Host Immunity and Pathogen Strain Contribute to Intestinal Disaccharidase Impairment following Gut Infection

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Infection or other inflammatory insults in the small intestine often result in reduced disaccharidase enzyme levels. Using a mouse model of giardiasis, we examined the role of host immunity and pathogen virulence in mediating disaccharidase deficiency post-infection (p.i.). C57BL/6J mice were infected with two strains, WB and GS, of the human parasite *Giardia duodenalis*. The levels of sucrase, maltase, and lactase decreased in wild-type mice p.i. with the GS strain but not with the WB strain. Both CD4-deficient and SCID mice failed to eliminate the infection and did not exhibit disaccharidase deficiency. β2-Microglobulin knockout animals controlled infections similar to wild-type mice but exhibited no decrease in disaccharidase activity. Analysis of cytokine production by spleen and mesenteric lymph node cells showed production of IL-4, IL-10, IL-13, IL-17, IL-22, TNF-α, and IFN-γ p.i. with both WB and GS, with IFN-γ being the dominant cytokine for both parasite strains. Mesenteric lymph node cells produced lower levels of cytokines compared with splenocytes in response to parasite extract, although the overall pattern was similar. These data suggest that T cell responses mediate parasite clearance whereas also contributing to pathogenesis. They also demonstrate that differences in pathogen strain can also determine the outcome of infection and further our understanding of the clinical variation seen in human giardiasis. *The Journal of Immunology*, 2011, 187: 000–000.
Materials and Methods

Mice
Six- to 8-wk-old female C57BL/6J, SCID (B6.CB17-Prkae<sup>−/−</sup> /Siz), β2-microglobulin (β2-m)-deficient (B6.129P2-P2m<sup>−/−</sup>/J), and CD4 knockout (B6.1292S-Cd4<sup>−/−</sup>/J) mice were purchased from The Jackson Laboratory (Harbor, ME). Mice were housed at the Georgetown University Animal Care Facility, and all experiments were carried out in accordance with guidelines approved by the Georgetown University Animal Care and Use Committee in compliance with the National Institutes of Health guidelines.

Parasites and infection protocols
The GS-M-83-H7 (ATCC 50581) and the WB clone-C6 (ATCC 50803) strains of G. duodenalis were used in these experiments. Parasites were axenically grown in TYI-S-33 media supplemented with adult bovine bile, t-cysteine, ascorbic acid, and antibiotics (all from Sigma-Aldrich, St. Louis, MO). Forty-eight hours before infection, mice were given antibiotics in drinking water ad libitum: amoxicillin (1.4 mg/ml; Durvet, Blue Spring, MO), cephalothin (1 mg/ml; Sigma-Aldrich), and vancomycin (1 mg/ml; Hospira, Lake Forest, IL) (21). Mice were gavaged with 10<sup>6</sup> trophozoites in 0.1 ml PBS (pH 7.4). Antibiotic use was maintained for the duration of the infection. To quantify parasite loads at different times p.i., we euthanized mice and discarded the first 3 cm of the small intestine (pylorus to ligament of Treitz). The next 2-cm section of the duodenum was removed, opened longitudinally, and minced in 4 ml ice-cold PBS (pH 7.4). Tissues were kept on ice for 15 min, and the numbers of trophozoites were counted using a hemocytometer.

Disaccharidase activity assay
Intestinal disaccharidase activity was measured using the method originally developed by Dahlqvist (22) with some modifications (23, 24). In brief, intestinal lysates were prepared using repeated freeze–thaw cycles (5×), and 50 μl of the homogenate were incubated at 37˚C for another hour. The glucose release was measured at 450 nm using a microplate reader (BioTek Instrument, Winooski, VT) with g-glucose oxidase-peroxidase was added to each well. The samples were incubated at 37˚C for another hour. The glucose release was measured at 450 nm using a microplate reader (BioTek Instrument, Winooski, VT) with g-glucose oxidase-peroxidase. The OD was read at 450 nm using a microplate reader (BioTek Instrument, Winooski, VT). The absorbance was converted to μmol of glucose produced per milligram of total protein per minute.

