Parasite-induced T$_{H}1$ cells and intestinal dysbiosis cooperate in IFN-$\gamma$-dependent elimination of Paneth cells

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Activation of Toll-like receptors (TLRs) by pathogens triggers cytokine production and T cell activation, immune defense mechanisms that are linked to immunopathology. Here we show that IFN-$\gamma$ production by CD4$^+$ T$_{H}1$ cells during mucosal responses to the protozoan parasite *Toxoplasma gondii* resulted in dysbiosis and the elimination of Paneth cells. Paneth cell death led to loss of antimicrobial peptides and occurred in conjunction with uncontrolled expansion of the *Enterobacteriaceae* family of Gram-negative bacteria. The expanded intestinal bacteria were required for the parasite-induced intestinal pathology. The investigation of cell type–specific factors regulating T$_{H}1$ polarization during *T. gondii* infection identified the T cell–intrinsic TLR pathway as a major regulator of IFN-$\gamma$ production in CD4$^+$ T cells responsible for Paneth cell death, dysbiosis and intestinal immunopathology.

Microbial and viral infections result in the potent activation of innate and adaptive immune cells required for host resistance to pathogens$^1$$^3$. Several classes of receptors are involved in the early detection of pathogens and the regulation of immunity. In most cases, the activation of Toll-like receptors (TLR) triggers inflammatory programs dependent on the adaptor MyD88 (ref. 1). Impaired TLR- and MyD88-dependent sensing of pathogens results in increased susceptibility to microbial and viral infections caused by uncontrolled pathogen dissemination$^1$$^3$. Inflammation driven by activation of the TLR pathway can also lead to severe and sometimes lethal tissue damage$^1$. The beneficial and detrimental effects of TLR-driven inflammation in the context of infectious diseases are particularly evident during immune responses to the protozoan parasite *T. gondii*. *T. gondii*–infected wild-type mice initiate potent interleukin 12 (IL-12)-dependent interferon-$\gamma$ (IFN-$\gamma$) responses, which are impaired by MyD88 inactivation$^4$$^7$. As a result, mice deficient in MyD88 die from uncontrolled parasite dissemination, with kinetics and pathogen loads that are practically indistinguishable from those seen in IL-12- and IFN-$\gamma$-deficient mice$^8$$^9$. However, overproduction of IFN-$\gamma$ in response to the same pathogen in mice that are unable to produce the immunoregulatory cytokine IL-10 also leads to rapid death, despite efficient control of the pathogen$^{11,12}$. Severe tissue damage caused by IFN-$\gamma$ seems to be responsible for the death of IL-10-deficient mice infected with *T. gondii*.$^{13}$ Thus, although IFN-$\gamma$-expressing T$_{H}1$ cells play an indispensable role in host resistance, these cells can also cause tissue destruction and lethal immunopathology.

Furthermore, even in wild-type mice, mucosal responses to the parasite result in detrimental T$_{H}1$ cell–driven intestinal pathology$^{14}$. Despite a well-appreciated role for MyD88 in driving T$_{H}1$ responses against the parasite$^{15,16}$ and the identification of TLR11 as a major activator of MyD88 during murine toxoplasmosis$^{17}$, little is known about cell type–specific MyD88-dependent programs dictating T$_{H}1$ polarization *in vivo*. Such insight is essential for understanding the mechanisms regulating T$_{H}1$–mediated host protection and the factors responsible for CD4$^+$ T cell–associated immunopathology. Here we showed that IFN-$\gamma$ production by CD4$^+$ T$_{H}1$ cells during mucosal responses to the protozoan parasite *T. gondii* is regulated by T cell–intrinsic MyD88 signaling pathway. CD4$^+$ T$_{H}1$ cells trigger intestinal immunopathology caused by IFN-$\gamma$–dependent Paneth cell death in conjunction with uncontrolled expansion of Gram-negative bacteria of the *Enterobacteriaceae* family.

**RESULTS**

*T. gondii* infection triggers intestinal dysbiosis

Infection with *T. gondii* results in severe intestinal inflammation that is associated with qualitative shifts in the composition of the intestinal microbiota$^{18,19}$. Quantitative analysis of intestinal bacteria showed transient expansion of Proteobacteria that peaked 7 d after *T. gondii* infection, and the simultaneous loss of Bacteroidetes, while the relative abundance of Firmicutes remained largely unchanged (Fig. 1a,b and Supplementary Fig. 1). The observed dysbiosis was transient and resolved at the end of the acute responses to the parasite.

