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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Trichomonas vaginalis triggers the release of THP-1 extracellular traps

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Abstract

Trichomonas vaginalis is responsible for the prevalence of trichomoniasis, which may be one of the most epidemic nonviral sexually transmitted pathogens. Extracellular traps (ET) are a unique form of innate immunity against infection; they bind to and kill microorganisms. However, the effect of *T. vaginalis* on ET release in the human monocytic cell line THP-1 remains unclear. In the present study, the morphology of ET derived from THP-1 in response to *T. vaginalis* was observed by scanning electron microscopy (SEM). The results demonstrated ET entangling *T. vaginalis*. Then, the colocalization of histone (H3) and myeloperoxidase (MPO) with DNA was observed via fluorescence confocal microscopy. Colocalization revealed the classic characteristics of DNA decorated with H3 and MPO. *T. vaginalis* significantly increased reactive oxygen species (ROS) and THP-1-derived ET. In addition, we measured the levels of lactic dehydrogenase (LDH) and the phosphorylation of the P38 and ERK1/2 MAPK signaling pathways. The results indicated that the formation of ET induced by *T. vaginalis* was related to phosphorylation of the P38 and ERK1/2 MAPK signaling pathways but not to LDH levels. These data confirmed the phenomenon of THP-1-derived ET being triggered by *T. vaginalis* in vitro; this process may play a pivotal role in innate immunity during defense against *T. vaginalis* infection.

Keywords *Trichomonas vaginalis* · Extracellular traps · THP-1 · Histones · Myeloperoxidase

Introduction

Trichomonas vaginalis (TV) is a protozoan parasite that is the most common nonviral sexually transmitted pathogen (Kissinger 2015). Each year, more than 170 million infections occur among individuals worldwide. Patients infected with TV have symptoms varying from urethral discharge, dysuria, and vaginal pruritus to irritation (Menezes and Tasca 2016). Moreover, TV infection can disrupt the production of secretory leukocyte protease inhibitor, which can reduce the transmission of HIV (Menezes and Tasca 2016), consistent with the observation that trichomoniasis is associated with an increased risk of acquiring HIV (McClelland et al. 2007; Quinlivan et al. 2012). Monocytes or neutrophils are important immune cells, contributing to innate immunity, and are among the first cells arriving at an infection site (Caro et al. 2014). The landmark discovery of ET by Brinkmann revealed a new mechanism of neutrophils in defense against bacteria and enriched the theory of innate immunity (Brinkmann et al. 2004). ET are elicited when pathogens are encountered, causing the release of DNA, which leads to the entrapment of pathogens. ET play a pivotal role in the acute phase of infection not only by entrapping the invading pathogens but also by killing bacteria and fungi (Brinkmann and Zychlinsky 2007; Urban et al. 2006). Thus far, ET have been reported to interact with parasites such as *Eimeria arloingi*, *Neospora caninum*, *Cryptosporidium parvum*, *Eimeria bovis*, *Leishmania*, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Strongyloides stercoralis*, and *Schistosoma japonicum* (Abi Abdallah et al. 2012; Bonne-Annee et al. 2014; Chuah et al. 2013;

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Guimaraes-Costa et al. 2009; Silva et al. 2014; Sousa-Rocha et al. 2015; Wei et al. 2016; Wei et al. 2018). However, few studies have been conducted on the ET induced by TV. Therefore, the purpose of the present study is to investigate the functions of ET elicited by TV and characterize the molecular mechanism underlying the release of ET induced by TV.

Materials and methods

Parasites

TV was cultured in glass tubes in liver extract medium containing 16% fetal bovine serum (BioInd, Israel) and 1% penicillin/streptomycin (BioInd, Israel). The temperature for the TV culture was 37 °C. Every 24–48 h, the TV was passaged. Live TV was put in tubes at 37 °C for 4 days to obtain dead TV. Prior to stimulating the cells, live TV cells and dead TV cells were washed three times with RPMI 1640 medium (Gibco, USA) (3000 rpm, 5 min) and suspended in the same medium (Gibco, USA).

