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Tumor Necrosis Factor α Contributes to Protection Against *Giardia lamblia* Infection in Mice

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Summary

Giardia lamblia is a ubiquitous parasite that causes diarrhea. Effective control of *Giardia* infections in mice has been shown to involve IgA, T cells, mast cells, and IL-6. We now show that TNF α also plays an important role in the early control of giardiasis. Mice treated with neutralizing anti-TNF α antibodies or genetically deficient in TNF α were infected with the *G. lamblia* clone GS/(M)-H7. In both cases, mice lacking TNF α had much higher parasite numbers than controls during the first two weeks of infections. However, anti-parasite IgA levels, mast cell responses and IL-4 and IL-6 mRNA levels do not appear significantly altered in the absence of TNF α . In addition, we show that mice infected with *G. lamblia* exhibit increased intestinal permeability, similar to human *Giardia* infection, and that this increase occurs in both wild-type and TNF α deficient mice. We conclude that TNF α is essential for host resistance to *Giardia lamblia* infection, and that it does not exert its effects through mechanisms previously implicated in control of this parasite.

Keywords

Giardia lamblia; TNF; Trans-epithelial electrical resistance

Introduction

Giardia lamblia (syn. *G. intestinalis*, *G. duodenalis*) is the most commonly diagnosed parasitic cause of diarrhea in the U.S. and infects ~200 million people world-wide. There is significant variability in the outcome of these infections (reviewed in (1-4)). Many infections are asymptomatic, while others result in severe diarrhea, cramps and nausea. Similarly, most infections are controlled by host immune responses, whereas others become chronic. *G. lamblia* infections in humans induce immune response characterized by production of large amounts of anti-parasite IgA (reviewed in (1,3)). IgA is required for prevention of chronic *G. muris*, a rodent specific species of *Giardia*, infections in mice (5-8). In contrast, in the adult mouse model of *G. lamblia* infection, antibodies are not required to eliminate the parasite and a cellular immune response is able to control the infection in the absence of antibodies (8-11). It remains to be determined if and how cellular immune responses might contribute to control of *G. lamblia* infections in humans.

The precise nature of the protective cellular response against *G. lamblia* in mice is unclear, but we and others have found that CD4⁺ T cells, mast cells and IL-6 are all required (10-13). Mice treated with depleting antibodies against CD4 or c-kit fail to eliminate this parasite (10,13).

Similarly, mice with mutations in c-kit or targeted deletion of IL-6 or the T cell receptor β gene also fail to control *G. lamblia* infections (10-12). However, unlike many other mouse models of parasitic infection, mice deficient in either IL-4 or IFN- γ had no defects in parasite control (10). Recently, while analyzing the role of nitric oxide during *G. lamblia* infections in mice, we noted that TNF α mRNA levels were lower in IL-6 deficient mice following *Giardia* infection than in wild-type mice, (14). We therefore decided to directly address the role of TNF α in the mouse model of *G. lamblia* infection.

Materials and Methods

Mice, parasites and infections

C57BL/6J, homozygous TNF α deficient mice on a B6X129 mixed background (B6;129S2-Tnf^{tm1Gkl}/J) and wild-type B6X129 F2/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *G. lamblia* strain GS/(M)-H7 was cultured to confluence and used for infections as previously described (10). Female mice aged between 5 and 8 weeks were used for all experiments. Briefly, mice were infected by gavage with 1×10^6 parasites each in 0.2 ml phosphate-buffered saline (PBS) on day 0 and killed on different days after infection. Parasite numbers were determined from 10 cm of the distal duodenum and proximal jejunum just below the ligament of Trietz. Tissue was minced in 4 ml ice-cold PBS and chilled for 15 minutes on ice and parasites were counted on a hemocytometer as previously described (13). The limit of sensitivity for this assay is 10,000 parasites / 10 cm. All animals were handled in accordance with protocols approved by the Georgetown University Animal Care and Use Committee.

Anti-TNF α treatment

TNF α was neutralized in vivo as previously described (15). C57BL/6J mice were injected i.p. with 1 mg of anti-TNF α (clone HT-11-22) or isotype control (clone GL113) on the same day as infection.

