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IL-17A promotes protective IgA responses and expression of other potential effectors against the lumen-dwelling enteric parasite *Giardia*

Sara M. Dann^{*,†}, Carolin F. Manthey[‡], Christine Le[‡], Yukiko Miyamoto[‡], Lauren Gima[‡], Andrew Abraham^{*}, Anthony T. Cao[†], Elaine M. Hanson[‡], Jay K. Kolls[§], Eyal Raz^{*}, Yingzi Cong^{†,¶}, and Lars Eckmann[‡]

^{*}Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX

[†]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX

[¶]Department of Pathology, University of Texas Medical Branch, Galveston, TX

[‡]Department of Medicine, University of California, San Diego, La Jolla, CA

[§]Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA

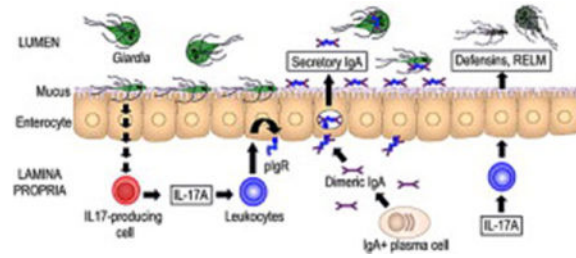
Abstract

Giardia lamblia is a leading protozoan cause of diarrheal disease worldwide. It colonizes the lumen and epithelial surface of the small intestine, but does not invade the mucosa. Acute infection causes only minimal mucosal inflammation. Effective immune defenses exist, yet their identity and mechanisms remain incompletely understood. Interleukin (IL)-17A has emerged as an important cytokine involved in inflammation and antimicrobial defense against bacterial pathogens at mucosal surfaces. In this study, we demonstrate that IL-17A has a crucial function in host defense against *Giardia* infection. Using murine infection models with *Giardia muris* and *G. lamblia*, we observed marked and selective induction of intestinal IL-17A with peak expression after two weeks. Th17 cells in the lamina propria and innate immune cells in the epithelial compartment of the small intestine were responsible for the IL-17A response. Experiments in gene-targeted mice revealed that the cytokine, and its cognate receptor IL-17RA, were required for eradication of the parasite. The actions of the cytokine were mediated by hematopoietic cells, and were required for the transport of IgA into the intestinal lumen, since IL-17A deficiency led to marked reduction of fecal IgA levels, as well as for increased intestinal expression of several other potential effectors, including β -defensin 1 and resistin-like molecule β . In contrast, intestinal hypermotility, another major anti-giardial defense mechanism, was not impacted by IL-17A loss. Taken together, these findings demonstrate that IL-17A and IL-17 receptor signaling are essential for intestinal defense against the important lumen-dwelling intestinal parasite *Giardia*.

Send correspondence to: Lars Eckmann, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, phone (858) 534-0683, fax (858) 534-3338, leckmann@ucsd.edu.

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Graphical abstract



Keywords

Protozoan parasites; mucosal immunity; small intestine; cytokines

INTRODUCTION

Giardiasis, caused by the parasitic protozoan, *Giardia lamblia*, is one of the most common waterborne diarrheal diseases in the world, with more than 280 million people annually infected (Baldursson and Karanis, 2011; Feng and Xiao, 2011; Upcroft and Upcroft, 2001). The parasite colonizes predominantly the upper small intestine, where it attaches to the surface of the epithelium and undergoes asexual replication, but does not penetrate the epithelial barrier. Symptomatic disease is characterized by watery diarrhea, abdominal cramps, bloating, malabsorption, and weight loss. Infection is generally self-limiting, but can become persistent and severe in high-risk groups, especially young children and pregnant women, and re-infections occur frequently in endemic areas (Lengerich, et al., 1994).

The normally self-limiting course of *Giardia* infection in humans, and studies in murine infection models, demonstrate that effective immune defenses exist against the parasite, yet they remain relatively poorly understood (Eckmann, 2003; Solaymani-Mohammadi and Singer, 2010). The parasite is located in the intestinal lumen and at the epithelial surface, suggesting that effective host defenses must be active at those “off-shore” sites. One important defensive mechanism that protects the epithelial surface is secretory IgA, which is produced by plasma cells in the lamina propria and transported to the lumen via the polymeric immunoglobulin receptor (pIgR) expressed on epithelial cells. Several murine studies have demonstrated essential functions of B cells (Stager and Muller, 1997), secretory IgA (Langford, et al., 2002), and pIgR (Davids, et al., 2006) in controlling parasite burden and eliminating infection. In contrast, in other mouse models, B cells were not required for clearance (Singer and Nash, 2000), suggesting that IgA can be dispensable in anti-giardial defense and that antibody-independent effectors such as α -defensins may be important (Tako, et al., 2013). In humans, giardiasis is associated with hypogammaglobulinemia due to common variable immunodeficiency or X-linked agammaglobulinemia (Agarwal and Mayer, 2013; Stark, et al., 2009), whereas the association between selective IgA deficiency and infection is mostly anecdotal and has not been systematically investigated (Eren, et al., 2007; Fisher, et al., 1975). Independent of secretory IgA, increased intestinal motility has been shown to contribute to clearance of *Giardia* (Andersen, et al., 2006; Li, et al., 2006),

presumably by reducing the chances of the parasite to attach to the epithelium and resist the luminal bulk flow.

Beyond direct effector mechanisms, several immune cells and regulators are known to be involved in anti-*Giardia* immune defense. Mast cells and CD4⁺ T cells, but not CD8⁺ T cells, are required for clearing *Giardia* infection (Heyworth, et al., 1987; Li, et al., 2004). CD4⁺ T cells may act in part by controlling anti-*Giardia* IgA responses (Heyworth, 1989), while their functions are not related to classical Th1 or Th2 subsets, since their signature cytokines, IFN- γ or IL-4, play no role in immune defense (Singer and Nash, 2000). In contrast, IL-6 is important in *Giardia* clearance (Bienz, et al., 2003; Zhou, et al., 2003). The cytokine appears to act by promoting dendritic cell functions during infection (Kamda, et al., 2012), although it has many other activities, including activation of neutrophils and monocytes, enhancement of follicular helper T cell responses, and stimulation of B cell proliferation and antibody production (Eto, et al., 2011; Mihara, et al., 2012). IL-6 is also a key inducer of Th17 cell responses and IL-17 production by innate and adaptive immune cells (Bettelli, et al., 2006; Geddes, et al., 2011; Mangan, et al., 2006; Passos, et al., 2010; Taylor, et al., 2014).

