Veterinary Parasitology 258 (2018) 1–7

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

Bovine macrophage-derived extracellular traps act as early effectors against the abortive parasite *Neospora caninum*

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ARTICLE INFO

Keywords:
Bovine
Macrophage
Extracellular traps
Apicomplexan parasite
Neospora caninum

ABSTRACT

Macrophages are multipurpose phagocytes and are considered to be irreplaceable during the early host innate immune response against microbial and parasitic pathogens. However, no report has investigated the novel anti-parasitic mechanism of macrophage-derived extracellular traps (ETs) against the abortive apicomplexan parasite *Neospora caninum* (*N. caninum*) in cattle. Scanning electron microscopy (SEM) was used to visualize and characterize *N. caninum* tachyzoite-induced macrophage-triggered ETs in exposed bovine macrophages. Fluorescence confocal microscopy was used to confirm the classical backbone structure of DNA embedded with histone 3 (H3) and myeloperoxidase (MPO) in *N. caninum* tachyzoite-induced macrophage-derived ETs. Furthermore, the lactate dehydrogenase (LDH) levels in the supernatants of parasite-exposed macrophages were detected by a LDH Cytotoxicity Assay® kit. The results clearly demonstrated that *N. caninum* tachyzoites triggered bovine macrophage-derived ET-like structures. Inhibiting assays revealed that *N. caninum* tachyzoite-induced macrophage-mediated ET formation may be an ERK 1/2- and p38 MAPK-dependent cell death process. In conclusion, the present study is the first report on the formation of ETs in bovine macrophages against *N. caninum* tachyzoites and adds new data on the possible role of macrophages *in vivo* infection by capturing invasive stages and exposing them to other leukocytes.

1. Introduction

Neosporosis is mainly characterized by causing abortion in cattle worldwide, leading to large economic losses in the cattle industry (Dubey, 1999; Reichel et al., 2013). Neosporosis is caused by the apicomplexan protozoa *Neospora caninum* which is closely related to *Toxoplasma gondii*. This organism has a wide range of natural hosts, including domestic and wild mammals (Dubey, 2003; Gondim, 2006). *N. caninum* in cattle mainly leads to abortion, neonatal mortality and congenital infection (Dubey et al., 2007). As an obligate intracellular parasite, a vital step of *N. caninum* is to rapidly invade host cells and proliferate, leading to bovine neosporosis. The innate immune system, mainly composed of professional phagocytes [i. e. polymorphonuclear neutrophils (PMN), monocytes and macrophages], is known to play a pivotal role in early host resistance to *N. caninum* infections (Abe et al., 2014; Mineo et al., 2010; Wei et al., 2016a). Macrophages, as multipurpose professional phagocytes, possess many functions, including adjusting inflammation and maintaining tissue homeostasis. Macrophages are also considered to be irreplaceable phagocytes in early innate immunity against microbial pathogens (Boe et al., 2015). Moreover, a previous study has shown that the rapid recruitment of macrophages into the area of *N. caninum* infection can significantly enhance host innate immune reactions against this invasive parasite (Dion et al., 2011b). Taken together, these researches further suggested the pivotal role of macrophages in host resistance to *N. caninum* infections

Since the first detailed description of extracellular trap (ET) formation extruded by PMNs (Brinkmann et al., 2004), there has been an increasing number of investigations into ETs subjected to invasive pathogens. Importantly, not only PMNs but also other immune cells, including eosinophils (Yousefi et al., 2008), mast cells (Köckritzblickwede et al., 2008), and macrophages (Chow et al., 2010), can use ETs as potent effectors. Subsequently, there have been reports on monocyte-triggered ETs in response to bacterial pathogens and apicomplexan parasites (Tamara et al., 2014; Webster et al., 2010). Furthermore, our recent studies on the effects of *N. caninum* on neutrophils-derived and

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https://doi.org/10.1016/j.vetpar.2018.06.002
Received 10 October 2017; Received in revised form 31 May 2018; Accepted 4 June 2018

monocyte-derived ETs showed that neutrophils tend to be easier to release ETs than monocytes (Villagra-Blanco et al., 2017; Wei et al., 2016a; Yang et al., 2017), which may be related to different immune cells undertaking different main roles though different immune cells have similar functions in host immune systems. Meanwhile, our studies also showed the same immune cells running similar functions upon to *N.caninum* stimulation in different host. Before 2010, a detailed study on macrophage-induced ET formation was conducted and reported the presence of neutrophil extracellular trap (NET)-like structures, named macrophage extracellular traps (METs) (Chow et al., 2010). However, there is no current report on the formation of *N. caninum* tachyzoite-induced ETs in bovine macrophages, despite the fact that these cells play a key role in neosporosis (Dion et al., 2011a). In the present study, we aimed to investigate the influence of *N. caninum* on bovine METs and to further characterize the most relevant molecular signaling pathways.

