2014; Kimball et al., 2016; Qi et al., 2017).

Furthermore, we investigated the effect of extracellular pH on *B. besnoiti* tachyzoites-exposed bovine PMN as extracellular pH modulates the functions of diverse immunocompetent cells (Kellum et al., 2004; Lardner, 2001), including PMN (Trevani et al., 1999), macrophages (Malayev and Nelson, 1995), and lymphocytes (Nakagawa et al., 2015). Extracellular acidosis is among the most common condition related to various physiological, pathological and infective situations, and most of studies focused on the effect of extracellular acidification in immune response more than on extracellular alkalinization. Early studies showed that acidic extracellular pH increased either PMN migration or diapedesis (Nahas et al., 1971; Rabinovich et al., 1980; Zigmond and Hargrove, 1981), and

activated PMN-mediated respiratory burst activities (Ahlin et al., 1995; Gabig et al., 1979; Leblebicioglu et al., 1996; Simchowitz, 1985). Another study revealed that extracellular acidification delayed human PMN apoptosis, enhanced endocytosis and inhibited bacteria killing (Cao et al., 2015). Likewise, intracellular bacterial killing of bovine PMN was also inhibited by extracellular pH acidification (Craven et al., 1986). Recently, it has been reported that extracellular acidification inhibited ROS-dependent formation of NETs (Behnen et al., 2017). Similarly, we found *B. besnoiti* tachyzoite-induced NETosis in bovine PMN being enhanced along with increasing extracellular pH (Zhou et al., 2019b), which is in accordance with other reports (Maueröder et al., 2016; Naffah de Souza et al., 2018). Extracellular acidification can rapidly reduce intracellular pH (Austin and Wray, 1993), and intracellular pH in PMN was also drastically enhanced with increasing extracellular pH (Naffah de Souza et al., 2018). Given that intracellular alkalinization induced cytosolic calcium flux (Li et al., 2012), and elevated calcium levels are required for proper PAD4-mediated citrullination of H3 and casting of NETosis (Naffah de Souza et al., 2018; Villagra-Blanco et al., 2017a), enhancement of *B. besnoiti* tachyzoite-induced NETosis under raising extracellular pH conditions might be due to calcium flux (Zhou et al., 2019a, b).

Host cells such as leukocytes, skeletal muscle, and most tumor cells, which all produce ATP as energy mainly from glycolysis pathways, must expel lactic acid to avoid intracellular acidosis (Merezhinskaya et al., 2004). Transport of lactic acid across PMN membrane is mainly mediated by different MCTs transporters. The MCT-family contains 14 members, among which only four members (MCT1-4) transport monocarboxylates (Halestrap and Meredith, 2004, 16), and MCT1 and MCT4 are the predominant transporters in lactate uptake- and efflux pathways in mammalian PMN (Dhup et al., 2012; Halestrap, 2013). However, our data revealed that the inhibition of MCT1 did not influence NETosis induced by *B. besnoiti* tachyzoites in stimulated cattle PMN (Zhou et al., 2019b). Given that MCT1 has high, and MCT4 rather low lactic acid affinity (Vinnakota and Beard, 2011), and being capable of lactate import and export, the less specific MCT4, also present in bovine PMN, might be involved in *B. besnoiti*-triggered NETosis. However, MCT1 is typically for lactate import in oxidative cells, and MCT4 is suited to export of lactate derived from glycolysis because it exhibits a lower affinity for pyruvate, which ensures continuous conversion of pyruvate into lactate and regeneration of cytosolic nicotinamide adenine dinucleotide (NAD^+) (Dimmer et al., 2000; Draoui and Feron, 2011; Fox et al., 2000). As we mentioned before, energy for many PMN functions is mainly derived from PMN glycolysis activities. Therefore,

MCT4 probably play a more vital role than MCT1 to export lactate in bovine PMN system as postulated elsewhere (Zhou et al., 2019b).

So far, no data is available on early reactions of bovine PMN, including oxidative burst activities (i. e. ROS production), phagocytosis and NETosis, after the confrontation with vital *B. besnoiti* bradyzoites neither *in vitro* nor *in vivo*. In this doctoral thesis, vital bradyzoites were successfully isolated from tissue cysts located in skin biopsies of a naturally infected animal presenting characteristic clinical signs of chronic bovine besnoitiosis (Zhou et al., 2019c). Histopathological examinations showed typical multifocal round to ovoid large-sized subdermal cysts within histiocytes, and unveiling a characteristic thick three-layer wall of *B. besnoiti*-cysts, which is in line with previous published reports (Álvarez-García et al., 2013a; Hornok et al., 2014; Jacquiet et al., 2010; Rostaher Ana et al., 2010). More importantly, we demonstrated for the first time that vital bradyzoites of *B. besnoiti* were also capable to induce strong NETosis in a similar manner to tachyzoites (Muñoz-Caro et al., 2014a), although being different parasitic stages and exhibiting very different antigens (Fernandez-Garcia et al., 2009; Schares et al., 2010). In line with these findings, other parasitic reports have also shown that different stages of the same parasite species are able to induce NETosis (Hermosilla et al., 2014; Muñoz-Caro et al., 2015a; Silva et al., 2014b; Villagra-Blanco et al., 2017a; Villagra-Blanco et al., 2017c). SEM analysis unveiled the presence of classical web-like structures released by PMN exposed to viable *B. besnoiti* bradyzoites, as previously observed for tachyzoites *in vitro* (Muñoz-Caro et al., 2014a; Maksimov et al., 2017). Additionally, the main components of NETosis [histones (H1, H2A/H2B, H3, H4) and NE] were here identified adorning extruded DNA filaments via immunostaining and proving that these web-like structures were NETs. Bradyzoites were entrapped in these extracellular structures, indicating that PMN might play a role in fast bovine innate immune responses against *B. besnoiti* bradyzoites being released actively through mechanical tissue cyst rupture. Accordingly, during chronic bovine besnoitiosis *in vivo* bradyzoites are liberated from tissue cysts after mechanical rupture and/or host immune suppression (Langenmayer et al., 2015; Schulz, 1960). Therefore, it has been speculated that released bradyzoites might become immediately potential targets for circulating PMN *in vivo* (Muñoz-Caro et al., 2014a; Langenmayer et al., 2015; Maksimov et al., 2017). Free-released bradyzoites of *B. besnoiti* might not immediately get into the bloodstream but PMN recruited to the site of bradyzoite release might initiate early antiparasitic reactions as postulated elsewhere (Brinkmann and Zychlinsky, 2012b; Hermosilla et al., 2014; Silva et al., 2016). During chronic phase of

bovine besnoitiosis intravascular circulation of bradyzoites might occur *in vivo* after mechanical rupture of tissue cysts located directly underneath vascular endothelium and/or after reactivation of tissue cysts and stage conversion to tachyzoites (Langenmayer et al.,. 2015). Irrespective of all these scenarios, bradyzoites would be in close contact with PMN *in vivo* and released NETosis could hamper dissemination of bradyzoites into other organs (Zhou et al., 2019a, b, c). Moreover, bradyzoites released from tissue cysts *in vivo* result in strong cellular reactions surrounding them and being mainly composed of professional phagocytes (Schulz, 1960). In addition, some studies indicated that tissue cysts seemed to be degenerated (Basson et al., 1970; Bigalke et al., 1966), and inflammatory cells were infiltrating these affected *B. besnoiti* tissue cysts. Interestingly, a past study on *B. jellisoni* infection in hamsters showed that bradyzoites liberated after spontaneous cyst rupture were found to be poorly stained and clearly controlled by PMN phagocytosis (Frenkel, 1955). Bradyzoites infections were less severe, and the most successful infections were obtained upon immunosuppression treatments with cortisone (Diesing et al., 1988), indicating that early host innate immune response has a strong protective effect against this parasitic stage (Frenkel, 1955; Basson et al., 1970; Bigalke et al., 1966; Diesing et al., 1988).