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Received 4 October; accepted 27 November; published online 23 December 2012; doi:10.1038/ni.2508
**Figure 1** *T. gondii* infection results in intestinal dysbiosis. (a) qRT-PCR analysis of Proteobacteria and Bacteroidetes loads in the lumens of small intestines of wild-type mice left untreated (d0) or infected orally with 20 cysts of the *T. gondii* ME49 strain per mouse at the indicated time after infection. The results are representative of four independent experiments, each involving at least five mice per group. *P < 0.05; **P < 0.01. NS, nonsignificant. (b) Genomic DNA analysis of microbial communities from the lumens of naive or *T. gondii*-infected wild-type mice on day 7 after infection (n = 5–7 mice per group). For each mouse, 192 colonies containing cloned 16S rRNA amplicons were processed for sequencing. The relative frequencies of Proteobacteria (pink), Bacteroidetes (turquoise), Firmicutes (blue) and other phyla (purple) are shown. Bacteroidetes were not detected in samples prepared from *T. gondii*-infected mice. (c) Heat map of the relative abundance of the 40 most dominant genera in the small intestines of naive or *T. gondii*-infected wild-type mice as determined by genomic DNA analysis by 454-based DNA pyrosequencing. Samples are sorted based on hierarchical clustering of weighted unifrac distances. (d) Bacterial loads in the lumens of the small intestines of uninfected (d0) or *T. gondii*-infected mice (d7) analyzed by plating bacteria on blood agar or CHROMagar plates under aerobic conditions. *P < 0.05; **P < 0.001. Error bars, s.d. (e,f) *In situ* hybridization of Proteobacteria (green) in uninfected (e) or *T. gondii*-infected mice (f). The results are representative of four independent experiments, each involving at least five mice per group.

(Fig. 1a and Supplementary Fig. 1a–c). 454 pyrosequencing of the intestinal bacteria of infected mice also confirmed the dominance of Proteobacteria that appeared to be *Enterobacteriaceae*, a large family of Gram-negative bacteria frequently associated with intestinal inflammation and dysbiosis (Fig. 1c and Supplementary Fig. 2)\(^{20,21}\). Culture-based 16S rRNA gene pyrosequencing and *in situ* hybridization techniques showed the expansion of *Enterobacteriaceae*, and especially *Escherichia* spp. and *Shigella* spp., caused by *T. gondii* infection (Fig. 1c–f and Supplementary Figs. 2 and 3). Co-housing of *T. gondii*-infected mice

**Figure 2** *T. gondii* infection results in loss of Paneth cells. (a) qRT-PCR analysis of relative *Lyz1*, *Defr1* (α-defensin-1), *Defa-rs1*, *Defa21* and CR2 expression, measured in the small intestines of mice that were untreated (n = 6, blue) or infected orally with 20 cysts per mouse of the ME49 on day 7 after infection (n = 6, purple). \(* P < 0.001.\) (b) qRT-PCR analysis of RegIII-γ expression in the small intestines of naive (n = 6) and infected (n = 6) mice. (c) Histological visualization of Paneth cells in the small intestines of naive and infected (d7) mice. The results are representative of four independent experiments, each involving at least six mice per group. Blue arrows indicate Paneth cells; black arrows point toward bases of the crypts lacking these cells. (d) Electron microscopy analysis of the bases of the crypts in naive and *T. gondii*-infected mice (three mice per group). Blue arrow indicates Paneth cells. The results are representative of four independent experiments. Scale bars, 5 μm.
with uninfected animals indicated that the dysbiosis was restricted to infected mice and was not transmissible to uninfected littermate controls (Supplementary Fig. 4). This indicates that the presence of the parasite is essential for the selective expansion of Enterobacteriaceae (Escherichia spp. and Shigella spp.) because the parasite itself is not transmissible by the fecal–oral route from infected to uninfected mice. These results show that infection with T. gondii results in a quantitative shift in microbiota characterized by Proteobacteria dominance in the lumen of the inflamed intestine.

T. gondii infection triggers Paneth cell loss

Intestinal bacteria are controlled by antimicrobial peptides produced by Paneth cells and enterocytes. To test whether T. gondii triggers dysbiosis via aberrant induction of antimicrobial peptides (AMPs), we analyzed a panel of AMPs produced in naive and infected mice. We observed a marked loss of lysozyme and defensin expression in the intestines of T. gondii–infected mice (Fig. 2a). This result was somewhat unexpected because TLR ligands were shown to elicit induction of β-defensins and the bactericidal C-type lectin RegIII-γ24-26, and the protozoan parasite T. gondii triggers potent TLR-dependent immune responses. Parasitic infection resulted in the selective loss of Paneth cell–specific AMPs, as expression of RegIII-γ produced by multiple epithelial cell lineages25 was unimpaired (Fig. 2b).