2. Materials and methods

2.1. Parasites and cells

The bovine macrophage cell line was kindly provided by Professor Aizhen Guo from the Huazhong Agricultural University of Wuhan, China, and grown as described previously (Stabel and Stabel, 1995). The expressions of NF-κB p65, Iκβα and ERK 1/2 phosphorylation, and apoptosis-related proteins Bax, Bcl-2, and caspase 3 in this bovine macrophage cells have been reported (Zhang et al., 2016). In our previous study, the mRNA levels of Arg-1, iNOS, IFN-γ,TNF-αand IL-10 induced by *N. caninum* have also been proved to express in this bovine macrophage cell line (data not published). The cell line was cultured in RPMI 1640 medium (Hyclone, USA) containing 10% fetal bovine serum (FBS, Biological Industries, Israel) and 1% penicillin/streptomycin (Hyclone, USA). VERO cells were cultured in RPMI 1640 medium containing 5% fetal bovine serum and 1% penicillin/streptomycin. *N. caninum* (Nc-1 strain) tachyzoites were passaged in VERO cells cultured in RPMI 1640 medium containing 5% fetal bovine serum and 1% penicillin/streptomycin. Purification of *N. caninum* tachyzoites was accomplished as described in a previous study (Wei et al., 2016b). In brief, *N. caninum* tachyzoites were harvested from infected-VERO cells by centrifugation at 1800 *g* for 10 min. The sediment was collected and re-suspended in the 1640 medium, and then successively passed through 20, 5, 1 ml syringe and a 27-gauge needle for three times. Finally, *N. caninum* tachyzoites were purified with 40% percoll by centrifugation at 1800 *g* for 30 min. After twice washed, the precipitates were re-suspended in RPMI 1640 medium.

2.2. Scanning Electron Microscopy (SEM)

Bovine macrophages were plated onto glass cover slides and stimulated with *N. caninum* tachyzoites (ratio 1:8 or 1:16, 90 min, 37 °C). The samples were treated as previously described (Wei et al., 2016a). Briefly, the glass slides were pre-treated as follows: they were coated with poly-$_L$-lysine (0.1 mg/ml, Sigma-Aldrich) for 12 h and then washed with distilled water three times before the cells were fixed with 4.0 % glutaraldehyde (Merck) for 24 h. Subsequently, the cells were washed with PBS and fixed in 1.0% osmium tetroxide (Merck). Thereafter, the samples were dehydrated in an ascending gradient of 30, 50, 70, 80, 90, and 1.0% ethanol; frozen in tertiary butyl alcohol at −20 °C; and sputtered with gold. Finally, images were generated with a scanning electron microscope (Hitachi S-3400 N, Japan).

2.3. Fluorescence confocal microscopy analyses

Bovine macrophages were plated on poly-$_L$-lysine (0.1 mg/ml, Sigma-Aldrich) pre-treated glass slides in 24-well plates and stimulated with *N. caninum* tachyzoites (ratio 1:1, 90 min, 37 °C). The samples were treated as previously described (Wei et al., 2016a). After cell adherence, the cells were fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature (RT) and washed with PBS three times. The samples were then permeabilized using 0.1% Triton X-100 for 15 min and blocked with 3% goat serum dissolved in PBS. Subsequently, the samples were incubated with specific primary antibodies (1:200, dissolved in 3% goat serum) at 4 °C overnight. In the present study, an anti-histone antibody (LS-C353149; Life Span BioSciences, Inc.) and anti-myeloperoxidase (MPO) antibody (Orb16003; Biorbyt) were chosen to detect histone 3 (H3) and MPO in *N. caninum* tachyzoite-induced ET-like extracellular structures. Then, samples were incubated with a secondary conjugated-antibody (goat anti-rabbit IgG-FITC conjugated, Bioworld Technology, Inc.), washed in PBS three times, stained with 5 μM Sytox Orange® (Invitrogen) and diluted in PBS for 10 min at RT. Finally, the samples were installed in anti-fading reagents (Beyotime Biotechnology, China) after being washed twice with PBS and examined with a scanning confocal microscope (Olympus FluoView FV1000).

2.4. Quantitation of macrophage-derived ETs

Bovine macrophages were stimulated with purified *N. caninum* tachyzoites (ratio 1:8 or 1:16) for 90 min at 37 °C. In the inhibiting experiments, UO126 (50 μM, Calbiochem) as inhibitor of extracellular signal-regulated kinase 1/2 (ERK 1/2), and SB202190 (10 μM, Sigma-Aldrich) as the inhibitor of p38 mitogen-activated protein kinase (p38 MAPK) were pretreated with macrophages for 30 min prior to *N. caninum* tachyzoites stimulation. Lysed or dead *N.caninum* was also used in parallel experiments. To prepare the stimuli of hot-lysed or cold-lysed *N.caninum*, live *N.caninum* was boiled at 100 °C for 15 min or treated with repeated thawing and refreezing at −80 °C every 10 min for three cycles. In these two processes, the structures of *N.caninum* tachyzoite have almost been lysed. And the dead-*N.caninum* tachyzoite retain its conservation of structure. Bovine macrophages without *N. caninum* stimulation were used as negative control. Zymosan (1 mg/ml) was widely served as positive control as described (Villagra-Blanco et al., 2017; Wei et al., 2018; Yang et al., 2017). Bovine macrophage-triggered ETs were quantified using Pico Green® (Invitrogen) and identified using a fluorometric reader Infiniti M200® (TECAN, Austria) under an excitation wavelength of 488 nm and emission wavelength of 523 nm.

2.5. Detection of reactive oxygen species (ROS)

N. caninum tachyzoites (ratio 1:8, 90 min) induced reactive oxygen species (ROS) production in exposed macrophages, and ROS secretion was determined with 2,7 dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) as described previously (Conejeros et al., 2011). The results were detected with an automatic fluorometric reader (TECAN, Austria) using an excitation wavelength of 485 nm and emission wavelength of 530 nm.

2.6. LDH detection

The LDH levels of the supernatant were detected using a commercially available LDH Cytotoxicity Assay kit® (Beyotime Biotechnology, China) in accordance with the manufacturer's instructions. Lysis buffer provided by the LDH Cytotoxicity Assay kit® was used as positive groups.

2.7. Statistical analysis

The data are expressed as the means ± SD. Differences between groups were calculated by one-way ANOVA with tukey multiple comparison test. P value < 0.05 with P value = 0.05 was considered to be significant. Data were analyzed using the GraphPad 6.0 software.