The IL-17 family of cytokines has six members (A–F), of which IL-17A is the founding and best studied one. IL-17A was initially identified as a pro-inflammatory cytokine that is produced by Th17 cells and stimulates neutrophils recruitment and production of inflammatory factors (Kolls and Khader, 2010; Onishi and Gaffen, 2010). In addition, IL-17A induces secretion of antimicrobial peptides (Onishi and Gaffen, 2010) and regulates intestinal pIgR expression and IgA production (Cao, et al., 2012; Hirota, et al., 2013). The cytokine can have both protective and pathogenic effects in different infection and inflammation models and organ systems (Onishi and Gaffen, 2010). In the intestinal tract, IL-17A is required for protective immunity against *Helicobacter pylori* (Algood, et al., 2009), *Citrobacter rodentium* (Ishigame, et al., 2009), *Salmonella enterica* serovar Typhimurium (Mayuzumi, et al., 2010; Raffatellu, et al., 2008), and enteroaggregative *Escherichia coli* (Philipson, et al., 2013), indicating that IL-17A is an important regulator of mucosal immune defenses associated with mucosal inflammation. Although *Giardia* infection is typically devoid of acute inflammatory events (Oberhuber, et al., 1997), a recent study found that IL-17A contributes to clearance of the murine pathogen *Giardia muris* (Dreesen, et al., 2014). The cellular source of IL-17A and the involvement of specific host defenses remain unknown. The present studies were conducted to elucidate the cellular requirements of IL-17A expression and signaling, and to determine how IL-17A signaling can impact intestinal defenses against different *Giardia* species.

MATERIALS AND METHODS

Mice and infection protocols

C57BL/6 (wild-type), *Rag2*^{-/-} and *Cd4*^{-/-} mice (The Jackson Laboratory), *Il17a*^{-/-} (Nakae, et al., 2002) and *Il17ra*^{-/-} (Ye, et al., 2001) were housed under specific pathogen-free conditions. At 8–12 weeks of age, mice were infected by oral gavage with either *G. muris* cysts (10⁴ cysts/mouse) or *G. lamblia* GS/M trophozoites (10⁶ trophozoites/mouse) as previously described (Davids, et al., 2006). Parasite loads were assessed at different times after infection by enumerating trophozoites in the small intestine (Langford, et al., 2002).

Animals were housed according to institutional guidelines, as detailed in the current Guide for the Care and Use of Laboratory Animals. All animal studies were reviewed and approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Bone marrow chimeras

Eight- to ten-week-old wild-type, *Il17a*^{-/-}, and *Il17ra*^{-/-} recipient mice received a single dose of 9 Gy total body irradiation from a ¹³⁷Cesium source. Eight hours later, mice were intravenously injected with 4–6 × 10⁶ bone marrow cells from sex-matched wild-type, *Il17a*^{-/-}, and *Il17ra*^{-/-} donor mice, and then rested for 8 weeks before use.

Analysis of mRNA levels

Whole tissue was collected from the mid-third region of the small intestine, snap-frozen, and stored at –80°C until use. Total RNA was isolated from the tissue using TRIzol reagent (Life Technologies). RNA samples were treated with Turbo DNA-free (Ambion), assessed for quantity and purity using a NanoDrop spectrophotometer (NanoDrop Technologies), and reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Microarray analysis was performed to assess changes in expression of IL-17 family member genes after infection. Total RNA was processed and hybridized to CodeLink Mouse Whole Genome Bioarrays (carrying 34,790 unique probes; GE Healthcare) as described before (Spehlmann, et al., 2009). Hybridized slides were stained with streptavidin-Alexa 647 conjugate (Invitrogen/Molecular Probes) and scanned at 10 μm resolution with an Axon 4000B Scanner (Molecular Devices). Background correction was performed using Codelink software. Assays were performed on RNA collected from uninfected mice (Week 0) and at weeks 1, 2, and 3 post-infection. For each array, RNA was pooled from six female C57BL/6 mice. Microarray studies were also performed to identify genes differentially expressed between C57BL/6 mice and *Il17ra*^{-/-} mice. For these studies, pooled total RNAs (six female mice/group) were analyzed using the Whole Mouse Gene Expression Microarray (4 × 44,000; Agilent Technologies), following the manufacturer's protocols. Intensities were normalized and the means of array replicates were calculated. The microarray data were deposited with the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information under accession number GSE68868.

Selected microarray data were confirmed by quantitative PCR analysis (qPCR) using Mesa Green 2 × SYBR mix (Eurogentc) in a real-time PCR machine. Primers and expected PCR product sizes were as follows: IL-17A, 5'- ACT ACC TCA ACC GTT CCA CG-3' (sense), 5'-TTC CCT CCG CAT TGA CAC AG-3' (antisense), 120 bp; IL-17E, 5'- CAG CAA AGA GCA AGA ACC CC-3' (sense), 5'- ACC CGA TTC AAG TCC CTG TC-3' (antisense), 180 bp; IL-17F, 5'- TGA AGT GCA CCC GTG AAA CA-3' (sense), 5'- CTG CTT TGG GGT TCT TCC GA-3' (antisense), 102 bp; GAPDH, 5'-TGT GAT GGG TGT GAA CCA CGA CAA-3' (sense), 5'-AGT GAT GGC ATG GAC TGT GGT CAT-3' (antisense), 209 bp. Relative changes in target mRNA levels were calculated by the 2^{-Ct} method, with GAPDH mRNA as the reference standard (CT values for GAPDH varied by <1 between different samples and experiments).

Isolation of epithelial and lamina propria cells

The mid-third region of the small intestine was opened, rinsed in PBS, and cut into 5 mm pieces, which were incubated in HBSS (without Ca and Mg) with 5 mM EDTA, 5% FCS, 10 mM HEPES (pH 7.3) twice for 20 min each at 37°C with gentle shaking. Detached cells within the epithelial compartment were passed through a nylon mesh strainer and collected by centrifugation. Cells were kept on ice prior to staining (see below). The remaining tissue pieces were washed in PBS, diced, and incubated three times at 37°C for 20 min each in RPMI 1640 medium containing 1 mg/ml collagenase D (Roche Applied Science) and 100 µg/ml DNase I (Worthington Biochemical). After each incubation, tissue suspensions were vortexed and passed through a nylon strainer. Cells from all digestions were combined and collected by centrifugation.

Flow cytometry and cell sorting

Isolated lamina propria cells were stimulated for 6 h with 50 ng/ml phorbol myristate acetate and 750 ng/ml ionomycin in complete RPMI 1640 medium at 37°C. GolgiStop (BD Biosciences) was added for the last 2 h of incubation. Stimulated cells were washed and stained with anti-CD4 PeCy5, fixed and permeabilized, and stained with PE-labeled anti-IL-17A. All antibodies were purchased from eBioscience. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) followed by data analysis with FlowJo software (TreeStar Inc.). For cell sorting, isolated cells were washed in PBS, FcR-blocked with anti-CD16/CD32 Mab (eBioscience) prior to incubation with anti-CD45 FITC and anti-EpCAM PE in PBS with 1% BSA for 30 min at 4°C. After washing in PBS, cells were resuspended in RPMI 1640 supplemented with 5% FBS and 1% penicillin/streptomycin, and sorted using a MoFLO XDP cell sorter (Beckman Coulter).

ELISA

For quantitation of tissue IL-17A levels, the mid-third region of the small intestine was divided into quadrants, and 0.5 cm long pieces were excised, snap-frozen, and stored at -80°C until use. The tissue was weighed and homogenized in PBS containing 1% Triton X-100 and Complete Protease Inhibitor (Roche). Following centrifugation, supernatants were collected and assayed by ELISA (Mouse IL-17A DuoSet, R&D Systems). Results were normalized to wet weight of tissue.

