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Tbet Expression by Regulatory T Cells Is Needed to Protect against Th1-Mediated Immunopathology during *Toxoplasma* Infection in Mice

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**ABSTRACT**

*Toxoplasma gondii* infection has proven to be an ideal model to understand the delicate balance between protective immunity and immune-mediated pathology during infection. Lethal infection causes a collapse of T regulatory cells (Tregs) mediated by the loss of IL-2 and conversion of Tregs to IFN-γ–producing cells. Importantly, these Tregs highly express the Th1 transcription factor Tbet. To determine the role of Tbet in Tregs, we infected *Tbx21f/f-Foxp3YFP−Cre* and control *Foxp3YFP−Cre* mice with the type II strain of *T. gondii*, ME49. The majority of *Tbx21f/f-Foxp3YFP−Cre* mice succumbed to a nonlethal dose. Notably, parasite burden was reduced in *Tbx21f/f-Foxp3YFP−Cre* compared with *Foxp3YFP−Cre* control mice. We found that *Tbx21f/f-Foxp3YFP−Cre* mice have significantly higher serum levels of proinflammatory cytokines IFN-γ and TNF-α, suggestive of a heightened immune response. To test if CD4+ T cells were driving immunopathology, we treated *Tbx21f/f-Foxp3YFP−Cre* mice with anti-CD4–depleting Abs and partially rescued these mice. Broad-spectrum antibiotic treatment also improved survival, demonstrating a role for commensal flora in immunopathology in *Tbx21f/f-Foxp3−Cre* mice. RNA sequencing analysis reinforced that Tbet regulates several key cellular pathways, including leukocyte activation, regulation of lymphocyte activation, and cell cycle progression, that help to maintain fitness in Tregs during Th1 responses. Taken together, our data show an important role for Tbet in Tregs in preventing lethal immunopathology during *T. gondii* infection, further highlighting the protective role of Treg plasticity in controlling immune responses to infection and the microbiota.


**INTRODUCTION**

The sustained presence of regulatory T cells (Tregs) is vital to maintaining immunological homeostasis and is required for host survival, as evidenced by the emergence of autoimmune diseases when the suppressive function of Tregs is disrupted (1, 2). Tregs not only regulate autoimmune responses and chronic inflammation, but they also modulate immune responses to invading pathogens, and defective Treg functionality has been implicated in a spectrum of pathologies (1, 3). As the immunological landscape rapidly changes during an infection, Tregs must adapt to the inflammatory environment. This adaptation, termed Treg...
plasticity, endows them with certain features of Th cells that are thought to enhance the survival and functionality of Tregs in diverse inflammatory conditions (4–11). For example, the acquisition of certain Th1 effector properties aids in enhancing suppression during Th1 inflammation, and the upregulation of CXCR3 confers precise homing and accumulation at these sites, however, not all of the acquired phenotypes are fully understood (12–15). Tregs in Th1 polarized inflammatory environments will also upregulate Tbet, the lineage-specifying transcription factor for Th1 cells (4–11). During both acute and chronic Toxoplasma gondii infection in mice, Tbet-expressing Tregs can be found throughout the host (12, 16–20), including the small intestine, brain, muscles, spleen, and lymph nodes (11, 12, 16–20). Notably, Tregs in skeletal muscle express equivalent levels of Tbet compared with CD4+ effector T cells (16). However, the functional role of Tbet in Tregs during T. gondii infection has not been explored.

Th1-like Tregs (Tbet+ Tregs) are a highly stable population of Tregs, suggesting their persistence is advantageous to the host if they possess a suppressive function and their specificity is toward self-antigen (14). Our laboratory previously showed that, during chronic T. gondii infections, Th1-like Tregs are perpetrators of muscle damage by promoting macrophages to maintain proinflammatory M1 properties instead of the tissue-regenerative M2 function (16). These disparate findings in immunoregulatory processes led us to question whether Tbet expression in Tregs was essential for host response and control of T. gondii. This is important because there is also debate on the role of Tbet in Tregs within the inflamed gastrointestinal tract and if Tbet is needed for Treg function (13, 21, 22). Taken together, these ambiguities led us to question whether Tbet expression in Tregs is required for host response and control of T. gondii infection.

To address this question, we infected Treg-specific, Tbet-deficient conditional mice (Tbx21fl−Foxp3YFPcre) and control Foxp3YFPcre mice with the type II strain ME49. In our attempt to understand if Tbet was driving the pathology in chronically infected muscle, we discovered that Tbet is required for the survival of infected muscle, we discovered that Tbet is required for the survival of the host during acute infection. Interestingly, although Tbet expression by Tregs was required for survival of acutely infected mice, it did not influence host control of the parasite. We found that both CD4+ T cells and the commensal flora partially drove the immunopathology observed in the Tbx21fl−Foxp3YFPcre mice during acute infection with T. gondii. DNA sequencing (RNA-seq) on sorted splenic Tregs from day 10–infected Tbx21fl−Foxp3YFPcre and Foxp3YFPcre mice highlighted several cellular pathways regulated by Tbx21 in Th1-Tregs. Among these were pathways related to leukocyte activation, regulation of lymphocyte activation, and cell cycle progression. Taken together, this reveals that Tbet plays a critical role in mediating the fitness of the Treg response to infection-induced dysbiosis and remains a vital determinant of host survival during oral T. gondii infection.

