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IL-17 producing gamma-delta T cells protect against *Clostridium difficile* infection

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Abstract

Colitis caused by *C. difficile* infection is an increasing cause of human morbidity and mortality, especially after antibiotic use in healthcare settings. The natural immunity of newborn infants and protective host immune mediators against *C. difficile* infection are not fully understood, with data suggesting that inflammation can be either protective or pathogenic. Here we show an essential role for IL-17A produced by gamma-delta T cells in host defense against *C. difficile* infection. Fecal extracts of children with *C. difficile* infection showed increased IL-17A and T cell receptor gamma-chain expression, and IL-17 production by intestinal gamma-delta T cells was efficiently induced after infection in mice. *C. difficile* induced tissue inflammation and mortality were each significantly increased in mice deficient in IL-17A or gamma-delta T cells. Neonatal mice, with naturally expanded ROR-γt+ gamma-delta T cells poised for IL-17 production were resistant to *C. difficile* infection, whereas eliminating gamma-delta T cells or IL-17A each efficiently overturned neonatal resistance against infection. These results reveal an expanded role for IL-17 producing gamma-delta T cells in neonatal host defense against infection and provide a mechanistic explanation for the clinically observed resistance of infants to *C. difficile* colitis.
Introduction

Clostridioides difficile (formerly Clostridium difficile) is a Gram-positive, spore-forming, anaerobic bacillus that colonizes the large intestine and causes colitis when normal microbiota communities are disrupted. C. difficile infection is a major healthcare-associated infection and is now recognized as the primary cause of infectious diarrhea after hospitalization and treatment with antibiotics (1). In the US, C. difficile was responsible for almost half a million infections and associated with approximately 29,000 deaths in 2011 (2). There is also rising incidence and severity of C. difficile infection (3-7), and community-acquired infection is increasingly recognized (8-10). Clinical symptoms of C. difficile infection range from mild diarrhea to a severe, life-threatening pseudomembranous colitis, toxic megacolon and death (11, 12). However, colonization with C. difficile is frequently asymptomatic, particularly in very young infants (13, 14).

Intestinal inflammation associated with C. difficile infection is primarily mediated by the major virulence factors of toxigenic C. difficile, toxins A (TcdA) and B (TcdB), on the intestinal epithelium (15). The immune components that protect against C. difficile infection are not fully understood, with data suggesting that inflammation can play both protective and pathogenic roles. Several studies have shown that mice with altered innate immune responses, including defects in innate lymphoid cells, IL-1β, and MyD88 signaling, have increased mortality after C. difficile infection (16-20). On the other hand, IL-23 deficient mice have decreased inflammation and disease severity (21). Further, we previously showed that persistent diarrhea in C. difficile infection correlates with intestinal inflammation and not fecal pathogen burden in adults and children with C. difficile infection (22, 23) which suggests that inflammation may also be responsible for clinically symptomatic infection. Thus, C. difficile infection likely involves a complex interplay between the organism, the intestinal microbiome and local immunological mediators, with disease resolution requiring a balanced inflammatory response that eradicates infection without causing collateral tissue damage (24-27).
Several known features of *C. difficile* epidemiology and pathogenesis led us to examine the role and source of IL-17A in defense against this pathogen. Firstly, an influx of neutrophils into the mucosa is a characteristic feature of *C. difficile* infection (28), and IL-17 signaling is important for neutrophil recruitment to local tissues during other bacterial infections (29-34). Furthermore, very young infants are highly protected against *C. difficile* infection (13, 14), which is in striking contrast to most other infectious diseases. Whereas immune components protective against microbial infection are typically hyporesponsive in neonates (reviewed in (35)), IL-17A producing gamma-delta T cells remain relatively abundant and may be particularly important mediators of mucosal defense during the initial stages of postnatal life (36-41). We hypothesize that the temporal and anatomic distribution of IL-17 producing gamma-delta T cells might contribute to *C. difficile* infection resistance in very young infants. Furthermore, the abundance of IL-17A producing gamma-delta T cells is diminished by antibiotic treatment (42), the major risk factor for *C. difficile* infection. Each of these correlative observations led us to investigate whether IL-17 and gamma-delta T cell are induced by *C. difficile* infection in children, and more definitive analysis on their potential role in protection.

In this article, we report that IL-17 arising from gamma-delta T cells is a major component of the response to *C. difficile* infection. Complementary transcripts encoding IL-17A and T cell receptor delta-chain were elevated in fecal extracts of infected children, highlighting that these immune components are induced during *C. difficile* infection. We also demonstrate IL-17-producing gamma-delta T-cells are naturally expanded in neonatal mice, and essential for enhanced protection against *C. difficile* infection in this developmental window. Together, these results reveal an essential role for IL-17 produced by gamma-delta T cells in defense against *C. difficile* infection.
Results

**IL-17 efficiently induced during C. difficile infection**

Various murine models of *C. difficile* infection have been described, with variations in inoculation dosage and antibiotic pre-treatment regimes required to achieve consistent infection, that likely reflect differences in commensal microbiota composition for mice in each institution (43-48). Experiments were performed in two institutions (Washington University in St. Louis and Cincinnati Children’s Hospital) where similar susceptibility to *C. difficile* was established after optimizing antibiotic treatment and infectious dose. In both facilities, age and gender-matched mice on the C57BL/6 background were exposed to a defined cocktail of antibiotics prior to oral gavage with *C. difficile* spores, and then monitored for weight loss and mortality. In adult mice we found doses ranging from $1 \times 10^4$ to $1 \times 10^6$ CFU caused symptoms of *C. difficile* disease, including ruffled fur, hunched posture, and weight loss, with dose-dependent mortality (**Figure 1A**). *C. difficile* intestinal burden was monitored by qPCR against the *tcdB* gene as this approach is more sensitive than culture (49), and detects endogenous strains of *C. difficile* found in some mouse strains (50, 51). We found that *C. difficile* was absent in antibiotic-treated mice prior to infection, peaked at day 2, then declined to nearly undetectable levels by day 8 after infection (**Figure 1B**). This tempo was further confirmed by histopathological analysis demonstrating the most severe epithelial damage, and edema in the cecum day 2 post-infection, followed by almost complete recovery by day 8 (**Figure 1C**). Likewise, leukocyte infiltration into the cecum and colon lamina propria, and expression of proinflammatory and anti-microbial genes peaks days 2 to 4 post-infection, and progressive decline during the recovery phase (**Figure 1D**, and **Supplemental Figure 1**).

Interestingly, intestinal inflammation after *C. difficile* infection was associated with selectively increased production of IL-17. Leukocytes recovered from the cecum of infected mice showed >20-fold increased percentage of IL-17A producing cells, but minimal changes in IFN-γ or IL-4 production (**Supplemental Figure 2**). This paralleled selectively increased *Il17a* and *Il17f*
expression in intestinal tissues and the draining mesenteric lymph node (mLN) (Figure 1E). IL-17A expression was increased 20-fold and 25-fold in the cecum and mLN respectively by day 2 post infection, whereas only marginal or non-significant shifts were found for Ifng and Il4. IL-17F is frequently co-produced with IL-17A, (52, 53) and Il17f expression is simultaneously up-regulated upon C. difficile infection (Figure 1E). In turn, sharply increased levels of all three dimeric forms of IL-17A/F protein were found in cecum, colon, and mLN beginning day 2 after C. difficile infection (Figure 1F). Thus, robust induction of IL-17 is already well underway at time points when mortality appears in mice treated with the highest C. difficile inoculum.

