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Mouse macrophages capture and kill *Giardia lamblia* by means of releasing extracellular trap

Ling Li[a,1], Xin Li[a,1], Guojiang Li[b,1], Pengtao Gong[a], Xichen Zhang[a], Zhengtao Yang[a], Ju Yang[a], Jianhua Li[a,b,*]

[a] Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, 5333 Xian Road, Changchun 130062, China
[b] Jilin Agricultural Science and Technology University, 77 Hanlin Road, Jilin 132101, China

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ABSTRACT

Giardia lamblia is one of the most prevalent parasites residing in the duodenum of human and many other mammals throughout the world which is transmitted via ingested cysts through contaminated food or water. The severity of disease may depend on multiple parasite and host factors. Commonly, children and immunologically compromised persons like AIDS patient exhibit severe diarrhea, malabsorption and weight loss, however, there are also some infected people who are asymptomatic or only exhibit mild clinical symptoms and can shed the *Giardia* cysts in the environment. Although many studies have indicated that the innate immune system is important for *Giardia* defense, however, whether the innate immune responses such extracellular traps (ETs) could be induced by *G. lamblia* is still unclear. In recent years, macrophage extracellular traps (METs) have been described as an effective defense mechanism against invading microorganisms. In the present study, the formation of METs triggered by *G. lamblia* trophozoites was investigated. The formation of METs induced by *G. lamblia* trophozoites of mouse macrophage was observed with Scanning Electron Microscopy (SEM). The main components DNA, H3 histone and MPO were confirmed by Sytox orange staining, DNase1 digestion, immunofluorescence staining and fluorescence confocal microscopy. Inhibitor assays suggested that *G. lamblia* trophozoites triggered METs formation through ERK1/2 and p38 MAPK signal pathways and was Store-operated Ca^{2+} entry (SOCE) dependent. In addition, the process of METs formation triggered by *G. lamblia* trophozoites was also time and dose-dependent. Furthermore, the production of Reactive Oxygen Species (ROS) in macrophages stimulated with *G. lamblia* trophozoites significantly increased whereas no significant changes were observed about LDH activity.

1. Introduction

G. lamblia is the causative agent of giardiasis, which can spread among people and other mammals through ingestion of *G. lamblia* cysts contaminated water and food. *G. lamblia* can also cause water borne outbreaks in developing countries (Daly et al., 2010). *Giardia* trophozoites are responsible for clinical manifestation of Giardiasis.

Approximately 20–80% of the human population with positive fecal specimen show severe symptoms clinically, however, some patients are asymptomatic or only exhibit mild clinical symptoms (Adam, 2001; Thompson, 2004; Troeger et al., 2007). People suffering from this disease are always characterized by diarrhea, dehydration, headache, colic and weight loss, with children and immunologically compromised persons like AIDS patient exhibit more severe symptoms (Büyükbaba et al., 2004; Muhsen and Levine, 2012; Stark et al., 2009). *Giardiasis* can lead to morbidity in human and economic losses in agricultural animals and has been included in the Neglected Diseases Initiative by the World Health Organization since 2006 (Olson et al., 1999; Savioli et al., 2006). Genetic characterization of *Giardia* isolates has revealed the existence of eight groups (assemblages A to H) which differ in their host distribution. Assemblages A and B are found in humans and in many other mammals (Sprong et al., 2009). However the mechanisms of host innate immunity against *G. lamblia* infection have not been fully understood.

Microorganisms can be initially recognized by the innate immune system through germline encoded pattern-recognition receptors (PRRs). Several classes of PRRs, including Toll-like receptors and Nod-like receptors could recognize distinct microbial components and directly activate immune cells (Akira et al., 2006). For example, the binding of

* Corresponding author. Jilin Agricultural Science and Technology University, 77 Hanlin Road, Jilin 132101, China.
E-mail address: Jianhuali7207@163.com (J. Li).
[1] These authors contributed equally.

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G. lamblia to immunoglobulin protein could trigger the maturation of dendritic cells via the activation of TLR4-MyD88-p38 and ERK1/2 MAPKs (Lee et al., 2015). TLR2 expressed in WT mouse peritoneal macrophages could recognize *Giardia* and regulate the secretion of cytokines (Xin Li et al., 2017). When the innate immune system senses *giardia*; dendritic cells, macrophages and epithelial cells produce the initial rapid response (Kamda, 2007; Ringqvist et al., 2008). Macrophages are the most abundant innate immune cells in the body, which participate in the induction of inflammatory responses caused by the parasites infection (Anthony et al., 2006; Chaussabel et al., 2003; Liew et al., 1990). Recently, ETs have been confirmed to be a novel mechanism against pathogenic microorganisms. ETs are meshworks of chromatin fiber structures extruded from neutrophils, macrophages, monocytes and mast cells. These structures always bound with antibacterial peptides and granule constituents such as neutrophil elastase, myeloperoxidase, lactoferrin and gelatinase (Aulik et al., 2010; Köckritzblickwede et al., 2008; Urban et al., 2009). Neutrophil extracellular traps (NETs) have been shown to trap and kill many bacteria and fungi (Brinkmann et al., 2004; Urban et al., 2006). NETs are also involved in the host defense against infection of several viruses (Hemmers et al., 2011; Jenne et al., 2013; Raftery et al., 2014; Saitoh et al., 2012; Wardini et al., 2010). In addition, parasitic protozoans including *Eimeria bovis, Toxoplasma gondii, Neospora, Leishmania amazonensis* have been shown to be the inducers of NETosis (Behrendt et al., 2010; GuimarÃ£escosta et al., 2009; Muñoz-Caro et al., 2015b; Reichel et al., 2015; Wei et al., 2016).

