THE ROLE OF FAS IN PATHOLOGICAL GIARDIASIS

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ABSTRACT

Diarrheal disease presents a debilitating burden on many people and economies around the world. Many of these cases are caused by the protozoan parasite Giardia sp., which colonizes the small intestine of a wide range of hosts and disrupts normal digestive and absorptive processes within the gut. Malnutrition caused by giardiasis is largely immune-dependent; the pathology commonly observed within the infected small intestine is likely triggered by activated CD8+ T cells.

Understanding the dynamic immune response to Giardia infection is paramount for eventually identifying therapeutic targets and improving preventative measures. We report an influx of FasL+ cells into the duodenum following infection in C57BL/6 mice. Ex vivo restimulation of splenic and mesenteric lymph node (MLN) lymphocytes revealed an IFN-γ dominant cytokine response, which is conducive for the expansion of activated, FasL expressing CD8+ T cell. This observation prompted us to explore the role of Fas as a potential mediator of Giardia-induced intestinal injury. We observed defective parasite clearance in mice lacking functional Fas (Fas\textsuperscript{lp}r). Despite a high parasite burden, Fas\textsuperscript{lp}r mice did not exhibit the hallmark signs of a Giardia-injured small intestine, such as reduced disaccharidase activity. Increased ezrin phosphorylation was observed to correlate with reduced disaccharidase in infected C57BL/6 but not Fas\textsuperscript{lp}r mice. Fas\textsuperscript{lp}r mice exhibited similar levels of enterocyte apoptosis compared to C57BL/6 mice throughout infection. Activated CD8+ T cells within the spleens of infected Fas\textsuperscript{lp}r mice were detected, which was likely a consequence of their lympho-proliferative phenotype as this phenomenon was absent in C57BL/6 mice.
TABLE OF CONTENTS

INTRODUCTION -page 1
MATERIALS AND METHODS -page 8
RESULTS -page 11
DISCUSSION -page 16
FUTURE DIRECTIONS/PROPOSAL -page 20
RESULTS (FIGURES) -page 42
REFERENCES -page 55
INTRODUCTION

The protozoan *Giardia* is abundant in most running water and is a major cause of parasitic diarrheal disease around the world. Infection with *Giardia* leads to a variety of pathological changes in the host small intestine resulting in the malabsorption of nutrients. The intestinal injury inflicted upon the host bears resemblance to several non-infectious intestinal disorders, such as Crohn’s and celiac disease as well as irritable bowel syndrome (Dunne et al., 1977; Shepherd and Gibson, 2006). This pathological overlap between giardiasis and other intestinal disorders suggests that similar mechanisms may be driving pathogenesis. It is well understood now that host immunity play a major role in the formation of these diseases. In fact, the activation of T cells within explanted fetal human intestinal cultures, with PWA, leads to pathology that resembles the *Giardia*-infected gut (MacDonald and Spencer, 1998). Understanding the underlying mechanisms that injure the *Giardia*-infected gut thus may help identify common therapeutic targets within a spectrum of intestinal disorders while expanding our overall understanding of pathogenic mucosal immunity.

Our hypothesis is that intestinal injury acquired during giardiasis is at least partially mediated by Fas signaling. We believe that the death receptor Fas, expressed on enterocytes, is triggered by host lymphocytes to induce the pathological breakdown of the intestinal epithelium following infection. Mechanistically, we believe that Fas signaling in the infected small intestine imposes altered post-translational modifications on important brush border proteins that facilitate the shortening of the microvilli. This microvilli shortening event reduces the absorptive surface area of the gut, leading to intestinal enzyme deficiencies and ultimately malnutrition. We expect that a loss-of-function mutation in Fas will at least partially rescue *Giardia*-induced intestinal injury. Furthermore, we hypothesize that activated CD8\(^+\) T cells trigger pathogenesis by up-regulating and signaling through FasL. Likewise, we expect that the cytokine environment in the spleen and mesenteric lymph nodes will be conducive
for CD8⁺ T cell responses following infection. Thus, this study makes progress towards defining the nature of pathogenic signaling in the small intestine of the *Giardia*-infected host. To this end, we increase our understanding of *Giardia* induced pathogenesis and potentially identify a novel target for therapeutic intervention in the clinical setting.

**The intestinal brush border and giardiasis**

The intestinal brush border is composed of complex membrane micro-domains enriched within actin-supported microvilli structures, which protrude into the intestinal lumen from the apical pole of mature enterocytes. Digestive enzymes are packaged into these sphingolipid/cholesterol rich lipid raft micro-domains, which are extremely abundant within the brush border membrane (Hansen et al., 2011; Alfalah et al., 1999). Scaffolding proteins associate with the lipid rafts and facilitate the trafficking of raft-embedded brush border enzymes. For example, annexin II is responsible for the trafficking of sucrase and ezrin to brush border lipid rafts (Heinz et al., 2011). Ezrin is a member of the ezrin-radixin-moesin (ERM) family of proteins that specialize in the formation cellular polarizing structures, including the microvilli (Gupta et al., 2006; Tomas et al., 2002). Indeed, ezrin⁺⁻ mice exhibit short, flat microvilli and poorly organized terminal webs compared to wild-type mice (Saotome et al., 2004). This function of ezrin is largely dependent upon it being phosphorylated at Th 567 as this modification liberates the protein from an auto-inhibited state (Fievet et al., 2004). Villin is another important brush border protein that, unlike ezrin, has the capacity to bundle and sever actin filaments (Ferrary et al., 1999). Intracellular calcium levels and tyrosine phosphorylation modulate villin, allowing it to assume such diverse functional roles within the brush border (Zhai et al., 2001). When transfected into CV-1 fibroblasts, villin alone is sufficient for the development of microvilli-like structures (Friederich et al., 1989).

A common indicator of *Giardia*-induced brush border breakdown is the reduction of disaccharidases and intestinal alkaline phosphatase (IAP) (Solaymani-Mohammadi and Singer, 2011;
Scott et al., 2000; Scott et al., 2004; Singh et al., 2000; Mohammed et al., 1995; Mahmood et al., 2005). These enzymatic deficiencies reflect a reduction in epithelial surface area and absorptive capacity due to shortened microvilli (Scott et al., 2004). Disaccharidases convert complex sugars into glucose and fructose, allowing their transport across the epithelium. Consistently, the replenishment of glucose following infection with *G. duodenalis* rescues Caco-2 cells from parasite triggered apoptosis (Yu et al., 2008). It has been repeatedly demonstrated that the immune system actively participates in disaccharidase reductions following *Giardia* infection (Solaymani-Mohammadi and Singer, 2011; Scott et al., 2004). The mechanisms by which *Giardia* alters these enzymes remain unclear. However, host immunity likely modulates ezrin and villin upstream of microvilli shortening and enzyme reductions.

**Protective immunity against *Giardia***

*Giardia* infection is self-limiting, largely because of effective antimicrobial, humoral and cell-mediated immune responses mounted by the host. *Giardia* has been shown to be susceptible to killing by the mannose binding lectin arm of complement as well as antimicrobial molecules like nitric oxide and defensins (Evens-Osses et al., 2010; Eckmann et al., 1999; Aley et al., 1994). Elevations in *Giardia* targeting IgA and IgG have been observed in mouse and human infections (Velazquez et al., 2005 / Langford et al., 2002). The parasite targets for these antibodies include surface proteins, flagella, the adhesive disk, giardins and heat shock proteins (Einfeld et al., 1984; Crossley et al., 1985; Char et al., 1992; Farthing et al., 1986; Crossley et al., 1983). Furthermore, anti-IgM treated mice are unable to clear infection with *G. muris* (Snider and Underdown, 1985). However, our lab has shown that mice lacking B cells do clear *G. duodenalis* infection, suggesting that there are different immune responses between mouse strains against different species of *Giardia* (Singer et al., 2000). Additionally, *Giardia* evades antibody responses by switching the expression of variant-specific surface proteins (VSPs) in a process called antigenic variation (Singer et al., 2001). Thus, antibody-independent mechanisms also likely play important roles in parasite clearance.
CD4+ T cells are essential for clearing *Giardia* infection. CD4+/- mice infected with *G. duodenalis* and anti-CD4 treated mice infected with *G. muris* fail to clear infection (Solaymani-Mohammadi et al., 2011; Heyworth et al., 1987). Infections in γδ TCR-/- and β2m-/- knockout mice are resolved, suggesting that αβ TCR CD4+ T cells are protective (Singer and Nash, 2000; Solaymani-Mohammadi et al., 2011). Furthermore, the depletion of CD4+ T cells in B cell-deficient mice infected with *G. duodenalis* leads to chronic infection suggesting that CD4+ T cells have a protective role apart from stimulating antibody production (Singer and Nash, 2000). Interestingly, mice lacking IL-4, IFN-γ, or STAT-6 manage to clear infection with *G. duodenalis*, although with varying kinetics. This suggests that either a T\(_{H1}\) or a T\(_{H2}\) response alone is sufficient to clear infection and that one response may compensate for the absence of the other. While it is clear that certain lymphocyte and antibody responses are involved in clearing infection, the mechanistic details remain poorly defined.

**Pathogenic immunity in giardiasis**

Infections in knockout mice suggest that CD4+ and CD8+ T cells play different roles in giardiasis. While CD4+ T cells function in controlling the infection, CD8+ T cells seem to participate in pathogenesis. Adoptive transfer of CD8+ T cells, but not CD4+ T cells, from *G. muris*-infected mice leads to reduced sucrase activity and microvilli shortening in athymic nude recipients (Scott et al., 2004). Reports from several labs support that *Giardia*-activated CD8+ T cells likely cause reductions in microvilli length, IAP and disaccharidase activity, thereby impairing intestinal absorptive function (Scott et al., 2004; Solaymani-Mohammadi and Singer 2010; Mahmood et al., 2005). Importantly, there is little histological evidence in support of CD8+ T cell cytotoxic effector function in the infected intestine. While certain *Giardia* strains have been shown to directly induce apoptosis *in vitro*, there has yet to be a report of immune mediated intestinal apoptosis following infection (Chin et al., 2000; Yu et al., 2008). Thus, CD8+ T cells may regulate intestinal brush border function through non-cytotoxic mechanisms during giardiasis.
The canonical CD8$^+$ T cell effector function does not seem to be suitable for controlling *Giardia* infection. Classically, CD8$^+$ T cells function, along with natural killer (NK) cells, in eliminating virus-infected and transformed cells by antigen receptor targeted cytotoxicity. *Giardia* is an extracellular parasite and CD8$^+$ T cell priming would likely rely on cross-presentation of exogenous antigens on Major Histocompatibility Complex class I (MHC-I). Since *Giardia* does not replicate in the cytosol of infected enterocytes, CD8$^+$ T cell cytotoxic effector function against *Giardia* antigen would likely be unproductive and could lead to unnecessary intestinal injury. CD8$^+$ T cell cytotoxicity is mediated by the release of intracellular granules containing lytic granzymes and perforins as well as apoptotic signaling to infected target cells via membrane bound FasL (Shanker et al., 2009; Migueles et al., 2008; Podack et al., 1984). FasL signaling from activated CD8$^+$ T cells can, however, occur in the absence of cytotoxic granule release.

**Apoptotic Signaling through Fas/FasL Interactions**

Fas (CD95/APO-1) belongs to the TNF family of apoptotic type I transmembrane proteins referred to as death receptors. An important functional feature of all death receptors is the presence of a conserved C-terminal ~80 amino acid long death domain (DD) within the cytoplasmic tail. The DD functions as an interface between Fas and the Fas associated death domain (FADD), which is an adaptor protein that recruits and assembles a caspase-rich complex of proteins referred to as the death-inducing signaling complex (DISC) (Walczak and Krammer, 2000). Upon ligation by FasL, the DISC is assembled at the cytoplasmic tail of Fas via the DD and FADD resulting in a proteolytic cascade that ultimately invokes the caspase-3-dependent execution pathway of apoptosis. The induction of apoptosis through Fas is essential for the homeostatic maintenance of cell populations within all tissue as well as the removal of infected or transformed cells by FasL expressing lymphocytes.