IgA responses

Anti-parasite IgA in intestinal fluid was detected as previously described (11). Intestinal fluid was collected from mice by flushing the distal jejunum with 3 ml cold PBS. In vitro cultures of *Giardia* were allowed to attach to glass slides and fixed with cold methanol-acetone (1:1) for 5'. Fixed parasites were blocked by incubation in 5% normal goat serum in PBS and serial dilutions of intestinal fluid was applied for 60'. After washing, anti-parasite IgA was detected using FITC conjugated goat anti-mouse IgA (Southern Biotechnology, Birmingham, AL). IgA anti-*Giardia* monoclonal antibodies (16) were used as positive controls. Slides were mounted with Vectashield + propidium iodide (Vector Laboratories, Burlingame, CA) and viewed with a Zeiss Axiophot microscope. Images were collected with a CoolSnap fx camera (Roper Scientific, Trenton, NJ) using OpenLab software (Improvision, Cambridge, MA). Images were processed with AdobePhotoshop (Adobe Systems, San Jose, CA).

Mast cell responses

Mast cells in the small intestine were detected using chloroacetate esterase activity as previously described (13). Distal jejunum was fixed overnight in 4% formaldehyde in PBS, embedded in paraffin and sectioned (5μ) onto glass slides. Sections were stained as described and viewed as above using brightfield imaging and a CRI color filter (Cambridge Research, Cambridge, MA).

Semi-Quantitative RT-PCR

Cytokine mRNA levels were determined as previously described (11). Total RNA was prepared from a 1–2 cm segment of the small intestine corresponding to the jejunal region immediately distal to that used for determining parasite counts. RNA was purified using RNA-STAT-60 (Tel-test, Inc., Midland, Tex.). RNA was quantified using a Nanodrop spectrophotometer and quality was assessed by MOPS-formaldehyde gel electrophoresis prior to cDNA synthesis. Five μg of total RNA from each mouse was used to generate double stranded cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). IL-4, IL-6 and HPRT cDNAs were amplified as described and analyzed on 1.5% agarose gels. PCR products were quantitated by densitometric analysis of agarose gels using AlphaEase Software and an AlphaImager (Alpha Innotech, San Leandro, CA).

Epithelial Permeability

We used a modified snap-well assay system described by Fasano and colleagues to measure electrical resistance of the intestinal mucosa (17). Briefly, segments of the jejunum were removed and the muscle layers carefully stripped away from the mucosa. For each mouse, three $\sim 1\text{ cm}^2$ pieces of mucosa were then mounted in snap-well chambers with a precisely machined aperture (Costar, Cambridge, MA) and cultured in DMEM without serum. Epithelial resistance was measured every 30 minutes using an EndOhm-24 and EVOM voltmeter (World Precision Instruments, Sarasota, FL). Resistance of the mucosae in these chambers typically increases immediately after tissues are mounted in the chambers, then stabilizes and eventually decreases over time. Two or three pieces of mucosa per mouse were analyzed in parallel.

Statistical Analysis

We applied a Student's t-test to test the hypothesis that the mean expression values for the control and infected groups were equal. Statistical probability of $P < 0.05$ was considered significant.

Results

The role of TNF α was initially assessed by infecting groups of wild-type C57BL/6 female mice (Jackson Laboratories, Bar Harbor, ME) with *G. lamblia* GS/(M)-H7 and determining parasite numbers following infection as described (10). Mice received either neutralizing anti-TNF α or control IgG on the day of infection (15). Parasite numbers in the small intestines of mice receiving anti-TNF α were more than ten-fold greater five days following infection than in mice receiving control IgG (Fig. 1A). Moreover, while control-treated mice had eliminated parasites by day 12, anti-TNF α treated mice still had parasite numbers greater than the limit of detection of our assay ($\sim 10,000$ parasites / mouse, dashed lines in Fig. 1). The reduced numbers of parasites on day 12 could reflect decreasing concentrations of anti-TNF α in these mice over time. We therefore infected genetically TNF α deficient mice on a B6X129 F2 genetic background and wild-type mice (Jackson Laboratories) and analyzed parasite numbers on days 5, 12 and 28 post-infection (Fig. 1B). Again, TNF α deficient mice had substantially more parasites than wild-type controls on days 5 and 12. However, parasite numbers on day 12 had significantly decreased in TNF α deficient mice, similar to what was seen in anti-TNF α treated mice, while control mice had no detectable parasites. By day 28, parasites were no longer detectable in TNF α deficient mice or wild-type controls. Thus, TNF α plays a critical role during the establishment and/or early control of this infection.

