Title: Th17 cells provide direct protective effects that limit stomach parasite burden following orogastric mucosal *Trypanosoma cruzi* infection

Short title: Th17 cells protect against mucosal *T. cruzi* infection

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Abstract

Trypanosoma cruzi is the intracellular parasite of Chagas disease, a chronic condition characterized by cardiac and gastrointestinal morbidity. Protective immunity requires CD4+ T cells, and Th1 cells and IFN-γ are important players in host defense. More recently, Th17 cells and IL-17 have been shown to exert protective functions in systemic *T. cruzi* infection. However, it remains unclear whether Th17 cells and IL-17A protect against mucosal infection, which is an important cause of human outbreaks. We found that IL-17RA knock-out (KO) mice are highly susceptible to orogastric infection, indicating an important function for this cytokine in mucosal immunity to *T. cruzi*. To investigate the specific role of Th17 cells for mucosal immunity, we reconstituted RAG1 KO mice with *T. cruzi*-specific T cell receptor transgenic Th17 cells prior to orogastric *T. cruzi* challenges. We found that Th17 cells provided protection against gastric mucosal *T. cruzi* infection, indicated by significantly lower stomach parasite burdens. *In vitro* macrophage infection assays revealed that protection by Th17 cells is reversed with IL-17A neutralization or loss of macrophage NADPH oxidase activity. Consistent with this, *in vivo*, mice lacking functional NADPH oxidase were not protected by Th17 cell transfer. These data are the first report that Th17 cells protect against mucosal *T. cruzi* infection, and identify a novel protective mechanism involving the induction of NADPH oxidase activity in macrophages by IL-17A. These studies provide important insights for Chagas vaccine development, and more broadly, increase our understanding of the diverse roles of Th17 cells in host defense.

Introduction

Chronic infection with the intracellular protozoan parasite *Trypanosoma cruzi* causes Chagas disease, a neglected tropical disease characterized by life-threatening cardiac and gastrointestinal pathology(1). The disease is endemic in Latin America with geographical spread into non-endemic areas, and it affects at least 8 million people(2). Infected reduviid “kissing bug” insects carry *T. cruzi* in their gastrointestinal tracts and deposit infectious parasites in their excreta after taking a blood meal. People become infected when parasite-containing excreta are accidentally ingested or inoculated into the eye or a break in the skin. Thus, the major routes of vector-borne transmission are mucosal and cutaneous.

During chronic *T. cruzi* infection, the parasite load is controlled but never completely eliminated. A robust T cell response is sufficient for parasite control in some models(3, 4), and lack of either CD4+ or CD8+ T cell responses increases susceptibility to infection(5-9). Among the CD4+ T cell subsets, Th1 cells have been demonstrated to protect against both systemic and mucosal infection(5, 10-12), while Th2 cells promote parasite persistence and mortality(12, 13). More recent studies have investigated the role of Th17 cells and IL-17A. We previously discovered using an adoptive cell transfer model that Th17 cells significantly reduce parasitemia and prevent mortality after a normally lethal challenge administered via subcutaneous injection of parasites (3). Other investigators have shown that mice deficient in IL-17A signaling due to genetic mutation of IL-17A (14) or its receptor(15), or through antibody neutralization(16), have increased susceptibility to an intraperitoneally administered *T. cruzi* challenge.

Despite evidence that IL-17A and Th17 cells protect against systemic parasite challenges, whether this type of response contributes to mucosal immunity is unknown. Orogastric infection has caused hundreds of outbreaks in humans(17-19). It has grown more common in recent years and is a leading route of transmission in some endemic areas(19). Oral ingestion is also considered to be a major route of transmission in other mammalian hosts such as domestic dogs, which are infected at high rates in endemic areas(20). Th17 cells are highly abundant at mucosal surfaces, especially in the intestinal gut, where they play a major role in homeostasis and immunity(21). Mainly through the secretion of IL-17A, these cells can recruit neutrophils, induce the expression of anti-microbial peptides, and upregulate factors that maintain epithelial integrity. These functions contribute to immunity in the intestinal mucosa(21) and may also be relevant for immunity in the gastric mucosa.

In this project, we investigated whether Th17 cells, previously demonstrated to confer immunity against systemic *T. cruzi* infection, can also drive immunity protective against mucosal infection. Ultimately, understanding of the full spectrum of functions of Th17 cells and IL-17A in *T. cruzi* infection will guide the development of vaccines inducing protective mucosal and systemic immunity.

Results

IL-17A signaling is important for mucosal immunity to *T. cruzi* infection

IL-17A is critical in systemic immunity against *T. cruzi* infection(14-16). To investigate how IL-17A functions in *T. cruzi* mucosal immunity, we performed orogastric infections in wild-type (WT) and IL-17RA knock-out (KO) BALB/c mice, which lack a

subunit of the heterodimeric receptor for IL-17A(21). Twelve days later, we performed hematoxylin and eosin staining of tissue sections taken where *T. cruzi* preferentially infects at the margo plicatus(22), the region of transition between the glandular corpus of the stomach and the non-glandular forestomach. At baseline, the stomachs of WT and IL-17RA KO mice were histologically similar, with the presence of well-differentiated glands (Supplemental Fig 1). After infection, only mild histological abnormalities were observed in WT mice (Fig 1A). However, infected IL-17RA KO mice exhibited pronounced inflammatory infiltrate and loss of specialized glandular cells representing atrophy(23), indicating increased susceptibility to gastric mucosal parasite challenge (Figs 1A and 1B). IL-17RA KO mice also had higher parasite burdens in the stomach and a greater proportion of infected spleen cells (Figs 1C and 1D), indicating comparably worse immune control. These data are the first evidence that IL-17 signaling plays a critical role in protective gastric mucosal immunity to *T. cruzi* infection.

Th17 cells can protect against gastric mucosal infection

Th17 cells are major CD4+ T cell producers of IL-17A. To investigate the role of Th17 cells in gastric mucosal *T. cruzi* infection, we generated Th17 cells specific for *T. cruzi.* This was done via *in vitro* Th17 differentiation of CD4+ T cells with transgenic T cell receptors (TCR Tg) recognizing an immunodominant epitope of the *T. cruzi trans-sialidase* antigen(3). We have previously characterized the phenotype and persistence of these TCR Tg Th17 cells in adoptive transfer models(3), and cells were confirmed to express the canonical Th17 cell markers RORγt and IL-17A prior to every transfer.