Cell culture

The human monocytic leukemia cell line THP-1 was cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (BioInd, Israel) and 1% penicillin/streptomycin (BioInd, Israel) at 37 °C and 5% CO_2. THP-1 cells were passaged every 48 h. The cells were washed three times in RPMI 1640 (Gibco, USA) before use.

Scanning electron microscopy

THP-1 cells were adhered onto glass coverslips precoated with poly-L-lysine (Sigma, 1 mg/mL) and incubated with TV (THP-1:TV = 2:1) for 1 h. Subsequently, the cultures were fixed with 4.0% glutaraldehyde (Merck) overnight. Thereafter, the coverslips were washed with PBS, postfixed with 1.0% osmium tetroxide (Merck), and dehydrated in an ascending ethanol series (30, 50, 70, 80, 90, and 100%); then, the specimens were covered with gold particles and analyzed by a Hitachi S-3400N scanning electron microscope.

Visualization of ET

THP-1 cells were washed three times with RPMI 1640 (Gibco, USA), and then the cells (6×10^6/mL) were plated onto coverslips coated with poly-L-lysine (1 mg/mL, Sigma) and incubated at 37 °C and 5% CO_2 for 2 h in a 24-well plate. The cells were then stimulated by TV (3×10^6/mL) for 1 h at 37 °C and 5% CO_2. Next, the THP-1 cells were fixed with 4% paraformaldehyde (15 min, room temperature) and then washed three times with PBS. THP-1 were subsequently permeabilized for 15 min (RT) with 0.2% Triton X-100 and then washed three times with PBS. The coverslips were then blocked with 3% goat serum (RT, 2 h) and incubated with anti-histone monoclonal antibodies (LS-C353149; LifeSpan BioSciences) and anti-MPO antibodies (Orb16003; Biorbyt) overnight (4 °C). The samples were washed three times with PBS and incubated for 1 h with secondary antibodies. For the detection of DNA, the coverslips were stained with 5-μM SYTOX Orange (dissolved in PBS, Invitrogen) for 10 min (RT) and gently washed three times with PBS. Briefly, the coverslips were mounted onto glass slides with antifading buffer and visualized by an Olympus FluoView FV1000 fluorescence microscope.

Quantification of THP-1-induced ROS

THP-1 cells were suspended in RPMI 1640 without phenol red (Solarbio, China) in the presence of DCFH-DA (2′,7′-dichlorofluorescein–diacetate; Solarbio; 1:1000) and incubated for 20 min at 37 °C and 5% CO_2. Subsequently, THP-1 cells were seeded onto a 96-well cell culture plate (6×10^6/mL) in triplicate. As a positive control, zymosan was added to the wells at a final concentration of 1 mg/mL. Additionally, the cells were stimulated with TV (3×10^6/mL) for 1 h. Lastly, the relative fluorescence units (RFU) were measured by using a spectrofluorometer at 485-nm excitation and 520-nm emission.

Quantification of DNA

THP-1 cells (2.5×10^5/mL) were resuspended in RPMI 1640 (without phenol red or serum) and plated onto a 96-well plate. The cells were incubated with zymosan (1 mg/mL), live TV (5×10^5/mL) and dead TV (5×10^5/mL), and medium at 37 °C and 5% CO_2 for 1 h and subsequently centrifuged (3000 rpm, 5 min). The supernatant (50 μL per well) from each sample was transferred to black 96-well flat-bottomed plates. Then, 50 μL 1 × TE and 100 μL PicoGreen (1:200 dilution by 1 × TE) were added to each well at room temperature for 4 min. Fluorescence intensities were measured at 485/535 nm.

Western blotting

The stimulation of THP-1 cells (6×10^6/mL) by TV (12×10^6/mL) was conducted at 37 °C and 5% CO_2 for 1 h. Protein was extracted by the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) according to the manufacturer's instructions and determined by BCA (Thermo Scientific). Total protein, in equal amounts, was fractionated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Then, the membranes were incubated with p-ERK1/2 antibody and p-p38 MAPK antibody (CST,

USA) at 4 °C overnight. After washing, the blot was incubated with HRP-conjugated anti-rabbit IgG antisera and visualized by electrochemiluminescence (ECL) western blotting reagents (Millipore Corporation, USA).