Antimicrobial ET is also being considered as an effector mechanism for macrophages. It has been shown that bacteria and fungi (e.g. *Staphylococcus aureus, Escherichia coli, Mannheimia haemolytica, Histophilus somni, Mycobacterium tuberculosis, Candida albicans*) can trigger the formation of METs (Chow et al., 2010; Hellenbrand et al., 2013; Liu et al., 2014) and *M. haemolytica* secreted leukotoxin can induce bovine alveolar macrophage to generate METs with bactericidal activity via the activation of NADPH oxidase. However, the functions of METs in response to parasitic protozoans have not been well studied.

Several studies have indicated that macrophages play a key role during *G. lamblia* infection (Hill and Pearson, 1987; Hill and Pohl, 1990). In the present study, the formation of METs induced by *G. lamblia* and the function of METs on trapping and killing the parasites were investigated.

2. Materials and methods

2.1. Ethics statement

Female C57BL/6 mice (Huafukang Experimental Animal Centre, Beijing, China) were housed in filter-top cages in an air-conditioned animal facility in the National Experimental Teaching Demonstration Centre of Jilin University (Changchun, China). Water and normal mice food were consumed ad libitum. All experimental procedures connected with *G. lamblia* were strictly operated in Biological Safety Cabinet and complied with Pathogenic Microorganisms Laboratory Biosafety Regulations of China. All animal experimental procedures were performed in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved through the State Council of People's Republic of China and with approval of the Animal Welfare and Research Ethics Committee at Jilin University (IACUC Permit Number: 201701009).

2.2. G. lamblia trophozoites cultivation and collection

Trophozoites of *G. lamblia* WB strain (ATCC30957; American Type Culture Collection, Manassas, VA, USA) were cultivated to the logarithmic growth phase in TYI-S-33 medium and collected by centrifugation at 1000*g* for 10 min after icing bath. After washed with culture medium RPMI 1640 (Hyclone, USA) the live trophozoites were resuspended in RPMI 1640. Furthermore the dead trophozoites were obtained by treatment at 65 °C for 50 min and the dead trophozoites were determined by trypan blue staining (Ringqvist et al., 2008).

2.3. Isolation of mouse peritoneal macrophages

Mice were injected with 3 ml of Thioglycollate Broth (Thermo Fisher, USA) three days before sacrifice. For macrophage collection, mice were euthanized with ether and soaked in 75% ethanol for 15 min. Peritoneal cavities were gently flushed twice with 15 ml 1 × PBS (Sangon Biotech, Shanghai, China) and cells were collected by centrifugation at 1000*g* for 10 min. Macrophages were washed twice with 20 ml 1 × PBS and resuspended in 1 ml RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin, then incubated overnight at 37 °C with 5% CO2. Cells were washed twice with PBS to remove the non-adherent cells, then the adherent cells were trypinized and analyzed by flow cytometry. Cells with CD11b expression over 95% were retained for further experimental procedures.

2.4. Scanning electron microscopy (SEM) observation

Mouse macrophages (1.5×10^6 cells/ml) pre-cultured on poly-L lysine (1 mg/ml, Sigma-Aldrich) coated cover-glass in 24-well culture plates (JET BIOFIL, China) overnight then incubated with live or dead *G. lamblia* (7.5×10^5 trophozoites/ml) at ratios of 6:1, 4:1, and 2:1 at 37 °C for 150 min, respectively, and non-adherent cells and trophozoites were washed off with RPMI 1640. Cells were then fixed with 4.0% glutaraldehyde (Merck, USA) for 24 h, gently washed with PBS and post-fixed in 1.0% osmium tetroxide (Merck, USA). The fixed samples were first dehydrated in ascending ethanol concentrations (30, 50, 70, 80, 90 and 100%) and then frozen in tertiary butyl alcohol at −20 °C and sputtered with Gold powder. Finally, specimens were observed using a scanning electron microscope (Hitachi S-3400N, Japan).

2.5. Determination of mouse METs composition

After incubation of mouse macrophages (1.5×10^6 cells/ml) pre-cultured on poly-L lysine with live or dead *G. lamblia* (7.5×10^5 trophozoites/ml) on poly-L-lysine-treated glass coverslips as mentioned above, the cell samples were fixed with 4% paraformaldehyde for 20 min at room temperature, then gently washed thrice with PBS, permeabilized with 0.1% Triton X-100 for 15 min and blocked in 1% BSA. For the detection of histones and MPO within ETs structures, the samples were incubated with anti-histone (H3) antibody (1:200, Life Span BioSciences, USA) or anti-MPO antibody (Alexa Fluor 488, Biorbyt, 1:200) at 4 °C overnight, then incubated with the FITC conjugated secondary goat anti-rabbit IgG (Boster, China). The web-like ETs structures released by macrophages were defined by staining with Sytox Orange (1:1000, Invitrogen, USA). DNA structures released from PMA-stimulated macrophages were stained with 5-μM Hoechst 33342 (Sagon Biotech, China) and 5-μM Sytox Orange (Invitrogen, USA) as positive control. After washing with sterile PBS the samples were mounted in Lab Vision™ PermaFluor™ (ThermoFisher, USA) and observed under a fluorescence confocal microscope (Olympus FluoView FV1000).

2.6. Quantitative analysis of mice METs

Mouse macrophages (7.5×10^5 cells/ml) pre-cultured in 96-well plates overnight, then stimulated with *G. lamblia* trophozoites at different ratios (4:1, 2:1, 1:1, 1:2) and times (60min, 90min, 120min, 150min), respectively. In parallel experiments, macrophages were pretreated with the NADPH oxidase inhibitor diphenylene iodonium (DPI, 20 μM, Sigma-Aldrich, USA), the myeloperoxidase (MPO) inhibitor aminobenzoic acid hydrazide (ABAH, 100 μM, Calbiochem,