Innate and Adaptive Immune Responses Shape the Outcome of *Clostridium difficile* Infection

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Thesis Abstract

Clostridium difficile infection (CDI) is the number one hospital-acquired gastrointestinal infection in the United States. *C. difficile* is listed as one of three urgent threats by the Centers for Disease Control and Prevention and is the cause of 29,000 deaths annually in the United States alone. Several risk factors have been associated with this infection, including the use of broad-spectrum antibiotics, long-term stays in healthcare settings and older age. In addition, several clinical studies have identified inflammatory bowel disease (IBD) in its two major forms (ulcerative colitis and Crohn's disease) as an independent risk factor for CDI. The incidence of CDI is higher in IBD patients compared to those without IBD, and this infection tends to be more severe as evidenced by higher mortality, endoscopy and bowel surgery rates. Although this increased susceptibility of IBD patients to the infection is well documented, the underlying mechanism was poorly understood. In order to understand the mechanism of increased severity of CDI in IBD patients, we utilized a Dextran Sulfate Sodium (DSS) murine model of inflammatory colitis. In support of clinical observations, our data showed that mice treated with DSS and then infected with *C. difficile* developed a more severe *C. difficile* disease compared to untreated mice. Increased severity of disease was measured by increased mortality and clinical scores. Importantly, disease severity did not correlate with *C. difficile* burden, toxin A/B levels or major differences in the microbiota, suggesting that increased severity of disease might be due to the host immune response to infection. Immunophenotyping of immune cells recruited to the colon at the peak of CDI

revealed increased levels of CD4+ T cells at the site of infection in mice with prior DSS colitis. Furthermore, depletion of CD4+ T cells using a monoclonal antibody protected mice with prior DSS colitis from severe *C. difficile* disease and adoptive transfer of these cells caused severe CDI in naïve mice. Later we identified that DSS caused a skew towards type 3 immunity. Type 3 immunity is mediated by RORγt+ lymphocytes that express IL-17A alone or co-express IL-22. These lymphocytes include CD4+ Th17 cells, CD8+ Tc17 cells and type 3 innate lymphoid cells (ILC3s). In this study, we found that DSS treatment led to increased Th17 cells that persisted beyond the resolution of inflammation. Adoptive transfer of these cells was sufficient to worsen the outcome of CDI and this was dependent on IL-17A production by Th17 cells. Previous work in our lab and others studied the roles of certain type 3 cytokines during CDI, including IL-6, IL-17A and IL-23, all of which have been associated with increased CDI severity. However, this study was one of few that assessed the role of adaptive immunity in general and CD4 T cells in particular during CDI. Furthermore, this work was the first to identify a role for colitis-induced Th17 cells in the pathogenesis of *C. difficile.* This is extremely important because an increase in Th17 cells has been documented in IBD patients and aberrant Th17 responses have been associated with the pathogenesis of IBD. Our work suggests that inhibiting Th17 cells or one or more of their effector cytokines may provide protection against CDI in IBD patients who are highly susceptible to the infection.

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Chapter One: Introduction to Type 3 Immunity during *Clostridium difficile* Infection

Part of this chapter has been adapted from “Type 3 Immunity during *Clostridium difficile* Infection: Too Much of a Good Thing?”

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1.1 *Clostridium difficile* biology and pathogenesis

*Clostridioides (*formerly known as *Clostridium) difficile* is a spore-forming, Gram-positive, anaerobic bacterium that has been described as the cause of pseudomembranous colitis since 1978 (Bartlett et al., 1978). *C. difficile* is the leading cause of hospital-acquired gastrointestinal infections in the United States (Magill et al., 2018). In a 2015 study funded by the Centers for Disease Control and Prevention, *C. difficile* caused almost 500,000 infections and 29,000 deaths in a single year in the United States alone (Lessa et al., 2015). The annual cost of *C. difficile* infection (CDI) cases in the United States is estimated at almost 4 billion dollars (Ananthakrishnan et al., 2011; Song et al., 2008). Several studies have reported a significant increase in the prevalence and severity of CDI over the last two decades and this increase is thought to be attributable to the emergence of the hypervirulent *C. difficile* transferase (CDT)-expressing strains such as ribotype 027 (Lucado et al., 2012; Pepin et al., 2004; Bacci et al., 2011).

C. difficile causes disease in hosts with dysbiosis, where a healthy gut microbiota is disrupted usually due to the use of broad-spectrum antibiotics (Ananthakrishnan, 2011). Typically, the infection is acquired in hospital settings although the incidence of community-acquired infections is also on the rise (Khanna et al., 2012). Some reports suggest that although the majority of community-acquired infections are associated with antibiotic use, 30-35% of infected patients have no prior antibiotic exposure (Khanna et al., 2012; Chitnis et al., 2013).

Since *C. difficile* is an obligate anaerobe, the vegetative form of this bacterium is unable to survive outside the host in an aerobic environment. Therefore, dissemination of *C. difficile* is mediated by dormant spores ingested through the oral-fecal route (Zhu et al. 2018). Once ingested, these spores germinate and the vegetative bacteria cause disease by toxin production. Several signals have been shown to be important for spore germination including bile acids, amino acids and Ca^{2+} (**Figure 1.1**). In humans, the two main primary bile acids are cholic acid and chenodeoxycholic acid. These bile acids are the end product of cholesterol metabolism and although they are mostly reabsorbed and recycled to the liver, they can also be found in the large intestine. There, bile salt hydrolases expressed by many colon microbiota species convert these primary bile acids to secondary bile acids. The primary bile acid cholate is a known inducer of germination, whereas chenodeoxycholate inhibits germination (Britton and Young, 2012). In a study of the effect of several secondary bile acids, Thanissery et al. (2017) showed that many secondary bile acids, namely deoxycholate (DCA), lithocholate (LCA), ursodeoxycholate (UDCA), Isodeoxycholate (iDCA), isolithocholate (iLCA), ω-muricholate (ωMCA) and hyodeoxycholate (HDCA), inhibited spore germination. *C. difficile* spores use the subtilisin-like receptor CspC pseudoprotease as the bile acid germinant receptor (Zhu et al. 2018).

Bile acids are necessary for *C. difficile* germination, but they are not sufficient on their own. Other signals are needed for germination, including amino

acid co-germinants. Glycine is the most effective co-germinant, although L-alanine, taurine and L-glutamine are also good co-germinants (Shrestha and Sorg, 2017). Finally, a role for Ca^{2+} has been described where *in vitro* media and *ex vivo* mouse ileal contents depleted for Ca^{2+} did not support *C. difficile* spore germination (Kochan et al., 2017).

Once *C. difficile* spores germinate into vegetative bacteria, they breach the mucosal barrier, adhere to epithelial cells and cause disease by producing Toxin A (TcdA) and Toxin B (TcdB) and in some strains a third toxin called *C. difficile* transferase (CDT) (Cowardin and Petri, 2014; Buonomo and Petri, 2016). TcdA and TcdB are glucosyltransferases that inactivate Rho family GTPases and lead to the disruption of the actin cytoskeleton of epithelial cells, cell rounding and cell death (Chumbler et al., 2012). CDT has been shown to enhance bacterial adhesion by inducing microtubule protrusions on host cells (Schwan et al., 2009).

TcdA and TcdB are considered *C. difficile*'s main virulence factors since strains that do not produce at least one of these toxins are avirulent (Awad et al., 2014). In addition to the toxins, however, *C. difficile* expresses other virulence factors that mediate colonization and adherence to epithelial cells. For example, all *C. difficile* strains express surface layer proteins (SLPs) that form the outermost layer of the bacterium. The surface layer of *C. difficile* is composed to two proteins (high molecular weight and low molecular weight proteins) that have been shown to be involved in adherence to host cells (Calabi et al., 2002). Other

C. difficile virulence factors include fibronectin-binding protein and the heat-shock protein GroEL, both of which are thought to be involved in enhanced adhesion of *C. difficile* to host cells (Awad et al., 2014).

C. difficile colonization, adhesion to epithelial cells and toxin production leads to the upregulation of many proinflammatory cytokines and the recruitment of neutrophils and other inflammatory immune cells. The influx of neutrophils, along with fibrin, mucin and cellular debris, causes the formation of the pseudomembranes that are characteristic of *C. difficile* colitis (**Figure 1.1**). Clinical presentation of disease ranges from mild diarrhea to toxic megacolon and host mortality (Poutanen and Simor, 2004). The standard of care for treatment of this infection is antibiotics, including vancomycin and fidaxomicin. While these antibiotics are usually effective at clearing the infection, further dysbiosis puts the host at risk for recurrence which affects 1 in 5 patients with *C. difficile* (Lessa et al., 2015). Fecal microbial transplants (FMT) have proven successful in 80-90% of patients with recurrent infection, but the mechanism of protection and the long-term effects these transplants might have on the host are not fully understood (Leslie et al., 2019).

Figure 1.1

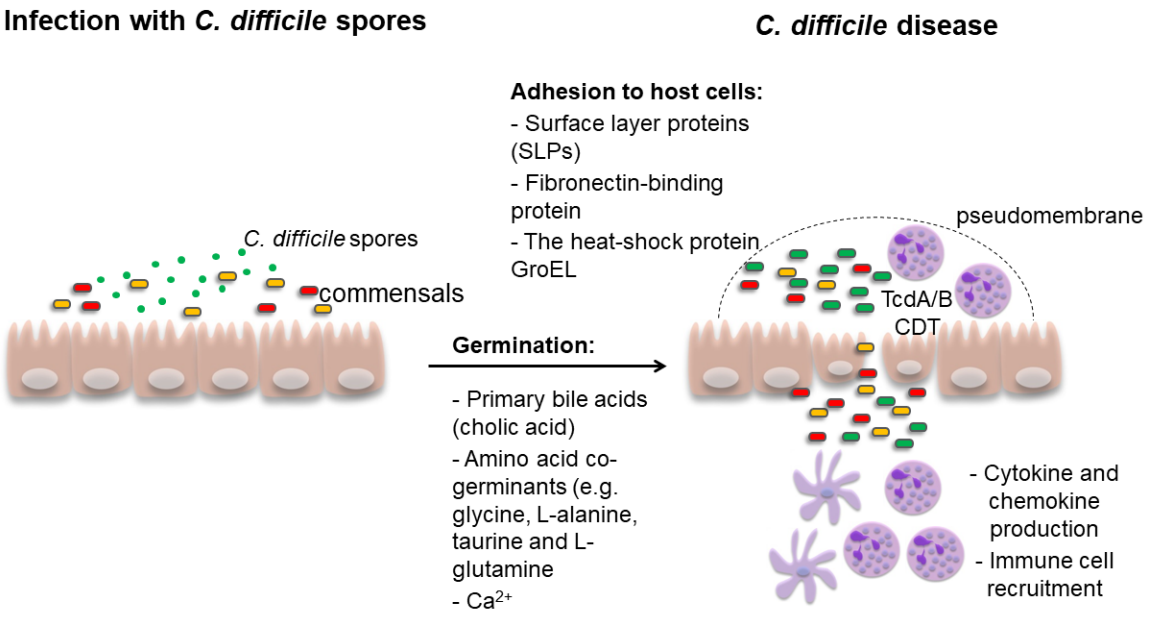

Infection with C. difficile spores
C. difficile disease
Adhesion to host cells:
- Surface layer proteins (SLPs)
- Fibronectin-binding protein
- The heat-shock protein GroEL
C. difficile spores
commensals
pseudomembrane
TcdA/B
CDT
Germination:
- Primary bile acids (cholic acid)
- Amino acid co-germinants (e.g. glycine, L-alanine, taurine and L-glutamine
- Ca2+
- Cytokine and chemokine production
- Immune cell recruitment

Figure 1.1: The pathogenesis of *C. difficile*. *C. difficile* spores infect hosts with dysbiosis where the healthy gut microbiota is disrupted usually due to the use of antibiotics. Once the spores are in the large intestine, several signals trigger germination including the primary bile acid cholic acid, amino acid co-germinants such as glycine, L-alanine, taurine and L-glutamine and Ca^{2+} ions. After germination, adhesion of vegetative cells is mediated by *C. difficile*'s surface layer proteins as well as the fibronectin-binding protein and the heat-shock protein GroEL. *C. difficile* disease is mediated by the production of its main virulence factors: toxins A and B (TcdA/B) and in some strains the binary toxin CDT. These toxins cause disruption of the actin cytoskeleton, epithelial cell rounding and cell death. The production of damage associated molecular patterns (DAMPs) and several cytokines and chemokines by epithelial cells leads to the recruitment of neutrophils and other immune cells. The influx of neutrophils, along with fibrin, mucin and cellular debris leads to the formation of pseudomemranes, which are characteristic of *C. difficile* colitis.

1.2 The role of the immune system during CDI

Growing evidence has supported the idea that the type of immune response mounted by the host against CDI directly affects the severity and outcome of disease. Therefore, a better understanding of protective and pathogenic immune responses during CDI will help identify new targets for future treatments and preventative strategies.

Intriguingly, some studies have shown that biomarkers of intestinal inflammation such as CXCL5 and IL-8 are better predictors of the outcome of CDI than bacterial burden (Feghaly et al., 2013 a&b). Both CXCL5 and IL-8 bind to the IL-8 receptor 2 and recruit and activate neutrophils. These biomarkers were elevated in diarrheal patients compared to controls and have been associated with severe CDI in human patients and mouse models (Feghaly et al., 2013b).

Data from our lab support the notion that manipulating the immune response can modulate the severity of CDI independently of *C. difficile* burden or toxin production. For example, we have shown that pre-treating mice with the Th2 cytokine interleukin 25 (IL-25) can protect mice against severe CDI via the induction of eosinophils and the downstream protection of epithelial integrity (Buonomo et al., 2016). In a related mechanism, we have also shown that CDT-expression by the hyperviulent R20291 strain of *C. difficile* causes severe CDI by inducing eosinophil apoptosis and leading to a decrease in eosinophil recruitment to the site of infection via a TLR2-dependent mechanism (Cowardin et al., 2016).

Finally, we have recently described a protective role for another Th2 cytokine, interleukin 33 (IL-33), during CDI that works to induce ILC2s and downstream eosinophils to protect against *C. difficile*-associated weight loss and mortality (Frisbee et al., under review). All of these studies have established a novel role for type 2 immunity in protection against CDI and have paved the way for future therapeutics that may target this pathway in patients with CDI.

Neutrophils, downstream of type 3 immune responses, have long been described as the hallmark of *C. difficile* infection. Type 3 immune responses include RORγt+ lymphocytes that have the ability to produce IL-17A alone or with IL-22 as their signature cytokines. These cells include CD4+ Th17 cells, CD8+ Tc17 cells and type 3 innate lymphoid cells (ILC3s). Because of the long-described association between CDI and type 3 immunity, many studies have interrogated this pathway and its role during infection. Below, we review innate and adaptive type 3 responses and describe their dual role in the pathogenesis of *C. difficile.*

1.2.1 Innate immunity and type 3 innate lymphoid cells

Innate lymphoid cells (ILCs) are a recently-described cell subset that shares a lymphoid cell morphology but lacks the somatically rearranged surface receptors expressed by T and B lymphocytes and therefore lacks antigen specificity. Like T cell lymphocytes, non-cytotoxic innate lymphoid cells are subdivided into three groups based on the transcription factors required for their development and the effector cytokines they produce. Group 1 ILCs (ILC1s)

express the transcription factor T-bet and produce IFNγ and TNF, group 2 ILCs (ILC2s) express the transcription factor GATA3 and produce IL-4, IL-5, IL-9 and IL-13 and group 3 ILCs (ILC3s) express the transcription factor RORγt and produce IL17A, IL-17F, IL-22, TNF and granulocyte macrophage colony-stimulating factor (GM-CSF). ILC3s play a role in defense against extracellular pathogens including *Citrobacter rodentium, Candida albicans* and *streptococcus pneumoniae.* In addition, IL-22-producing ILC3s drive pathogenesis in T cell-independent mouse models of IBD and IL-17 expression by ILC3s is increased in patients with Crohn's disease. IL-17- and IL-22- producing ILC3s are also associated with psoriasis vulgaris (Miossec et al., 2009; Artis and Spits, 2015). ILCs share many effector cytokines and therefore functions with T cell lymphocytes, including protection against infections, tissue healing and repair and autoimmunity and inflammation. However, the main difference between ILCs and T cell lymphocytes is the lack of antigen specificity and immunological memory.

Like all lymphocytes, ILCs are derived from a common lymphoid progenitor (CLP) in the bone marrow. A cell population similar in phenotype to CLPs but expresses the integrin α4β7 gives rise to all ILCs and NK cells but not T and B cells. Downstream of this cell type are two populations that express the transcriptional repressor Id2. Id2+ cells that do not express the transcription factor promyeloid leukemia zinc finger (PLZF) can give rise to lymphoid tissue inducer (LTi) cells. Whereas Id2+ cells that co-express PLZF are restricted to

ILC1, ILC2 and ILC3 lineages (Artis and Spits, 2015). Once these cells differentiate in the bone marrow, they can be found in various tissues with different frequencies including peripheral blood, the gut, lung and skin (Hazenberg and Spits, 2014).

In the context of *C. difficile* infection, there are two reports assessing the roles of ILCs during CDI. First, Geiger et al. (2014) showed that Nfil3-/- mice, which have a deficit in all three ILC subsets, were more susceptible to infection with spores of *C. difficile* strain VPI 10463. These mice lack the bZIP transcription factor Nfil3, previously known to be required for NK cell and ILC1 development. Geiger et al. showed that these mice also had significant deficiencies in intestinal ILC3s and lung and visceral adipose tissue ILC2s. They showed that Nfil3 is required for development of these ILCs, but not necessarily for the maintenance of mature ILC3s since a conditional knockout of Nfil3 in mature NKp46+ ILC3s did not diminish their frequencies.

In a later study by the same group, Abt et al. (2015) investigated directly the protective or pathogenic role of each ILC subset during CDI. The authors used Rag2 common gamma chain double knockout mice (Ragγc-/-) which lack all ILC subsets in addition to T and B cells. They showed that Ragγc-/- mice develop more severe CDI than WT mice and Rag1-/- mice, only lacking T and B cells. Next, the authors showed that transferring ILCs is sufficient to protect Ragγc-/- from increased severity of disease. Furthermore, by using Rag1 IFNγ double knockout and Rag1 Tbet double knockout mice, they demonstrated a role

for T-bet+, IFNγ-producing ILC1s in protection from CDI early during infection. The authors also observed that IL22-/- mice had higher weight loss rates when infected with spores and significantly higher mortality when infected with a spore/vegetative mixed inoculum. When IFNγ was neutralized in the Rag1 IL-22 double knockout mice, 100% of the mice succumbed to the infection suggesting a combined role for IFNγ- producing ILC1s and IL-22- producing ILC3s in providing early protection from CDI. Interestingly, the authors found that neutralizing IL-17A in Rag1-/- mice did not change the outcome of infection, suggesting no role for IL-17A-producing ILCs in this model. It is important to note that the double knockout mice do not test exclusively the role of these cytokines produced by ILCs since other innate immune cell subset are capable of producing IFNγ, IL-22 and IL-17A.

In support of the protective role for IL-22 during CDI, Hasegawa et al. (2014) found that CDI induces the production of IL-22 in the colon, liver and lung. The authors found that the CDI-induced upregulation of IL-22 is independent of RAG1, suggesting that the source of IL-22 is innate immune cells, predominantly ILCs (Sonnenberg et al., 2011). Hasegawa and colleagues found that IL-22-/- mice develop more severe disease and that this increased severity is independent of adaptive and neutrophilic immune responses. Instead, severe disease was due to translocation of pathobiont commensal strains across the damaged epithelium following CDI. The authors show nicely that during CDI, increased IL-22 leads to the induction of the complement C3, the deposition of

C3 on the surface of pathobionts and the downstream phagocytosis of these bacteria by neutrophils and macrophages. Sadighi Akha et al. (2015) reported no increased weight loss in mice treated with anti-IL-22 antibody post CDI. This contrary result is perhaps due to the use of a neutralizing antibody against IL-22, rather than a knockout model, which could mean less efficient abrogation of IL-22 signaling. Nonetheless, Hasegawa et al. (2014) and Abt et al. (2015) independently showed a protective role for IL-22 during CDI.

1.2.2 Adaptive immunity and Th17 cells

Th17 cells were first described by Harrington et al. (2005) that found a subset of T cells that does not fit the dichotomy of Th1 and Th2 cells that was the dogma at the time. The differentiation of these cells was inhibited by IFNγ and IL-4, but the absence of these cytokines and the presence of IL-23 led to a cell population that expresses the transcription factor RORγt and is capable of producing IL-17A (Harrington et al., 2005). Since then, Th17 cells have been shown to produce IL-17F, IFNγ, IL-21, IL-22, TNF and GM-CSF. Through cytokine production, Th17 cells have downstream effects on neutrophil and mononuclear phagocyte recruitment as well as on antimicrobial peptide production by epithelial cells. While Th17 cells are an important defense against extracellular bacteria and fungi, they have also been implicated in the pathogenesis of autoimmune diseases such as psoriasis and inflammatory bowel disease (Miossec et al., 2009; Annunziato et al., 2015).