The canonical death cascade that follows Fas engagement can be avoided by the differential assembly of downstream adaptor proteins. The stoichiometry of the DISC is an important determinant
in the outcome of Fas signaling (Scaffidi et al., 2000; Bäumler et al., 2003). The caspase-8 content of the DISC is regulated by the cellular FADD-like IL-1β-converting enzyme inhibitory protein (cFLIP_L). There is considerable homology between cFLIP_L and caspases with the exception that cFLIP_L lacks proteolytic activity. cFLIP_L knockdown by RNAi was shown to sensitize colonic epithelial HT-29 cells to Fas triggered apoptosis (Zang et al., 2007). In the absence of cFLIP_L knockdown, HT-29 cells are resistant to Fas-triggered apoptosis and instead produce cytokines like IL-8 (Abreu-Martin et al., 1995; Zang et al., 2007). Thus, cFLIP_L competes with caspase-8 for FADD binding within the DISC and raises the threshold for committing to apoptosis. RNAi knockdown and co-immunoprecipitation experiments in HT-29 (Colonic epithelial), Jurkat (T cell) and CEM (T cell) cells have demonstrated that ezrin bridges the gap between Fas and the actin cytoskeleton, facilitating the propagation of signals by relocalizing ligated Fas to lipid rafts (Rebillard et al., 2010; Hébert et al., 2008; Parlato et al., 2000). It is likely that Fas affects the intestinal brush border through non-apoptotic signals propagated by ezrin in the Giardia-infected host.

**T-Cell produced cytokines**

Throughout the course of infection, CD8^+ effector T cells produce large quantities of cytokines that have pleiotropic effects on neighboring cells. Not only do these cytokines promote the survival of nearby lymphocytes but they also activate the vascular endothelium as well as the intestinal epithelium. Activation of endothelial and epithelial cells by T cell cytokines enhances antigen presentation as well as the expression of adhesion molecules, facilitating T cell attachment (Osborn et al., 1989; Graber et al., 1990). The classical CD8^+ T cell cytokines are IFN-γ and IL-12, which are also produced by the T_{H1} subset of activated CD4^+ T cells. Interestingly, much of the CD8^+ T cell differentiation program is driven by the transcription factor T-bet, which is also responsible for the differentiation of T_{H1} CD4^+ T cells (Shnyreva et al., 2004). This redundancy supports CD8^+ T cell responses with CD4^+ T cell help. Thus, we expect that a T_{H1} CD4^+ T cell response is dominant in our model as it would foster the
activation of CD8\(^+\) T cells following infection with *Giardia*.

Hypothesis: Fas signaling induces intestinal injury following infection with *G. duodenalis*.

1. FasL/CD8\(^+\) T cell responses are initiated following infection with *G. duodenalis*.
   - Infections in C57BL/6 mice lead to an influx of FasL\(^+\) cells in the small intestine.
   - CD8\(^+\) T cell responses are supported by T\(_H1\) polarized CD4\(^+\) T cell help.
   - Following infection, CD8\(^+\) T cells become activated and express markers like FasL.

2. Infection with *G. duodenalis* leads to reduced intestinal sucrase and lactase activity in a Fas dependent manner.
   - Infections in C57BL/6 mice result in reduced sucrase and lactase activity.
   - Infected Fas\(^{lpr}\) mice exhibit relatively higher sucrase and lactase activity than infected C57BL/6 mice.

3. *G. duodenalis*-triggered disaccharidase reduction is downstream of post-translational modifications to ezrin.
   - Infections in C57BL/6 mice lead to altered intestinal ezrin phosphorylation.
   - Infected Fas\(^{lpr}\) mice do not exhibit altered ezrin phosphorylation to the same extent as C57BL/6 mice.
MATERIALS AND METHODS

Parasites

*G. duodenalis* strain GS/M-83-H7 was obtained from the ATCC (Manassas, VA). Trophozoites were cultured in standard TYI-S-33 media supplemented with bovine bile, L-cysteine, ascorbic acid, and antibiotic/antimycotic. Two days before infection, mice were given drinking water containing neomycin oral solution (1.4 mg/ml; Durvet, Blue Spring, MO), ampicillin (1 mg/ml; Sigma-Aldrich), and vancomycin (1 mg/ml; Hospira, Lake Forest, IL). Prior to infection, the parasites were detached from culture flasks by icing in PBS for ~15 minutes. The parasites were washed three times in ice-cold PBS and fed to mice at 1 x 10^6 parasites in 0.1 ml PBS / mouse by oral gavage.

Mice

C57BL/6 and Fas<sup>lpr</sup> (B6.MRL-Fas<sup>lpr</sup>/J) mice, between 6 and 8 weeks of age, were obtained from the Jackson laboratory (Bar Harbor, ME). The mice were housed and cared for in adherence to animal protocols approved by the Animal Care and Use Committees of Georgetown University.

Cell culture

HT-29 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 5% antibiotic/antimycotic obtained from Invitrogen (Carlsbad, CA). Trypsinized cells were seeded in 24 well plates and treated with either media or anti-Fas IgM (200 ng/ml, clone CH-11) obtained from MBL international (Woburn, MA). For immunofluorescent microscopy experiments, the cells were grown on 6 well chamber slides obtained from Invitrogen (Carlsbad, CA).

Immunocytochemistry

Following the Fas-ligation time course, the cells were washed in PBS and fixed in 4% paraformaldehyde for 30 min. Following fixation, the cells were permeabilized in 0.5% Triton-X for 30 min. The cells were then washed in 0.1% Triton-X and blocked with 5% goat serum and 1% BSA.
The cells were stained using an anti-phospho-ezrin antibody obtained from Cell Signaling Technologies (Danvers, MA). Fluorescent-microscopy was used to detect stained cells via an anti-rabbit-FITC secondary antibody obtained from SouthernBiotech (Birmingham, AL).

**Sucrase and lactase activity assay**

Sucrase and lactase activity was measured according to a previously described protocol (Dahlqvist et al., 1968). Briefly, 10 cm jejunal sections were extracted upon euthanasia and the mucosa were collected by scraping. Purified mucosa was homogenized in 1 ml MilliQ water supplemented with protease inhibitor cocktail III obtained from Calbiochem (La Jolla, CA). Sucrose or lactose (56 mM) was co-incubated with homogenates in maleic buffer (0.1 M, pH 6.0) for 1 hr at 37°C. Chromogenic buffer containing Tris, glucose oxidase, and peroxidase was used to detect the production of glucose following the co-incubation of substrate and enzyme. Samples were scanned by a BioTek Instruments microplate reader (Winooski, VT) at 450 nm. Glucose production was normalized to the protein content of each tested sample. Protein content of each sample was quantified by Bio-Rad Bradford assay (Hercules, CA).

**Immunohistochemistry**

Antibodies against FasL and cleaved caspase-3 were obtained from Santa Cruz Biotech (Santa Cruz, CA) and Cell Signaling Technology (Danvers, MA), respectively. Following euthanasia, a 3 cm duodenal section was removed from each mouse and fixed in 10% formalin for 24 hours. The tissue as then placed in 70% ethanol for another 24 hours and embedded in paraffin and sectioned. The 5 μm thick tissue sections were deparaffinized in xylene and rehydrated by passing through decreasing concentrations of ethanol followed by water. Antigen retrieval was carried out by microwaving the sections in citrate buffer obtained from Vector laboratories (Burlingame, CA) for 20 minutes. The samples were then blocked in PBS containing 5% goat serum and 1% BSA and stained with antibodies. Fluorescent microscopy was used to detect stained cells via goat anti-rabbit-FITC and rabbit anti-
mouse-TRITC conjugated secondary antibodies obtained from SouthernBiotech (Birmingham, AL).

**Western Blot**

Antibodies against ezrin, villin β-tubulin and β-actin were obtained from Santa Cruz Biotech (Santa Cruz, CA). An anti-phospho-ezrin antibody was obtained from Cell Signaling Technologies (Danvers, MA). Following euthanasia, 10 cm jejunal/ileal segments were lysed and homogenized in 1 ml NP40 lysis buffer (250 mM NaCl, 5 mM HEPES, 2 mM EDTA, 10% glycerol, 0.5 % NP40). Protein concentration was determined using the Bradford reagent obtained from Bio-Rad (Hercules, CA). A total of 100 µg of protein from each mouse was separated by SDS-PAGE and transferred on to a PVDF membrane. The membrane was then blotted using the above mentioned antibodies and detected using anti-mouse and anti-rabbit HRP conjugated secondary antibodies obtained from Sigma-Aldrich (St. Louis, MO).

**Flow Cytometry**

Fluorophore conjugated antibodies against CD3, CD4, CD8, CD69, IL-2Rα, FasL, and CCR9 were obtained from BioLegend (San Diego, CA). LIVE/DEAD Fixable Yellow Stain was obtained from Invitrogen (Carlsbad, CA). The spleens and MLNs were collected in Hank’s buffered saline solution (HBSS) supplemented with 5% FBS and 25 mM HEPES and passed through a 70 µm strainer. Red blood cells were eliminated using an NH₄Cl based buffer. Lymphocytes (1 x 10⁶) were washed in PBS and stained with LIVE/DEAD stain for 45 minutes at 4°C in the dark. The cells were then washed in PBS and stained with the appropriate antibodies for 1 hour at 4 °C in the dark. Antibody labeled cells were then washed in PBS and fixed in 1% paraformaldehyde overnight at 4 °C. The stained cells were then analyzed using a Becton Dickinson FACStar Plus dual laser system (Franklin Lakes, NJ) and FCS express version 4.0 software from DeNovo Software (Los Angeles, CA).

**Ex vivo restimulation of lymphocytes**

Spleen and MLN lymphocytes were collected in HBSS supplemented with 5% fetal bovine
serum and 25 mM HEPES and strained through a 70 μm strainer. Lymphocytes were cultured in RPMI 1640 supplemented with FBS, antibiotic, L-glutamine, and 2-mercaptoethanol. Red blood cells were eliminated using an NH₄Cl based buffer. Lymphocytes (5 x 10⁶) were cultured with or without 100 μg/ml Giardia extract to for 48 hours at 37°C and 5% CO₂. After 48 hours, the supernatants were collected and analyzed by ELISA in adherence to the protocols provided by the manufacturer. ELISA kits for IFN-γ, IL-4, IL-10, IL-17, and TNF-α were obtained from ebioscience (San Diego, CA).

RESULTS

**Infection with G. duodenalis leads to an influx of FasL⁺ cells into the small intestine.**

The immune effector mechanisms that facilitate intestinal injury associated with giardiasis are poorly understood. We suspected that Fas signaling in the intestinal epithelium may contribute to the pathophysiology of Giardia-induced malnutrition. To test this, we infected C57BL/6 mice with G. duodenalis strain GS for 7 and 14 days. After infection, we monitored the presence of FasL⁺ cells within the lamina propria of the small intestine by immunohistochemistry. Uninfected, saline receiving, mice were analyzed in parallel. As illustrated in figure 1, a sharp increase (~3 fold) of FasL⁺ cells was observed in the lamina propria of infected mice. These cells were consistently found to infiltrate the luminal regions of villi in infected mice, whereas uninfected mice exhibited modest staining primarily near the crypts. Thus, Giardia infection recruits FasL⁺ cells to the lamina propria that infiltrate the villi. These data support the hypothesis that Giardia infection leads to increased Fas signaling within the gut.