MATERIALS AND METHODS

**Mice**

B6.129(Cg)-Foxp3tm4(YFP/cre)Ayr/J (Foxp3YFPcre) and B6.129-Tbx21flm22mr/J (Tbx21fl−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed to generate Tbx21fl−Foxp3YFPcre mice. All experimental mice were raised in specific pathogen-free conditions, were sex and age matched, and were used at 8–10 wk old at the time of infection. Both males and females were used in the experiments. All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee at the State University of New York at Buffalo.

**Isolation of tissue lymphocytes from organs**

Spleen and mesenteric lymphocytes were harvested by passing through Falcon 70-μm cell strainers to create single suspensions in 2% media (RPMI with 2% FBS, 25 mM HEPES, 0.1% β-mercaptoethanol, 1% penicillin-streptomycin, and 1% L-glutamine). RBCs were lysed as needed on ice for 3 min and resuspended in 10% media (RPMI with 10% FBS, 25 mM HEPES, 0.1% β-mercaptoethanol, 1% penicillin-streptomycin, and 1% L-glutamine).

Liver was collected and finely minced in digestion media (RPMI, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 0.1% β-mercaptoethanol, 25 mM HEPES, and 150 μg/ml DNase I [Sigma-Aldrich], 0.5 mg/ml Liberase [Roche]) and then slow spun at 50 x g for 5 mins (11). Small intestinal lamina propria (SILp) was isolated as previously described (11). SILp was harvested, cut into 0.5-cm pieces, and predigested in 3% media with 5 mM EDTA and 0.145 mg/ml DTT for 20 min at 37°C. The lamina propria was isolated from flow through epithelium by shaking in 2 mM EDTA, 25 mM HEPES, and 1% penicillin-streptomycin media. The tissue was then finely minced in digestion media containing Liberase and DNase I and incubated for 30 min at 37°C (11). Digested tissue was passed through a 70-μM and then 40-μM strainer. SILp single-cell suspensions were resuspended in 10% media (RPMI with 10% FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 0.1% β-mercaptoethanol, and 25 mM HEPES).

**Extracellular and intracellular flow cytometric analysis of tissue lymphocytes**

Single-cell suspensions were stained with HBSS containing extracellular surface Abs and LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies). After extracellular staining for 25 min on ice, cells were fixed and permeabilized overnight (Intracellular Fixation and Permeabilization Buffer Set, Thermo Fisher Scientific/eBioscience). Cells were then washed and stained with the eBioscience Permeabilization Buffer containing intracellular Abs for 45 min on ice. Samples were washed and resuspended in FACS Buffer (PBS, 1% BSA [Sigma-Aldrich], and 2 mM EDTA [Life Technologies]) for acquisition on a BD Fortessa SORP. Absolute numbers were derived from cell
counts using a hemocytometer (Life Technologies) or Count-Bright Absolute Counting Beads (Invitrogen).

**T. gondii–specific tetramer staining**

Single-cell suspensions of tissue lymphocytes were stained with allogeneic–conjugated MHC class II tetramers bound to *T. gondii* ME49 hypothetical antigenic peptide I-A(b) (A韦EHRPVPGTAPPSS) obtained through the National Institutes of Health Tetramer Facility (Atlanta, GA). Cells were incubated with the *T. gondii*–specific tetramer or CLIP tetramer for 1 h at room temperature. After *T. gondii*–specific staining, cells were stained with extracellular and intracellular Abs as described previously.

**Abs**

Abs used in flow cytometric analysis were: anti-TCRβ–allophycocyanin–Cy7 (BD Pharmingen, clone H57-597), anti-CD4–PE–Cy7 (BD Pharmingen, clone RM4-5), anti-CD4–V450 (clone RM4-5, BD Pharmingen), anti-CD8b–PerCp–Cy5.5 (clone H35-17.2, BD Pharmingen), anti-CD11a–BV650 (clone 2D7, BD Pharmingen), anti-CD49d–BV421 (clone R1-2, BD Pharmingen), anti-Foxp3–FITC (eBioscience, clone FJK-16s), anti-Thet–PE (eBioscience, clone eBio4B10), and anti-Ki67–AF700 (BD Pharmingen, clone B56). Flow cytometry data were acquired using a BD LSRII Fortessa Cell Analyzer and analyzed using FlowJo version 10.0.8 (Tree Star, Ashland, OR).