In contrast to the roles of innate and humoral responses during *C. difficile* infection, the role of CD4 T cells has been vastly understudied. Some evidence for the protective role CD4 T cells might play against CDI comes from studies in HIV patients. Sanchez et al. (2005) reported higher rates of *C. difficile* infection in patients with advanced stages of HIV, compared to HIV-infected patients without AIDS. Similarly, Jha et al. (2012) found that in Indian HIV patients, the rate of *C. difficile* infection correlated with HIV seropositivity as well as with low CD4 T cell counts. Other studies reporting at least a 2 fold increase of CDI in AIDS patients and correlations with low CD4 T cell counts are reviewed nicely by Colloni et al. (2013).

In a mouse study of the protective role CD4 T cells might play during CDI, Johnston et al. (2014) observed that wildtype C57BL/6 mice challenged with *C. difficile* were protected from a subsequent challenge with the same *C. difficile* strain. They found that re-challenged mice had protective colonic IgA and systemic IgG responses against *C. difficile* toxins A and B. To test the role of T cell help in generating protective humoral responses, the authors used CD4-/- mice, which lack the expression of the CD4 co-receptor, have a deficit in CD4 T cells and lack IgG class switching. Upon re-challenge, CD4-/- mice were protected from CDI and this protection was long-lived. These mice generated IgA responses, but no IgG response as predicted. Interestingly, when the authors used MHCII-/- mice, which have a dramatic decrease in CD4 T cells and a deficiency in IgA and IgG class switching, these mice were not protected against

re-challenge. The authors found no anti-toxin antibodies in the sera or mucosa of these mice. Altogether, these data suggest that T cell help is needed in protection against recurrent CDI and that this protection correlates with anti-toxin IgA or IgG antibody generation. As the authors point out, however, this study does not address the differences in the microbiota between the knockout and wildtype strains by co-housing, 16S rRNA sequencing or using littermate controls. These differences likely play a role in protection against re-challenge in some knockout strains, especially considering the drop in the bacterial burden in these mice. Another limitation is that the production of IgA antibody did not require CD4+ T cell help. The authors postulate that CD4- T cells might help with the generation of anti-toxin IgA antibodies without providing data to support this hypothesis.

In another report, Ryan et al. (2011) investigated the ability of the immune system to recognize *C. difficile* surface layer proteins (SLPs) and the downstream cytokine profile. They found that pulsing bone marrow-derived dendritic cells (BMDCs) with SLPs led to the production of several cytokines such as IL-12p70, IL-23, IL-1β, TNFα and IL-10 and that this recognition is dependent on TLR4 signaling. They also observed that co-culturing these BMDCs with CD4 T cells led to the induction of T helper responses and the production of IL-17, IFNγ and IL-4 by these T cells. Finally, the authors found that TLR4-/- and Myd88-/- mice develop more severe disease than WT mice. However, they do not show directly that this phenotype *in vivo* is dependent on recognition of SLPs.

This increase severity of disease in TLR4-/- mice is also unlikely to be dependent on CD4 T cells because differences in weight loss between TLR4-/- and WT mice are observed as early as day 1 of infection, before a T cell response against *C. difficile* can be generated. Nonetheless, this report establishes a TLR4-dependent recognition of *C. difficile* proteins and the downstream induction of T helper cells. This leads the way to studying the protective role of *C. difficile*-specific T cells in longer or recurrent models of disease.

On the other hand, there is also evidence that certain subsets of CD4 T cells play a pathogenic role during infection. In a study focused on the severity of *C. difficile* infection following treatment with the nonsteroidal anti-inflammatory drug (NSAID) indomethacin, Maseda et al. (2019) found that indomethacin mice develop more severe CDI compared to untreated controls. This increased severity of disease was associated with increased numbers of neutrophils, CD4 T cells and RORγt+ CD4 T cells in the colon lamina propria during infection. Indomethacin-treated mice also had higher levels of the Th17 cytokines IL-6 and IL-1β in colonic tissue compare to untreated controls. Finally, indomethacin treatment also led to alterations in the composition of the gut microbiota that the authors hypothesize might play a role in exacerbating CDI severity in these mice. Although this study shows a correlation between enhanced Th17 responses in mice with more severe CDI, it remains unclear whether Th17 cells alone are necessary and sufficient to exacerbate CDI severity in indomethacin-treated mice.

The only study to look at human T cell responses to *C. difficile* infection was done by Yacyshyn et al. in 2014. In this study, the authors used flow cytometry to characterize IL-17, IFNγ and FoxP3 expression in PBMCs of *C. difficile* patients. The patient cohort included 20 patients with initial *C. difficile* infection, 6 patients that developed recurrent disease during the study, 14 patients with known previous episodes of CDI, 20 inpatient controls and 16 healthy controls. Due to the small number of patients in each groups, many of the results in the study failed to reach statistical significance. Nonetheless, the authors observed that patients with CDI had slightly higher numbers of circulating IL-17+ CD4 T cells than healthy controls; however, there was no difference in these cells between CDI patients and inpatient controls. The authors also made the qualitative observation that 5 of the 6 patients that developed recurrent disease during the course of the study had higher IL-17+, IFNγ+ and FoxP3+ T cells and they postulate that Th17 cells in these patients may be co-expressing two or more of these proteins. However, as the authors note, the staining for IL-17, IFNγ and FoxP3 was done separately so directly assessing co-expression was not feasible. Despite the limitations of this study, it does raise the question of the role of Th17 cells, and perhaps IFNγ-producing Th17 cells, in *C. difficile* recurrence. Future studies with larger patient numbers and a deeper phenotypic characterization of T cell subsets are needed to address this fascinating question.

1.2.3 Effector cytokines and downstream responses

IL-23

Interleukin 23 (IL-23) is a cytokine that was first described by Oppmann et al. in 2000. IL-23 shares the p40 subunit with IL-12, but has a unique p19 subunit that is distantly related to the p35 subunit of IL-12. Prior to the discovery of IL-23, it was thought that IL-12 plays a major role during autoimmune diseases because many of the antibodies targeting IL-12 were against the shared p40 subunit. Since then, it has been shown that IL-23 plays a major role in the pathogenesis of inflammatory bowel disease (IBD) and other autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE). Polymorphisms in the *IL-23R* gene have been linked to IBD (McGovern and Powrie, 2007). Later it was described that IL-23 causes colitis in a T cell transfer model by directly acting on Th17 cells via the IL-23 receptor and promoting their survival as well as inducing the production of IL-17A and other Th17-derived cytokines (Ahern et al., 2010). IL-23 has also been shown to play a role in T cell-independent colitis induced by infection with *Helicobacter hepaticus.* Buonocore et al. (2010) showed that induction of colitis in this model was dependent on a RORγt-expressing innate lymphoid cell subset (ILC3s) and the production of IL-17A and IFNγ. In a separate report, Powell et al. (2012) showed that IL-23 can promote IL-17A production by ILCs where T-bet signaling is lost, and induce more severe colitis. Taken together, these studies show an effect of IL-23 signaling on both T cell

and ILC populations leading to the production of IL-17A and contributing to the pathogenesis of inflammatory bowel disease.

Because of the long-appreciated role for the IL-23 axis during IBD, several studies have evaluated the involvement of this cytokine in *C. difficile* colitis. Buonomo et al. (2013) found higher levels of IL-23 in colonic tissue biopsies isolated from *C. difficile* positive patients compared to biopsies from *C. difficile* negative patients. Similarly, Darkoh et al. (2014) found higher levels of IL-23, as well as IL-8, in the stool of patients with *C. difficile* diarrhea compared to other diarrheal patients.

In a mouse model of CDI, it has been shown that abrogation of IL-23 signaling by a gene knockout or antibody neutralization protects mice from severe CDI (Buonomo et al., 2013). Using the same mouse genotype in two separate reports, McDermott et al. (2016, 2017) found that IL-23 knockout mice had reduced recruitment of neutrophils and Ly6C^{hi} monocytes to the colon and a slight, but not statistically significant, reduction in histopathological scores during infection. The authors, however, did not assess the effect of knocking out IL-23 on survival or clinical signs of disease severity. Finally, in a study aimed at understanding the mechanism of IL-23 induction during CDI, Cowardin et al. (2015) found that *C. difficile* toxins A/B can act synergistically with pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to activate the inflammasome, leading to the production of IL-1β and the downstream induction of IL-23.

Taken together, these studies establish that CDI induces IL-23 and that elevated IL-23 levels cause severe disease. None of these studies, however, describe the cellular mechanism of increased severity of disease. More investigation is needed to evaluate whether this effect is T cell-dependent or independent and which downstream signals and effector cells are required for exacerbation of CDI severity.

IL-17A

IL-17A was first described in 1988 and was shown to induce inflammation by triggering the release of IL-6, IL-8, G-CSF, IL-1β and other inflammatory cytokines by various target cell types (Sud et al., 2018). Then, it was shown that injection of IL-17A into the airway *in vivo* triggered neutrophil recruitment to the lung by the induction of CXC cytokines (Laan et al., 1999). Later, lymphocytes expressing the transcription factor RORγt, including Th17, ILC3 and Tc17 cells, were shown to produce IL-17A and the roles of these cells in bacterial and fungal infections as well as autoimmune diseases started to be investigated.

In a study directly testing the role of IL-17 signaling during CDI, Nakagawa et al. (2016) reported enhanced survival in IL-17A and IL-17F double knockout mice (IL17 KO). This enhanced survival correlated with a dampening in type 17 cytokines such as IL-1β and IL-6 as well as a decrease in neutrophil recruitment to the colon during infection. This study suggests that IL-17 signaling plays a pathogenic role during CDI, although the authors do not investigate the role of IL-17A and IL-17F separately. A role for IL-17F during CDI is yet to be examined.

However, a separate study has also described an association with increased IL-17A and worsening of *C. difficile* disease. Wang et al. (2018) found that increased levels of IL-17A and IL-23 in the absence of IL-27 signaling led to increased mortality post infection.

In human *C. difficile* patients, Jafari et al. described the production of many Th17 cytokines, including IL-8, IL-6, IL-1β, IFNγ, IL-17A and IL-22 by colonic biopsies from *C. difficile*-negative donors in response to stimulation with *C. difficile* strains R20291 and 630. Yu et al. (2017) found elevated levels of IL-17A, IL-6, IL-1β, IL-8 and IFNγ among many other cytokines in the serum of *C. difficile* patients compared to healthy controls, as might be expected in an inflamed mucosa. However, when they compared the levels of these cytokines between patients with mild/moderate disease and those with severe disease, they did not find a correlation between high serum type 17 cytokines and increased severity of disease. The authors claim that severe disease was associated with a decrease in the ratio of IFNγ/IL-17A in the serum, however their data suggest that this is strictly due to a decrease in IFNγ in severe CDI patients rather than an increase in IL-17A. The absence of a correlation between any of the type 17 cytokines in the serum and disease severity is perhaps due to the small number of patients (16-20). Moreover, assessment of these cytokine profiles in colonic tissue of CDI patients is yet to be determined and might reveal a correlation between high IL-17A levels and severe disease, which is yet to be described in human CDI.

Neutrophilic responses in the defense against *C. difficile*

Further downstream of IL-23 signaling and the induction of IL-17A production by T cells and ILC3s, some studies have evaluated the role of neutrophils during CDI. IL-17A and other cytokines in the IL-17 family, including IL-17B, IL-17C and IL17F, have been shown to promote neutrophil recruitment. These cytokines bind to receptors on immune cells (such as monocytes and macrophages) and nonimmune cells (such as epithelial and endothelial cells and fibroblasts). In response, these cells produce granulocyte colony-stimulating factor (G-CSF) and chemokines such as CXCL1, CXCL2 and CXCL5 leading to enhanced production of neutrophils in the bone marrow and recruitment to the tissue, respectively (Iwakura et al., 2011; Matsuzaki and Umemura, 2018).

In the context of CDI, studies have shown a dual role for neutrophils during infection. Reports that suggest a protective role during infection include a study that found that in patients with Leukemia, neutropenia was one of the factors associated with CDI occurrence (Luo et al., 2015). In another study of hematopoietic stem cell transplant patients, neutropenia was the only independent predictor of recurrent CDI (Huang et al., 2014). Mouse data that support this beneficial role for neutrophils come from a study of the effects of toll-like receptor and MyD88 signaling during CDI. Jarchum et al. (2012) found that MyD88 signaling protects against severe CDI by recruiting neutrophils to the site of infection. Depletion of neutrophils in this model led to increased CDI-associated mortality. Finally, Hasegawa et al. (2011) showed that loss of Nod1

signaling in response to CDI led to increased mortality due to defective CXCL1-dependent recruitment of neutrophils.

On the other hand, some studies found correlations between enhanced neutrophilic responses and increased severity of CDI. In general, leukocytosis and high white blood cell counts have been associated with increased CDI-induced mortality (Bauer et al., 2012; Solomon et al., 2013). More specifically, in an analysis of fecal samples from children with and without symptomatic *C. difficile* diarrhea, elevated levels of the neutrophil recruiters CXCL5 and IL-8 correlated with persistent diarrhea. Furthermore, in diarrheal patients, time-to-diarrhea resolution was significantly increased in those with high CXCL5 and IL-8 levels in the stool (El Feghaly et al., 2013). Additionally, blocking neutrophil recruitment with an anti-CD18 antibody resulted in reduced tissue pathology following injection of *C. difficile* toxin A into rabbit ileal loops (Kelly et al., 1994). Similarly, reduced neutrophil recruitment in the context of IL-23 blockade correlated with reduced histopathology in a mouse model of infection (McDermott et al., 2016). A more comprehensive review of the dual role of neutrophils during CDI was done by Jose and Madan (2016).

In summary, *C. difficile* infections are a major cause of infectious disease mortalities in the United States. Although antibiotic and FMT treatments exist for the infection, the high level of recurrence and the emergence of hypervirulent strains highlight the importance of developing new therapies. Targeting the immune system in many animal models has proven successful at preventing

CDI-associated mortality and many of these approaches focus on type 3 immune responses. Overall, the host immune system seems to be a double-edged sword in the context of *C. difficile* infection. For example, blocking neutrophil recruitment downstream of Myd88 signaling leads to increased mortality. However, in humans, higher levels of the known neutrophil recruiters IL-8 and CXCL-5 correlate with increased mortality. Similarly, MHCII-/- mice do worse in a relapsing *C. difficile* models because of defects in anti-toxin antibodies due to the lack of CD4 T cell help. However, in other models, increased CD4 T cell responses correlate with severe disease (**Figure 1.2**). Therefore, a deeper understanding of the type and magnitude of the different arms of immunity is needed before these discoveries can be translated into therapies that harness the power of a patient's own immune system to protect them against severe disease.

Figure 1.2

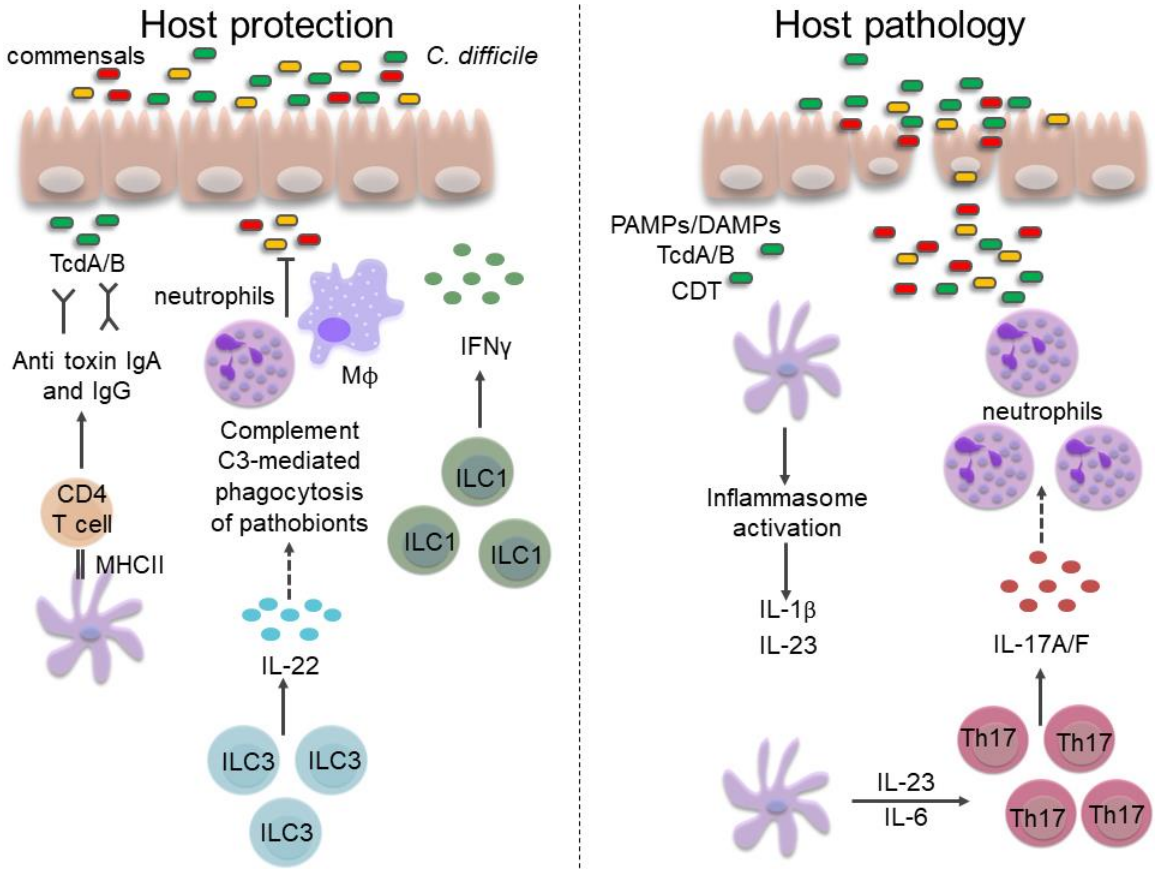

Host protection
commensals
C. difficile
TcdA/B
Anti toxin IgA
and IgG
CD4
T cell
MHCII
neutrophils
Mϕ
Complement
C3-mediated
phagocytosis
of pathobionts
IL-22
ILC3
ILC3
ILC3
IFNγ
ILC1
ILC1
ILC1
Host pathology
PAMPs/DAMPs
TcdA/B
CDT
Inflammasome
activation
IL-1β
IL-23
neutrophils
IL-17A/F
IL-23
IL-6
Th17
Th17
Th17
Th17

Figure 1.2: Type 3 immunity during *C. difficile* infection. Several *in vitro, in vivo* and human studies have assessed the role of type 3 immunity during CDI. The type and magnitude of this response directly influences the outcome of disease. While some inflammatory and neutrophilic responses are needed to control the infection, toxin production and translocation of pathobionts, excess inflammation can have off-target effects on the host and lead to increased pathology and mortality.

1.3 Inflammatory Bowel Disease as a Risk Factor for *C. difficile* Infection

Inflammatory Bowel Disease (IBD) is a set of conditions that involve inflammation of the gastrointestinal (GI) tract. The two main types of IBD are Crohn's disease and ulcerative colitis. Symptoms for both diseases include abdominal pain, fatigue, fever, weight loss, diarrhea and rectal bleeding (Strober et al., 2007). Crohn's disease can affect any part of the GI tract from the mouth to the anus, whereas ulcerative colitis is restricted to the colon and rectum (Baumgart and Sandborn, 2007). Although our understanding of the causes of IBD is limited, genome wide analyses have identified several susceptibility loci for Crohn's disease including *NOD2* (also designated *CARD15* and *IBD1*), *IBD5, IL23R* and *ATG16L1* (Xavier and Podolsky, 2007). Familial and twin aggregation studies also provide evidence for a genetic component for both Crohn's disease and ulcerative colitis. However, the concordance of Crohn's disease in monozygotic twins is higher (37.3%) than ulcerative colitis (10%) suggesting a stronger genetic component for the pathogenesis of Crohn's disease (Baumgart and Carding, 2007). In addition, IBD has been correlated with several environmental factors including diet, living in an urban setting and over-sanitation in developed countries (Baumgart and Carding, 2007). Generally, it is accepted that a disruption of the interaction between bacterial microflora and the mucosal immune system underlies IBD. In a large, multi-state study of about 9 million Americans, Kappelman *et al.* found that the prevalence rate was 201 per 100,000 for Crohn's disease and 238 per 100,000 for ulcerative colitis. They estimate that

nearly 1 million Americans suffer from IBD (Kappelman et al., 2007). Treatment of IBD conditions involves the use of anti-inflammatory and immunosuppressive drugs, such as 5-aminosalycilic acid (mesalazine), α-TNF (infliximab) and corticosteroids, which help to ameliorate the symptoms (Baumgart and Sandborn, 2007). Ulcerative colitis can be cured surgically with a proctocolectomy, although this leaves the patient with a permanent ileostomy. Surgery in Crohn's disease cases can treat complications such as bowel blockage and fistula formation; however, currently there is no cure for the disease (Strober et al., 2007; Baumgart and Sandborn, 2007).