Ex vivo restimulation of splenocytes and mesenteric lymph node cells
G. duodenalis trophozoites were axenically grown in TYI-S-33 media as described earlier. Confluent tissue flasks were kept on ice for 30 min, and detached trophozoites were washed five times with ice-cold PBS (pH 7.4). Whole-cell lysate was prepared using repeated freeze–thaw cycles (5×), and 50 μl of the homogenate were incubated at 37˚C for another hour. The glucose release was measured at 450 nm using a microplate reader (BioTek Instrument, Winooski, VT) with g-glucose oxidase-peroxidase. The OD was read at 450 nm using a microplate reader (BioTek Instrument, Winooski, VT). The absorbance was converted to μmol of glucose produced per milligram of total protein per minute.

Flow cytometry
For flow cytometry, MLNs and spleens were collected in HBSS supplemented with 5% FBS (HyClone) and 25 mM HEPES and strained through a 70-μm nylon membrane (BD Falcon). Live/dead cell labeling was performed using a LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Invitrogen) for 45 min at 4˚C in the dark according to the manufacturer’s instructions. Cells were incubated in HBSS supplemented with 5% FBS and 25 mM HEPES to reduce nonspecific binding. Cells (10<sup>5</sup>/sample) were stained with anti–CD3-PerCP (clone 145-2C11), anti-CD4-PE (clone RM4-5), and anti–CD8-allophycocyanin (clone 53-6.6; all from BioLegend) for 45 min at 4˚C and were fixed with 1% paraformaldehyde. Cells were analyzed using a Becton Dickinson FACSAria (BD Biosciences) and FACS Express Version 4.0 software (DeNovo Software, Los Angeles, CA).

Statistical analyses
Data were analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA) and expressed as mean ± SEM. For statistical analyses, a two-tailed Mann–Whitney U test was used, and p < 0.05 was considered statistically significant.

Results
CD8<sup>+</sup> T cells are required for disaccharidase deficiency but not control of infection
To investigate the role of different T cell subsets in control of infection and in mediating intestinal pathology, we used a murine model system. We infected adult mice with the GS strain of G. duodenalis (26). Wild-type (WT) mice and mice lacking all adaptive immune responses (SCID), CD4<sup>+</sup> T cells only (CD4<sup>−/−</sup>), or CD8<sup>+</sup> T cells (β2-m<sup>−/−</sup>) were infected and parasite loads were enumerated at days 5 and 18 p.i. Results showed that all mice were heavily infected at day 5, and that WT mice eliminated almost all parasites by day 18 p.i. (Fig. 1A). In SCID and CD4<sup>−/−</sup> mice, however, parasite loads were even higher at day 5 p.i. and significant parasite elimination was not seen by day 18 (Fig. 1B, 1D), indicating the vital role of host immunity in clearing this infection. In contrast, β2-m<sup>−/−</sup> mice eliminated parasites with essentially the same kinetics as WT mice (Fig. 1C), indicating that although CD4<sup>+</sup> T cells are required to control this infection, CD8<sup>+</sup> T cells are not.

Disaccharidase deficiency has been documented p.i. and after other inflammatory conditions (7, 27). We hypothesized that

![FIGURE 1. CD4<sup>+</sup> T cells are required for the clearance of infections in a mouse model of disaccharidase deficiency. C57BL/6J (A), SCID (B), β2-m<sup>−/−</sup> (C), and CD4<sup>−/−</sup> (D) mice were infected on day 0 with the GS strain of G. duodenalis. Mice were euthanized on the indicated days p.i., and parasite loads in the small intestine were enumerated as described in Materials and Methods. Each bar indicates the mean ± SEM of four mice per time point. Data are representative of two independent experiments.](http://www.jimmunol.org/)

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infection of WT C57BL/6J mice with *G. duodenalis* would result in disaccharidase deficiency as previously shown for the murine species of *Giardia, G. muris* (11). To test this hypothesis, we measured disaccharidase enzyme activity in the jejunum of infected mice. We found that the levels of sucrase, maltase, and lactase, decreased 40, 29, and 37%, respectively, on day 5 p.i. with the GS strain of the parasite (Fig. 2A–C). The decrease in trehalase levels was not statistically significant (Fig. 2D). All enzyme levels returned to normal by day 18 when the hosts had cleared the infections.

