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Short communication

Macrophages expressing arginase 1 and nitric oxide synthase 2 accumulate in the small intestine during *Giardia lamblia* infection

Jenny Maloney[1], Aleksander Keselman[2], Erqiu Li[3], Steven M. Singer*

Georgetown University, Dept. of Biology, 37th and O Sts NW Reiss 406, Washington DC 20057, USA

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Abstract

Nitric oxide (NO) has been shown to inhibit *Giardia lamblia* in vitro and in vivo. This study sought to determine if *Giardia* infection induces arginase 1 (ARG1) expression in host macrophages to reduce NO production. Stimulations of RAW 264.7 macrophage-like cells with *Giardia* extract induced arginase activity. Real-time PCR and immunohistochemistry showed increased ARG1 and nitric oxide synthase 2 (NOS2) expression in mouse intestine following infection. Flow cytometry demonstrated increased numbers of macrophages positive for both ARG1 and NOS2 in lamina propria following infection, but there was no evidence of increased expression of ARG1 in these cells.
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Keywords: Giardia lamblia; Arginase 1; Macrophages

1. Introduction

The protozoan parasite *Giardia lamblia* (syn. *Giardia intestinalis*, *Giardia duodenalis*) is a common cause of diarrheal disease worldwide. *G. lamblia* can infect humans and many other mammals, with prevalence rates in humans that range from 2 to 7% in developed countries to 20–30% in developing countries [1]. *G. lamblia* is transmitted through faecal contamination of food or water. As such infection rates are highest in countries where water purification is limited, leading to *G. lamblia* being included in the World Health Organization's Neglected Disease Initiative since 2004 [2]. Intestinal pathology during infection is driven in part by the host immune system [3]. Yet the immunological processes that control infection and pathology are not entirely identified.

Arginine has diverse functions in mammalian physiology and plays an important role in host immunity. The production of Nitric Oxide (NO) by Nitric Oxide Synthase 2 (NOS2) from arginine is a key element of the innate immune response as NO is toxic to many pathogens. In fact arginine is the sole amino acid substrate for NO production [4]. The depletion of arginine as a means of limiting NO production is a survival strategy employed by many pathogenic organisms including viruses, bacteria, fungi, and protists [5]. Arginine limitation can be achieved through a number of pathways that include pathogen production of arginase (ARG), pathogen mediated induction of host ARG in macrophages, or consumption of host arginine by the pathogen [6,7]. Nitric oxide is known to be cytostatic and potentially cytotoxic to *G. lamblia* in culture [8–11]. Yet the activation of host ARG as an evasive mechanism of *G. lamblia* has not been explored in vivo.

Host macrophages are often divided into two classes based on the expression of ARG1 and NOS2: classically activated M1 macrophages which express NOS2 and alternatively activated M2 macrophages which express ARG1 [5,12]. These cells are thought to have antagonistic roles in the immune response with M1 macrophages being involved in pathogen control and M2 macrophages serving to limit excessive NO

* Corresponding author. Tel.: +1 202 687 9884; fax: +1 202 687 5662.
E-mail addresses: jgm56@georgetown.edu (J. Maloney), ak476@georgetown.edu (A. Keselman), el44@georgetown.edu (E. Li), sms3@georgetown.edu (S.M. Singer).
[1] Tel.: +202 687 1852; fax: +202 687 5662.
[2] Tel.: +202 687 1852; fax: +202 687 5662.
[3] Tel.: +202 687 1852; fax: +202 687 5662.

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production and support healing. The ability of some pathogens to influence the expression of ARG1 and NOS2 can play an important role in immune subversion and parasite survival.

Previous work has shown that host NO can inhibit *G. lamblia* survival in vitro, and that arginine limitation negates this effect [9]. Recent studies on the influence of *G. lamblia* on epithelial cell ARG activity report a down regulation of host ARG in response to *G. lamblia* [13]. However these studies exclusively used in vitro culture systems of non-immune cells. In this study we aimed to determine if *G. lamblia* can directly induce macrophage ARG1 in vitro and if infection can lead to an increase in ARG1 expression in host macrophages in vivo.

2. Materials and methods

2.1. Mice and infections

C57BL/6J mice were obtained from Jackson Laboratories and kept under specific-pathogen-free conditions at Georgetown University. All experiments were performed with the approval of the Georgetown University Animal Care and Use Committee. *G. lamblia* (strain GS/M/H7) was cultured and used for infections as previously described [14]. Mice were infected via gavage with 1×10^6 parasites in phosphate buffered saline.

