Modulation of immune responses dictate the severity of *Clostridium difficile* infection

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Thesis Abstract

Clostridium difficile infection (CDI) occurs when the microbiome is disrupted, most often through the use of antibiotics. Previous studies have demonstrated that increased inflammatory markers, such as IL-8, are better at predicting poor patient outcome than enhanced bacterial burden. This finding, coupled with the neutrophilia observed in CDI patients, supports the hypothesis that the severity of disease may be correlated with intensity of host response. Thus, we aim to elucidate mediators in the immune response, which may regulate a healthy immune response. We have established a pathogenic role for IL-23 in CDI through the observation that mortality is decreased in two distinct mouse models where IL-23 signaling is absent. The absence of IL-23 resulted in decreased expression of downstream type 17 cytokines IL-22, IL-17A and neutrophilia. The role of these mediators remains to be examined, yet mice lacking IL-23 signaling had enhanced integrity of the epithelial barrier suggesting IL-23 drives tissue pathology.

Moving forward, our goal was to determine immune mediators that regulate beneficial inflammation during CDI. Previous studies have demonstrated an inverse relationship in the signaling of cytokines IL-23 and IL-25. Additionally, IL-25 expression is decreased in antibiotic treated and germ free mice. The ability of *C. difficile* to cause active infection after antibiotic treatment stresses the beneficial role of commensal bacteria. Despite this knowledge, the role of the microbiota to modulate disease severity by influencing the host immune response has yet to be explored. As a result, we hypothesized that IL-25 was

protective during infection. Here, we show that IL-25 production, a cytokine regulated by the microbiota, is suppressed during CDI in humans and in a mouse model. Administration of IL-25 protein protected mice from CDI-associated mortality without affecting the amount of *C. difficile* bacteria or toxin in the gut. We identified eosinophils as the cellular mechanism by which IL-25 protects using two distinct mouse models lacking eosinophils. In both instances, IL-25 was unable to reduce mortality or morbidity or prevent gut barrier disruption in the absence of eosinophils. Thus, our study provides a novel mechanism by which induction of innate immune responses, specifically microbiota-regulated IL-25 actions on eosinophils, can reduce the severity of CDI and protect the gut barrier.

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Chapter One: Introduction to *Clostridium difficile* and host responses

Part of this chapter has been adapted from "The microbiota and immune responses during *Clostridium difficile* infections"
Submitted at Anaerobe

1.1 *Clostridium difficile* epidemiology

Clostridium difficile is a spore forming, Gram-positive, toxin-producing anaerobe that causes diarrhea and colitis when the natural gut flora has been disrupted, primarily through use of antibiotics. It is currently the leading cause of nosocomial infection in the United States, surpassing methicillin resistant *Staphylococcus aureus* (MRSA) [1–3]. The Centers for Disease Control and Prevention (CDC) lists *C. difficile* as one of three urgent threats in the United States and it is estimated to cause approximately 453,000 infections per year with 29,300 related deaths [4]. Moreover, a 30-day mortality rate has been observed in up to 15% of *C.difficile* patients [5]. Disease can range from asymptomatic colonization to mild diarrhea, pseudomembranous colitis, and life threatening toxic megacolon. Treatment for *Clostridium difficile* infection (CDI) costs the US health care system an estimated $4.8 billion annually in acute health settings alone, with an additional substantial burden seen in long-term care facilities [6]. Despite therapy, recurrent disease is seen in 10-35% of patients after initial infection and in secondary relapses are found in 35-65% of patients after primary reoccurrence [7–9]. Risk factors include antibiotic exposure, acute or long term care facility exposure, advanced age, comorbidities such as inflammatory bowel disease, and use of proton pump inhibitors [6,10]. The prevalence of *C. difficile* cases have been increasing annually in both health care and community settings, and hypervirulent strains of *C. difficile*, most

notably BI/NAP1/Ribotype 027 strains, are also becoming more common [11]. Additionally, the ability of *C. difficile* spores to survive in harsh conditions, including resistance to alcohol-based cleaners, contributes to disease transmissibility. In the past ten years, there has been a five-fold rise in disease incidence in the North American population, emphasizing the need for better treatment and management strategies [12,13].