LDH detection

The levels of LDH produced by TV were tested by the LDH Cytotoxicity Assay kit (Beyotime Biotechnology, China). In the group of lysis, cells were lysed by breaking agent of cell membrane offered by the LDH Cytotoxicity Assay kit.

Statistical analysis

The analysis was conducted using GraphPad 5.0 software. The data are shown as the means with SEM. We performed the statistical analysis by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests among different groups. We performed the statistical analysis by using *t* test between two groups. $P \leq 0.05$ was considered a significant difference.

Results

TV is captured by THP-1 cells

THP-1 cells were incubated with TV on glass coverslips precoated with poly-L-lysine for 1 h. Then, we analyzed the thicker and thinner networks in which TV was hampered originating from THP-1 cells using scanning electron microscopy. Upon stimulation, the drawn-out filaments extruded by THP-1 cells form extracellular fibers that bind TV. As illustrated in Fig. 1a, the exposure of TV to a large number of adjacent THP-1 cells resulted in the formation of rather chunky mesh that was firmly attached to the parasites and completely covered the TV. However, as shown in Fig. 1b, the morphology of the TV remained normal.

Immunofluorescence microscopy

To determine whether TV elicits the release of ET from THP-1, we stimulated THP-1 with TV. The TV-induced ET was identified as containing DNA by SYTOX Orange staining (Fig. 2a, d). Since ET are derived from the colocalization of DNA decorated with MPO and histone H3 in parasite-entrapping structures, we confirmed these structures using specific antibodies to stain MPO and H3 (Fig. 2b, e).

Quantitation of ROS

We evaluated the fluorescence intensity of THP-1 cells stimulated by TV after 1 h. Compared with the control, the fluorescence intensity revealed that the exposure of THP-1 cells to TV was significantly increased (Fig. 3).

TV-induced ET formation is independent of LDH activity

We measured LDH release as the confirmation of a new cell death mechanism different from necrosis, apoptosis, and autophagy. We investigated the levels of LDH release from THP-1 cells exposed to zymosan, RPMI 1640, and TV for 2, 1.5, 1, and 0.5 h, which were not upregulated, whereas the LDH levels in the lysis group (cells were lysed by breaking agent of cell membrane offered by the LDH Cytotoxicity Assay kit) were markedly elevated (Fig. 4).

Measurements of ET

To determine the components of ET, THP-1 cells were incubated with TV for 1 h, and the ET release was estimated using PicoGreen (Thermo Fisher Scientific, USA). The measurement of ET in the supernatant indicated that the exposure of THP-1 cells to live TV at different ratios was accompanied by significant upregulation in a dose-dependent manner (Fig. 5). The results showed that dead *T. vaginalis* also induced ET formation, which indicated that both live TV and dead TV can induce THP-1-derived ET (Fig. 6).

P38 and ERK1/2 MAPK are involved in TV-triggered ET formation

ET formation has been shown to rely on the activation of the P38 and ERK1/2 MAPK signaling pathways (Munoz-Caro et al. 2015b). To explore whether TV-triggered ET were controlled by these signaling pathways, we measured the activity of P38 MAPK and ERK1/2. After 1 h of stimulation, TV significantly increased the phosphorylation of P38 and ERK1/2 (Fig. 7).