**Infection with G. duodenalis leads to an IFN-γ dominant cytokine environment within secondary lymphoid tissue.**

FasL, as an effector molecule, is typically associated with T₉₁ CD4⁺ and CD8⁺ T cell responses. We wanted to characterize the cytokine environment within secondary lymphoid tissue
(spleen and MLN) following infection in order to determine if FasL responses would be supported. We chose a panel of cytokines that are exclusively distinct to T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, and T\textsubscript{REG} CD4\textsuperscript{+} T cell responses. C57BL/6 mice were infected with \textit{G. duodenalis} strain GS for 7 and 14 days along with uninfected mice receiving saline rather than parasites. After euthanasia, spleen and MLN lymphocytes were collected and cultured with or without \textit{Giardia}-extract for 48 hrs. \textit{Ex vivo} restimulation of spleen and MLN lymphocytes revealed a sharp increase of IFN-\textgamma, a T\textsubscript{H}1 cytokine following infection in both immune compartments (Figure 2 B and Figure 3). To a lesser extent, splenic and MLN T\textsubscript{H}17 (IL-17) and T\textsubscript{H}2 (IL-4, IL-13) cytokines were also produced following infection and \textit{ex vivo} restimulation (Figure 2 and 3). The T\textsubscript{REG} / T\textsubscript{H}2 cytokine IL-10 was also produced following infection but very modestly. This cytokine profile is consistent with a polarization toward a T\textsubscript{H}1 cell response as evident by the dominant production of IFN-\textgamma. The cytokines that initiate and maintain T\textsubscript{H}1 responses are known to be necessary for CD8\textsuperscript{+} T cell activation as these T cell responses occur in parallel. Thus, the cytokine environment within the secondary lymphoid tissue of infected mice is conducive to T cell responses that utilize Fas signaling as an effector mechanism.

**Fas deficiency rescues intestinal disaccharidase reduction caused by \textit{G. duodenalis}.**

Our lab and others have reported a reduction in intestinal digestive enzymes like the disaccharidases sucrase and lactase following infection with \textit{G. duodenalis}. We aimed to determine whether abrogated Fas signaling within the infected small intestine would alleviate these enzyme deficiencies. Fas\textsuperscript{lpv} mice, harboring a loss-of-function Fas mutation, were infected with \textit{G. duodenalis} in parallel with Fas sufficient C57BL/6 mice. After 7 and 14 days of infection, the mice were euthanized and intestinal disaccharidase activity was measured. Fas\textsuperscript{lpv} mice were unable to reduce parasite numbers during a two week infection course (Figure 4). Despite having an increased parasite burden, compared to C57BL/6 mice, Fas\textsuperscript{lpv} mice did not exhibit reductions in sucrase nor lactase activity (Figure 5). The C57BL/6 mice did however exhibit a significant reduction of sucrase (p =
0.0286) and lactase ($p = 0.0027$) activity on day 7 of infection with *G. duodenalis*. This reduction persisted throughout the 14 day infection course in C57BL/6 mice.

**Ezrin is phosphorylated and proteolysed following infection with *G. duodenalis* in C57BL/6 mice.**

We suspected that post-translational regulation of ezrin may play an important role in facilitating the dismantling of microvilli structures, thereby reducing sucrase and lactase activity. To test this, we infected C57BL/6 mice with *G. duodenalis* strain GS for 7 and 14 days. Infected and uninfected mice were euthanized and a segment of ileum/jejunum was analyzed by western blot. As illustrated in figure 6, ezrin is phosphorylated on day 7 p.i. in C57BL/6 mice. This correlates with the emergence of sucrase and lactase deficiency. This phosphorylation event is concurrent with the proteolytic processing of ezrin, which peaks at day 14. Interestingly, full-length ezrin was not detectable in Fas$^{lpr}$ mice while the proteolysed products were abundant (Figure 6). Consistently, there was no phosphorylated ezrin detected in the Fas$^{lpr}$ mice throughout the course of infection.

It was tempting to speculate that Fas ligation may directly promote ezrin phosphorylation during *Giardia* infection. A number of studies have shown that ezrin is required for Fas mediated apoptosis and that Fas engagement alters ezrin localization (Rebillard et al., 2010; Hébert et al., 2008; Parlato et al., 2000). However, apoptosis is not a hallmark of giardiasis. HT-29 colonic epithelial cells are resistant to Fas-induced apoptosis but undergo alternative responses, such as cytokine production (Abreu-Martin et al., 1995). This provided an appropriate *in vitro* model to test the effects of Fas ligation on ezrin phosphorylation in the absence of apoptosis. When HT-29 cells were acutely exposed to an anti-Fas IgM, ezrin exhibited a rapid phosphorylation event (Figure 7). This phenomenon was transient, peaking after 30 minutes.

Along with ezrin, villin may also play an important role in microvilli shortening following infection with *G. duodenalis*. Villin expression was stable during the course of infection in C57BL/6 mice (Figure 8). A mild increase in a proteolytic product was observed, although the full-length protein
remained unaltered. Interestingly, Fas\textsuperscript{lpr} mice exhibited decreased villin expression compared to the C57BL/6 mice, in the absence of infection. Villin expression increased substantially following infection in Fas\textsuperscript{lpr} mice. This phenomenon does not correlate with enzyme deficiencies and likely does not impact pathology.

**T-cell activation following *Giardia* infection**

We monitored total splenocytes as well as CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell numbers throughout the course of infection. C57BL/6 and Fas\textsuperscript{lpr} mice were infected with *G. duodenalis* strain GS for 7 and 14 days. Upon euthanasia the spleens and MLN of the animals were collected. After red blood cell lysis, the remaining lymphocytes were counted. Interestingly, total splenocyte numbers in C57BL/6 mice remained constant throughout infection whereas a significant reduction ($p = 0.0155$) was detected in Fas\textsuperscript{lpr} mice after 7 days (Figure 9). Splenocyte numbers remained low throughout the two week infection period, correlating with the failure of Fas\textsuperscript{lpr} mice to control parasite load. Interestingly, although total splenocyte numbers decreased in infected Fas\textsuperscript{lpr} mice, the CD4\textsuperscript{+} T cell composition within the spleens of these mice increased following infection ($p = 0.0284$) (Figure 10 B). Relative increases in CD4\textsuperscript{+} T cells were also observed in the MLN of day 7 infected Fas\textsuperscript{lpr} mice ($p = 0.0085$) (Figure 10 D). The CD8\textsuperscript{+} T cell populations remained unaltered in Fas\textsuperscript{lpr} mice in both the spleen and MLN following infection (Figure 10 B and D). C57BL/6 CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell numbers were relatively stable throughout the course of infection in both the spleen and MLN (Figure 10 A and C).

In order to characterize the activated phenotype of splenic CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells following infection with *G. duodenalis*, we monitored the expression of several activation markers on splenocytes. Strain GS-infected C57BL/6 and Fas\textsuperscript{lpr} mice were euthanized after 7 days, along with uninfected mice. The spleens of these mice were collected and stained by flow cytometry. We expected to find differences in the activated phenotypes of CD4\textsuperscript{+} T cells between C57BL/6 and Fas\textsuperscript{lpr} mice following infection as only the C57BL/6 mice were able to establish protective immunity.
Furthermore, we hoped to detect increased numbers of CD8$^+$ T cells expressing FasL and other activation markers in both mice. We did not detect significant increases in CD4$^+$ T cells expressing CD69 nor IL-2R$\alpha$ following infection in either the C57BL/6 nor Fas$^{lpr}$ mice (Figure 11). Increased CCR9$^+$CD4$^+$ T cells were, however, detected following infection in both mice (Figure 11 B, $p = 0.0372$ [C57BL/6] and $p = 0.0345$ [Fas$^{lpr}$]). These CCR9 expressing T cells were likely homing to the small intestine where CCL25, the ligand for CCR9, is constitutively produced by epithelial cells. CCR9$^+$ CD8$^+$ T cells, however, did not increase following infection (Figure 12 C). FasL and CD69 expressing CD8$^+$ T cells populations also remained constant in C57BL/6 and Fas$^{lpr}$ spleens throughout infection (Figure 12 A and D). Interestingly, we did observe an increase in IL-2R$\alpha$ expressing CD8$^+$ T cells in the Fas$^{lpr}$ mice following infection ($p = 0.0286$) (Figure 12 B). This response was not detected in subsequent infections, however.

Because the spleen is distal to the small intestine, we monitored T cell activation within the MLNs as they are the direct draining sites for intestinal antigens and may be more involved in initiating immune responses against *Giardia*. We did not detect shifts in CD4$^+$ T cell populations expressing activation markers such as IL-2R$\alpha$, CCR9, and CD69 following infection within the MLN (Figure 13). Infected Fas$^{lpr}$ mice did exhibit reduced IL-2R$\alpha$ expressing CD8$^+$ T cells, however, within the MLN (Figure 14 B). We were unable to detect shifts in CD8$^+$ T cells expressing other activation markers (Figure 14).

**Infection with *G. duodenalis* does not induce enterocyte apoptosis.**

The potential for *Giardia* to kill intestinal epithelial cells has widely been explored, resulting in an abundance of reports demonstrating the direct killing capacity of *Giardia* trophozoites and their secreted products (Chin et al., 2002; Panaro et al., 2007, Yu et al., 2008). These reports are predominantly *in vitro* studies using monolayers of transformed colonic cell-lines that are exposed to high parasite numbers, ultimately resulting in caspase-3 mediated cell death. Importantly, *in vitro*
systems do not accommodate an immune presence and thus these reports strictly reflect the ability of the parasite to directly induce cell death. Reports of increased apoptosis associated with infection, *in vivo*, are largely lacking. We assessed the potential for increased enterocyte apoptosis following infection as this may be a consequence of activated CD8$^+$ T cells as well as parasite factors. C57BL/6 and Fas$^{lpr}$ mice were infected with *G. duodenalis* strain GS for 7 and 14 days. Upon euthanasia, a 3 cm section of the duodenum of infected and uninfected mice was extracted, fixed and paraffin embedded. Immunofluorescent staining for cleaved caspase-3 did not reveal increased apoptosis following infection in neither C57BL/6 nor Fas$^{lpr}$ mice (Figure 15). In contrast, an apparent reduction in apoptosis was observed following infection, although this phenomenon was not statistically significant.

DISCUSSION

There is currently very little known about the nature of host-parasite interactions within the small intestine during giardiasis. It is clear that host immunity is effective at controlling infection as giardiasis is largely self-limiting in immune-competent humans and mice. Although host immunity provides protection, it also bears responsibility for injury sustained to the gut during infection. This pathological aspect of host immunity accounts for the sometimes debilitating symptomatic disease that presents in the clinical setting and does not seem to be protective at all. Our lab has shown that β2m$^{-/-}$ mice clear infection with similar kinetics to wild-type mice but without sustaining intestinal injury as indicated by enzyme deficiency. Thus, protective immunity seems to be carried out independently of pathogenic immunity, which is largely CD8$^+$ T cell dependent. Understanding the mechanisms facilitating enteropathy and identifying the cell types involved is important progress in the field. Here, we report the involvement of Fas signaling, potentially by activated CD8$^+$ T cells, in pathogenesis following *Giardia* infection.
The cytokine environment within the spleen and MLNs of *Giardia*-infected mice is conducive for CD8⁺ T cell activation. The *ex vivo* restimulation of splenic and MLN lymphocytes with *Giardia* antigen revealed a diverse array of cytokines representing all of the T₄ responses. This was largely expected as a typical immune response evokes all of these cell types but ultimately leads to a polarization event in which one helper subset is preferentially dominant. Large amounts of IFN-γ were detected in both the spleens and MLN of infected mice. This cytokine is representative of both T₄₁ CD4⁺ T cell polarization and CD8⁺ T cell activation. Flow cytometry analysis revealed increased CCR9⁺ CD4⁺ T cells within the spleens of infected mice, suggesting increased migration of splenic CD4⁺ T cells to the infected small intestine. This gut-homing event was, however, not exhibited by splenic CD8⁺ T cells. CD8⁺ T cell activation may follow different kinetics than CD4⁺ T cells or may occur at a site other than the spleen.