IBD has been identified as an independent risk factor for CDI in numerous clinical studies. In a single-center study of the Barnes –Jewish hospital in St. Louis, Missouri, Rodemann *et al.* analyzed 357,242 admissions over a period of 7 years (1996-2003) to deduce any correlations between IBD and susceptibility to CDI (Rodemann et al., 2007). Their results showed a significantly higher incidence of CDI in IBD patients (23.3/1000 admissions) in comparison to non-IBD patients (12.3/1000 admissions, $P<0.001$). They also found that IBD patients, on average, had reduced times from admission to *C. difficile* infection. This data is also supported by other single-center studies in the United States and Europe, including Issa *et al.* (2007) who reported a significantly higher proportion of IBD patients with CDI compared to non-IBD patients at the Medical College of Wisconsin (Issa et al., 2007; Cojocariu et al., 2015).

In addition to these single-center studies, analyses of nationwide patient data have also shown evidence for increased incidence of CDI in IBD patients. Based on an analysis of the National Inpatient Sample (NIS), the largest short-stay hospital discharge database in the United States, Nguyen *et al.* reported higher rates of CDI in ulcerative colitis patients (37.3 per 1000) and Crohn's disease patients (10.9 per 1000) when compared to non-IBD patients (4.5-4.8 per 1000), $P<0.0001$ (Nguyen et al., 2008). The same study showed that *C. difficile* infection significantly increased in-hospital mortality rates for ulcerative colitis patients. *C. difficile* infection also correlated with longer hospital stays and higher healthcare charges for both Crohn's disease (65%) and ulcerative colitis (46%) patients. These data suggests that CDI is more prevalent and severe in patients with IBD. However, our understanding of the mechanism of increased incidence and severity is limited.

1.4 Conclusions

Given the clinical correlation between IBD and susceptibility to severe CDI and our limited understanding of the underlying mechanism, we decided to investigate the cellular mechanism of increased severity of CDI in IBD patients. Using a dextran sulfate sodium (DSS) murine model of colitis, we found that DSS-treated mice developed more severe CDI despite full recovery from DSS colitis. This increased severity of disease was not associated with enhanced *C. difficile* burden, toxin production or major differences in the composition of the gut microbiota. Instead, severe disease in DSS mice was dependent on CD4 T cells

and depletion of CD4-expressing cells before infection protected these mice from severe CDI. Using adoptive transfer studies, we showed that Th17 cells alone are sufficient to exacerbate the severity of CDI very early during infection. Additionally, we showed that blocking IL-17 signaling either by neutralizing the IL-17A cytokine or blocking the IL-17 receptor alpha provided protection against severe disease in the early stages of infection. Finally, in patients with CDI, we showed that serum levels of two Th17 cytokines (IL-6 and IL-23) were associated with severe CDI. Patients with high levels of IL-6 in the serum were significantly more likely to succumb to the infection comparted to those with low serum IL-6. This is consistent with a previous report indicating higher IL-6 levels in the serum of 8 patients with severe CDI (Rao et al. 2018). Taken together, our study was the first to directly demonstrate that Th17 cells alone are sufficient to worsen the outcome of CDI highlighting the importance of further studies into the role of these cells during disease. The following chapter of this dissertation focuses on describing the data we generated that led to the discovery of a Th17-dependent exacerbation of CDI severity. Then, the final chapter describes the implications of this work on the field and the remaining questions and future directions in this area of research.

Chapter Two: Colitis-Induced Th17 Cells Increase the Risk for Severe Subsequent *Clostridium difficile* Infection

This chapter was adapted from: Saleh, M. M., Frisbee, A. L., Leslie, J. L., Buonomo, E. L., Cowardin, C. A., Ma, J. Z., Simpson, M. E., Scully, K. W., Abhyankar, M. M. and Petri, W. A. Jr. (2019). Colitis-induced Th17 cells increase the risk for severe subsequent *Clostridium difficile* infection. *Cell Host & Microbe*. In Press.

M.M.S. conceived, designed and performed the experiments and wrote the manuscript. M.M.S. conducted the human data analysis with J.M. A.L.F., E.L.B., C.A.C. and M.E.S. provided valuable advice, reviewed the manuscript and helped with tissue processing. J.L.L. conducted the 16S rRNA analysis of the microbiome. M.M.S., M.M.A. and K.W.C. conducted the studies in humans. W.A.P. supported all aspects of the work.

2.1 Summary

Clostridium difficile infection (CDI) is the number one hospital-acquired infection in the United States. CDI is more common and severe in inflammatory bowel disease patients. Here we studied the mechanism by which prior colitis exacerbates CDI. Mice were given Dextran Sulfate Sodium (DSS)-colitis, recovered for two weeks and infected with *C. difficile*. Mortality and CDI severity were increased in DSS-treated mice compared to controls. Severe CDI was dependent on CD4+ T cells, highlighting the importance of studying their role in the pathogenesis of *C. difficile*. Adoptive transfer of Th17 cells was sufficient to increase CDI- associated mortality. Finally, in humans, the Th17 cytokines IL-6 and IL-23 were associated with severe CDI and patients with high serum IL-6 were 7.6 times more likely to die post-infection. These findings establish a central role for Th17 cells in CDI pathogenesis following colitis and identify them as a potential target for preventing severe disease.

2.2 Introduction

Clostridium difficile is a spore-forming, Gram-positive, anaerobic bacterium that was identified as the cause of antibiotic-associated pseudomembranous colitis in 1978 (Bartlett et al., 1978). Despite an overall decline in infectious disease mortalities over the last 3.5 decades, mortality due to diarrheal diseases in the U.S. continues to rise (Bcheraoui et al., 2018). This trend is attributed to the spread of hypervirulent strains of *C. difficile* over the last decade (Bacci et al., 2011; Shuman and Malani, 2018), including the epidemic ribotype 027 strain used in our studies. The Center for Disease Control estimates that *C. difficile* causes almost 500,000 infections and 29,000 deaths annually in the United States alone (Lessa et al., 2015). These reports and others highlight the importance of studying *C. difficile* infection (CDI) and identifying novel targets for reducing disease severity.

Inflammatory Bowel Disease (IBD) is a set of conditions, including Crohn's disease and ulcerative colitis, characterized by gastrointestinal tract inflammation due to dysregulation of the immune response to commensal bacteria (Baumgart and Carding, 2007). IBD is an independent risk factor for CDI (Issa, et al., 2007; Nguyen et al., 2008; Cojocariu et al., 2015). Studies from the National Inpatient Sample, the largest short-stay hospital discharge database in the U.S., as well as single-center studies have reported a higher incidence of CDI in IBD patients compared to non-IBD patients (Rodemann et al., 2007; Issa, et al., 2007; Nguyen et al., 2008). Moreover, CDI in IBD patients is more severe as characterized by

reduced times from admission to CDI, longer hospital stays, increased surgery and in-hospital mortality rates (Rodemann et al., 2007; Nguyen et al., 2008; Ananthakrishnan et al., 2008). However, the cause of increased incidence and severity of CDI in IBD patients remains poorly understood.

We hypothesized that in IBD patients, aberrant immune responses resulting in gut inflammation are the cause of increased CDI severity. Indeed, studies in mouse models as well as human patients have shown that the type of immune response mounted against CDI by the host can determine the severity and outcome of infection (Feghaly et al., 2013; Buonomo et al., 2016; Cowardin et al., 2016). In this study, we used Dextran Sulfate Sodium (DSS) colitis to model how prior gut inflammation due to IBD can influence the outcome of subsequent CDI by altering the host immune response. Our results show that colitis induces Th17 cells that are still present in the mesenteric lymph nodes after full recovery from colitis. We demonstrated by adoptive transfer that these Th17 cells are sufficient to increase mortality after challenge with *C. difficile*. Prior to this study, previous reports showed similar CDI-associated mortality in WT and Rag1-/- mice and so a role for T cells during primary CDI has been overlooked (Abt et al., 2015). Our findings describe an important role for CD4 T cells in general, and Th17 cells in particular, during CDI and identify them as a potential therapeutic target for patients with IBD who are at risk for severe disease.

2.3 Results

Increased CDI-associated mortality in mice with prior DSS colitis

To determine whether prior gut inflammation can predispose the host to severe subsequent CDI, we designed a mouse model where we treated mice with 2% DSS for 6 days to induce gut inflammation. After a two-week recovery period, mice were challenged with *C. difficile* (**Figure 2.1 A**). The recovery period was sufficient to reverse the DSS-induced weight loss, as well as restore gut barrier integrity as measured by permeability to FITC dextran (**Figure 2.1, B-C**). Examination of hematoxylin and eosin (H&E) stained histopathological sections of the caeca revealed that although DSS treatment caused an acute disruption of the epithelial barrier, the recovery period was sufficient to restore epithelial integrity (**Figure 2.2 A**). Furthermore, several studies have shown that DSS treatment causes the upregulation of several inflammatory cytokines, including TNFα, IFNγ, KC, IL-6, IL-1α and IL-1β (Alex et al., 2009; Arai et al., 1998 and Jeengar et al., 2017). Our data showed that although these cytokines were elevated during acute DSS colitis, the levels of these inflammatory cytokines were comparable between the untreated and DSS-treated groups after the recovery period (**Figure 2.2 B**). Despite the resolution of acute colitis, mice previously treated with DSS had higher CDI-associated mortality when compared to previously untreated controls (**Figure 2.1 D**). Mice with prior DSS colitis also had more severe CDI when scored for: weight loss, coat appearance, eyes/nose discharge, activity, posture and diarrhea (**Figure 2.1, E-F**). We observed that

Figure 2.1

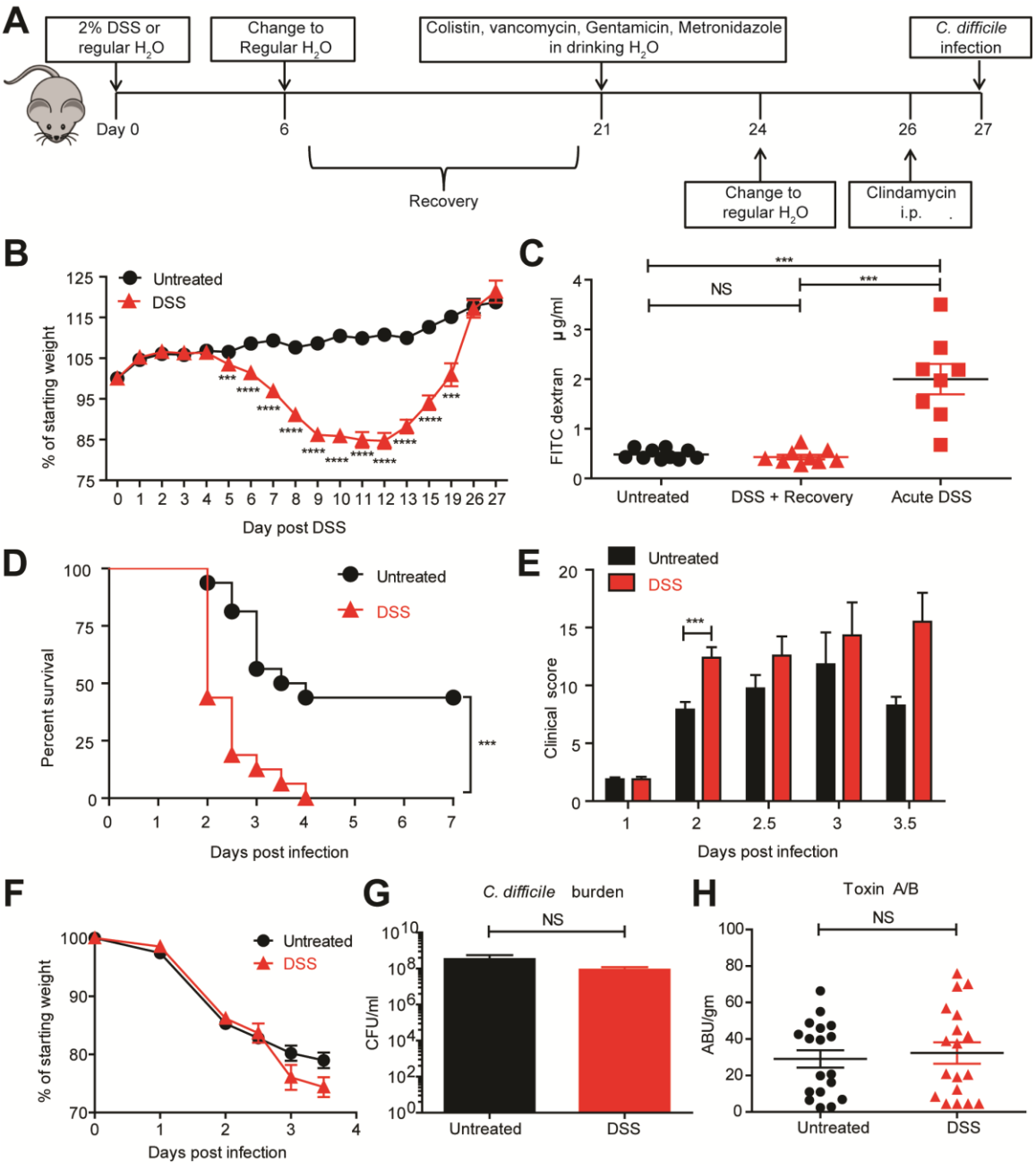

A
2% DSS or regular H2O
Change to Regular H2O
Colistin, vancomycin, Gentamicin, Metronidazole in drinking H2O
C. difficile infection
Day 0
6
21
24
26
27
Recovery
Change to regular H2O
Clindamycin i.p.
B
Untreated
DSS
% of starting weight
Day post DSS
C
FITC dextran µg/ml
NS
Untreated
DSS + Recovery
Acute DSS
D
Percent survival
Days post infection
E
Clinical score
Days post infection
F
% of starting weight
Days post infection
G
C. difficile burden
CFU/ml
NS
H
Toxin A/B
ABU/gm
NS

Figure 2.1: Increased CDI-associated mortality in mice with prior DSS colitis. 6-week old C57Bl/6J mice were treated with 2% DSS or no treatment in the drinking water for 6 days, then allowed to recover for 2 weeks. Both groups were treated with antibiotics and infected with $1x10^6$-$1x10^7$ CFU of *C. difficile* strain R20291. (**A**) Model for CDI after recovery from DSS colitis. (**B**) DSS-induced weight loss and recovery before *C. difficile* infection (n=18-19 per group). (**C**) FITC dextran detection assay in the serum was used to determine gut permeability in untreated mice, during acute DSS colitis, and after recovery from DSS colitis (n=8-10 per group). After infection, survival (**D**), clinical scores: weight loss, coat appearance, eyes/nose discharge, activity, posture and diarrhea (**E**) and weight loss (**F**) of the two groups were assessed twice a day for 7 days (n=16 per group). Day 0 post infection in (**D-F**) refers to Day 27 in the timeline in (**A**). (**G**) Caecal contents were collected from mice with/without prior DSS colitis on day 2. The samples were homogenized and plated anaerobically on *C. difficile*- selective agar plates (n=10-14 per group). (**H**) Toxins were detected within caecal contents using the *C. difficile* TOXA/B enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (n=18 per group). Data represent mean ± SEM.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by two-tailed t-test (**B, F-H**), one-way analysis of variance (**C**), Logrank statistical test (**D**) and Mann-Whitney test (**E**). These data are combined from two independent experiments.

Figure 2.2

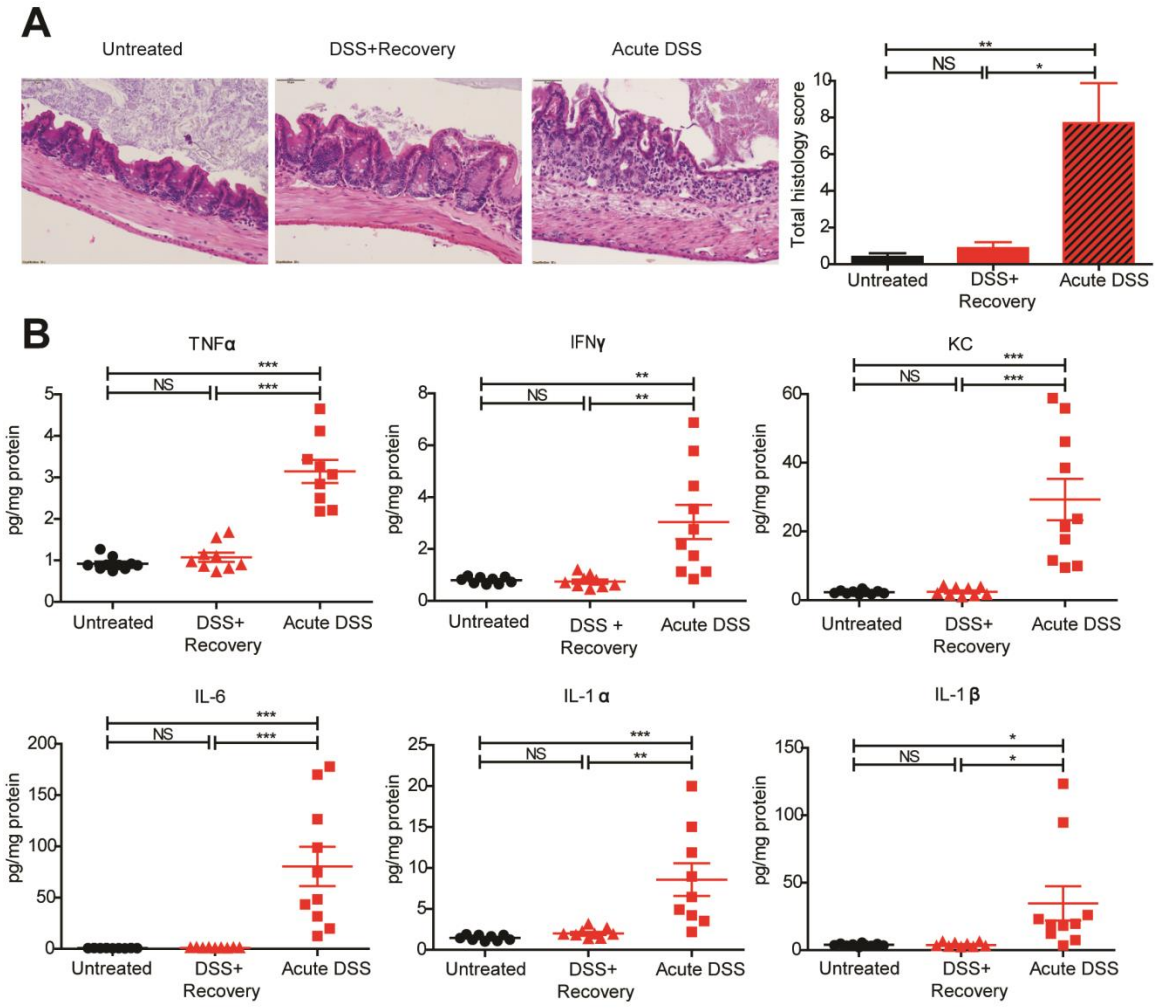

A
Untreated
DSS+Recovery
Acute DSS
Total histology score
NS
**
*
Untreated
DSS+ Recovery
Acute DSS
B
TNFα
IFNγ
KC
IL-6
IL-1 α
IL-1 β
pg/mg protein
NS
***
**
*
Untreated
DSS+ Recovery
DSS + Recovery
Acute DSS

Figure 2.2: **Recovery from DSS colitis**. Mice were given untreated or DSS-treated water and caecal tissue was collected either during acute DSS or after recovery. (**A**) caecal sections were isolated and fixed in Bouin's solution for 24 hours before paraffin embedding and H&E staining. Samples were then blindly scored by two independent scorers based on epithelial disruption, submucosal edema, inflammatory infiltrate, mucosal thickening and luminal exudates. (**B**) caecal tissue was homogenized using a bead beater, cytokine levels were determined using a Luminex MAGPIX bead-based multiplex analyzer and normalized to total protein quantified using a Pierce BCA Assay. Data represent mean ± SEM.*$p<0.05$, **$p<0.01$, ***$p<0.001$ using a one-way analysis of variance. Data are representative of two independent experiments.

DSS mice developed severe disease early starting at day 2 of infection. Interestingly, when we measured *C. difficile* burden at the peak of infection on day 2, no differences were found between DSS mice and untreated mice (**Figure 2.1 G**). Additionally, *C. difficile* toxin A/B levels during infection were comparable between the two groups (**Figure 2.1 H**). Next, we tested whether increased severity of disease in the DSS-treated mice was due to DSS-induced changes in the gut microbiota. To characterize changes in the bacterial communities in the gut, we utilized 16S rRNA gene sequencing of the V4 region. We found that after two weeks of recovery, the DSS-treated mice had a significantly different fecal microbiota from the untreated mice (**Figure 2.3 A**). However, when we analyzed the caecal communities after antibiotic treatment, we found that the two groups were no longer significantly different suggesting that antibiotic treatment normalizes the changes in the gut microbiota caused by DSS (**Figure 2.3 C-D**). Finally, to test the role of the microbiota in this model, we co-housed DSS-treated mice with untreated mice before *C. difficile* infection. We found that co-housing was not sufficient to protect DSS mice from severe CDI or to worsen disease severity for untreated mice (**Figure 2.3 B**). Given the similarities in *C. difficile* colonization and toxin production and the lack of protection after co-housing, we hypothesized that increased severity of CDI in DSS mice is due to differences in the host immune response to infection.

