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Research paper

Role of NETs in the difference in host susceptibility to *Toxoplasma gondii* between sheep and cattle

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ABSTRACT

The main aim of this study was to compare extracellular traps (NETs) formation by polymorphonuclear neutrophils (PMNs) of cattle and sheep when exposed to *T. gondii* tachyzoites *in vitro*. The effects of parasite concentrations and different incubation periods on NETs development in cattle and sheep PMNs were studied. The effect of NET structures on host cell invasion by tachyzoites was also studied. This is the first report of NETs development by sheep and cattle PMNs against *T. gondii in vitro*. *T. gondii*-induced extracellular DNA production from PMNs was dependent on tachyzoite concentrations and incubation time in both sheep and cattle. Many nuclear and cytoplasmic changes were observed in sheep and cattle PMNs after exposure to *T. gondii* tachyzoites. The typical appearance of NETs, with MPO, NE and histone (H3) attached to extracellular DNA, was observed. Tachyzoites were entrapped within this structure. Myeloperoxidase (MPO) activity was higher in the cattle PMN-tachyzoite co-cultures than sheep. NETs structures released from sheep PMNs caused mechanical immobilisation of *T. gondii* tachyzoites, however, NET structures released from cattle PMNs may be lethal to tachyzoites. Bovine MPO may have a lethal effect on *T. gondii* tachyzoites *in vitro* during a 3 h incubation. Besides other mechanisms that effect on host susceptibility to *T. gondii* in sheep and cattle, extracellular traps formation as a part of immunological reactions may be play a role in host susceptibility to *T. gondii*.

1. Introduction

Toxoplasma gondii is one of the most prevalent zoonotic parasites in the world (Tenter et al., 2000). *T. gondii* has three infectious stages, namely tachyzoite, bradyzoite and sporozoite (Weiss and Kim, 2007). Tachyzoites are able to infect all cell types and proliferate intracellularly by endodyogeny (Hill et al., 2005). The members of the Felidae family are the final host for *T. gondii* but many animal species, including cats and man, can act as intermediate hosts (Dubey, 2010). Some hosts are very sensitive for development of *T. gondii* (Dubey, 2010). Sheep are one of the most sensitive hosts. When ewes are infected during pregnancy *T. gondii* may cause serious pathology such as early embryonic death, abortion and stillbirth dependent on the stage of pregnancy (Dubey, 2010). Whilst cattle can be infected with *T. gondii,* the parasite is often eliminated without causing clinical signs – perhaps because of innate immunity (Dubey, 2010).

Innate immunity is a primary defence mechanism that provides rapid protection against pathogens entering the body (Wilson 2012). The primary role of innate immunity is to contain pathogens in the area of initial infection and thus prevent systemic spread (Papayannopoulos and Zychlinsky, 2009; Mesa and Vasquez, 2013). Innate immunity is a complex phenomenon involving several mechanisms (Kumar and Sharma, 2010). A major component of the innate immune system are neutrophils, which are produced in the bone marrow and, although short lived, rapidly congregate in the area of infection and use a variety of strategies to fight pathogens (Mantovani et al., 2011). The primary function of the neutrophil is phagocytosis and, after uptake, the pathogen is killed in the phagolysosome by enzymes and proteins (Brinkmann and Zychlinsky, 2012). Some of these factors may also be released by exocytosis into the extracellular environment (Wakelin, 1996). Netosis, is a novel defence strategy employed by neutrophils, and plays an important role in the host's early immune response (Brinkmann et al., 2004).