To measure fecal antibodies, stool pellets were collected, homogenized in PBS containing 0.04 mg/ml soybean trypsin inhibitor, 20 mM EDTA, and 2 mM PMSF, and centrifuged to remove bacteria and debris (Cong, et al., 1998). Two-fold dilutions in PBS were added to 96-well plates coated with 1 µg/ml anti-IgA (Kirkegaard & Perry Labs), and incubated for 2 h at room temperature. Biotinylated anti-IgA (KPL; 0.25 µg/ml) was added for 1 h, followed by incubation with HRP-conjugated streptavidin. TMB substrate was used to visualize specific antibody binding. Optical density (OD) was determined at 450 nm, and results were normalized to the total protein concentrations determined by BCA assay (BioRad). We found in preliminary studies that protein levels are stable between individual animals (8.7 ± 2.1 µg protein/mg feces; n=5) and are therefore suitable for standardization.

To determine anti-*Giardia* IgA titers in serum, microtiter plates were coated with whole trophozoites by overnight drying and glutaraldehyde fixation as described (Davids, et al., 2006). After quenching with 0.15 M glycine and blocking with 5% dried nonfat milk and 1% goat serum in PBS, plates were incubated with 1:10 diluted sera (in PBS with 1% BSA) for 1 h at room temperature, washed with PBS, and further incubated with HRP-labeled goat anti-mouse IgA (Southern Biotech; 1:1,000 dilution in 1% goat serum in PBS) for 1 h at room temperature. Bound HRP was detected with H₂O₂ and TMB in 0.1 M sodium acetate buffer (pH 6.0), followed by addition of 1.2 M sulfuric acid and OD reading at 450 nm.

Assessment of intestinal motility

Intestinal motility was assessed by the transit of carmine dye through the intestine (Andersen, et al., 2006). Mice were fasted overnight and given a single oral dose of 0.2 ml of carmine solution (6% carmine dye and 5% gum arabic in PBS). Twenty minutes later, the small intestine was removed, and the position of the carmine dye front and the length of the entire small intestine were recorded. The distance traveled by the dye front was expressed as a percentage of the entire length of the small intestine.

Statistical analysis

Trophozoite numbers were log₁₀ transformed, and mean and SEM were calculated. Samples without detectable trophozoites were assigned a log value of 3, which is equivalent to half of the detection limit for the assay (which was ~2 × 10³ trophozoites/small intestine). Mann-Whitney rank sum test was used to assess differences in trophozoite counts between groups of mice. Student's *t* test was used for all other comparisons. Differences with a *p*-value of <0.05 were considered significant.

RESULTS

G. muris infection induces intestinal IL-17A expression

To begin to define the role of IL-17 in the mucosal response to *Giardia* infection, we inoculated adult C57BL/6 mice with *G. muris* cysts by the natural oral route. Maximal trophozoite numbers in this model are observed by one week in the small intestine, which is followed by gradual clearance, with a >100 fold reduction in the infectious load by three weeks after infection (Langford, et al., 2002). Total RNA was extracted from the mucosa of the middle third of the small intestine (where the majority of *Giardia* trophozoites are localized) at different times after infection, and assayed for expression of IL-17 family members by microarray analysis. Levels of IL-17A mRNA increased after one week and peaked after two weeks (Fig. 1A). IL-17F, which is structurally and functionally related to IL-17A, was also induced two weeks after infection, but the increase was more modest compared to IL-17A (Fig. 1A). Other IL-17 cytokine family members, IL-17B, IL-17C, and IL-17D, were only minimally or not at all altered after infection (Fig. 1A).

Because IL-17A was the most strongly induced IL-17 family member after infection, we focused on this cytokine in subsequent studies. Increased intestinal expression was confirmed by qPCR, with maximal induction again seen after two weeks (Fig. 1B). Moreover, IL-17A protein levels were significantly elevated in tissue extracts after two

weeks (Fig. 1C). Together, these results demonstrate that *G. muris* infection induces intestinal expression of IL-17A, and to a lesser extent IL-17F, with peak levels at two weeks, a time that corresponds to the maximal immune response to infection and the greatest reduction in parasite load.

Cellular sources of IL-17A after *G. muris* infection

IL-17A is the signature cytokine of Th17 cells. To determine whether they were responsible for the IL-17A response after infection, we prepared single-cell suspensions from the lamina propria of the small intestine and analyzed the CD4⁺ T cell population for IL-17A production by flow cytometry. Infection induced a moderate (2–3 fold) increase in IL-17A⁺ CD4⁺ T cells after two weeks (Fig. 2A), suggesting that Th17 cells in the lamina propria account for some but perhaps not all of the observed increase in IL-17A expression after infection.

To determine whether other cells might also be involved in the IL-17A response, we isolated cells from the epithelial compartment and determined cytokine expression by qPCR. A marked increase in IL-17A was observed in the cells by two weeks after infection (Fig. 2B). Furthermore, flow cytometric analysis of these cells demonstrated IL-17A induction among the CD45⁺ cells, suggesting that IL-17A was increased in leukocytes within the epithelial compartment (Fig. 2C,D). Because Th17 cells are not known to be present in the epithelial compartment, we speculated that other leukocytes, such as innate immune cells (Cua and Tato, 2010), may be involved in the IL-17A response to infection. To address this possibility, we infected CD4 deficient (*Cd4*^{-/-}) mice, which lack Th17 and other Th subsets, and cannot clear *Giardia* infection (Solaymani-Mohammadi and Singer, 2010), and mice deficient in recombination-activating gene 2 (*Rag2*^{-/-}), which lack all T and B cells but have intact innate immune cells (Shinkai, et al., 1992). Marked induction of intestinal IL-17A expression was observed after infection in both groups of mutant mice (Fig. 2E). Collectively, these data suggest that non-Th17 leukocytes, probably innate immune cells residing within the small intestinal epithelium, contribute to the intestinal IL-17A response after *G. muris* infection.

IL-17A deficiency impairs *G. muris* clearance

We next sought to determine the physiological function of IL-17A in host defense against *G. muris*. Mice deficient for IL-17A (*Il17a*^{-/-}) were infected with *G. muris* cysts, and trophozoite numbers were determined in the small intestine at different times after infection. Parasite levels were not significantly different from wild-type controls after one week, but *Il17a*^{-/-} mice had a marked clearance defect thereafter, with 100- to 1,000-fold higher trophozoite numbers at week 7 (Fig. 3A). Furthermore, IL-17A production by hematopoietic cells was important for mediating its functions in host protection against *Giardia*, because bone marrow chimeric mice lacking IL-17A in hematopoietic cells displayed a significant defect in clearing *G. muris* after 7 weeks, whereas chimeric mice lacking IL-17A in non-hematopoietic cells had only a modest clearance defect that did not reach significance after 7 weeks (Fig. 3B). These data provide functional support for our earlier observation that Th17 cells as well as non-Th17, innate immune cells are responsible for the IL-17A response to infection.