Interestingly, our present results demonstrated that autophagy was associated to both tachyzoite- and bradyzoite-triggered bovine NETosis, and recent data on *N. caninum* tachyzoite-mediated NETosis with concomitant autophagy (Zhou et al., manuscript in preparation) corresponded well to these results. Cellular autophagy is a highly conserved, an essential intracellular degradation mechanism to regulate protein and organelle turnover in many living cells thereby maintaining cell homeostasis and intracellular energy balance (Levine and Kroemer, 2008). During autophagy, unnecessary cytoplasm and/or damaged organelles are engulfed by double-membrane vesicles called autophagosomes which are characteristic markers of autophagy. These intracellular autophagosomes ultimately fuse with lysosomes to degrade and recycle the inside cargo of autophagosomes (Bernard and Klionsky, 2013). LC3 is a small soluble protein which is distributed ubiquitously in mammalian tissues and cultured cells and known to form stable associations with the membrane of autophagosomes (Tanida et al., 2008). As such, LC3-I (a cytosolic form of LC3) is conjugated to phosphatidylethanolamine to form LC3-II, which is then recruited to autophagosomal membranes (Park et al., 2017). Thus, LC3 is widely used as a classical marker for detection of intracellular autophagosomes. The LC3 gene family has three members LC3A, LC3B and LC3C, and most autophagy-related investigations used

endogenous autophagic marker is LC3B (Koukourakis et al., 2015). Autophagy in human PMN was found to be induced in both phagocytosis-dependent and -independent manner, and was also found to be involved in other relevant functions of PMN (Skendros et al., 2018). Upon phagocytosis of bacteria by macrophages, autophagy induction was shown to be crucial in eliminating intracellular pathogens like *L. monocytogenes*, *M. tuberculosis*, *S. enterica*, *S. flexneri*, and *S. pyogenes* (Dorn et al., 2002). Moreover, recent studies have revealed that autophagy is required for proper NETosis extrusion (Remijsen et al., 2011; Ullah et al., 2017) and that autophagy itself can result in the induction of NET formation (Park et al., 2017). In accordance with these previous studies, autophagy occurred in both tachyzoite- and bradyzoite-triggered NETosis observed by confocal analyses, and these two processes had a positive correlation by Spearmann test, showing a potential role of autophagy in bovine PMN-derived immune responses against *B. besnoiti* (Zhou et al., 2019a)

While autophagy process has been tightly associated with NETosis, the exact molecular mechanisms and pathways are still not completely clear (Skendros et al., 2018). mTOR, a serine/threonine kinase, is a crucial regulator of cell growth and proliferation, and also playing a central role in regulating autophagy (Kim and Guan, 2015). Thus, it is also reported that autophagy modulated NETosis via mTOR pathway is occurring (Itakura and McCarty, 2013). Farther, Park et al. (2017) showed that rapamycin pretreatments primed human PMN enhancing NETosis in response to PMA using a Sytox Green-related assay for NETosis quantification (Park et al., 2017). In this current thesis, rapamycin treatments alone did not influence tachyzoite-induced NETosis. Using *B. besnoiti* tachyzoites instead of PMA, rapamycin treatments did not influence the degree of parasite-triggered bovine NETosis when using Pico Green-based analyses on total NETs, "anchored" NETs and "cell-free" NETs (Zhou et al., 2019a). In addition, treatments with the autophagy-inhibitor wortmannin failed to affect parasite-triggered NETosis. This observation was complemented with the use of the PI3K inhibitor LY294002, observing a non-significant decrease of "anchored"-NETs. Same result was observed by the use of NF-κB inhibitor. However, when estimating early NETosis via nuclear decondensation [i. e. nuclear area expansion, (NAE)] analysis, we found that rapamycin pretreatments indeed primed bovine PMN for enhanced NET formation in response to *B. besnoiti* tachyzoites. The discrepancy between the different methods for NETosis detection may explain this by two factors: firstly, autophagy appears to precede NETosis being rather linked to early NETosis- than late NETosis, and secondly NAE-based assays appeared more sensitive for detection of tachyzoite-triggered NETosis therefore have

produced an improved resolution of obtained data. We therefore assume that early tachyzoite-triggered NETosis is indeed linked to autophagy in bovine PMN (Zhou et al., 2019a).

In addition, cellular autophagy is generally activated by nutrient deprivation, oxidative stress, and ultraviolet radiation, but it is also associated with physiological and pathological processes such as development, differentiation, neurodegenerative diseases, stress, infection, and cancer (Kesidou et al., 2013; Nikoletopoulou et al., 2015). A vast of cellular stress signals converge to mTOR complex 1 (mTORC1) to regulate autophagy upstream of the core machinery. mTORC1 is a critical regulator of autophagy induction, with mTORC1 activation by Akt and MAPK signaling suppressing autophagy (Dibble and Manning, 2013), and mTORC1 inhibition by AMPK and p53 signaling promoting this cell process (Alers et al., 2012). AMPKα is a key metabolic master regulator in eukaryotes with high impact on several important cellular mechanisms. AMPKα activation is initiated by changes in the metabolic status which result from inhibition of ATP generation during hypoxia, glucose deprivation and increased ATP consumption (Zhao et al., 2008). Previous observations in PMN showed that AMPK activation decreased PMA-induced ROS production in human PMN (Alba et al., 2004), but enhanced PMN chemotaxis, bacterial killing, and phagocytosis (Park et al., 2013). Moreover, AMPK promotes autophagy by directly activating Ulk1 which is a mTOR downstream enzyme during autophagosome formation (Kim et al., 2011). On the other hand, inhibition of AMPK in mice model induced H3 secretion, suggesting that AMPK activation contributed to murine NETosis (Jiang et al., 2014). Since autophagy is a complex process and it can be initiated via various signaling pathways, we tried to check whether AMPK pathway was also involved in *B. besnoiti* tachyzoite-induced autophagy. Our current data showed that confrontation of PMN with *B. besnoiti* tachyzoites clearly induced AMPKα activation in a time-dependent manner. Thus, AMPKα phosphorylation was rapidly induced in exposed PMN from the very beginning of parasite-PMN interactions (until 30 min; Zhou et al., 2019a). So far, it is unclear if enhanced AMPKα activation is linked either to autophagy or NETosis alone or to both concomitantly in tachyzoite-exposed PMN, but this will be a matter for further research.

In summary, this current work provides a better understanding on the relevance of metabolic pathways, purinergic signaling and pH conditions involved in *B.besnoiti* tachyzoite-induced NETosis. Moreover, concomitant NETosis and autophagosome formation were found to occur simultaneously in tachyzoite-exposed PMN. Autophagy was accompanied by rapid phosphorylation of AMPKα in exposed *B. besnoiti* PMN. In addition, we describe for the

first time the ability of bovine PMN to cast NETosis against motile *B. besnoiti* bradyzoites evidencing the importance of this ancient and well-conserved effector mechanism of early host innate immune system in cattle. Furthermore, LC3B-stained autophagosomes were detected in *B. besnoiti* bradyzoite-exposed PMN casting NETs resulting in a significant positive correlation of autophagy and parasite-induced suicidal NETosis. However, further autophagy-related investigations should elucidate whether other molecular mechanisms in this cell pathway might occur. Finally, *B. besnoiti*-mediated vital NETosis resulted in a rapid extrusion and retraction of a 'chameleon tongue-like' structure, which is the first hint for this type of NETosis against apicomplexan parasites. Exact machinery, *B. besnoiti*-specific antigens and PMN receptors leading to fast parasite-triggered vital NETosis need further investigations.