Netosis leads to nuclear and cytoplasmic changes in neutrophils (Guimaraes-Costa et al., 2012). Reactive oxygen species are produced when neutrophils encounter pathogens (Kaplan and Radic, 2012). The

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neutrophil nucleus then loses eu- and heterochromatin discrimination and its characteristic lobular form disappears, the nuclear membrane swells and granule membranes break, so nuclear, cytoplasmic and granular content become mixed with one another (Papayannopoulos and Zychlinsky, 2009). Finally, extracellular traps augmented with myeloperoxidase (MPO), elastase (NE) and histones (H1, H2A, H2B, H3, H4) are excreted from neutrophils into extracellular areas (Papayannopoulos et al., 2010; Kaplan and Radic, 2014). Netosis is different from other cytoxic mechanisms such as necrosis and apoptosis (Fuchs et al., 2007). After they were first discovered by Brinkmann et al. in 2004, NETs were reported in many species including man, mice (Abi Abdallah et al., 2011), cattle (Munoz-Caro et al., 2014a), goats (Silva et al., 2014), cats (Wardini et al., 2010), dogs (Jeffery et al., 2015; Wei et al., 2016), sheep (Pisanu et al., 2015), fish (Palic et al., 2007), chickens (Chuammitri et al., 2009) and shrimps (Koiwai et al., 2016). Extracellular traps can also be produced by other granulocytic cells including eosinophils (Yousefi et al., 2012) and monocytes (Kaplan and Radic, 2014; Munoz-Caro et al., 2014b; Reichel, 2015 Munoz-Caro et al., 2014b; Munoz-Caro et al., 2014bKaplan and Radic, 2014; Munoz-Caro et al., 2014b; Reichel, 2015). Some authors called this process etosis instead of netosis (Guimaraes-Costa et al., 2012). Netosis develops both *in vitro* (Silva et al., 2014; Munoz-Caro et al., 2014a; Reichel et al., 2015; Wei et al., 2016) and *in vivo* (Abi Abdallah et al., 2011; Munoz-Caro et al., 2016).

Some bacteria, viruses, fungi and parasites are known to trigger netosis (Kaplan and Radic, 2012; Branzk and Papayannopoulos, 2013). *Plasmodium falciparum* was the first parasite reported to trigger NETs formation (Baker et al., 2008). NETs formation was subsequently observed in other protozoon parasite species such as *Leishmania amazonensis*, *Eimeria bovis, T. gondii, Besnoitia besnoiti, Eimeria arloingi, Neospora caninum, Cryptosporidium parvum* and *Entamoeba histolytica* (Guimaraes-Costa et al., 2009; Behrendt et al., 2010; Abi Abdallah et al., 2011; Munoz-Caro et al., 2014a; Silva et al., 2014; Munoz-Caro et al., 2015b; Wei et al., 2016; Avila et al., 2016) and also some helminth species (Chuah et al., 2014; Bonne-Annee et al., 2014; Munoz-Caro et al., 2015a).

Development of NET structures is the culmination of a series of chemical reactions in PMNs that have been exposed to infectious stages of parasites including tachyzoites, sporozoites, promastigotes and amastigotes (Hermosilla et al., 2014). NETs can bind to parasites and them immobilize within the extracellular compartment (Baums and von Köckritz-Blickwede, 2015). The theory of the lethal effect of NETs on parasites is still controversial (Lu et al., 2012; Menegazzi et al., 2012).

Toxoplasma gondii tachyzoites trigger the formation of extracellular trap structures from murine and human neutrophils (Abi Abdallah et al., 2011), and from harbour seal PMNs and monocytes (Reichel et al., 2015). In addition to the physical entrapment of tachyzoites which prevents host cell invasion, NETs also have a negative effect on the viability of entrapped tachyzoites (Abi Abdallah et al., 2011). Abi Abdallah et al. (2011) reported that nearly 25% tachyzoites died after exposure to mouse PMNs. Infection rates reduced significantly when *T. gondii* tachyzoites were exposed to PMNs and monocytes from harbour seals (Reichel et al., 2015). However, the role of neutrophils in *T. gondii* infection is still controversial and their role on control of the infection is not well defined (Han, 2012).

Sheep are excellent intermediate hosts, being easily infected with *T. gondii* (Dubey, 2010). However, in cattle the parasite is often eliminated after infection, possibly due to an innate immune response (Dubey, 2010). Understanding of the role of NETs as an important effector mechanism of the host early immune response may help explain the host difference between sheep and cattle against *T. gondii*. The main aim of the study was to detect the formation of extracellular traps developed in cattle and sheep PMNs when exposed to *T. gondii* tachyzoites *in vitro*. The effects of different incubation periods and parasite concentrations on NETs development in cattle and sheep PMNs were studied. The effect of NET structures on host cell invasion by *T. gondii* was also studied.