To investigate the loss of AMP expression in T. gondii–infected mice, we isolated small intestines from naive and infected mice and analyzed Paneth cells in both groups. We observed that the acute response to the parasite resulted in the disappearance of Paneth cells, which were practically undetectable by day 7 after infection (Fig. 2c). Loss of Paneth cells was confirmed by electron microscopy experiments (Fig. 2d), suggesting that reduced expression of Paneth cell–specific AMPs was due to the disappearance of these cells. The disappearance of the Paneth cells was transient, with kinetics that closely recapitulated the dysbiosis observed in T. gondii–infected mice (Supplementary Fig. 5). Electron microscopic analysis of the Paneth cells showed that the death of these cells was not the result of apoptosis but rather was associated with mitochondrial damage (Supplementary Fig. 6). The ultrastructural analysis of dying Paneth cells during T. gondii infection also ruled out necrosis as a mechanism of Paneth cell elimination (Supplementary Fig. 6).

Morphologically necrotic cells show a decrease in cell electron density and complete degradation of organelles and membranes, neither of which was seen in Paneth cells during the course of T. gondii infection (Supplementary Fig. 6). Instead, at least some death of Paneth cells may be executed through necroptosis or a related mechanism of cell death associated with impaired mitochondrial functions. These results suggest that the reduction in AMPs triggered by T. gondii infection was due to the physical loss of Paneth cells.

Microbiota is required for the intestinal pathology

To examine the significance of the parasite-induced dysbiosis, we investigated whether mice lacking intestinal bacteria developed intestinal damage when infected with T. gondii. We used germ-free mice that are microbiologically sterile and lack the intestinal microbiota, along with germ-free mice colonized with a normal intestinal bacterial flora (conventionalized germ-free mice) as controls. In contrast to conventional and conventionalized mice (data not shown), T. gondii–infected germ-free mice did not develop intestinal pathology (Fig. 3a), and their Paneth cells were intact (Fig. 3b), suggesting that intestinal bacteria contribute to the development of intestinal inflammation.

To specifically address whether T. gondii–triggered dysbiosis characterized by expansion of Proteobacteria (Enterobacteriaceae) is involved in intestinal pathology and Paneth cell death, we isolated Enterobacteriaceae (Escherichia coli) from T. gondii–infected mice using E. coli–selective CHROMagar bacterial plates (Supplementary Fig. 3a) and adoptively transferred the bacteria into germ-free mice, alone or in combination with T. gondii. As controls, naive and T. gondii–infected germ-free mice were colonized with Bacteroides fragilis, a prominent intestinal Bacteroides species in mice. Transfer of Enterobacteriaceae isolates resulted in intestinal pathology in the presence of T. gondii parasitic infection, whereas transfer of B. fragilis did not induce pathology in either condition (Fig. 3a). The observed intestinal pathology was associated with loss of Paneth cells (Fig. 3b). These results indicated that T. gondii–induced dysbiosis characterized by expansion of Enterobacteriaceae contributes to intestinal pathology and loss of Paneth cells.

T. gondii–induced Paneth cell loss is mediated by TLR11

Because TLR11 exerts an important role in initial parasite sensing through the detection of T. gondii profilin and induction of intestinal pathology during mucosal immune responses to the pathogen17,28,29, we investigated whether TLR11 and its downstream adaptor protein, Myd88, contributed to the loss of Paneth cells caused by toxoplasmosis. Unlike for wild-type mice, we observed minimal loss of Paneth cells in Tlr11−/− or Myd88−/− mice infected with T. gondii (Figs. 2 and 4a). Histological analysis of Myd88−/− mice revealed that Paneth cells were present at the base of each crypt, whereas Tlr11−/− mice infected with
the parasite showed some loss of Paneth cells (Fig. 4a). Quantitative analysis of AMP expression showed minor loss (by a factor of 2–3) of Paneth cell–specific proteins in TLR11- or MyD88-deficient mice (Fig. 4b), whereas Paneth cell–specific AMPs were undetectable (less numerous by a factor of >100) in wild-type mice (Fig. 2a).

In addition, quantification of Proteobacteria and Bacteroidetes showed normal distribution of these taxa in infected Myd88−/− and Tlr11−/− mice (Fig. 4c). These results demonstrate that activation of TLR11 and MyD88 is required for the loss of Paneth cells triggered by the parasitic infection.

IFN-γ contributes to Paneth cell loss and intestinal dysbiosis

An important downstream effect of TLR-dependent recognition of pathogens is the induction of IFN-γ, which is required for host protection against parasites but can also lead to severe immunopathology. Because TLR11 and MyD88 are important regulators of IFN-γ production in T. gondii infections6,7,17, we next assessed whether the TLR11- and MyD88-mediated loss of Paneth cells was dependent on IFN-γ. Anti-IFN-γ, but not an isotype control antibody, completely prevented the loss of Paneth cells in T. gondii–infected mice (Fig. 5a). Furthermore, Paneth cells were intact in IFN-γ-deficient mice infected with T. gondii, even though markedly elevated parasite loads were detected in the intestines of these mice (Supplementary Fig. 7), suggesting that IFN-γ was required for Paneth cell loss.