**IL-17A is essential for host protection against C. difficile infection**

To determine the contribution of IL-17A to host protection against C. difficile infection, potential differences in susceptibility of IL-17A-deficient mice were evaluated. We found sharply increased mortality after infection of IL-17-deficient compared with isogenic C57BL/6 control mice (Figure 2A). Increased susceptibility paralleled more profound tissue damage, particularly edema and ulceration in the cecum after C. difficile infection in IL-17-deficient compared with control mice (Figure 2B). Significantly increased C. difficile recovery was also found in the intestinal contents (Figure 2C), along with increased intestinal permeability measured by systemic recovery of orally administered FITC dextran after C. difficile infection in IL-17A-deficient compared with wild-type control mice (Figure 2D). There was significantly higher neutrophil infiltration in the colon of IL-17A-deficient mice, likely reflecting the greater severity of disease in these animals (Supplemental Figure 3A). Interestingly however, depletion of granulocytes using the anti-Ly6G antibody did not impact susceptibility, suggesting neither the presence of neutrophils nor their recruitment through IL-17a dependent pathways are needed for protection in our model of C. difficile infection (Supplemental Figure 3B).

Importantly, these differences in susceptibility could not be explained by potential differences in the intestinal microbiome of these unique mouse strains since the bedding between cages of
these mice were regularly mixed before and after \textit{C. difficile} inoculation. Likewise, increased susceptibility was observed amongst genetically identical co-housed mice after IL-17A functional neutralization using antibodies (Figure 2E). Taken together, these findings indicate an essential role for IL-17A in protection against epithelial and tissue injury during \textit{C. difficile} infection.

\textit{Gamma-delta T cells are the major source of IL-17A following \textit{C. difficile} infection}

Given the critical role of IL-17A in our model of \textit{C. difficile} infection, we sought to determine the cellular source of this cytokine. IL-17A can be produced by multiple cell types, including conventional CD4$^+$ Th17 alpha-beta T cells, CD8$^+$ T cells, gamma-delta T cells, innate lymphoid cells, NK cells and epithelial cells. We found both alpha-beta and gamma-delta T cells infiltrated the lamina propria of infection mice, with progressively increased accumulation of each cell T cell subsets in the first week after infection (Figure 3A). Interestingly, expression of the activation marker, CD69, was significantly higher in intestinal gamma-delta compared with alpha-beta T cells after \textit{C. difficile} infection (Figure 3B), while only background expression levels were found for each cell type prior to \textit{C. difficile} infection (Supplemental Figure 4). Furthermore, gamma-delta T cells in the mesenteric lymph nodes (mLN) showed increased proliferation upon infection, whereas alpha-beta T cells remained at baseline, based on Ki67 expression levels (Figure 3C).

Flow cytometry and intracellular staining further showed that gamma-delta T cells constitute the dominant source of this cytokine in the cecum, colon and mLN, accounting for \textasciitilde80\% of all IL-17A-producing cells, while \textalpha\textbeta T cells constituted less than 10\% in each tissue day 2 post-infection (Figure 4A). Expanding this analysis showed that IL-17A production was near exclusive to intestinal gamma-delta T cells within the first week after infection (Figure 4B). Sharply increased IL-17A production was found for mLN gamma-delta T cells by day 2 post-infection, whereas cells in the cecum and colon showed delayed kinetics not reaching peak
levels until day 6 post-infection (Figure 4B). By contrast, only a small fraction of alpha-beta T cells produced IL-17A within the first 6 days after C. difficile infection (Figure 4B). Consistent with the role of gamma-delta T cells in the immediate response to C. difficile, Il17a transcripts were unchanged (cecum, colon) or significantly reduced (mLN) after infection in TCR-δ knock-out mice (Tcrd-/-), which lack all mature gamma-delta T cells (54) (Figure 4C). In turn, Tcrd-/- compared with wild-type control mice showed significantly increased susceptibility following C. difficile infection (Figure 4D). Taken together, these data implicate gamma-delta T cells as the primary source of protective IL-17A during C. difficile infection.

To investigate whether IL-17A producing gamma-delta cells may represent a similar component of the clinical response to C. difficile infection, we performed qRT-PCR to quantitate the relative abundance of il17a and trdv transcripts encoding IL-17A and the T-cell receptor delta chain, in fecal extracts from children with C. difficile infection compared with uninfected controls. Whereas il17a mRNA was detected in only 2 of 16 control samples, 8 of 15 children with C. difficile infection had elevated il17a expression. Similarly, this analysis showed detectable trdv expression in the fecal extracts of all (15 of 15) children with C. difficile infection, but in only 3 of 16 control children (Figure 5). Thus, IL-17 and gamma-delta T cells are both induced by C. difficile clinical infection in humans and mice.

C. difficile infection induced activation of gamma-delta T cells

The importance of IL-17 producing gamma-delta cells in mucosal barrier protection is increasingly recognized (52, 55-57). However, the signals mediating their activation and unique molecular features of these cells are not fully described, particularly during response to infection. Previous studies have shown that gamma-delta T cells are activated in part through non clonal receptors, such as NK cell receptors and Toll like receptors (58, 59). However, we found that expression of NK receptors (NK1.1, NKG2A, NKG2D, NKp46) by gamma-delta T cells in the
intestines of *C. difficile* infected mice was sharply reduced compared with the IL-17 promoting transcriptional regulator RORgammat (**Figure 6A**). Other potential activation signals for gamma-delta T cells include stimulation through each cell’s respective TCR (60). However, despite their potential for responding to a broad array of antigens through somatic rearrangement of V (variable), D (diversity) and J (joining) gene segments, oligoclonal subsets sharing the same TCR-gamma and TCR-delta chains in specific tissues are often described, likely having populated distinct sites during embryonal and postnatal development (61, 62). For example, gamma-delta T cells in the dermal layers primary express Trgv5 (International ImMunoGeneTics (IMGT) (http://www.imgt.org/) nomenclature nomenclature is used throughout (63-65)), whereas gamma-delta intraepithelial cells (IELs) primary express Trgv7 (64, 66). IL-17 producing gamma-delta cells predominantly bear Trgv6 (61, 67), although under certain circumstances, IL-17 producing gamma-delta cells may produce Trgv4 or rarely Trgv2 or Trgv3 instead of Trgv6.