The current paradigm in the field is that CD8⁺ T cells become activated following infection and target the intestinal epithelium to reduce microvilli length and disaccharidase activity. The mechanisms that facilitate these pathological alterations within the small intestine remain largely unknown. We hypothesized that Fas signaling within the infected small intestine may trigger cytoskeletal rearranging events that shorten microvilli and thereby down-regulate disaccharidases within the brush border. Our hypothesis was supported by two initial findings. First, immuno-fluorescent staining revealed a large influx of FasL⁺ cells to the small intestine of infected C57BL/6 mice. Second, we did not detect increased enterocyte apoptosis following infection. This is important as it suggests that activated CD8⁺ T cells do not target *Giardia*-infected enterocytes for killing via the conventional perforin/granzyme pathway. This is consistent with the finding that *G. duodenalis* infection in humans leads to increased CD8⁺ IELs but that these infiltrating lymphocytes do not express granzyme (Oberhuber et al., 1996). Furthermore, studies in human colonic epithelial cells suggest that these cells are resistant to Fas triggered apoptosis. Thus, we felt justified in exploring the role of Fas in
pathological giardiasis.

The pathological reduction of intestinal disaccharidase enzymes requires cytoskeletal rearrangement that is initiated by Fas signaling. Fas signaling has widely been shown to alter cytoskeletal dynamics in a variety of cell types. Furthermore, the actin-binding protein ezrin has been shown to be indispensable for propagating Fas signals by facilitating the localization of Fas to lipid rafts upon ligation (Rebillard et al., 2010; Hébert et al., 2008; Parlato et al., 2000). The intestinal brush border membrane is largely composed of lipid rafts. Thus, we expected that Fas signaling within the infected small intestine, while not being apoptotic, would lead to altered ezrin dynamics. Indeed, we found that on day 7 p.i., ezrin was phosphorylated at residue Th567 in C57BL/6 mice. We also found this residue to be phosphorylated upon ligation of Fas on colonic epithelial HT-29 cells. This phosphorylation event in, C57BL/6 mice, coincided with the influx of FasL$^+$ cells and reduced sucrase and lactase activity on day 7 p.i.. On day 14 p.i., sucrase and lactase activity remained low and FasL$^+$ cells were still present in the gut but phosphorylated ezrin was no longer detectable. This was due to the fact that almost all the full-length ezrin was proteolytically cleaved at day 14. The biological relevance of ezrin cleavage is unclear but it has been repeatedly observed in our lab and seems to be immune-mediated as SCID mice do not exhibit this phenomenon (data not published).

To further assess the role of Fas signaling in eliciting enteropathy associated with giardiasis, we conducted infections in Fas$^{lpr}$ mice. These mice harbor a spontaneous mutation that renders Fas unresponsive to ligation. Infections with *G. duodenalis* did not reduce sucrase nor lactase activity below the uninfected baseline. Interestingly, an increase in sucrase and lactase activity was observed in infected Fas$^{lpr}$ mice on day 14. This may suggest that a restitution event that restores proper digestive function following parasite clearance is initiated independently of enzyme reductions. No phosphorylated ezrin was detected in these mice at any point during the course of infection, supporting the role of ezrin in pathological enzyme reductions. Interestingly, Fas$^{lpr}$ mice exhibited a progressive
induction of villin following infection. Yet, full-length ezrin was extremely scarce in these mice even in the absence of infection. This may account for the discrepancy in disaccharidase activity between uninfected C57BL/6 and Fas<sup>lpr</sup> mice. The lack of full-length ezrin in Fas<sup>lpr</sup> mice is likely a consequence of abrogated Fas signaling. Likewise, Fas<sup>lpr</sup> mice exhibited an immune defect as they failed to reduce parasite numbers following infection with <i>G. duodenalis</i>. The failure to control infection correlated with reduced splenocytes in the infected Fas<sup>lpr</sup> mice. Despite having reduced splenocytes, the Fas<sup>lpr</sup> mice exhibited an increased proportion of CD4<sup>+</sup> T cells within the spleen and MLN following infection. This is not surprising as Fas is required for regulating expanding T cells in a process termed activation induced cell death.

We did not detect overt CD4<sup>+</sup> nor CD8<sup>+</sup> T cell activation following infection in either C57BL/6 or Fas<sup>lpr</sup> mice. This was likely due to a large pool of T cells that are not antigen specific and remain resting during infection. This limits our ability to isolate a small antigen specific population of T cells by flow cytometry. We were able to detect slight increases in IL-2Rα<sup>+</sup> CD8<sup>+</sup> T cells within the spleen of infected Fas<sup>lpr</sup> mice. The expansion of IL-2Rα<sup>+</sup> CD8<sup>+</sup> T cells was, however, difficult to reproduce in a statistically significant manner in subsequent infections. This highlights the necessity for a more sensitive approach, such as an ovalbumin model utilizing transgenic T cells expressing TCRs specific for the OVA peptide, which is in turn expressed by transgenic <i>Giardia</i>. However, if this IL-2Rα response is real, it is likely that <i>Giardia</i> antigens are cross-presented on MHC-I by antigen presenting cells. This is the first example of a protozoan pathogen that is entirely extracellular throughout its life cycle triggering CD8<sup>+</sup> T cell responses through cross-presentation. IL-2Rα is induced by costimulatory signaling through CD28, which follows antigen specific T cell activation (Ford et al., 2011). In fact, bystander activated CD8<sup>+</sup> T cells to not exhibit increased IL-2Rα expression (Tough et al., 1996). Thus this is likely not the result of bystander activation whereby CD8<sup>+</sup> T cells are activated by T<sub>H</sub>1 CD4<sup>+</sup> T cell. Further work is required to determine the validity of this result.
FUTURE DIRECTIONS: PROPOSAL

Having demonstrated that Fas signaling in the infected small intestine contributes to the enteropathy associated with giardiasis, we will focus our efforts on the defining role of CD8$^+$ T cells in delivering these pathogenic signals. Our lab has previously demonstrated that intestinal enzymes such as sucrase and lactase exhibit reduced activity following infection with *G. duodenalis* in an immune-dependent manner. Furthermore, these deficiencies are absent in infected β2m$^{-/-}$ mice (Solaymani-Mohammadi and Singer, 2011). However, β2m is a common chain component of MHC-I that is shared among classical and non-classical complexes (Treiner et al., 2003). As a result, these mice have defective NK cell responses that may also contribute to enzyme deficiency (Hoglund et al., 1991). Therefore, it is imperative to directly target CD8$^+$ T cells in our model while avoiding other CD8 expressing cells like NK cells and dendritic cells. This is especially important since increases in both CD8$^+$ T cells and Leu-7$^+$ NK cells, within the intestinal epithelium, have been reported in histological biopsies of giardiasis patients (Oberhuber et al., 1996).

The potential for CD8$^+$ T cells involvement in pathogenesis following *Giardia* infection has been suggested by data emerging from our lab and other labs. The adoptive transfer of CD8$^+$ T cells from *G. muris*-infected mice into athymic naïve recipients is sufficient for reducing sucrase activity (Scott et al., 2004). Our lab found that neither CD4$^{-/-}$ nor β2m$^{-/-}$ mice exhibit reduced sucrase activity following infection with *G. duodenalis* (Solaymani-Mohammadi and Singer, 2011). This suggests that CD4$^+$ T cells help support the activation of CD8$^+$ T cells in *G. duodenalis* infection. In the proposed study, we wish to delineate the role of CD8$^+$ T cells in the development of intestinal pathology following infection with *G. duodenalis*. We propose to use an *in vivo* model that allows us to selectively deplete CD8$^+$ T cells prior to infection, thus avoiding the disruption of other CD8 expressing cells. Furthermore, we propose to explore the antigen specific nature of CD8$^+$ T cell
responses against *G. duodenalis* in an *in vivo* model using transgenic T cells that recognize an antigenic protein ectopically expressed in *Giardia*.

**Ovalbumin-specific T cell adoptive transfer system**

In our previous attempts, we were unable to detect the activation of T cells by flow cytometry following infection with *G. duodenalis*. We expected that a very small population of antigen specific CD8$^+$ T cells would be activated following infection. Therefore, the proportionality of activated antigen specific CD8$^+$ T cells to resting cells would be small and perhaps outside the range of detection by flow cytometry. The same was true for CD4$^+$ T cells making it difficult to characterize the activated phenotype of T cells following infection. However, there is no doubt that CD4$^+$ T cells are activated following infection as they are necessary for parasite clearance (Singer and Nash, 2000). To resolve this issue, we plan to employ a model of infection in which non-antigen specific T cells will be removed and replaced with *Giardia*-specific T cells.

In order to increase the relative abundance of antigen specific T cells in our infection model, we will use *G. duodenalis* expressing ovalbumin (OVA) to infect mice containing OVA-specific T cells. Ovalbumin is the major component of egg white. Commercially available OVA-specific, class I-restricted TCR transgenic (OT-1) mice expressing a V$\alpha$2/V$\beta$5T cell receptor that recognizes a peptide derivative of OVA (SIINFEKL) in the context of MHC-I (haplotype H-2K$^b$) provide a useful tool for studying antigen specific responses *in vivo*. This transgenic model has been instrumental in expanding our understanding of T cell development in the thymus and their functions in the periphery. Furthermore, this system has been useful in studies focusing on the dynamics of TCR-MHC interactions. It has also permitted the characterization of antigen-specific T cells in a variety of infectious models, where the low abundance of endogenous antigen-specific T cells was once a limitation.
Hypothesis: Infection with *Giardia* leads to antigen-specific CD8⁺ T cell activation and CD8⁺ T cell mediated intestinal pathology.

1. CD8⁺ T cells are activated and cause intestinal enzyme deficiencies and brush border breakdown following infection with *G. duodenalis*.
   - Infection with *G. duodenalis* does not elicit disaccharidase and IAP deficiencies nor does it increase brush border ezrin and villin phosphorylation in the absence of CD8⁺ T cells.
   - Infection with *G. duodenalis* activates CD8⁺ T cells in an antigen-specific manner.
   - CD8⁺ T cell activation following *Giardia* infection requires the presence of CD4⁺ T cells.

2. FasL expression by CD8⁺ T cells contributes to pathogenesis during giardiasis.
   - The adoptive transfer of FasL-deficient CD8⁺ T cells into TCRβ⁻ recipients prevents *G. duodenalis*-triggered disaccharidase and IAP deficiencies and increased brush border ezrin and villin phosphorylation.
   - Ligation of Fas leads to reduced intestinal enzymes and increases ezrin and villin phosphorylation in the brush border of Caco-2 cells.
   - Fas induced disaccharidase and IAP deficiency is at least partially mediated by ezrin and villin.

3. Infection with *G. duodenalis* leads to activation of intra-epithelial, lamina propria and Peyer’s patch CD8⁺ T cells.
   - CD8⁺ IELs express activation markers like FasL following infection with *G. duodenalis*. 
• Lamina propria CD8⁺ T cells express activation markers like FasL following infection with *G. duodenalis*.