Because Paneth cells were also in T. gondii–infected Ragg−/− mice (data not shown), we focused on CD4+ and CD8+ T cells as cellular sources of IFN-γ. Depletion of CD4+ T cells in wild-type mice largely prevented the loss of Paneth cells triggered by T. gondii infection (Fig. 5a). Quantitative analysis of AMPs in the intestines of mice infected with the parasite and treated with antibodies against IFN-γ or CD4 showed that elimination of Paneth cells was dependent on IFN-γ and CD4+ T cells (Fig. 5b). Depletion of CD8+ T cells reduced Paneth cell loss, though less so than CD4+ antibody–mediated depletion (Fig. 5a,b). Wild-type CD4+ T cells adoptively transferred into Rag1−/− or Ifng−/− hosts infected with T. gondii induced the elimination of Paneth cells (Supplementary Fig. 8), whereas adoptively transferred CD8+ T cells had only a minor role in T. gondii–triggered loss of Paneth cells (Supplementary Fig. 8). Finally, IFN-γ blockade in Tlr11−/− and Myd88−/− mice completely prevented the loss of AMPs triggered by T. gondii (data not shown). Thus, IFN-γ regulated by TLR11 and MyD88 was predominantly, even though incompletely, responsible for the parasite-induced loss of Paneth cells.

MyD88 signaling in T cells regulates an IFN-γ response

We next addressed the mechanisms by which CD4+ T cells contribute to the IFN-γ and TLR11–MyD88–dependent disappearance of Paneth cells. Despite a well-established role for dendritic cells (DCs) in the activation of T cells, mice with a DC-specific MyD88 deficiency have only minor defects in establishing systemic Tg1 immunity against T. gondii16. Similarly, mice with a DC-specific MyD88 deficiency

Figure 4 TLR11-mediated activation of MyD88 triggers Paneth cell death and intestinal dysbiosis. (a) Wild-type (not shown), Tlr11−/− and Myd88−/− mice (five mice per group) were left untreated or were infected orally with 20 cysts per mouse of the ME49 strain of T. gondii. Histological visualization of Paneth cells in the small intestines was performed on day 7 after infection. Blue arrows indicate Paneth cells; black arrows point toward bases of the crypts lacking these cells. (b) qRT-PCR analysis of relative Defcr1 (α-defensin-1), Defa1, Defa21, CR2 and Reglll−γ expression, measured by qRT-PCR in the small intestines of wild-type (WT; n = 3), Tlr11−/− (n = 6) and Myd88−/− (n = 4) mice on day 7 after infection. ∗P < 0.05; **P < 0.01; NS, nonsignificant. (c) qRT-PCR analysis of Proteobacteria and Bacteroidetes in the lumens of small intestines of naive (blue) or T. gondii–infected (purple) mice. The data shown are the means ± s.d. ∗P < 0.05; **P < 0.01; ***P < 0.001. The results are representative of 12 independent experiments, each involving 3–7 mice per group.

Figure 5 IFN-γ mediates loss of Paneth cells. (a) Wild-type (WT) mice were treated with isotype control (IC) antibodies, anti-IFN-γ, anti-CD4 or anti-CD8 and then infected orally with 20 cysts per mouse of the ME49 strain of T. gondii. Histological analyses of Paneth cells in small intestines were performed on day 7 after infection. (b) qRT-PCR analysis of relative Lyz1, Defcr1 (α-defensin-1), Defa1, Defa21, CR2 and Reglll−γ expression, measured in the small intestines of naive (blue) or T. gondii–infected WT mice treated with IC, anti-IFN-γ, anti-CD4 or anti-CD8 (purple). ∗P < 0.05; **P < 0.01. The results are representative of three independent experiments, each involving 4–7 mice per group.
CD4+ T cells was analyzed by flow cytometry. The data shown are representative of five independent experiments each involving 4–6 mice per group. (b) Frequency (b) and absolute quantification (c) of T_{H}1 cells (CD4+IFN-γ) in the mLN's of mice infected orally with the parasite. The data shown are representative of five independent experiments each involving 4–6 mice per group. NS, nonsignificant; * P < 0.01; ** P < 0.001. Error bars, s.d.

MyD88 signaling in macrophages (as assessed in Mlys-Cre Myd88fl/fl mice) and epithelial cells (as assessed in Villin-Cre Myd88fl/fl mice) was also dispensable for the induction of IFN-γ-secreting CD4+ T cells during mucosal responses to T. gondii (Fig. 6a–c). Simultaneous inactivation of MyD88 in DCs and macrophages in CD11c-Cre × Mlys-Cre Myd88fl/fl mice or in DCs and epithelial cells in CD11c-Cre × Villin-Cre Myd88fl/fl mice still resulted in induction of a high frequency and high absolute numbers of IFN-γ-producing CD4+ T cells after T. gondii infection (Fig. 6). Furthermore, mice lacking MyD88 in DCs, macrophages or epithelial cells also lost Paneth cells during experimental toxoplasmosis (Fig. 7a,b and data not shown), indicating that MyD88 signaling in other cell type(s) apart from DCs, macrophages and epithelial cells induces T_{H}1 immunity and intestinal pathology.