We transferred these TCR Tg Th17 cells into RAG1 KO mice lacking endogenous T cells, with or without *T. cruzi*-naïve polyclonal CD8+ T cells. Control

115 animals received CD8+ T cells alone or no T cell transfer. The following day, we
116 induced stomach infection via oral gavage of parasites. On day 12 post-infection,
117 previously identified as the time of peak parasite burden, we sacrificed the mice for
118 quantification of parasite burden in the stomach using qPCR and in the spleen using
119 parasite outgrowth assays (Fig 2A). Mice receiving Th17 cells with CD8+ T cells had
120 significantly reduced *T. cruzi* DNA in the stomach compared to control mice (Fig 2B). In
121 addition, these mice had a smaller proportion of infected spleen cells compared to
122 control mice, indicating significantly improved overall control of the infection (Fig 2C).
123 We previously demonstrated that in systemic infection, co-transfer of CD8+ T
124 cells is required for the protective effects of Th17 cells. Mechanistically, parasite-specific
125 Th17 cells protected by providing help to CD8+ T cells via IL-21 signaling(3).
126 Surprisingly, Th17 cells alone provided significant mucosal protection, and the addition
127 of CD8+ T cells did not improve protection (Figs 2B and 2C). These data indicate that
128 Th17-mediated mucosal protection operates via a different mechanism, involving direct
129 protective functions rather than helper effects on CD8+ T cells.
130 Th1 cells are known to provide direct protective effects that can control *T. cruzi*
131 infection independent of CD8+ T cells, primarily through IFN-γ-mediated activation of
132 macrophages(24). We next reconstituted RAG1 KO mice with either TCR Tg Th1 or
133 Th17 cells to compare the direct protective effects of these CD4+ T cell subsets. Both
134 Th1 and Th17 cells were able to confer direct protective effects against a gastric
135 challenge, as indicated by low levels of parasite DNA in the stomach after infection (Fig
136 2D). Th1 and Th17 cells similarly reduced the proportion of infected spleen cells
137 compared to control mice given no T cell transfer (Fig 2E), indicating that both Th1 and

Th17 cells protect against a mucosal *T. cruzi* infection even in a CD8+ T cell and B cell deficient environment.

Th17 cells provide direct protective effects *in vitro* via IL-17A

To investigate direct protective effects of Th17 cells, we infected macrophages with *T. cruzi in vitro*, and then co-cultured them with either parasite-specific Th1 or Th17 cells before enumerating the number of infected macrophages arising after 2 days (Fig 3A). Both Th1 or Th17 cells resulted in a significantly reduced number of infected cells (Fig 3B). Adding an anti-IL-17A neutralizing antibody partially reversed the protective effects of Th17 cells (Fig 3C), suggesting that the protection is cytokine-mediated. Consistent with this, treatment with IFN-γ or IL-17A, representing the major cytokines produced by Th1 and Th17 cells, respectively, also significantly reduced the number of infected cells in both murine (Fig 3D) and human macrophages (Fig 3E). These data confirm that Th17 cells provide direct protective effects via IL-17A and suggest a similar effect may exist in humans.

IL-17A induces NADPH oxidase activity in infected macrophages

Th1 cells prime macrophage activation for the killing of intracellular microorganisms through IFN-γ-mediated induction of iNOS, which results in the generation of microbicidal nitric oxide (NO)(10, 24). We confirmed that treatment of infected BMDMs with IFN-γ resulted in an increase in NO concentration (Fig 3F). In contrast, treatment with IL-17A had no effect on increasing NO levels over control cells (Fig 3F). Reactive oxygen species (ROS) are generated by NADPH oxidase during the phagocyte respiratory burst response, and similar to NO, they can inhibit microbial growth. To evaluate whether ROS were induced by IL-17A, we incubated IL-17A-treated

and infected BMDMs with DCFDA, a probe that can be oxidized into a fluorescent substrate. *T. cruzi* infection in the presence of IL-17A induced a 3.5-fold increase in the mean fluorescence intensity of oxidized DCFDA over uninfected cells, indicating a higher oxidation state under these conditions (Fig 3G). However, the amount of oxidized DCFDA did not increase among gp91phox KO cells lacking functional NADPH oxidase enzyme under the same conditions, reflecting the expected defect in ROS production (Fig 3G). Further, this deficiency of functional NADPH oxidase had no effect on IFN-γ-mediated protection, but reversed IL-17A-mediated protection (Fig 3H). These data indicate that direct protection by Th17 cells requires IL-17A signaling and NADPH oxidase activity.

Th17 cells induce oxidation in mucosal immune cells *in vivo*

To determine whether parasite-specific Th17 cells given by adoptive transfer could be detected in the stomach, we recovered cells from the gastric mucosa of mice given no T cells or Th17 cells after orogastric infection with *T. cruzi*. We confirmed that parasite-specific Th17 cells were present in the gastric mucosa post-infection (Fig 4A). Proportions of other immune cell subsets, including neutrophils, which can be recruited by IL-17A, were not significantly altered compared to control mice receiving no T cell transfer (Fig 4A).

We next asked whether parasite-specific Th17 cells induced NADPH oxidase activity in the gastric mucosa during *T. cruzi* infection *in vivo*. We adoptively transferred Th17 cells into mice and, then orogastrically infected them and recovered gastric mucosal cells for DCFDA staining. We determined that macrophages and neutrophils recovered from the gastric mucosa of mice given Th17 cells had significantly higher

oxidation states compared to control mice (Fig 4B), indicating an increase in reactive oxygen species. These results are consistent with phagocytic cells being the primary cell types undergoing the respiratory burst response and support the hypothesis that Th17 cells protect against orogastric infection by inducing increased expression of ROS in certain cells.

NADPH oxidase activity is required for Th17-mediated mucosal protection

Based on the *in vitro* infection assays demonstrating protection by Th17 cells operates via secretion of IL-17A and induction of NADPH oxidase in target cells, we asked if these are also required for *in vivo* protection. We administered an anti-IL-17A neutralizing antibody every other day in Th17-reconstituted and orogastrically infected mice (Supplemental Fig 2). This partially reduced serum levels of IL-17A (Supplemental Fig 2A), but it did not reverse Th17-mediated protection (Supplemental Fig 2B), likely due to the incomplete abrogation of IL-17A activity (Supplemental Fig 2A).

To further study the role of NADPH oxidase *in vivo*, we transferred parasite-specific Th17 cells into RAG1 KO and gp91phox x RAG1 double KO (dKO) mice prior to orogastric challenge. While RAG1 KO mice had significantly improved resistance to orogastric *T. cruzi* infection following Th17 cell transfer (mean 2,783 parasite mEq per 100ng gastric DNA with Th17 cell transfer, versus 12,548 without transfer, $P<0.005$), Th17 cells did not confer measurable protection in mice lacking functional NADPH oxidase (mean 12,690 parasite mEq per 100ng gastric DNA with Th17 cell transfer, versus 20,252 without transfer, $P=0.4304$). These data indicate a critical role for this enzyme in IL-17A-mediated immunity (Fig 5).

Discussion

Although Chagas disease is an important illness causing significant morbidity and mortality in the Western hemisphere, few truly effective treatments exist. Drugs are limited by side effects and poor efficacy during the chronic stage of infection(25), and no vaccines are being tested in humans. The development of an effective vaccine would be an economically sound approach (26) and requires a thorough understanding of the protective immune response to this parasite, including mucosal immunity.