1.2. Pathogenesis of CDI

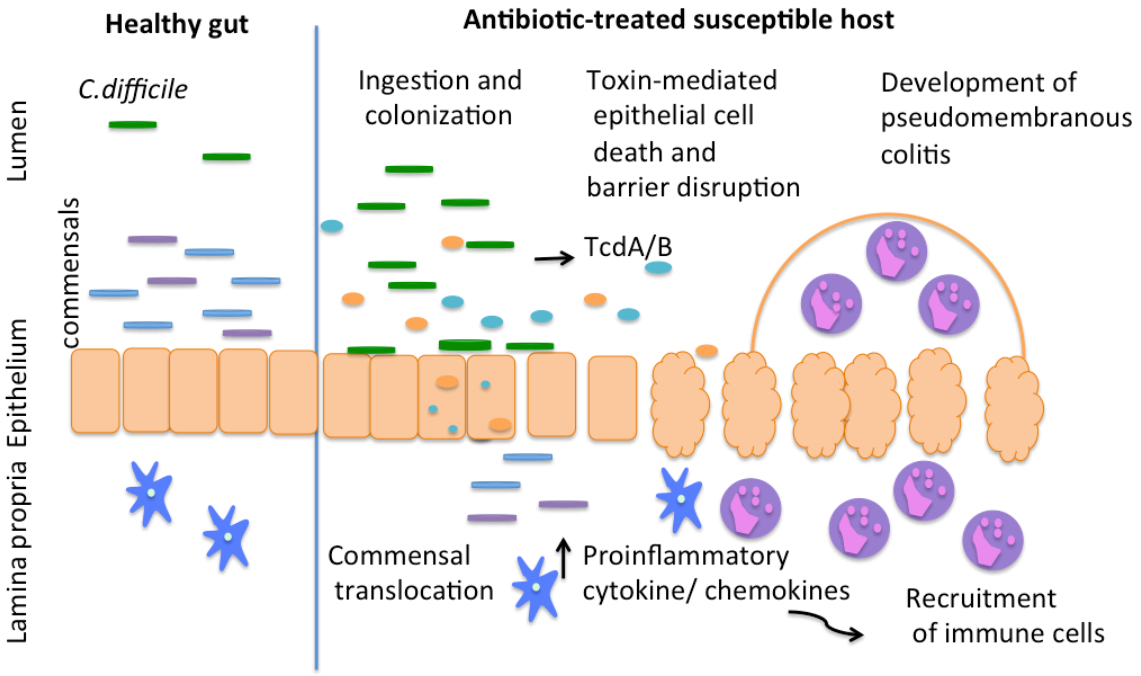
Disruption of the host's endogenous microbiota, a state called dysbiosis, provides an ideal environment for CDI to occur. Several components of a healthy microbiota contribute to preventing host susceptibility to infection, outlining the importance of commensal bacteria to combat *C. difficile*. Bacterial spores are transmitted through the fecal-oral route and germinate into vegetative cells in the intestine of susceptible hosts. These cells infiltrate the mucus layer surrounding the epithelial cell layer and adhere to its surface [14]. Once adhered, the bacteria produce toxins that mediate a robust inflammatory response. Toxin A (TcdA) and toxin B (TcdB) are the primary virulence factors of *C. difficile* and are released during the late log phase and stationary phase of vegetative growth [15]. TcdA and TcdB are able to glucosylate and inactivate Rho and Ras family small GTPases causing disruption of the actin cytoskeleton, cell rounding, inhibition of cell division and cell death [16,17]. This process is especially harmful to the integrity of the epithelial barrier. The breakdown of the epithelium causes permeability of the barrier and allows for both pathogenic and commensal

bacteria to translocate into the lamina propria. Collectively, these actions induce the release of proinflammatory mediators from epithelial and immune cells in the lamina propria that subsequently recruit additional immune effector cells to the site of infection [16,18–20]. Neutrophils are the hallmark cell subset recruited to the intestinal barrier during infection and are found in pseudomembranous lesions during severe disease. However, the role of the immune response during infection remains incompletely understood as there is evidence to support both protective and pathogenic roles during CDI. This combination of this observation with the knowledge that a healthy microbiota prevents infection demonstrates the importance of both commensal bacteria and the host inflammatory response during CDI.