Targeted inactivation of MyD88 in T cells in Lck-Cre Myd88fl/fl mice resulted in a reduction of IFN-γ-producing CD4+ T cells almost identical to that observed in Myd88−/− animals (Fig. 6). Furthermore, the Paneth cells were largely intact in T. gondii–infected Lck-Cre Myd88fl/fl mice (Fig. 7a,b), and these mice did not show the significant dysbiosis or intestinal pathology seen in the other MyD88-deficient mice analyzed (Fig. 7c and data not shown). However, quantitative analysis of T_{H}1 cells and microbiota showed that Lck-Cre Myd88fl/fl mice were not identical to Myd88−/− mice after T. gondii infection.

Figure 7  T cell–intrinsic MyD88 signaling mediates loss of Paneth cells intestinal dysbiosis. (a) qRT-PCR analysis of relative expression of Lyz1, Defcr1 (α-defensin-1), Defa-rs1, Defa21 in the small intestines of wild-type (WT), Myd88−/− and cell type–specific Myd88-deficient mice on day 7 after infection. The results are representative of five independent experiments each involving 4 or 5 mice per group. (b) Histological visualization of Paneth cells in small intestines of DC-Myd88−/−, T-Myd88−/− and DC&T-Myd88−/− mice compared to complete Myd88-deficient mice (Myd88−/−). The results are representative of five independent experiments each involving 4 or 5 mice per group. (c) Bacterial loads in the lumens of small intestines of naive (blue) or infected WT, Myd88−/− and cell type–specific Myd88−/− mice (purple) were analyzed by plating bacteria on blood agar plates on day 7 after infection. CFU, colony-forming units. * P < 0.05; ** P < 0.01. NS, nonsignificant. The results are representative of five independent experiments each involving 4 or 5 mice per group. Error bars, s.d.
infection (Figs. 6b,c and 7c). Mice lacking Myd88 in both DCs and T cells (CD11c-Cre × Lck-Cre Myd88<sup>fl/fl</sup>) had frequencies and absolute numbers of \( \text{T}_{\text{H}1} \) cells similar to those in Myd88<sup>−/−</sup> animals (Fig. 6b,c). We also observed that IL-1R-deficient mice, similarly to \( \text{Casp}1^{−/−} \) mice<sup>28</sup>, showed normal \( \text{T}_{\text{H}1} \) immunity to \( T. \text{gondii} \) (data not shown). Thus, we speculate that T cell–intrinsic TLR signaling or additional IL-1R- and IL-1R-independent T cell–intrinsic functions of Myd88 regulate IFN-\( \gamma \) secretion by CD4<sup>+</sup> T cells. In addition, T cell–intrinsic Myd88 signaling is linked to IFN-\( \gamma \)-stability in T cells<sup>30,31</sup>. Taken together, these results indicated that the Myd88 signaling pathways in T cells is required for the induction of \( \text{T}_{\text{H}1} \) responses against the parasite and subsequent intestinal dysbiosis, where DC Myd88 plays a supplemental role in triggering IFN-\( \gamma \) production by CD4<sup>+</sup> T cells.

**DISCUSSION**

Using a comprehensive panel of cell type–specific Myd88-deficient mice, we show that T cell–intrinsic Myd88 signaling, rather than Myd88 activation in antigen-presenting cells, had a major role in \( \text{T}_{\text{H}1} \) polarization during \( T. \text{gondii} \) infection. T cell–specific elimination of Myd88 reduced IFN-\( \gamma \) secretion by CD4<sup>+</sup> T cells. Moreover, \( T. \text{gondii} \)-infected T cell–specific Myd88-deficient mice, similarly to completely Myd88-deficient animals, did not develop the IFN-\( \gamma \) and CD4<sup>+</sup> T cell–mediated dysbiosis responsible for the intestinal pathology seen in orally infected wild-type mice. These results are in contrast to those for both DC-specific Myd88-deficient mice and mice lacking Myd88 in both DCs and macrophages, which showed a modest reduction in \( \text{T}_{\text{H}1} \) cells as compared with wild-type controls. Furthermore, lack of Myd88 in DCs and macrophages had no effect on \( T. \text{gondii} \)-induced intestinal dysbiosis and immunopathology characterized, as shown here, by the death of Paneth cells. This further dismisses the role of TLR signaling in antigen-presenting cells for \( \text{T}_{\text{H}1} \)-mediated immunity and immunopathology.