We examined TCR receptor expression using commercially available antibodies recognizing Trgv1, Trgv4, Trgv5 and Trgv7, and these did not label a majority of IL-17 producing gamma-delta T cells in the intestines of *C. difficile* infected mice (**Supplemental Figure 5**). To bypass this limitation, RNA-seq was used to evaluate TCR gene usage amongst sort purified IL-17A⁺ and IL-17A⁻ gamma-delta T cells from mice after *C. difficile* infection (**Supplemental Figure 6**). This analysis showed a limited distribution of TCR usage amongst gamma-delta T cells recovered from the mLN. Comparison of TCR gene usage between IL-17A⁺ and IL-17A⁻ gamma-delta T cells revealed that nearly all cytokine producing cells expressed the genes encoding Trgv6 (58%) or Trgv2 (26%) in association with Trdv4 (98%; Trdv4 was formerly known as Vδ1 in prior nomenclature (68)) (**Figure 6B**). Thus, IL-17 producing gamma-delta T cells responsive to *C. difficile* infection show a highly constrained oligoclonal repertoire dominated by Trgv6/Trdv4 and Trgv2/Trdv4 TCRs. These findings are similar to the recently described clonal expansion of Vγ6⁺Vδ4⁺ cells that provide immunity against *Staphylococcus aureus* infection (69).
To investigate the relative contribution of TCR stimulation for IL-17 production by gamma-delta T cells, cytokine production was evaluated after stimulation with defined anti-TCR antibodies. This analysis showed >100-fold and >30-fold increased production of IL-17A/A and IL-17A/F, respectively, by gamma-delta T cells recovered from the mLN of C. difficile infected mice in response to anti-CD3 or anti-TCR-gamma-delta stimulation (Figure 6C). In turn, several studies have reported that IL-1β/IL-23 can also independently drive IL-17A production by gamma-delta T cells (29, 70-72). We found similar results since comparable production of IL-17 was achieved after stimulation with IL-1β/IL-23 compared with anti-TCR antibodies (Figure 6C). Interestingly however, IL-17 production increased dramatically with combined IL-1β/IL-23 and anti-TCR stimulation, highlighting synergistic effects of these proinflammatory cytokines and cognate antigen TCR stimulation in promoting activation of gamma-delta T cells. These results are consistent with increased IL-1β and IL-23 responsiveness after gamma-delta TCR stimulation, which in turn results in amplified IL-17 production (60).

Gamma-delta T cells are produced in waves during embryonal development with TCR chain expression closely tracking stage of development. Those expressing Trgv6 are produced in the thymus exclusively during embryogenesis whereas those bearing Trgv2 develop later in gestation or the early newborn period (58, 61). IL-17 effector fate of Trgv6-producing cells is determined prior to thymic egress while development of IL-17 effector fate for other TCR-expressing gamma-delta subsets is less well described. Recently several studies have demonstrated characteristic features of gamma-delta cells that acquire IL-17 effector fate during embryogenesis (57, 68, 73-76). Cell surface labeling demonstrated them to be negative for tumor necrosis factor receptor family member CD27 (77) and positive for the IL-7 receptor CD127 (78) as expected for IL-17 producing gamma-delta cells (Figure 6D). The aforementioned RNA-seq data was further analyzed, and as expected, IL-17 producing gamma-delta T cells isolated from C. difficile infected animals expressed high levels of Rorc, Blk, Sox13, Ztb16 transcription factors, Il17a and Il17f
cytokines, and cytokine receptors Il1r1 and Il23r (Figure 6E), all of which have been described to promote developing IL-17 producing gamma-delta T cells and effector function (65, 79-81). Conversely, Tcf7, a negative regulator which is down-regulated during Tgamma-delta17 cell development (64, 65) was markedly repressed (Figure 6E). As expected, expression of Stat3, Irf4 and Batf, transcription factors essential for promoting Th17 differentiation in conventional CD4+ alpha-beta cells, was also reduced, whereas expression of the Th17 repressor Maf, essential for IL-17 producing gamma-delta cell development (82), was up-regulated in IL-17A+ gamma-delta T cells (Figure 6E). Thus, gamma-delta cells responding to C. difficile infection are characteristic of those previously demonstrated to acquiring effector fate in utero.

**Neonatal resistance to C. difficile infection is dependent on IL-17 and gamma-delta cells**

Human newborn infants are naturally resistant to C. difficile infection (13, 14). To investigate whether natural immunity against C. difficile during the neonatal period occurs similarly in mice, the susceptibility of 7-day old neonate compared with 6-8 week old adult mice was evaluated. Remarkably, we found at least >100-fold increased resistance to C. difficile infection amongst neonatal compared with adult mice. Adult mice showed progressively increased susceptibility after infection with 10^4, 10^5 and 10^6 CFUs, whereas mortality was not observed for neonatal mice infected with these same C. difficile inocula (Figure 7A).

Given the critical role for IL-17A during C. difficile, as well as identifying gamma-delta T cells as the source of IL-17A, we hypothesized that newborns have greater capacity to expand this cell population on encountering C. difficile which may account for the known resistance of very young infants against C. difficile infection. To explore this further, we examined the relative abundance of gamma-delta T compared with alpha-beta T cells in the intestinal tissues of neonatal compared with adult mice. We found gamma-delta T cells to be significantly enriched amongst the intestinal lamina propria and mLN of neonatal compared with adult mice, with only
marginal difference in percentage alpha-beta T cells (Figure 7B). Importantly, a majority of gamma-delta T cells in neonatal mice were RORyt positive prior to infection (Figure 7C), and IL-17 production was sharply increased after C. difficile infection amongst gamma-delta T cells in neonatal compared with adult mice (Figure 7D).

Complementary loss of function approaches were used to investigate the necessity of gamma-delta T cells to enhanced resistance of neonatal mice to C. difficile infection. Neonatal Il17a-/- and Tcrd-/- mice each compared with age matched wild-type control mice were highly susceptible to infection (Figure 7E). We also found sharply increased susceptibility in neonatal mice after the administration of anti-IL17A or anti-TCR gamma-delta antibodies, whereas the normal resistance of neonatal mice was not affected in littermate control neonatal mice treated with anti-TCR alpha-beta or isotype control antibodies (Figure 7F). In line with prior studies in adult mice (83), gamma-delta T cells were not depleted in neonatal mice treated with anti-TCR gamma-delta antibody (clone UC7-13D5). However, TCR was functionally neutralized since staining with an alternative anti-TCR gamma-delta antibody clone (GL3) was efficiently eliminated, and the increased susceptibility phenotype is identical to Tcrd-/- neonatal mice (Figures 7E and 7F). Importantly, susceptibility of neonatal mice did not further increase amongst neonatal mice simultaneously administered IL-17A and gamma-delta T cell neutralizing antibodies (Figure 7G). These non-additive effects highlight gamma-delta T cells as an important cellular source of IL-17 that protects neonatal mice against C. difficile infection.
Discussion

IL-17 has been implicated in the development of chronic inflammation and autoimmunity. Preclinical infection models show it is also essential for host defense against bacterial and fungal pathogens and in maintaining homeostasis, particularly at mucoepithelial surfaces (55, 84). The dual roles of IL-17 appear to be conserved in humans, as heightened levels of expression have been linked to the development of Crohn’s disease and psoriasis, while patients genetically deficient in IL17RA or expressing a dominant negative form of IL17F are susceptible to mucocutaneous Candidiasis. Protective roles for IL-17 in defense against bacterial infections, including E. coli, B. subtilis, and L. monocytogenes are also well described (29, 85, 86).