Role of IL-17A in host defense against *G. lamblia*

To expand the relevance of our findings, we tested another *Giardia* species, *G. lamblia*, which is a natural human pathogen. After oral infection of wild-type mice with *G. lamblia* GS/M trophozoites, small intestinal IL-17A mRNA levels were significantly increased 5 days after infection and remained elevated after two weeks (Fig. 4A; note that *G. lamblia* is known to have an accelerated time course of infection compared to *G. muris*). No significant changes were observed in IL-17E or IL-17F mRNA expression (with relative peak levels of 1.5 and 1.4, respectively, occurring after 5 days compared to uninfected controls). Oral infection of *Il17a*^{-/-} and wild-type mice with *G. lamblia* GS/M revealed similar parasite numbers early (day 5) after infection, but *Il17a*^{-/-} mice were markedly impaired in their ability to clear *G. lamblia* after two weeks (Fig. 4B). Together, these results indicate that IL-17A is critical in immune defense against two different *Giardia* species, including the human pathogen *G. lamblia*.

Importance of IL-17RA in host defense against *G. muris*

IL-17A has multiple effects on different cell types, including epithelial cells, endothelial cells, lymphocytes and granulocytes (Onishi and Gaffen, 2010), raising the question which of these may be important for mediating its functions in anti-*Giardia* defense. To begin to address this question, we utilized mice lacking a critical subunit, IL-17RA, of the cognate IL-17 receptor (Song and Qian, 2013). IL-17RA-deficient (*Il17ra*^{-/-}) mice were strongly compromised in their ability to control and clear infection, as demonstrated by >9,000-fold higher *G. muris* numbers in the small intestine after 7 weeks compared to wild-type controls (Fig. 5A). Next, we generated bone marrow chimeric mice deficient in IL-17RA expression on either hematopoietic or non-hematopoietic cells. IL-17RA deficient mice reconstituted with wild-type hematopoietic cells exhibited no impairment in *G. muris* elimination, whereas wild-type mice reconstituted with IL-17RA-deficient hematopoietic cells showed a significant clearance defect after 5 weeks (Fig. 5B). These results indicate that IL-17RA signaling in hematopoietic cells is required for mediating immune protection against *G. muris*.

IL-17A deficiency compromises IgA transport into the lumen but not intestinal motility

Having established that IL-17A and IL-17RA signaling are required for *Giardia* clearance, we sought to elucidate the underlying mechanisms. To date, only a small number of effectors have been identified in anti-*Giardia* immune defense, including intestinal hypermotility (Andersen, et al., 2006; Li, et al., 2006), secretory IgA (Davids, et al., 2006; Langford, et al., 2002), α -defensins (Aley, et al., 1994; Tako, et al., 2013), and nitric oxide (NO) (Eckmann, et al., 2000; Tako, et al., 2013), although their relative importance under different conditions remains to be clarified (Langford, et al., 2002; Singer and Nash, 2000; Solaymani-Mohammadi and Singer, 2010). To address the potential role of intestinal hypermotility in IL-17 dependent defense, we assayed small intestinal motility in *Il17a*^{-/-} and wild-type mice after *G. muris* infection. Both strains of mice displayed enhanced small intestinal transit two weeks after infection, but no significant difference was observed between the groups (Fig. 6A). Thus, intestinal hypermotility was unlikely to be responsible for impaired parasite clearance in *Il17a*^{-/-} mice.

We then examined secretory IgA as another potential effector mechanism. Fecal extracts were analyzed for IgA levels by ELISA. *Il17a*^{-/-} mice had lower levels of fecal IgA prior to and after *G. muris* infection in comparison to wild-type controls (Fig. 6B). In contrast, levels of anti-giardial IgA in the serum were higher in *Il17a*^{-/-} mice compared to controls (median OD₄₅₀ = 0.50 vs. 0.15, respectively; 1:10 dilution, n=3–4 mice/group). These data indicate that the loss of fecal IgA in *Il17a*^{-/-} mice was not related to diminished induction of antigen-specific IgA, but probably caused by impaired IgA delivery into the intestinal lumen due to reduced pIgR expression in the absence of IL-17A (Cao, et al., 2012; Davids, et al., 2006).

To determine whether other known mechanisms may be involved in IL-17A-dependent anti-giardial defense, we examined the expression of α -defensins and NO synthase (NOS) 2 (Tako, et al., 2013). Microarray analysis showed that the absence of IL-17RA signaling had no differential impact on the expression of genes directly involved in NO synthesis, or genes whose products regulate NO production or are affected by it (Fig. 6C). Minor differences were observed in the expression of matrix metalloproteinase (MMP) 7 (also known as matrilysin), which is required for activating epithelial α -defensins (Wilson, et al., 1999), and several other members of the MMP family of endopeptidases potentially involved in host defense (Fig. 6D). Thus, differential MMP7-dependent activation of α -defensins may make a minor contribution to the IL-17A functions in this system.

Further analysis of the microarray data revealed that wild-type mice had an increase in β -defensin 1 expression that was absent in *Il17ra*^{-/-} mice after infection (Fig. 6D). In addition, several other genes were preferentially induced in wild-type but not *Il17ra*^{-/-} mice (Table 1). Interestingly, the strongest induction was observed for the genes *Retnlb*, *Saa1*, and *Saa2*, whose respective products, resistin-like molecule β , serum amyloid A1, and serum amyloid A2, contribute to antimicrobial defense (Eckhardt, et al., 2010; Herbert, et al., 2009). Conversely, a number of genes were preferentially induced in *Giardia*-infected *Il17ra*^{-/-} mice, which shows that the absence of IL-17R signaling can lead to compensatory gene expression (Table 1). Furthermore, several genes were similarly induced in both groups of mice, underlining the specificity of the observed expression differences (Table 1). Taken together, our findings suggest that IL-17A signaling is not required for intestinal hypermotility or NOS expression after infection, but is critical for secretion of protective IgA into the intestinal lumen and expression of several other candidate effectors.

DISCUSSION

IL-17A is a multi-functional cytokine involved in regulating mucosal homeostasis, inflammation, and immunity (Kolls and Khader, 2010; Onishi and Gaffen, 2010). Although the cytokine has been shown to be critical for host protection against various pulmonary and urogenital pathogens, efforts to determine the importance of IL-17A in defense against enteric pathogens have yielded varying results (Cypowyj, et al., 2012). Our studies, extending the findings in a recent report (Dreesen, et al., 2014), demonstrate that IL-17A plays a critical role in mucosal defense against both *Giardia* species, *G. muris* and *G. lamblia*. Protective functions of IL-17A have also been reported for several enteric bacterial pathogens, including *C. rodentium* and *S. Typhimurium* (Ishigame, et al., 2009; Mayuzumi,

et al., 2010; Raffatellu, et al., 2008), although these pathogens differ substantially from *Giardia* in their interactions with the host. Both bacterial species are invasive and cause inflammatory responses in the intestinal tract (Mittrucker and Kaufmann, 2000; Mundy, et al., 2005). In contrast, *Giardia* is non-invasive and acute infection causes only minimal inflammation (Eckmann, 2003; Oberhuber, et al., 1997). Despite these differences, our data, together with prior findings (Dreesen, et al., 2014; Ishigame, et al., 2009; Mayuzumi, et al., 2010; Raffatellu, et al., 2008), demonstrate that IL-17A is a critical mediator of intestinal defense against both bacterial and protozoan pathogens.