4. ZUSAMMENFASSUNG

Besnoitia besnoiti ist der Erreger der Rinderbesnoitiose, einer Erkrankung, die die Tierproduktivität beeinträchtigt und auch tierschutz-relevant ist. Die Bildung sogenannter ‚neutrophile extracellular traps' (NETs) stellt einen wichtigen angeborenen Effektormechanismus von Neutrophilen dar, der gegen verschiedene Pathogene wirkt. Vor kurzem wurde über die Freisetzung von NETs, die auch als NETosis bezeichnet wird, als Reaktion auf *B. besnoiti*-Tachyzoiten berichtet. Über die Rolle von Stoffwechselwegen bei durch Parasiten ausgelöster NETosis ist jedoch nur ein begrenztes Wissen verfügbar. In der vorliegenden Arbeit wurden Stoffwechselsignaturen von Tachyzoiten-exponierter Neutrophilen (PMN) analysiert und Experimente zur funktionellen NETosis-Hemmung durchgeführt. Weiterhin wurde die Bedeutung verschiedener PMN-abgeleiteter Stoffwechselwege für eine durch *B. besnoiti*-Tachyzoiten und -Bradyzoiten induzierte NETosis untersucht. Insgesamt führte die Exposition gegenüber hitzeinaktivierten Tachyzoiten zu einem Anstieg des Glukose- und Serin-Verbrauchs, einem Rückgang des Glutamin-Verbrauchs und einer Steigerung der Glutamat- und Alanin-Produktion in exponierten Rinder-PMN. Darüber hinaus wurde die Tachyzoiten-induzierte Bildung zellfreier NETs durch PMN-Vorbehandlung mit Dichloracetat (Pyruvatdehydrogenase-Inhibitor), Oxythiamin (Inhibitor der Pyruvatdehydrogenase-Kinase) und Oxamat (Inhibitor der Lactatdehydrogenase) signifikant reduziert, was auf eine Schlüsselrolle der Pyruvat- und Laktat-vermittelten Stoffwechselwege für die richtige Tachyzoit-vermittelte NETosis hinweist. In der Folge wurde die NETosis-Bildung auch durch einen erhöhten pH-Wert induziert, jedoch konnten Laktat-Transporter-Blocker (AR-C141900, AR-C151858) die NETosis nicht beeinflussen. Eine signifikante Reduktion der Tachyzoiten-induzierten NET-Bildung wurde auch durch Behandlung mit Oligomycin (Inhibitor der ATP-Synthase) und NF449 (purinergischer Rezeptor P2X1-Antagonist) erreicht, was auf eine zentrale Rolle der Verfügbarkeit von ATP hindeutet. Im Gegensatz dazu beeinflussten Behandlungen mit Inhibitoren der frühen Schritte der Glykolyse oder Glutaminolyse die durch Parasiten ausgelöste NETosis nicht. Diese genauen Daten vermitteln ein besseres Verständnis der Stoffwechselwege, die an der Bildung von durch *B. besnoiti*-Tachyzoiten induzierten NETosis beteiligt sind.

Weiterhin induzierten Tachyzoiten von *B. besnoiti* die Bildung von LC3B-verwandten Autophagosomen parallel zur NETosis in Rinder-PMN. Bemerkenswerterweise beeinflusste weder eine Rapamycin- noch eine Wortmannin-Behandlung die durch *B. besnoiti* ausgelöste

NET-Bildung und die Bildung von Autophagosomen. Auch isolierte NETs induzierten keine Autophagie, was die Unabhängigkeit zwischen beiden zellulären Prozessen nahelegt. Interessanterweise wurde innerhalb der ersten Minuten der Wechselwirkung in Tachyzoiten-exponierten Rinder-PMN eine verstärkte Phosphorylierung von AMPKα, einem der wichtigsten Regulatormoleküle der Autophagie, beobachtet. Dies unterstreicht, dass die durch *B. besnoiti* ausgelöste NET-Bildung tatsächlich parallel zur Autophagie auftritt.

Außerdem wurden frühe Effektormechanismen von Rinder-PMN, die vitalen, aus Hautgewebszysten eines infizierten Tieres isolierten *B. besnoiti*-Bradyzoiten ausgesetzt waren untersucht. Histopathologische Untersuchungen bestätigten das Vorhandensein von typischen runden, großformatigen Zysten in Hautbiopsien. Nach PMN:*B.besnoiti*-Bradyzoiten Co-Kulturen, Rasterelektronen- und Fluoreszenzmikroskopieanalysen zeigten ein feines Netzwerk NETosis-ähnlicher Strukturen, die von PMN von Rindern und verstrickten Bradyzoiten freigesetzt wurden. Klassische NETosis-Komponenten wurden in diesen Strukturen durch Immunfluoreszenzanalysen an extrazellulärer DNA, Histone (H1-H4) und Neutrophilen-Elastase (NE)-Co-Lokalisation, bestätigt. Neben NETosis wurde in Bradyzoiten-stimulierten PMN auch eine erhöhte Bildung von Autophagosomen (sichtbar durch spezifische LC3B-Färbung) beobachtet. Statistische Analysen zeigten eine signifikante positive Korrelation (p = 0,042) zwischen dem NETosis-Auftreten und der Autophagie in diesen angeborenen Immunzellen. Diese Ergebnisse legen nahe, dass NETosis eine zentrale Rolle bei frühen angeborenen Wirtsreaktionen gegen *B. besnoiti*-Bradyzoiten spielt und liefern darüber hinaus erste Beweise für die Autophagie, die mit der durch *B. besnoiti*-Bradyzoiten induzierten NET-Bildung assoziiert ist.

5. SUMMARY

Besnoitia besnoiti is the causative agent of bovine besnoitiosis, a disease that affects both, animal welfare and cattle productivity. Neutrophil extracellular trap (NET) formation represents an important innate effector mechanism of polymorphonuclear neutrophils (PMN) acting against various pathogens. Recently, NETs release was reported in response to *B. besnoiti* tachyzoites. However, limited knowledge is available on the role of metabolic pathways during parasite-triggered NET formation, nowadays known as NETosis. By analysing metabolic signatures of tachyzoite-exposed PMN and applying functional inhibition experiments, we here aimed to investigate the importance of distinct PMN-derived metabolic pathways for effective *B. besnoiti* tachyzoite-induced NETosis. Overall, exposure to heat-inactivated tachyzoites induced an increase in glucose and serine consumption, a drop in glutamine consumption and an enhancement of glutamate and alanine production in bovine PMN. Moreover, tachyzoite-induced formation of cell free NETs was significantly diminished via PMN pretreatments with dichloroacetate (pyruvate dehydrogenase inhibitor), oxythiamine (inhibitor of pyruvate dehydrogenase kinase) and oxamate (inhibitor of lactate dehydrogenase), thereby indicating a key role of pyruvate- and lactate-mediated metabolic pathways for proper tachyzoite-mediated NETosis. In line, NET formation was also induced by enhanced pH, however, blockers (AR-C141900, AR-C151858) of lactate transporters failed to influence NETosis. Moreover, a significant reduction of tachyzoite-induced NET formation was also achieved by treatments with oligomycin (inhibitor of ATP synthase) and NF449 (purinergic receptor P2X1 antagonist) which suggested a pivotal role of ATP availability in this effector mechanism. In contrast, treatments with inhibitors of early steps of glycolysis or glutaminolysis did not affect parasite-triggered NETosis. These current data will provide a better understanding of metabolic pathways involved in *B. besnoiti* tachyzoite-induced NETosis.

Moreover, tachyzoites of *B. besnoiti* induced LC3B-related autophagosome formation in parallel to NETosis in bovine PMN. Notably, both rapamycin- and wortmannin-treatments failed to influence *B. besnoiti*-triggered NETosis and autophagosome formation. Also, isolated NETosis failed to induce autophagy suggesting independence between both cellular processes. Interestingly, enhanced phosphorylation of AMPKα, a key regulator molecule of autophagy, was observed within the first minutes of interaction in tachyzoite-exposed bovine

PMN thereby emphasizing that *B. besnoiti*-triggered NETosis indeed occurred in parallel to autophagy.

In addition, we investigated early effector mechanisms of bovine PMN being exposed to vital *B. besnoiti* bradyzoite stages, which were isolated from skin tissue cysts of a naturally infected animal presenting characteristic symptoms of bovine besnoitiosis. Histopathological examinations confirmed the presence of typical roundish, large-sized cysts in subdermal biopsies. After PMN:*B. besnoiti* bradyzoites co-cultures, scanning electron microscopy (SEM)- and epifluorescence microscopy-analyses demonstrated a fine network of NET-like structures being released by bovine PMN and efficiently ensnaring bradyzoites. Classical NETosis-associated components were confirmed in these extracellular structures via immunofluorescence analyses on extracellular DNA, histone (H1-H4) and neutrophil elastase (NE) colocalization. Besides NETosis, an increased formation of autophagosomes (visualized by specific-LC3B staining) was observed in bradyzoite-stimulated PMN. Statistical analyses revealed a significant positive correlation ($p = 0.042$) between the occurrence of NETosis and autophagy in these immunocompetent cells. These findings suggest NETosis plays a pivotal role in early innate host responses against bradyzoite stages and furthermore deliver first evidence on autophagy being associated with *B. besnoiti* bradyzoite-induced NETosis.