Previous studies using IL-6 deficient mice also demonstrated a significant defect in early control of *G. lamblia* infections (11,12). Indeed, the parasite numbers seen in both IL-6 and TNF α deficient mice at days 5 and 12 post-infection are quite similar (Fig. 1 and ref. 13). Interestingly, while IL-6 deficient mice required 60 days to reduce parasite numbers to

undetectable levels, the TNF α deficient mice are able to eliminate infections by day 28. This suggests that the defect in IL-6 deficient mice is more severe than just the lack of TNF α production.

To determine if the absence of TNF α led to changes in other immune responses that might explain the high parasite loads early in infections, anti-parasite IgA, mast cell responses, and mRNA levels for IL4 and IL-6 were examined. While IgA is not required to control the acute phase of a *G. lamblia* infection in mice, IgA could still contribute to parasite elimination. Intestinal fluid was therefore collected from infected mice and analyzed for the presence of parasite-specific IgA. No IgA was detected in uninfected mice or mice infected for 5 days, regardless of genotype (Fig. 2). Detectable IgA responses were seen in both wild-type and TNF α deficient mice on both day 12 and day 28 post-infection (Fig. 2). Thus, major differences in IgA responses do not account for the different parasite loads seen in wild-type and IL-6 deficient mice,

Mast cells have been shown to be important for control of both *G. lamblia* and *G. muris* infections in mice (13,18). Moreover, TNF α has recently been shown to be important for mast cell development in vitro and in vivo (19). Mast cells began were first detected in the mucosa of both wild-type and TNF α deficient mice 12 days post-infection (Fig. 3), indicating that mast cell accumulation begins to occur between days 5 and 12 post-infection. No differences in mast cell numbers were seen at any time post-infection. However, at day 28 post-infection, mast cells in TNF α deficient mice appeared to be localized more in the villus regions and less in the crypt regions of the jejunum compared to mast cells in wild-type mice (Fig. 3). Given that parasites had already been eliminated, it is unclear if this difference is important for resolution of the infection. Thus, the lack of TNF α did not cause a profound reduction in two pathways known to be involved in control of *Giardia* infection.

Like TNF α deficient mice, IL-6 deficient mice have much greater parasite burdens than wild-type mice early during infections (11,12). A major difference in immune response between wild-type and IL-6 deficient mice is the larger increase in IL-4 mRNA levels seen in IL-6 deficient mice (12). We therefore used semi-quantitative RT-PCR to examine induction of both IL-4 and IL-6 mRNA levels in the TNF α deficient mice. IL-4 mRNA levels were elevated in both wild-type and TNF α deficient mice 5 and 12 days post-infection, compared to uninfected mice (Fig. 4 A and B). Unlike in IL-6 deficient mice, however, the IL-4 mRNA does not appear to be significantly more abundant than in wild-type mice. Furthermore, IL-4 mRNA levels were decreased in both wild-type and TNF α deficient mice on day 28. Thus, while the strong induction of IL-4 responses might contribute to the defect in parasite control observed in IL-6 deficient mice, it is unlikely to explain the high parasite numbers seen in TNF α deficient mice.

More significant differences in IL-6 mRNA levels were seen when comparing wild-type and TNF α deficient mice (Fig. 4 A and C). Five days post-infection the TNF α deficient mice had intestinal IL-6 mRNA levels that were higher than those seen in wild-type controls. The higher parasite numbers in TNF α deficient mice at this time cannot, therefore, be attributed to a failure to induce IL-6. In contrast, IL-6 mRNA levels in wild-type mice were greater than those in TNF deficient mice 12 days and 28 days post-infection. While these differences were statistically significant, the biological relevance of this is unclear, particularly because IL-6 mRNA levels in uninfected wild-type mice were also greater than those in uninfected TNF deficient mice.

Finally, giardiasis in humans causes nutrient malabsorption and one possible contributing mechanism has been reported to be decreased epithelial barrier function and increased permeability (20-22). Since TNF α is well known to increase epithelial permeability in several in vitro and in vivo systems (reviewed in (23,24) we decided to examine permeability in wild-

type and TNF α deficient mice infected with *Giardia*. We used a recently developed method for rapid determination of trans-epithelial electrical resistance to examine intestinal permeability in the mucosa of infected and uninfected mice (17). Mucosae from infected mice exhibited significantly lower resistance than mucosae from uninfected mice (Fig. 4A). Moreover, this decrease occurred whether or not the mice had an intact TNF α gene (Fig. 4B). These data also demonstrate that *Giardia lamblia* infected mice exhibit a pathologic response to infection that is characteristic of human infections (21,22).