The timing and magnitude of the IL-17A response to *Giardia* were compatible with the kinetics of a prototypic adaptive Th17 immune response, and Th17 cell numbers were accordingly increased after infection. However, using Rag-2-deficient mice that lack T and B cells, we discovered that innate immune cells residing within the epithelial compartment are additional producers of IL-17A in response to infection. Consistent with this observation, several subsets of Rag 2-independent cells, including innate lymphoid cells, neutrophils, NK cells, and mast cells, have been shown to express IL-17A in the mucosa under different conditions (Cua and Tato, 2010). The localization of these cells within the epithelium may enable them to interact with *Giardia* attached to the apical epithelial surface, or with factors released locally by the parasite, and respond by activating IL-17A expression. This idea is supported by our observation that the IL-17A response was localized to the site of infection and not observed in mesenteric lymph nodes (data not shown). Expression of the cytokine in most innate immune cells, except mast cells, is dependent on IL-6 and the nuclear receptor ROR γ t (Cua and Tato, 2010). Consistent with this, we found in preliminary studies that IL-6-deficient mice and mice lacking functional ROR γ t failed to induce IL-17A expression in response to *Giardia* infection, indicating that the canonical pathway is involved in IL-17A induction in this situation. This observation might also provide a connection between the known role of IL-6 in anti-*giardial* defense (Bienz, et al., 2003; Zhou, et al., 2003) and our findings on IL-17A involvement.

Deficiency in IL-17A signaling had little impact on parasite burden in the first week after *Giardia* infection, suggesting that innate defenses, such as antimicrobial peptides produced by Paneth cells or neutrophils, which may control initial infection (Aley, et al., 1994), are not IL-17A dependent. Instead, the cytokine had a critical function 2–3 weeks after infection, suggesting that it mediates protection through controlling adaptive immune responses. Indeed, IL-17RA expression on bone marrow-derived hematopoietic cells was required for parasite clearance. Although the underlying effector mechanisms are not known, IL-17A was not important for controlling intestinal NOS expression, which can contribute to anti-*giardial* defense (Tako, et al., 2013), or for mediating intestinal hypermotility, a key effector of anti-*giardial* immune defense (Andersen, et al., 2006; Li, et al., 2006). This is in contrast to the findings with another lumen-dwelling parasite, the nematode *Trichinella spiralis*, where IL-17A promotes hypermotility during infection (Fu, et al., 2009), underlining that the actions of IL-17A can be mediated by different immune effectors.

Another important, albeit controversial, effector mechanism of anti-*giardial* defense is secretory IgA (Davids, et al., 2006; Langford, et al., 2002; Singer and Nash, 2000). Our data

suggest that IL-17A is required for optimal mucosal IgA secretion, because fecal IgA was markedly diminished in IL-17A-deficient mice, while antigen-specific IgA was increased in the serum. This observation is consistent with the observation that the cytokine regulates expression of the IgA transport receptor, pIgR, required for IgA secretion into the intestinal lumen (Cao, et al., 2012; Hirota, et al., 2013), although the regulation is predictably indirect since we found that the cognate IL-17 receptor is required on hematopoietic but not non-hematopoietic cells, such as pIgR-expressing epithelial cells. Beyond IgA, we found that IL-17A signaling may play a minor role in controlling MMP7 dependent α -defensin actions, which have been shown to contribute to controlling *Giardia* infection (Tako, et al., 2013), but is critical for expression of several other potential effectors, including β -defensin 1 and resistin-like molecule β , which can contribute to antimicrobial defenses in the intestine (Eckhardt, et al., 2010; Herbert, et al., 2009), although this remains to be established for *Giardia*.

The observed anti-giardial function of IL-17A, which is apparently active in the absence of mucosal inflammation (Oberhuber, et al., 1997), broadens the understanding of the complexity of IL-17-mediated immunological protection against enteric infections. Importantly, we demonstrated that a lumen-dwelling pathogen can activate immune cells in the mucosa and within the epithelium to produce IL-17A, which helps to orchestrate protective intestinal immune defenses. The insights gained from these studies provide new mechanistic ideas about the pathophysiology and immunology of giardiasis, and are expected to be important for designing future mucosal vaccination strategies against this common worldwide cause of diarrheal disease.

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Abbreviations

pIgR	polymeric immunoglobulin receptor
NO	nitric oxide
Rag 2	recombination-activating gene 2

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Highlights

- The protozoan parasite *Giardia* dwells in the intestinal lumen and causes diarrhea
- Mucosal defenses exist against the parasite but remain poorly understood
- We found that intestinal IL-17A is induced by infection and needed for eradication
- IL-17A signaling promotes transport of protective IgA into the intestinal lumen
- It also induces other possible effectors, β -defensin 1 and resistin-like molecule β

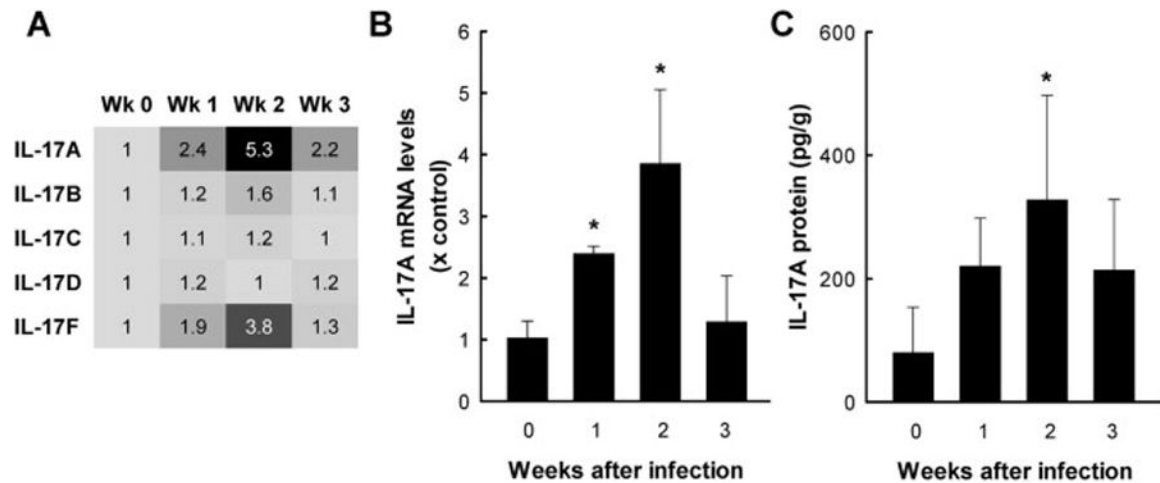


Figure 1. Increased IL-17A expression after *G. muris* infection

C57BL/6 mice were orally infected with 10^4 *G. muris* cysts, and the small intestine was removed at the indicated times. Uninfected mice were used as controls (Week 0). (A) Heat map of IL-17 family member mRNA expression determined by microarray analysis. Numbers represent the fold increases relative to uninfected controls (Wk 0). Data represent RNA pooled from 6 mice/group. (B) mRNA expression was analyzed by qPCR for IL-17A. Levels are presented as mean + SE of three separate experiments, which each experiment performed on total RNA pooled from 3–5 mice/time point. (C) Levels of IL-17A protein were assessed by ELISA in tissue extracts of the small intestine. Data represent mean + SE of 5–6 mice/time point. Data are representative of results from two independent experiments. * $p < 0.05$ relative to uninfected controls.