C. difficile infection induces profound damage of the intestinal mucosa in susceptible individuals, suggesting that IL-17 defenses may impact on the outcome of this disease. Indeed, we demonstrated that IL-17 was selectively produced in intestinal tissues following C. difficile infection. IL-17 message and protein were both sharply induced within 2 days of infection, the time point at which infected animals begin to become ill and at higher infectious doses succumb to disease. Strikingly, animals with impaired IL-17 responses through gene knockout or antibody neutralization had increased mortality after C. difficile infection, directly implicating this cytokine in protection against disease.

IL-17 can arise from several cell types in the intestine, including CD4 positive alpha-beta T-cells (Th17 cells), innate lymphoid cells (ILC3 cells), and gamma-delta T-cells. We investigated the cellular source of intestinal IL-17 at the critical early time points during C. difficile infection and found that gamma-delta cells accounted for almost the entire IL-17 staining population. In turn, il17a and tcrd mRNA, encoding IL-17A and T-cell receptor delta chain, respectively, were present in most children with C. difficile infection while rarely detected in uninfected children. Correspondingly, T-cell receptor delta chain deficient animals unable to produce gamma-delta cells were almost devoid of intestinal IL-17 gene expression following C. difficile infection. As expected, given their lack of IL-17 responsiveness, these animals had sharply increased
susceptibility to *C. difficile*, with near identical mortality kinetics compared with IL-17A-deficient mice.

The principal function of IL-17A has largely been attributed to neutrophil recruitment to inflammatory sites, and recent reports examining the susceptibility of Nod1-/- and Myd88-/- mice have linked impaired neutrophil recruitment with worse outcome following *C. difficile* infection (17, 18). In our model, Il17a-/- mice showed no defect in neutrophil infiltration into the intestines. Further, we examined the role of neutrophils in defense against infection in our model and found no impact on survival after neutrophil depletion. Our results are similar to those of McDermott, et. al. which reported no impact on outcome of *C. difficile* infection following neutrophil depletion with the anti-GR1 antibody used in our studies. Similarly, GM-CSF treatment decreased neutrophil infiltration into the intestines of *C. difficile* infected mice but did not impact on survival (87). It should be noted that our results are in contrast with other publications, including those by Nakagawa et al, which showed Il17a-/- mice on the BALB/c genetic background were protected from *C. difficile* infection (88). Further, Jarchum et. al. found increased mortality in *C. difficile* infected mice after neutrophil depletion using antibody 1A8, independent of IL-17 manipulation (17). Although the factors responsible for these discrepant results are unclear, we surmise that differences in mouse strain, experimental protocol, and commensal flora (19), may be contributing factors. The contribution of host immunity to defense against *C. difficile* infection is exceptionally complex, as might be expected for an infection that causes widespread injury to the intestinal mucosa. Components of the innate and adaptive immune systems have been shown to contribute to defense, and yet marked inflammation is a hallmark of the disease, and accounts for the clinical features of severe *C. difficile* colitis (22, 23). Murine models of *C. difficile* disease highlight the multifactorial roles of innate responses in defense against acute infection while also demonstrating the same responses may be protective in some experimental conditions while harmful in others. For example, Myd88 and Il-1b are protective against infection (17, 19), yet specific blockade of inflammasome activation and IL-1β were protective against intestinal
inflammation and injury (89). Finally, we show here that gamma-delta cells mediate defense via release of IL-17A, yet prior studies demonstrated that Rag1-deficient mice lacking all alpha-beta and gamma-delta T cells were not more susceptible to *C. difficile* infection (16, 90, 91). In those publications innate lymphoid cells (ILC) were shown to mediate protection. Rag1-deficient mice are have expanded numbers of IL-17 and IL-22 producing RORγT-positive ILC which produce excessive amounts of cytokine and antimicrobial peptides (92, 93) and therefore likely masked the contribution of IL-17 producing gamma-delta cells to defense.

The developmental features and physiologic functions of IL-17 producing gamma-delta cells are coming into focus through much recent research (52, 68, 73-75, 82, 94-96). A point of interest has been the relative contribution of TCR stimulation and cytokine signaling in activation. IL-1β and IL-23 are known to activate IL-17 production in developmentally programmed gamma-delta cells whereas TCR signaling has typically been regarded as inessential (57, 62, 97, 98). We found that cytokine and TCR stimulation alone stimulated comparable levels of IL-17 production from *C. difficile* responsive gamma-delta cells. However, combined stimulation resulted in markedly increased IL-17 production, consistent recent study suggests that TCR signaling may enhance activation through upregulated expression of cytokine receptor expression, effectively licensing cells for high level activation (60, 77, 99). The TCR may also contribute to the recently identified capacity for gamma-delta cells to develop a memory phenotype and expand in response to reinfection when they often have the capacity to co-produce IL-17A and TNFα (100, 101)

The contribution of TCR signaling to IL-17 release prompted us to investigate TCR gene usage by these cells. We found that the vast majority expressed gamma chains Trgv6 or Trgv2 in association with delta chain Trdv4. Trgv6/Trdv4 expressing cells are known to arise during early embryogenesis and acquire effector function prior to thymic egress. These cell demonstrate a distinctive expression pattern of transcription factors and cytokine receptors enabling eventual IL-17 production (38, 64, 65, 102). We demonstrate by flow cytometry and
transcriptional analysis that *C. difficile*-responsive IL-17 producing gamma-delta T cells share characteristic features of embryonal derived IL-17 producing gamma-delta T-cells (64, 65, 102), including their CD27^D127^RORγt^+ phenotype and increased expressions of *Il1r1, Il1r2, Il23r, Rorc, Sox13, Zbtb16, and Cd163l1*.

IL-17 producing gamma-delta T-cells arise in the embryonic thymus and populate the periphery during a confined stage of *in utero* development (73-75, 94). During challenge, such as mucosal infection, IL-17 producing gamma-delta T-cells response typically occurs via proliferation from a pre-existing population of fully programed progenitor cells. In mice, the size of the IL-17 producing gamma-delta T-cell progenitor pool wanes with age, leading to decreased capacity for IL-17 secretion from the gamma-delta pool after the newborn period. Similar findings have been observed in humans (36-39, 41, 103). For example, IL-17-producing gamma-delta T cells are much more abundant in peripheral blood of newborns as compared to adults (103), and gamma-delta T cells are found at greatest abundance in the intestines of full-term newborn infants (41). It has long been recognized that adults are more prone to severe disease and poor outcome than young children infected with *C. difficile*. Remarkably, up to 65% of children are colonized with *C. difficile* within the first year of life but fail to manifest disease, while by age three, presence of *C. difficile* generally causes intestinal inflammation and injury (104). We hypothesized that the greater abundance of intestinal IL-17 producing gamma-delta T-cells during the first several months of life may account, at least in part, for the age dependent-changes in *C. difficile* susceptibility during childhood. To test this hypothesis, we first demonstrate that neonatal mice, like human infants, are markedly resistant to *C. difficile* infection. Consistent with enhanced number of IL-17 producing gamma-delta cells observed in newborn humans, we found higher levels of these cells in intestines of neonatal compared with adult mice. Complementary loss of function approaches using neonatal mice with targeted
genetic defects in IL-17A or TCR-delta, or functional neutralization of each with antibodies confirm the necessity of each in enhanced neonatal resistance to *C. difficile* infection.