Discussion

G. lamblia infections in humans are marked by considerable variation in their outcomes, both in the symptoms of disease and the duration of infection. We now show that TNF α is an important cytokine for determining the parasite burden and duration of *G. lamblia* infection in mice. We also show that mice infected with *G. lamblia* develop increased epithelial permeability, a characteristic of human giardiasis. Furthermore, we show that TNF α does not contribute to control of infections through any previously described mechanism, i.e. IgA, mast cells, IL-4 or IL-6, suggesting that other effector responses downstream of this cytokine are important. Finally, we show that TNF α is not required for the increase in epithelial permeability observed in this system.

It is becoming clear that mechanisms used by the immune system to control *G. lamblia* infections in mice are not identical to those used to control *G. muris*. While antibodies, particularly IgA are crucial for control of *G. muris* infections in mice, cellular responses are able to control the human parasite, *G. lamblia* (5-8,10). This is consistent with clinical observations in humans, where chronic *Giardia* infections have been associated with two particular hypogammaglobulinemia syndromes, common variable immunodeficiency (CVID) and Bruton's X-linked agammaglobulinemia (XLA), but not with selective IgA deficiency (reviewed in (1-3). It has become clear that the molecular defects in both XLA and CVID affect cells in addition to B cells (25-27), and it may be associated cellular responses that are responsible for the failure to control *Giardia* in these patients. For example, mutations in the btk kinase in XLA lead to reduced TNF α production by peripheral blood mononuclear cells in response to TLR signaling (25). Studies of the cellular mechanisms responsible for control of *G. lamblia* using mouse models are therefore highly valuable.

The two cytokines shown to have a major impact on *Giardia* infections in mice are IL-6 and TNF α . The defect in IL-6 deficient mice is more severe in terms of the persistence of infection. TNF α deficient mice clear their infections by day 28 whereas IL-6 deficient mice needed 60 days (11). It is possible that some of the differences observed between IL-6 and TNF deficient mice might also reflect the different genetic backgrounds used in these studies. The TNF deficient mice used in this study were on a mixed C57BL/6 X 129 genetic background, while the IL-6 deficient mice used previously (11) were inbred C57BL/6 mice. Inbred C57BL/6 mice were also used in the current study for anti-TNF α treatment. Several lines of evidence suggest that IL-6 appears to act upstream of TNF α in this *Giardia* model system. For example, in IL-6 deficient mice, IL-4 mRNA levels are increased following infection while TNF α mRNA levels are reduced (12,14). Thus, IL-6 appears necessary for normal TNF α responses. Furthermore, in the absence of TNF α , mRNA levels for IL-6 and IL-4 were only mildly affected. Indeed, at day 5 post-infection, IL-6 levels were higher in TNF α deficient mice than in wild-type controls. Thus, TNF α is not needed for IL-6 responses during this infection. One possible explanation is that the greater IL-6 expression at day 5 in TNF α deficient mice reflects the greater parasite load present at this time. As recently noted, intestinal epithelial cells appear to be a significant source of IL-6 during mouse infections (28). Higher parasite burdens would therefore be expected to increase epithelial cell responses. It is likely that IL-6 and TNF α are produced by multiple cell types during *G. lamblia* infections in mice and experiments using tissue specific

gene targeting and/or chimeric mice will be required to identify sources of these cytokines necessary for parasite elimination.

Interestingly, both IL-6 and TNF α are considered pro-inflammatory cytokines, helping to recruit macrophages and neutrophils to sites of inflammation. However, *G. lamblia* infections in humans and mice are typically not marked by significant infiltration of neutrophils or macrophages (29,30). This suggests that regulatory responses in the intestinal tract may be active during *Giardia* infections in order to reduce immune-mediated pathology. Indeed, infections in the gastrointestinal tract are well known for inducing regulatory responses (31). Many immune effector mechanisms, e.g. acute phase responses and macrophage activation, are regulated by IL-6 and TNF α and it remains to be determined which are responsible for elimination of *Giardia* in this model.