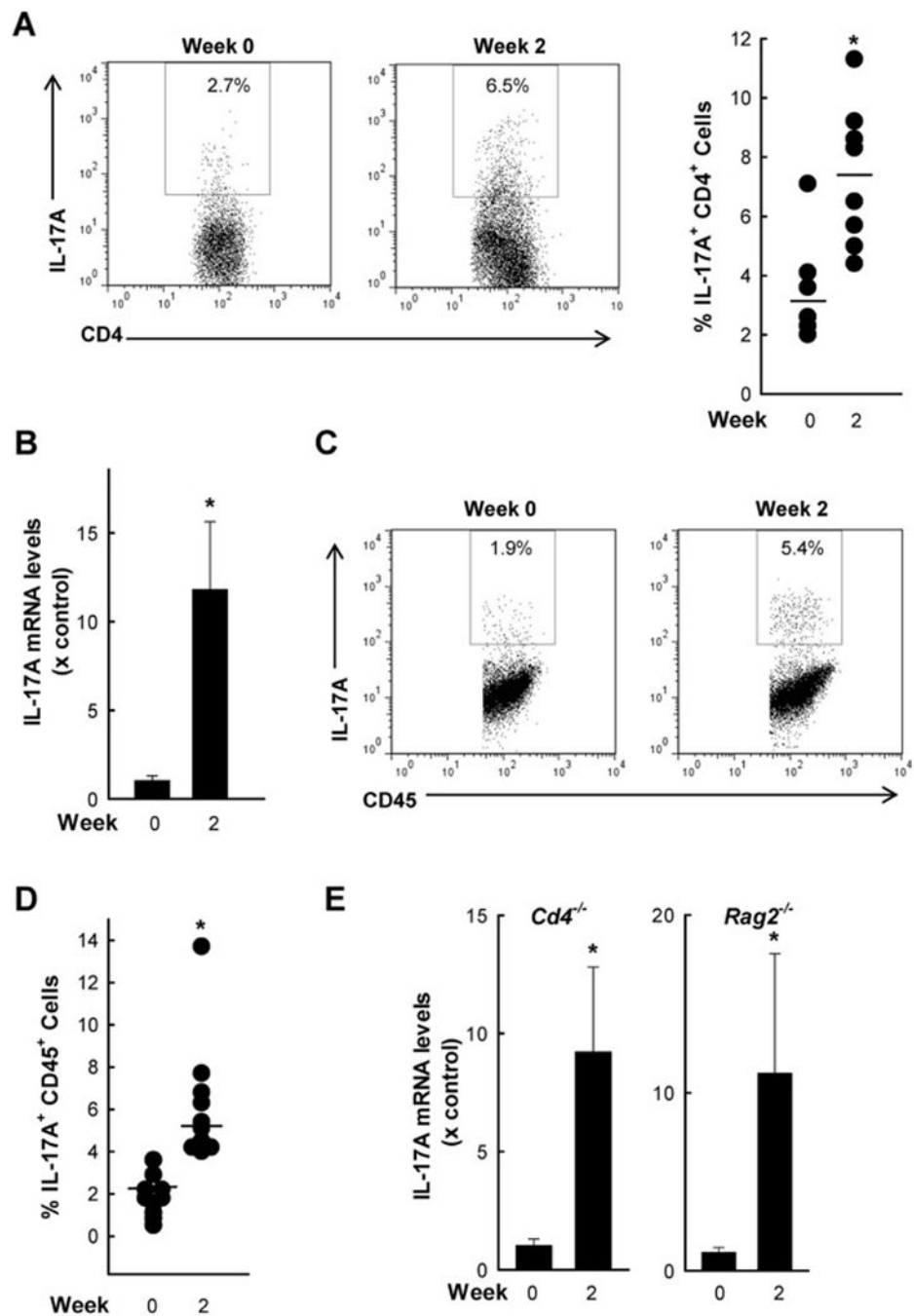


Figure 2. Cellular sources of intestinal IL-17A in response to *G. muris* infection

C57 mice were orally infected with *G. muris* cysts and examined after two weeks.

Uninfected mice were used as a control. (A) CD4⁺ T cells were isolated from the lamina propria of the small intestine and analyzed by flow cytometry for CD4 and IL-17A expression. Representative FACS dot plots (left) and percentages of IL-17A producing CD4⁺ cells of individual mice (right). Horizontal bars indicate means. (B–D) Cells were isolated from the epithelial compartment of the small intestine of groups of 3–4 mice, and assessed for IL-17A mRNA expression by qPCR (B; bar graphs represent mean + SE for

three independent experiments) and by flow cytometry for co-staining of IL-17A and CD45 (representative FACS dot plot in C, and data on cell percentages from 8–10 individual mice in D; Horizontal bars indicate means). (E) Quantitative PCR analysis of IL-17A mRNA in the small intestine of CD4-deficient (*Cd4^{-/-}*) and Rag 2-deficient (*Rag2^{-/-}*) mice infected with *G muris*. Bar graphs show fold induction (mean + SE; n=3–4) compared to uninfected controls. Data from A–D are representative of the results from three independent experiments; and data from E is representative of the results from two independent experiments; **p* <0.05 compared to uninfected controls.