**Detection of serum cytokines**

Sera were isolated from mice either by cardiac puncture or collection by submandibular vein puncture at various time points. Assessment of the sera cytokines was performed by ELISA to detect IFN-γ and TNF-α (eBioscience) at 10–12 d postinfection. Cytokines were also quantified using Cytometric Bead Array (CBA, BD Biosciences) before infection and 9–10 d postinfection.

**Toxoplasma infection**

Brains were isolated from chronic *T. gondii*–infected mice (30–60 d postinfection). The brain was homogenized in PBS (pH 7.2) for oral infection (12). RFP-expressing ME49 cysts from brain homogenates were enumerated using a fluorescence microscope to detect RFP and counted in triplicate. Mice were orally gavaged with five ME49 RFP cysts in 200 μL PBS. Mouse weight, activity, and posture were scored every other day for the first 8 d of infection and were monitored daily during acute infection at day 8 through day 14 and then weekly thereafter.

**Ab depletion of CD4+ T cells in vivo**

Groups of *Tbx21*Δ/Δ–Foxp3γFPCre and Foxp3γFPCre mice were treated with either 0.5 mg of anti-mouse CD4 (clone GK1.5) or rat IgG2b isotype control anti-keyhole limpet hemocyanin, (clone LTF-2) (Bio X Cell’s InVivoMab). Mice were i.p. injected with a depleting Ab or isotype control on days −5, −1, and 0 preinfection and on days 5, 14, and 19 postinfection.

**Antibiotic treatment**

To deplete gut microbiota, groups of Foxp3γFPCre and *Tbx21*Δ/Δ, Foxp3γFPCre mice were given broad-spectrum antibiotics in their drinking water (23). The following antibiotics were added to autoclaved tap water: ampicillin (1 g/L), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L) (Sigma-Aldrich, St. Louis, MO); and administered ad libitum using drinking bottles. A set of mice from each group were designated as untreated controls and received water supplied from the animal facility. Antibiotic treatment started two weeks prior to infection with *T. gondii* and was administered until three weeks postinfection, a total of five weeks of treatment.

**Analysis of bacterial 16S genes**

Feces were collected prior to antibiotic water administration and at various time points thereafter. Fecal pellets were collected directly into microcentrifuge tubes and immediately placed on dry ice until storage in a −80°C freezer. Bacterial DNA was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen). Quantification of DNA was done using a nanodrop. Quantitative PCR (qPCR) was performed using the iTaq Universal SYBR Green Mastermix (BioRad) on a StepOne Real-Time PCR System. Bacterial detection was analyzed using amplification of the 16s ribosomal subunit of specific populations. The selected bacteria to be analyzed by qPCR were Eubacteria, Enterobacteriaceae, *Escherichia coli*, Bacteroides, and the Eubacterium rectale/Clostridium cocoides group (ERE) (24).

**Quantification of parasite burden**

Mouse organ tissues were harvested and preserved in RNAlater (Invitrogen) for DNA isolation using a DNeasy Blood & Tissue Kit (Qiagen). DNA was quantified using a Nanodrop Spectrophotometer for quality and concentration. An amount of 200 ng of various tissue DNA was used in PCR amplification of the *T. gondii*–specific repetitive gene Bi (forward: 5′-TCCCGCTTGCTGCGAAATG-3′, reverse: 5′-AGCCGTTGGTTGCACTATCGATG-3′). Parasite burden was calculated using a comparison of a standard curve of known *T. gondii* genomic DNA amplified with Bi–specific primers.

**Blood chemistry assays**

Blood was collected from *T. gondii*–infected mice by cardiac puncture after euthanasia. The blood was placed in tubes without anticoagulants and then placed on ice until analysis. University at Buffalo Laboratory Animal Facilities veterinary staff analyzed blood chemistry using an Element DC Veterinary Chemistry Analyzer (Heska).

**RNA-Seq**

Tregs were sort purified from *Tbx21*Δ/Δ–Foxp3γFPCre and Foxp3γFPCre mice 9 d after being infected with five ME49 cysts. Total RNA to be sequenced was extracted from sort-purified Tregs using TRIzol (Thermo Fisher). RNA-seq was performed and analyzed at the University at Buffalo Genomics and Bioinformatics Core Facility using the R package DESeq2 to...
determine differentially expressed genes between Tregs from infected Tbx21^β−Foxp3^{YFP_{cre}} and Foxp3^{YFP_{cre}} mice. Gene ontology (GO) analysis was performed using the GOseq Bioconductor package, version 1.42.0. GOseq performed GO analysis while addressing biases present in RNA-seq data that are not found using other techniques, namely that expected read counts for a transcript are based on both the gene’s level of expression and the length of the transcript. The Wallenius approximation is used by GOSeq to approximate GO category enrichment and calculate p values for each GO category being overrepresented among genes that were differentially expressed (DESeq2 \( p_{adj} \leq 0.05 \)). These enrichment p values were corrected for multiple testing using the Benjamini–Hochberg procedure.