We next investigated whether host immunity could mediate the depression of intestinal enzyme activity after gut infections. To test this, we infected SCID mice with the GS strain of the parasite and measured disaccharidase activities in the jejunum. Although infection in SCID mice resulted in increased parasite burdens and failure to eliminate the infection by the host (Fig. 1B), no significant impairment of intestinal enzymes was observed (p < 0.05; Fig. 3). These findings suggest that disaccharidase impairment is mediated by host immunity.

Having established that disaccharidase impairment is immune mediated, we examined specific immune cell populations that could contribute to disaccharidase impairment during *Giardia* infection. In β2m−/− mice infected with *Giardia*, there were no significant changes in the levels of sucrase activity at day 5 p.i. (p < 0.05; Fig. 4A). Similarly, no significant changes were observed in the activity levels of maltase, lactase, and trehalase 5 d p.i. compared with uninfected animals (Fig. 4B–D). Results showed that the absence of CD4+ T cells in infected mice did not induce lower enzymatic levels compared with uninfected controls (Fig. 4). These findings clearly suggest that the pathology seen in the intestine during the *Giardia* infections is T cell dependent.

**Cytokine responses by spleen and MLN cells p.i.**

Our results suggest that T cell responses are required for elimination of infection and induction of disaccharidase deficiency. We were therefore interested to determine whether the proportion of CD4+ and CD8+ T cells would change p.i. and to determine the cytokines being produced by T cells. Flow cytometry analysis showed that the composition of CD4+ and CD8+ T cells in the spleen and MLNs remained constant in mice infected with the GS strain of *G. duodenalis* 5 and 7 d p.i. compared with uninfected mice (Fig. 5A,5B). Analysis of cytokine production by cells from spleens and MLNs of mice infected with the GS strain showed that the peak of cytokine production occurred at day 7 p.i., and that the levels of cytokines produced by splenocytes were generally higher than the levels of cytokines produced by MLN cells (Fig. 5C–H). IFN-γ was the dominant cytokine produced by both splenocytes and

**FIGURE 2.** Disaccharidase enzymes are depressed in the intestines of WT C57BL/6J mice after gut infections. Disaccharidase activity assays were performed on jejunal homogenates from mice infected in Fig. 1. Levels of sucrase (A), maltase (B), lactase (C), and trehalase (D) were determined using the appropriate disaccharide. Data are shown as mean and SEM for four mice per group. *p < 0.05 by Mann–Whitney U test versus uninfected mice. Data are representative of three independent experiments. ns, not significant.

**FIGURE 3.** Lack of adaptive immunity prevents SCID mice from exhibiting impaired intestinal disaccharidase activity p.i. Sucrase (A), maltase (B), lactase (C), and trehalase (D) activities were measured in jejunal homogenates from SCID C57BL/6J mice infected with 10⁶ trophozoites of the GS strain or uninfected SCID mice. Data represent means and SEM for four mice per group. Data are representative of two independent experiments.

**FIGURE 4.** Lack of CD8+ T cells prevents mice from exhibiting impaired intestinal disaccharidase activity p.i. β2m−/− and CD4−/− mice were orally inoculated with 10⁶ trophozoites of the GS strain or uninfected mice. Sucrase (A), maltase (B), lactase (C), and trehalase (D) activities were measured in jejunal homogenates from mice infected for 5 or 18 d or uninfected mice. Data represent means and SEM for four mice per group. Data are representative of two independent experiments.
MLN cells in response to parasite Ags, followed by IL-10. Lesser amounts of TNF-α, IL-4, IL-17, and IL-22 were also detected.