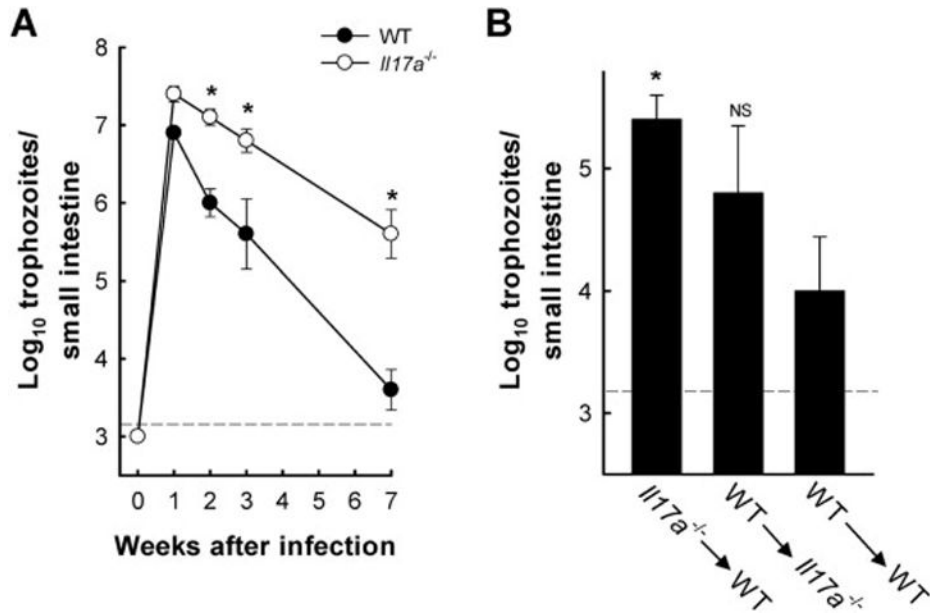


Figure 3. *G. muris* clearance is impaired in the absence of IL-17A

(A) IL-17A-deficient (open circles) and wild-type (closed circles) mice were infected with *G. muris*, and trophozoite numbers in the small intestine were determined at the indicated times. Data are mean ± SE (n = 6 mice/time point) from three independent experiments; **p* < 0.05 compared to wild-type controls. (B) Bone marrow chimeric mice were generated by isolating and transferring bone marrow cells from *Il17a*^{-/-} mice into C57BL/6 (WT) mice (*Il17a*^{-/-} → WT), from WT to *Il17a*^{-/-} mice (WT → *Il17a*^{-/-}), or as controls from WT to WT mice (WT → WT). Following an 8 week reconstitution period, mice were infected orally with *G. muris* cysts. Trophozoite numbers were determined 7 weeks after infection. Data are mean + SE (n = 6 mice/time point) from two independent experiments; **p* < 0.05 relative to WT → WT controls. The black dashed lines depict the detection limit of the assays.

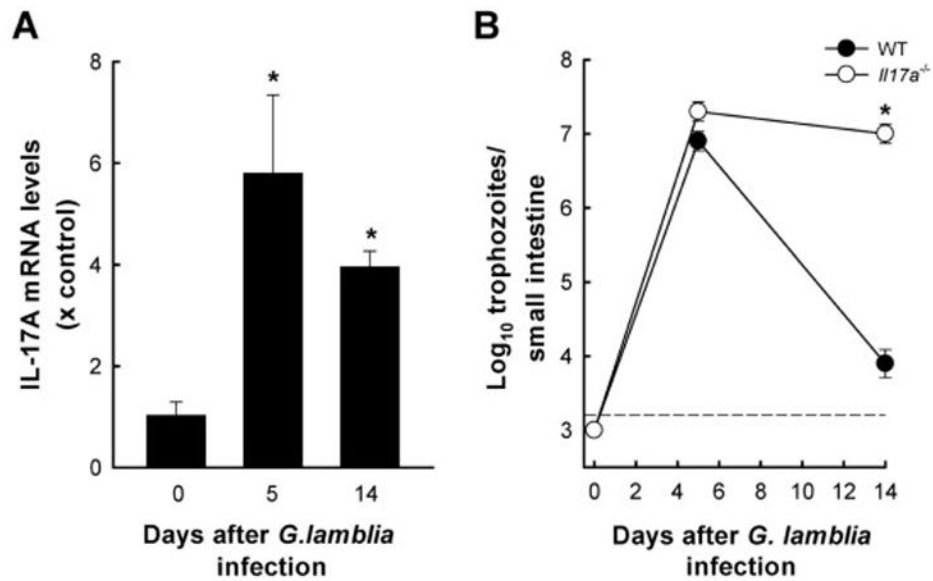


Figure 4. IL-17A deficiency impairs eradication of *G. lamblia*

(A) Wild-type C57BL/6 mice were orally infected with 10^6 *G. lamblia* GS/M trophozoites. Total RNA was extracted from the small intestine and analyzed for IL-17A mRNA expression by qPCR. Data are mean + SE (n=3–4 mice/time point) from two independent experiments, * $p < 0.05$ relative to uninfected controls (Day 0). (B) Mice deficient in IL-17A (*Il17a*^{-/-}, open circles) and wild-type (WT, closed circles) mice were infected with *G. lamblia*, and trophozoite numbers were determined at the indicated times. Data are mean ± SE (n=3–4 mice/time point); * $p < 0.05$ relative to wild-type controls. The black dashed line depicts the detection limit of the assay.

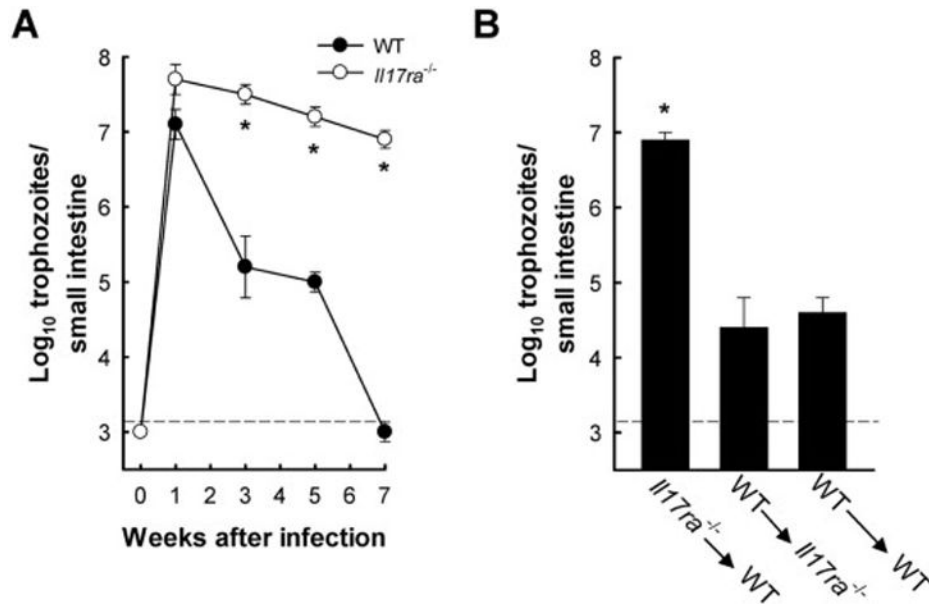


Figure 5. IL-17RA signaling in hematopoietic cells is essential for *G. muris* eradication
 (A) Mice deficient in IL-17RA (*IL17ra*^{-/-}, open circles) and wild-type (WT, closed circles) mice were infected with 10⁴ *G. muris* cysts, and trophozoite numbers in the small intestine were determined at the indicated times. Data are mean ± SE (n=3–4 mice/time point) from three independent experiments; **p*<0.05 relative to uninfected controls (Day 0). (B) Bone marrow chimeric mice were generated by isolating and transferring bone marrow cells from *IL17ra*^{-/-} mice into C57BL/6 (WT) mice (*IL17ra*^{-/-}→WT), from WT to *IL17ra*^{-/-} mice (WT→*IL17ra*^{-/-}), or as controls from WT to WT mice (WT→WT). Following an 8 week reconstitution period, mice were infected orally with *G. muris* cysts, and trophozoite numbers were determined after 5 weeks. Data are mean + SE (n = 6 mice/time point) from three independent experiments; **p*<0.05 relative to WT→WT chimeras. The black dashed lines depict the detection limit of the assays.