1.3. The role of the microbiota during *Clostridium difficile* infection

Antibiotic exposure remains the leading risk factor of disease, stressing the beneficial role of the microbiota in host protection [5]. Disruption of a ‘healthy’ microbiota or the reduction of its diversity is directly linked to host susceptibility to CDI. The microbiota of patients in the hospital are commonly in a dysbiotic state due to increased incidence of antibiotic treatment, modulation of diet, and other treatments such as chemotherapy. Dysbiosis coupled with enhanced exposure to resistant *C. difficile* spores in the hospitals may explain why the majority of CDI cases are associated with health care facilities. The loss of disease resistance

Figure 1.1: The pathogenesis of *Clostridium difficile* infection. *C. difficile* infects the gut when the healthy microbiota has been disrupted. Once dysbiosis occurs, *C. difficile* adheres to the epithelial layer and secretes toxins A and B, its main virulence factors. The action of toxins results in cell death and breaks in the epithelial barrier. This allows for translocation of toxins, pathogenic, and commensal bacteria into the lamina propria which results in the activation of a proinflammatory cascade and culminates in neutrophilia and colitis.


Healthy gut
Antibiotic-treated susceptible host
C.difficile
Lumen
commensals
Epithelium
Lamina propria
Ingestion and colonization
Toxin-mediated epithelial cell death and barrier disruption
Development of pseudomembranous colitis
TcdA/B
Commensal translocation
Proinflammatory cytokine/ chemokines
Recruitment of immune cells

associated with alterations of the endogenous flora is an important initial step in the pathogenesis of CDI (Figure 1). The necessity of antibiotic pretreatment to render mice susceptible to CDI has since been established in mouse models [21]. The microbiota has been shown to protect against infection through a process called colonization resistance, which involves outcompeting the pathogen for space and nutrients in the intestine [22]. Wilson and colleagues originally described colonization resistance by demonstrating that the transfer of cecal contents from an untreated hamster to a vancomycin-treated hamster effectively prevented susceptibility to CDI [23]. It was later shown that bacteria with similar nutrient and spatial demands are capable of excluding *C. difficile.* In fact, a series of experiments demonstrated that pre-infection with a non-toxigenic strain of *C. difficile* was capable of successfully protecting hamsters from subsequent infection with a toxigenic *C. difficile* strain [24]. Although, the immune response to non-toxigenic *C. difficile* was not examined as a potential mechanism of disease prevention, the authors conclude that similar niche requirements utilized by non-toxigenic *C. difficile* results in protection from CDI [24]. Furthermore, recent studies have identified that alterations in the microbiota in response to antibiotic treatment induced spikes in succinate and sialic acid which are then exploited by *C. difficile* to facilitate its expansion in the gut [25,26]. Together, this data support a role for the microbiota to outcompete *C. difficile* resulting in inhibition of infection.

In addition to colonization resistance through competition for space and nutrients, the microbiota has also been observed to regulate bile salts and inhibit *C. difficile* outgrowth by blocking the germination of spores into the vegetative cells that cause disease. The primary bile salt taurocholate was identified as an *in vivo* germinant of *C. difficile* spores in the small intestine [27]. It was later shown that derivatives of cholate activate spore germination when combined with glycine, whereas derivatives of chenodeoxycholate suppress germination, supporting a role for bile salts in regulating the outgrowth of *C. difficile* [28,29]. Interestingly, antibiotic treatment and subsequent changes in the endogenous microbiota lead to increased taurocholate in the cecum and reduced levels of the inhibitory secondary bile salt deoxycholate, which is toxic to vegetative cells [30]. Thus, antibiotic treatment supports the outgrowth of *C. difficile* by inducing bile salts that enhance germination and reducing bile salts that suppress the expansion of vegetative cells. In fact, transfer of bacteria from the cecal contents of antibiotic treated mice supported expansion of *C. difficile in vivo*, while transfer of contents from untreated mice increased host resistance to infection [31]. Buffie et al. associated protection observed in mice receiving cecal contents from untreated mice with the presence of enhanced secondary bile acids [31]. The elevation of secondary bile acids in protected mice could be achieved by transferring a cocktail of four specific bacteria, with a primary role for bacterium *Clostridium scindens* [31]. This study supports the hypothesis that specific components of the microbiota have the ability to protect against CDI.