Discussion

Monocytes, macrophages, and polymorphonuclear neutrophils are considered the most important immune cells in innate immunity, playing a pivotal role in the first line of defense against pathogens such as bacteria (Aulik et al. 2012), viruses (Wardini et al. 2010), fungi (Bruns et al. 2010), and parasites (Munoz-Caro et al. 2015a; Yang et al. 2017). In addition to the secretion of antimicrobials and phagocytosis, polymorphonuclear neutrophils employ a novel defense against pathogens, namely, extracellular traps, in which DNA released from the nucleus hampers and ensnares the microbes. These extracellular DNA fibrils have been observed in several ET originating from neutrophils exposed to *Eimeria bovis* (Munoz-Caro

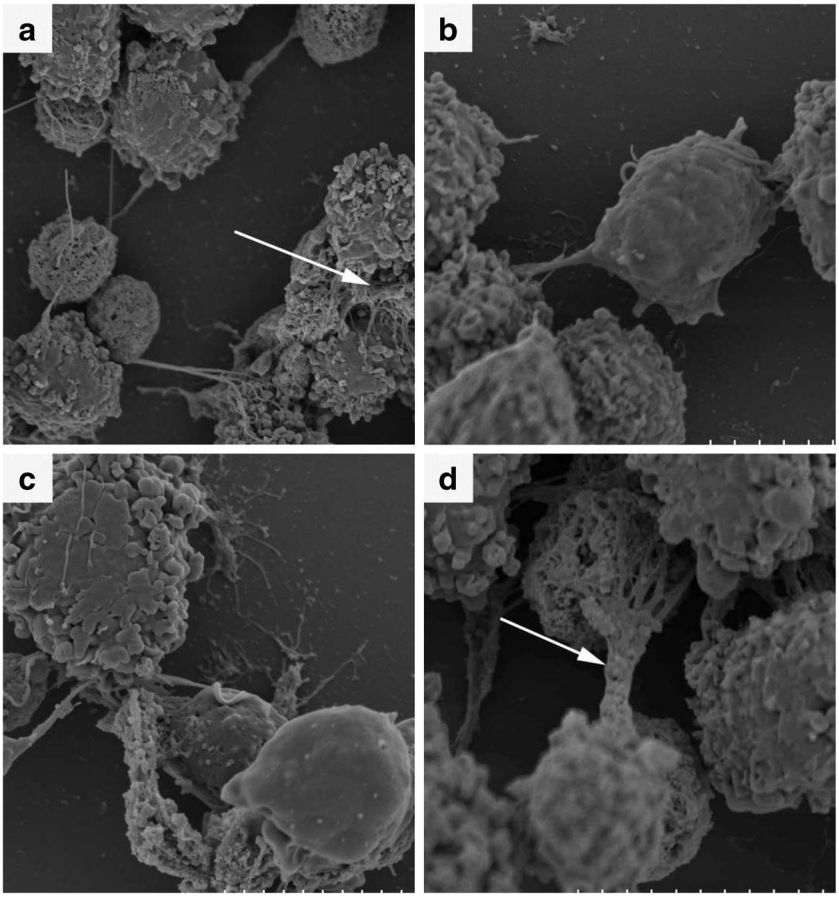

Fig. 1 The observation of THP-1 exposed to TV by SEM. **a** TV was surrounded by nuclear DNA released from THP-1 cells. **b**, **c** The ET entrapped TV, and the morphology of TV was not affected. **d** The fibers originated from THP-1 immobilized the TV and were stronger and longer than those of NET. The white arrow represents ET induced by TV. We conducted each experiment at least 3 times

et al. 2015b; Behrendt et al. 2010), *Leishmania* spp. (Guimaraes-Costa et al. 2012), *Plasmodium falciparum* (Baker et al. 2008), *Neospora caninum* (Wei et al. 2016), *Toxoplasma gondii* (Abi Abdallah et al. 2012), *E. arloingi* (Silva et al. 2014), *Schistosoma japonicum* (Chuah et al. 2013), and *Strongyloides stercoralis* (Bonne-Annee et al. 2014); monocytes exposed to tachyzoites