Figure 2.3

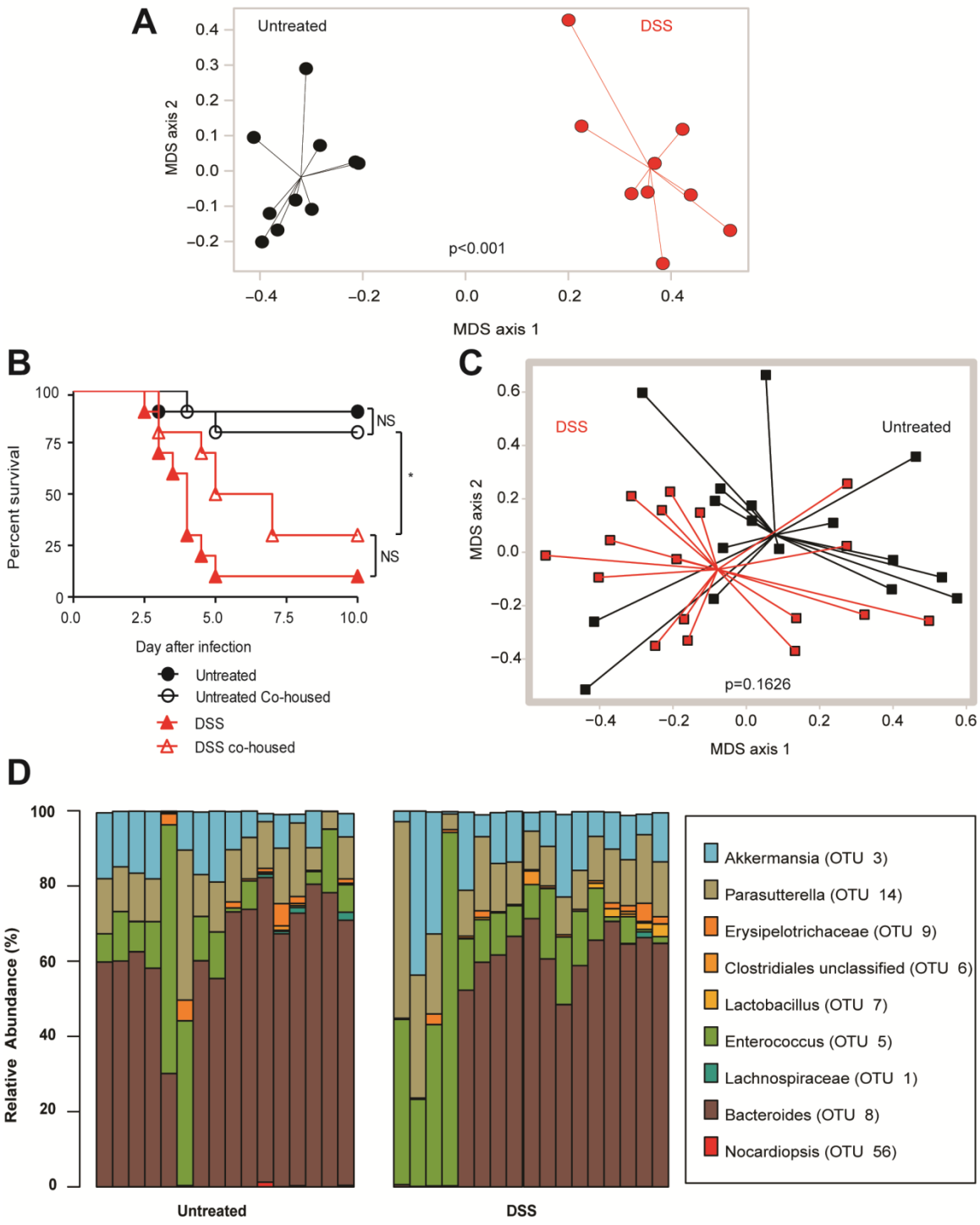

A
Untreated
DSS
MDS axis 2
MDS axis 1
p<0.001
B
Percent survival
Day after infection
NS
NS
*
Untreated
Untreated Co-housed
DSS
DSS co-housed
C
DSS
Untreated
MDS axis 2
MDS axis 1
p=0.1626
D
Relative Abundance (%)
Untreated
DSS
Akkermansia (OTU 3)
Parasutterella (OTU 14)
Erysipelotrichaceae (OTU 9)
Clostridiales unclassified (OTU 6)
Lactobacillus (OTU 7)
Enterococcus (OTU 5)
Lachnospiraceae (OTU 1)
Bacteroides (OTU 8)
Nocardiopsis (OTU 56)

Figure 2.3: **DSS-induced changes in the gut microbiota alone are not sufficient to increase CDI severity**. Mice were given untreated or DSS-treated water (**A**) Multidimensional scaling (MDS) plot of Bray-Curtis dissimilarity index comparing fecal communities from mice after recovery from DSS colitis vs untreated mice, before antibiotic treatment. (**B**) Survival curve of untreated and DSS-treated mice that were co-housed or housed separately for three weeks then infected with *C. difficile*. (**C**) MDS plot of Bray-Curtis dissimilarity index from caecal samples of DSS-treated and untreated mice post antibiotic treatment and before *C. difficile* infection. (**D**) Bar chart displaying relative abundance of family level OTUs in the caecal communities of DSS-treated and untreated mice post antibiotic treatment and before *C. difficile* infection.*$p<0.05$, statistical comparisons were made using permutational multivariate analysis of variance (PERMANOVA) (**A,C**) and a Logrank statistical test (**B**).

CD4+ T cells increase CDI severity in mice with prior DSS colitis

To understand the differences in the host immune response to CDI between DSS and untreated mice that might influence disease severity, we used flow cytometry to characterize immune cell recruitment to the colon on day 2, the peak of infection. We found no significant difference in the numbers of neutrophils, eosinophils, Ly6C^{hi} and Ly6C^{lo} monocytes between the two groups (**Figure 2.4 A**). Surprisingly, CD4+ T cell numbers were consistently and significantly higher in DSS mice compared to untreated mice during CDI, both as a total number and as a percentage of TCRβ+ T cells (**Figure 2.5, A-B**). Given the robust increase in CD4+ T cells, we hypothesized that these cells are the cause of increased severity of CDI in DSS mice. To test this hypothesis, first we depleted CD4-expressing cells using an anti-CD4 depleting antibody. We used flow cytometry to confirm successful depletion of CD4+ T cells from the colon lamina propria using the anti-CD4 antibody (**Figure 2.5 C**). Interestingly, depletion of CD4-expressing cells from DSS mice prior to CDI protected them from increased severity of disease as measured by reduced mortality, weight loss and clinical scores (**Figure 2.5, D-F**). Since CD4 can also be expressed by NK T cells, splenic DCs and lymphoid tissue inducer (LTi) cells in mice, we tested directly whether DSS-induced CD4+ T cells are sufficient to exacerbate CDI severity. To do this, we isolated CD4+ T cells from the colon lamina propria and mesenteric lymph nodes of both DSS-treated and untreated mice using a negative selection microbeads isolation kit. We used flow cytometry to confirm

that the isolated cells were CD3+ CD4+ T cells (with a purity of 98.8%) (**Figure 2.5 G**) and transferred them into naïve recipients that were subsequently infected with *C. difficile.* Mice receiving CD4+ T cells from DSS-treated donors had significantly lower survival rates post-infection compared to mice receiving CD4+ T cells from untreated donors and mice that did not receive a T cell transfer (**Figure 2.5 H**). Taken together, these studies show that in mice with prior gut inflammation, CD4+ T cells are necessary and sufficient to increase CDI-associated mortality.

Figure 2.4

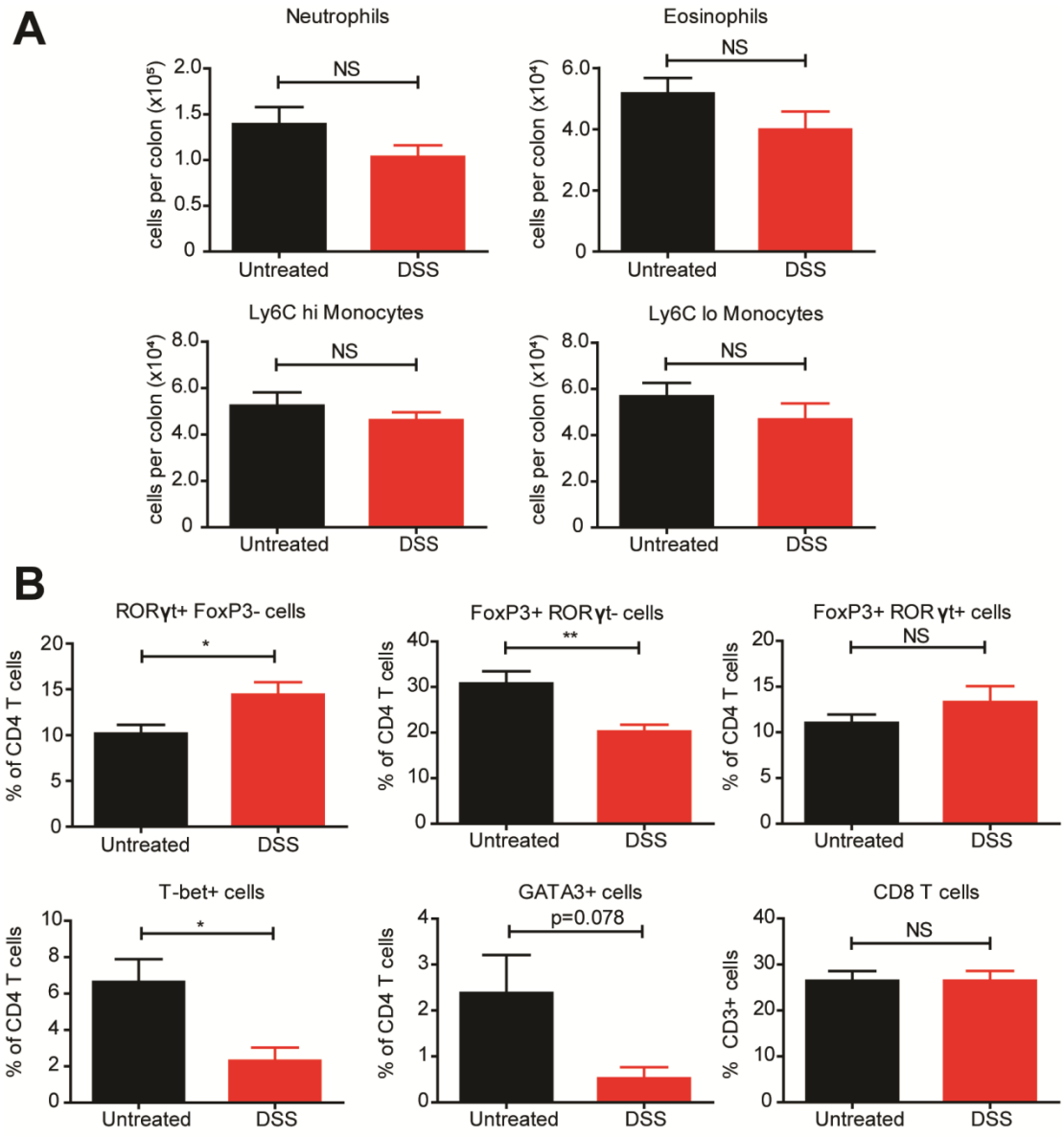

A
Neutrophils
cells per colon (x10⁵)
NS
Untreated
DSS
Eosinophils
cells per colon (x10⁴)
NS
Untreated
DSS
Ly6C hi Monocytes
cells per colon (x10⁴)
NS
Untreated
DSS
Ly6C lo Monocytes
cells per colon (x10⁴)
NS
Untreated
DSS
B
RORγt+ FoxP3- cells
% of CD4 T cells
*
Untreated
DSS
FoxP3+ RORγt- cells
% of CD4 T cells
**
Untreated
DSS
FoxP3+ RORγt+ cells
% of CD4 T cells
NS
Untreated
DSS
T-bet+ cells
% of CD4 T cells
*
Untreated
DSS
GATA3+ cells
% of CD4 T cells
p=0.078
Untreated
DSS
CD8 T cells
% CD3+ cells
NS
Untreated
DSS

Figure 2.4: **Characterization of immune responses to CDI in untreated and DSS-treated mice**. Mice were given DSS or regular water (as described before), recovered for 2 weeks and infected with *C. difficile*. On day 2 of infection, Colon tissue was isolated, processed into single-cell suspension and stained for flow cytometry. (**A**) Quantification of neutrophils (CD45+ CD11b+ Ly6G+), eosinophils (CD45+ CD11b+ SiglecF+), Ly6C^{hi} and Ly6C^{lo} monocytes (CD45+ CD11b+ Ly6C+) (n=9-10 per group per experiment). (**B**) Quantification of different (CD45+ TCRβ+/CD3+) CD4+ T cell subsets, as well as CD8+ T cells (n=5-6 per group per experiment). Data represent mean ± SEM.*$p<0.05$, **$p<0.01$ by a two-tailed student t-test. Data are representative of at least two independent experiments.

Figure 2.5

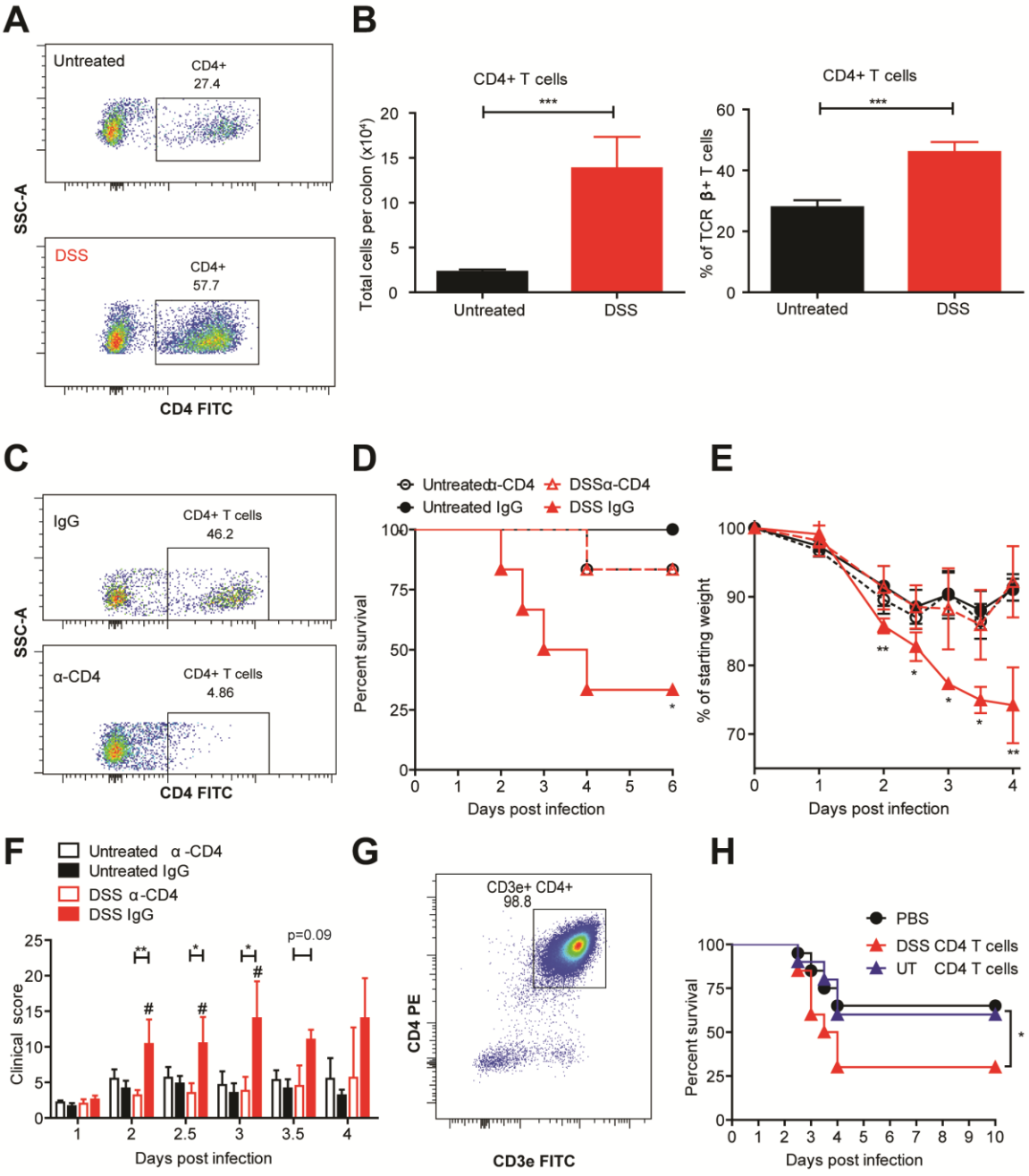

A
Untreated
CD4+
27.4
DSS
CD4+
57.7
SSC-A
CD4 FITC
B
CD4+ T cells
***
Total cells per colon (x10⁴)
Untreated
DSS
CD4+ T cells
***
% of TCR β+ T cells
Untreated
DSS
C
IgG
CD4+ T cells
46.2
α-CD4
CD4+ T cells
4.86
SSC-A
CD4 FITC
D
Untreatedα-CD4
DSSα-CD4
Untreated IgG
DSS IgG
Percent survival
Days post infection
E
% of starting weight
Days post infection
F
Untreated α-CD4
Untreated IgG
DSS α-CD4
DSS IgG
p=0.09
Clinical score
Days post infection
G
CD3e+ CD4+
98.8
CD4 PE
CD3e FITC
H
PBS
DSS CD4 T cells
UT CD4 T cells
Percent survival
Days post infection

Figure 2.5: CD4+ T cells increase *C. difficile* disease severity in mice with prior DSS colitis. Mice were given DSS or regular water (as described before), recovered for 2 weeks and infected with *C. difficile*. Colon tissue was isolated, processed into single-cell suspension and stained for flow cytometry. (**A**) Representative flow plots depicting colonic CD4+ T cells (CD45+ TCRβ+). (**B**) Quantification of colonic CD4+ T cells (n=10-12 per group). (**C-F**) Mice were injected i.p. with 400μg α-CD4 or IgG isotype control on days -6, -3 and on the day of infection with *C. difficile*. (**C**) Representative flow plots showing depletion of colonic CD4 T cells. (**D-F**) Survival, weight loss and clinical scores were assessed twice daily for 6 days (n=6 per group). (**G**) Donor mice were given 2% DSS or regular water for 6 days. On day 7, CD4+ T cells were isolated from the colon and mesenteric lymph nodes using negative selection magnetic beads and the purity was checked by flow cytometry. (**H**) $1x10^6$ CD4+ T cells were transferred into naïve, antibiotic-treated recipients. The following day, recipient mice were infected with *C. difficile.* Survival curves were compared using a Logrank statistical test. Weight loss and clinical scores were compared using a one-way analysis of variance and Kruskal Wallis test, respectively (n=10 per group per experiment). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. # p<0.05: DSS IgG compared to Untreated IgG. Data are combined from two independent experiments.

Increased Th17 responses in mice with prior DSS colitis during CDI

Given our discovery that CD4+ T cells play an essential role during CDI in mice with prior gut inflammation, we aimed to further characterize the T cell subset that might exacerbate CDI severity. Using flow cytometry, we found a significant increase in Th17 cells both in absolute numbers and as a percentage of CD4+ T cells in DSS mice during CDI (**Figure 2.6 A**). Similarly, using IL-17A-GFP reporter mice we found higher numbers of IL-17A-producing CD4+ T cells during CDI in DSS mice compared to untreated controls (**Figure 2.6 B**). Furthermore, when we quantified colonic Th1, Th2, and T_{reg} cells, we did not find a compensatory increase in these cell subsets. Instead, our data showed a disruption in the homeostatic Th17/T_{reg} balance and a significant skew towards Th17 responses in DSS mice (**Figure 2.4 B**). Therefore, we focused our studies on understanding the role of Th17 cells during CDI.