• Peyer’s patch CD8⁺ T cells express activation markers like FasL following infection with *G. duodenalis*.

AIM 1: Infection with *Giardia* leads to antigen-specific CD8⁺ T cell activation and CD8⁺ T cell mediated intestinal pathololgy.

Sub-aim 1a: Determine whether CD8⁺ T cells are required for the pathological reduction of disaccharidases and IAP as well as increased ezrin and villin phosphorylation following infection with *G. duodenalis*.

To test the role of CD8⁺ T cells in giardiasis, we will compare the pathological outcome of infections in CD8⁺ T cell sufficient and deficient mice. We will compare *Giardia*-triggered pathogenesis in TCRβ⁻/⁻ mice having received either CD4⁺ T (Group 1) or CD8⁺ and CD4⁺ T (Group 2) cells by adoptive transfer, prior to infection. TCRβ⁻/⁻ mice receiving sham-saline transfers (Group 3) will be used as controls along with unaltered C57BL/6 mice (Group 4). These mice will be infected with *G. duodenalis* strain GS by oral gavage for 7 days. Intestinal IAP and disaccharidase enzymes (sucrase, maltase and lactase) will be measured along with brush border p-ezrin and p-villin at day 7 p.i., as well as in uninfected mice for each group. We will also measure enterocyte apoptosis following infection to rule out the potential for CD8⁺ T cell cytotoxicity in our model. We expect that TCRβ⁻/⁻ mice replenished only with CD4⁺ T cells (Group 1) will fail to develop the pathological markers described above. Group 2 and 4 mice should develop IAP and disaccharidase reductions as well as altered ezrin and villin dynamics. The potential finding that *Giardia*-triggered enteropathy is independent of both CD8⁺ and CD4⁺ T cells would contradict reports from several labs, including our own. Thus, we do not expect that Group 3 mice will exhibit reduced disaccharidase and IAP activity.
along with enhanced ezrin and villin phosphorylation. However, in the case that it does, we will infect these mice with *G. muris* as this species has also been demonstrated to elicit CD8$^+$ T cell dependent pathology following infection (Scott et al., 2004). This control experiment will be done to confirm that other reports are reproducible in our hands and that our model and assays are indeed reliable. As another alternative experiment, we will adoptively transfer activated CD8$^+$ T cells from infected mice into naïve TCR$\beta^{-/-}$ recipients and monitor enzymes and brush border proteins in order to determine if these T cells are armed with effector molecules and thus have the capacity to induce intestinal injury.

**Methods:** CD8$^+$ and CD4$^+$ T cells for adoptive transfers will be purified from the spleens of uninfected C57BL/6 by immune-column based mediated negative selection. TCR$\beta^{-/-}$ mice will receive injections in the orbital cavity containing either CD4$^+$ T cells (10$^7$/0.1 ml) alone or together with CD8$^+$ T cells (10$^7$/0.1 ml). PBS injections will be used to perform sham transfers. Five days after the adoptive transfer, the mice will be placed on antibiotics two days prior to infection with *G. duodenalis* trophozoites (10$^6$/0.1ml) or euthanized as uninfected controls. The antibiotic regimen will be kept throughout the course of infection. Antibiotic doses are described above.

At day 7 p.i., the mice will be euthanized along with uninfected controls. A 10 cm section of the jejunum will be collected to assay for sucrase, lactase and IAP activity. The section will be washed in PBS, cut longitudinally and the musoca will be stripped off of the underlying muscle tissue and collected. Another 10 cm section of ileum and jejunum will be collected for immunoprecipitation and western blot analysis to determine the phosphorylation state of ezrin and villin.

Intestinal disaccharidase activity will be assayed as previously described (Dahlqvist, 1968). Briefly, intestinal mucosal homogenates will be incubated with the relevant disaccharidase substrate (56 mM) in maleic buffer (0.1 M, pH 6.0) for 1 hr at 37 °C. Tris, glucose-oxidase and peroxidase containing buffer will be used to quantitate glucose production relative to a D-glucose standard.
Samples will be scanned on a BioTek Instruments microplate reader (Winooski, VT) at 450 nm and the disaccharidase activity will be normalized to total protein content as measured by the Bradford reagent obtained from Bio-Rad (Hercules, CA).

Alkaline phosphatase activity of intestinal mucosal homogenates will be measured using a kit obtained from Abcam (Cambridge, MA). This colorimetric kit uses \( p \)-nitrophenyl phosphate as the phosphatase substrate. After a brief incubation, the color change, indicating activity, is detected by a BioTek Instruments microplate reader (Winooski, VT) reader at 405 nm. Optical density (OD) values for each sample will then be fitted to a \( p \)-Nitrophenol standard curve. IAP activity will be normalized to total protein content as determined by the Bradford reagent obtained from Bio-Rad (Hercules, CA).

The phosphorylation of ezrin and villin will be monitored by western blot. Ileal/jejunal sections (10 cm) will be homogenized in NP40 buffer (250 mM NaCl, 5 mM HEPES, 2 mM EDTA, 10% glycerol, 0.5 % NP40) supplemented with a protease inhibitor cocktail III obtained from Calbiochem (La Jolla, CA). The tissue will be fractionated by centrifugation at 14000 rpm for 5 min, at 4°C. The protein content of each sample will be determined using the Bradford reagent obtained from Bio-Rad (Hercules, CA). Equal amounts of supernatant from each mouse will be loaded onto 4-12% SDS-PAGE gels obtained from Invitrogen (Carlsbud, CA). The protein will then be transferred onto a PVDF membrane obtained from Millipore (Bedford, MA). Immunoblotting will be carried out under standard protocols. Phosphorylated ezrin will be detected using a rabbit anti-phospho-ezrin primary antibody obtained from Cell Signaling Technologies (Danvers, MA) and a goat anti-rabbit-HRP secondary antibody obtained from Santa Cruz Biotech (Santa Cruz, CA). Immuno-precipitation of villin (500\( \mu \)g protein/IP) will be carried out in NP40 buffer using 2 \( \mu \)g mouse anti-villin antibody obtained from Santa Cruz Biotech (Santa Cruz, CA). Villin will be enriched using protein A/G coated agarose beads and several PBS washes. Western blot analysis will follow using a PY20 anti-phospho-tyrosine antibody from Santa Cruz Biotech (Santa Cruz, CA).
Fluorescent microscopy will be used to confirm western blot data. Following euthanasia, a 3 cm duodenal section will be removed from each mouse and fixed in 10% formalin for 24 hours. The sections will then be paraffin embedded and sectioned. The 5 μm thick tissue sections will then deparaffinized in xylene and rehydrated. Antigen retrieval will be carried out by microwaving the tissue sections in citrate buffer obtained from Vector laboratories (Burlingame, CA) for 20 minutes. The samples were then blocked in PBS containing 5% goat serum and 1% BSA and stained with a phopho-ezrin recognizing antibody and detected with a goat anti-rabbit-FITC conjugated secondary antibody obtained from SouthernBiotech (Birmingham, AL).

Enterocyte apoptosis will be measured by Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) as well as cleaved-caspase-3 staining and detected by fluorescent microscopy. Caspase-3 staining will be carried out using an antibody specific for cleaved-caspase-3 obtained from Cell Signaling Technologies (Danvers, MA). TUNEL staining will be used to detect nicked DNA by the addition of dUTPs by TdT. Modified nucleotides will be detected by fluorescent microscopy, as previously described, using either anti-dUTP antibodies or fluorophore labeled dUTP.

<table>
<thead>
<tr>
<th>Sub-aim 1a</th>
<th></th>
<th></th>
<th>Assays to be conducted on day 0 and day 7 (Expected outcomes in day 7 mice compared to day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td><strong>Recipients</strong></td>
<td><strong>Donors</strong></td>
<td><strong>Parasites</strong></td>
</tr>
<tr>
<td>1: (N = 8)</td>
<td>TCRβ⁻</td>
<td>CD4 (WT)</td>
<td>Normal</td>
</tr>
<tr>
<td>2: (N = 8)</td>
<td>TCRβ⁺⁻</td>
<td>CD4 (WT) +CD8 (WT)</td>
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<tr>
<td>3: (N = 8)</td>
<td>TCRβ⁻⁻</td>
<td>None</td>
<td>High</td>
</tr>
<tr>
<td>4: (N = 8)</td>
<td>B6-WT</td>
<td>None</td>
<td>Normal</td>
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Sub-aim 1b: Determine if CD8\(^+\) T cell responses after infection with *G. duodenalis* are antigen specific.

Since we have previously failed to detect CD8\(^+\) T cell activation in *G. duodenalis*-infected C57BL/6 mice, we will employ the adoptive transfer utilizing OT-1 CD8\(^+\) T cells that target *Giardia*-OVA. We will analyze and compare splenic and MLN CD8\(^+\) T cell activation in infected CD4\(^+\) T cell-replenished TCR\(\beta^{+/-}\) mice having received either C57BL/6 CD8\(^+\) T cells (Group 1) or OT-1 CD8\(^+\) T cells (Group 2) by adoptive transfer. T cell activation will be indicated by expression of markers like FasL, CD69, IL-2Rx, VLA-4, IL-7Rx and CCR9. As controls, we will conduct infections in TCR\(\beta^{+/-}\) mice receiving only CD4\(^+\) T cell transfers (Group 3) and unaltered C57BL/6 mice (Group 4). Uninfected mice will also be analyzed for each group to establish a baseline. Importantly, the infecting strain of *G. duodenalis* will be expressing OVA, which is recognized by OT-1 but not C57BL/6 CD8\(^+\) T cells. Thus, if *Giardia* antigen is recognized by CD8\(^+\) T cells *in vivo*, we expect that only OT-1 cells belonging to infected Group 2 mice will exhibit an activated phenotype. Consistently, we do not expect that *Giardia*-OVA infected Group 1 mice will exhibit CD8\(^+\) T cell activation compared to uninfected mice. If we find that OT-1 CD8\(^+\) T cells (Group 2) and C57BL/6 CD8\(^+\) T cells (Group 1) are activated to a similar extent in the lymphoid tissues of *Giardia*-OVA infected mice, then we will immunize these mice with OVA and look at T cell activation by flow cytometry to show that the transgenic model is not flawed. To confirm our results, we will compare infections in Group 2 mice using *Giardia*-OVA and OVA lacking *Giardia*. We expect that infections with *Giardia*-OVA will selectively activate OT-1 CD8\(^+\) T cells whereas OVA lacking *Giardia* will fail to do so.

**Methods:** For adoptive transfer experiments into TCR\(\beta^{+/-}\) recipient mice, splenic wild-type CD8\(^+\) T cells will be obtained from uninfected C57BL/6 and OT-1 CD8\(^+\) T cells will be obtained from OT-1 mice as described in sub-aim 1a. CD4\(^+\) T cells transfers from C57BL/6 mice into TCR\(\beta^{+/-}\) recipients
will also be conducted as described in sub-aim 1a. TCRβ−/− mice will receive either CD4+/CD8+ T cells or CD4+/OT-1 CD8+ T cells prior to infection with *G. duodenalis*-OVA. At day 7 p.i., the mice will be euthanized and the MLNs and spleens collected in HBSS supplemented with 5% FBS and 25 mM HEPES and strained through a 70 μm filter. Uninfected mice will be treated in parallel. After lysis of red blood cells in NH₄Cl buffer, lymphocyte suspensions will be counted and stained with LIVE/DEAD Fixable Yellow Stain to determine viability. Fluorophore-conjugated antibodies against CD3, CD4, and CD8 will be used to identify CD8+ T cells while FasL, IL-2Rα, IL-7Rα, CD69, VLA-4, and CCR9 targeting antibodies will be used to characterize T cells after infection. After staining, the cells will be fixed in 1% paraformaldehyde overnight at 4°C. Antibody bound cells will be detected using a Becton Dickinson FACStar Plus dual laser system (Franklin Lakes, NJ) and FCS express version 4.0 software from DeNovo Software (Los Angeles, CA).