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7. SUPPLEMENTARY DATA

7.1 *Dirofilaria immitis* microfilariae and third-stage larvae induce canine NETosis resulting in different types of neutrophil extracellular traps

Muñoz-Caro, T., Conejeros, I., **Zhou, E**., Pikhovych, A., Gärtner, U., Hermosilla, C. & Taubert, A. (2018). Dirofilaria immitis Microfilariae and Third-stage larvae induce canine neTosis resulting in Different Types of neutrophil extracellular Traps. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.00968

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Dirofilaria immitis Microfilariae and Third-Stage Larvae Induce Canine NETosis Resulting in Different Types of Neutrophil Extracellular Traps

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Heartworm disease is a zoonotic vector-borne disease caused by *Dirofilaria immitis* mainly affecting canids. Infectious third-stage larvae (L3) are transmitted to the definitive hosts *via* culicid mosquitoes; adult nematodes reside in the pulmonary arteries and in the right heart releasing unsheathed first-stage larvae (microfilariae) into the bloodstream leading to chronic and sometimes fatal disease. So far, early innate immune reactions triggered by these different *D. immitis* stages in the canine host have scarcely been investigated. Therefore, *D. immitis* microfilariae and L3 were analyzed for their capacity to induce neutrophil extracellular traps (NETs) in canine polymorphonuclear neutrophils (PMN). Overall, scanning electron microscopy analysis revealed both larval stages as strong inducers of canine NETosis. Co-localization of PMN-derived extracellular DNA with granulocytic histones, neutrophil elastase, or myeloperoxidase in parasite-entrapping structures confirmed the classical characteristics of NETosis. Quantitative analyses showed that both larval stages triggered canine NETs in a time-dependent but dose-independent manner. Moreover, parasite-induced NET formation was not influenced by the parasites viability since heat-inactivated microfilariae and L3 also induced NETs. In addition, parasite/PMN confrontation promoted significant entrapment but not killing of microfilariae and L3. Both, NETosis and larval entrapment was significantly reversed *via* DNase I treatments while treatments with the NADPH oxidase inhibitor diphenyleneiodonium failed to significantly influence these reactions. Interestingly, different types of NETs were induced by microfilariae and L3 since microfilarial stages merely induced spread and diffuse NETs while the larger L3 additionally triggered aggregated NET formation.

Keywords: *Dirofilaria immitis*, neutrophil extracellular traps, innate immunity, NETosis, canine polymorphonuclear neutrophils

INTRODUCTION

Cardiopulmonary dirofilariosis or heartworm disease is a zoonotic vector-borne disease caused by the parasitic nematode *Dirofilaria immitis* affecting mainly dogs in warm-temperate regions, particularly in the southern United States, Central and South America, East Asia, and Mediterranean regions of Europe (1). Parasites are transmitted as third-stage larvae (L3) by infected culicid female mosquitoes of the genera *Culex*, *Aedes*, and *Anopheles* during the blood meal. Adult *D. immitis*

Dirofilaria immitis Microfilariae and Third-Stage Larvae Induce Canine NETosis Resulting in Different Types of Neutrophil Extracellular Traps

nematodes reside in the pulmonary arteries and the right heart of definitive hosts where mature viviparous females release unsheathed first-stage larvae (microfilariae) into the bloodstream. Initial inflammatory responses occur in the walls of the pulmonary vasculature and are critical for pathogenesis and further development of the disease (2). This chronic and progressive infection may have a fatal outcome for definitive hosts and clinical cardiopulmonary disease has been reported in several carnivores, including domestic and wild canids [e.g., foxes, wolves (3)] and felids. Feline infections are increasingly reported from areas endemic for canine dirofilariosis, but generally show a low parasite burden and asymptomatic infections. In some cases, cats may present severe disease or even sudden death even in the presence of a small number of adult worms (4–8). Currently, cardiopulmonary dirofilariosis is considered as an emerging disease in Europe, most probably due to climate change favoring intermediate host expansion, inadequate management of pets, human intervention in the environment, and the existence of wild reservoirs (9–11).

So far, little data are available on innate immune reactions against *D. immitis*. The fact, that polymorphonuclear neutrophils (PMN) are critically involved in the killing and clearance of microfilariae *via* antibody-dependent cell-mediated cytotoxicity *in vitro* and from dogs with occult *D. immitis* infections (12, 13), indicates an important role of this innate immune cell type. PMN showed enhanced oxidative burst and degranulation activities in response to opsonized microfilariae (14) and adhered to *D. immitis* microfilariae in the absence of immune serum and presence of ivermectin (15, 16). In addition, PMN infiltration was observed in various organs and tissues, including kidney and arterial walls from *D. immitis* infected canids (17, 18). Accordingly, several parasite-derived chemotactic factors were demonstrated to attract PMN (19). Interestingly, endosymbiotic bacteria of the genus *Wolbachia* residing within *D. immitis* adults (20) were shown to activate canine PMN leading to enhanced production of IL-8 (21). Concerning other filarial parasites, PMN were shown to promote the killing of *Onchocerca volvulus* microfilariae (unsheathed) and L3 *in vitro* (22, 23) and played a major role in the early control of *Litomosoides sigmodontis* L3 stages in the skin leading to enhanced oxidative burst and degranulation (24).

The effector mechanism of NETosis is performed by PMN and other immune cells and results in the cellular release of granule proteins and chromatin upon activation, forming sticky extracellular fibers capable of binding and killing Gram-positive and -negative bacteria, fungi, virus, and protozoan/metazoan parasites (25–28). So far, the capacity of extracellular trap formation has been attributed to PMN (25), mast cells (29), macrophages (30), eosinophils (31), and monocytes (32–34). NETosis is most of the time mediated by NADPH oxidase (NOX)-dependent ROS production (35, 36) and efficient neutrophil extracellular trap (NET) extrusion is a cell death-dependent process in the majority of cases. However, non-lethal NET release was also reported by Yousefi et al. (31, 37) who demonstrated that eosinophils and certain PMN subpopulations release NETs of mitochondrial origin without dying. In addition, recent studies verified that PMN were still viable and retained their capability to engulf bacteria *via* phagocytosis even after performing NETosis (38).

So far, most studies on parasite-triggered NETosis focused on protozoan parasites (38–45). By contrast, little data exist on metazoan-triggered NET formation (46–50). In the case of filariae, the reports on NETosis come exclusively from *Brugia malayi* and *L. sigmodontis*, in which microfilariae/L3 were recently demonstrated as potent triggers of NETs leading to parasite entrapment but not killing *in vitro* (24, 50). It is worth noting that adult *D. immitis* worms survive for an average of 5 years within the definitive host, releasing microfilariae into the bloodstream (51). However, the immune mechanisms that drive host tolerance and the modulation of host innate immune responses driven by blood circulating microfilariae are not yet fully understood. In addition, no data are available on the capacity of *D. immitis* stages as NET inducers in the canine host.

In this study, we show that microfilariae and L3 from the *D. immitis* Missouri strain, USA, significantly induce NET formation and trigger different forms of NETs in a stage-specific manner promoting additional and novel innate immune reactions of canine PMN against this parasite.

MATERIALS AND METHODS

Parasites

For all here described experiments, the *D. immitis* Missouri strain, USA, was used. This strain originated from a dog sheltered in an animal pound in Missouri in the year 2000. Beginning in 2005, the strain was maintained and passaged in beagle dogs at the University of Georgia (Athens, GA, USA) (52). From 2012 onward, the strain was maintained at the laboratories of Bayer Animal Health, Monheim, Germany. Here, all animal procedures were approved by the local animal care and use committee and by governmental authorities (LANUV #200/A176 and #200/A154). For this study, microfilaremic blood was sampled from four dogs (Beagle, Marshall BioResources, two males and two females) by cephalic vein puncture. Microfilariae were purified from the blood samples according to the protocol provided by the NIH/NIAID Filariasis Research Reagent Resource Center (FR3; College of Veterinary Medicine, University of Georgia, Athens, GA, USA) (http://www.filariasiscenter.org/protocols/Protocols/purification-of-microfilariae-by-filtration). L3 were obtained by feeding microfilaremic canine blood to *Aedes aegypti* (black-eyed Liverpool strain). Fifteen days after feeding, L3 were isolated from infected *A. aegypti* according to the protocol by Evans et al. (52).

Canine PMN Isolation

In total, blood sampling for PMN isolation was performed on six adult dogs (Beagle, Marshall BioResources) by jugular vein puncture. In each experimental setting, at least three different blood donors were included. 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 gently resuspended and shaken in 25 ml distilled water for 20 s to lyse erythrocytes. Then, osmolarity was adjusted by adding 5 ml of 10× Hanks Salt Solution (HBSS, Biochrom AG). Afterward, PMN were washed twice (400 × *g*, 10 min), resuspended in RPMI medium, counted in a Neubauer

Dirofilaria immitis Microfilariae and Third-Stage Larvae Induce Canine NETosis Resulting in Different Types of Neutrophil Extracellular Traps

hemocytometer chamber, and incubated at 37°C and 5% CO_2 for at least 30 min before experimental use.