2. Materials and methods

2.1. PMN isolation from sheep and cattle

Animal handling was performed according to guidelines from the Kirikkale University Ethics Commission (10.07.2014, no:14/63). The methodology for this study was a modified version of Munoz-Caro et al. (2014a). Briefly, jugular blood samples (20 mL) were collected from clinically healthy sheep (n:3) and cattle (n:3) into heparinised tubes. The blood samples were mixed with equally volumes of sterile 0.02% PBS-EDTA and then layered into sterile tubes (Falcon) with Biocoll Separating Solution (Biochrom). After centrifugation (800 × *g*, with no brake, 45 min, 22 °C) (Thermo Scientific 16SR) the plasma, lymphocytes and monocytes were removed, and erythrocytes were lysed by addition of 25 mL ultrapure sterile water followed, 30 s later, by 3 mL sterile HBSS (x10, Sigma). PMNs were centrifuged with RPMI 1640 (without phenol red, Sigma) (400xg, 10 min, 4 °C). Neutrophil purity was determined by light microscopy (Leica DM750) examination after staining with Diff-Quick stain (Bio Optica, Italy). Viability was assessed using the trypan blue dye test. PMNs were counted using a Neubauer chamber under light microscopy and then reconstituted as 1×10^5/100 µL in RPMI 1640.

2.2. Toxoplasma gondii tachyzoites

Toxoplasma gondii RH strain tachyzoites were maintained in Swiss mice by intraperitoneal passages at the Parasitology Lab of Public Health Institution of Turkey. Tachyzoites were collected from the peritoneal fluid of experimentally infected mice 48 h later. The intraperitoneal fluid was centrifuged at 200 × *g* for 10 min at room temperature to remove cells and debris. The supernatant was collected and centrifuged at 1000 × *g* for 10 min. The pellet obtained was washed with sterile saline solution (pH 7.2). Tachyzoite counts were performed using a Neubauer chamber and tachyzoites were resuspended in RPMI 1640. Tachyzoite viability was evaluated using trypan blue dye.

2.3. Quantitative analysis of efficacy of NETs against T. gondii

Both sheep and cattle PMNs (1×10^5/100 µL) – tachyzoite co-cultures were taken into sterile reaction tubes at a parasite-to-PMN ratio of 1:1, 1:2, 1:3 and incubated for 1 h (37 °C, 5% CO_2). Micrococcal nuclease (5 U, NEB) was added to PMN-tachyzoite cultures and incubated for 15 min under the same conditions. After centrifugation (300 × *g*, 5 min), the supernatants were transferred into wells of a flat bottom immunplate (Nunc, Sigma-Aldrich), each sample was tested in duplicate. Sytox Green Dye (5 µM, Invitrogen) was added to the wells and incubated in the dark (15 min, room temperature), the fluorescence level was measured with a fluorometer (Fluoroskan Ascent FL, Thermo Scientific) (485 nm excitation/538 nm emision). Phorbol 12-myristate 13-acetate (PMA) (50 nM, Sigma-Aldrich) and samples containing only PMNs were used as positive and negative controls, respectively.

At a PMN-tachyzoite ratio of (1:2) samples from both sheep and cattle showed similar fluorescence intensity. This ratio was selected for further studies where incubation times were altered. Sheep and cattle PMN-tachyzoite cultures were incubated for 30, 60, 120 and 300 min (37 °C, 5% CO_2). Then, micrococcal nuclease (5 U, NEB) was added to the culture and incubated for 15 min. After centrifugation (300 × *g*, 5 min), the supernatants were transferred into wells of immunplates, each sample being tested in duplicate. Sytox Green Dye (5 µM, Invitrogen) was added to the wells, and incubated for 15 min in the dark. Fluorescence levels were measured with a fluorometer (485 nm excitation/538 nm emision). PMA and samples containing only PMNs were used as positive and negative controls respectively.