### Sub-aim 1b

<table>
<thead>
<tr>
<th>Group</th>
<th>Recipients</th>
<th>Donors</th>
<th>Parasites</th>
<th>CD8+ T cell activ. markers</th>
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</thead>
<tbody>
<tr>
<td>1: (N = 8)</td>
<td>TCRβ−/−</td>
<td>CD4 (WT) +CD8 (WT)</td>
<td>Normal</td>
<td>Low</td>
</tr>
<tr>
<td>2: (N = 8)</td>
<td>TCRβ−/−</td>
<td>CD4 (WT) +CD8 (OT-1)</td>
<td>Normal</td>
<td>High</td>
</tr>
<tr>
<td>3: (N = 8)</td>
<td>TCRβ−/−</td>
<td>CD4 (WT)</td>
<td>Normal</td>
<td>Undetectable</td>
</tr>
<tr>
<td>4: (N = 8)</td>
<td>TCRβ−/−</td>
<td>None</td>
<td>High</td>
<td>Undetectable</td>
</tr>
<tr>
<td>5: (N = 4)</td>
<td>TCRβ−/−</td>
<td>CD4 (WT) +CD8 (OT-1)</td>
<td>Normal</td>
<td>High</td>
</tr>
</tbody>
</table>

### Sub-aim 1c: Determine if CD8+ T cell responses require the presence of CD4+ T cells.

We aim to determine whether CD8+ T cells are sufficient for *Giardia*-triggered intestinal IAP
and disaccharidase reduction and increased brush border ezrin and villin phosphorylation. We will test the requirement of CD4+ T cell help in activating CD8+ T cells following infection. After adoptive transfer of OT-1 CD8+ T cells with (Group 2) or without (Group 1) CD4+ T cells, TCRβ−/− mice will be infected with *G. duodenalis*-OVA. At day 7 p.i., the spleens and MLNs of infected mice will be analyzed for CD8+ T cell activation. Uninfected mice will be analyzed for each group in parallel in order to establish a baseline. As controls, we will examine infected and uninfected TCRβ−/− mice having received only CD4+ T cells by adoptive transfer (Group 3) as well as unaltered C57BL/6 mice (Group 4). We expect that CD8+ T cells will not be activated without CD4+ T cell help (Group 1). Thus, we expect that *G. duodenalis*-OVA infected OT-1 CD8+ T cell recipient TCRβ−/− mice (Group 2) will express activation markers within lymphoid tissue only in the presence of CD4+ T cells. Being that Group 3 animals lack CD8+ T cells altogether; we do not expect to detect activated CD8+ T cells in this group following infection. We have previously demonstrated that infected C57BL/6 mice do not exhibit activated CD8+ T cells following infection and thus we do not expect group 4 animals to do so. If OT-1 CD8+ T cells (Group 2) fail to be activated by *Giardia*-OVA, we will use alternative approaches to indirectly detect CD8+ T cell function in the intestinal epithelium. Along with CD8+ T cell activation, we will monitor enzyme deficiencies and brush border integrity following infection as downstream markers of CD8+ T cell activity. The direct (flow cytometry) and indirect (intestinal enzyme assays / brush border protein phosphorylation) approaches proposed here will permit the assessment of CD4+ T cell requirement for CD8+ T cell-induced pathology even if the CD8+ T cell responses are not antigen-specific. Should CD4+ T cells be required for activating CD8+ T cells during giardiasis, we will consider testing the role of IFN-γ producing CD4+ T cells in eliciting CD8+ T cell-dependent pathology. To do so, we will compare the extent of CD8+ T cell activation in infected OT-1 recipient TCRβ−/− mice replenished with either C57BL/6 or IFN-γ−/− CD4+ T cells. Since IFN-γ is shared among Th1 CD4+ T cells and CD8+ T cells, we expect that this cytokine is very important in eliciting gut pathology.
following infection.

**Methods:** For adoptive transfers, splenic CD4\(^+\) T cells will be purified from uninfected C57BL/6 or IFN-\(\gamma\) \(-/-\) mice and splenic CD8\(^+\) T cells will be purified from OT-1 mice. Uninfected TCR\(\beta\) \(-/-\) mice will receive orbital cavity injections containing either CD4\(^+\) T cells (10\(^7\)/0.1 ml) along with OT-1 CD8\(^+\) T cells (10\(^7\)/0.1 ml) or OT-1 CD8\(^+\) T (10\(^7\)/0.1 ml) cells alone. Sham recipients will be injected with PBS. Adoptive transfer and infection will be carried out as in sub-aim 1. At day 7 p.i., the mice will be euthanized and the splenic and MLN CD8\(^+\) T cells will be stained for activation markers by flow cytometry as in sub-aim 1b. Uninfected mice will be treated in parallel. CD8\(^+\) T cell activation will be compared between infected and uninfected mice in each T cell recipient group as well as between groups. If necessary, a section of the jejunum will be collected and assayed for enzymatic activity. Another section will be obtained for western blot analysis to determine the relative p-ezrin and p-villin levels in the brush border. Pathological markers among the mice will be compared in the same manner as CD8\(^+\) T cell activation.

<table>
<thead>
<tr>
<th>Sub-aim 1c</th>
<th>Assays to be conducted on day 0 and day 7 (Expected outcomes in day 7 mice compared to day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td><strong>Recipients</strong></td>
</tr>
<tr>
<td>1: (N = 8)</td>
<td>TCR(\beta)(^-/-)</td>
</tr>
<tr>
<td>2: (N = 8)</td>
<td>TCR(\beta)(^-/-)</td>
</tr>
<tr>
<td>3: (N = 8)</td>
<td>TCR(\beta)(^-/-)</td>
</tr>
<tr>
<td>4: (N = 8)</td>
<td>TCR(\beta)(^-/-)</td>
</tr>
</tbody>
</table>

**Aim 2:** FasL expression by CD8\(^+\) T cells contributes to pathogenesis during giardiasis.
Sub-aim 2a: Determine the role of FasL in CD8⁺ T cell mediated intestinal pathology following infection with *G. duodenalis*.

In order to test the hypothesis that FasL expression on CD8⁺ T cells contributes to intestinal IAP and disaccharidase reduction and increased brush border ezrin and villin phosphorylation following infection with *G. duodenalis*, we will compare infection outcomes in CD4⁺ T cell-replenished TCRβ⁻⁻ mice having received either C57BL/6 (Group 1) or FasL<sup>gld</sup> (Group 2) CD8⁺ T cells by adoptive transfer. As controls, we will analyze infection outcome in CD4⁺ T cell-replenished TCRβ⁻⁻ mice receiving sham transfer rather than CD8⁺ T cells (Group 3). Another control infection will be carried out in C57BL/6 mice as we know these mice to develop pathology following infection (Group 4). Uninfected mice will be analyzed for each group in parallel in order to establish a baseline. We expect that FasL deficiency (in Fas<sup>gld</sup> mice) will partially relieve CD8⁺ T cell mediated intestinal IAP and disaccharidase reduction as well as increased brush border ezrin and villin phosphorylation following infection with *G. duodenalis*. This would confirm that Fas signaling in the infected small intestine partially contributes to pathology associated with giardiasis. Furthermore, this would establish that FasL expressed on CD8⁺ T cells initiates this enteropathy. As an alternative experiment, we will compare the pathological outcomes of *Giardia* infection in Fas-loxp<sup>villin</sup>-Cre and C57BL/6 mice without adoptive transfer. Fas-loxp<sup>villin</sup>-Cre mice have a selective Fas deficiency driven by the expression of villin, which is largely restricted to the intestinal epithelium. This would allow us to disrupt Fas signaling within the intestinal epithelium while leaving other tissues unaffected. This approach would not, however, directly link pathology to Fas signals initiated by CD8⁺ T cells. Although, if we find in Aim sub-aim 1a that all of the pathology associated with giardiasis is CD8⁺ T cell mediated, these cells would certainly be implicated in infected Fas-loxp<sup>villin</sup>-Cre mice.

**Method:** CD4⁺ T cells obtained from uninfected C57BL/6 will be used to replenish uninfected TCRβ⁻⁻. CD8⁺ T cells from uninfected C57BL/6 and Fas<sup>gld</sup> will be adoptively transferred into TCRβ⁻⁻.
in concert with CD4\(^+\) replenishment. Adoptive transfers will be carried out as described in sub-aim 1a. Following a 7 day infection with *G. duodenalis*, the mice will be euthanized and assayed for intestinal disaccharidase and IAP activity as well as p-ezrin and p-villin levels in the brush border as described in sub-aim 1a. The pathological outcomes will be compared in infected and uninfected mice as well as between CD8\(^+\) T cell recipients. Should infections in Fas-lox\(^p\) villin-Cre mice be necessary, we will treat the mice in an identical manner after euthanasia.

### Sub-aim 2a

<table>
<thead>
<tr>
<th>Group</th>
<th>Recipients</th>
<th>Donors</th>
<th>Assays to be conducted on day 0 and day 7 (Expected outcomes in day 7 mice compared to day 0)</th>
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<tr>
<td></td>
<td></td>
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<td>Parasites</td>
</tr>
<tr>
<td>1: (N = 8)</td>
<td>TCR(\beta^+)</td>
<td>CD4 (WT) + CD8 (WT)</td>
<td>Normal</td>
</tr>
<tr>
<td>2: (N = 8)</td>
<td>TCR(\beta^+)</td>
<td>CD4 (WT) + CD8 (Fas(^{gld}))</td>
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</tr>
<tr>
<td>3: (N = 8)</td>
<td>TCR(\beta^+)</td>
<td>None</td>
<td>High</td>
</tr>
<tr>
<td>4: (N = 8)</td>
<td>B6-WT</td>
<td>None</td>
<td>Normal</td>
</tr>
</tbody>
</table>

### Sub-aim 2b: Ligation of Fas leads to intestinal enzyme deficiency and altered brush border architecture in Caco-2 cells.

We hypothesize that signaling through FasL on activated CD8\(^+\) T cells leads to intestinal injury following infection with *G. duodenalis*. In order to test this hypothesis, we will employ an *in vitro* model using the human colon adenocarcinoma Caco-2 cell-line. These cells exhibit enhanced polarization and thus increased expression of brush border enzymes like sucrase and IAP, compared to other colonic cell-lines (Chantret et al., 1994). We have observed increased ezrin phosphorylation.
following ligation of Fas with a monoclonal IgM) in the colonic adenocarcinoma cell-line HT-29. Thus, we wish to explore the consequences of Fas ligation in Caco-2 cells with respect to sucrase, lactase, and IAP activity and the phosphorylation of ezrin and villin. Differentiated Caco-2 cells will either be treated with an anti-Fas IgM (Group 4) or with media control (Group 3) and assayed for disaccharidase and IAP reductions as well as increased ezrin and villin phosphorylation. Undifferentiated media (Group 1) and IgM treated (Group 2) cells will also be included as controls. Fas triggered ezrin phosphorylation in HT-29 cells peaks at 30 minutes and so we will assay ezrin and villin phosphorylation at short time points. The effects of Fas ligation on sucrase, lactase, and IAP levels would likely require longer incubations to detect. We will conduct time course experiments in order to optimize our stimulations. We expect that Fas ligation induces ezrin and villin phosphorylation followed by a reduction of disaccharidase and IAP activity. Should we find that Fas ligation does not lead to ezrin or villin phosphorylation in Caco-2 cells, we will conduct these experiments in HT-29 cells where we initially described this phenomenon. HT-29 cells express less sucrase than Caco-2 cells and are more challenging to differentiate so they will be reserved as an alternative cell-line.