Th17 cells are well-established as important players against various fungi and extracellular bacteria(21) but are only recently gaining recognition as effectors in the immune response to intracellular pathogens like *T. cruzi*. Several studies over the past decade have described a role for IL-17A in the protective response against systemic *T. cruzi* parasite infection in mice(14, 16), and more recent work suggests a protective role for this cytokine in human Chagas disease as well(27). However, none of these studies has specifically examined the role of IL-17A in mucosal immunity against *T. cruzi*.

In this study, we demonstrate for the first time that Th17 cells and IL-17A contribute immunity against a gastric mucosal *T. cruzi* infection. Although CD8+ T cells are critical for Th17-mediated protection in systemic infection (3), they are dispensable in mucosal immunity. Instead, Th17 cells provide direct protective effects via secretion of IL-17A and induction of NADPH oxidase. While systemic *T. cruzi* infection involves multiple tissues and requires CD8+ T cell responses, local direct protective effects of IL-17A may be sufficient in mucosal infection. In addition, in systemic infection models, Th17 cells provided more protection against mortality compared to Th1 cells due to improved helper effects. However, protection by Th1 and Th17 cells was comparable at the gastric mucosa, where these cells act directly.

In summary, we identify a role for Th17 cells and IL-17A in mucosal immunity against *Trypanosoma cruzi* and we describe a novel protective mechanism of IL-17A against an intracellular pathogen. Th17 cells may have more broad protective functions than previously believed, and these studies in *T. cruzi* could also provide insights into infections with similar mucosally transmitted intracellular protozoa. Ultimately, these findings should be assessed for translational potential through studies targeting the induction of Th17 cells through mucosal vaccination, with the goal of reducing the significant morbidity and mortality associated with this disease.

Materials and Methods

Mice. *T. cruzi*-specific BALB/c mice containing a transgenic CD4+ TCR specific for p7, an immunodominant CD4 epitope of the trans-sialidase antigen, were generated in the Hoft laboratory as previously described(3). WT BALB/c mice (NCI Charles River Laboratories), RAG1 KO BALB/c mice (The Jackson Laboratory), IL-17RA KO BALB/c mice (Amgen), WT C57BL/6 (NCI Charles River), RAG1 KO C57BL/6 (The Jackson Laboratory), and B6.129S6-Cybbtm1Din/J mice lacking the gp91phox catalytic subunit of NADPH oxidase (gp91phox KO, The Jackson Laboratory) were obtained directly from the vendor or maintained as breeding colonies within the Hoft laboratory. Gp91phox KO mice were also bred to RAG1 KO C57BL/6 mice to generate RAG1 x gp91phox dKO C57BL/6 mice. All studies were approved by the Saint Louis University Institutional Animal Care and Use Committee (IACUC) under protocol #1106 and conducted in an AAALAC accredited facility at Saint Louis University. Euthanasia was performed using

CO2 narcosis according to the American Veterinary Medical Association guidelines on euthanasia.

Parasites and challenges. Tulahuèn strain parasites were maintained by *in vivo* passage through *Dipetalogaster maximus* insects and WT BALB/c mice and *in vitro* passage in LDNT+, an H_2O-based medium containing Liver Digest Neutralized Tryptose broth (Oxford). Culture-derived metacyclic trypomastigotes (CMT) were generated by *in vitro* differentiation in supplemented Grace's insect medium (Sigma) and maintained in a 26°C parasite incubator. For orogastric infections, mice were fasted for three hours and then fed 500 µl of a 1.5% sodium bicarbonate solution using a 22-gauge animal feeding needle, followed by 1 x 10^7 *T. cruzi* CMT parasites in 100 µl of 1% glucose in phosphate buffered saline (PBS). Extent of infection was assessed via quantification of parasite load in the stomach and spleen. Because orogastrically infected mice do not typically mount very high blood parasite burdens compared to systemically infected mice or die due to orogastric infection, these parameters were not routinely measured in our study.

Parasite DNA qPCR: Twelve days after orogastric infection, stomachs were dissected, cut along the greater curvature, rinsed in PBS to remove food contents, and minced with scissors. DNA was extracted from stomach tissue using DNeasy Blood and Tissue kits according to the manufacturer's instructions (Qiagen). Primers and TaqMan probes specific for *T. cruzi* genomic DNA were used to measure parasite load in the stomach as previously described (28). When histological analyses were performed in the same experiment, the stomachs were cut along both curvatures and dorsal portions used for qPCR.

Histology. Longitudinal strips 2-3 mm in width were cut from the esophageal to the pyloric ends of the ventral stomach, placed between pre-wetted foam pads in biopsy cassettes (Leica), and fixed by submersion in 10% neutral buffered formalin. Several hours later, the tissue was embedded, sectioned, affixed to slides and stained with hematoxylin and eosin at the Saint Louis University Research and Histology Microscopy Core. Images were acquired on an Olympus BX41 or Leica epifluorescent microscope. Inflammation scores were assigned according to a qualitative 0-4 scale by a blinded evaluator based on a scoring system adapted from Rogers et al(29). Briefly, inflammation was scored based upon the presence of leukocytes and the extent of their infiltration into the gastric mucosa. A score of 0 would indicate only the number of leukocytes present in the tissue at baseline (i.e. in a normal uninfected mouse with no inflammation), while a score of 4 indicates severe transmural inflammation with the presence of numerous infiltrating leukocytes.

Generation of Th1 and Th17 cells: *T. cruzi*-specific Th1 and Th17 cells were generated *in vitro* as previously described (3). Briefly, TCR Tg CD4+ T cells were stimulated with irradiated, cognate peptide-pulsed dendritic cells in the presence of α-IL-4 and IL-12 (to generate Th1 cells), or α-IL-4, α-IFN-γ, IL-6, TGF-β, and IL-23 (to generate Th17 cells). Media was refreshed on days 3 and 6 with IL-2 for Th1 cells or IL-23 for Th17 cells. The cells underwent two rounds of this differentiation (re-stimulation with peptide-pulsed APCs on day 7 followed by maintenance cytokines on days 10 and 13). Differentiation was confirmed by intracellular cytokine staining (ICS) for transcription factors and cytokines, and the cells were used on day 14. The phenotypes and stability of these Th1 and Th17 cells is previously documented(3).