**Data availability**

The data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession number GSE183731; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE183731).

**Statistics**

All statistics were generated using Graphpad Prism version 6.0h.

## RESULTS

**Tbet expression in Tregs is required for survival of acute T. gondii infection**

We investigated the role of Tbet in Tregs during Toxoplasma infection by administering five ME49 T. gondii cysts via oral gavage to both Foxp3^{YFP_{cre}} and Tbx21^β−Foxp3^{YFP_{cre}} mice. The acute phase of infection began in the intestinal tract, and was marked by weight loss. We found similar weight loss between infected Foxp3^{YFP_{cre}} and Tbx21^β−Foxp3^{YFP_{cre}} mice (Fig. 1A). However, infected Tbx21^β−Foxp3^{YFP_{cre}} mice succumbed to infection at a significantly higher rate (87%) than infected Foxp3^{YFP_{cre}} mice, the majority of which survived through acute infection (Fig. 1B). We first asked if Tbx21^β−Foxp3^{YFP_{cre}} mice were succumbing to parasitemia because of an inability to control the infection. To this end, we harvested tissue from day 12 postinfection mice and used qPCR to quantify T. gondii DNA using the tandem repeat gene B1. No differences were observed in parasite burden between mice in the thymus, lung, heart, and ileum. There was significantly less parasite in the brains of Tbx21^β−Foxp3^{YFP_{cre}} mice versus Foxp3^{YFP_{cre}} controls (Fig. 1C). These findings show that Tbet expression in Tregs is necessary for survival of acute T. gondii infection and that it does not negatively impact immune control of T. gondii infection.

**Tbx21^β−Foxp3^{YFP_{cre}} mice exhibit severe pathology during acute T. gondii infection**

Because uncontrolled parasite growth was not observed in Tbx21^β−Foxp3^{YFP_{cre}} mice, we turned to the host response to understand how Tbet expression in Tregs could promote host survival during acute infection. We analyzed biomarkers in serum associated with excessive inflammation to ascertain if acute organ failure was occurring in the absence of Treg-specific Tbet expression. A comprehensive metabolic panel was

![FIGURE 1. Tbet expression in Tregs is required for survival of T. gondii infection.](https://doi.org/10.4049/immunohorizons.2100080)
performed on blood freshly isolated from Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice and Foxp3<sup>YFP</sup>cre control mice on day 10 postinfection. Significantly decreased levels of glucose and albumin were observed in the Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice. In addition, significantly higher levels of inorganic phosphate, cholesterol, and blood urea nitrogen were measured in the Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice (Fig. 2). These differences in the serum may be tied to liver and kidney damage, however, when measuring alanine/aspartate aminotransferase levels, we found no significant differences between the two groups of mice (data not shown). These data suggest that despite the immune response controlling the infection, there was potentially systemic lethal immunopathology in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice.

**Treg-specific Tbet deficiency elevates systemic IFN-γ and TNF-α, but not Th1 populations during acute T. gondii infection**

We next examined the CD4<sup>+</sup> T cell response in T. gondii–infected Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with control Foxp3<sup>YFP</sup>cre mice. Analysis of serum levels of IFN-γ and TNF-α, critical Th1 inflammatory cytokines that are important in host survival of T. gondii (25–29), were significantly increased in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with Foxp3<sup>YFP</sup>cre mice (Fig. 3A). Next, CD4<sup>+</sup> T cells were examined by FACS analysis for phenotype and absolute numbers in the spleen, mesenteric lymph nodes (mlns), and SILp. There was no difference in the absolute number of CD4<sup>+</sup> T cells in the spleen between Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with Foxp3<sup>YFP</sup>cre mice (Fig. 3B) or their expression of Ki67<sup>+</sup> (Fig. 3C, left panel). The frequency of Tbet in CD4<sup>+</sup>Tcells was similar in the mlns and SILp, but was reduced in the spleen in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with Foxp3<sup>YFP</sup>cre mice (Fig. 3C, right panel). We assessed the binding of the ASI5 tetramer, which identifies T. gondii Ag-specific CD4<sup>+</sup> T cells (Fig. 3D). We found no difference in the frequency or absolute number of ASI5 tetramer–positive CD4<sup>+</sup> T cells in the mlns and SILp, but both were reduced in the spleen in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with Foxp3<sup>YFP</sup>cre mice (Fig. 3D, 3E). These results suggest that although expression of Tbet by Tregs is important for the restraint of widespread, fatal inflammation, it is largely dispensable for limiting the size and activity of the Th1 cell pool and parasite–specific CD4<sup>+</sup> T cell responses in the gastrointestinal tract during acute infection.