Taken together, our findings highlight the importance of IL-17 production by gamma-delta T cells in host defense against *C. difficile* infection. The naturally expanded pool of these cells in intestinal tissue in neonates explains the unique resistance of infants to *C. difficile* infection. Thus, IL-17 producing gamma-delta T cell abundance dictates *C. difficile* infection susceptibility and enhancing the accumulation of these cells represents an exciting new therapeutic approach for preventing infection by this emerging human pathogen.
Methods

Stool collection

Stool samples from *C. difficile* culture-positive children were collected at the St Louis Children’s Hospital (SLCH), a tertiary pediatric center in St Louis, MO, between June 2011 and Jule 2012, and stored at -80°C. SLCH microbiology laboratory uses a flowchart for the diagnosis of *C. difficile*, starting with a glutamate dehydrogenase (GDH) enzyme immunoassay (EIA) (the Wampole C. diff Quik Chek, Orlando, FL). If this test is positive, it is confirmed with the GeneXpert *C. difficile* polymerase chain reaction (PCR) (Cepheid, Sunnyvale, CA). We excluded children whose residual stools were <1mL in volume. We included inpatient, outpatient and emergency department visits, and had no limitations on patient age or underlying disease. We also enrolled a convenience sample of symptomatic controls: a study team member, when present, stored available stool samples from children diagnosed with bacterial gastroenteritis or who had diarrheal stools with negative bacterial cultures, within 48 hours of receipt.

Mice

All mice used were adults 7-9 weeks of age, unless otherwise specified. C57BL/6 mice were purchased from Jackson Laboratory. *Il17a-/-* mice were generously provided by Dr. Yoichiro Iwakura. *Tcrd-/-* mice were generously provided by Dr. Anthony French.

All mice were bred and maintained in the specific pathogen-free animal facility of Washington University School of Medicine or the specific pathogen-free animal facility at Cincinnati Children's Hospital. At Washington University, animals were housed in static polysulfone microisolators (Allentown), where their bedding type was 1/8” Bed-o-cobs Corncob bedding (Andersons Lab Breeding; Maumee, OH). Animals were fed Picolab Rodent Diet 20 (Purina 5053) and received autoclaved sterile city tap water. At Cincinnati Children’s Hospital animals were housed in static microisolator cages (Alternative Design Manufacturing & Supply,
Inc; Siloam Springs, AR) where bedding was ¼” Bed-o’Cobs (Andersons Lab Breeding; Maumee, OH) and animals were fed Laboratory Autoclavable Rodent Diet 5010 (LabDiet; St. Louis, MO) and received autoclaved water purified by reverse osmosis. All cage components (bottom, lid, wire hopper, feed), bedding, water bottles, nestlets and enrichment were sterilized by autoclave. NuAire Class II Type A2 Biological Safety Cabinets were used for cage change. Health Surveillance was performed using 6 to 8-week-old, outbred, female sentinels (CD-1 or SW from Charles River or Taconic, respectively) that received dirty bedding from each cage on the rack during weekly or bi-weekly cage change. One sentinel cage was placed per side of ventilated rack, approximately every 70-80 cages. Every twelve weeks a sentinel was bled for serology to assess antibodies to: *Mycoplasma pulmonis*, CAR bacillus, Ectromelia, EDIM, Hantaan, K virus, LCMV, MAV1, MAV2, MCMV, MHV, MPV, MTV, MVM, Polyoma, PVM, REO3, Sendai, TMEV, and *Encephalitozoon cuniculi*. Feces and fur swabs were also collected every 12 weeks for PCR evaluation for *Aspiculuris tetraptera*, *Myocoptes*, *Radfordia/Myobia*, *Syphacia muris*, and *Syphacia obvelata*.

**Antibodies**

The following antibodies were purchased from Biolegend: Alexa Fluor 700 anti-CD45 (30-F11), APC/Cy7 anti-CD3ε (145-2C11), APC/Cy7 anti-CD8α (53-6.7), APC/Cy7 anti-CD19 (6D5), APC/Cy7 anti-CD45 (30-F11), FITC anti-CD11b (M1/70)), FITC anti-IL-17A (TC11-18H10.1), FITC anti-TCRgamma-delta (GL3), FITC anti-CD3ε (145-2C11), FITC Rat IgG1, κ isotype (RTK2071), PE Armenian hamster IgG isotype (HTK888), PE Rat IgG1, κ isotype (RTK2071), PE Rat IgG2a, κ isotype (RTK2578), PE Rat IgG2b, κ isotype (RTK4530), PE Mouse IgG1, κ isotype (MOPC- 21), PE anti-NK1.1 (PK136), PE anti-NKG2D (A10), PE anti-NKp46 (29A1.4) PE anti-Ly6G (1A8), PE anti-Ly49D (4E5), PE anti-NKG2A (16A11), PE anti-CD8α (53-6.7), PE anti-CD69 (H1.2F3), PE anti-IFNy (XMG1.2), PE anti-IL-4 (11b11), PE anti-IL-17F (9D3.1C8),
PE anti-CD27 (LG.3A10), PE anti-Vγ1 (2.11), PE anti-Vγ4 (UC3-10A6), PE anti-Vγ5 (536), PE/Cy7 anti-CD11b (M1/70), PerCP/Cy5.5 anti-TCRβ (H57-597), PerCP/Cy5.5 anti-CD19 (6D5), APC anti-TCRgamma-delta (GL3), LEAF-purified anti-CD3ε (143-2C11) and LEAF-purified anti-TCRgamma-delta (GL3). Anti-CD16/CD32 Fc Block (2.4G2) and BV650 anti-RORgt (Q31-378) was purchased from BD Biosciences. FITC anti-Ki-67 (SolA15) and PE anti-RORg(t) (B2D) were purchased from eBioscience. The following antibodies were purchased from BioXCell: Rat IgG2a isotype control (2A3), anti-Ly6G (1A8), mouse IgG1 isotype control (MOPC-21), anti-IL-17A (17F3), Armenian hamster IgG isotype control, anti-TCRgamma-delta (UC7-13D5), and anti-TCRbeta (H57-597). PE anti-Vγ7 (F2.67) was a gift from Dr. Pablo Pereira.

C. difficile infection

C. difficile spores were prepared as previously described (94). Briefly, C. difficile (VPI 10463) were streaked on anaerobic blood agar plates and grown anaerobically using the GasPak system for 6 days to induce sporulation. Collected spores were then washed in PBS and heat-shocked at 56°C for 10 minutes to kill remaining vegetative organisms. The spores were centrifuged and resuspended in DMEM and frozen in aliquots at -80°C. Spores were quantified by plating serial dilutions onto taurocholate-fructose-agar plates.