A key finding in this study is the fact that mice with *G. lamblia* infections exhibit increased intestinal permeability. Increased permeability of intestinal epithelium has been described in vitro and in human infections (20,22,32,33). Changes in intestinal permeability, however, have not been described previously in mouse infections. The modified “Snap-well” assay described here is a simple, fast and reproducible way to measure changes in trans-epithelial electrical resistance (TEER) that does not require sophisticated equipment like Ussing chambers. Our results with this system indicate that while *Giardia* infection causes a decrease in TEER, TNF α is not responsible. This is consistent with reduced TEER seen in vitro using only parasites and epithelial cell lines (20,32,33). Interestingly, the magnitude of the TEER decrease reported here was similar in wild-type and TNF α knockout mice 5 days post-infection, despite the presence of roughly 10-fold more parasites being present in the TNF α deficient mice. We and others have also recently reported that *G. lamblia* infected mice have altered intestinal motility and that these changes are absent in SCID mice (14,34). Together with the altered intestinal permeability reported here, this indicates that although these mice do not have diarrhea, they do develop pathology representative of human disease.

TNF α is important for protective immune responses to many infections and also for pathological responses in inflammatory diseases like Crohn's disease and rheumatoid arthritis. These are the first data reporting a role for TNF α in protection against *G. lamblia* infections. Furthermore, the defect in TNF α deficient mice does not appear to be related to mechanisms previously shown to control *Giardia* infections, including IgA production, mast cell responses, IL-6 or IL-4 expression. Together with the observation that *G. lamblia* infected mice have increased intestinal permeability, these results confirm the utility of studying *G. lamblia* infections in adult mice as a model of human giardiasis for understanding both pathogenesis and protective immunity against this infection.

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Figure 1.

TNF α is required for efficient control of *Giardia* infection. A. C57BL/6 mice were infected with *G. lamblia* and treated with anti-TNF α (hatched bars) or control rat IgG (open bars) on day 0. Parasite numbers were determined on different days post-infection. B. TNF α deficient mice (hatched bars) and B6X129 F2 controls (open bars) were infected on day 0 and parasite numbers were determined on different days post-infection. N = 4 / group. Data are presented as means with S.D. * p < 0.05 by Mann-Whitney test. The limit of detection in this assay is 10⁴ trophozoites / mouse and is marked by the dashed horizontal line. Data are representative of three experiments each with anti-TNF α treatment and TNF α deficient mice.

Figure 2.

IgA responses in TNF α deficient mice. Wild-type and TNF α deficient B6X129 F2 mice were infected with *G. lamblia* and euthanized on days 5, 12 and 28 as indicated. Uninfected mice (Day 0) were included as controls. Intestinal fluid was used to stain in vitro cultures of *G. lamblia* and IgA was detected by indirect immunofluorescence. Original magnification 200X.

Figure 3.

Mast cell responses in TNF α deficient mice. Wild-type and TNF α deficient B6X129 F2 mice were infected with *G. lamblia* and euthanized as in Fig. 2. Jejunal specimens were stained for chloroacetate esterase activity to detect mast cells (red cells). Original magnification 200X.

Figure 4.

Intestinal gene expression in *G. lamblia* infected mice. cDNA was prepared from intestinal RNA of wild-type and TNF α deficient B6X129 F2 mice infected with *G. lamblia*. A. IL-4, IL-6 and HPRT were amplified and analyzed on 1.5% agarose gels (A). M- 100 bp ladder. No cDNA PCR control is shown in the far right lane (-). B and C. Densitometric analysis of the images in A. Data are presented as the ratio of pixel intensities for IL-4 vs HPRT and IL-6 vs HPRT. * $p < 0.05$ by student t-test.

Figure 5.

Epithelial permeability is increased in both wild-type and TNF α deficient mice. A. Mucosae from jejunum of C57BL/6 mice were mounted in modified snap-well chambers and electrical resistance measured every 30 minutes. Two pieces of tissue were analyzed / mouse and 5 mice / group were analyzed. Data are presented as means and S.D. * $p < 0.05$ by student t-test. B. Electrical resistance was measured as in A for infected and uninfected wild-type and TNF α deficient B6X129 F2 mice. Three pieces of tissue were analyzed / mouse and 4 mice / group were analyzed. Data shown are for the 90 minute measurement. Mice in B were 6 weeks of age and those in A were 12 weeks, likely explaining the lower TEER values seen in uninfected mice in B.