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Infection dynamics of *Toxoplasma gondii* in gut-associated tissues after oral infection: The role of Peyer's patches☆

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ABSTRACT

Toxoplasma gondii is a common perorally transmitted parasite; however, its immunopathogenesis in gut-associated tissues remains unclear. Here, we compared disease manifestation in C57BL/6 immunocompetent wild type (WT) mice and immunocompromised interferon (IFN)-γ-deficient (GKO) mice after peroral infection (PI) with *T. gondii* cysts (Fukaya strain). Strong PI-induced Th1 cytokine expression was detected in WT mice. Moreover, bradyzoite-specific *T.g.HSP30/bag1* mRNA was detected in the ileum parenchyma and Peyer's patches (PP), but not in the mesenteric lymph nodes, at 7 days post-infection in WT mice, and was significantly higher than that in GKO mice. Nested PCR showed that parasites existed in ileum parenchyma at days 1 and 1.5 post-PI in GKO and WT mice, respectively. In addition, quantitative competitive-PCR indicated that *T. gondii* first colonized the PP (day 3 post-PI), followed by the ileum parenchyma and mesenteric lymph nodes, spleen, and portal and aortic blood (day 7 post-PI). Although parasites were consistently more abundant in GKO mice, similar invasion and dissemination patterns were observed in the two hosts. Collectively, these data suggest that some zoites differentiate from tachyzoites to bradyzoites in the ileum and that *T. gondii* initially invades the ileum parenchyma, and then accumulates and proliferates in the PP before disseminating through the lymphatic systems of both GKO and WT hosts.

1. Introduction

The obligate intracellular protozoan parasite *Toxoplasma gondii* is a food- and water-borne pathogen that can cause disease in humans and animals. *T. gondii* generally invades the host through the lining of the intestine after peroral infection (PI) by bradyzoites or oocysts [1], which can lead to lethal ileitis [2] and colitis [3]. The gut mucosal immune system serves as the frontline of defense against this parasite [4]. Following antigenic stimulation, the mucosa-associated lymphoid tissues (MALTs) can induce an immune response characterized by lymphocyte proliferation and differentiation. These lymphocytes then egress from the secondary lymphoid tissues into the bloodstream [5,6]. Although Peyer's patches (PPs) are MALTs and are thus inductive sites of mucosal immunity [7], the precise roles mediated by the PP in response to peroral *T. gondii* infection remain unclear.

Pathogens employ a variety of mechanisms to escape detection by host defense systems as they disseminate either lymphogenously or hematogenously [8]. Many reports have described the kinetics of parasitic dissemination routes after *T. gondii* PI [1,8–13], which differ based on strain, mouse genotype, and inoculation (oral gavage or natural feeding) and detection methods. In addition, a rigorous analysis of stage conversion in gut-associated tissues has not been performed to date.

Recent studies have revealed that *T. gondii* infection is a significant cause of morbidity and mortality in immunocompromised hosts, such as patients with acquired immunodeficiency syndrome (AIDS) or organ transplant patients treated with immunosuppressive drugs [14,15]. As such, the infection dynamics of *T. gondii* in both immunocompromised and immunocompetent hosts need to be clarified. Control of toxoplasmosis has been extensively studied in mouse models, and it has

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been demonstrated that interferon (IFN)-γ responses are critical for protection against this parasite [16,17]. As *IFN*-γ gene-deficient (GKO) mice are unable to resist *T. gondii* infection, they can therefore be considered as a representative model of immunocompromised hosts for *T. gondii* infection.

In this study, we have performed the cytokine levels, stage-conversion status (tachyzoites vs. bradyzoites), and histopathology of the digestive tract tissues from C57BL/6 immunocompetent wild type (WT) and immunocompromised GKO mice during acute phase of infection. Furthermore, very early invasion route was assessed by nested polymerase chain reaction (PCR) targeting *B1* gene [18], and dissemination route by quantitative competitive (QC) PCR targeting *SAG1* gene [10].

2. Material and methods

2.1. Parasites

Cysts derived from the avirulent *T. gondii* Fukaya strain (type II genotype) [19] were prepared from B10.A (4R) mice that had been orally infected with five cysts containing the Fukaya strain, 6 weeks previously [10].

2.2. Animals

Eight- to twelve-week-old C57BL/6 WT (SLC, Hamamatsu, Japan) and same background GKO (a gift from Prof. Yoichiro Iwakura, Center for Animal Disease Models, Research Institute for Biomedical Sciences, Tokyo University of Science, Tokyo, Japan) mice were used for our analyses. GKO mice were maintained in our laboratory and genotyped by PCR [20]. Infection status was assessed in mice euthanized by intraperitoneal injection of 2 mg ketamine hydrochloride at 1, 1.5, and 2 days post-PI. To assess infection dynamics, mice were orally infected with 10 *T. gondii* cysts administered using a syringe fitted with a rounded 19-gauge needle and were euthanized at 1, 3, 5, and 7 days after PI. The abdomen was incised and the peritoneal lining was opened to expose the intestines. The portal vein was ligated at the porta hepatica, and portal blood was collected using a 24-gauge venous cannula. Aortic blood was collected by cardiopuncture. The distal side of the small intestine was removed and washed with cold phosphate-buffered saline (PBS) before being divided longitudinally. All visible PPs were removed using shaped scissors and the remaining tissues were defined as the ileum parenchyma. The mesenteric lymph node (MLN) and spleen were also extracted.