Scanning Electron Microscopy (SEM)

Canine PMN ($n = 3.5 \times 10^5$) were cocultured with vital *D. immitis* L3 (10 larvae/sample) or microfilariae (20 larvae/sample) on poly-L-lysine (Sigma-Aldrich) pre-coated coverslips (60 min, 37°C). After incubation, the samples were fixed in 2.5% glutaraldehyde [60 min, room temperature (RT), Merck], post-fixed in 1% osmium tetroxide (Merck), washed in distilled water, dehydrated, critical point dried by CO_2 treatment, and spayed with gold. Thereafter, samples were examined with a Philips XL30 scanning electron microscope at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany.

NET Visualization by Immunofluorescence Analysis

Canine PMN ($n = 3$; 2×10^5/300 µl) were seeded on 15 mm poly-L-lysine-treated glass coverslips placed in 12-well plates in RPMI 1640 medium (without phenol red, supplemented with 1% penicillin/streptomycin, Sigma-Aldrich) and cocultured with *D. immitis* microfilariae (50 larvae/well; 37°C, 5% CO_2, 60 min). After the incubation period, the samples were fixed in 4% paraformaldehyde (Merck) and kept at 4°C until further use. Canine NET structures were visualized by staining extracellular DNA with Sytox Orange® (1:1,000, 15 min, Invitrogen) according to Martinelli et al. (53). For the detection of histones, myeloperoxidase (MPO) and neutrophil elastase (NE) within NET structures the following antibodies were used: anti-histone (H1, H2A/H2B, H3, H4) (clone H11-4, 1:1,000; Merck Millipore), anti-MPO (orb11073, 1:1,000, Byorbit), and anti-NE (AB68672, 1:1,000, Abcam). Briefly, the samples were washed thrice in PBS, blocked with 2% bovine serum albumin (Sigma-Aldrich; 30 min, RT) and incubated in antibody solutions (60 min, RT). Thereafter, the samples were washed thrice with PBS and incubated in secondary antibody solutions (Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG, both Life Technologies, 60 min, 1:1,000, RT). Finally, the samples were washed thrice in PBS and mounted in anti-fading buffer (ProLong Gold Antifade Mountant®; Thermo Fisher Scientific). Visualization was achieved using an inverted Olympus IX81 fluorescence microscope equipped with a digital camera (Olympus).

Quantification of Extracellular DNA Release

Using 96-well flat bottom plates (Nunc), canine PMN ($n = 3$, 2×10^5 in duplicates) were cocultured with *D. immitis* microfilariae (100 and 300 larvae per sample) or L3 (10 and 20 larvae per sample) in RPMI 1640 medium (1% penicillin/streptomycin, without phenol red) in a final volume of 200 µl. In parallel, PMN were pretreated with the NADPH inhibitor diphenyleneiodonium (DPI, 10 µM 30 min; Sigma-Aldrich) before exposure to microfilariae. To resolve NET formation, treatment with DNase I (90 U/sample, Roche Diagnostics) was used. For heat-induced killing (HI), microfilariae or L3 were incubated for 60 min at 60°C. Following coculture, 3 µM Sytox Orange® Nucleic Acid Stain (Life Technologies, final concentration) was added to each well, and measurements were performed every 30 min for up to 8 h. Sytox Orange-derived fluorescence intensities were estimated by spectrofluorometric analyses at an excitation wavelength of 547 nm and emission wavelength 570 nm using an automated plate monochrome reader (Varioskan Flash®; Thermo Scientific). For negative controls, PMN in plain medium were used. Stimulation of PMN with zymosan (1 mg/ml; Invitrogen) served as positive control.

Attachment and Entrapment Assay

Canine PMN ($n = 3$, 2×10^5 in duplicates) were seeded on poly-L-lysine pre-coated coverslips and exposed to *D. immitis* microfilariae (50 larvae/sample) or L3 (10 larvae/sample) in a final volume of 200 µl for 60 and 180 min (37°C, 5% CO_2). In parallel settings, PMN were exposed to heat-killed (HI) microfilariae or pretreated with 10 µM DPI (Sigma-Aldrich) for 30 min before exposure to microfilariae. Treatments of PMN with 90U DNase I (Fisher Scientific) at the moment of parasite exposure were used to dissolve NETs. Following incubation, all samples were fixed (4% paraformaldehyde), and PMN-entrapped larvae were scored microscopically by using an inverted DMIRB® phase-contrast microscope (Leica). Larvae were considered as entrapped when PMN, and/or PMN-derived NETs were attached to the larvae. The data were expressed as percentage of entrapped larvae relative to the total amount of larvae.

Assessment of Different Types of NETs

For the quantification of different types of NETs, canine PMN ($n = 3$, 2×10^5 in duplicates) were seeded on poly-L-lysine pre-coated coverslips and exposed to *D. immitis* microfilariae (50 larvae/sample) and L3 (10 larvae/sample) for 60 and 180 min (37°C, 5% CO_2). Thereafter, the samples were fixed (4% paraformaldehyde) for further analysis. NET structures were visualized by staining extracellular DNA with Sytox Orange® (1:1,000, 15 min; Invitrogen) and were reacted with an anti-global histone antibody (clone H11-4 monoclonal antibody, 1:1,000; Merck Millipore) as previously described. Thereafter, four randomly selected images from each experimental condition were analyzed microscopically based on typical morphological characteristics according to Muñoz-Caro et al. (48) and Lange et al. (49): (i) "diffuse" NETs (*diff*NETs), which are composed of a complex of extracellular decondensed chromatin mesh being decorated with antimicrobial proteins with globular and compact form, defined by a size of 15–20 µm diameter, (ii) "spread" NETs (*spr*NETs) consisting of smooth and elongated web-like structures of decondensed chromatin and antimicrobial proteins and being composed exclusively of thin fibers with a diameter of 15–17 µm, and (iii) "aggregated" NETs (*agg*NETs), being defined as clusters of NET-like structures with a "ball of yarn" morphology and a size of more than 20 µm in diameter. Within each sample, all structures with the described characteristics were counted.

Analysis of NET-Derived Larval Killing

Canine PMN ($n = 3$, 2×10^5) were seeded in 12-well culture well plates and exposed to *D. immitis* microfilariae (50 larvae/sample) or L3 (10 larvae/sample) for 24, 36, 48, and 72 h (37°C, 5% CO_2). Larval survival was determined microscopically based on the presence or absence of larval motility and monitored *via*

Dirofilaria immitis Microfilariae and Third-Stage Larvae Induce Canine NETosis Resulting in Different Types of Neutrophil Extracellular Traps

video recording. For reference samples, the equal number of non-exposed larvae was used.

Statistical Analysis

Given that the data were normally distributed, coculture/stimulation conditions were compared by one- or two-factorial analyses of variance (ANOVA) with repeated measures. Differences were regarded as significant at a level of $p \leq 0.05$ and analyzed by GraphPad Prism® programme. To analyze time dependency, a 2-way repeated measures ANOVA, followed by a Tukey's multiple comparisons test were performed. The repeated measure category was time, and the α value to consider *p* significant between the experimental conditions was 0.05. Data were plotted as mean (SD).

RESULTS

D. immitis Microfilaria and L3 Induce Canine NETosis in a Time-Dependent Manner

Scanning electron microscopy analyses revealed that exposure of canine PMN to *D. immitis* microfilariae resulted in rapid and strong formation of NETs since fine and filamentous but also robust DNA-derived structures contacted and ensnared these unsheathed microfilarial stages (**Figure 1**).

To confirm the classical NETs components in parasite-triggered structures, fluorescence-based analyses were performed. Thus, Sytox Orange® staining (**Figures 2A,D,G,J**) proved the DNA nature of extracellular NET-like structures being formed by canine PMN upon exposure to *D. immitis* parasites. In addition, co-localization studies revealed the simultaneous presence of MPO, NE and histones (H1, H2A/H2B, H3, and H4) (**Figures 2B,E,H,K**) in DNA-positive parasite-induced NET structures (**Figures 2C,F,I,L**). It is noteworthy that a fast induction of NETs was observed *via* both stages, microfilariae and L3, since respective reactions were visible already 10 min after coculture (Image S1 (A and B) in Supplementary Material).

