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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.01.001

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

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ARTICLE INFO

*Article history:*
Received 17 Nov 2015
Received in revised form 27 Nov, 2nd revised form 30 Nov 2015
Accepted 28 Dec 2015
Available online 8 Jan 2016

*Keywords:*
*Eimeria bovis*
*Eimeria arloingi*
Neutrophil extracellular traps
Apicomplexa
Coccidiosis

ABSTRACT

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

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

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

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

## 1. Introduction

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

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All animal procedures were performed according to the Justus Liebig University Animal Care Ethic Committee and in accordance to the current European Animal Welfare Legislation: ART13TFEU.

Foundation Project: Supported by the German Research Foundation (DFG; Grant No. TA 219/4-1).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

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

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

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

## 2. Materials and methods

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

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

### 2.2. Immunohistochemical detection of NETs

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

### 2.3. Fluorescence-based detection of NETs

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

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

## 3. Results

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

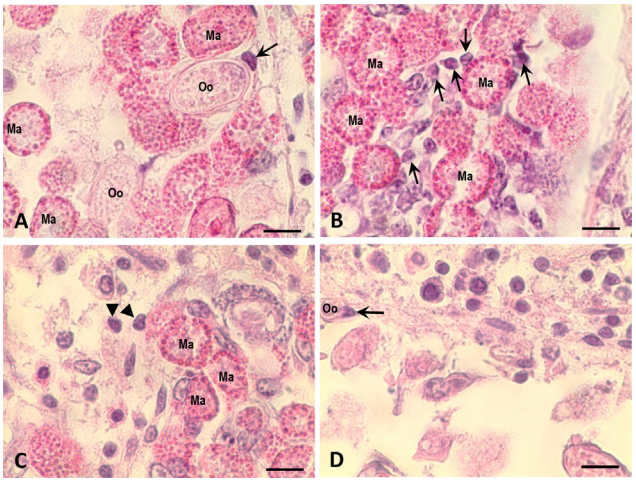


**Figure 1.** Haematoxylin-eosin staining of E. *bovis*-infected intestinal tissue.
A: Intestinal leucocyte contacting *E. bovis* oocysts (arrow). B and C: Intestinal leucocyte contacting *E. bovis* macrogamonts (arrows). D: Intestinal leucocyte contacting an *E. bovis* oocyst in lumen (arrow). Oo: Oocysts; Ma: Macrogamonts. Scale bars = 20 μm.

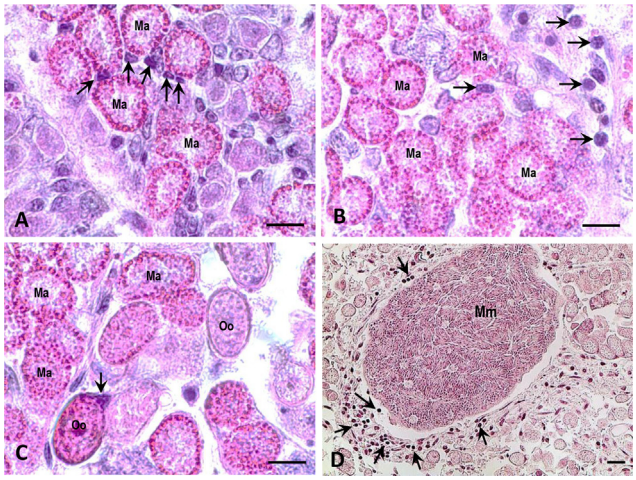


**Figure 2.** Haematoxylin-eosin staining of *E. arloingi*-infected intestinal tissue.
A and B: Leucocyte infiltration contacting *E. arloingi* macrogamont stages (arrows); C: Leucocyte infiltration contacting *E. arloingi* oocysts stages (arrows); D: *E. arloingi* macromeront being surrounded by leucocyte infiltration (arrows). Mm: Macromeront; Oo: Oocysts; Ma: Macrogamonts. Scale bars = 20 μm.

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

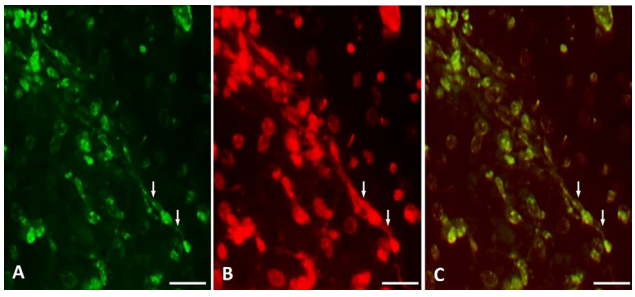


**Figure 3.** Co-localization of extracellular DNA and histones in E. arloingi-triggered NET structures in infected intestinal tissue.
Intestinal tissue (jejunum) sections from *E. arloingi*-infected animals were used for immunofluorescence analysis in order to identify NETs by (monoclonal) antibody-based detection of histones (H1, H2A, H2B, H3 and H4, in green). DNA was stained with Sytox orange (in red). A: Anti-histone staining of H1, H2A, H2B, H3 and H4; B: Sytox orange staining of DNA; C: Overlay of A and B. White arrows indicate NET structures being extruded from PMN. Scale bars = 20 μm.