**Tbet is required for the proliferation and accumulation of Tregs in the SILp during acute T. gondii infection**

We next examined CD4<sup>+</sup> Tregs in the SILp, mlns, and spleens of infected mice by FACS analysis. We found there was a reduced frequency of Tregs in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with Foxp3<sup>YFP</sup>cre mice in the mlns, however, no difference in the total number of Tregs was observed (Fig. 4A). Tregs in the SILp were reduced in absolute numbers (Fig. 4A, right panel). Tregs from the SILp had reduced frequency of the proliferation marker Ki67 (Fig. 4B, left panel) and a reduced absolute number of Ki67-expressing Tregs (Fig. 4B, right panel) in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice compared with Foxp3<sup>YFP</sup>cre mice. As expected, Tregs from Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice expressed significantly less Tbet compared with Foxp3<sup>YFP</sup>cre mice (Fig. 4C).

Because the increased mortality and immunopathology in the Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice were potentially indicative of inflammation associated with multiorgan failure, we also examined the T cell response in the liver. We found a small but significant decrease in the frequency of CD4<sup>+</sup> TCRβ<sup>+</sup>Foxp3<sup>+</sup> T cells, but no difference in the total numbers. We found no difference in the number of Ag-experienced CD4<sup>+</sup> T cells (CD11a<sup>+</sup>CD49d<sup>+</sup>), their expression of Tbet, or the proliferative marker, Ki67, between Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice and Foxp3<sup>YFP</sup>cre mice (Fig. 5A). Analysis of CD8<sup>+</sup> TCRβ<sup>+</sup> T cells in the liver showed no differences between the two groups of mice in frequency, numbers, activation status, and proliferative capacity (Fig. 5B). We examined the same parameters on Tregs in the livers of infected mice. Notably, we found that although SILp Tregs from Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice were reduced in both number and proliferation, there was a significant increase of both the frequency and number of Tregs in the liver (Fig. 5C). These data show that Tbet is needed for Treg accumulation at primary sites of acute infection, but secondary sites may not rely on Tbet for Treg accumulation.

**CD4<sup>+</sup> T cells participate in the enhanced immunopathology from acute T. gondii infection in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice**

We next asked if the morbidity and mortality observed in the Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice were potentially indicative of inflammation associated with multiorgan failure, and also examined the T cell response in the liver. We found a small but significant decrease in the frequency of CD4<sup>+</sup> TCRβ<sup>+</sup>Foxp3<sup>+</sup> T cells, but no difference in the total numbers. We found no difference in the number of Ag-experienced CD4<sup>+</sup> T cells (CD11a<sup>+</sup>CD49d<sup>+</sup>), their expression of Tbet, or the proliferative marker, Ki67, between Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice and Foxp3<sup>YFP</sup>cre mice (Fig. 5A). Analysis of CD8<sup>+</sup> TCRβ<sup>+</sup> T cells in the liver showed no differences between the two groups of mice in frequency, numbers, activation status, and proliferative capacity (Fig. 5B). We examined the same parameters on Tregs in the livers of infected mice. Notably, we found that although SILp Tregs from Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice were reduced in both number and proliferation, there was a significant increase of both the frequency and number of Tregs in the liver (Fig. 5C). These data show that Tbet is needed for Treg accumulation at primary sites of acute infection, but secondary sites may not rely on Tbet for Treg accumulation.

**FIGURE 2. Acute T. gondii infection causes immunopathology and a metabolic shift indicative of systemic damage in Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice.**

Groups of Foxp3<sup>YFP</sup>cre and Tbx21<sup>£</sup>-Foxp3<sup>YFP</sup>cre mice were orally infected with five ME49RFP T. gondii cysts. On day 10 postinfection, fresh blood was isolated by cardiac puncture to perform a comprehensive metabolic panel. Blood chemistry assay was performed 10 d postinfection looking at liver and kidney function. Dashed lines are reference values for uninfected mice. Results are cumulative of two experiments with n ≥ 5 per group per experiment; cumulative total of n ≥ 10 per group; error bars = SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Student t test.
using the anti-CD4 mAb, GK1.5. Mice were i.p. injected with 0.5 mg/ml anti-CD4–depleting or isotype Abs (clone LTF-2) before and during infection (30). Infected Foxp3 YFPCre mice that received anti-CD4 Abs had similar survival compared with isotype control–treated, infected Foxp3 YFPCre mice. However, Tbx21f/f Foxp3 YFPCre mice that received anti-CD4–depleting Abs were partially rescued from T. gondii infection compared with isotype control–treated Tbx21f/f Foxp3 YFPCre mice (Fig. 6A). We found no difference between Foxp3 YFPCre or Tbx21f/f Foxp3 YFPCre mice treated with either isotype control or anti-CD4 Abs and parasite burden (Fig. 6B). We next determined if there was a reduction in the serum levels of IFN-$\gamma$ or TNF-$\alpha$ in Foxp3 YFPCre mice compared with Tbx21f/f Foxp3 YFPCre mice treated with isotype control or anti-CD4 Abs. As observed previously, Tbx21f/f Foxp3 YFPCre mice had significantly higher serum levels of IFN-$\gamma$ as well as heightened TNF-$\alpha$ levels compared with Foxp3 YFPCre mice. However, no marked differences in the levels of these cytokines were seen upon CD4 depletion (Fig. 6C). The increased levels of IFN-$\gamma$ and TNF-$\alpha$ in Tbx21f/f Foxp3 YFPCre mice, despite anti-CD4 treatment, highlights the importance of fully functional Tregs to not only control Th cells, but also other arms of immunity. The partial rescue of CD4-depleted Tbx21f/f Foxp3 YFPCre mice implicates a role for CD4$^+$ T cells in driving lethal immunopathology when Tregs cannot express Tbet during T. gondii infection.