Previous studies have established that Myd88<sup>−/−</sup> mice, which cannot signal through IL-1 family receptors and most TLRs<sup>1</sup>, including TLR11 (ref. 17), are acutely susceptible to \( T. \text{gondii} \) infection<sup>7</sup>. Myd88<sup>−/−</sup> mice fail to activate T cells and produce IFN-\( \gamma \) essential for host resistance to the parasite<sup>2,15</sup>. In vitro CD4<sup>+</sup> T cell polarization assays using transgenic naïve CD4<sup>+</sup> T cells show that wild-type DCs have a superior ability to drive \( \text{T}_{\text{H}1} \) differentiation from the same precursor pool as compared with Myd88<sup>−/−</sup> DCs<sup>15</sup>. The combination of in vivo phenotypes of Myd88<sup>−/−</sup> mice, in vitro \( \text{T}_{\text{H}1} \) polarization assays and the essential role for DCs in antigen-presentation<sup>22</sup> suggest the possibility that activation of TLRs and the downstream adaptor protein Myd88 in antigen-presenting cells, specifically in DCs trigger \( \text{T}_{\text{H}1} \) responses<sup>2</sup>. The proposed model of innate control of adaptive immunity implies that Myd88 activation in DCs is solely responsible for induction of IFN-\( \gamma \)-production by CD4<sup>+</sup> T cells. A major limitation of this concept is the lack of in vivo evidence since the conditional Myd88-deficient mice were unavailable. Instead, the phenotypes of Myd88<sup>−/−</sup> mice were extrapolated to TLR defects in innate immune cells<sup>2</sup>. Recent generation of Myd88<sup>fl/fl</sup> mice<sup>33</sup> allowed investigation of cell type–specific Myd88 functions involved in host defense against microbial pathogens.

Our recent analysis of DC-specific Myd88-deficient mice systemically infected with \( T. \text{gondii} \) unexpectedly showed that Myd88 signaling in DCs is dispensable for the engagement of T cell responses<sup>16</sup>. Although both Myd88<sup>−/−</sup> and DC-specific Myd88-deficient mice are highly susceptible to the parasitic infection, activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells is impaired only in Myd88<sup>−/−</sup> mice and is largely intact in mice lacking Myd88 only in DCs. The examination of DC-specific Myd88-deficient mice indicated that TLR activation in DCs promotes IFN-\( \gamma \) production by NK cells rather than T cells. Myd88 signaling in DCs is required for early IFN-\( \gamma \) production by NK cells, which is critical for limiting the infection until T cells can contribute to immune control of the parasite<sup>16</sup>. These results strongly implicated a major defect in the innate immune response as being responsible for the acute susceptibility of DC-specific Myd88-deficient mice to \( T. \text{gondii} \) infection<sup>16</sup>, but they left unanswered a fundamental question regarding cell type–specific requirements for Myd88 in the regulation of \( \text{T}_{\text{H}1} \) immunity. This question is of particular relevance for mucosal responses to \( T. \text{gondii} \). In addition to leukocytes, mucosal epithelial cells are involved in TLR-mediated sensing of both the parasite and commensal bacteria<sup>28</sup>. Specifically, it is the intestinal microbiota that potentiates IFN-\( \gamma \)-production by CD4<sup>+</sup> T cells in response to \( T. \text{gondii} \) infection<sup>28</sup>. It appears that TLR11-mediated parasite recognition combines with the TLR4- and TLR9-dependent effects of intestinal bacteria to influence the outcome of the IFN-\( \gamma \)-production and intestinal pathology<sup>28</sup>, but the cellular biology of TLR-dependent Myd88 activation responsible for \( \text{T}_{\text{H}1} \) mediated immunity and immunopathology is not known. These points further underline the importance of understanding the mechanisms regulating IFN-\( \gamma \) production by CD4<sup>+</sup> T cells in response to TLR-mediated activation. Our current in vivo experiments formally established a T cell–intrinsic Myd88 signaling pathway in regulation of \( \text{T}_{\text{H}1} \) effector choice. Furthermore, our results also ruled out a role for Myd88 in epithelial cells, macrophages and B cells, in addition to DCs, for controlling IFN-\( \gamma \)-production by CD4<sup>+</sup> T cells. These observations indicate that we have just begun to understand the extent to which Myd88 signaling in cells of both innate and adaptive immunity contributed to host defense. Myd88 in epithelial cells regulates their proliferation and expression of AMPs<sup>24,26,34–36</sup>; B cell–intrinsic Myd88 is involved in augmenting IgM secretion<sup>27</sup> and in T cell–dependent antibody production in response to viral particles<sup>38</sup>; and, as established in this work, T cell–intrinsic Myd88 regulated \( \text{T}_{\text{H}1} \) polarization. Altogether, these experiments suggest remarkable cell type–specific Myd88 functions, but further work is needed to identify the relevant Myd88 activators in different cell types.