298 **Flow cytometry and intracellular cytokine staining (ICS).** To obtain gastric
299 mucosal immune cells, stomachs were dissected open, a syringe needle was
300 introduced into the mucosal layers, and the region flushed with large volumes of media.
301 The recovered cells were cultured with 1 µl/ml of GolgiPlug containing monensin (BD
302 Pharmingen) and 0.67 µl/ml of GolgiStop containing Brefeldin A (BD Pharmingen) for 3
303 hours at 37°C. Cells were then stained with Live Dead fixable aqua, followed by surface
304 antibodies directed against CD3, CD4, CD11b, CD11c, Ly6G, F4/80, NKp46 (all BD or
305 eBiosciences). Cells were fixed and permeabilized for intracellular cytokine staining
306 using a Foxp3 transcription buffer staining set (eBiosciences). Cells were washed with
307 1% FBS in Dulbecco's PBS between all steps. All incubations were performed for 30
308 minutes at 4°C. For oxidation staining, cells were incubated for 20 minutes at 37°C with
309 100 nM of H_2DCFDA (Invitrogen) after surface staining. All samples were acquired on a
310 BD LSRII flow cytometer at the Saint Louis University flow cytometry core facility and
311 analyzed on FlowJo software (Treestar, Inc.).
312 **Splenic parasite outgrowth assay:** Total spleen cells were mechanically
313 isolated from dissected spleens 12 days after orogastric infection. Spleen cells were re-
314 suspended in LDNT+ parasite medium, plated in a limiting dilution fashion in 96-well
315 plates, and incubated in a 26°C parasite incubator. One month later, all wells were
316 examined for live parasites. The last well with parasite outgrowth was used to estimate
317 the number of parasites per million cells (e.g., live parasites emerging from 100 plated
318 cells = at least 1 parasite per 100 cells) (28). Because a single cell suspension can be
319 obtained from the spleen, this method was selected over qPCR for its ability to quantify

the presence of live parasites, though it may underestimate the true burden due to the detection of only parasites that can differentiate into trypomastigotes.

Generation of peritoneal exudate macrophages (PEMs), bone-marrow derived macrophages (BMDMs), and human monocyte-derived macrophages: PEMs were generated from BALB/c strain mice, and BMDMs were generated from C57BL/6 strain mice. For PEMs, mice were injected intraperitoneally with 100 µg of concanavalin A (Sigma). PEMs were recovered 3-4 days later via peritoneal lavage using a syringe and needle. To generate BMDM, bone marrow cells harvested from mouse femurs were plated in supplemented RPMI medium with 20 ng/ml M-CSF (eBioscience), refreshed on day 3. BMDMs were harvested for use on day 7. For human monocyte-derived macrophages, peripheral blood mononuclear cells were cultured in 96-well plates for 7 days to induce maturation. Adherent cells were removed and re-plated in 8-well chamber slides (Lab-Tek) for infection assays.

***In vitro* infection assay:** Macrophages were plated at 200,000 cells/well of 8-well chamber slides and infected with CMT (MOI=10). After 3 hours, extracellular parasites were removed by repeated washing. In some experiments, macrophages were co-cultured with purified recombinant murine or human IFN-γ (Genentech) at 1000 U/ml or IL-17A (R&D) at 100 ng/ml, or an anti-IL-17A neutralizing antibody (TC11-18H10, BD Pharmingen) was added at a concentration of 10 µg/ml. Nitric oxide concentrations in supernatants were measured using the Griess Reagent System (Promega). Slides were stained with Diff-Quik (IMEB, Inc.) after 2 days, and infected cells were enumerated microscopically.

***In vivo* IL-17A neutralization:** Mice were given 100 µg of an anti-IL-17A neutralizing antibody (17F3, Bio X Cell) or an IgG1 isotype control antibody (MOPC-21, Bio X Cell) every 2 days by intraperitoneal injection. Serum IL-17A levels were analyzed via Mouse IL-17A ELISA MAX Standard kits (BD).

Statistics: All analyses were performed in Prism version 8 (GraphPad Software) using a significance level of 5%.

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Visualization: CWC

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Competing interests

The authors have declared that no competing interests exist.

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FIGURE LEGENDS

Fig 1. IL-17A signaling is required for mucosal immunity against gastric *T. cruzi* infection. WT and IL-17RA KO mice were orogastrically infected with *T. cruzi* parasites, then sacrificed for tissue studies 12 days later. (A) Hematoxylin and eosin staining of stomach tissue sections taken near the margo plicatus reveals greater histological disturbances and inflammation in IL-17RA KO mice. Arrows indicate areas of immune cell infiltrate, and brackets capture areas with loss of specialized cells (e.g. chief cells) at the bases of the glands (B) IL-17RA KO mice had higher inflammation scores in the gastric mucosa, based on the extent of mononuclear cell infiltrate. (C-D) IL-17RA KO mice had higher parasite DNA levels in stomach as measured by qPCR (C), and greater frequencies of infected splenocytes (D), reflecting decreased control of infection. **p<0.01, *p<0.05 by two-tailed Student t test compared to WT mice.

Fig 2. Th17 cells can provide direct protection against *T. cruzi* gastric mucosal infection. (A) RAG1 KO BALB/c mice were given various combinations of cells by adoptive transfer, orogastrically infected with *T. cruzi* parasites the following day, then sacrificed 12 days post-infection for studies of tissue parasite burden. (B-C) Mice given parasite-specific Th17 cells before challenge had decreased parasite DNA burdens in the stomach measured by qPCR (B) and a smaller frequency of infected splenocytes

468 measured by limiting dilution parasite outgrowth assay (C). The addition of CD8+ T cells
469 did not significantly improve this Th17-mediated protection (B-C). (D) Both Th1 and
470 Th17 cells significantly lowered stomach parasite burdens arising after mucosal
471 infection. (E) Th1 cells and Th17 cells comparably decreased the burden of infection in
472 the spleen. **p<0.01, *p<0.05 by two-tailed Student t test compared to CD8 only group
473 (B) or no tx group (C-E). Results are representative of two separate experiments.
474
475 **Fig 3. Th17 cells and IL-17A alone can inhibit *T. cruzi* intracellular growth in**
476 **macrophages *in vitro* by inducing NADPH oxidase activity.** (A) Macrophages were
477 infected *in vitro* with *T. cruzi* parasites and co-cultured with T cells or cytokines. The
478 number of infected macrophages was counted after 2 days. (B) The addition of either
479 parasite-specific Th1 or Th17 cells reduced the number of infected cells. (C) The
480 protective action of Th17 cells was reduced in the presence of an anti-IL-17A
481 neutralizing antibody, indicating a cytokine-mediated effect. (D-E) IFN-γ or IL-17A
482 treatment also decreased the number of infected cells among both murine (D) and
483 human (E) macrophages. (F) Treatment of infected macrophages with IFN-γ, but not IL-
484 17A, induced expression of nitric oxide. (G) The mean fluorescence intensity (MFI) of
485 oxidized DCFDA, a measure of intracellular ROS, increased approximately 3.5-fold with
486 *T. cruzi* infection and IL-17A treatment in WT macrophages, but not gp91phox KO
487 macrophages. (H) IFN-γ treatment could protect both WT and gp91phox KO
488 macrophages, while IL-17A was only able to significantly reduce the number of infected
489 cells among WT macrophages, indicating that functional NADPH oxidase is required for
490 IL-17A-mediated protection. Experiments in panels B-F were performed using BALB/c

mice; those in panels G-H were performed in C57BL/6 background mice. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 by two-tailed Student t test compared to medium alone. Results are representative of multiple separate experiments.