In addition to its well-defined role in preventing host susceptibility, there is emerging evidence for the ability of the microbiota to resolve active CDI. Fecal microbiota transplant (FMT) involves the transfer of 'healthy' donor stool to *C. difficile* infected patients with the goal of restoring bacterial diversity in the colon and expelling *C. difficile*. Studies in mice are now beginning to explore the ability of a transferred microbiota to clear CDI from mice and prevent relapsing disease. In fact, the transfer of six phylogenetically diverse intestinal bacteria was sufficient to clear CDI in these mice [32]. Moreover, Tvede and Rask-Madsen successfully performed FMT in six CDI patients in 1989, although the treatment had not received mainstream attention until recent years [33]. A recent study from the New England Journal of Medicine observed an approximate 90% cure rate in relapsing CDI patients [34], indicating that the microbiota has a beneficial role to play at both the resistance and resolution stages of disease. Furthermore, restoration of microbiota through FMT has shown more promise in preventing disease reoccurrence then vancomycin treatment [34]. This may be due to the ability of vancomycin to target beneficial members of the microbiota in addition to *C. difficile* and prevent the recovery of a healthy microbiota and patient recovery.

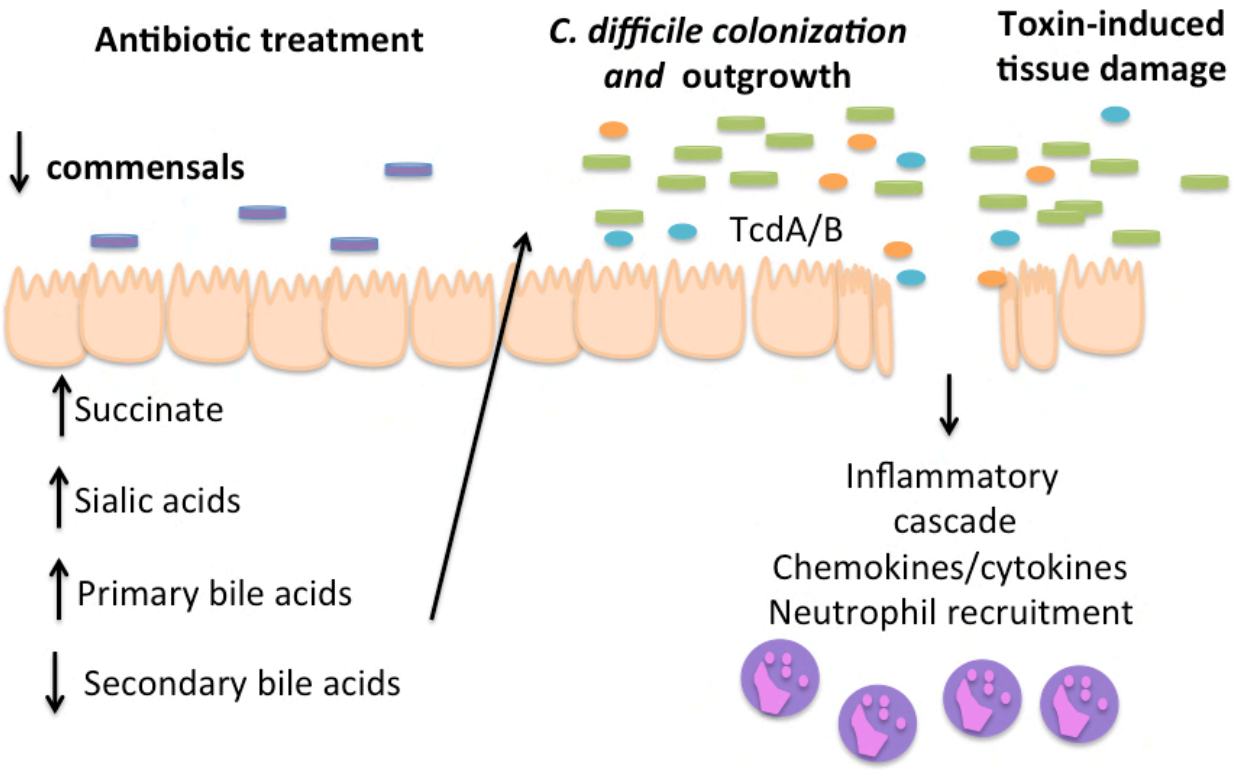
1.4. The dual role of the immune response during *Clostridium difficile* infection