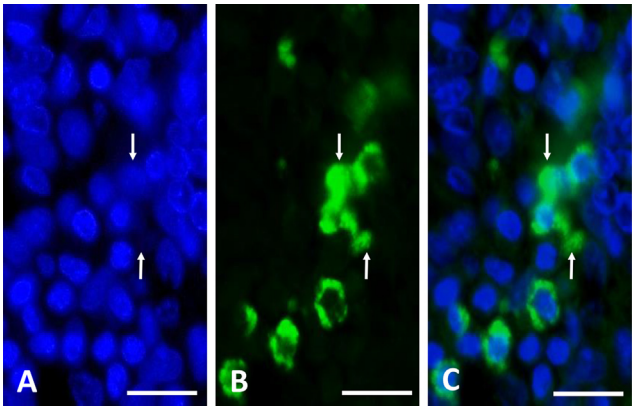


**Figure 4.** Co-localization of extracellular DNA and NE in *E. arloingi*-triggered NET structures in infected intestinal tissue.
Intestinal tissue (jejunum) sections from *E. arloingi* infected animals were used for immunofluorescence analysis in order to identify NETs by (polyclonal) antibody-based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

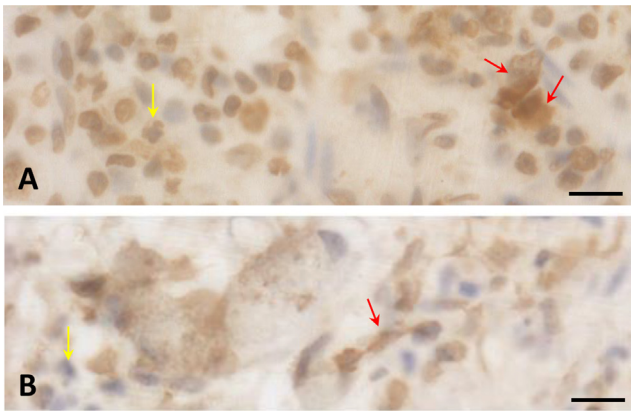


**Figure 5.** Histone detection in *E. bovis*- (A) and *E. arloingi*-infected (B) intestinal samples. NETs were identified by combining haematoxylin staining (in blue) with the (monoclonal) antibody-based detection of histone H3 [Cell Signaling, 1:100 (in brown)]. Red arrows indicate PMN releasing NET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

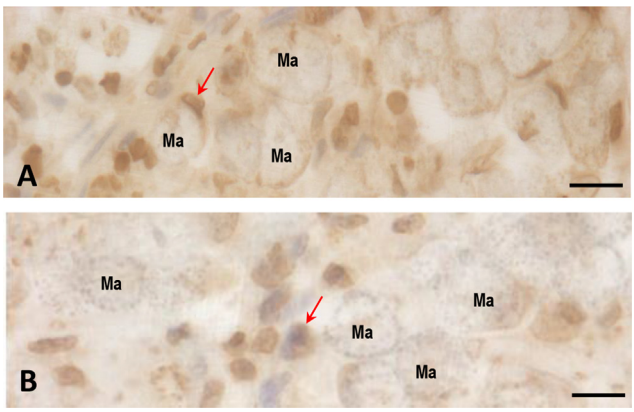


**Figure 6.** Histone detection in *E. arloingi*-infected intestinal samples.
A: PMN contacting *E. arloingi* macrogamont (arrow); B: PMN releasing NETs in close proximity to macrogamonts of *E. arloingi* (arrow). NETs were identified by combining haematoxylin staining (in blue) with the (monoclonal)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

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

## 4. Discussion

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

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

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

easier in the future to obtain evidence of parasites entrapped in NETs in *Eimeria*-infected rodent models, such as *Eimeria falciformis* or *Eimeria vermiformis* [50].

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

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

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

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

We would like to thank Gabriele Fuchs-Moll (Laboratory Section of Experimental Surgery, Justus Liebig University Giessen, Germany) for her excellent technical assistance in immunohistochemistry analysis. We are also deeply thankful to the Portuguese Association of Serpentina Goat Farmers for supplying samples and technical support during field studies. We would like to thank Luísa Rosendo Fialho (Department of Veterinary Medicine, University of Evora, Portugal), Christin Ritter (Institute of Parasitology, Justus Liebig University Giessen, Germany) and Ricardo Hartley (Laboratory of Cryobiology and Analysis of Spermatic Functionality, Institute of Animal Science, Faculty of Veterinary Sciences, Austral University of Chile) for their excellent technical support. This work was supported and financed by the German Research Foundation (DFG; Grant No. TA 219/4-1).

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