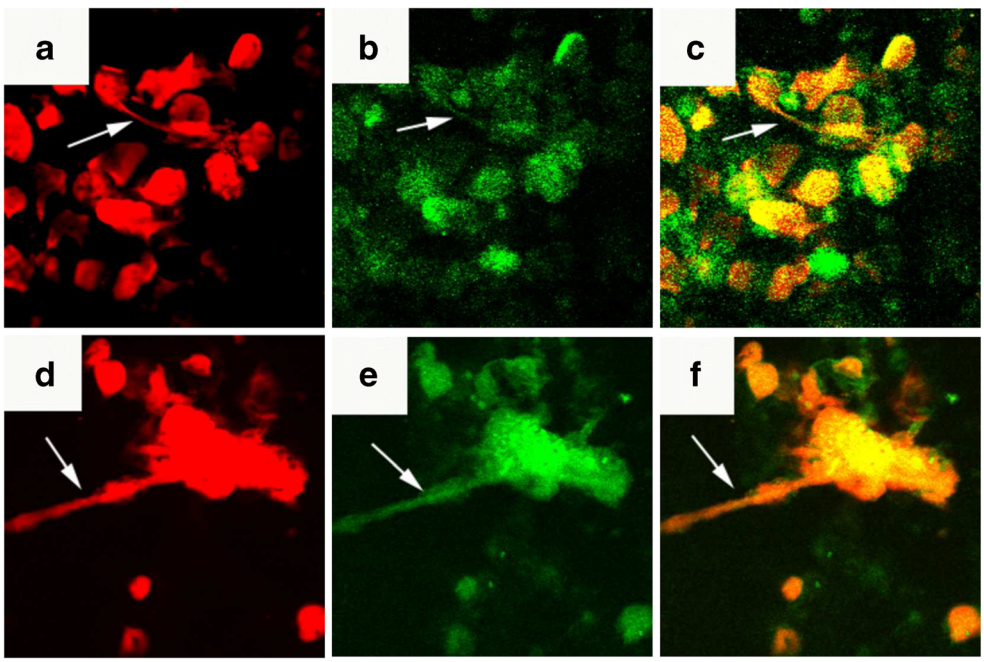

Fig. 2 The confirmation of ET studded with H3 and MPO by immunofluorescence microscopy. The fibers of nuclear DNA triggered by TV were detected by SYTOX Orange (**a**, **d**), using anti-H3 (**e**) and MPO (**b**) antibodies. **c**, **f** The merges. The white arrow represents ET induced by TV. We conducted each experiment at least 3 times

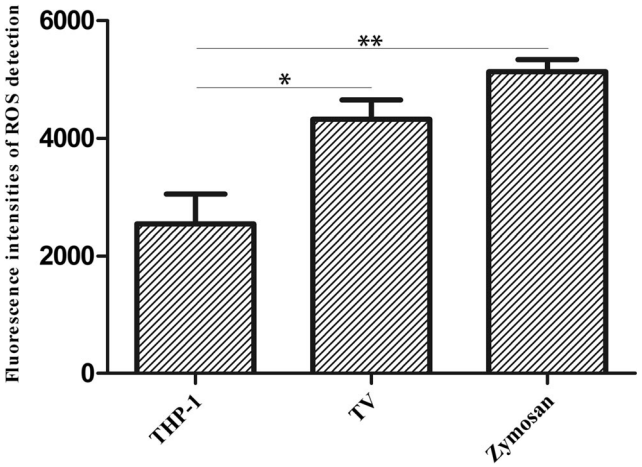

Fig. 3 The examination of ROS levels in THP-1-derived ET. THP-1 cells were stimulated by TV (THP-1:TV = 2:1) and zymosan (1 mg/mL) for 1 h, and then, the ROS levels were tested by the Reactive Oxygen Species Assay Kit. We conducted each experiment at least 3 times. We performed the statistical analysis by one-way analysis of variance (ANOVA) among different groups. $P \leq 0.05$ was considered a significant difference (*$P \leq$ 0.05, **$P \leq 0.01$, and ***$P \leq 0.001$)