The role of the immune response during *C. difficile* infection remains controversial as there is evidence to support protective and pathogenic phenotypes during disease. In animal models of *C. difficile* infection, the absence

of an intact immune response is disadvantageous to the host. The importance of neutrophils is supported by evidence that mice infected with *C. difficile* that lack the capability to recruit neutrophils to the gut suffered from enhanced mortality compared to controls. [35,36]. In fact, $TLR4^{-/-}$ and $MyD88^{-/-}$ mice experienced enhanced morbidity, likely through the observed decrease in MyD88-dependent neutrophil recruitment to the gut [35–38]. Likewise, leptin signaling, a hormone that is associated with innate and adaptive immune regulation, enhances *C. difficile* clearance and is associated with increased inflammatory mediators during CDI in mice [39]. In addition, innate lymphoid cells (ILCs), primarily group 1 ILCs, have been recognized as critical cell type involved in resolving infection by producing IFN-γ, a type 1 proinflammatory cytokine, to reduce CDI-associated mortality [40,41].The activation of IL-22, a type 17 proinflammatory cytokine that also has tissue repair capabilities, has also been shown to enhance survival during CDI mice. IL-22 functioned to enhance complement-mediated bacterial phagocytosis, which targeted pathobionts to limit the detrimental systemic translocation of these bacteria [42]. The culmination of these data support the hypothesis that an intact inflammatory response is beneficial to combat bacterial burden and resolve the infection.

A hallmark of *C. difficile* infection is the trafficking of neutrophils to the site of infection. While the recruitment of neutrophils appears to be essential to combat the pathogen and resolve infection, it has been postulated that these cells may also exacerbate disease severity through off-target influences that lead

Figure 1.2: A healthy microbiota is important to inhibit host susceptibility to *Clostridium difficile* infection. Antibiotic treatment reduces microbial abundance and diversity which results in enhanced nutrients and regulation of primary and secondary bile acids that favor *C. difficile* outgrowth. Once established in the colon, *C. difficile* releases toxins that lead to cell death, disruption to the epithelial barrier, and induction of proinflammatory responses.


Antibiotic treatment
C. difficile colonization and outgrowth
Toxin-induced tissue damage
commensals
TcdA/B
Succinate
Sialic acids
Primary bile acids
Secondary bile acids
Inflammatory cascade
Chemokines/cytokines
Neutrophil recruitment

to tissue damage and the formation of pseudomembranous colitis. In support of this hypothesis, leukocytosis, an indicator of systemic inflammatory response, correlates to increased disease severity and a negative prognosis in hospitalized patients with diarrhea [43]. Additionally, recent studies have indicated that increases observed in inflammatory markers are a more accurate way to predict poor patient outcome than increased *C. difficile* bacterial burden. For instance, a slower recovery rate in adults and children was associated with higher levels of the intestinal mediators CXCL5 and IL-8, which both function to recruit neutrophils to the gut. Interestingly, the burden of *C. difficile* bacteria in the gut did not correlate with delayed patient recovery [44,45]. Furthermore, a polymorphism resulting in increased expression of the IL-8 gene was associated with enhanced recurrence of CDI, highlighting the potential role for overabundant inflammation as a mechanism of severe infection. [46]. Evidence that induction of certain immune mediators may enhance disease severity was directly demonstrated in our work in a mouse model of CDI investigating the role of IL-23, a cytokine with potent neutrophil recruiting capabilities. In this study, mice were significantly protected from CDI-associated mortality and morbidity in two distinct models which lacked IL-23 signaling [47]. Moreover, *in vitro* studies observed an increase in IL-23p19 mRNA and protein expression from cells stimulated by two individual strains of *C. difficile* [48]. Together, these studies indicate that *C. difficile* induced IL-23 signaling may tip the immune balance towards a robust proinflammatory environment that results in enhanced intestinal damage and pathology during CDI. In addition to data derived from human and mouse