Fig 4. Th17 cells traffic to the gastric mucosa and induce increased expression of intracellular ROS in macrophages. RAG1 KO BALB/c mice were reconstituted with Th17 cells and orogastrically infected with *T. cruzi* the next day. Twelve days after infection, mice were sacrificed and immune cells were isolated from the gastric mucosa for flow cytometric analysis. (A) CD4+ T cells were recovered from the gastric mucosa of mice reconstituted with Th17 cells, demonstrating that these cells migrated to the site of infection. No significant differences in proportions of other immune cell subsets were detected between mice given Th17 cells or not. (B) A significantly higher oxidation state was observed in gastric mucosal macrophages and neutrophils recovered from mice given Th17 cell adoptive transfer compared to controls. n=3/group, *p<0.05 by two-tailed Student t test compared to mice given no T cell transfer. Cells were defined by the following surface markers: F4/80+ (macrophages), CD11b+Ly6G+ (neutrophils), CD3+ (CD4+ T cells), CD3-NKp46+ (NK cells), Live/Dead aqua- (all live cells), EpCAM+ (epithelial cells).

Fig 5. Th17 cells cannot protect in the absence of functional NADPH oxidase. RAG1 KO mice and gp91phox x RAG1 dKO C57BL/6 mice lacking functional NADPH oxidase were given parasite-specific Th17 cells or not, orogastrically challenged with *T. cruzi* the next day, then sacrificed for tissue studies 12 days after infection. Only RAG1

KO mice and not gp91phox x RAG1 dKO mice were protected from orogastric *T. cruzi* infection by parasite-specific Th17 cells, as indicated by no significant decrease in stomach parasite DNA load. *p<0.05 by two-tailed Student t test. Results depict pooled data from three independent experiments.

Supplemental Fig 1. Stomachs of WT and IL-17RA KO BALB/c mice are similar at baseline. Prior to infection, both WT (A) and IL-17RA KO (B) mice demonstrated the presence of well-differentiated gastric glands and no significant immune cell infiltrate in the stomach mucosa.

Supplemental Fig 2. IL-17A neutralization does not reverse protection by Th17 cells *in vivo*. RAG1 KO BALB/c mice reconstituted with parasite-specific Th17 cells were orogastrically infected with *T. cruzi* the next day and treated with an anti-IL-17A neutralizing antibody or an isotype control antibody every other day until sacrifice on day 12 post-infection for tissue studies. (A) Pooled sera of mice treated with anti-IL-17A show reduced but not abolished serum IL-17A. (B) Treatment with anti-IL-17A had no effect on Th17-mediated suppression of gastric parasite burden after orogastric infection.

Figure 1

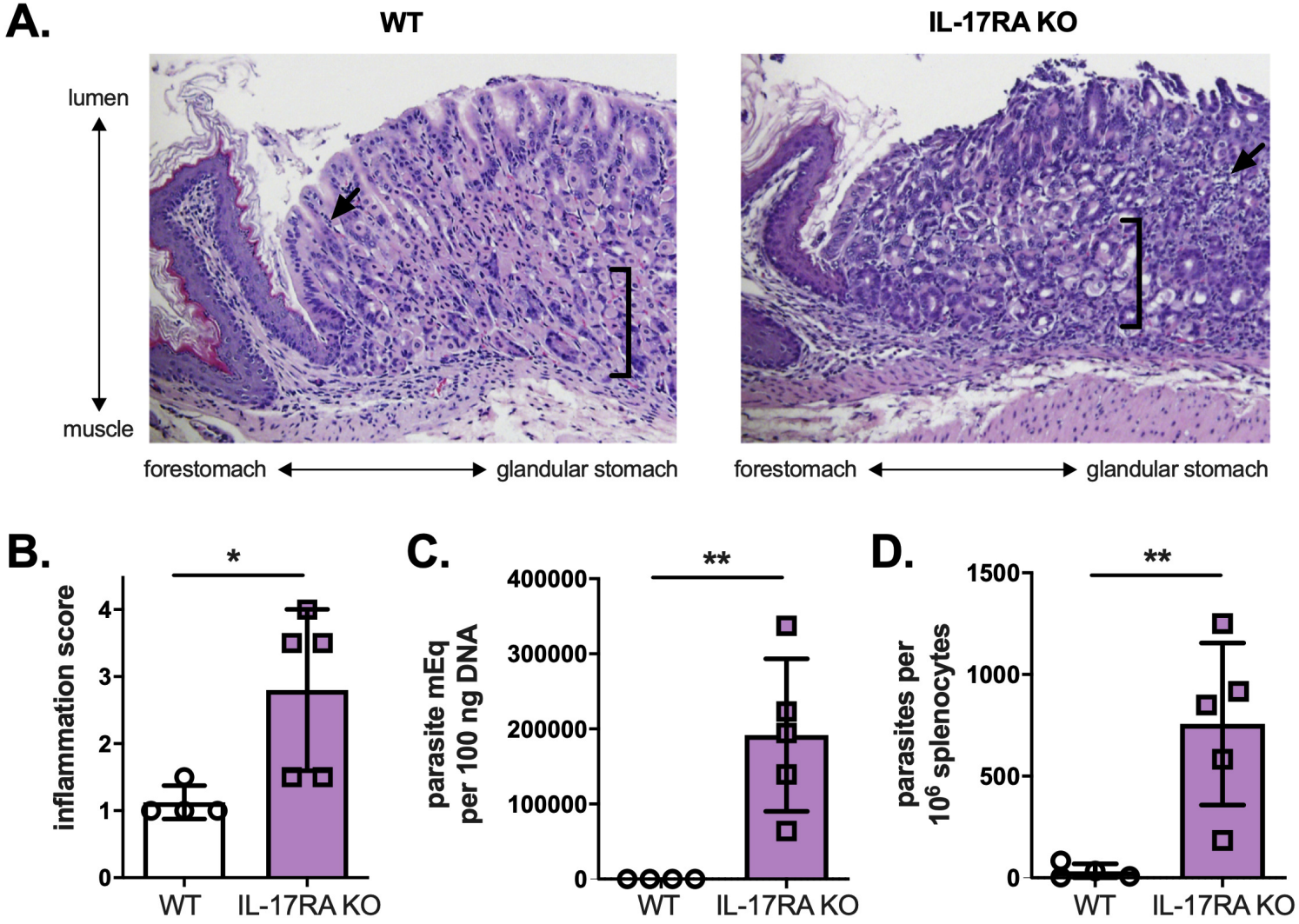

A.
WT
IL-17RA KO
lumen
muscle
forestomach
glandular stomach
forestomach
glandular stomach
B.
inflammation score
*
WT
IL-17RA KO
C.
parasite mEq per 100 ng DNA
**
WT
IL-17RA KO
D.
parasites per 10^6 splenocytes
**
WT
IL-17RA KO