Th17 cells express the IL-23 receptor, and the presence of IL-23 during Th17 differentiation has been shown to lead to a pathogenic Th17 phenotype that has been implicated in autoimmunity, inflammation and infection (Langrish et al., 2005; Lee et al. 2012). Furthermore, the IL-23 axis upstream of Th17 cells has long been thought to play a role in the pathogenesis of IBD (McGovern and Powrie, 2007; Ahern et al., 2008). Finally, studies in our lab and others have shown that blocking IL-23 signaling during CDI protects from severe disease (Buonomo et al., 2013). Therefore, we investigated whether mice with DSS colitis have higher IL-23 levels before and after CDI. Indeed, we found higher levels of

IL-23 protein in the caeca of DSS mice on days 0 and 2 of infection. Additionally, we found higher levels of the Th17 cytokine IL-17A in the caeca of DSS mice during infection (**Figure 2.6 C**). Finally, our data showed a direct correlation between IL-23 levels in the cecum during infection and CDI severity (**Figure 2.6 D**). Taken together, these findings indicated that a colitis-induced skew towards Th17 responses was associated with worse disease outcome during CDI.

In addition to the production of IL-17A, Th17 cells can produce other cytokines including IL-17F, IL-22, IFNγ and GMCSF. In order to determine which of these cytokines are important for the Th17-mediated exacerbation of CDI severity, we used flow cytometry and ELISA assays to compare the levels of these cytokines between DSS and untreated mice. We found that the majority of IL-17A+ T cells do not co-express IFNγ or IL-22. Our analysis showed that on Day 0 before infection, DSS mice had elevated levels of IL-17A+ IFNγ- and IL-17A+ IL-22- T cells in the mesenteric lymph nodes but not in the colon. On Day 2 during infection, however, we found significantly increased numbers of these cells in the mesenteric lymph nodes as well as the colon (**Figure 2.7 A**). Additionally, we found an increase in the levels of IL-17F and IL-22 caecal proteins in DSS mice during infection and no difference in GMCSF and IFNγ (**Figure 2.7 B**).

Because of the significant increase in IL-17A and IL-17F protein levels in the tissue and because TH17 cells in DSS mice were predominantly negative for IFNγ and IL-22, we hypothesized that IL-17 signaling is the mechanism by which

Th17 cells cause increased severity of disease. To test this hypothesis, we used a blocking antibody against the IL-17RA, which binds to IL-17A homodimers and with less affinity to IL-17A IL-17F heterodimers and IL-17F homodimers. Our data showed that blocking IL-17RA protected DSS mice from severe disease early during infection (**Figure 2.6 E**). Similarly, neutralizing IL-17A provided significant protection early during infection (**Figure 2.7 C**). Together, these data suggest that IL-17 signaling is important for Th17-mediated exacerbation of CDI severity in DSS mice.

Figure 2.6

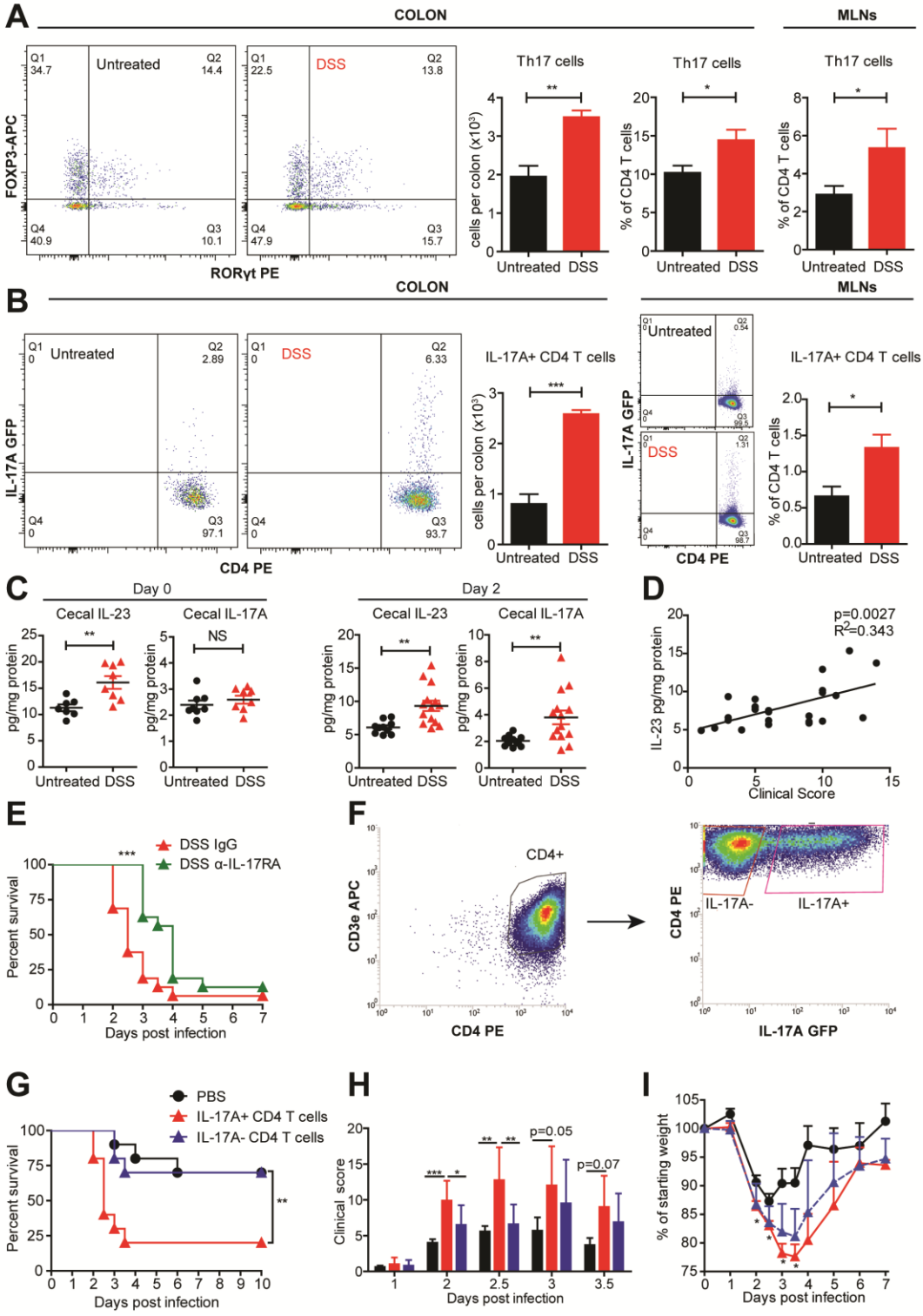

A
COLON
MLNs
Untreated
DSS
FOXP3-APC
RORγt PE
Th17 cells
cells per colon (x10³)
% of CD4 T cells
Untreated DSS
B
IL-17A+ CD4 T cells
IL-17A GFP
CD4 PE
C
Day 0
Day 2
Cecal IL-23
Cecal IL-17A
pg/mg protein
NS
D
p=0.0027
R²=0.343
IL-23 pg/mg protein
Clinical Score
E
DSS IgG
DSS α-IL-17RA
Percent survival
Days post infection
F
CD4+
CD3e APC
CD4 PE
IL-17A-
IL-17A+
IL-17A GFP
G
PBS
IL-17A+ CD4 T cells
IL-17A- CD4 T cells
H
Clinical score
p=0.05
p=0.07
I
% of starting weight

Figure 2.6: Increased Th17 responses in mice with prior DSS colitis cause severe subsequent CDI. (A-D) Mice were given DSS or regular water (as described before), recovered for 2 weeks and infected with *C. difficile*. On day 2 of infection, colon and mesenteric lymph node tissue was isolated, processed into single-cell suspension and stained for flow cytometry. (**A**) Representative flow plots and quantification of Th17 cells (CD45+ TCRβ+ CD4+ RORγt+ FOXP3-) (n=5-6 per group per experiment). (**B**) IL-17-GFP reporter mice were used to determine the number and frequency of IL-17A+ CD4 T cells during infection by flow cytometry (n=5 per group per experiment). (**C**) For protein data, caecal tissue was homogenized using a bead beater, data is normalized to total protein quantified using a Pierce BCA Assay (n=8-14 per group). (**D**) caecal IL-23 protein levels plotted against clinical scores. (**E**) Mice were injected i.p. with 125μg α-IL-17RA or IgG isotype control on days -1, 1 and 2 of infection (n=16 per group). (**F-I**) Naïve CD4+ T cells were isolated from the spleen of IL17A-GFP reporter mice and differentiated into Th17 cells *ex vivo* in the presence of TGFβ and IL-6. (**F**) CD3e+CD4+IL-17A+ and CD3e+CD4+IL-17A- T cells were sorted and $1x10^6$ cells were transferred i.p. to antibiotic-treated recipients. (**G-I**) One day following the T cell transfer, recipients were infected with *C. difficile.* Survival, weight loss and clinical scores were assessed twice daily (n= 10 per group). Data represent mean ± SEM.*p<0.05, **p<0.01, ***p<0.001 by a two-tailed student t-test (**A-C**), Pearson correlation statistical test (**D**), Logrank statistical test (**E,G**),

Kruskal-Wallis test (**H**) and one-way analysis of variance (**I**). Data are representative of two independent experiments.

Figure 2.7

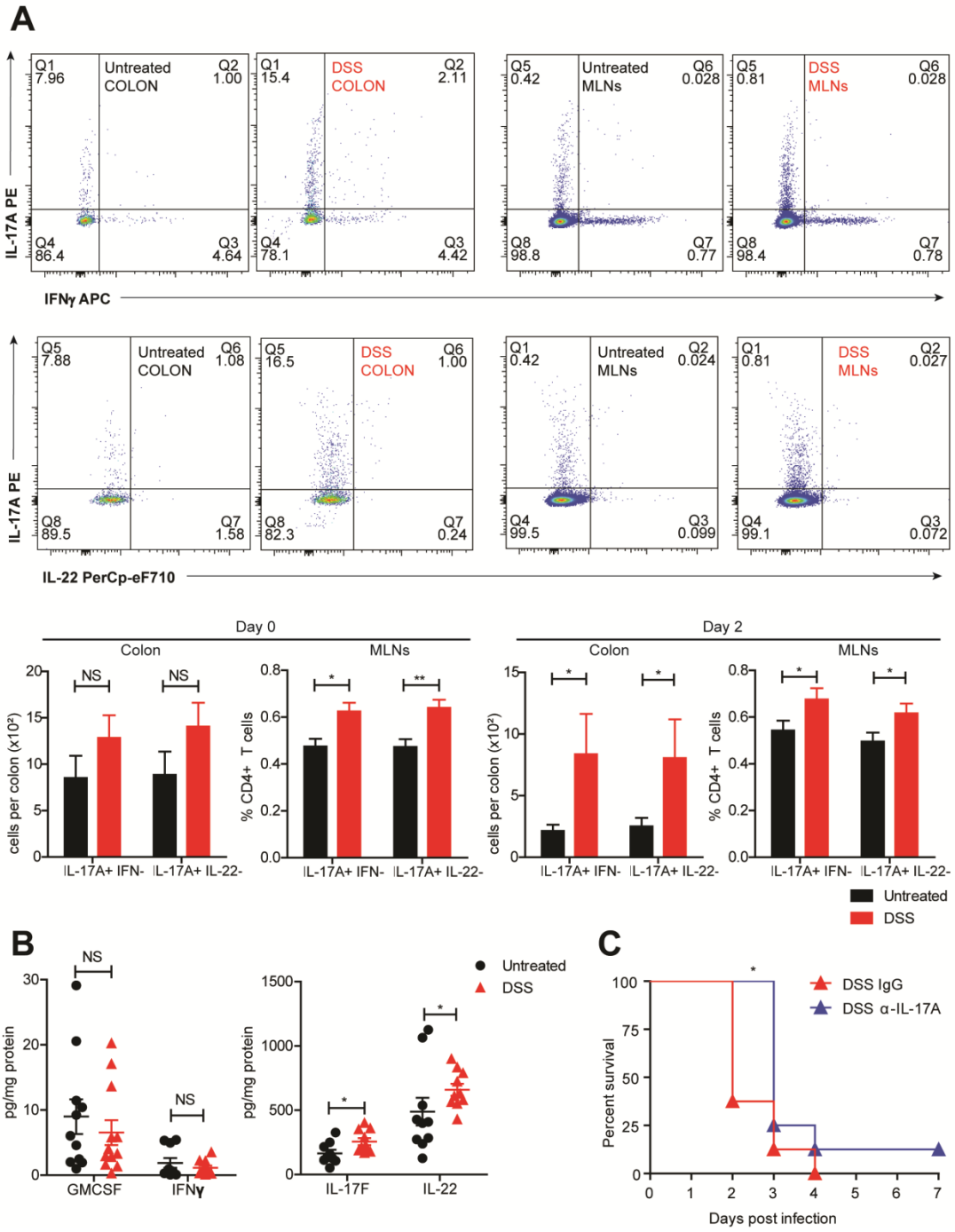

A
Q1 7.96
Untreated COLON
Q2 1.00
Q4 86.4
Q3 4.64
Q1 15.4
DSS COLON
Q2 2.11
Q4 78.1
Q3 4.42
Q5 0.42
Untreated MLNs
Q6 0.028
Q8 98.8
Q7 0.77
Q5 0.81
DSS MLNs
Q6 0.028
Q8 98.4
Q7 0.78
IL-17A PE
IFNγ APC
Q5 7.88
Untreated COLON
Q6 1.08
Q8 89.5
Q7 1.58
Q5 16.5
DSS COLON
Q6 1.00
Q8 82.3
Q7 0.24
Q1 0.42
Untreated MLNs
Q2 0.024
Q4 99.5
Q3 0.099
Q1 0.81
DSS MLNs
Q2 0.027
Q4 99.1
Q3 0.072
IL-17A PE
IL-22 PerCp-eF710
Day 0
Day 2
Colon
MLNs
cells per colon (x10³)
% CD4+ T cells
NS
*
**
IL-17A+ IFN-
IL-17A+ IL-22-
Untreated
DSS
B
pg/mg protein
GMCSF
IFNγ
IL-17F
IL-22
C
Percent survival
Days post infection
DSS IgG
DSS α-IL-17A

Figure 2.7: **Pathogenic Th17 cells in DSS mice are predominantly IFNγ- and IL-22-**. Mice were given DSS or regular water (as described before) and recovered for 2 weeks. On day 0 before infection and day 2 after infection, colon and mesenteric lymph node tissue was isolated, processed into single-cell suspension, stimulated with PMA/ionomycin and stained for flow cytometry. (**A**) Representative flow plots and quantification of Th17 cells (CD45+ TCRβ+ CD4+) on days 0 and 2 of infection, (n=6-10 per group per experiment). (**B**) On day 2 of infection, caecal tissue was homogenized using a bead beater, protein levels were measured by ELISA or a Luminex bead-based multiplex assay. Data is normalized to total protein quantified using a Pierce BCA assay (n=10-12 per group). (**C**) Survival analysis of mice injected i.p. with 125μg α-IL-17A or IgG isotype control on days -1, 1 and 2 of infection with *C. difficile* (n=8 per group). Data represent mean ± SEM.*p<0.05, **p<0.01 by a two-tailed student t-test or Mann-Whitney statistical test (**A, B**) and Logrank statistical test (**C**).

Adoptive transfer of Th17 cells is sufficient to increase CDI severity

Given the significant Th17 skew in DSS mice and our discovery that CD4+ T cells are necessary and sufficient to cause severe CDI in these mice, we wanted to directly test whether Th17 cells alone can exacerbate CDI severity. To do this, we isolated naïve CD4+ T cells from the spleens of IL-17-GFP reporter mice and differentiated them *ex vivo* into Th17 cells. On the day before infection, we sorted CD3+ CD4+ T cells into IL-17A+ and IL-17A- cells (**Figure 2.6 F**). The sorted cells were transferred into naïve recipient mice that were subsequently infected with *C. difficile.* After infection, mice that received IL-17A+ CD4+ T cell transfer had higher CDI-associated mortality rates, weight loss and clinical scores compared to mice that either received IL-17A- CD4+ T cells or no T cell transfer (**Figure 2.6, G-I**). It is important to note that while recipients of the IL-17A- CD4+ T cells had similar survival and clinical scores to the PBS controls, they lost more weight post infection. This modest effect is likely due to some IL-17A- CD4 T cells gaining IL-17A expression post-transfer. Indeed, when we looked by flow cytometry after infection, we found some GFP+ cells in the colons of these mice. Taken together, these results establish a central role for Th17 cells in determining the severity and outcome of CDI following colitis.

Serum IL-6 and IL-23 in *C. difficile* patients correlate with severe disease

In light of our data establishing a role for Th17 cells during CDI in a mouse model, we wished to explore whether Th17 responses are also important during

human CDI. Serum samples from *C. difficile* patients were analyzed for IL-6, IL-23, IL-17A and IL-4 protein levels. Severe CDI was defined with a white blood cell (WBC) count ≥15,000 per microliter as previously described (Shivashankar et al. 2013; Yu et al. 2017). We used a Kaplan-Meier survival analysis to confirm that the severe CDI group had significantly lower survival probability (**Figure 2.8 A**). When patients were categorized based on disease severity, those with severe CDI had significantly higher serum IL-6 and IL-23 levels compared to those with non-severe CDI. We found no difference in serum IL-17A levels between the two groups (**Figure 2.8 B**). In addition to its role in Th17 differentiation, IL-6 can promote IL-4 production by Th2 cells and lead to enhanced Th2 differentiation through an IL-4 feedback loop (Dienz and Rincon, 2009). However, when we measured the levels of IL-4 in the human serum samples, we actually found a decrease in IL-4 levels in patients with severe CDI suggesting no enhanced Th2 responses downstream of IL-6 (**Figure 2.8 B**). Strikingly, when we divided the patients into four quartiles based on IL-6 levels in the serum, patients with higher IL-6 (second, third and fourth quartiles) were significantly less likely to survive post-infection than patients in the lowest quartile (**Figure 2.8 C**). After adjustment for age, sex, race, comorbidities and ICU admission, patients in the highest quartile for serum IL-6 were 7.6 times less likely to survive than those in the lowest quartile (p=0.0009) (**Table 1**). A survival analysis based on serum IL-23 levels could not be completed due to the high number of samples under the assay's limit of detection, which limited the statistical power of the survival studies for this cytokine. Taken together, these

data suggest that serum IL-6 and IL-23, two cytokines upstream of pathogenic Th17 cells, likely play a role in increasing CDI- associated mortality in human patients.

Figure 2.8

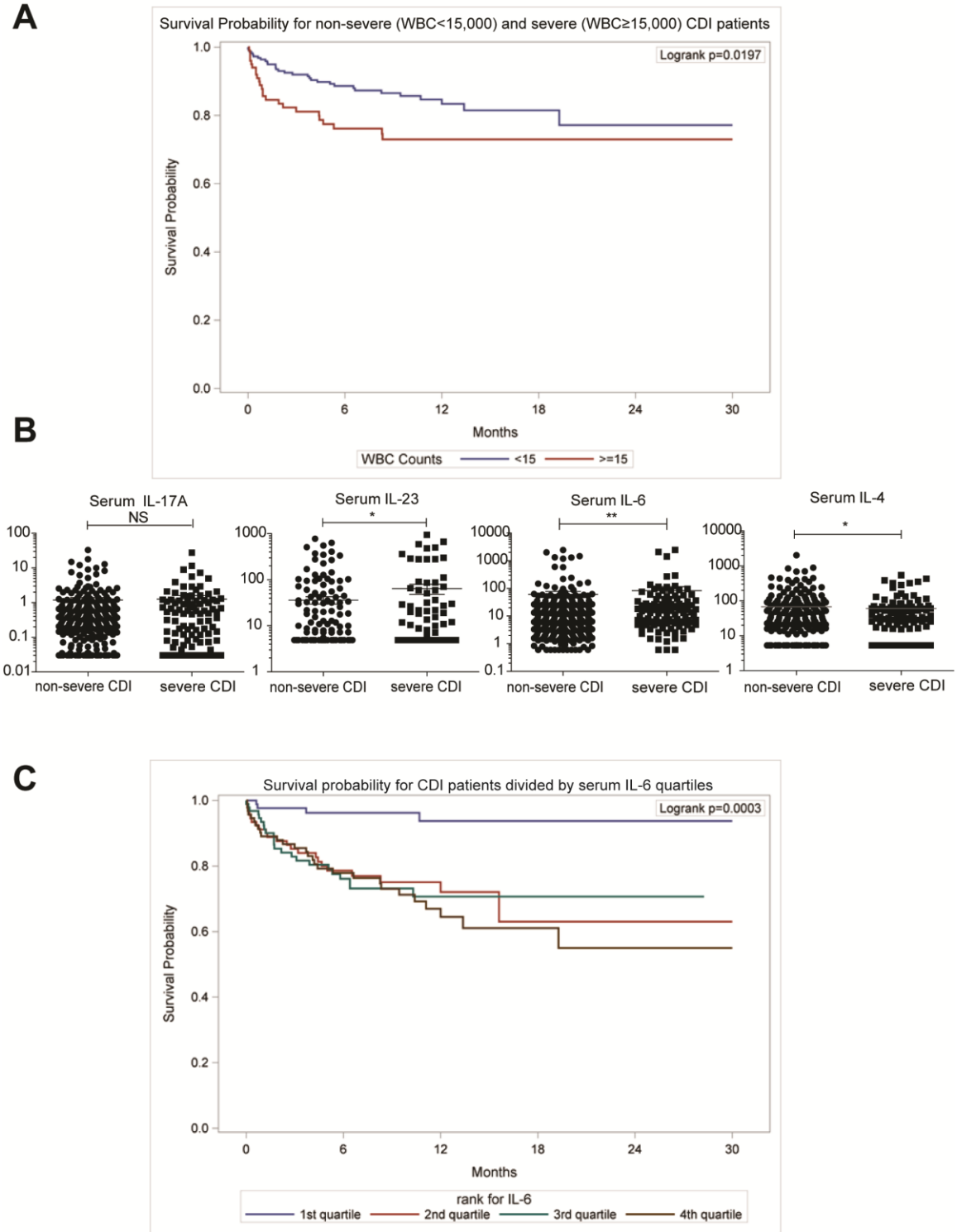

A
Survival Probability for non-severe (WBC<15,000) and severe (WBC≥15,000) CDI patients
Logrank p=0.0197
Survival Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
30
Months
WBC Counts
<15
>=15
B
Serum IL-17A
NS
Serum IL-23
*
Serum IL-6
**
Serum IL-4
*
100
10
1
0.1
0.01
1000
100
10
1
10000
1000
100
10
1
0.1
non-severe CDI
severe CDI
C
Survival probability for CDI patients divided by serum IL-6 quartiles
Logrank p=0.0003
Survival Probability
Months
rank for IL-6
1st quartile
2nd quartile
3rd quartile
4th quartile

Figure 2.8: The Th17 cytokines IL-6 and IL-23 in the serum of *C. difficile* patients correlate with severe disease. IL-23 and IL-17A in the serum of *C. difficile* patients were quantified using high sensitivity ELISAs from R&D. IL-6 and IL-4 were quantified using a Luminex bead-based multiplex assay. (**A**) Kaplan-Meier survival curve post CDI diagnosis for patients with non-severe CDI (WBC<15,000 per microliter, n=226) and severe CDI (WBC≥15,000 per microliter, n=100). (**B**) serum IL-17A, IL-23, IL-6 and IL-4 for patients with non-severe and severe CDI, n=323, 323 and 362, 379 respectively. (**C**) Kaplan-Meier survival curve post CDI diagnosis for patients categorized into quartiles based on IL-6 serum levels, n=92-94 per quartile. Data represent mean ± SEM.*p<0.05, **p<0.01, ***p<0.001 using Logrank (**A,C**) and Mann-Whitney (**B**) statistical tests.