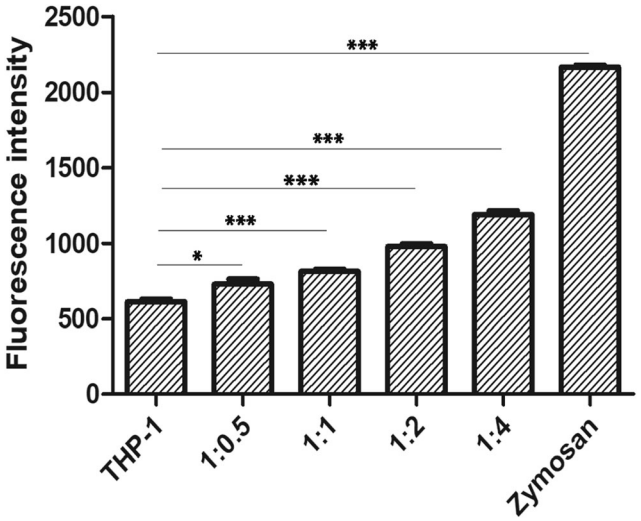

Fig. 5 Quantification of THP-1-derived ET. THP-1 in response to TV (THP-1:TV = 1:1, 1:2, and 1:4) for 1 h. The 485/535-nm wavelength was used to measure the fluorescence intensities. We conducted each experiment at least 3 times. We performed the statistical analysis by one-way analysis of variance (ANOVA) among different groups. $P \leq 0.05$ was considered a significant difference (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$)

of *Besnoitia besnoiti* (Caro et al. 2014); and monocytes from harbor seal exposed to tachyzoites of *T. gondii* (Reichel et al. 2015). Furthermore, a study has demonstrated that neutrophils rapidly killing *T. vaginalis* occur via trogocytosis but not in a neutrophil extracellular traps (NET)-independent manner (Mercer et al. 2018). However, whether *T. vaginalis* can trigger ET in the process remains unknown. Thus, in the present study, we comprehensively analyzed monocyte-derived ET production stimulated by TV for the first time, using scanning electron microscopy, immunofluorescence microscopy, quantification of reactive oxygen species, measurement of the activity of LDH, and examination of the MAPK signaling pathway.

SEM is considered the most potent evidence confirming the release of ET induced by TV. Since the landmark discovery of ET by Brinkmann et al. 2004, the ET formed in response to pathogens have been found to exhibit different morphologies. Observation of neutrophils exposed to bacteria

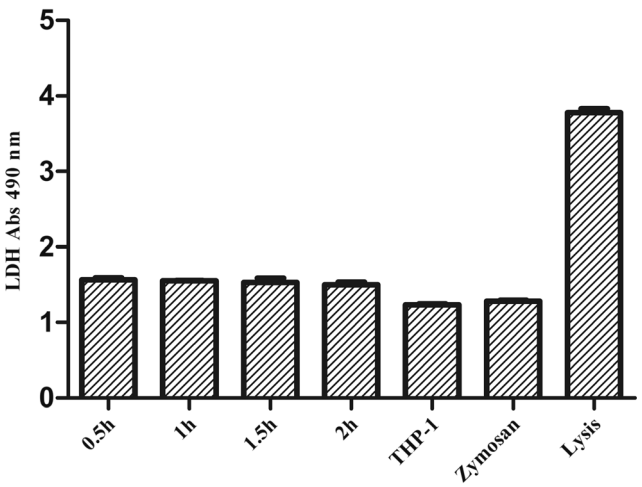

Fig. 4 LDH levels in THP-1-derived ET. THP-1 cells were exposed to TV (THP-1:TV = 2:1) for 30, 60, 90, and 120 min. The concentration of LDH was examined by the LDH Cytotoxicity Assay kit. In the group of lysis, cells were lysed by breaking agent of cell membrane offered by LDH Cytotoxicity Assay kit. We conducted each experiment at least 3 times. We performed the statistical analysis by one-way analysis of variance (ANOVA) among different groups. $P \leq 0.05$ was considered a significant difference (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$)