**Broad-spectrum antibiotics ameliorates immunopathology from acute T. gondii infection in Tbx21f/f Foxp3 YFPCre mice**

Mice infected perorally with a high dose of T. gondii succumbed to severe ileitis and small intestine immunopathology through Th1-associated proinflammatory cytokines (32–34). This acute Th1 immune response causes a shift in the gut commensal communities and can contribute to the morbidity and mortality of mice during acute T. gondii in genetically susceptible hosts. Dysbiosis is attributed to specifically accumulated populations of the Gram-negative bacteria Escherichia coli, Bacteroides spp., and other proinflammatory-associated bacteria (35, 36). The use of broad-spectrum antibiotics to deplete commensal gut flora rescues mice from systemic immunopathology during the acute stages of T. gondii infection (35). To test if the lethal immunopathology in Tbx21f/f Foxp3 YFPCre mice was mediated by an overexuberant immune response to commensal flora, we administered water supplemented with broad-spectrum antibiotics to both strains of mice 14 d prior to infection.
with *T. gondii*. Control groups of mice received water without antibiotics. All mice were orally gavaged with five ME49 cysts and received control water or continued antibiotic treatment until 21 d postinfection. Foxp3^YFP^Cre mice showed no difference in survival between control water and antibiotic water (Fig. 7A). The *Tbx21^f/f*-Foxp3^YFP^Cre cohort that was administered antibiotic water survived significantly longer than the *Tbx21^f/f*-Foxp3^YFP^Cre controls. Of note, 50% of the mice receiving antibiotics in the *Tbx21^f/f*-Foxp3^YFP^Cre group survived until day 65 postinfection (Fig. 7A). In addition to extended survival, antibiotic-treated *Tbx21^f/f*-Foxp3^YFP^Cre mice had reduced cyst burden compared with Foxp3^YFP^Cre mice treated with antibiotic water (Fig. 7B).

Because antibiotic treatment partially rescued the *Tbx21^f/f*-Foxp3^YFP^Cre mice, we wanted to investigate the microbiota landscape and how subsequent dysbiosis caused by infection was altered between antibiotic-treated and nontreated groups. To verify if the overall bacterial load was depleted, we compared 16s levels from before antibiotic treatment to the levels on day 7 postinfection (Supplemental Fig. 1). After 3 wk of antibiotic administration in both Foxp3^YFP^Cre and *Tbx21^f/f*-Foxp3^YFP^Cre groups, 16s was significantly lower at the day 7 postinfection timepoint. This result confirms that bacterial load in the antibiotic-treated groups was decreased as expected. To parse out the specific changes in bacterial populations, qPCR was performed using primers targeting the unique 16s rRNA subunit gene in DNA to discriminate global bacterial changes of the Enterobacteriaceae family, *Bacteroides* family, EREC, and *E. coli* species (24). Quantifying the changes of the microbiota was achieved by comparing preinfection levels at day 0 with levels at day 14 postinfection. Unfortunately, due to the fact that *Tbx21^f/f*-Foxp3^YFP^Cre mice succumbed to infection, we lacked comparisons with the *Tbx21^f/f*-Foxp3^YFP^Cre water control group at day 14. We included the *Tbx21^f/f*-Foxp3^YFP^Cre mice in the graphs, but they were excluded from the statistical comparisons between groups. We first asked if there were baseline differences between the four groups of mice prior to infection at day 0, after mice had been pretreated for 2 wk with antibiotic water. Our results show a significant outgrowth of Enterobacteriaceae and a species of
that family, \textit{E. coli}, in both antibiotic-treated groups (Fig. 7C, lower left and right graphs, respectively). This increase was present in both antibiotic groups, but the Foxp3^{YFPCre} antibiotic group had an even greater increase in Enterobacteriaceae and \textit{E. coli} when compared with the \textit{Tbx21f/f}-Foxp3^{YFPCre} antibiotic-treated samples. In the \textit{Tbx21f/f}-Foxp3^{YFPCre} control water group, EREC was reduced in comparison with Foxp3^{YFPCre} controls at day 0. Antibiotic water treatment reduced EREC to a similar degree in both genotypes of mice (Fig. 7C, upper right graph). We found similar levels of \textit{Bacteroides} between Foxp3^{YFPCre} controls and \textit{Tbx21f/f}-Foxp3^{YFPCre} controls and the respective antibiotic-treated groups prior to infection at day 0 (Fig. 7C, upper left graph).