Table 1: mortality risk of *C. difficile* patients based on IL-6 serum levels.

IL-6 quartile	Mortality risk (hazard ratio)	95% CI	p value
1st n= (51 M, 41 F)	Ref	-	-
2nd n= (48 M, 44 F)	7.09	(2.10, 23.92)	0.0016
3rd n= (40 M, 54 F)	6.07	(1.80, 20.52)	0.0037
4th n= (44 M, 50 F)	7.60	(2.30, 25.17)	0.0009

[a] Correlation between IL-6 levels and survival assessed via a Cox proportional hazards model. M= male, F= female.

[b] Mortality risk (hazard ratio) adjusted for age, sex, race, Charlson comorbidity index and ICU admission

2.4 Discussion

Altogether, our study demonstrates that in a colitis setting, Th17 cells are an important source of IL-17A and these cells alone can increase the risk for severe CDI. Our findings are particularly important in light of new evidence showing that IBD patients have higher numbers of Th17 cells in the colon and circulation (Hegazy et al., 2017), which might explain their increased risk for severe *C. difficile* colitis. In addition to *C. difficile* infection, *Salmonella* infections have also been linked to IBD (Gradel et al., 2009); Hegazy et al. found more *S. typhimurium*-specific Th17 cells in PBMCs from patients with IBD compared to controls. There is also evidence that suppressing T cell responses during *S. typhimurium* infection can protect the host from severe disease by preventing the downstream neutrophil recruitment and off-target tissue pathology (Godinez, 2008). This suggests that aberrant Th17 responses in IBD patients may generally put them at risk for severe gastrointestinal infections.

Previous studies have found that targeting IL-23 and IL-17A can protect mice from *C. difficile*-associated epithelial damage and mortality (Buonomo et al., 2013; McDermott et al., 2016; Tateda et al., 2016). In our study, we found that neutralizing IL-17A and blocking the IL-17RA receptor protected DSS mice from severe disease early on during infection. Blocking the receptor was more robust at providing protection suggesting that IL-17F, also elevated in DSS mice, might play a role in exacerbating CDI severity. Blocking IL-17 signaling did not provide protection later during infection, suggesting that perhaps some IL-17 signaling is

required to control the infection. Indeed, other studies have shown that although increased neutrophilia downstream of IL-17A is associated with severe disease, complete depletion of neutrophils does not protect mice from disease because of their essential role in clearing the infection (Jarchum et al., 2012 and Feghaly et al., 2013). The lack of complete protection by IL-17 signaling blockade might suggest that other products of Th17 cells perhaps play a role in the exacerbation of CDI severity. Although we did not see striking differences in the production of IFNγ or IL-22 by Th17 cells in DSS mice, further studies are needed to determine whether any other Th17 cytokines work synergistically with IL-17A to cause severe disease.

Interestingly, in a T cell transfer model of colitis, Wedebye et al. (2013) found that blocking IL-17A and IL-17F simultaneously was more successful at ameliorating colitis than blocking either cytokine individually. Furthermore, targeting Th17 cells with pharmacologic inhibitors of RORγt was successful in reducing gut inflammation in two different murine models of IBD (Withers et al., 2016). The findings of our study suggest that specifically targeting Th17 cells and their effector cytokines in IBD patients may also protect them from acquiring severe CDI.

The limitations of this study include the use of DSS colitis as a way to induce gut inflammation prior to CDI. DSS colitis does not perfectly model human IBD because it is chemically-induced colitis. However, for the purposes of this study, DSS colitis did cause a skew towards Th17 responses that persisted

beyond the resolution of acute colitis and allowed us to study the effect of the Th17 axis on subsequent CDI. Therefore, the model we developed can be used for future studies to identify the downstream mechanisms that are required for the Th17-mediated exacerbation of CDI. Another strength of the DSS model for our studies is that it is an acute form of colitis, which allows for full recovery before CDI. Recently, Zhou et al. (2018) showed that mice with acute DSS colitis are more susceptible to *C. difficile* infection. However, the cause of this increased susceptibility is unclear and could be attributed to the physical damage to the epithelial barrier during acute DSS colitis. In patients, time-to-CDI diagnosis data suggest that a high percentage of IBD patients acquire CDI during remission and not during an IBD flare (Rodemann et al., 2007). For this reason, we designed a model where mice are recovered from acute colitis before CDI and we suggest that this model might better reflect what happens in IBD patients.

Consistent with a previous report by Yu et al. (2017), we found no difference in serum IL-17A between the patients with severe vs. non-severe disease. Yu and colleagues, however, found no difference in serum IL-6, but the two studies were done on two different cohorts and our study included 362 patients, compared to 36 in the Yu et al. report. While we found differences in IL-6 and IL-23 systemically in the serum, perhaps the effects on IL-17A production are localized to colonic Th17 cells and evaluation of tissue IL-17A levels might reveal differences between the two patient groups.

Future experimentation is needed to understand how Th17 cells exacerbate CDI severity. Th17 cells produce IL-17A, F and IL-22 that can act on epithelial cells, which in turn produce several chemokines that ultimately lead to neutrophil recruitment (Ouyang et al., 2008). While neutrophils are important for defense against bacterial infections, they often can have off-target damaging effects on host tissue. Our studies on day 2 did not show enhanced neutrophil recruitment in DSS mice, but future studies can further characterize the effects of Th17 cells on neutrophil activation and function during CDI.

The incidence of *C. difficile* over the last two decades has continued to rise and the CDC identifies *C. difficile* as an urgent threat. Although antibiotic treatment is the first line of defense against CDI, the antibiotic-induced disruption of the gut microbiota contributes to disease relapse in 1 of 5 patients. Therefore, discovering new therapies is essential for treating this life-threatening infection. Previous studies have reported a role for CD4 T cells in protection against CDI via a TLR4-dependent recognition of *C. difficile* surface layer proteins (Ryan et al., 2011). However, these cells' role during infection remains underexplored. Using adoptive transfer studies, we have directly shown that Th17 cells are sufficient to increase CDI severity. Furthermore, by analyzing serum samples from *C. difficile* patients, we found that IL-23 and IL-6 correlate with disease severity and that patients with high IL-6 levels are significantly more likely to die after infection. This is supported by a previous study that described higher levels of IL-6 in 8 patients with severe CDI (Rao et al. 2014). These mouse studies and

their human correlate suggest than in patients with increased pathogenic Th17 responses, such as IBD, targeting these cells may not only ameliorate IBD symptoms but also reduce their risk of developing severe CDI.

2.5 Materials and Methods

Mice

C57BL6 and IL17-GFP reporter mice (strain: C57BL/6-Il17atm1Bcgen/J, Cat# JAX: 018472, RRID:IMSR_JAX:018472) were ordered from Jackson Laboratories. All mice were housed under specific pathogen-free conditions at the University of Virginia's animal facility. All experiments were performed according to provisions of the Animal Welfare Act of 1996 and were approved by the University of Virginia Institutional Animal Care and Use Committee. Littermates were randomly assigned to experimental groups. Male mice were used that were 6 weeks old at the start of each experiment. During the infection, mice were weighed and scored daily and euthanized if they lost more than 25% of the starting body weight, or had a clinical score of 14 or above.

Human serum samples

Information on the sex and sample size of the human subjects in each experimental group is listed in Table S1. Ages ranged from 12-97 years, with a mean of 60 years and a median of 63 years. Cox proportional hazards model was used to adjust for age among other variables including sex, race, Charlson comorbidity index and ICU admission.

Data collection and analysis were approved by the UVA Institutional Review Board (IRB-HSR #16926). The Institutional Review Board for Health Sciences Research confirms that this project meets the criteria of research involving coded private information or biological specimens. According to the Office for Human Research Protections (OHRP) guidance, this project is considered to not involve human subjects.

DSS treatment and *C. difficile* infection

For DSS treatment, mice were given 2% Dextran Sulfate Sodium (DSS, Thermo Fisher Scientific, Cat# AAJ1448922) in the drinking water for 6 days, then switched to regular drinking water and allowed to recover. During DSS colitis, weight loss was monitored and mice were euthanized in the rare instance that they developed severe DSS colitis. For *C. difficile* infections, age and sex-matched mice were given 45 mg/L vancomycin (Mylan), 35 mg/L colistin (Sigma), 35 mg/L gentamicin (Sigma) and 215 mg/L metronidazole (Hospira) *ad libitum* for 3 days. Then, mice were switched to regular drinking water for two days. On the day before infection, mice were injected intraperitoneally with 0.016 mg/g of clindamycin (Hospira). To prepare the inoculum, *C. difficile* strain R20291 was grown from a frozen glycerol stock onto Brain Heart Infusion (BHI) agar plates supplemented with cycloserine and cefoxitin (*C. difficile* supplement, Sigma) overnight at 37^{o}C in an anaerobic chamber (Shel Labs). A single colony was then transferred into a tube containing BHI liquid media and grown overnight at 37^{o}C anaerobically. The next day, the liquid culture was centrifuged and the pellet was

washed twice with PBS, the optical density determined using a spectrophotometer and the inoculum was diluted to $5x10^7$ CFU/ml using sterile, anaerobic PBS. The inoculum was loaded into sterile syringes and transported in sealed biohazard bags to the animal facility. The concentration of each inoculum was confirmed by counting CFUs after plating on BHI plates supplemented with 1% sodium taurocholate and grown overnight at 37^{o}C anaerobically. Mice were orally gavaged with $5x10^6$ CFU/mouse in randomized order. During the infection, mice were weighed and scored daily and euthanized if they developed severe disease based on the scoring criteria. Clinical scores are based on weight loss, coat appearance, eyes/nose discharge, activity, posture and diarrhea. To quantify *C. difficile* burden during infection, caecal contents were suspended using sterile, anaerobic PBS, serially diluted and plated on BHI plates supplemented with cycloserine and cefoxitin (*C. difficile* supplement, Sigma). After an overnight incubation at 37^{o}C in an anaerobic chamber, CFUs of *C. difficile* were counted and normalized to stool weight. Toxins A/B were quantified using the ELISA *C. difficile* TOX A/B II kit from Techlab according to the manufacturer's instructions and normalized to stool weight.

FITC dextran Assay

Mice were gavaged with 40mg/100g body weight Fluorescein isothiocyanate (FITC)–dextran solution (Sigma, Cat# 46944-500MG-F). 4 hours later, mice were sacrificed and a spectrophotometer was used to detect FITC in the serum at 485/530 nm.

LP Isolation and Flow Cytometry

To isolate the lamina propria, colons were removed, cut longitudinally and rinsed thoroughly in Hank's balanced salt solution (HBSS) supplemented with 5% FBS and 25 mM HEPES. Epithelial cells were removed with two 20-minute incubations in pre-warmed HBSS with 15 mM HEPES, 5 mM EDTA, 10% FBS and 1 mM dithiothreitol in a 37^{o}C shaking incubator. The colons were then cut into small sections and incubated in pre-warmed RPMI media containing 0.17 mg/ml liberase TL (Sigma) and 30 mg/ml DNase (Sigma) in a 37^{o}C shaking incubator. Following the digestion step, the tissue was passed through 40 and 100 μM cell strainers, respectively, counted and re-suspended to a concentration of 1x10^{7} cells/ml in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FBS). 1x10^{6} cells were plated in a 96-well plate and stained for flow cytometry. After an Fc blocking step (anti-mouse CD16/32 TruStain, BioLegend Cat #101320, RRID:AB_1574975) and staining with a fixable viability dye (Zombie Aqua, Biolegend Cat# 423102), the following antibodies were used for staining: CD11b-APC (M1/70, Biolegend Cat# 101212, RRID:AB_312795), CD45-APC-Cy7 (30-F11, Biolegend Cat# 103116, RRID:AB_312981), CD11c-BV421 (N418, Biolegend Cat# 117330, RRID:AB_11219593), CD3e-APC (500A2, Biolegend Cat# 152306, RRID:AB_2632669), CD4-PE (RM4-4, Biolegend Cat# 116006, RRID:AB_313691), CD45-PE/Cy7 (30-F11, Biolegend Cat# 103114, RRID:AB_312979), T-bet-BV421 (4B10, Biolegend 644815, RRID:AB_10896427), TCRβ-PerCp/Cy5.5 (H57-597, Biolegend Cat# 109228,

RRID:AB_1575173), CD4-FITC (GK1.5, Cat# 100406, RRID:AB_312691), CD3e-FITC (500A2, Biolegend Cat# 152304, RRID:AB_2632667),CD45-BV421 (30-F11, Biolegend Cat# 103134, RRID:AB_2562559), CD4-PE/Cy7 (GK1.5, Biolegend Cat# 100422, RRID:AB_312707), GATA3-AF647 (16E10A23, Biolegend Cat# 653810, RRID:AB_2563217), TCRβ-FITC (H57-597, Biolegend Cat# 109206, RRID:AB_313429), IL-17A-PE (TC11-18H10.1, Biolegend Cat# 506904, RRID:AB_315464), lFNγ-APC (XMG1.2, Biolegend Cat# 505810, RRID:AB_315404), Ly6G-PeCy7 (1A8, Biolegend Cat# 127618, RRID:AB_1877261) , Ly6C-FITC (HK1.4, Biolegend Cat# 128006, RRID:AB_1186135), SiglecF-PE (E50-2440) (BD Biosciences Cat# 552126, RRID:AB_394341), FOXP3-APC (FJK-16s Thermo Fisher Scientific Cat# 17-5773-82, RRID:AB_469457), RORγt-PE (B2D, Thermo Fisher Scientific Cat# 12-6981-82, RRID:AB_10807092), IL-22-PerCP-eFluor 710 (IL22JOP, Thermo Fisher Scientific Cat# 46-7222-82, RRID:AB_2573839).

For intracellular transcription factor staining, a FOXP3/Transcription Factor Staining Buffer Set (eBioscience, catalog# 00-5523-00) was used according to the manufacturer's instructions. For intracellular cytokine staining, isolated colonic cells were re-suspended in 5 ml 10% percoll (Sigma, catalog# P1644) and layered over 80% percoll, centrifuged at 1000xg for 15 minutes at RT with maximum acceleration and no brakes. Lymphocytes were collected at the interphase, washed with RPMI, plated at $1x10^{5-6}$ cells per well. Cells were stimulated in T cell culture media (RPMI Meida 1640, 10% FBS, 2mM L-

glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5mM 2-β-mercaptoethanol, BD GolgiPlug containing Brefeldin A (BD Cytofix/Cytoperm kit cat#555028), 50ng/ml PMA, 750ng/ml ionomycin) for 4 hours at 37^{o}C with 5% CO_2. Following the stimulation, cells were stained using Cytofix/Cytoperm™ Plus kit (BD, catalog# 555028) according to the manufacturer's protocol. Flow cytometry data was acquired using an LSR Fortessa cytometer (BD Biosciences) and data analysis was performed via FlowJo.

Tissue protein and cytokine analysis

Caecal tissue was isolated and rinsed gently with PBS, then homogenized by bead-beating in 400 µl of a buffer containing 1× HALT protease inhibitor (Pierce) and 5 mM HEPES. The homogenate was incubated on ice for 30 minutes after the addition of 400ul of another buffer containing 1× HALT protease inhibitor (Pierce), 5 mM HEPES and 2% Triton X-100. Finally, the lysates were spun at 13,000g for 5 minutes and the supernatants were transferred to a new tube and used for protein analysis. Mouse tissue IL-23, IL-17A, IL-17F and IL-22 ELISAs were done according to the manufacturer's instructions (DuoSet, R&D). Protein levels were normalized to the total protein concentration determined using a Pierce BCA Protein Assay (Thermo Fisher Scientific). For TNFα, IFNγ, KC, IL-6, IL-1α, IL-1β, GMCSF and IFNγ caecal tissue was processed using the same protocol and total protein was quantified using a Pierce BCA Protein Assay. Cytokine levels were determined using a Luminex MAGPIX bead-based multiplex analyzer through the University of Virginia's Flow Cytometry Core Facility.

Human serum IL-23 and IL-17A were measured using the Human IL-23 Quantikine ELISA Kit and Human IL-17 Quantikine HS ELISA Kit from R&D, respectively, according to the manufacturer's instructions. Human serum IL-6 and IL-4 were measured using a Luminex bead-based multiplex assay from R&D according to the manufacturer's instructions.

Cell depletion and antibody neutralization

For CD4 cell depletion: DSS-treated and untreated mice were injected i.p. with 400μg α-CD4 (clone GK1.5, BioXcell Cat# BE0003-1, RRID:AB_1107636) or IgG isotype control (BE0090, BioXcell Cat# BE0090, RRID:AB_1107780) on days -6, -3 and on the day of infection with *C. difficile* as previously reported (Moynihan et al., 2016). Flow cytometry was used to confirm depletion of CD4-expressing T cells from the colon with 90% efficiency.

For IL-17RA blockade and IL-17A neutralization: DSS-treated mice were injected i.p. with 125μg α-IL-17RA (R&D Systems, Cat# MAB4481, RRID:AB_10891109), 125μg α-IL-17A (R&D Systems, Cat# MAB421, RRID:AB_2125018) or IgG isotype control (R&D Systems, Cat# MAB006, RRID:AB_357349) on days -1, 1 and 2 of *C. difficile* infection.

T cell transfers

For the adoptive transfer of CD4+ T cells, mice were either treated with DSS or regular drinking water. On day 7 after DSS treatment, colons and mesenteric lymph nodes were removed and processed into single-cell suspensions as

described above. Then, CD4+ T cells were isolated using a negative selection CD4+ T cell isolation kit from Miltenyi Biotec (Cat# 130-104-454). The purity of the cells isolated was checked by flow cytometry. $1x10^6$ cells were transferred i.p. into naïve recipients on the day before infection with *C. difficile.*

For the adoptive transfer of Th17 cells, splenocytes were isolated from IL17A-GFP reporter mice and cultured *ex vivo* using a CellXVivo Mouse Th17 Cell Differentiation Kit (R&D Systems, Cat# CDK017) according to the manufacturer's instructions. After differentiation, the cells were stained for CD3e and CD4 expression and viability was determined using 7-AAD Viability Staining Solution (BioLegend, Cat# 420404). IL-17A+ and IL-17A- CD4+ T cells were sorted using an Influx Cell Sorter (BD Biosciences) and $1x10^6$ cells were transferred into naïve recipients on the day before *C. difficile* infection.