infection studies, the negative role of the immune response is most evident in mouse models of intoxication with purified *C. difficile* virulence factors TcdA and TcdB. Using these models researchers can specifically investigate the influence of *C. difficile* virulence factors on host tissue *in vivo*. Intoxication with TcdA in IFNγ- deficient mice displayed dampened inflammation and less severe pathology compared to control mice [49]. Likewise, TcdA/B intoxication in mice treated with an IL-1R antagonist had reduced inflammation and exhibited a similar protective phenotype [50]. Together, there is evidence to support a deleterious role for certain immune mediators in both models of intoxication and infection.

The role of the immune response during CDI is likely multifaceted, as there is evidence that inflammation can be both protective and pathogenic (Figure 2). Thus, maintaining a balanced inflammatory response to combat infection while limiting tissue damage is likely beneficial during CDI. The varying roles for inflammation in providing protection or increasing disease severity indicate that the host response in part regulates disease outcome and supports the notion that the severity of disease suffered by the patient hinges on intensity or the type of immune response elicited.

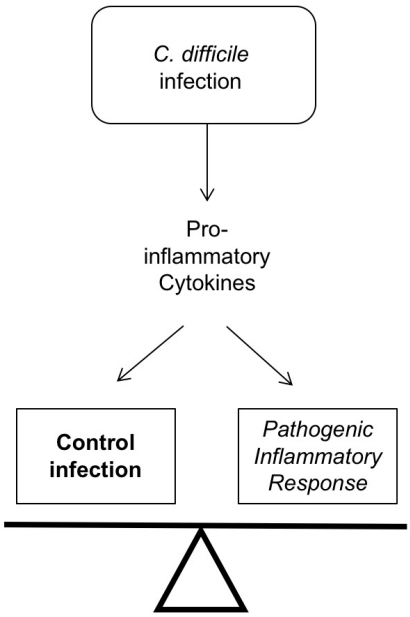
1.5 Interleukin-23 (IL-23)

IL-23 is comprised of two subunits, p19 and p40, which signals through a distinct heterodimer receptor containing subunits IL-12Rβ1 and IL-23R [51] Binding of p19 and p40 subunits to the receptor will cause the phosphorylation of JAK2/Tyk2, which leads to the induction of primary downstream proinflammatory

cytokines Interleukin 22, 17A and F (IL-17A, IL-17F) [51,52]. IL-23 signaling can be pathogenic or protective depending on the context in which it is activated. IL-23 signaling is a crucial modulator in the development of most autoimmune diseases [51,52]. Contradictory to its pathogenic role in autoimmune diseases, many bacterial infection models including *Klebsiella pneumonia, Mycoplasma pneumonia, Mycobacterium tuberculosis*, *Borderella pertussis*, and intestinal *Citrobacter rodentium* demonstrate a protective role for IL-23 [53–59]. Silencing of IL-23 in these models result in host susceptibility and severe disease. Further exploration into how IL-23 is regulated and how its influences downstream mediators may elucidate its role in mediating host protection or pathogenesis.

Activation of IL-23 and its successive signaling leads to a robust pro-inflammatory environment. This phenotype results from its ability to induce pro-inflammatory cytokines, such as IL-17A, 17F, and IL-22, from both adaptive and innate immune cells, as well as its ability to sustain inflammation through the inhibition of anti-inflammatory IL-10 production [60]. Various innate immune cell subsets have been shown to rapidly respond to IL-23 secretion. IL-23R is constitutively expressed on innate Natural Killer T cells (NKT), γδ T Cells, innate lymphoid cells (ILCs), and lymphoid tissue inducer-like cells (LTi-like) [61–65]. Although the role of LTi-like cells in response to IL-23 signaling in the mucosa has not been evaluated, splenic LTi-like cells are shown to immediately produce both IL-17 and IL-22 in response to IL-23 [65].