Figure 2

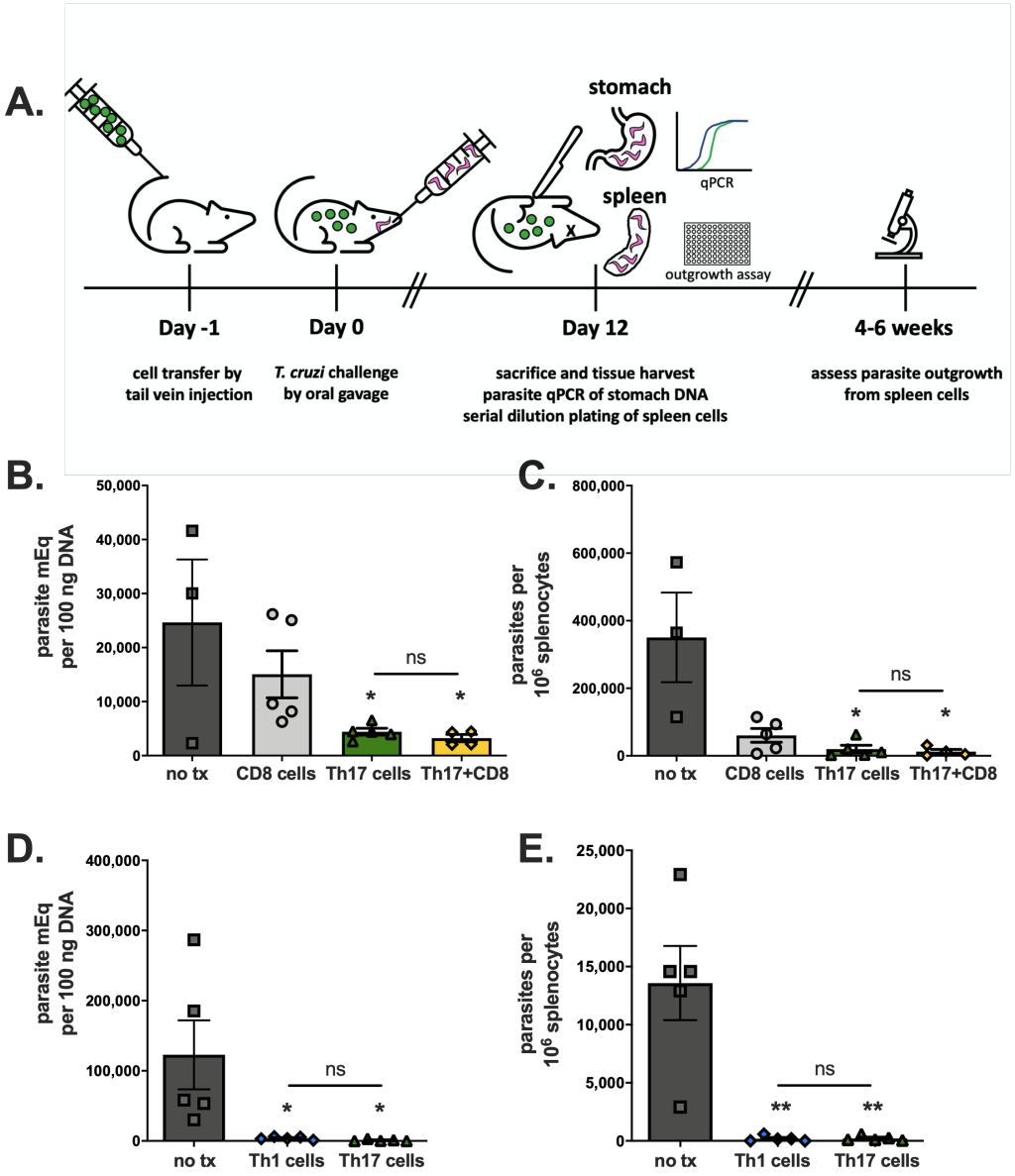

A.
stomach
spleen
qPCR
outgrowth assay
Day -1
Day 0
Day 12
4-6 weeks
cell transfer by tail vein injection
T. cruzi challenge by oral gavage
sacrifice and tissue harvest
parasite qPCR of stomach DNA
serial dilution plating of spleen cells
assess parasite outgrowth from spleen cells
B.
parasite mEq per 100 ng DNA
50,000
40,000
30,000
20,000
10,000
0
ns
*
*
no tx
CD8 cells
Th17 cells
Th17+CD8
C.
parasites per 10^6 splenocytes
800,000
600,000
400,000
200,000
0
ns
*
*
no tx
CD8 cells
Th17 cells
Th17+CD8
D.
parasite mEq per 100 ng DNA
400,000
300,000
200,000
100,000
0
ns
*
*
no tx
Th1 cells
Th17 cells
E.
parasites per 10^6 splenocytes
25,000
20,000
15,000
10,000
5,000
0
ns
**
**
no tx
Th1 cells
Th17 cells