16S rRNA Sequencing

Fecal pellets were collected from mice given untreated or DSS-treated water after a two-week recovery period and before antibiotics treatment, on day 21 in **Figure 1A**. Caecal contents were collected from untreated or DSS-treated mice after antibiotics treatment, on day 27 in **Figure 1A**, and diluted 1:2 with sterile PBS. DNA was extracted using Qiagen MagAttract PowerMicrobiome kit DNA/RNA kit (Qiagen, catalog no. 27500-4-EP). The V4 region of the 16S rRNA gene was amplified from each sample using the dual indexing sequencing strategy as described previously (Kozich et al., 2013). Sequencing was done on

the Illumina MiSeq platform, using a MiSeq Reagent Kit V2 500 cycles (Illumina cat# MS102-2003), according to the manufacturer's instructions with modifications found in the Schloss SOP: https://github.com/SchlossLab/MiSeq_WetLab_SOP. The mock community produced ZymoBIOMICS Microbial Community DNA Standard (Zymo Research cat# D6306) was sequenced to monitor sequencing error. The overall error rate was 0.019%.

Sequence Curation and Analysis

Raw sequences were curated using the software package mothur version 1.39.5 (Schloss et al., 2009) following the Illumina MiSeq standard operating procedure. Briefly, paired end reads were assembled into contigs and aligned to the V4 region using the SLIVA 16S rRNA sequence database (Quast et al., 2013), sequences that failed to align or were flagged as possible chimeras were removed. A naïve Bayesian classifier using the Ribosomal Database Project (Wang et al., 2007) classified sequences. Operational Taxonomic Units (OTUs) using a 97% similarity cutoff were generated using the Opticlust clustering algorithm (Westcott et al., 2017).

The number of sequences in each sample was then rarefied to 25,000 sequences to minimize bias due to uneven sampling. Following curation in mothur, further data analysis and figure generation was carried out in R (v 3.4.1) using the package vegan (Oksanen et al, 2018). This includes determining the

axes for the multidimensional scaling (MDS) plots using Bray-Curtis dissimilarity calculated from sequence abundance. Additionally, vegan was used to determine significance between groups using PERMANOVA. The sequences associated with analysis were deposited to the SRA under the bioproject PRJNA475161. Final figures were modified and arranged in Adobe Illustrator CC.

Histology

Caecal tissue was isolated and fixed in Bouin's solution for 24 hours then transferred to 70% ethanol, paraffin embedded and stained with haematoxylin and eosin (H&E). The tissue was sectioned onto slides and scored by two independent, blinded observers. Each sample was given a score of 0-3 for each of the following parameters: epithelial disruption, submucosal edema, inflammatory infiltrate, mucosal thickening and luminal exudates as described previously (Pawlowski et al., 2010).

Quantification and statistical analysis

For mouse data, survival curves were compared using a Log-Rank (Mantel-Cox) statistical test. Comparisons between two groups were assessed using a student t-test or Mann-Whitney test depending on whether the data were normally distributed. Statistical significance between multiple groups was tested using a one-way multiple analysis of variance (ANOVA) or Kruskal-Wallis. For human data, patients were categorized into quartiles based on IL-6 serum levels. Survival curves were compared using a Kaplan-Meyer test, then further

evaluated by a Cox proportional hazards model to adjust for age, sex, race, Charlson comorbidity index and ICU admission. Statistical significance between severe and non-severe patients was assessed using a Mann-Whitney test. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA) and SAS 9.4 (SAS Institute, Cary, NC). Information on the statistical test used, number of subjects “n”, definition of center and dispersion and precision measures are included in each figure legend.

Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Data and software availability

The sequences associated with the 16S microbiota analysis were deposited to the SRA under the bioproject PRJNA475161.

Chapter Three: Conclusions and Future Directions

3.1 Prior colitis puts the host at risk for severe *C. difficile* infection

Given the strong clinical evidence for increased incidence and severity of CDI in IBD patients, we set out to understand the underlying mechanism in a mouse model of infection. When designing the model, we suspected that mice with acute DSS colitis would develop more severe CDI because of the loss of epithelial integrity associated with IBD. This, we hypothesized, would allow for the dissemination of *C. difficile* and its toxins past the epithelial barrier leading to enhanced host tissue damage and increased inflammation compared to mice without active DSS colitis. Therefore, we designed the infection model to include a recovery period to allow for the restoration of the epithelial barrier and the resolution of inflammation before CDI. By facilitating the study of long-term effects colitis might have on susceptibility to a subsequent infection, this model allowed us to study adaptive immune responses to infection and to discover a role for Th17 cells in exacerbating CDI disease severity.

When we asked the question why previously DSS-treated mice develop more severe CDI, we started with the hypothesis that it might be due to differences in innate immune responses. However, after characterizing myeloid cell recruitment (eosinophils, neutrophils and Ly6C monocytes) we found no difference in the numbers of these cells between DSS and untreated mice during infection. This was surprising because of the existing literature that implicates each of these cell types in determining the outcome of infection (Jarchum et al, 2012; Buonomo et al., 2016; Cowardin et al., 2016; McDermott et al., 2017). This

further stresses the fact that many of the existing *C. difficile* models underestimate the contributions of adaptive immunity because they are usually short (2-4 days of severe disease) and done in specific pathogen free (SPF) mice with no prior exposure to pathogens or other gut insults. *C. difficile* patients often have other comorbidities, including IBD, which lead them to the hospital in the first place. Therefore, our longer-term, more complicated model allowed us to study adaptive immunity during CDI, a highly understudied area of disease. Whereas shorter disease models have focused on manipulating innate cells, such as ILCs and neutrophils, to protect against CDI, the findings of this study identify T cells as an important cell population. We found that CD4 T cells, which we later identified as IL-17A-producing Th17 cells, were necessary and sufficient to cause severe disease. Therefore, new therapeutics targeting these cells may prove effective in preventing severe CDI, especially in the context of IBD.

3.2 Th17 cells exacerbate the severity of CDI in DSS mice

Early on in this study, we made the observation of increased CD4 T cells in the colons and mesenteric lymph nodes of mice with prior DSS colitis. To test the necessity of these cells for exacerbation of CDI severity, we designed an experiment where we treated MHCII-/- mice with DSS or regular water, allowed them to recover and then infected them with *C. difficile*. We hypothesized that since these mice have major deficiencies in CD4 T cells, disease severity would be similar between the two groups. However, the DSS-treated MHCII-/- mice had significantly higher post-infection mortality and clinical scores (**Figure 3.1**).

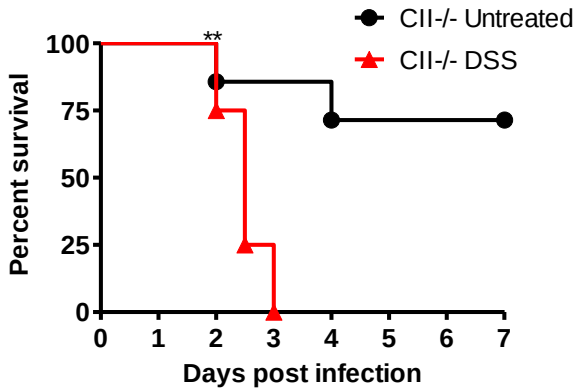
Although this was an unexpected result, we postulate that DSS-treated MHCII-/- mice were impaired in their recovery from DSS colitis. Indeed, there is evidence that the absence of T regulatory cells exacerbates the severity of DSS colitis and causes a prolonged weight loss, disease index, histological damage and colonic inflammation (Boehm et al., 2012; Wang et al., 2015). Where this model might be interesting to study DSS colitis and the role for different CD4 T cells in recovery from colitis, it was not the best model to study the role for these cells in *C. difficile* infection. A more sophisticated approach would be to use diphtheria toxin receptor-expressing CD4 T cells (CD4-DTR) to deplete CD4 T cells after recovery from DSS colitis and before infection with *C. difficile.* Better yet, an inducible knockout of RORγt-expressing cells under the control of the CD4 promotor could be used to selectively deplete Th17 cells before infection and test the necessity of these cells to cause severe disease.

Using a depleting antibody against CD4-expressing cells we showed the necessity and using adoptive transfer experiments we showed the sufficiency of Th17 cells for exacerbation of disease severity. This experiment also suggested no role for ILC3 or CD8+ Tc17 cells in this model since depleting CD4-expressing cells protected DSS mice to the same levels as untreated controls. ILC3s and Tc17 cells do not express CD4, with the exception of a small subset of ILC3s that express CD4 (LTi cells) that are important for lymphoid tissue formation during development. However, because of the described pathogenic role for ILC3s in mouse models of IBD (Buonocore et al., 2010; Powell et al., 2012) we cannot

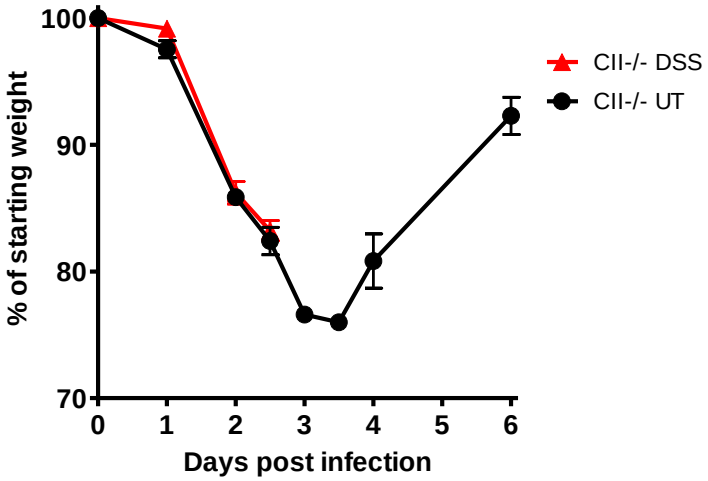
rule out a contribution for these cells in the pathogenesis of CDI in IBD patients. Perhaps the role of these cells during CDI can be interrogated using other models of IBD or by drawing correlations in human studies.

Figure 3.1

A



B



C

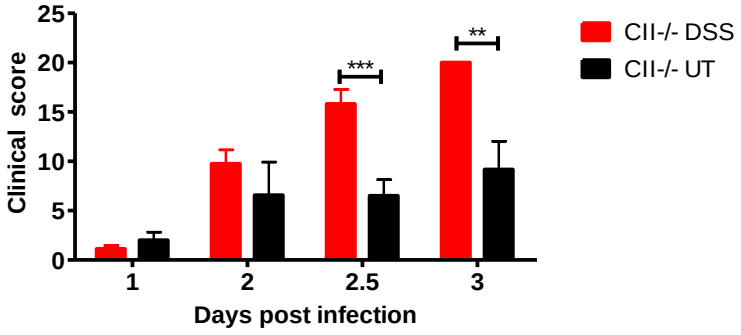


Figure 3.1: MHCII-/- mice treated with DSS develop severe CDI after recovery. MHCII-/- littermates were divided randomly into two groups. One group was treated with 2% DSS for 6 days and the other was given regular water. After a two-week recovery, all mice were infected with *C. difficile*. Survival (**A**), weight loss (**B**) and clinical scores: weight loss, coat appearance, eyes/nose discharge, activity, posture and diarrhea (**C**) of the two groups were assessed twice a day for 7 days (n=7-8 per group). **p<0.01, ***p<0.001 using a Logrank statistical test (**A**) and a student t-test (**C**).

3.3 Remaining questions

The role of T cell memory in severe CDI post DSS treatment

The highlight of our study is that we have shown that colitis induces long-persisting Th17 cells that put the host at risk for severe CDI long after recovery from acute colitis. In our co-housing study, mice remained susceptible to infection (whether housed separately or together with untreated mice) one month after DSS treatment was stopped. It remains to be determined how long-lasting this susceptibility is. To understand this, future studies are needed to determine if T cell memory is involved in increased disease severity in the DSS mice. In mice, several markers are used to identify the different types of memory T cells. Central memory T cells (Tcm) are CD44+ CD62L+ CCR7+ and they can be found in the lymph nodes. Effector memory T cells (Tem) are CD44+ CD62L- CCR7- and they can be found in the circulation and tissue. Gut tissue resident memory T cells are CD69+ CD103+ and can be found in the tissue. (Henao-Tamayo et al., 2010; Carbone et al., 2015; Thome and Farber, 2015). Our study found more CD4 T cells capable of producing IL-17A upon stimulation with PMA/ionomycin in the lymph nodes before infection. The difference in colonic IL-17A+ T cells was only found after infection, suggesting that the persisting memory T cells home to the lymph nodes and might be central memory cells. However, more phenotyping of these cells using the markers mentioned above is required to understand which memory subset they are. In a study of memory T cells in IBD patients, Hegazy et al. (2017) found higher numbers of commensal-

specific Th17 cells in colonic tissue and the circulation of IBD patients compared to non-IBD controls. In this study, the authors found commensal-specific T cells that are central memory cells, effector memory cells as well as cells that express tissue-resident and gut-related markers including CD69. This suggests that multiple types of memory Th17 cells exist in IBD patients and poses the question: which of these cells play a role during a subsequent *C. difficile* infection?

T cell antigen specificity and recognition of commensals

One of the biggest remaining questions this project has led to is the antigen specificity of the Th17 cells. Our leading hypothesis is that these cells are specific to commensal antigen. This is based on the evidence described above that in patients with IBD there is an increase in commensal-specific Th17 cells in colonic tissue as well as the circulation. Interestingly, these cells recognize bacterial species that are present in the healthy gut microbiota, supporting the idea that IBD is caused by aberrant responses to members of the normal microbiota (Hegazy et al., 2017). Similarly, our 16S rRNA analysis showed that after antibiotic treatment, the caecal communities between DSS and untreated groups are not significantly different. This leads us to believe that the Th17 cells driving CDI severity in DSS mice recognize bacterial species present in both DSS mice as well as untreated mice.

In an attempt to test whether exposure to commensal antigen during DSS colitis is required for the Th17 skew that later exacerbates CDI, we designed an experiment where we treated one group of mice with our antibiotic cocktail and

another group with regular water before we gave them DSS. We hypothesized that although antibiotic treatment would not abrogate all commensals; perhaps it would clear some of the Th17-inducing bacterial strains and result in reduced commensal-specific Th17 cells therefore reducing the severity of subsequent CDI. However, giving DSS to antibiotic-treated mice resulted in 40% DSS-induced mortality compared to 0% mortality in mice not given antibiotics before DSS treatment (**Figure 3.2**). This observation is supported by other reports that have described increased severity of DSS colitis in germ-free mice and antibiotic-treated mice (Rakoff-Nahoum et al., 2004; Hernández-Chirlaque et al., 2016). While this poses an interesting question about the role of commensals in protection against DSS colitis, we decided not to pursue this model because the differences in the development of and recovery from DSS colitis would confound any conclusions we try to draw about a subsequent infection with *C. difficile*. Similarly, the use of germ-free mice would make the interpretation of such experiment difficult because they develop hemorrhaging and epithelial injury, which might result in more severe subsequent CDI independently of commensal-specific Th17 cells (Hernández-Chirlaque et al., 2016).

Figure 3.2

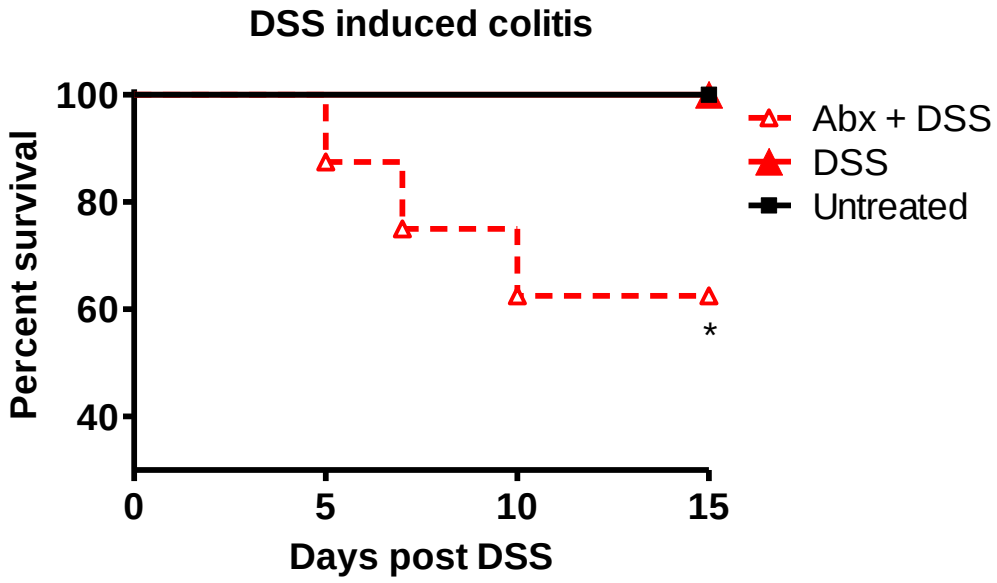

DSS induced colitis
Percent survival
100
80
60
40
0
5
10
15
Days post DSS
*
Abx + DSS
DSS
Untreated

Figure 3.2: Antibiotics before DSS treatment cause severe DSS colitis. The "Abx+DSS" group was treated with the antibiotic regimen described in chapter 2 immediately prior to DSS treatment. Then, the "Abx+DSS" and "DSS" groups were treated with 2% DSS, while the "untreated" group was given regular water. Survival post DSS treatment was monitored daily for 15 days (n=8 per group). *$p<0.05$ using a Logrank statistical test.

Two approaches can be taken to test the commensal-specificity of Th17 cells in the DSS mice. The first and simplest would be to isolate CD4 T cells from the colons and mesenteric lymph nodes of untreated and DSS treated mice after recovery and before *C. difficile* infection. The proliferation of these cells to commensal antigen can be tested by co-culturing them with dendritic cells pulsed with combined fecal extracts from untreated and DSS mice. If the Th17 cells that we have shown persisting in the mesenteric lymph nodes after recovery are commensal-specific, we would expect to see more cell proliferation and IL-17A production of the cells isolated from DSS mice in response to stimulation compared to those isolated from untreated mice.

The second approach would be to use TCR-transgenic mice to test this hypothesis. For example, OT-II transgenic mice with T cell receptors specific for ovalbumin can be treated with DSS or regular water, allowed to recover and then infected with *C. difficile.* In the absence of T cell antigen stimulation, we would expect the DSS mice would not have enhanced Th17 responses, and therefore would not develop more severe CDI. Recently, Harrison et al. (2019) published a study where they used TCR transgenic mice with commensal-specific T cells that recognize *Staphylococcus epidermidis* to study the effects of this skin commensal on Th17 and Tc17-mediated wound healing. In a similar approach, gut commensal-specific transgenic T cells can be used to assess T cell proliferation and IL-17A production during DSS colitis and during *C. difficile* infection in response to stimulation with commensal antigen.

Altogether, while some associations have been made between commensal-specific Th17 responses and the development of IBD, the cause of these aberrant responses remains to be determined (Gradel et al., 2009; Hegazy et al., 2017). In our study, we showed that induction of Th17 cells is detrimental to the host in the context of *C. difficile* infection regardless of antigen specificity. This is supported by the fact that the transfer of polyclonal splenic *in vitro*-differentiated Th17 cells is sufficient to cause severe disease. Understanding which commensals or pathobionts initiate the onset of IBD and put the host at risk for severe CDI is a major question in the IBD field and will continue to be studied for years to come. Identification of such bacterial species in the future might guide therapeutic approaches that specifically target them to prevent the development of IBD in genetically susceptible hosts.

The upstream mechanism required for induction of Th17 cells

While we understand the role of Th17 cells in enhancing disease severity, our understanding of the upstream signals leading to the Th17 skew remains incomplete. We have shown that DSS treatment induces IL-6 and IL-23 expression; two cytokines implicated in the differentiation and maintenance of pathogenic IL-17A-producing T cells. IL-6 is mainly produced by macrophages and monocytes while IL-23 is made by antigen presenting cells, including dendritic cells and epithelial cells (Hunter, 2005; Gabay, 2006; Macho-Fernandez et al., 2015). We have shown that enhanced production of IL-23 persists after recovery from DSS colitis and we believe this contributes to the maintenance of

the Th17 skew in the DSS mice. To better understand whether the source of IL-23 is epithelial or from bone marrow-derived immune cells, we treated mice with regular water or with DSS. We isolated bone marrow cells from untreated mice, mice with acute DSS and mice that have recovered from DSS colitis. We differentiated the isolated bone marrow cells into bone marrow-derived dendritic cells (BMDCs) and stimulated them with *C. difficile* culture supernatant. We measured IL-23 production by the BMDCs isolated from the three groups of mice and found that BMDCs from mice with acute DSS colitis and those from mice that recovered from DSS colitis both produce significantly higher levels of IL-23 (**Figure 3.3**). This is interesting because it suggests that DSS treatment might cause long-lived changes in the bone marrow that lead to the persistent skew to type 3 immunity. In other studies, our lab has reported that Segmented Filamentous Bacteria (SFB) colonization leads to increased IL-23 production by BMDCs and that these BMDCs alone provide protection from *Entamoeba histolytica* infection via an IL-17A and neutrophil-dependent mechanism (Burgess et al, 2014). Later, we showed that SFB-mediated protection from *E. histolytica* infection correlated with an upregulation of serum amyloid A (SAA), an induction of the H3K27 histone demethylase Jmjd3, and an expansion in granulocyte monocyte precursors (GMPs) (Burgess et al., 2016). This study showed that altering the gut by SFB colonization led to epigenetic changes in the bone marrow that affected susceptibility to a subsequent infection. Interestingly, we also found elevated SAA levels in the serum of acute DSS mice but not after recovery (**Figure 3.4**). Given the acute induction of SAA and the fact that BMDCs

derived from mice after recovery from DSS colitis continued to produce more IL-23, it is plausible that bone marrow precursors had been epigenetically altered. This can be directly tested by bone-marrow transplant experiments. Further experimentation is also needed to determine the cellular sources of IL-6 and IL-23, whether they are important for DSS-mediated exacerbation of CDI severity and whether neutralization of either cytokine at different time points during this model can prevent severe disease.