Age- and gender-matched mice were provided with water containing kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), vancomycin (0.045 mg/ml), colistin (0.057 mg/ml) and metronidazole (0.215 mg/ml) for 5 days. A single dose of clindamycin-2-phosphate (30 mg/kg) was given 48 hours later by intraperitoneal (i.p.) injection. Mice were then orally gavaged 48 hours later with C. difficile spores diluted in 400 ul of DMEM. Mice were monitored daily for mortality and symptoms including diarrhea, weight loss, hunched posture and ruffled furs. To minimize differences in the microbiome, beddings were mixed randomly between experimental cages for two weeks prior to C. difficile inoculation.
Neonatal infections were performed by initiation treatment with water supplemented with the same antibiotics (kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), vancomycin (0.045 mg/ml), colistin (0.057 mg/ml) and metronidazole (0.215 mg/ml)) to nursing mothers on the day of delivery for 5 days. On day 5 after delivery (and initiation of antibiotic drinking water supplementation), mothers received a single dose of clindamycin-2-phosphate (30 mg/kg). On day 7 after birth, neonatal mice were orally gavaged with \textit{C. difficile} (1 x 10^6 spores in 50 ul of DMEM per pup). On the day of infection, a nursing foster mother (on the wild-type C57BL/6 background) who had not received antibiotics was placed in the same cage. Infant pups were monitored twice daily from the day of infection until 28 days of life (21 days post-infection).

\textit{Neutrophil Depletion in adult mice}
Mice were injected i.p. with 1 mg of isotype control (2A3) or anti-Ly6G (1A8) beginning one day prior to infection, followed by 500 ug every 48 hours thereafter. Neutrophil depletion was confirmed by flow cytometry of cecum and colon tissues 2 days post infection.

\textit{IL-17A neutralization in adult mice}
Mice were injected i.p. with 1 mg of isotype control (MOPC-21) or anti-IL-17A (17F3) beginning one day prior to infection, followed by 500 ug every 48 hours thereafter.

\textit{IL-17A neutralization and gamma-delta T cell depletion in neonatal mice}
Neonatal mice were injected with each depleting/neutralizing or isotype control antibody (100 ug/50 ul/ pup) beginning two days prior to \textit{C. difficile} spore inoculation, and on the day of infection. Survival of infected neonatal mice was monitored for 21 days post-infection.
**Cell preparation**

For isolation of lamina propria cells, whole cecum and colon tissues were excised and washed in ice-cold PBS to remove digestive contents. Tissues were then cut into 5 mm pieces and incubated in PBS supplemented with 1 mM DTT, 5 mM EDTA and 3% FCS for 30 minutes at 37°C to remove epithelial cells. The remaining tissues were collected by straining through 100 um filter and homogenized by razor blades, followed by incubation in RPMI supplemented with collagenase type VIII (1 mg/ml) and DNAase I (50U) for 40 minutes. Cells were strained through 40 um filter into complete RPMI media and collected by centrifugation. For isolation of mesenteric lymph node (mLN) cells, whole mLNs were excised and crushed using sterile glass slides, followed by straining through 40 um filter and collected by centrifugation.

**Flow cytometry**

Cells were kept at 4°C throughout the procedure. Single cell preparations were incubated with 5% FCS, 2% rat serum and Fc block for 30 minutes. Without washing, fluorophore-conjugated antibodies were added at the recommended concentration and incubated for an additional 30 minutes in the dark. After two washes, dead cells were labeled using LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen) according to manufacturer’s protocol. Cells were then fixed with 2% paraformaldehyde for 30 minutes prior to analysis. Data was acquired on BD LSRII or BD FACSCanto and analyzed by FlowJo software.

**Intracellular staining**
Cytokine staining was performed using BD Cytofix/Cytoperm (BD Biosciences). Briefly, cells were suspended in media and stimulated with PMA/ionomycin for 5 hours at 37°C. Surface labeling was performed as above, followed by fixation and permeabilization according to manufacturer's protocol. Nuclear staining was performed using FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's protocol.

**Histology**

Cecum and colon tissues were excised and washed in ice-cold PBS to remove digestive contents and opened longitudinally with surgical scissors. Tissues were then mounted on filter paper and fixed in 4% paraformaldehyde for 30 minutes at 4°C. Following incubations in 30% sucrose and 30% sucrose:OCT (Tissue-Tek), tissues were placed in cryomolds and frozen in OCT on slurry of dry ice and 2-methyl-butane. Tissue blocks were sectioned using Leica CM1850 cryostat (Leica Biosystems) and stained with hematoxylin/eosin.

**Isolation of gamma-delta T cells**

Cells from mLNs were harvested from day 4-infected mice and stimulated with PMA/ionomycin in vitro for 3 hours at 37°C. IL-17A-producing cells were labeled using Mouse IL-17 Secretion Assay (Miltenyi Biotec) and additionally stained with APC-TCRgamma-delta, PerCP/Cy5.5-TCRβ, PerCP/Cy5.5-CD19 and PO-PRO-1 (Invitrogen) to exclude dead cells. Labeled cells were sorted on BD Aria-II (BD Biosciences).

**RNA isolation and cDNA synthesis**
Following dissection and washing, whole cecum and colon tissues were cut into 5 mm pieces and immediately homogenized in RNA Bee (Amsbio) using Omni GLH homogenizer with disposable RNase-free probes (Omni International) at 4°C. RNA was extracted according to manufacturer’s protocol. Total RNA was further purified using RNeasy Mini Kit (Qiagen). RNA concentration and purity were measured using Nanodrop 1000 (Thermo Scientific). cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit, according to manufacturer’s protocol. For isolated cells, RNA was extracted using RNeasy Mini Kit according to manufacturer’s protocol.

**Real-time PCR**

Quantitative real-time PCR of gene expressions were performed using Taqman assays according to manufacturer’s protocol. Taqman Gene Expression Master Mix (Applied Biosystems) was used for amplification, and Mouse GAPDH Endogenous Control (Applied Biosystems) was used as endogenous control. Data collection was performed using 7500 Fast System and analyzed by SDS v2.4 (Applied Biosystems). For list of Taqman assays used, see supplementary information.

**TcdB assay**

Cecal content were collected and weighed, and total nucleic acid was isolated using Bacteremia DNA Isolation Kit (BiOstic) according to manufacturer’s protocol. TcdB was amplified using Fast SYBR Green Master Mix (Applied Biosystems) with the following primers: 5’-ACGGACAAGCAGTTGAAT-3’; 5’-ATTAATACCTTTGCATGCT-3’.

**Tissue explant culture and ELISA**
Whole cecum and colon were excised and washed in ice-cold PBS to remove digestive content, and rinsed with penicillin/streptomycin. Tissues were weighed, cut into 5 mm pieces and cultured in complete RPMI at 100 mg/ml for 24 hours. Culture supernatant were collected and analyzed by ELISA for IL-17A/A, IL-17A/F and IL-17F/F (eBioscience). For mLNs, total cells were stimulated with plate-bound anti-CD3ε and cultured in complete RPMI at 2x10^6 cells/ml for 72 hours. Culture supernatant were collected and analyzed by ELISA as above. For culture of gamma-delta T cells, mLNs were harvested from day 4-infected mice, and gamma-delta T cells were isolated using TCRγ/δ^+ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer’s protocol. Purified gamma-delta T cells were cultured in complete RPMI at 2x10^6 cells/ml for 72 hours, under the indicated conditions.