Figure 3

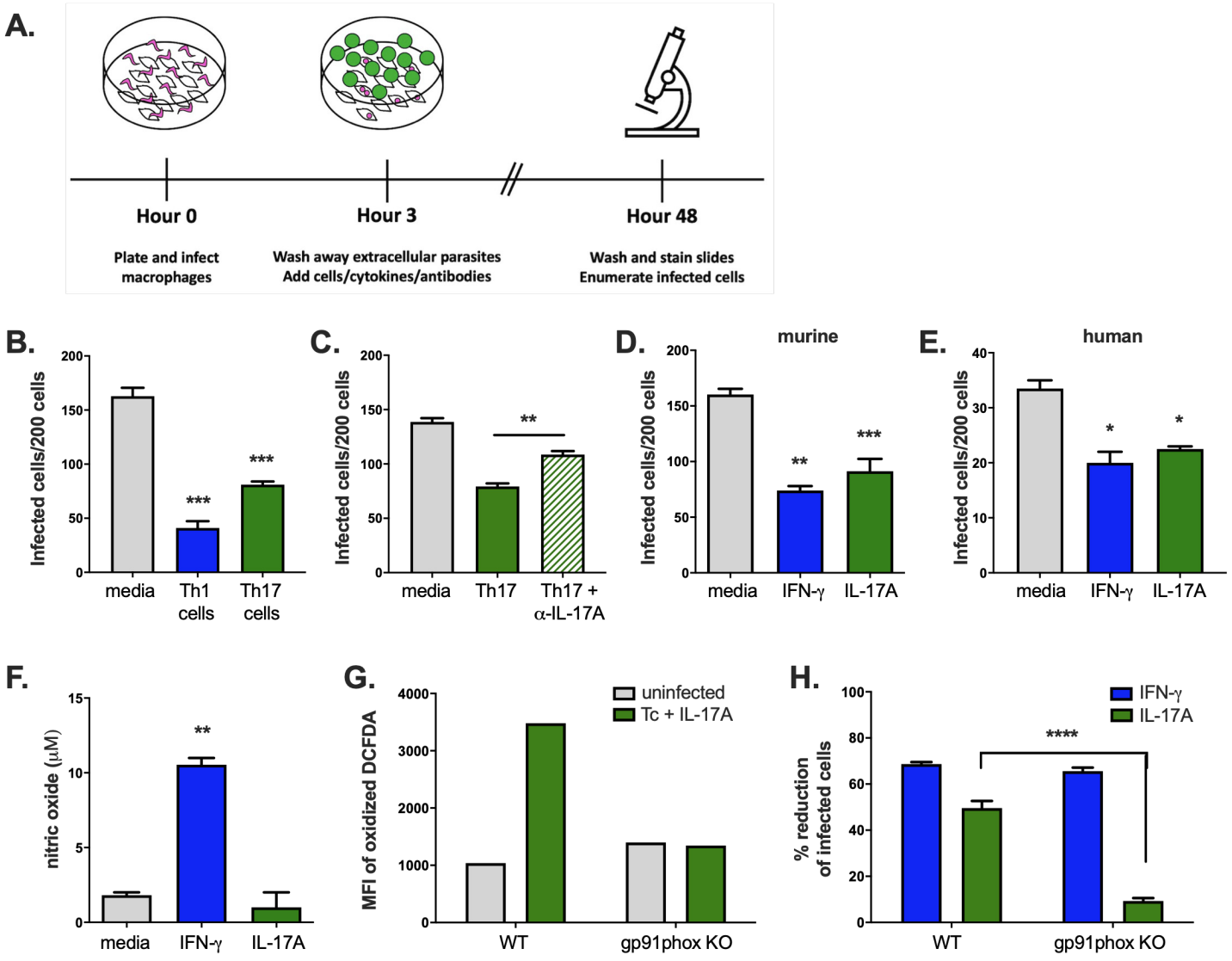

A.
Hour 0
Hour 3
Hour 48
Plate and infect macrophages
Wash away extracellular parasites
Add cells/cytokines/antibodies
Wash and stain slides
Enumerate infected cells
B.
Infected cells/200 cells
media
Th1 cells
Th17 cells
C.
Infected cells/200 cells
media
Th17
Th17 + α-IL-17A
D.
murine
Infected cells/200 cells
media
IFN-γ
IL-17A
E.
human
Infected cells/200 cells
media
IFN-γ
IL-17A
F.
nitric oxide (μM)
media
IFN-γ
IL-17A
G.
MFI of oxidized DCFDA
uninfected
Tc + IL-17A
WT
gp91phox KO
H.
IFN-γ
IL-17A
% reduction of infected cells
WT
gp91phox KO