To assess the survival rate, the mice were orally infected with 5 or 10 *T. gondii* cysts. Survival was monitored daily to assess mortality. Animals were treated according to the guidelines established by the Chiba University Animal Ethics Committee.

2.3. Reverse transcriptase (RT)-PCR

mRNA was purified from the ileum parenchyma, PP, and MLN tissues to assess tissue cytokine mRNA expression at 7 days after PI by RT-PCR as described previously [21]. In brief, GITC buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% N-laurylsarcosine) was added to homogenized tissue samples to promote lysis. RNA was then extracted using water-saturated phenol and chloroform/isoamyl alcohol. The prepared mRNA (1 μg) was reverse transcribed in a 20 μL reaction volume using an RNA PCR kit (Takara, Shiga, Japan) according to the manufacturer's instructions. PCR was performed using 5 μL of the resulting cDNA product. Similarly, the expression of *SAG1* (used as a tachyzoite marker) [22] and *T. gondii* (*T.g.*) *Hsp30/bag1* (used as a bradyzoite marker) mRNA [23] was also investigated by RT-PCR 7 days after PI. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA expression was used as an internal control. The expression levels of mRNA were calculated using the ratio of the respective densities of their RT-PCR products to that of *Gapdh* mRNA.

2.4. Nested PCR and QC-PCR

Total genomic (g) DNA was isolated from the ileum parenchyma, PP, and MLN and used to assess very early infection status by nested PCR with primers targeting *B1* gene [18]. Parasite load and dissemination were determined by assessing *SAG1* DNA in the ileum parenchyma, PP, MLN, spleen, portal blood, and aortic blood by QC-PCR as described previously [10]. In brief, digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate, and 0.1 mg/mL proteinase K) was used to homogenize tissues before digestion at 55 °C in a shaking water bath overnight. DNA was extracted as described previously [10]. Prepared gDNA (1 μg) was co-amplified with a constant concentration of truncated *SAG1* DNA, which competitively binds to oligo primers for WT *SAG1* [10]. The amplified cDNA was electrophoresed on a 1% agarose gel containing ethidium bromide. The WT to competitor ratio for amplified *SAG1* DNA (T/C) was measured using an IPLab Gel densitometer (Signal Analytical Corp., VA, USA). The abundance of *T. gondii* was calculated as described previously [10].

2.5. Primers

The primers used were as follows: *IFN*-γ, forward (5′-TGA ACG CTA CAC ACT GCA TCT TGG-3′), and reverse (5'-CGA CTC CTT TTC CGC TTC CTG AG-3′); IL-12, forward (5'-CGT GCT CAT GGC TGG TGC AAA G-3′), and reverse (5′-GAT GAA GAA GCT GGT GCT G-3′); IL-4, forward (5′-GAA TGT ACC AGG AGC CAT ATC-3′), and reverse (5'-CTC AGT ACT ACG AGT AAT CCA-3′); IL-10, forward (5'-CGG GAA GAC AAT AAC TG-3′), and reverse (5'-CAT TTC CGA TAA GGC TTG G-3′); SAG1, forward (5'-GGC ATA TGT CGG ATC CCC CTC TTG TTG C-3′), and reverse (5'-GGC TCG AGC TCC AGT TTC ACG GTA CAG T-3′); T.g.Hsp30/bag1, forward (5′-GGG AAT TCA TGG CGC CGT CAG CAT C-3′), and reverse (5′-GGG CGG CCG CCT ACT TCA CGC TGA TTT GTT-3′); Gapdh, forward (5'-ACC ACA GTC CAT GCC ATC AC-3′), and reverse (5'-TCC ACC ACC CTG TTG CTG TA-3′). Two PCR primer pairs were used for *B1* gene nested PCR as follows: S1, 5′-TGT TCT GTC CTA TCG CAA CG-3′ and AS1, 5'-ACG GAT GCA GTT CCT TTC TG-3′; S2, 5'-TCT TCC CAG ACG TGG ATT TC-3′ and AS2, 5'-CTC GAC AAT ACG CTG CTT GA-3′ [18].

2.6. Histopathology

The ileum and MLN were harvested from WT and GKO mice euthanized 8 days after PI with 10 cysts and analyzed histopathologically. The extracted ileum was washed twice in PBS to remove the mucus before being cut longitudinally along the side that adhered to the mesentery. Tissues were fixed in 20% buffered formalin and embedded in paraffin. The paraffin embedded ileum was then cut orthogonally to the axis and stained with hematoxylin and eosin (HE). Images were acquired on a microscope (BX41, Olympus, Tokyo, Japan) equipped with a charge-coupled device camera (FX630, Olympus).

2.7. Drugs

Polymyxin B sulfate (Pfizer Japan Inc., Tokyo, Japan) and metronidazole (Shionogi Co., Ltd., Osaka, Japan) were administered separately or concomitantly in drinking water at 250 U/mL and 50 μg/mL, respectively. Metronidazole was initially dissolved in diluted hydrochloric acid to facilitate water absorption before adjusting the pH to 7 with 2 N NaOH.

2.8. Statistical analysis

Differences between groups were determined by the Mann-Whitney