In addition to identifying Myd88 in T cells as a central molecule regulating IFN-\( \gamma \) by CD4<sup>+</sup> T cells, our experiments have revealed a mechanism responsible for severe intestinal inflammation mediated by \( \text{T}_{\text{H}1} \) cells. We showed that IFN-\( \gamma \)-production primarily by CD4<sup>+</sup> T cells during \( T. \text{gondii} \) infection resulted in the death of Paneth cells, loss of their AMPs and the subsequent expansion of intestinal bacteria, the majority of which appeared to be Enterobacteriaceae. These results add to a growing body of literature indicating that IFN-\( \gamma \), as an essential host protection factor, is also responsible for host tissue damage<sup>39,40</sup>. We also established that \( T. \text{gondii} \) infection in germ-free mice, in the absence of intestinal bacteria, was not sufficient for intestinal inflammation and the loss of Paneth cells. Instead, the pathogen-induced intestinal dysbiosis, and in particular the presence of Proteobacteria, were crucial for the development of intestinal inflammation and the IFN-\( \gamma \)-dependent death of Paneth cells during parasitic infection. These results are in agreement with the ability of intestinal bacteria to potentiate a \( \text{T}_{\text{H}1} \) response to the parasite<sup>28</sup> that, although required for host protection, is also involved in intestinal pathology characterized by Paneth cell death. Overall, our investigation of cell type–specific factors regulating \( \text{T}_{\text{H}1} \) polarization during \( T. \text{gondii} \) infection identified a T cell–intrinsic Myd88 pathway as a major regulator of IFN-\( \gamma \)-production by CD4<sup>+</sup> T cells leading to lethal intestinal pathology triggered by the parasite.
**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary information is available in the online version of the paper.

**ACKNOWLEDGMENTS**

This research was supported by US National Institutes of Health (NIH) grants R01 AI085263 to E.Y. and R01 DK070855 to L.V.H. and by the Howard Hughes Medical Institute (L.V.H.). C.R.S. was supported in part by NIH grant A1005284-33. We would also like to thank C. Behrendt and C. Clements for germ-free mouse husbandry.

**AUTHOR CONTRIBUTIONS**

E.Y. conceived the project. M.R., S.-h.H., C.L.W., D.K., A.B., C.R.S., J.M., S.V. and F.Y. analyzed data. M.R., C.L.W. and F.Y. wrote the manuscript, with all authors contributing to the writing and providing advice.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
**ONLINE METHODS**

**Mice.** C57BL/6 mice were obtained from the University of Texas Southwestern (UTSW) Medical Center Mouse Breeding Core Facility. Rag2−/− and Ifng−/− mice were obtained from Jackson Laboratory. Triflurymide and Myd88−/− mice have been previously described in experimental toxoplasmosis studies. Myd88fl/fl mice have been previously described in experimental toxoplasmosis and in the context of TLR11+−/− mice. Myd88fl/fl × Villin-Cre (E-Myd88−/−), Myd88fl/fl × CD19-Cre (B-Myd88−/−), Myd88fl/fl × Lck-Cre (T-Myd88−/−) and Myd88fl/fl × LysM-Cre (M-Myd88−/−) mice have been described previously. In this study, the Myd88fl/fl mice were additionally crossed to CD11c-Cre × Lck-Cre and LysM-Cre × CD11c-Cre deleter mice. RegIIIγ-deficient (Reg3γ−/−) mice were previously described.

Control and experimental mice were age-matched within individual experiments. All mice were maintained at the American Association of Laboratory Animal Care–accredited animal facility at the UTSW Medical Center. All of the animals that were used were age- and sex-matched. All mice were maintained in the same animal room.

**Toxoplasma gondii infections, histopathology and RT-PCR.** All mice were infected orally with an average of 20 T. gondii cysts (ME49 strain). At days 3, 5, 7, 10 and 14 after infection, the animals were necropsied, and portions of the small intestines were fixed in Carnoy’s fixative, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin or Alcian blue or used for in situ hybridization. For in situ hybridization, after deparaffinization and rehydration in hybridization buffer (0.9 M NaCl, 0.1% SDS and 20 mM Tris-HCl, pH 7.4), the small intestines were incubated overnight at 50 °C in the dark with Alexa–488-conjugated Enterobacteriaceae-specific ENTBAC (5′-catgataacatcagttgacgcg-3′) and Alexa–532-conjugated eubacteria UEB338 (5′-gctgcttctgcgttaggtg-3′) probes for bacterial 16S rRNA genes. The probes were diluted to final concentrations of 1 ng/µl in hybridization solution. The sections were then washed three times with a hybridization solution for 15 min, counterstained with SYTO62 and mounted using ProLong Gold Antifade Reagent (Invitrogen). The sections were imaged using a Leica SPE system fitted with a Leica 63X objective NA 1.4. The data sets were processed using Leica Advanced Fluorescence software (Leica).