Quantitative analyses confirmed that both, microfilariae and L3 significantly induced NET formation in canine PMN ($p \leq 0.01$, **Figures 3A–C**). As expected, these reactions were significantly and almost entirely resolved by DNase I treatments ($p \leq 0.001$, **Figure 3A**). To elucidate the role of NOX in *D. immitis*-triggered NETosis, functional inhibition experiments were performed using DPI as blocker of NOX. Here, DPI treatments failed to significantly reduce microfilariae-induced NET formation (**Figure 3A**), suggesting that this was a NOX-independent process. Furthermore, microfilariae- and L3-induced NET formation proved as independent on the parasite vitality since heat-killed microfilariae (MF-HI) and L3 (L3-HI) induced comparable levels of NETs when compared with non-treated parasite stages (**Figures 3B,C**). In addition, microfilariae- and L3-triggered NET formation revealed as dose-independent since enhanced numbers of parasitic stages did not significantly alter the level of NET induction (**Figures 3B,C**). Kinetic analysis on *D. immitis* microfilariae- and L3-induced NETosis showed a time-dependent pattern since NET formation was found increasingly enhanced (**Figure 4A**) in the case of 100 microfilariae ($p \leq 0,05$; 4 h) and 300 microfilariae ($p \leq 0.05$; 3 h) as well as 10 ($p \leq 0.05$; 3 h) and 20 L3 ($p \leq 0.05$; 3 h) (**Figure 4B**). Again, no dose-dependency was detected in these assays.

In all experiments, the stimulation with zymosan proved as reliable positive control (zymosan treatment vs. PMN in plain medium: $p \leq 0.0001$). Microfilariae and L3 stages alone as well as PMN alone showed almost no or low reactions in this experimental setting, respectively.

D. immitis Microfilariae and L3 Induce Different Types of NETs

Overall, NETs may be displayed in different morphological forms, i.e., as diffuse NETs (*diff*NETs), aggregated NETs (*agg*NETs), and spread NETs (*spr*NETs). Thus, we here analyzed microscopically if microfilariae and L3 stages of *D. immitis* induced different types of NETs (as exemplary illustrations, please refer to Images S2 and S3 in Supplementary Material). In principle, all types of NETs were observed in PMN/microfilariae cocultures (**Figure 5**). Quantitative assessment of the different NET types revealed that *diff*NETs and *spr*NETs were induced by both parasitic stages, while *agg*NETs were mainly triggered by the larger larval stages (L3) (**Figure 5**). This stage-dependent difference was highly significant (*agg*NETs/microfilariae vs. *agg*NET/L3: $p \leq 0.01$).

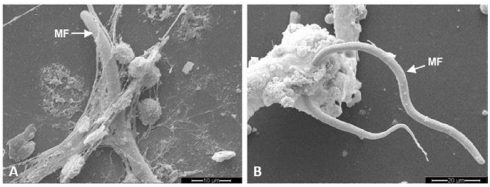

FIGURE 1 | *Dirofilaria immitis*-induced neutrophil extracellular trap (NET) formation analyzed *via* scanning electron microscopy analysis. Coculture of canine polymorphonuclear neutrophils with *D. immitis* microfilariae (MF). **(A)** Fine spread NETs entrapping microfilariae. **(B)** Meshwork of aggregated NETs (*agg*NETs) entrapping microfilariae.

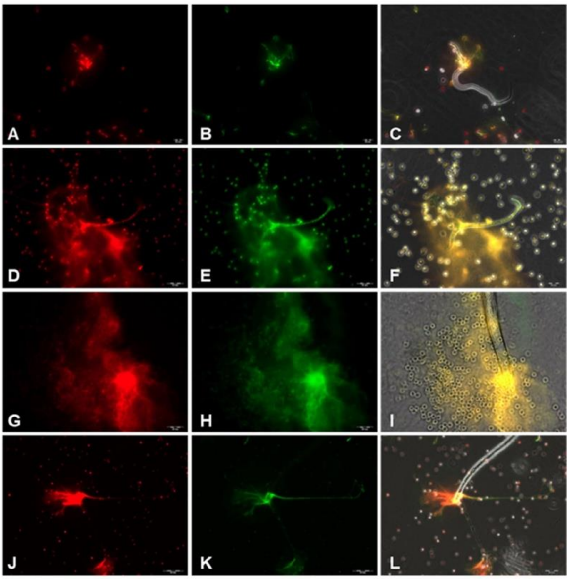

FIGURE 2 | Immunofluorescence analyses on *Dirofilaria immitis* microfilariae-induced neutrophil extracellular trap (NET) formation. Co-localization experiments on extracellular DNA and histones, myeloperoxidase (MPO), and neutrophil elastase (NE) in *D. immitis* microfilariae-induced NET structures using DNA-marker Sytox Orange and anti-histone H1, H2A/H2B, H3, H4, anti-MPO, and anti-NE antibodies. **(A,D,G,J)** Extracellular DNA stained with Sytox Orange (red). **(B)** Anti-histone (green). **(E,H)** Anti-NE (green). **(K)** Anti-MPO (green). **(C,F,I,L)** Merges.

Furthermore, microfilarial stages more frequently induced *spr*NETs than *diff*NETs ($p \leq 0.01$) or *agg*NETs ($p \leq 0.001$). For *spr*NET and *diff*NET formation, these reactions revealed as time-dependent and increased significantly with longer duration of coculture ($p \leq 0.05$) (**Figure 5**). By contrast, the case of L3 all forms of NETs were induced at comparable levels but also showed a time-dependent pattern for all three types of NETs (*diff* NETs: $p \leq 0.05$; *spr*NETs and *agg*NETs: $p \leq 0.01$) (**Figure 5**).

D. immitis Stages Are Entrapped *via* NETs in a Time-Dependent Manner

Since *D. immitis* microfilariae and L3 were proven as NET inducers in quantitative experiments, we here analyzed which proportion of these larval stages were physically contacted by PMN and entrapped by NET structures. Here, a time-dependent increase of parasite entrapment was observed for both stages. As such, a significant increase of parasite entrapment was observed in PMN/microfilariae cocultures at 60 and 180 min exposure ($p \leq 0.01$, **Figure 6A**) leading to a 28 and 52% larval entrapment, respectively. Similar reactions were observed for L3 stages since 62 and 95% were found entrapped at 60 and 180 min of incubation ($p \leq 0.05$, **Figure 6B**). When comparing the two larval stages, L3 were generally contacted and entrapped to a higher degree than microfilariae [28% (microfilariae) vs. 62% (L3) at 60 min, and 52% (microfilariae) vs. 95% (L3) at 180 min, both $p \leq 0.05$, **Figure 6B**]. The rather strong L3 entrapment is also illustrated in **Figure 6C**1 (60 min) and **Figure 6C**2 (180 min). We also observed the formation of "clasp"-like NET structures sticking mainly to the anterior part of the larvae (**Figure 6C**2, white

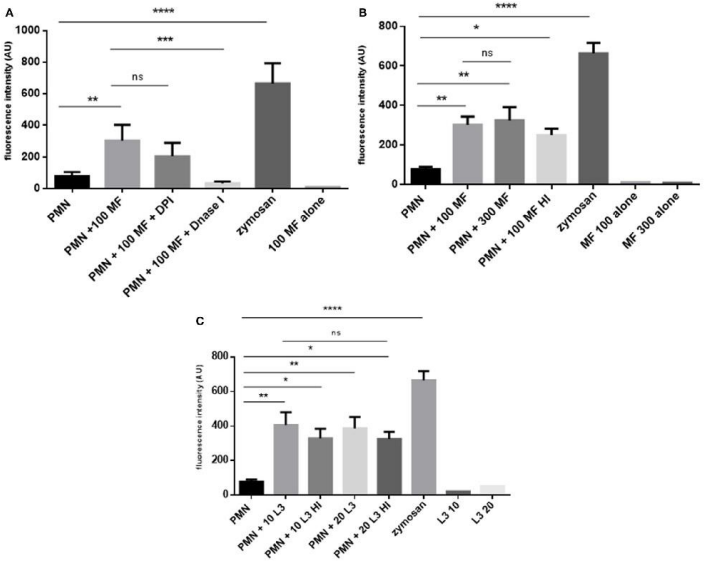

FIGURE 3 | *Dirofilaria immitis*-induced dose- and viability- and diphenyleneiodonium (DPI)-independent neutrophil extracellular trap (NET) formation. **(A)** *D. immitis* microfilariae (MF; 100) were cocultured with canine polymorphonuclear neutrophils (PMN) for 180 min. For NADPH oxidase inhibition, DPI pre-treatment was used. To resolve NET formation, DNase I was added to coculture. **(B)** Canine PMN were cocultured with vital or heat-inactivated microfilariae (MF-HI). **(C)** Vital and heat-inactivated *D. immitis* L3 were exposed to canine PMN. Sytox Orange-derived fluorescence intensities were analyzed by spectrofluorometric analysis at an excitation wavelength of 547 nm and emission wavelength 570 nm using an automated plate monochrome reader. As negative control, PMN in plain medium were used. PMN stimulated with zymosan served as positive control.

arrow) and here we provide as supplementary data bright field- as well as fluorescence microscopy-based videos on L3 stages being entrapped by DNA-positive *agg*NET structures (Video S1 in Supplementary Material). These observations may also be linked to the induction of different types of NET by microfilarial and L3 stages. While microfilariae only induced spread and diffuse NETs, L3 additionally triggered the formation of the most robust NET type, i.e., aggNETs. Especially the latter type of NETs, which consists of rigid clusters of NET-like structures of >20 μm in diameter, may function superior to the other NET types in case of parasite entrapment of large parasite stages.