**Methods:** Caco-2 cells will be cultured in standard 10% FBS containing DMEM/F12 media. These cells require 21 days of culture as a confluent monolayer to fully polarize and express large amounts of brush border enzymes. Monoclonal anti-Fas IgM (CH-11) will be used to engage Fas. A brief 10 min exposure (200 ng/ml) was sufficient in phosphorylating ezrin, and thus likely villin, in our previous experiments using HT-29 cells. A time course and dose curve will be conducted to establish the optimal exposure conditions. The reduction of the brush border is a very dynamic process and likely requires a lag period between Fas ligation and analysis. These parameters will be established in preliminary experiments. Following treatment with CH-11, the cells will be lysed under non-denaturing conditions and sucrase and IAP activity will be assayed as described in sub-aim1a. Ezrin
and villin phosphorylation will be assayed in parallel by western blot analysis (sub-aim1a). Villin will be immuno-precipitated prior to analysis by western blot (sub-aim1a).

<table>
<thead>
<tr>
<th>Sub-aim 2b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td><strong>DS activity</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

Sub-aim 2c: Determine if Fas induced reduction of intestinal disaccharidase and IAP activity is downstream of ezrin and villin phosphorylation.

We hypothesize that post-translational modification of ezrin and villin functions upstream of the pathological reduction of brush border disaccharidases and IAP activity following infection with *G. duodenalis*. We will test the outcome of Fas engagement on sucrase, lactase, and IAP activity using CH-11 treated Caco-2 cells in the presence and absence of specific ezrin and villin phosphorylation inhibiting compounds. Confluent monolayers of fully differentiated cells will be exposed to either culture media (Group 1) or anti-Fas IgM (Group 2). For comparison, another group of cells will be exposed to either culture media (Group 3) or anti-Fas IgM (Group 4) and co-incubated with antagonistic compounds preventing the phosphorylation of ezrin (compound #8) and villin (PP2). Ezrin and villin phosphorylation will be inhibited individually as well as together. We will assess
sucrase, lactase and IAP activity following exposure. We expect that inhibited phosphorylation of ezrin, villin, or both will interfere with sucrase, lactase, and IAP deficiency following Fas engagement. Ezrin and villin phosphorylation will also be monitored to ensure that our inhibitors are functional. Should we observe that our inhibitors have no effect on Fas-induced sucrase, lactase, and IAP activity, we will test a knockdown model *in vitro*. Using an RNAi approach, we will knockdown ezrin and villin, individually and together, in Caco-2 cells and repeat the Fas ligation experiments. We expect that ezrin and villin knockdown will prevent the Fas signal from eliciting intestinal injury by way of reducing sucrase, lactase, and IAP activity. One setback associated with using a knockdown approach is that it may disrupt the formation of the brush border in these cells irrespectively of Fas ligation. Ezrin^−/−^ mice fail to develop normal villi and exhibit short, defective microvilli structures in intestinal epithelial cells (Saotome et al., 2004). Thus knocking these proteins down will likely impair brush border formation.

**Methods:** Fas engagement will be carried out as described in sub-aim 2b. Ezrin phosphorylation will be blocked by compound #8, provided by Dr. Uren, Georgetown University. Villin phosphorylation will be blocked by PP2, a Src kinase inhibitor. Caco-2 cells will be pretreated with compound 8/PP2 30 min prior to Fas ligation. As previously stated, the optimal time between Fas ligation and sucrase deficiency will be determined by time- and dose- curves. Inhibitions will be carried out individually as well as together. After the appropriate incubation time, the cells will be lysed and assayed for sucrase, lactase and IAP activity following Fas ligation.
### Aim 3: Infection with G. duodenalis leads to activation of intra-epithelial, lamina propria and Peyer’s patch CD8$^+$ T cells.

#### Sub-aim 3a: Determine the abundance of activated CD8$^+$ T cells within the intestinal epithelium following infection with *G. duodenalis*.

In order to test the hypothesis that infection with *G. duodenalis* activates intra-epithelial CD8$^+$ T cells, we will purify CD8$^+$ IELs following infection and determine their effector phenotype by flow cytometry analysis. CD4$^+$ T and OT-1 CD8$^+$ T cell-replenished TCR$\beta^{-/-}$ recipient mice (Group 1) will be infected with *G. duodenalis*-OVA for one week and these mice will be compared with uninfected mice. For assessing the antigen-specific nature of T cell responses, we will compare Group 1 mice to infected and uninfected CD4$^+$ T cell-replenished TCR$\beta^{-/-}$ recipient mice having received C57BL/6 CD8$^+$ T cells by adoptive transfer (Group 2). Control mice will be analyzed similarly before and after infection having only CD4$^+$ T cell present (Group 3). Another control group will receive sham saline transfer (Group 4). Unaltered C57BL/6 mice will also be infected with *G. duodenalis*-OVA (Group 5). After 7 days, the mice will be euthanized and the small intestine collected. IELs will be purified by mucosal purification and percoll centrifugation. For efficient enrichment, the small intestines of the four mice within each experimental group may have to be pooled. If this is the case, the infections will

<table>
<thead>
<tr>
<th>Group</th>
<th>Maturity</th>
<th>Inhibition</th>
<th>Treatment</th>
<th>Assays and expected results</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DS activity</td>
<td>p-Ezrin/p-villin</td>
</tr>
<tr>
<td>1</td>
<td>Differentiated</td>
<td>–</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
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<td>–</td>
<td>α-Fas IgM</td>
<td>Low</td>
</tr>
<tr>
<td>3</td>
<td>Differentiated</td>
<td>#8/PP2</td>
<td>None</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>Differentiated</td>
<td>#8/PP2</td>
<td>α-Fas IgM</td>
<td>Normal</td>
</tr>
</tbody>
</table>
be repeated at least three times. CD8+ T cell activation will be assessed by flow cytometry analysis as previously described. We expect that the intestinal epithelium of infected mice will contain significantly higher levels of activated CD8+ T cells compared to uninfected mice. Furthermore, this CD8+ T cell activation is largely antigen-specific and so we expect that Group 2 mice will exhibit less T cell activation than Group 1 mice. Group 3 and 4 mice should not possess CD8+ T cells. We may also detect activated IELs in group 5 mice but we do not expect these mice to exhibit similar levels of CD8+ T cell activation as group 1 mice. Should we not detect activated CD8+ T cells within the intestinal epithelium following infection, we will test earlier time points as much of the pathology that we observe occurs by day 7 p.i. It is possible that activated CD8+ T cells induce pathology and leave the intestinal epithelium at this point. Other intestinal compartments will also be analyzed as described in the following aims.

**Methods:** Adoptive transfer of OT-1 CD8+ T cells into CD4+ T cell-replenished TCRβ−/− recipient mice will be carried out as in sub-aim 1a and b. Infections with *G. duodenalis*-OVA will be conducted as described in sub-aim 1b. At day 7 p.i., the mice will be euthanized and the entire small intestine will be collected and pooled. Uninfected mice will be euthanized and processed in parallel. The luminal content of the intestines will be flushed and the Peyer’s patches will be removed. The tissue will be disrupted with EDTA containing buffer and the epithelium will be stripped by a short incubation of the tissue in EDTA and DTT containing buffer at 37°C. The epithelium will be collected by passing the disrupted tissue through a strainer. The epithelium will be then be resuspended in 40% percoll overlaid with DPBS and centrifugated at 600g for 20 min. The percoll will then be neutralized with 20% FBS and the cells will be collected and counted. Flow cytometry analysis will be carried out as described in Aim1 sub-aim 1b.
<table>
<thead>
<tr>
<th>Group</th>
<th>Recipients</th>
<th>Donors</th>
<th>Parasites</th>
<th>CD8^+ T cell active, Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: (N = 8)</td>
<td>TCRβ^-</td>
<td>CD4 (WT) + CD8 (OT-1)</td>
<td>Normal</td>
<td>Moderately high in IELs</td>
</tr>
<tr>
<td>2: (N = 8)</td>
<td>TCRβ^-</td>
<td>CD4 (WT) + CD8 (WT)</td>
<td>Normal</td>
<td>Low in IELs</td>
</tr>
<tr>
<td>3: (N = 8)</td>
<td>TCRβ^-</td>
<td>CD4 (WT)</td>
<td>Normal</td>
<td>Undetectable</td>
</tr>
<tr>
<td>4: (N = 8)</td>
<td>TCRβ^-</td>
<td>None</td>
<td>High</td>
<td>Undetectable</td>
</tr>
<tr>
<td>5: (N = 8)</td>
<td>B6-WT</td>
<td>None</td>
<td>Normal</td>
<td>Low in IELs</td>
</tr>
</tbody>
</table>

**Sub-aim 3a:** Determine the abundance of activated CD8^+ T cells within the lamina propria following infection with *G. duodenalis*.

We aim to test the hypothesis that CD8^+ T cells are activated following infection with *G. duodenalis* and infiltrate the lamina propria. This would be consistent with our previous observation that FasL^+ cells infiltrate the lamina propria in *G. duodenalis* infected mice. We will compare the abundance of activated CD8^+ T cells within lamina propria purifications of infected and uninfected mice as discussed in sub-aim 3a. CD4^+ T cell-replenished OT-1 CD8^+ T cell recipient TCRβ^- mice will be infected with *G. duodenalis*-OVA. The same CD4^+ T cell-replenished and control mice will also be tested as described in sub-aim 3a. After 7 days, the mice will be euthanized and the small intestines collected and pooled. Uninfected mice will be processed in parallel. Upon extraction and purification of the lamina propria, the abundance of activated CD8^+ T cells will be accessed by flow cytometry. We expect that the lamina propria of infected mice will exhibit increased numbers of activated CD8^+ T cells as compared to that of uninfected mice. Furthermore, we expect this CD8^+ T cell response to be antigen-specific as indicated by an increased abundance of activated T cells in group 1 mice compared to...
to groups 2 and 5. Should we not successfully detect CD8⁺ T cell activation, we will test earlier time points. We will also monitor CD8⁺ T cell activation within the Peyer’s patches.

**Methods:** Infection and extraction of small intestines will be conducted as described in sub-aim 3a. However, after stripping of the intestinal epithelium, the remaining tissue will be collected. The tissue will be disrupted with collagenase and DNAse treatment and strained. The strained tissue will then be subjected to a percoll gradient and analyzed by flow cytometry as described in sub-aim 1b.

<table>
<thead>
<tr>
<th>Sub-aim 3b</th>
<th>Group</th>
<th>Recipients</th>
<th>Donors</th>
<th>Assays to be conducted on day 0 and day 7 (Expected outcomes in day 7 mice compared to day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1: (N = 8)</td>
<td>TCRβ⁺⁺</td>
<td>CD4 (WT) + CD8 (OT-1)</td>
<td>Parasites</td>
</tr>
<tr>
<td></td>
<td>2: (N = 8)</td>
<td>TCRβ⁺⁺</td>
<td>CD4 (WT) + CD8 (WT)</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>3: (N = 8)</td>
<td>TCRβ⁺⁺</td>
<td>CD4 (WT)</td>
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<tr>
<td></td>
<td>4: (N = 8)</td>
<td>TCRβ⁺⁺</td>
<td>None</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5: (N = 8)</td>
<td>B6-WT</td>
<td>None</td>
<td>Normal</td>
</tr>
</tbody>
</table>

**Sub-aim 3c:** Determine the abundance of activated CD8⁺ T cells within the Peyer’s patches following infection with *G. duodenalis*.