Disaccharidase deficiency in WT mice is strain dependent

Human giardiasis can cause a wide range of clinical symptoms ranging from asymptomatic infections to severe diarrhea and cramps, which can result in maldigestion and stunted growth, especially in pediatric patients (1). It has been suggested that distinct assemblages of *G. duodenalis* differ in their capacity to cause symptomatic disease. We therefore asked whether the changes we observed in C57BL/6J mice infected with the GS strain of the parasite, the prototype for assemblage B, would occur during infection with the prototype strain for assemblage A. Because initial studies indicated that mouse infections with strain WB, the prototype for assemblage A, were unsuccessful (26), we developed a revised protocol for mouse infections to allow us to work with this strain. To determine the kinetics of infection and disaccharidase activity p.i. in mice with both the GS and WB strains, we infected WT mice with each strain

![Figure 5](image1.png)

**FIGURE 5.** Cytokine production by cells from spleens and MLNs of WT mice infected with the GS strain of *G. duodenalis*. WT mice were infected with the GS strain of *G. duodenalis* and euthanized at days 5 and 7 p.i. The proportions of CD4+ and CD8+ cells in spleens (A) and MLNs (B) were determined in uninfected and infected mice during the course of infection. Production of IFN-γ (C), TNF-α (D), IL-4 (E), IL-10 (F), IL-17 (G), and IL-22 (H) by spleens and MLNs of infected mice was determined using ELISA. Cell viability after 48 h of stimulation was ~50 and 70% in stimulated and unstimulated preparations, respectively. Data presented are means and SEM for four mice per time point.

![Figure 6](image2.png)

**FIGURE 6.** Infection and disaccharidase kinetics and cytokine production profile in WT mice infected with WB and GS strain of *G. duodenalis*. WT C57BL/6J mice were infected with 10^6* trophozoites of either the WB or GS strain. Mice were euthanized at 5, 7, 10, 14, and 18 d p.i. Parasite burdens (A) and disaccharidase activities (B) were determined at each time point for both strains (A). Spleen cells were restimulated in vitro with *Giardia* extracts prepared from the same strain used for infection, and supernatants were collected after 48 h. Levels of IFN-γ (C), TNF-α (D), IL-4 (E), IL-10 (F), IL-13 (G), IL-17 (H), and IL-22 (I) were measured by ELISA. Intestines and splenocytes from individual mice were assayed in duplicate, and the data presented are means and SEM for four mice per time point (*p < 0.05, **p < 0.005, compared with uninfected mice). Data are representative of three independent experiments.
of *G. duodenalis* separately, and the infection and disaccharidase activity kinetics were measured at days 5, 7, 10, 14, and 18 p.i. Parasitological examinations revealed substantial colonization of the C57BL/6J mouse intestine by the WB strain of *G. duodenalis*. Although parasite burdens in the intestines of mice infected with the WB strain were generally slightly lower compared with mice infected with the GS strain of the parasite, the differences in parasite burdens between these strains were not statistically significant (Fig. 6A). In contrast, our analyses showed that the WB strain of *G. duodenalis* was not able to induce disaccharidase deficiency, whereas WT mice infected with the GS strain exhibited enzyme impairment p.i.; the reduced enzyme activity in the GS-infected mice started 5 d p.i., remained low 7 d p.i., and returned to normal levels by day 18 p.i. (Fig. 6B). These data suggest a strain-dependent basis for disaccharidase impairment.

**Cytokine production by T cells p.i.**

We next sought to determine whether T cell cytokine responses were significantly different p.i. between these two strains. Cytokine production by spleen cells from mice infected with each strain of the parasite was measured ex vivo at six different time points (i.e., days 0, 5, 7, 10, 14, 18). Our analysis showed that both strains of the parasite elicited robust cytokine production in response to parasite extract, with detectable levels of IFN-γ, TNF-α, IL-4, IL-10, IL-13, IL-17, and IL-22 being produced at day 7 p.i. with either strain (Fig. 6C–I). IFN-γ was the dominant cytokine secreted p.i. with both parasite strains. An average of 5000 and 2500 pg/ml was observed at 7 d p.i. in mice infected with the WB and GS strains, respectively (Fig. 6C). Interestingly, the levels of all cytokines assayed in mice infected with the WB strain of *G. duodenalis* were somewhat higher than those infected with the GS strain of the parasite.