As expected, DNase I treatment significantly abolished microfilariae entrapment ($p \leq 0.001$, **Figure 6D**) and minimized PMN attachment proving that NETs promoted these reactions. In contrast to NET formation, larval entrapment depended on parasite motility since dead (heat-treated) stages were entrapped to a less degree than vital ones (52 vs. 38%, respectively; $p \leq 0.05$, **Figure 6D**). As also reported for NET induction, DPI treatments failed to influence parasite entrapment and led to insignificant differences when compared with untreated controls ($p = 0.104$).

To elucidate whether *D. immitis* microfilariae or L3 were killed by NETs, parasite survival was monitored over 72 h by microscopic observation of larvae motility. Overall, neither *D. immitis* microfilariae nor L3 were adversely affected by NETs within this time frame, since no differences in parasite movements were observed in comparison to non-exposed stages (please refer to L3 exposed to PMN after 72 h in Video S3 in Supplementary Material).

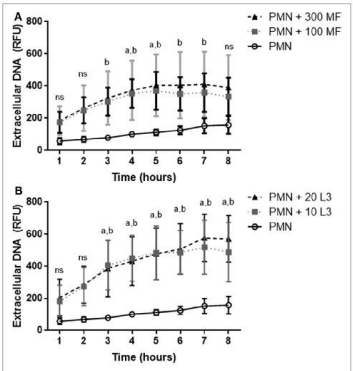

FIGURE 4 | Time dependency of *Dirofilaria immitis*-induced neutrophil extracellular traps. Canine polymorphonuclear neutrophils (PMN) were cocultured with microfilariae **(A)** or L3 **(B)** for 8 h. Sytox Orange-positive signals were analyzed every hour by spectrofluorometric analysis using an automated plate monochrome reader. **(A)** a: significant difference between PMN and 100 microfilariae; b: significant difference between PMN and 300 microfilariae. **(B)** a: significant difference between PMN and 10 L3; b: significant difference PMN and 20 L3. Abbreviation: ns, not significant.

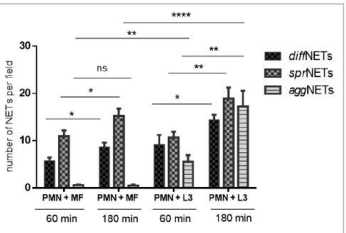

FIGURE 5 | Quantification of different types of neutrophil extracellular traps (NETs) induced by *Dirofilaria immitis* stages. Canine polymorphonuclear neutrophils (PMN) ($n = 3$, 2×10^5) we exposed to microfilariae (MF; 50 larvae) and L3 (10 larvae) on poly-L-lysine pre-coated coverslips for 1 and 3 h. After fixation, immunofluorescence analysis was performed using Sytox Orange as DNA-marker along with anti-histone antibodies. The types of NETs (*diff*NETs, *spr*NETs, and *agg*NETs) were identified microscopically and counted.

Nevertheless, since strong NET entrapment of microfilariae was observed (see also Video S2 in the Supplementary Material), it can be speculated that in the *in vivo* scenario, either the infectivity of *D. immitis* larvae from mosquitoes might be affected due do dampened migratory capacity of these stages or the immune cell-mediated larval attack within the definitive host might be facilitated *via* this "presentation."

DISCUSSION

In contrast to the vast majority of parasitic nematodes, *D. immitis* stages parasitize within the right heart and blood vessels and are thereby permanently exposed to an adverse environment, mainly composed of cells of the innate and adaptive immune system (e.g., PMN, monocytes, T cells, and NK cells), complement factors, antibodies, and cytokines/chemokines, or other soluble factors. However, investigations on early canine innate immune reactions against this parasite have scarcely been performed. Therefore, we here analyzed parasite-induced NETosis as early effector mechanism of PMN. Besides microfilariae we also included *D. immitis* L3 since this stage is transmitted to the dog as infective stage. Overall, we here provide first evidence on *D. immitis*-triggered NET release as part of early innate immune responses of canine PMN directed against microfilarial and L3 stages.

NETs are mainly formed of decondensed chromatin along with nuclear histones (H1, H2A/H2B, H3, and H4) and enzymatic granular components, such as neutrophil elastase (NE), MPO, lactoferrin, cathepsin, pentraxin, LL37, and gelatinase among others (25, 54, 55). Thus, typical NET characteristics were here confirmed for *D. immitis*-induced NETs by co-localization experiments on microfilariae and L3-triggered release of extracellular DNA being adorned with histones, NE and MPO. In accordance to studies on other metazoan parasites, L3 and microfilaria-triggered NETosis revealed as time dependent (47–50, 56). In line with data on *B. malayi*, *Haemonchus contortus*, *Strongyloides stercoralis*, and other metastrongyloid species (47–50), NETs were dissolved *via* DNase I treatment thereby proving the DNA nature of these structures. Moreover, we here observed that heat-inactivated microfilariae and L3 triggered NETs at a comparable level as vital ones, indicating that the parasites viability or integrity is not a crucial factor for NET induction. In contrast to *B. malayi* microfilariae (50), *D. immitis* microfilariae- and L3-induced NETosis appeared to be NOX-independent since DPI treatments failed to significantly inhibit NET formation.

Overall, it is noteworthy that filarial blood microfilariae appear to induce NETs independent of their ensheathment status. The microfilarial sheath is generally regarded as parasite-derived tool for improved immune evasion and was described to contain sheath-specific antigens (57, 58). The fact that both, ensheathed [*B. malayi* (50)] and non-ensheathed (*D. immitis*, this study) blood microfilariae significantly induce NET formation raises the question on surface-derived triggering molecules of these stages. However, no parasite-derived NET-inducing factors have been identified from filarial parasites, so far. Considering the fact, that microfilariae are present in blood vessels and trigger NET formation, it also appears of interest, that *D. immitis*-induced NETs may also exhibit adverse effects on the surrounding endothelium. In this context, NET-induced endothelial dysfunction and endothelial cell death was recently reported (59–61). To date, it remains unclear, whether microfilariae-triggered NET formation may

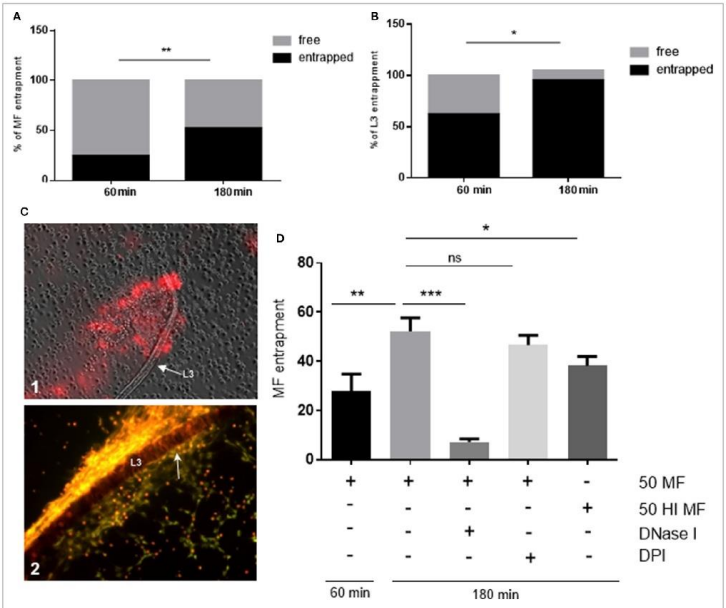

FIGURE 6 | *Dirofilaria immitis*-induced parasite entrapment. Canine polymorphonuclear neutrophils (PMN) were exposed to vital *D. immitis* microfilariae **(A)** or L3 **(B,C)** for 60 and 180 min. **(D)** In parallel settings, the same number of PMN was incubated either with heat-inactivated (HI) microfilariae (HI MF) or pretreated with diphenyleneiodonium (DPI) before exposure to vital microfilariae. Furthermore, DNase I was added at the moment of exposure to vital microfilariae. Larvae were considered as entrapped when PMN and/or PMN-derived neutrophil extracellular traps (NETs) were in contact with larvae. The data are expressed as percentage of entrapped larvae relative to the total amount of larvae per condition. The formation of "clasp"-like NET structures sticking mainly to the anterior part of the larvae is displayed in white arrow: image **[(C)**, 2].

also contribute to *D. immitis*-related pathogenesis with respect to vascular damage, progressive arteritis and coagulopathies, which, so far, were mainly attributed to adult stages of in chronic canine heartworm disease.