Since *Giardia* trophozoites are restricted to the intestinal lumen during infection, it is likely that *Giardia* antigen is recognized within the Peyer’s patches. This is because Peyer’s patches lay below specialized M cells that specialize in the uptake and transport of intestinal antigens to the underlying
immune presence. Thus we wish to test the hypothesis that CD8+ T cells are activated within the Peyer’s patches following infection. Our experimental design will be identical to sub-aim 3a. At 7 days p.i., the mice will be euthanized and the small intestines pooled. The Peyer’s patches will be extracted from the small intestine and subject to disruption and analysis by flow cytometry. We expect that large numbers of activated CD8+ T cells will be found within the Peyer’s patches of infected mice compared to uninfected mice. We also expect a more intense response from G. duodenalis-OVA infected OT-1 recipients (Group1) than C57BL/6 recipients (Group 2) and unaltered C57BL/6 mice (Group 5). We do not expect Group 4 mice to have detectable CD8+ T cells within the Peyer’s patches at any point. Should we not detect increased CD8+ T cell activation in Group 1 mice compared to Group 2 mice, we will test other time points as activated CD8+ T cells may migrate away from the Peyer’s patches by 7 days p.i. Should other time points fail to yield activated CD8+ T cells within the Peyer’s patches, we will conclude that the Peyer’s patches are not the primary site of CD8+ T cell priming against Giardia antigen.

Methods: Infections in CD4+ T cell-replenished OT-1 recipient TCRβ−/− mice will be conducted as described in sub-aim 3a. After euthanasia, the Peyer’s patches of the four mice in each experimental group will be extracted. The Peyer’s patches will be subject to straining and flow cytometry analysis.
### Sub-aim 3c

<table>
<thead>
<tr>
<th>Group</th>
<th>Recipients</th>
<th>Donors</th>
<th>Assays to be conducted on day 0 and day 7 (Expected outcomes in day 7 mice compared to day 0)</th>
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<tbody>
<tr>
<td>1: (N = 8)</td>
<td>TCRβ&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CD4 (WT) + CD8 (OT-1)</td>
<td>Parasites: Normal; CD8&lt;sup&gt;+&lt;/sup&gt; T cell activ. markers: High in Peyer’s patches</td>
</tr>
<tr>
<td>2: (N = 8)</td>
<td>TCRβ&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CD4 (WT) + CD8 (WT)</td>
<td>Parasites: Normal; CD8&lt;sup&gt;+&lt;/sup&gt; T cell activ. markers: Low in Peyer’s patches</td>
</tr>
<tr>
<td>3: (N = 8)</td>
<td>TCRβ&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CD4 (WT)</td>
<td>Parasites: Normal; CD8&lt;sup&gt;+&lt;/sup&gt; T cell activ. markers: Undetectable</td>
</tr>
<tr>
<td>4: (N = 8)</td>
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<td>Parasites: Normal; CD8&lt;sup&gt;+&lt;/sup&gt; T cell activ. markers: High; Undetectable</td>
</tr>
<tr>
<td>5: (N = 8)</td>
<td>B6-WT</td>
<td>None</td>
<td>Parasites: Normal; CD8&lt;sup&gt;+&lt;/sup&gt; T cell activ. markers: Low in Peyer’s patches</td>
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### TIME-TABLE

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<tbody>
<tr>
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<td>2 months</td>
<td>February – April 2012</td>
</tr>
<tr>
<td>1 (c)</td>
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<td>2 (a)</td>
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<td>3 (a)</td>
<td>2 months</td>
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</tr>
<tr>
<td>3 (c)</td>
<td>2 months</td>
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</tbody>
</table>
RESULTS

Figure 1: Intestinal infiltration of FasL$^+$ cells following infection with *G. duodenalis*. C57BL/6 mice were infected with *G. duodenalis* strain GS. Uninfected, day 7 p.i., and day 14 p.i. mice were euthanized and 3 cm duodenal segments were paraffin embedded and sectioned at 5 μm. FasL$^+$ cells were detected by immuno-fluorescent microscopy using an anti-FasL primary antibody followed by a TRITC conjugated secondary antibody. DAPI staining was used to reveal intestinal nuclei. FasL$^+$ cells were counted and the average stained cells per 20x field per mouse are shown above. An unpaired Student’s t test was used to determine statistical significance.
Figure 2: T cell cytokine responses in the spleen of *G. duodenalis* infected C57BL/6 mice. C57BL/6 mice were infected with *G. duodenalis* strain GS for 7 and 14 days. Cytokine production was measured by ELISA following *ex vivo* restimulation of splenic lymphocytes with 100 µg/ml of *Giardia*-extract for 48 hrs. Mean cytokines (pg/ml) produced are represented from 4 mice per time point above along with SEM.
Figure 3: Ex vivo restimulation of MLN lymphocytes from C57BL/6 mice infected with G. duodenalis. C57BL/6 mice were infected with G. duodenalis strain GS for 7 days. Cytokine production was measured by ELISA following ex vivo restimulation of splenic lymphocytes with 100 µg/ml of Giardia extract for 48 hrs. Mean cytokines (pg/ml) produced are represented above from 4 mice, in which MLN cells were pooled for each time point along with SEM.
Figure 4: Fas<sup>lpr</sup> mice exhibit a defect in parasite clearance. C57BL/6 (A) and Fas<sup>lpr</sup> (B) mice were infected with *G. duodenalis* strain GS for 7 and 14 days. After infection, the mice were euthanized and the parasites counted within the small intestine. The mean numbers of parasites found within a 3 cm duodenal section obtained from 4 mice for each indicated time point are presented above along with SEM. An unpaired Student’s t test was used to determine statistical significance.
Figure 5: Disaccharidase activity is reduced within the small intestine following infection in C57BL/6 mice but not Fas\textsuperscript{lp}r mice. Sucrase and lactase activity was measured within the jejunum of uninfected, day 7 p.i., and day 14 p.i. mice. Sucrase activity is presented in relative units for C57BL/6 mice (A) and Fas\textsuperscript{lp}r (B) mice. Lactase activity is also presented in relative units for C57BL/6 mice (C) and Fas\textsuperscript{lp}r (D). Mean enzyme activity is presented above for 4 mice per time point along with SEM. Baseline C57BL/6 sucrase and lactase activity were 19.49 and 5.59 nmoles Glucose / µg protein / hr, respectively. Baseline Fas\textsuperscript{lp}r sucrase and lactase activity were 4.41 and 0.94 nmoles Glucose / µg protein / hr, respectively. An unpaired Student’s t test was used to determine statistical significance.
Figure 6: Post-translational modification of ezrin following infection. C57BL/6 and Fas\textsuperscript{lp} mice were infected with \textit{G. duodenalis} strain GS for 7 and 14 days. After infection, the mice were euthanized along with uninfected mice and 10 cm ileal/jejunal sections were lysed and homogenized in NP40 lysis buffer. The protein samples were separated by SDS-PAGE and analyzed by western blot. A representative immunoblot is shown above.

Figure 7: Fas ligation leads to enhanced ezrin phosphorylation in HT-29 cells. Human colonic epithelial HT-29 cells were treated with an anti-Fas IgM for the indicated time points. Immunofluorescent microscopy (A) and western blot (B) was used to detect p-ezrin levels following Fas ligation. Representative figures are shown above.
Figure 9: Splenocyte numbers are reduced in Faslpr mice following infection with *G. duodenalis*. Spleens were obtained from uninfected, day 7 p.i., and day 14 p.i., (□) C57BL/6 and (■) Faslpr mice. Splenocytes were counted using a hemocytometer. Mean splenocyte numbers of 4 mice per time point are represented above along with SEM. An unpaired Student’s t-test was used to determine statistical significance.

Figure 8: Villin expression and proteolysis following infection. C57BL/6 and Faslpr mice were infected with *G. duodenalis* strain GS for 7 and 14 days. After infection, the mice were euthanized along with uninfected mice and 10 cm intestinal sections were lysed and homogenized in NP40 lysis buffer. The protein samples were separated by SDS-PAGE and analyzed by western blot. A representative immuno-blotted is shown above.
Figure 10: The abundance of T cell subsets within the spleen and MLN following infection with *G. duodenalis*. CD8⁺ and CD4⁺ T cells were relatively quantified within the spleens (A and B) and MLN (C and D) of Day 7 *G. duodenalis* infected (red) and uninfected (blue) C57BL/6 (A and C) and Fas⁻/⁻ (B and D) mice. Flow cytometry analysis was used to determine the relative abundance of T cell subsets within the lymphoid tissue. Each T cell subset was normalized to total splenocytes and represented above as the mean and SEM from 4 mice in each group. An unpaired Student’s t test was used to determine statistical significance.
Figure 11: Splenic CD4\(^+\) T cell responses following infection with *G. duodenalis*. CD4\(^+\) T cells obtained from the spleens of *G. duodenalis* infected (red) and uninfected (blue) C57BL/6 and Fas\(^{lp}r\) mice were analyzed for the expression of activation markers. The relative expression of IL-2R\(\alpha\) (A), CCR9 (B) and CD69 (C) were monitored after 7 days p.i. and in uninfected mice by flow cytometry analysis as indicated above by the mean and SEM from 4 mice in each group. The number of CD8\(^+\) T cells expressing the above markers was normalized to total CD8\(^+\) T cells. An unpaired Student’s t test was used to determine statistical significance.
Figure 12: Splenic CD8\(^+\) T cell responses following infection with *G. duodenalis*. CD8\(^+\) T cells obtained from the spleens of *G. duodenalis* infected (red) and uninfected (blue) C57BL/6 and Fas\(^{lp}r\) mice were analyzed for the expression of activation markers. The relative expression of FasL (A), IL-2R\(\alpha\) (B), CCR9 (C) and CD69 (D) were monitored after 7 days p.i. and in uninfected mice by flow cytometry analysis as indicated above by the mean and SEM from 4 mice in each group. The number of CD8\(^+\) T cells expressing the above markers was normalized to total CD8\(^+\) T cells. An unpaired Student’s t test was used to determine statistical significance.
Figure 13: CD4+ T cell responses in the MLN of infected mice. CD4+ T cells obtained from the MLN of day 7 *G. duodenalis* infected (■) and uninfected (□) C57BL/6 and Faslp mice were analyzed for the expression of activation markers. The relative expression of IL-2Rα (A), CCR9 (B) and CD69 (C) were monitored after 7 days p.i. and in uninfected mice by flow cytometry analysis as indicated above by the mean and SEM from 4 mice in each group. The number of CD8+ T cells expressing the above markers was normalized to total CD8+ T cells.
Figure 14: CD8+ T cell responses in the MLN of infected mice. CD8+ T cells obtained from the MLN of G. duodenalis infected (■) and uninfected (□) C57BL/6 and Fas<sup>lpr</sup> mice were analyzed for the expression of activation markers. The relative expression of FasL (A), IL-2Rα (B), CCR9 (C), CD69 (D) were monitored after 7 days p.i. and in uninfected mice by flow cytometry analysis as indicated above by the mean and SEM from 4 mice in each group. The number of CD8+ T cells expressing the above numbers was normalized to total CD8+ T cells. An unpaired Student’s t test was used to determine statistical significance.
Figure 15: Enterocyte apoptosis is not affected in Fas deficient mice following Giardia infection. Duodenal sections were analyzed for cleaved caspase-3 levels by fluorescent microscopy. Apoptosis was measured in uninfected, day 7 and day 14 p.i., C57BL/6 (A) and Fas<sup>lpr</sup> (B) mice. Stained cells were counted in each intestinal section. The mean apoptotic cell counts from 4 mice per time point are presented above along with SEM. An unpaired Student’s t test was used to determine statistical significance.
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