**CD4+ cells are the main cytokine-secreting T cells in mice infected with WB and GS strains**

Considering the essential roles of CD4+ T cells in resolving the intestinal infection in our model, we were interested in finding to what extent CD4+ cells contributed to the production of cytokines. WT mice were infected with either WB or GS and euthanized on day 7. Parasite burdens were similar to what was observed in previous experiments (Fig. 7A). Splenocytes were stimulated in vitro with *Giardia* extract, and CD4+ responses were blocked with an anti-CD4 IgG. Analyses of supernatants showed that CD4+ T cells were the major cytokine-secreting cells p.i. with either strain WB or GS. Blocking CD4 significantly reduced the production of IFN-γ, IL-4, IL-13, IL-17, and IL-22 (Fig. 7B, 7D, 7F–H). CD4+ T cell involvement in the production of TNF-α was only 30 and 34% for WB and GS strains, respectively (Fig. 7C), consistent with our previous findings that dendritic cells contributed to the secretion of TNF-α during infection with *G. duodenalis* (28). The production of IL-10 in mice infected with each strain was reduced drastically (87.5 and 37.5% for WB and GS strains, respectively), and it seemed that non-CD4 sources continued to secrete IL-10 in mice infected with the GS strain of the infection despite CD4 blockade (Fig. 7E).

**Discussion**

The major findings of this study are that levels of disaccharidase enzymes such as sucrase, maltase, and lactase decline significantly after murine infections with the GS strain of the human parasite *G. duodenalis*. We also showed that in the absence of adaptive immunity in SCID mice, there was no reduction of intestinal enzyme activity. Infections in CD4−/− and β2m−/− mice further showed that this impairment was an immune-based event requiring both CD4+ and CD8+ T cells. The results of this study also revealed an important difference in the ability of different pathogen strains to induce intestinal enzymatic deficiency in the mouse model of human giardiasis; C57BL/6J mice infected with the GS strain of the parasite displayed enzymatic deficiency, whereas mice infected with the WB strain of the parasite did not.

Similar to results from previous studies, our results indicate that CD4+ T cells are required for parasite elimination, whereas CD8+ T cells are not required. Interestingly, although β2m−/− eliminate infections normally, they fail to exhibit disaccharidase deficiency. Scott et al. (10) showed that CD8+ T cells from MLNs of *G. muris*-infected mice could induce disaccharidase deficiency after adoptive transfer into nude recipients. Our results are consistent with an important role for CD8+ T cells in causing disaccharidase deficiency. Our results further suggest that CD4+ T cells have a role in inducing disaccharidase deficiency because no disaccharidase deficiency was seen in CD4−/− mice. This role may be indirect, however; for example, CD4+ T cells may be necessary to generate an effector CD8+ T cell population. In summary, these results suggest that immunotherapies or vaccines that promote CD4+ T cell responses without generating CD8+ T cell responses could lead to protection without resulting in disaccharidase deficiency and contributing to nutrient malabsorption.

Different mechanisms have been regarded as the cause of disaccharidase deficiency p.i. and after other intestinal insults. These include direct damage to the epithelium caused by the parasite,
changes in gene expression within epithelial cells, and altered rates of EC maturation p.i. (1). Our results clearly exclude direct damage as a cause of disaccharidase deficiency in this model because no change in disaccharidase levels were seen in either SCID or CD4<sup>–/–</sup> mice despite having prolonged infections with very heavy parasite burdens. Although our data suggest that CD8<sup>+</sup> T cells are important in mediating pathology, they do not discriminate between specific changes in gene expression (i.e., reduced transcription of the sucrase/isoamaltase or lactase/phlorizin hydrolase genes) within epithelial cells or more global changes in epithelial cell biology (i.e., an increased proportion of immature ECs). Indeed, changes in the maturation of epithelial cells can involve both transcriptional and posttranscriptional regulation of disaccharidase enzymes (8, 29).