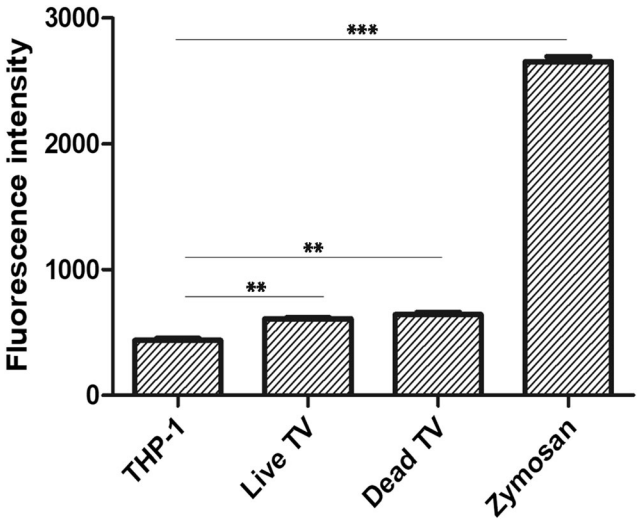

Fig. 6 Quantification of dead THP-1-derived ET. THP-1 in response to live TV and dead TV (THP-1:TV = 1:2) for 1 h. The 485/535-nm wavelength was used to measure the fluorescence intensities. We conducted each experiment at least 3 times. We performed the statistical analysis by one-way analysis of variance (ANOVA) among different groups. $P \leq 0.05$ was considered a significant difference (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$)

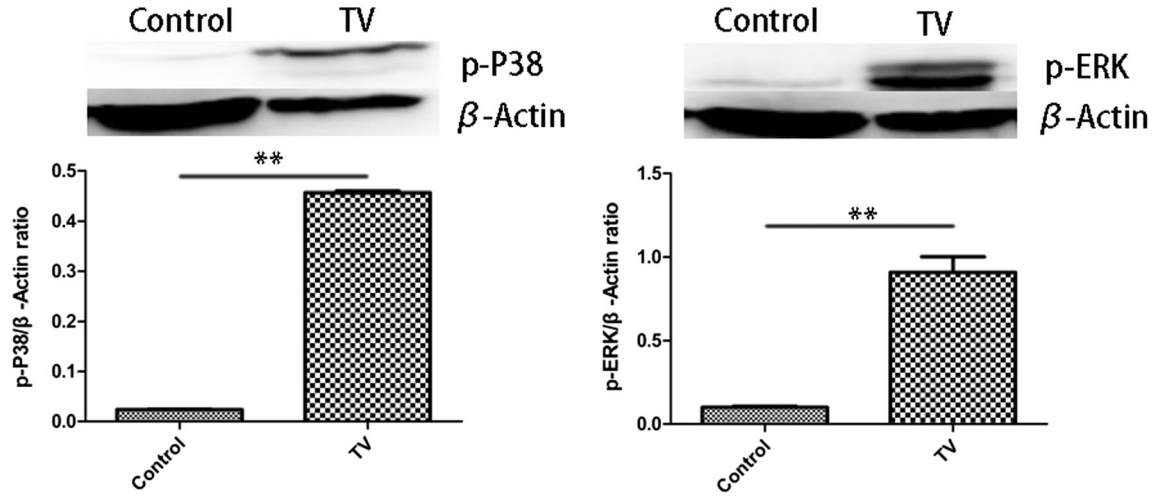

Fig. 7 The expression of phosphorylated P38 and ERK1/2 MAPK signaling pathway proteins in THP-1-derived ET. The phosphorylation of the P38 and ERK1/2 MAPK signaling pathways was detected by western blotting after THP-1 cells were exposed to TV (THP-1:TV = 1:2) for 1 h. We conducted each experiment at least 3 times. We performed the statistical analysis by using *t* test. $P \leq 0.05$ was considered a significant difference (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$)

revealed that the ET presented a thin line of fibers with protrusions consisting of a globular domain (Brinkmann et al. 2004). SEM analysis of *Leishmania* promastigotes-ET interactions revealed that *Leishmania* promastigotes were covered in flat mesh containing several irregular small holes (Guimaraes-Costa et al. 2009). Activated THP-1 cells stimulated by TV released ET, demonstrating a distinctive morphology in which stronger ET than NET aggregated into strands of fibers concomitant with ensnared TV (Fig. 1b, c).