2.2. RAW 264.7 cell culture

The murine macrophage cell line RAW 264.7 was maintained in complete Dulbecco's modified Eagle's medium (Invitrogen). Only cells between passage 5 and 25 were used for arginase activity assays. For all experiments cells were grown to confluence in 12 well culture plates. Fresh media containing 10 ng/ml IL-13 (R&D Systems) or 100 µg/ml *G. lamblia* lysate was added to culture wells. Lysate was generated by washing trophozoites extensively in sterile PBS to remove culture media followed by repeated freeze thaw cycles to lyse trophozoites. Freeze thaw cycles were performed by freezing the trophozoite pellet in liquid nitrogen. The pellet was then thawed in a 37 °C water bath and vortexed for 15 s. This process was repeated 6 times, and total cell extract was used for all assays. The protein concentration of the lysate was measure via Bradford assay (Biorad) and the absence of LPS contamination was confirmed using a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit per the manufacturer's protocol (Thermo Fischer). Following an 8 h incubation, macrophages were lysed in 10 mM Tris–HCL pH 7.4, 0.4% w/v Triton X-100 containing protease inhibitor cocktail (Calbiochem), and arginase activity was measured using the Quantichrom Arginase Assay Kit (Bioassay Systems) per the manufacturer's protocol. NO production was quantified by measuring nitrite, a stable breakdown product of NO, in culture supernatants using the Griess Reagent System per the manufacturer's protocol (Promega).

2.3. Real-time PCR

Small intestinal tissue was collected from mice at days 0, 3, and 7 following infection, and total RNA was extracted and reverse transcribed to cDNA using a reverse transcription kit (Applied Biosystems). For ARG1 the primers were F-CAGAAGAATGGAAGAGTCAG R-CAGATATGCAGGG AGTCACC and yielded a 250 bp product. For NOS2 the primers were F-TGCATGGACCAGTATAAGGCAAGC R-GCTTCTGGTCGATGTCATGAGCAA and yielded a 223 bp product. For GADPH the primers were F-ACCCAGAA-GACTGTGGATGG R-TCAGCTCTGGGATGACCTTG and yielded a 124 bp product. Real-time PCR was performed on cDNA with the primer pairs listed above on a CFX 96 cycler (Biorad). For each PCR reaction 2 ul cDNA, 900 nM primers, and 10 µl Syber-Green master mix (Sensifast) and an 65 °C anneal/extend were used. All samples were assayed in triplicate and analyzed using the ΔΔCT method to assess relative fold change in ARG1 and NOS2.

2.4. Immunohistochemistry

Small intestine tissue was fixed in 10% formalin, embedded in paraffin, and sectioned. Tissue sections were dewaxed in xylene and rehydrated. Antigen retrieval was performed by boiling slides in antigen unmasking solution (Vector Labs). Tissue sections were stained with antibodies against ARG1 and NOS2 followed by appropriate biotin conjugated secondary antibodies (all Santa Cruz Biotech). The tissue was labeled with Strep-Avidin HRP and visualized using a DAB peroxidase kit (Vector Labs). All tissue was counterstained with hematoxylin (Vector Labs).

2.5. Isolation of intestinal lamina propria cells

Ten cm duodenal segments were obtained and pooled from 4 mice per experimental group. The pooled duodena were fractionated to collect lamina propria cells as described [15]. Briefly, the Peyer's patches were removed and the remaining intestinal fragments were washed with 1 mM DTE in order to remove the epithelial layer. The tissue was washed with 5 mM EDTA to strip off the epithelium, leaving an intact lamina propria fraction to be digested by liberase TL (Roche). After enzymatic digestion, the lamina propria fraction was separated on a Percoll (Sigma–Aldrich) gradient in order to enrich for leukocytes and remove dead cells.

2.6. Flow cytometry

Lamina propria cell preparations were washed in PBS, and 10×10^6 cells were stained with LIVE/DEAD Fixable Yellow Stain (Invitrogen) for 30 min at room temperature in the dark. Surface stains with fluorophore conjugated antibodies against F4/80 and CD11b (Biolegend) were performed for 30 min at 4 °C. Cells were fixed in 4% paraformaldehyde and permeabilized using Perm Buffer (Biolegend). Intracellular staining with fluorophore conjugated antibodies ARG1 (R&D systems) and NOS2 (Santa Cruz Biotech) were performed for 1 h at room temperature. Staining with antibodies was done in the presence of a CD16 blocking antibody (TruStain fcX, Biolegend). Stained cells were analyzed using a FACStar Plus

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