Different isolates of *G. duodenalis* have been shown to differ in their abilities to trigger pathological changes in vitro. For example, some strains of the parasite (i.e., NF and S2) were able to induce apoptosis in a nontransformed epithelial cell line (SCBN), whereas other strains (i.e., WB and PB) could not (19). A more recent study showed that the WB strain could actually facilitate caspase-dependent apoptosis in the epithelial cell line HCT-8 (30). Consistent with previous reports describing important biological differences between WB and GS (18), we found that the induction of disaccharidase deficiency and the dynamics and kinetics of cytokine production in mice infected with each strain were distinct. Whether these differences in cytokine response are connected with the absence of disaccharidase deficiency p.i. with strain WB, however, remains to be determined.

To our knowledge, this study is the first to describe the cytokines produced by CD4<sup>+</sup> T cells in response to *Giardia* infection. Spleen cells and MLNs stimulated ex vivo with *Giardia* extracts produced IFN-γ, α, IL-4, IL-10, IL-13, IL-17, and IL-22. Blocking CD4 eliminated production of almost all of these cytokines, indicating that CD4<sup>+</sup> T cells are the likely source of these cytokines. The lone exception was TNF-α, which we have previously shown can be produced in small amounts by dendritic cells stimulated with *Giardia* extracts (28). Previous work in the *G. muris* model was unable to demonstrate cytokine production in response to parasite Ags, although some changes in mitogen-driven cytokine production were noted (31).

Among the cytokines produced, IFN-γ was the most abundant. However, previous work using GS infection in adult mice lacking IFN-γ suggested that parasite elimination could occur normally in the absence of this cytokine. In contrast, anti-IFN-γ treatment of C57BL/10 mice resulted in prolonged infections with *G. muris* (31). There may be differences in the effector mechanisms that are needed to eliminate the different pathogens. For example, NO, antimicrobial peptides, IgA, bile salts, and intestinal hypermotility have all been shown to participate in parasite clearance, and it is likely that significant redundancy results in the absence of strong effects after elimination of single cytokines or pathways.

Interestingly, WB-infected mice exhibited two distinct cytokine peaks, at 7 and 14 d p.i. Furthermore, all of the cytokines tested were produced at higher levels in mice infected with the WB strain of *G. duodenalis* than in mice infected with the GS strain. This may explain why mice infected with the WB strain of *G. duodenalis* showed consistently lower parasite burdens (Fig. 6A). Alternately, the differences in levels of cytokines produced could reflect different levels of immunogenicity associated with the two strains. In mice infected with either strain, marked cytokine production at day 7 p.i. seems to correlate with the peak of parasite burden within the small intestine. In mice infected with the GS strain of *G. duodenalis*, cytokine production by spleen T cells was observed at high levels on day 7 p.i. and at lower levels on day 10 p.i., whereas spleen cells from the WB-infected mice exhibited two distinct peaks of cytokine production. Potential explanations for the differences in cytokine production kinetics could be attributed to different patterns of antigenic variation between the strains or to different patterns of T cell migration into and out of the spleen p.i.

Disaccharidase deficiency can result from numerous situations such as intestinal infection or inflammation. Disaccharidase deficiency can contribute to diarrhea and nutrient malabsorption in these situations. Our results clearly indicate an important role for host T cell responses in contributing to this pathology. This study also marks important advances in our understanding of giardiasis. To our knowledge, it is the first report to directly show cytokine production by CD4<sup>+</sup> T cells p.i. It also establishes a model for direct, controlled comparisons among different parasite strains to examine their ability to stimulate immune responses and to reproduce aspects of the clinical syndrome seen in human disease.

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**Disclosures**

The authors have no financial interests of interest.

**References**