Next, we examined the differences in bacterial populations at day 14 postinfection to assess changes within the infected mice. Whereas the \textit{Bacteroides} population was similar at day 0 between all control groups, antibiotic treatment greatly reduced it prior to infection. Notably, it was significantly increased only in the \textit{Tbx21f/f}-Foxp3^{YFPCre} antibiotic group 14 d postinfection (Fig. 7C, upper left). We found a significant decrease of EREC in both antibiotic groups at day 14 when compared with Foxp3^{YFPCre} controls. Lastly, we found the increase in Enterobacteriaceae and \textit{E. coli} from antibiotic treatment was reduced during infection in the Foxp3^{YFPCre} antibiotic group compared with the Foxp3^{YFPCre} controls. We did not observe a similar decrease between the \textit{Tbx21f/f}-Foxp3^{YFPCre} mice treated with or without antibiotics. The increase in these bacterial populations prior to infection could be due to antibiotics depleting the majority of bacteria and supports \textit{E. coli} opportunistically blooming in comparison with other resident commensals (35, 36). Taken together, our data support the idea that immunopathology induced by dysbiosis is exacerbated in \textit{Tbx21f/f}-Foxp3^{YFPCre} mice infected with \textit{T. gondii}.

**Tbet promotes Treg cell cycle progression and fitness**

We sought to investigate global genetic changes in Tregs during infection and how Tbet influences Treg gene expression. We isolated total RNA from FACS-sorted splenic Tregs (CD4^{+}YFP^{+}) 10 d postinfection from \textit{Tbx21f/f}-Foxp3^{YFPCre} and Foxp3^{YFPCre} mice for RNA-seq. There were 399 genes that were upregulated and 576 that were downregulated in Tregs from \textit{Tbx21f/f}-Foxp3^{YFPCre} mice compared with Foxp3^{YFPCre} mice infected with \textit{T. gondii}.

FIGURE 5. Differences in Tregs but not CD4^{+} or CD8^{+} T cells in the liver during acute infection. (A) Quantification of Th cells gated on live CD4^{+}TCRB^{-}Foxp3^{+} in the liver on day 12 dpi, (B) CD8^{+}TCRB^{-}, or (C) CD4^{+}TCRB^{-}Foxp3^{+} Tregs. Results are cumulative of two experiments with \( n = 3 \) in each group. Error bars = SD. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.0001 \), Student t test.

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immune system response leukocyte activation and regulation of lymphocyte activation had distinct gene expression patterns between wild type (WT) and Tbet-deficient Tregs (Fig. 8C, left and middle panels). Notably, we found that Tbet-deficient Tregs had reduced expression of genes in the cell cycle transition pathway (Fig. 8C, right panel), which is consistent with the reduction in the proliferation and accumulation of Tbet-deficient Tregs in Fig. 3 and Ref. 5. Taken together, Tbet expression drives the expression of several aspects of fitness, in addition to homing molecules (5), that promote the ability of Tregs to dampen the immunopathology caused by dysbiosis during acute T. gondii infection.

DISCUSSION

We show that the capacity to express Tbet in Tregs is necessary for survival of the host during acute T. gondii infection, as the majority of Tbx21<sup>f/f</sup>-Foxp3<sup>YFPcre</sup> mice succumbed to the acute infection. Importantly, this increased susceptibility to infection was not due to uncontrolled parasite replication; in fact, Tbx21<sup>f/f</sup>-Foxp3<sup>YFPcre</sup> mice have enhanced control as evidenced by reduced parasite burden. These findings suggest that Tbet-expressing Tregs are not required for regulating the protective immune response directed at the parasite. Although there were minimal differences between the Th cells found in the spleen, mlns, and SIPp, mice harboring Tbet-deficient Tregs showed significantly reduced frequency and numbers of Tregs in the mlns and SIPp, respectively, which is in agreement with previous findings (5). We also observed that the frequency of K67 expression in Tregs was reduced in the SIPp of Tbx21<sup>f/f</sup>-Foxp3<sup>YFPcre</sup> mice compared with Foxp3<sup>YFPcre</sup> mice. Interestingly, although we found decreased accumulation of Tbet-deficient Tregs in the SIPp, this was not true for the liver. It remains to be determined how accumulation within different tissues differs in its dependence on Tbet during infection. Notably, serum IFN-γ and TNF-α levels were significantly elevated in infected Tbx21<sup>f/f</sup>-Foxp3<sup>YFPcre</sup> mice compared with Foxp3<sup>YFPcre</sup> mice. These data present distinct differences when compared with global Tbet knockout mice, namely the loss of control of T. gondii replication and dissemination at peripheral sites during acute infection, and similar IFN-γ production compared with WT mice (37).