**Intestinal permeability assay**

Intestinal permeability was measured in *C. difficile*-infected wild-type and *Il17a/-/-* mice by performing oral gavages with FITC-labelled dextran (FD4) and measuring translocation of fluorescence into the plasma. In brief, mice were starved for 6 hours, then orally gavaged with 0.6 mg/g of FITC-dextran (FD4, Sigma). Blood was collected via retro-orbital bleed 3 hours later and allowed to clot for 30 minutes at room temperature. Samples were centrifuged to remove clot, and serum fluorescence was measured on Synergy HT Microplate Reader (BioTek).

**RNA-seq and bioinformatics**

Library preparation, sequencing and analysis were performed by Genome Technology Access Center at Washington University School of Medicine. Sequencing was performed on HiSeq 2000 (Illumina), and aligned to the transcriptome with TopHat 1.4.1. Transcript abundances were
determined using Cufflinks 2.0.2, and differential gene expression between samples was analyzed by EdgeR and filtered for transcripts passing multiple-testing corrections. Data has been submitted to GEO with accession number GSE143124.

Statistics

Two-tailed unpaired t-test was used unless otherwise noted. P-value of less than 0.05 was considered statistically significant. Bar graphs and scatter plots show mean±SEM. For all figures, *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.0001; ns, not significant.

Study approval

The prospective cohort study was performed at St Louis Children's Hospital (SLCH), St. Louis, MO after obtaining approval from the Institutional Review Board of the Washington University School of Medicine. All animal experiments at Washington University and Cincinnati Children’s Hospital were IACUC approved and performed at AAALAC accredited institutions.
Author contributions

YSC, GP, TYS, SSW and DBH designed the experiments and analyzed the data. YSC, GP, and TYS performed, analyzed and interpreted experiments. IBC analyzed and interpreted RNA-seq data. YSC, SSW, and DBH wrote the manuscript.
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Figure 1

A. Percent survival over days post-infection.

B. C. difficile tcdB levels post-infection.

C. Histological images of Cecum and Colon over days post-infection.

D. Percentage CD45+ cells in Cecum and Colon over days post-infection.

E. Fold change of Il17a, Il17f, Ilfn, and Il4 in Cecum, Colon, and mLNs over days post-infection.

F. Concentration of IL-17A/A, IL-17A/F, and IL-17F/F in Cecum, Colon, and mLNs over days post-infection.
Figure 1: IL-17A expression is increased in intestines of mice with *C. difficile*. (A) Percent survival of adult WT C57BL/6 mice infected with various CFU of *C. difficile*. (B) *C. difficile* burden was monitored by qPCR of *tcdB* gene in total cecal content (5x10⁵ CFU; N=6-8 per time point). qPCR results were normalized to a standard curve to calculate pg *tcdB* in the input sample. (C) Longitudinal sections of cecum and colon stained with H&E (5x10⁵ CFU; 20X magnification; scale bar, 50um). (D) Infiltrating leukocytes were monitored by flow cytometry following *C. difficile* (5x10⁵ CFU). Gated on live CD45+ cells (N=4 per time point). (E) Total tissues from cecum, colon and mLN of *C. difficile*-infected mice (5x10⁵ CFU) were harvested, analyzed by qRT-PCR and normalized to day 0 samples with GAPDH as endogenous control (N=3-5 per time point). (F) Total cecum and colon tissues were harvested from *C. difficile*-infected mice (5x10⁵ CFU), dissociated and cultured for 24 hours, and culture supernatant was collected and analyzed by ELISA. Single-cell suspension of mLN samples from *C. difficile*-infected mice (5x10⁵ CFU), were stimulated with plate-bound anti-CD3ε for 72 hours, and similarly analyzed by ELISA (N=4 per time point).
Figure 2

A

Percent survival

Days post-infection

WT (N=28)

Il17a-/-(N=32)

B

C57BL/6

IL17a-/-

C

D

C. difficile tcdB (pg g cecal content)

FD4 (µg/ml)

WT

Il17a-/-

E

Percent survival

Days post-infection

Isotype Ctrl. (N=9)

anti-IL-17A (N=12)
Figure 2: IL-17A is essential for host-protection during *C. difficile*. (A) Percent survival of WT and *Il17a*−/− mice following *C. difficile* (4x105 CFU; p<0.0001, log-rank test; data combined from three experiments). (B) Longitudinal sections of cecum and colon of WT and *Il17a*−/− mice at day 2 post-infection, stained with H&E (4x105 CFU; 10X magnification; bar, 50 um; data representative of three experiments) (C) *C. difficile* burden in WT and *Il17a*−/− mice was monitored by qPCR of *tcdB* gene in total cecal content at day 2 post-infection (4x105 CFU). qPCR results were normalized to a standard curve to calculate pg *tcdB* in the input sample (N=6 for WT; N=9 for *Il17a*−/−). (D) WT and *Il17a*−/− mice at day 2 post-infection (4x105 CFU) were orally gavaged with FITC-Dextran (4 kDa). Blood was collected via retro-orbital bleed 3 hours later and serum fluorescence was measured (data combined from two experiments; N=10 for WT; N=11 for *Il17a*−/−). (E) Percent survival of WT littermate mice treated with isotype control (MOPC-21) or anti-IL-17A (17F3) after *C. difficile* (4x10^6 CFU; p<0.05, log-rank test). Mice were treated with 1 mg of antibody on day -1, followed by 0.5 mg every 48 hours thereafter.
Figure 3

A

CD8+ αβ T cells

CD8- αβ T cells

γδ T cells

Cecum, % among CD45+CD3+

Colon, % among CD45+CD3+

Days post infection

B

αβ T cells

γδ T cells

Cecum

Colon

Day 0

Day 4

CD69

Ki67

C

αβ T cells

γδ T cells

Day 0

Day 4

Figure 3
Figure 3: Gamma-delta T cells respond rapidly to *C. difficile*. (A) alpha-beta T cell and gamma-delta T cell infiltration into the cecum and colon following *C. difficile* was analyzed by flow cytometry. Gated on live CD45+ CD3ε+ CD8α+/- TCRβ+ cells or live CD45+ CD3ε+ TCR gamma-delta+ cells (N=4 per time point). (B) Surface expression of CD69 in alpha-beta T cells and gamma-delta T cells at day 4 post-infection (4x10^5 CFU). Filled histograms represent isotype control staining. Gated on live CD45+ CD3ε+ CD4+ TCRβ+ cells or live CD45+ CD3ε+ TCR gamma-delta+ cells (results representative of two experiments). (C) Ki-67 expression of alpha-beta T cells and gamma-delta T cells from mLN of naïve and day 4-infected mice (4x10^5 CFU). Results representative of two experiments. Gated as in (B).
Figure 4