Figure 3.3

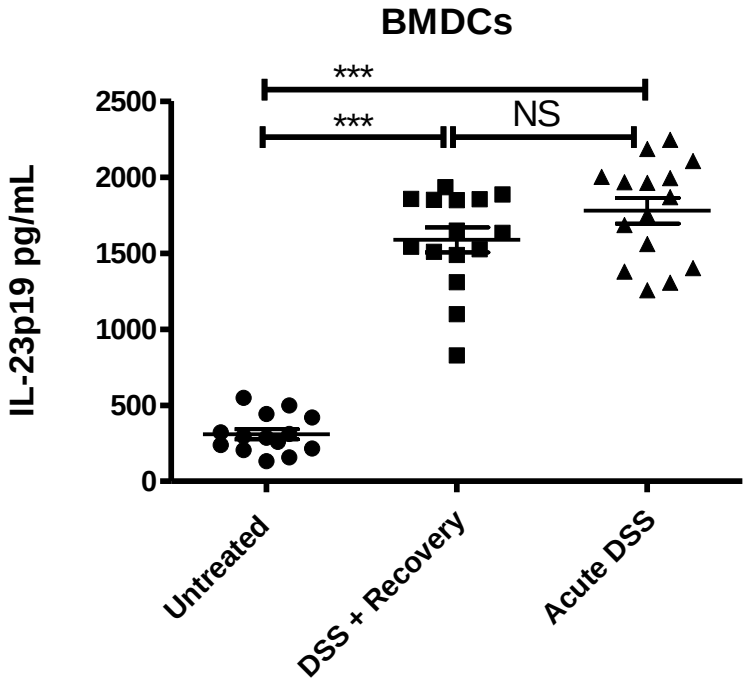

BMDCs
***
***
NS
2500
2000
1500
1000
500
0
IL-23p19 pg/mL
Untreated
DSS + Recovery
Acute DSS

Figure 3.3: Bone marrow-derived dendritic cells (BMDCs) from DSS treated mice produce more IL-23 even after recovery from DSS colitis. 6 week old C57Bl/6J mice were treated with 2.5% DSS and allowed to recover for 3 weeks. A second group was treated with 2.5% DSS without recovery. A third group was only given regular water. The bone marrow was harvested from all three groups and BMDCs were generated as previously described and stimulated using *C. difficile* culture filtrate (Cowardin et al., 2015). IL-23 protein levels were measured using an R&D ELISA kit according to the manufacturer's instructions. (n= 15 per group) ***$p<0.001$ using a one-way ANOVA.

Figure 3.4

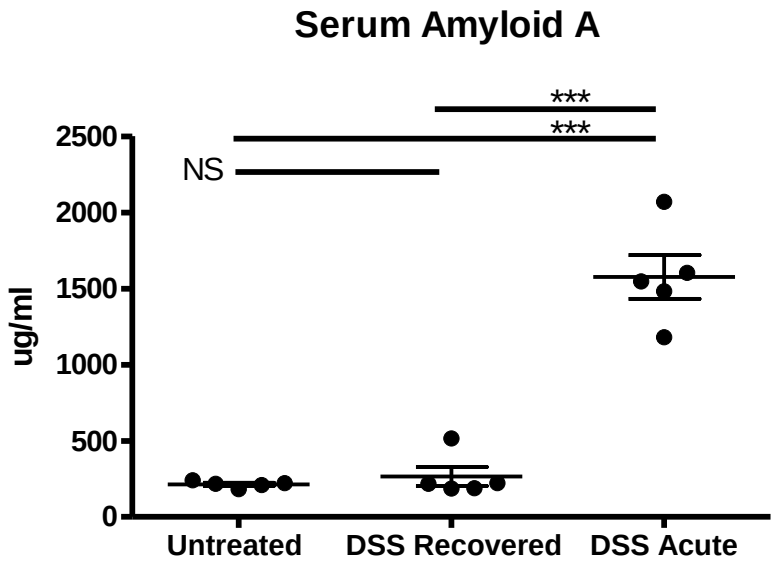

Serum Amyloid A
***
***
NS
2500
2000
1500
1000
500
0
ug/ml
Untreated
DSS Recovered
DSS Acute

Figure 3.4: DSS treatment causes acute induction of serum amyloid A (SAA). 6 week old C57Bl/6J mice were treated with 2.5% DSS and allowed to recover for 3 weeks. A second group was treated with 2.5% DSS without recovery. A third group was only given regular water. All three groups were harvested on the same day and the amount of SAA in the serum was measured using an Abcam (ab157723) ELISA kit according to the manufacturer's instructions. (n= 5 per group) ***$p<0.001$ using a one-way ANOVA.

The mechanisms downstream of IL-17 signaling

Future studies can also focus on delineating the downstream mechanisms of Th17-induced severity of disease. By inhibiting IL-17RA and IL-17A we have shown that IL-17 signaling is important in the early exacerbation of disease severity starting on day 2. However, the downstream signals remain unknown. IL-17 is a known inducer of IL-6, IL-1β and TNF, which constitute a positive feedback loop leading to enhanced IL-17 production. IL-17 also induces GM-CSF and G-CSF, which promote the expansion and survival of polymorphonuclear cells. Furthermore, IL-17 leads to the upregulation of CCL2, CCL7, CXCL1, CXCL2, CXCL5 and CXCL8 which are chemoattractants for neutrophils and monocytes. This, combined with the ability to induce several matrix metalloproteinases and vasodilation, allows for the recruitment of neutrophils and monocytes to the infection site (Veldhoen, 2017; Sud et al., 2018). Neutrophil recruitment, combined with production of antimicrobial proteins and IgA downstream of IL-17, play important roles in protection from infections. This beneficial role for the IL-17 pathway might explain why blocking it in our model did not have long-lasting protection from CDI. Perhaps complete blockade of IL-17 results in impaired clearance of *C. difficile* or other translocating pathobionts leading to host mortality later during infection.

Because of the lack of specificity of neutrophils to pathogens, they can have detrimental off-target effects on the host. Interestingly, however, when we measured neutrophil numbers during infection we found no difference between

DSS and untreated mice. There is evidence of heterogeneity in the neutrophil population in humans. Some of these neutrophil types include immature and mature neutrophils; immunosuppressive low density neutrophils (LDNs), also known as granulocytic-myeloid derived suppressor cells (G-MDSCs); proinflammatory LDNs, also known as low density granulocytes (LDGs) and proinflammatory and immunosuppressive normal density neutrophils (NDNs) (Ley et al., 2018). Immunosuppressive LDNs have been found in patients with solid tumors, HIV, sepsis, graft-versus-host disease and trauma. These cells can suppress T cell responses and cytokine production through arginase 1 and reactive oxygen species (ROS) production. Proinflammatory LDGs have been isolated from patients with autoimmune diseases such as lupus erythematosus and rheumatoid arthritis. These cells show enhanced production of the inflammatory cytokines IL-17A, IFNα, IFNγ and TNFα as well as neutrophil extracellular traps (NETs). Finally, immunosuppressive NDNs have been observed in HIV patients and can inhibit T cell function through PD-L1/PD-1 interaction and ROS production (Scapini et al., 2016). The lack of specific markers for each of these cells makes it difficult to determine whether they are distinct subsets and more work is needed to describe their development. In our model, future studies can describe functional differences in the neutrophils present at the site of infection in DSS mice compared to untreated mice. This can include measurement of myeloperoxidase, ROS or NET formation. Studies can also include RNA sequencing techniques of isolated neutrophils to describe

differences in the phenotype and function that might exist between the two groups of mice.

Growing evidence supports a role for IL-17A in the pathogenesis of sepsis. In a murine sepsis model, Li et al. (2012) found that sepsis induces IL-17A locally in the peritoneum as well as systemically in the blood. Sepsis-induced mortality was reduced after antibody neutralization of IL-17A and was associated with reduced neutrophilia in the lung as well as reduced lung injury. Similarly, in a genome-wide analysis of blood mRNA of septic human neonates, Wynn et al. (2016) found that the IL-17RA network was the most critical regulatory network dysregulated in septic neonates. The authors also found elevated IL-17A levels in the gut and lung of septic neonatal mice and neutralization of IL-17A, as well as a genetic knockout, protected from sepsis-induced mortality. Finally, in an *in vitro* study Atefi et al. (2011) showed that cardiomyocytes from septic rats produced more cardiosuppressive inflammatory cytokines and this was reduced by neutralizing IL-17A. The authors suggest that IL-17A signaling might be involved in sepsis-associated cardiac failure.

Currently, we do not know whether sepsis contributes to mortality in DSS mice after CDI. Future studies can determine whether IL-17A is induced systemically post infection, whether it causes increased neutrophilia and tissue damage in the lung, and whether it contributes to sepsis-induced cardiac failure. All of these studies will help identify systemic effects the Th17 skew in DSS mice might have that contribute to mortality away from the site of *C. difficile* infection.

Whether CDI occurs during an IBD flare or during remission

We later decided to test whether DSS mice develop more severe disease without a recovery period. Interestingly, we found that mice with acute DSS developed less severe disease than untreated controls when infected with *C. difficile* during active DSS colitis (**Figure 3.5**). Whether DSS mice were given the antibiotic cocktail before infection or not, they had significantly less CDI-associated mortality compared to untreated mice. This result was surprising to us, but several hypotheses could explain this phenotype. First, DSS colitis is associated with hyperinflammation, as we showed by characterizing inflammatory cytokines during acute colitis, and an influx of neutrophils into the colon (Ranganathan et al., 2013). This inflammation and neutrophil recruitment could have provided protection against *C. difficile* colonization since neutrophils have been shown to be important in bacterial clearance (Jarchum et al., 2012). Another hypothesis is that DSS induces changes in the gut microbiota. Although we characterized the composition of the gut microbiota in DSS and untreated mice after recovery and after antibiotic treatment, we did not characterize it during acute DSS colitis. The differences between the two groups during acute colitis might explain why a DSS microbiota is protective against colonization by *C. difficile*.

Later on, a study by Zhou et al. (2018) described increased susceptibility of mice with acute DSS to CDI in a slightly different model of infection. This model differs in the dose of DSS given, the strain of *C. difficile,* the infection with

spores and likely the mouse microbiota. All of these variables likely affect the course and outcome of CDI. Zhou and colleagues did not delineate the mechanism behind their phenotype. There is evidence that IBD patients have shorter time-to-CDI diagnosis times compared to non-IBD patients (Rodemann et al., 2007). The authors found that the majority of IBD patients are diagnosed with *C. difficile* in the first 24 of hospitalization; a time period too short for a hospital-acquired infection. This supports the idea that IBD patients may acquire CDI during remission, which subsequently induces an IBD flare. Our mouse data also supports this notion of susceptibility after the resolution of acute inflammation. However, this remains to be an unanswered question in IBD the field and future studies are needed to investigate susceptibility to infection during acute colitis.

A summary of the remaining open questions is described in **Figure 3.6** and highlighted in red font.

Figure 3.5

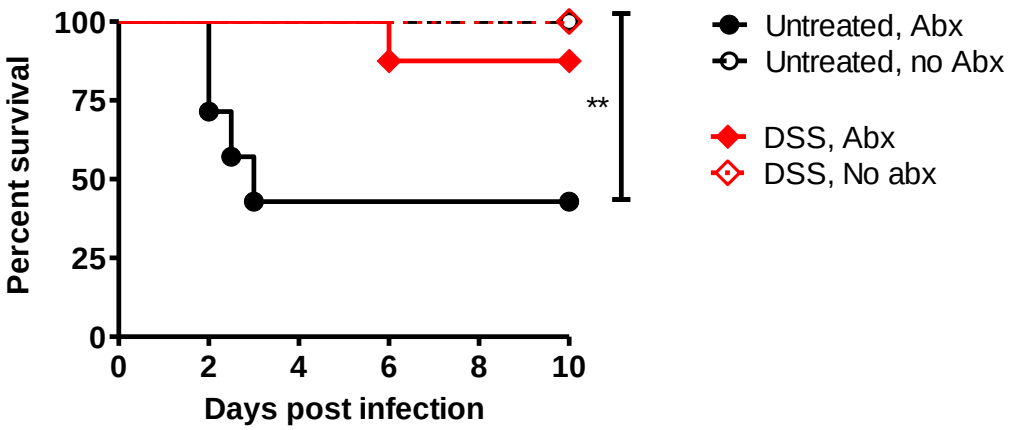
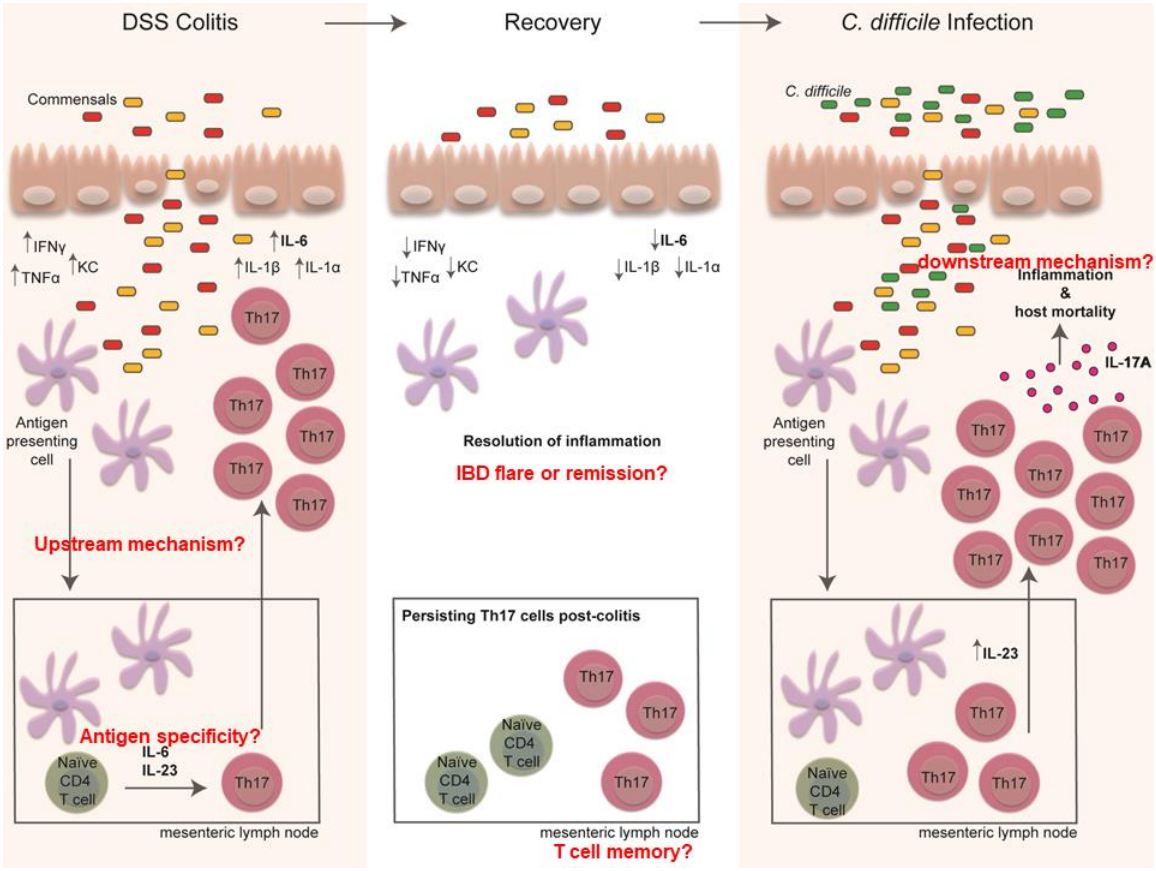

Percent survival
100
75
50
25
0
0
2
4
6
8
10
Days post infection
**
Untreated, Abx
Untreated, no Abx
DSS, Abx
DSS, No abx

Figure 3.5: mice with acute DSS are not susceptible to CDI. For the antibiotics groups (Abx): 6-9 week old C57Bl/6J mice were treated with 1.5% DSS or no treatment in the drinking water for 5 days. On day 6, both groups were treated with a cocktail of antibiotics as described in Chapter 2 and then infected with 1.35 x10^5 CFU of *C. difficile* strain R20291. For the other two groups (no Abx), mice were treated with 1.5% DSS or no treatment in the drinking water for 5 days and then immediately infected with 1.35 x10^5 CFU of *C. difficile* on the same day as the Abx mice. Survival was assessed twice daily for 10 days. n=7-8 per group. **$p<0.01$ using a Logrank statistical test.

Figure 3.6: remaining questions



3.4 Impact

Through this work, we were the first to directly show that Th17 cells can determine the outcome of *C. difficile* infection. The importance of this work to the field is highlighted by the fact that only a handful of studies have evaluated the role of CD4 T cells during infection. As described in Chapter 1, some studies described a correlation between CD4 T cells and protection against *C. difficile* infection in the context of HIV and recurrent CDI. One study correlated severe disease with a higher number of Th17 cells in the colon in the context of treatment with nonsteroidal anti-inflammatory drugs. Through depletion and adoptive transfer experiments, we were the first to show the necessity and sufficiency of these cells in determining the severity of CDI.

Our findings are particularly important in the context of IBD. There is concrete evidence that IBD patients have more microbiota-specific Th17 cells locally in the colon and systemically compared to healthy controls. These patients also have elevated IL-6 and IL-23 levels (Hegazy et al., 2017). We have described that the Th17 pathway, which has long been associated with the pathogenesis of IBD, also plays an important role during CDI. The elucidation of this mechanism using a mouse model underscores the importance of targeting this pathway not only for treatment of IBD, but also for protection against severe subsequent CDI.

C. difficile infections present a major health problem in the IBD patient population. In both Crohn's disease and ulcerative colitis patients, *C. difficile*

infections explained more than 80% of the increase in infection-associated IBD hospitalizations in the United States between years 1998 and 2014 (Barber et al., 2018). IBD patients with CDI had higher mortality, hospital charges and length of stay than those without CDI (Barber et al., 2018). In addition to CDI, other infections have been associated with IBD including *Salmonella* and *E. coli* infections (Gradel et al., 2009; Hegazy et al., 2017; Barber et al., 2018). Interestingly, suppressing T cell responses during *Salmonella typhimurium* infection protects the host from severe disease by preventing the downstream neutrophil recruitment and off-target tissue pathology (Godinez, 2008). This suggests that our observation that IBD-associated Th17 cells play a pathogenic role during CDI might also extend to susceptibility to other gastrointestinal infections.

It is important to note that the correlation we found between serum IL-6 and IL-23 and severe CDI was done outside the context of IBD due to a limited number of serum samples from IBD patients. However, this suggests that Th17 cytokines, and perhaps Th17 cells, are generally important during CDI irrespective of IBD status. These cytokines can then be used as biomarkers for severe disease and thus may be considered in a healthcare provider's decision on the best treatment plan for each patient. If patients with high IL-6 and/or IL-23 are considered "high-risk" for developing complications, these patients can be admitted to the hospital or the intensive care unit to prevent the development of severe disease.

Finally, the impact of this work might also extend to studying the role of Th17 cells during recurrent infection. Our lab and others have shown increased Th17 cytokine levels, including IL-23 and IL-6, in colonic tissue and circulation of CDI patients compared to controls (Buonomo et al., 2013; Rao et al., 2013; Yu et al., 2017). If these signals lead to enhanced Th17 differentiation and result in Th17 memory cells, they may contribute to the severity of a recurrent infection. Currently, no studies have been done to evaluate the presence or role of Th17 cells in recurrent CDI.

C. difficile infections are a major health threat in many countries, including the United States. Despite efforts to reduce infection rates, the incidence of *C. difficile* cases continues to rise. Most studies have focused on *C. difficile* sporulation and toxin production and antibiotic therapy is the standard of care for this infection. Although these antibiotics are successful at clearing the bacteria, many patients succumb to the infection due to disease complications. Substantial evidence suggests that the host's own immune response is a factor in determining the outcome of disease (Feghaly et al., 2013 a-b; Kulaylat et al., 2018). Therefore, a deeper immunological understanding is needed to design therapies that prevent severe disease and reduce patient mortality. Our study identifies a novel role for Th17 cells in worsening the outcome of this infection in hosts with prior colitis. This brings us one step closer to understanding and targeting immunological pathways to prevent severe disease in highly susceptible patient populations.

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