For electron microscopy analysis, the small intestines were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, followed by 1% osmium tetroxide in 0.1 M sodium cacodylate. The samples were embedded in epoxy resin (Electron Microscopy Sciences) and polymerized at 60 °C. Ultrathin sections were sectioned at 5 nm thick on a Leica Ultracut S ultramicrotome (Leica Microsystems) and collected on 400-mesh copper grids. The grids were stained with 5% uranyl acetate in 50% ethanol and lead citrate. Sections were examined at 120 KV with a Tecnai G2 Spirit transmission electron microscope (FEI Company), and the images were recorded on a Gatan USC1000 Detection System with taxon-specific or universal 16S rRNA gene primers. To complement the relative qPCR approach developed initially, a plasmid-based method for quantification of intestinal dysbiosis was used. Specifically, absolute bacterial abundance was determined using standard curves produced from serial dilutions of cloned bacterial DNA corresponding to a short segment of the 16S rRNA gene that was amplified using conserved 16S rRNA gene specific primers as described previously. Briefly, the abundance of Proteobacteria, Bacteroidetes and Firmicutes were determined by the use of taxon-specific 16S rRNA gene primers that were also used for relative abundance determination (Proteobacteria, forward primer 5′-gctgacgmcggcggtaag-3′ and reverse primer 5′-gctcaagggccatgatg-3′; Bacteroidetes, forward primer 5′-gcttgaagagggctgccg-3′, and reverse primer 5′-gctgctactagggatg-3′; Firmicutes, forward primer 5′-gctgctactagggatg-3′). Threshold cycle (Ct) values were plotted against plasmid copy number to produce each standard curve; GraphPad Prism analysis of each semilog line by nonlinear regression produced an equation relating Ct value to 16S rRNA gene copy number. The 16S rRNA gene copy number was calculated by multiplying the amount of DNA present in the sample. It should be noted that this qPCR method measures 16S rRNA gene copies per sample, not actual bacterial numbers or colony-forming units. Percentage of taxa in the small intestine was determined by dividing the taxon-specific 16S rRNA gene copy number by the eubacterial (universal) 16S rRNA gene copy number.

**Ex vivo measurement of CD4+ T cell responses.** To assay the responses of mice infected with T. gondii, the mesenteric lymph nodes were harvested from WT, Myd88−/− and cell type–specific MyD88-deficient mice on day 7 after infection. Single cell suspensions were re-stimulated with 1 µg/ml CD3 (BD Biosciences) for 5 h in the presence of GolgiPlug (Bredelinf A, BD Biosciences). After in vitro re-stimulation, the cells were washed once in PBS + 1% FBS and stained with fluorochrome-conjugated antibodies. Cell fluorescence was measured using a FACS Calibur or LSRII flow cytometer, and data were analyzed using FlowJo software (Tree Star). To quantify the depletion efficiency of Myd88−/− T cells in the T-Myd88−/− mice, genomic DNA was extracted from sort-purified T cells. The residual amount of bacterial genomic DNA was extracted from the small intestinal luminal content on days 3, 5, 7, 10 and 14 after infection using a QiaGen DNAeasy Kit according to the manufacturer’s instructions. Broad-range 16S rDNA PCR (forward primer, 5′-tagtttgatytgagcgtc-3′ and reverse primer, 5′-aggtcttgcaagcgtt-3′) was used to amplify and clone the bacterial DNA present in the individual samples without any prior cultivation. The cloned inserts were identified through sequencing of the 16S rDNA gene fragments using the pCR2.1 vector–specific M13 reverse and T7 promoter primers. The DNA sequences were interpreted by comparing the retrieved sequences with those stored in the GenBank database using the basic local alignment software tool (BLAST; National Center for Biotechnology Information) and the Seqmatch tool (Ribosomal Database Project).
the **loxP**-flanked (‘floxed’) region was quantified by Taqman PCR using primers (forward 5′-gttgtgtgtgctccagcc-3′, reverse 5′-tctcaattagctggtgca-3′) and a Taqman probe 5′-FAM-gtcggacctgtgctgg-Blackhole-3′ on an ABI PRISM 7300 sequence detection system as described previously51. Depletion of M\text{yD88} in T cells achieved by the Lck-Cre deleter was at least 96%. The genomic DNA from Lck-Cre–negative Myd88\textsuperscript{fl/fl} mice was used for the no-deletion control.

**Statistical analysis.** All data were analyzed with Prism (Version 5; GraphPad). Data were considered statistically significant with \( P \) values <0.05 obtained using a two-tailed \( t \)-test.

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