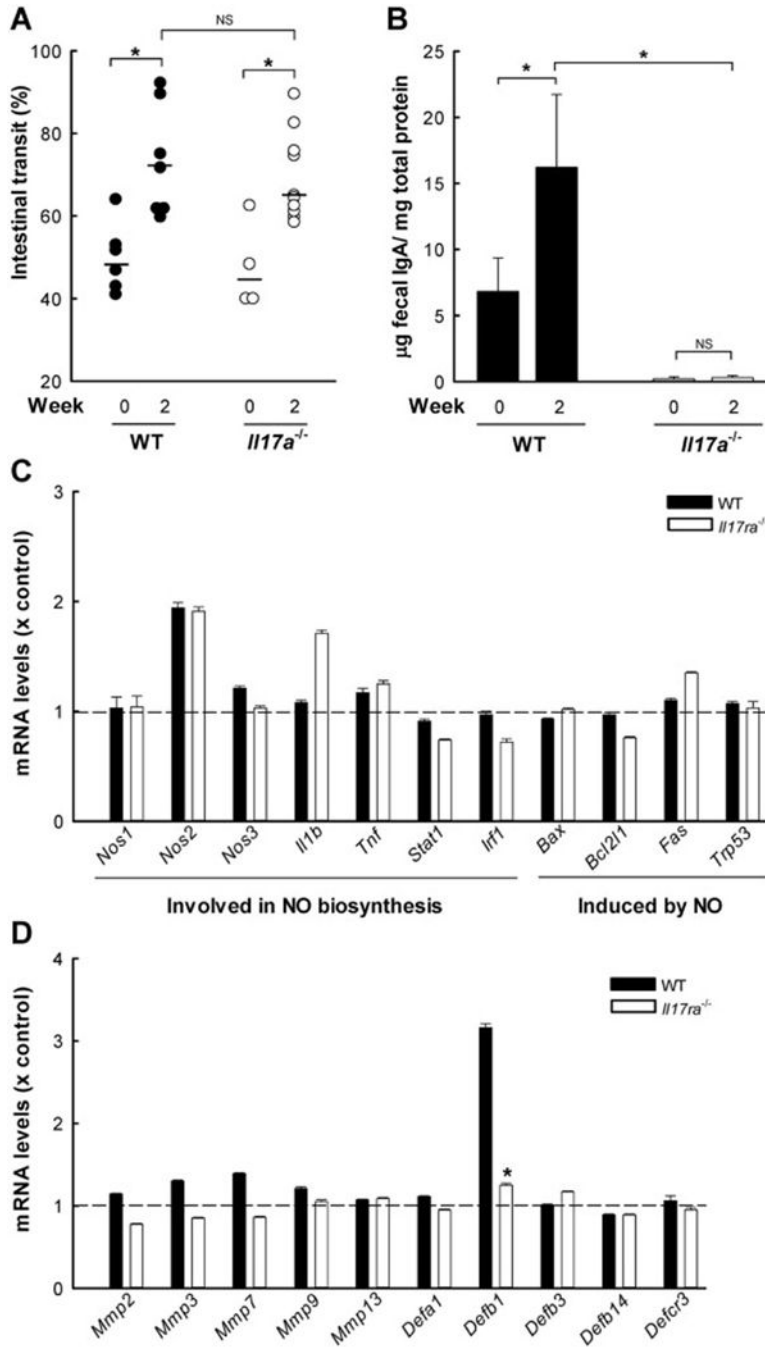


Figure 6. Mechanisms of IL-17A signaling-dependent anti-giardial defense

IL-17A-deficient (*Il17a*^{-/-}, open circles and bars in A and B), 17RA-deficient mice (*Il17ra*^{-/-}, open bars in C and D), and wild-type mice (WT, closed circles and bars in A–D) were infected with 10⁴ *G. muris* cysts or left uninfected as controls (Week 0), and analyzed two weeks after infection. (A) Small intestinal motility was evaluated by determining the distance traveled by a carmine dye-containing test meal relative to the length of the entire small intestine over a 20-minute period. Each point represents an individual animal from three independent experiments. Horizontal bars indicate medians; **p*<0.05 relative to

indicated control; NS, not significant compared to wild-type controls. (B) IgA levels were determined in stool homogenates by ELISA and normalized to total protein. Results are mean + SE (n=5–6 mice/time point) from two independent experiments; * $p < 0.05$ relative to uninfected controls. (C, D) Microarray analysis of small intestinal expression of the indicated genes after *G. muris* infection. Bars show relative expression compared to the respective uninfected control mice. Error bars represent SD for genes with array replicates (n=3–12 replicates/gene); * $p < 0.05$ relative to wild-type controls.

Table 1
Differentially expressed genes in WT and *IL17ra*^{-/-} mice two weeks after *G. muris* infection

Gene Symbol	GenBank ID	Fold Induction		Gene Product	Functions
		WT	<i>IL17ra</i> ^{-/-}		
Preferentially Induced in WT					
<i>Retnib</i>	NM_023881	77.9	5.9	Resistin-like molecule β	Hormone activity, intestinal innate immunity
<i>Saa2</i>	NM_011314	26.2	5.8	Serum amyloid A2	Chemoattractant activity, G-protein coupled receptor binding
<i>Saa1</i>	NM_009117	24.8	5.0	Serum amyloid A1	Chemoattractant activity, G-protein coupled receptor binding
U90926	U90926	21.5	1.2	Putative TNF-resistance related protein	Not known
<i>Pla2g4c</i>	NM_001004762	17.4	1.0	Phospholipase A2, group IVC	Glycerophospholipid, catabolic process
Preferentially Induced in <i>IL17ra</i>^{-/-}					
<i>Ms4a1</i>	NM_007641	1.1	20.5	Membrane spanning 4-domain, subfamily A, member 1/CD40	Development and differentiation of B cells into plasma cells
<i>Ighg</i>	BC092269	4.1	13.2	Immunoglobulin heavy chain (gamma polypeptide)	Antigen binding
<i>Blr1</i>	NM_007551	1.1	11.2	Burkitt lymphoma receptor 1/C-X-C chemokine receptor type 5 (CXCR5)	G-protein coupled receptor, has role in B cell migration
<i>Oxt</i>	NM_011025	1.7	10.4	Oxytocin	Neuromodulator, intestinal motility
<i>Tnfrsf13c</i>	NM_028075	1.3	9.2	Tumor necrosis factor receptor superfamily, member 13c/B-cell activating factor receptor (BAFF-R)	B cell regulator
Similarly Induced in WT and <i>IL17ra</i>^{-/-}					
L20961	L20961	8.2	5.8	Ig rearranged H-chain	Antigen binding
<i>S100a9</i>	NM_009114	4.2	3.0	S100 calcium binding protein A9 (calgranulin B)	Complexes with S100A8 to regulate myeloid cell function
<i>Egln3</i>	NM_028133	3.7	2.6	Egl-9 family hypoxia-inducible factor 3	Cellular oxygen sensor
Not induced in WT or <i>IL17ra</i>^{-/-}					
<i>Ccl1</i>	NM_011329	1.1	1.2	Chemokine (C-C motif) ligand 1	Chemotactic for monocytes, NK cells, immature B cells, and dendritic cells
<i>Ccl3</i>	NM_011337	0.9	0.9	Chemokine (C-C motif) ligand 3/macrophage inflammatory protein 1 α (MIP-1 α)	Recruitment and activation of polymorphonuclear leukocytes
<i>Ccl5</i>	NM_013653	0.8	0.9	Chemokine (C-C motif) ligand 5/RANTES (regulated on activation, normal T cell expressed and secreted)	Chemotactic for T cells, eosinophils, and basophils