Figure 4

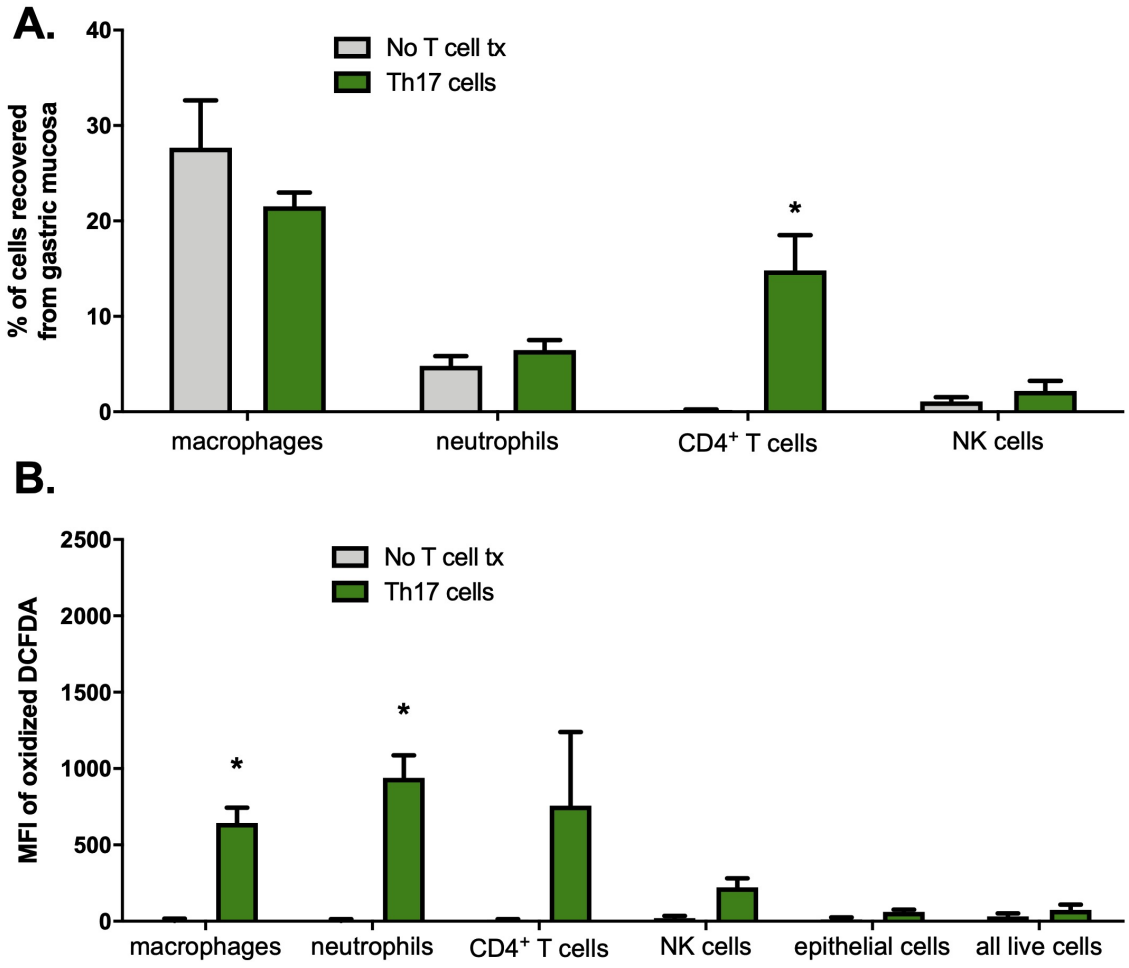
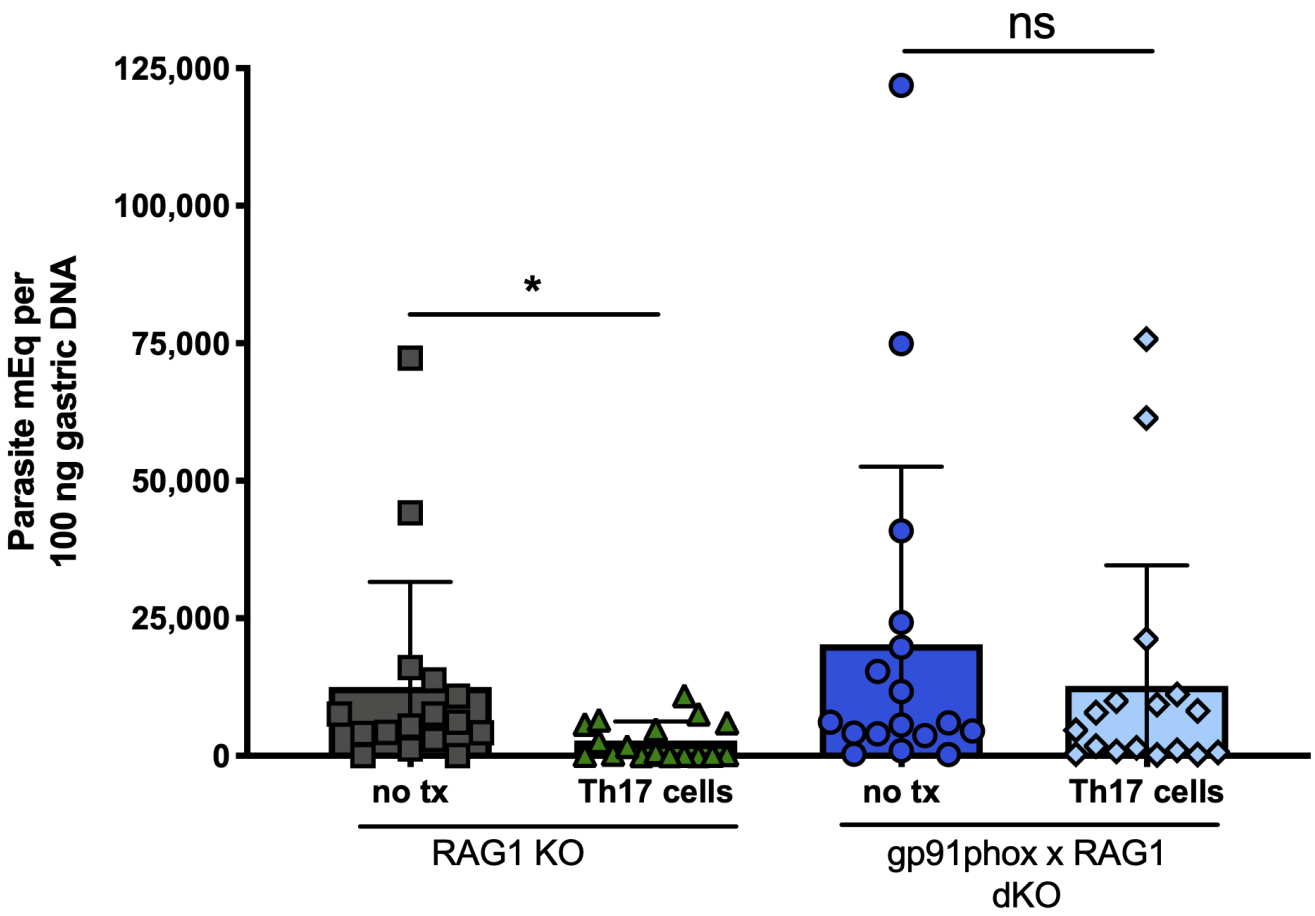
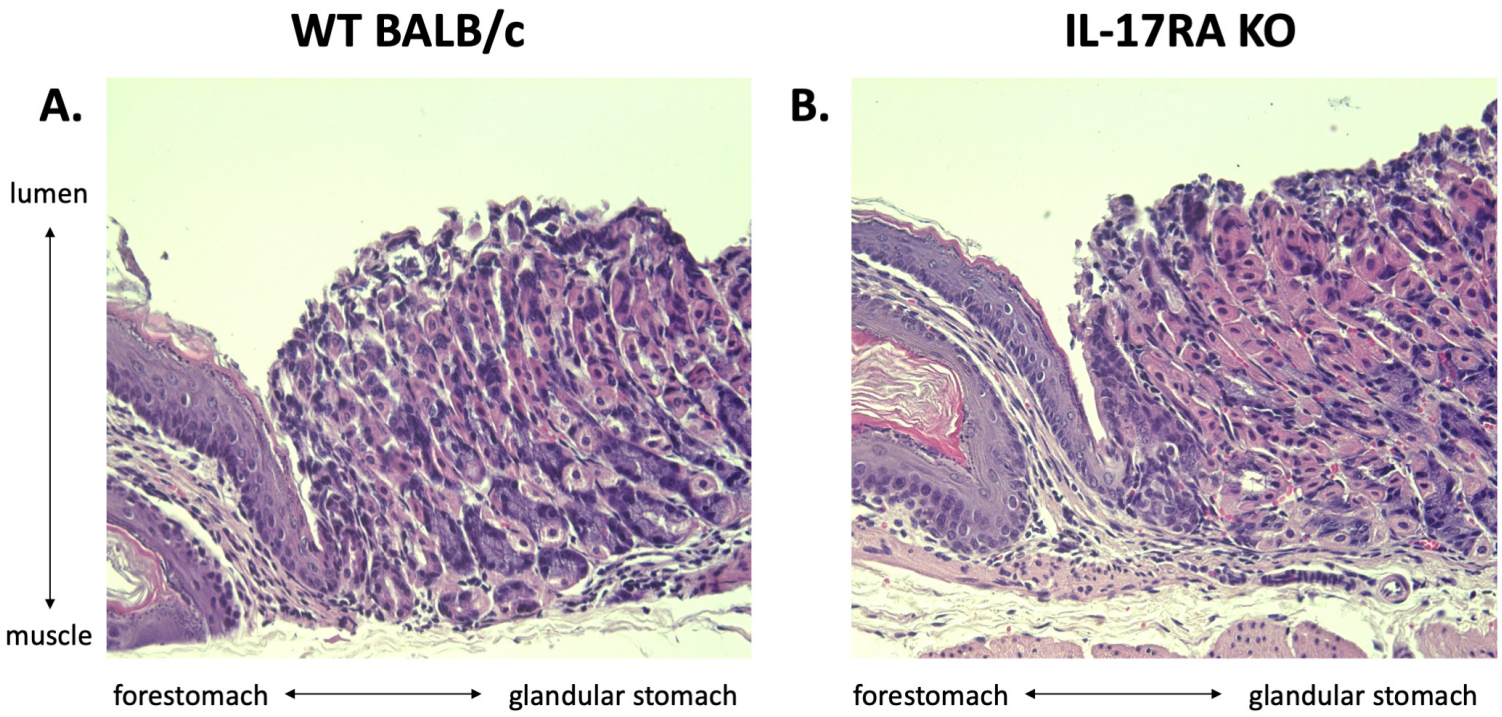

A.
No T cell tx
Th17 cells
% of cells recovered from gastric mucosa
40
30
20
10
0
*
macrophages
neutrophils
CD4+ T cells
NK cells
B.
No T cell tx
Th17 cells
MFI of oxidized DCFDA
2500
2000
1500
1000
500
0
*
*
macrophages
neutrophils
CD4+ T cells
NK cells
epithelial cells
all live cells

Figure 5


ns
*
125,000
100,000
75,000
50,000
25,000
0
Parasite mEq per
100 ng gastric DNA
no tx
Th17 cells
no tx
Th17 cells
RAG1 KO
gp91phox x RAG1
dKO

Supplemental Figure 1



Supplemental Figure 2