As reported in a previous study, neutrophils encounter bacteria, viruses, fungi, and parasites and form ET, which cause the release of nuclear DNA and the release of cytoplasmic contents into the extracellular environment, particularly H3, MPO, gelatinase, lactoferrin, and neutrophil elastase (NE) (Silva et al. 2016). To further examine the composition of ET induced by TV, immunofluorescence was used for component analysis. The results emphasized the classical characteristics of the colocalization of H3 and MPO with DNA into ET. Moreover, to investigate whether ET were correlated with the activity of NADPH oxidase, we quantified the ROS released from THP-1 cells. ROS release was significantly increased by TV, which was similar to the results of our previous research in *N. caninum* (Wei et al. 2018; Yang et al. 2017). In addition, THP-1-derived ET were induced by live TV in a dose-dependent manner. When cells were stimulated with *T. vaginalis*, several molecules were secreted, such as TNFα and IL-1β (Fiori et al. 2013; Han et al. 2009), and these molecules have been demonstrated to induce ET formation. To investigate whether only living parasites are mandatory in the release of NET, we used dead TV as a control (Fig. 6). The results showed that dead TV also induced ET formation, which indicated that both live TV and dead TV can induce THP-1-derived ET. However, which kinds of proteins or molecules from TV can trigger the release of ET still needs further investigation. Furthermore, the results obtained from LDH release also indicated that TV-induced ET are a typical death pathway of "NETosis."

The MAPK signaling pathway plays a prominent role in cellular programs, and there have been an increasing number of studies associated with the diverse biological functions of MAPK such as transformation, development, and proliferation (Roux and Blenis 2004). ERK1/2, P38-MAPK, c-Jun, and ERK5 are the main subfamilies of MAPK (Cargnello and Roux 2011). An array of elegant studies has been performed to identify the role of P38 MAPK in the activation of the serine/threonine kinase pathway, which has been associated with inflammatory disease, cardiovascular disease, neurodegenerative disease, inflammation, differentiation, and tumorigenesis. Studies have shown that ERK1/2 plays a key role as a regulator of cell proliferation (Roux and Blenis 2004). In recent years, Hakkim et al. indicated that store-operated calcium entry is regulated via ERK1/2, which is tightly associated with the formation of ET (Hakkim et al. 2011; Munoz-Caro et al. 2015b). To determine whether THP-1 stimulation by TV would induce results similar to *Eimeria bovis*-triggered NET, we investigated the activation of the ERK1/2 and P38 MAPK signaling pathways. THP-1 exposure to TV resulted in the formation of ET and activated the ERK1/2 and P38 MAPK signaling pathways.

There are some similarities between NET and monocyte-derived ET. Monocyte-derived ET is similar to NET; both have a DNA backbone, the classical characteristics of the colocalization of H3 and MPO and ROS dependency. Similar to NET, monocyte-derived ET also seem to be non-stage-specific with respect to the parasite. Neutrophils have a stronger tendency to induce ET than monocytes do. NET

result in considerable killing of *E. ninakohlyakimovae* sporozoites. The lethality of NET is higher than that of monocyte-derived ET (Perez et al. 2016). Furthermore, the morphology of monocyte-derived ET is distinct from that of NET. NET show both strong and thin fibers, as shown in *Neospora caninum*-induced ET (Wei et al. 2016) and *Eimeria bovis*-induced ET (Munoz-Caro et al. 2015b). Monocyte-derived ET, such as caprine monocyte-derived ET (Yang et al. 2017) and *B. besnoiti*-induced ET (Munoz-Caro et al. 2014), consist of more strong fibers.

Overall, the formation of THP-1-derived ET in response to TV was identified for the first time in the present study. Based on these findings, the exposure of human-derived monocytes to TV indicated the presence of ET, which was correlated with the activation of the P38 and ERK1/2 MAPK signaling pathways. Considering the studies of NET, there is sufficient evidence to support the idea that THP-1-derived ET can entrap *T. vaginalis*. While the phenomenon of ET elicitation by TV was confirmed in the present study, the role of ET in vivo needs further research.

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