Moreover, it has been shown previously that Tbet-deficient Tregs have similar in vitro suppressive capacities compared with WT Th1-Tregs and no difference in IL-10 production (12, 21, 38, 39). We did not find significant differences in Il10 expression between Tbet-deficient Tregs and WT Tregs in our RNA-seq analysis. Interestingly, in models of T cell–mediated colitis, Tbet is not required for their suppressive capacity in vivo (21, 22, 38). This is in line with studies demonstrating that RoR-γt–expressing Tregs are commensal specific and control intestinal inflammation (40, 41). Adding another layer to the understanding of Tbet-expressing Tregs, a recent study demonstrated that IFN-γ<sup>Tbet</sup> Tregs promoted Th1 intestinal inflammation in the DSS colitis model (13). The differences...
between our findings and others could be explained by potential differences in the commensal flora between animal facilities, instigating inflammatory stimuli, or the additional complexity of an ongoing infection in the gastrointestinal tract. Furthermore, Tbet is highly expressed in Tregs during *T. gondii* infection compared with other infections, which may alter its function in Tregs compared with Tregs expressing lower levels of Tbet (5, 6, 12, 14, 16, 19, 20). This is supported by data showing that Tregs in a *T. gondii*–infected environment are sensitive to IL-12 stimulation, resulting in Stat4 phosphorylation, whereas Tregs in a naive setting, or *Listeria monocytogenes*, are not IL-12 sensitive (6, 12). This sensitivity is Stat1-dependent and due to the sustained production of IL-12 and IL-27 in *T. gondii* infection (19). Prolonged Tbet expression in Tregs, however, appears to result in pathogenic Treg function and impairs tissue remodeling programs during chronic *T. gondii* infection of skeletal muscle (16–18). Together, our data show distinct roles for Tregs during the acute response compared with the chronic response to *T. gondii* infection. It still remains to be fully explored how this prolonged Tbet expression may reprogram Tregs during later stages of infection.

The driving force of immune-mediated pathology in acute *T. gondii* infection is dysbiosis of the microbiota in the gastrointestinal tract (24, 32, 35, 42–45). *Toxoplasma* infection can perturb the integrity of the epithelial barrier and cause leaky gut (35, 42, 46, 47). This overexuberant immune response is thought to be largely driven by CD4\(^+\) T cells. In agreement with this, both depletion of CD4\(^+\) T cells and treatment with broad-spectrum antibiotics improved the disease course in *Tbx21\(^{f/f}\*Fop3\(^{YFP}\) mice compared with *Foxp3\(^{YFP}\) mice. It is now well recognized that Tregs must adapt within the inflammatory environment to function and prevent this collateral damage. Their role in suppressing this damage from the parasite-specific, Th1-mediated immune response during *T. gondii* infection has been documented (19, 48–50). Interestingly, several studies have found that Tbet-deficient Treg function is not altered...
in the gastrointestinal tract, nor are production of IL-10 or other immune regulatory functions (21, 22, 38). However, we found that in our RNA-seq analysis, Tbet suppresses the expression of amphiregulin, a key cytokine that promotes wound healing. Tregs deficient in Areg have a reduced ability to promote wound repair from influenza infection, and a lack of functional Tregs can be overcome with Areg supplementation (17, 51, 52). The role of Areg during acute \textit{T. gondii} infection has not been explored, however, our data suggest that excessive levels of Tbet could reduce Areg expression and may explain the pathogenic Treg function during chronic infection. As for why Tbet-deficient Tregs are not capable of controlling the immune-mediated pathology due to the flora, we found pathways enriched in lymphocyte activation and the regulation of lymphocyte activation. The broad impact of Tbet on the ability of Tregs to be properly activated, proliferate, and survive likely leads to their inability to control the excessive inflammation observed during \textit{T. gondii} infection in the gastrointestinal tract. This is in agreement with the concept that Treg plasticity is needed for control of the specific inflammatory environment. Our work is important because it highlights the role of Tregs in driving several aspects of fitness that promote the ability of Tregs to dampen the immunopathology caused by dysbiosis during acute \textit{T. gondii} infection. The role of Tbet expression in Tregs during the chronic phase of infection in skeletal muscle and other tissues remains to be determined.

**DISCLOSURES**

The authors have no financial conflicts of interest.
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Supplemental Figure 1. Antibiotic water depletes commensal flora in both Foxp3^{YFPcre} and Tbx21^{if-}/Foxp3^{YFPcre} mice. (A) 16S Quantification by qRT-PCR at day 0 of infection. Results are cumulative of 2 experiments with n ≥ 3 in each group. Error bars = SD. *p<0.5, **p<.01, ****p<0.0001, Students t-Test.
Supplemental Figure 2. GoSeq Analysis and top 20 DEG in Foxp3^{YFPcre} and Tbx21^{ff}-Foxp3^{YFPcre} in Tregs from infected mice. (A) GoSeq analysis of the top pathways in biological processes, cell cycle and metabolic function.