A

CD45

TCRγδ

TCRαβ

IL-17A

B

αβ T cells

γδ T cells

Day 0
Day 2
Day 4
Day 6

C

Il17a, fold change

Days post infection

Cecum

Colon

mLN

D

Percent survival

Days post-infection

WT (n=22)

Tcrδ-/- (n=16)
Figure 4: Gamma-delta T cells are the major source of IL-17A and are essential for host defense. (A) Single-cell suspensions of tissues from day-4 infected mice (4x105 CFU) were stimulated with PMA/ionomycin *in vitro* followed by intracellular staining and analyzed by flow cytometry. Gated on live CD45+ cells (result representative of two experiments). (B) Single-cell suspensions from tissues of infected mice (4x105 CFU) were stimulated with PMA/ionomycin *in vitro* followed by intracellular staining and analyzed by flow cytometry. Gated on live CD45+ CD3ε+ CD8α- TCRβ+ cells or live CD45+ CD3ε+ TCRgamma-delta+ cells. (C) Total tissues from cecum, colon and mLN of naïve and day-2 infected mice (4x105 CFU) were harvested and analyzed for gene expression by qPCR (solid circles, WT; open circles, *Tcrd*−/−). Normalized to day 0 sample with GAPDH as endogenous control (N=4 per time point per genotype). (D) Percent survival of WT and *Tcrd*−/− mice following *C. difficile* (4x105 CFU; p<0.0001, log-rank test; data combined from two experiments).
Figure 5
Figure 5: IL-17 and T-cell receptor (TCR) gamma chain transcripts are detected in fecal extracts of children with C. difficile infection. Total nucleic acid from stool samples of C. difficile culture-positive patients and control patients were analyzed by qPCR. Normalized to GAPDH (red circle, C. difficile+ patients, N=15; blue circle, control patients, N=15).
Figure 6

A

B

C

D

E

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Figure 6: *C. difficile*-responsive IL-17A+ gamma-delta T cells bear a restricted subset of TCR and demonstrate distinctive phenotype. (A) Single-cell suspensions from cecum and colon of day 4-infected mice (4x10^5 CFU) were analyzed by flow cytometry. Plots shown are gated on live CD45+ CD3ε+ TCRgamma-delta+ cells. Filled histogram represent isotype control staining. (B) Distribution of Vγ and Vδ gene usage of IL-17A- and IL-17A+ mLN gamma-delta T cells from day-4 infected mice (4x10^5 CFU). mLN gamma-delta T cells were isolated (Supplemental Figure 6) and stimulated *in vitro* with PMA/ionomycin and labeled by surface cytokine capture. Gene expression was analyzed by RNA-seq. (C) gamma-delta T cells from mLN of day 4-infected mice (4x10^5 CFU) were isolated by magnetic beads and cultured for 72 hours with the indicated stimuli (anti-CD3ε, anti-TCRgamma-delta, 10 µg/ml; IL-1β/IL-23, 10 ng/ml; N=4 per group). Supernatants were then collected analyzed by ELISA. (D) Single-cell suspensions from cecum and colon of day 4-infected mice (4x10^5 CFU) were stimulated with PMA/ionomycin *in vitro* followed by intracellular staining and analyzed by flow cytometry. Gated on live CD45+ CD3ε+ TCRgamma-delta+ cells. (E) Heat map representation of selected genes from RNA-seq analysis of sorted mLN gamma-delta T cells from day 4-infected mice (4x10^5 CFU). Cells were stimulated *in vitro* with PMA/ionomycin and labeled by surface cytokine capture and sorted as in Supplemental Figure 6. I, transcription factors; II, surface receptors; III, effector molecules; IV, cytokine receptors; V, chemokine receptors; VI, TLRs.
Figure 7

A) Percent survival of neonates and adults infected with C. difficile. 
- 10^6 CFUs neonate (n=13)
- 10^4 CFUs adult (n=10)
- 10^5 CFUs adult (n=10)
- 10^6 CFUs adult (n=10)

B) Intestinal % of CD45+ cells expressing IL-17A among T cells.
- No infection (day 0)
- C. difficile (day 2)

C) Intestinal % of CD45+ cells expressing TCR gamma-delta among T cells.
- Adult
- Neonate

D) Intestinal % of CD45+ cells expressing TCR alpha-beta among T cells.
- Adult
- Neonate

E) Percent survival following treatment with different antibodies.
- C57BL/6 (n=14)
- l17a-/- (n=13)
- TCRd-/- (n=15)

F) Percent survival of neonates and adults infected with C. difficile.
- Isotype control (mouse IgG1) (n=10)
- Anti-IL-17A (n=10)

G) Percent survival of neonates and adults infected with C. difficile.
- Anti-TCR gamma-delta (n=10)
- Anti-IL-17A (n=10)
- Anti-TCR alpha-beta (n=6)
- Anti-TCR gamma-delta + anti-IL-17A (n=10)
Figure 7: IL-17 and gamma-delta T-cells are more abundant in newborn mice and essential for enhanced protection against C. difficile infection compared to adult mice. (A) Percent survival of WT C57BL/6 adult and neonatal mice infected with various CFU of C. difficile. (B) Alpha-beta T cell and gamma-delta T cell in uninfected adult and neonatal mice were analyzed by flow cytometry. Gated on live CD45+ CD3ε+ TCRβ+ cells or live CD45+ CD3ε+ TCRgamma-delta+ cells (N=4 per group). (C) RORγt expression in alpha-beta T cell and gamma-delta T cell in uninfected adult and neonatal mice were analyzed by flow cytometry. Gated on live CD45+ CD3ε+ TCRβ+ cells or live CD45+ CD3ε+ TCRgamma-delta+ cells. Result representative of two experiments. (D) Single-cell suspensions from intestine of adult and neonatal day 2-infected mice (1x10^6 CFU) were stimulated with PMA/ionomycin in vitro followed by intracellular staining and analyzed by flow cytometry. Gated on live CD45+ CD3ε+ TCRgamma-delta+ IL17A+ cells (N=4-6 per group). (E) Percent survival of neonatal WT, Il17a-/-, and Tcrd-/- mice following C. difficile (1x10^6 CFU; WT vs. Il17a-/- and WT vs. Tcrd-/-, p<0.0001, log-rank test). (F) Percent survival of WT neonatal littermate mice treated with isotype control (MOPC-21 or Armenian hamster IgG), anti-IL-17A (17F3), anti-TCRgamma-delta (UC7-13D5), or anti-TCRbeta (H57-597) after C. difficile (1x10^6 CFU; p<0.05, log-rank test) infection. Neonatal mice received 100 ug of antibody on day -2, followed by second dose on day 0. (G) Percent survival of WT neonatal littermate mice treated with isotype controls (MOPC-21 and Armenian hamster IgG), anti-IL-17A (17F3), and anti-IL-17A plus anti-TCRgamma-delta (UC7-13D5) after C. difficile (1x10^6 CFU; anti-IL-17A vs. anti-TCRgamma-delta vs. anti-IL-17A plus anti-TCRgamma-delta, p=0.20, log-rank test). Neonatal mice received 100 ug of antibody on day -2, followed by second dose on day 0.