In contrast to previous NET-related reports on bacteria and selected parasites (25, 39) but in accordance to data on *B. malayi*, *S. stercoralis*, or *H. contortus* (47, 48, 50), *D. immitis*-triggered NETs did not promote the killing of parasite stages. As such, even after a prolonged incubation of 72 h no NET-mediated lethal effects were observed thereby rather suggesting an immobilization effect on larval stages as key mechanism of *D. immitis*-induced NETosis. So far, most studies on metazoan parasite-triggered NETs highlight the strength and efficiency of entrapment of motile large-sized pathogens when compared with bacteria, virus, fungi or protozoa. Respective features were described for *S. stercoralis*, *H. contortus*, *Angiostrongylus vasorum*, *Aelurostrongylus abstrusus*, *Troglostrongylus brevior*, and *B. malayi in vitro* as well as *in vivo* (47–50) indicating that PMN are able to recognize motile and large-sized pathogens and explicitly use NET formation as specific effector mechanism against such pathogens, as recently reported (62).

NET-mediated immobilization of *D. immitis* microfilarial stages may indeed influence the outcome of this infection since the presence of microfilariae—besides adult stages—is directly

related to the pathogenesis of the disease. Thus, heartworm infections in dogs are often accompanied by pathologic alterations driven by antigen–antibody complexes (e.g., glomerulonephritis) that rely on the long-lasting circulation of microfilariae in the blood stream (17). Interestingly, circulating microfilariae drive the initiation of innate immune reactions, e.g., by a CXCR2/IL-17-dependent PMN recruitment (63). In general, the immunopathology of filarial diseases in humans and domestic animals is complex and clinical manifestations depend on the type of host immune response mounted against filarial nematodes (64). Human dirofilariosis include pulmonary granulomatous reactions especially in immune-deficient patients (9).

Interestingly, many filarial species harbor obligate bacterial endosymbionts (*Wolbachia*) which were recently shown to trigger NETosis (65). So far, it remains unclear whether microfilariae-triggered NETosis may interfere with antifilarial treatments. Efficient treatment of *D. immitis* infections in domestic dogs is currently based on macrocyclic lactones for the removal of circulating microfilariae from the blood system in addition to adulticide treatments (66). However, since evidence of resistance to this drug class was already reported in the USA (67, 68), efficacious prevention of canine dirofilariosis might be endangered in future. In this context, a strong adherence of PMN to *B. malayi* microfilariae in the presence of low ivermectin concentration and enhanced microfilarial killing by PMN or peripheral blood mononuclear cells by ivermectin treatments has been reported (69–71). Interestingly, receptor sites for macrocyclic lactones are exclusively located proximate of the excretory–secretory (ES) apparatus, which is the main site of microfilarial protein release (72). ES proteins are well recognized for their immunomodulatory properties (73) allowing parasites to evade the host immune system. Given that macrocyclic lactone administration appears to hamper ES protein release from microfilariae (71) and since attached NETs may also dampen ES release by mechanical issues, both mechanisms may contribute to an improved host systemic immune response (71). In addition, we here postulate that NET release might also facilitate larval killing by other immunocompetent cells being recruited to the site of NET formation. Previous data show that, besides PMN, other innate immune cells such as macrophages and monocytes are involved in the innate immune responses against nematode parasites. It is well known that filarial parasites induce eosinophilia and eosinophil blood count is commonly used as a screening tool (74). *In vitro* studies have demonstrated the ability of IFN-γ-activated macrophages to kill *B. malayi* microfilariae (75). Furthermore, human PMN also promote lethal effects on *B. malayi* microfilariae in the presence of autologous serum *in vitro* and extrude NETs in response to these ensheathed microfilariae thereby entangling these stages (50).

A striking feature of this study was the fact that different types of canine NETs, i.e., *diff*NETs, *spr*NETs, and *agg*NETs were observed upon contact to *D. immitis*, all of them promoting larval entrapment. Consistently, a similar phenomenon was also observed for *H. contortus*- and lungworm-induced NETs (48, 49) showing a tight immobilization of L3 by these NET structures. However, concerning L3-induced *agg*NETs, it may also be speculated that they function in the prevention of proper L3 exsheathment into L4 stages within the definitive host and thereby hamper the ongoing development of *D. immitis*.

The here reported types of *D. immitis*-mediated NETs might be linked to the recently described capacity of mammalian PMN to sense small and large-sized pathogens and to selectively release NETs in response to large pathogens Branzk et al. (62). Given that especially L3 stages, but also microfilariae of *D. immitis*, represent large-sized pathogens, it appears obvious that PMN rather react by NETosis than by phagocytosis which will be ineffective against large multicellular pathogens.

Overall, we here present new insights into the early host innate immune response driven against the zoonotic parasite *D. immitis*. We demonstrate for the first time that both, microfilariae and L3 are potent inducers of different types of canine NETs. Overall, canine NETs do not kill the parasitic stages but might facilitate their killing by other leukocytes circulating in the blood system as postulated elsewhere. So far, it remains unclear whether excessive NET formation may also have adverse effects for the definitive host, such as vascular damage or coagulopathies. Consequently, we here call for more investigations not only on the *in vivo* evidence of *D. immitis*-triggered NETosis but also on its intravascular consequences with regards on pathogenesis and outcome of disease.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the local animal care and use committee and by governmental authorities (LANUV #200/A176 and #200/A154).

AUTHOR CONTRIBUTIONS

CH, AT, DK, TMC, and IC designed the project and experiments. DK and AP isolated, purified, and provided the parasites as well as the blood for this study. TMC, IC, EZ, and UG carried out most of the experiments. TMC, AT, CH, and IC prepared the manuscript. All the authors reviewed the manuscript.

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Dirofilaria immitis Microfilariae and Third-Stage Larvae Induce Canine NETosis Resulting in Different Types of Neutrophil Extracellular Traps

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00968/full#supplementary-material.

VIDEO S1 | *D. immitis* L3 entrapped in NETs structures. Video performed using fluorescence microscopy analysis along with the DNA-marker Sytox Orange to stain NETs-DNA contacting *D. immitis* L3.

VIDEO S2 | *D. immitis* microfilariae entrapped in NETs. By using bright field microscopy, *D. immitis* microfilariae is observed entrapped in NETs.

VIDEO S3 | *D. immitis* L3 are not killed by NETs after 72 h exposure to canine PMN. By using bright field microscopy, *D. immitis* L3 is observed still motile entrapped in *agg*NETs after 72 h of coculture with canine PMN.

IMAGE S1 | **(A,B)** *D. immitis*-induced NETosis after 10 min of incubation with canine PMN. Merge images on bright field and fluorescence microscopy obtained by using DNA-staining Sytox Orange and an anti-histone antibody.

IMAGE S2 | *D. immitis* microfilariae induce *agg*NETs and *diff*NETs. As indicated by arrows, the formation of different types of NETs is demonstrated as seen in merge image using bright field and fluorescence microscopy analysis with DNA-staining Sytox Orange and an anti-histone antibody.

IMAGE S3 | *D. immitis* microfilariae induce *spr*NETs. As indicated by arrows, the formation of different types of NETs is demonstrated as seen in merge image using bright field and fluorescence microscopy analysis with DNA-staining Sytox Orange and an anti-histone antibody.

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Dirofilaria immitis Microfilariae and Third-Stage Larvae Induce Canine NETosis Resulting in Different Types of Neutrophil Extracellular Traps

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8. DECLARATION

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

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

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