Figure 1.3: Multifaceted role of the immune response during *Clostridium difficile* infection. There is evidence to support both pathogenic and protective roles for the immune response during CDI. Thus, an immune response which controls infection while limiting off target effects that lead to tissue pathology is likely beneficial to disease outcome



Molecule	Role during CDI	Reference
TLR4 and MyD88	**Reduce morbidity in mice**	**Ryan et al (2011), Jarchum et al (2011), Lawley, 2009**
IL-22/complement	**Control bacterial translocation; reduce mortality in mice**	**Hasegawa et al (2014)**
Innate Lymphoid Cells (ILCs)	**Ablation of ILCs lead to severe mortality in mice**	**Abt et al (2015), Geiger et al (2014)**
IL-23	*Increases mortality/ morbidity*	*Buonomo et al (2013)*
IL-8 / CXCL5	*Predicts poor prognosis better then C. difficile burden in humans*	*El Feghaly et al (2013A &B)*

γδ T cells are enriched in the epithelial layer of the gut and skin at steady-state which suggest they are may be first responders to IL-23 signaling in these tissues [66,67]. Buonocore *et al* established that bacterial driven innate colitis is dependent on the ability of ILCs to produce both IL-17 and IFNγ in an IL-23 dependent manner [64]. The study also indicates the ILCs are responsive to IL-23 and contribute to pathology in T cell dependent colitis models [64]. Production of type 17 cytokines from these cell types signal to epithelial, fibroblast, and endothelial cells to secrete chemokines and cytokines that recruit neutrophils to respond with either a protective or destructive outcome. There is a large gap in knowledge in regards to the role of IL-23 in innate pathologies, although these data indicate subsets of interest when evaluating its signaling in *C. difficile* colitis.

1.6 Interleukin 25 (IL-25)

Interleukin-25 (IL-25) (also called IL-17E) is a member of the IL-17 family, although it is not a downstream mediator of IL-23 and does not share common functions with other cytokines in the IL-17 family. IL-25 facilitates a type 2 response and is induced in response to exogenous stimulus such as allergens, and helminth and bacterial antigens. IL-25 has been demonstrated to function as a pathogenic, protective, or homeostatic mediator suggesting that the context in which it is elicited dictates the role it will play in an individual scenario [68]. IL-25 is primarily produced by epithelial cells, but has also been detected originating from cecal CD4+ and CD8+ T cells, mast cells, endothelial and eosinophils. The downstream consequence of IL-25 induction is the production of type 2 cytokines

(IL-4, IL-5 and IL-13), IgA, IgG, and IgE antibody responses, increased mucus, and the recruitment of eosinophils, mast cells, and basophils [68,69]. IL-25 signals through IL-17RB. This receptor shared with IL-17B, although IL-25 has increased binding affinity [70]. Cells expressing its receptor are not completely defined, although it has been observed on intraepithelial lymphocytes, epithelial cells, macrophages, eosinophils, basophils, NKT, innate lymphoid cells, smooth muscle tissue, and Th2 CD4 cells [68].

The role of IL-25 has been most thoroughly examined in the context of asthma and allergy where it functions to drive disease. IL-25 signaling is deleterious in many allergy and asthma models, potentially due to its ability to recruit eosinophils, mast cells, and basophils which are key modulators of disease. Although, recent studies have identified IL-25 as a key cytokine responsible for protection against nematode infection by stimulating type 2 cytokines production from innate cell populations [69]. IL-25 has also been demonstrated to facilitate the expulsion of gut helminths through IL-13 mediated induction of goblet cells to produce mucus [71]. Interestingly, recent data suggest IL-25 signaling functions as a negative regulator of type 17 responses. A study examining the role of IL-25 signaling in experimental autoimmune encephalomyelitis indicated that IL-25$^{-/-}$ mice had increased levels of type 17 cytokines and were more susceptible to disease [72]. Additionally, a 2008 study observed that commensal bacteria increased IL-25 production from epithelial intestinal cells and resulted in decreased IL-23 expression and subsequent

decrease of type 17 cytokines. The study also showed that germ free mice which lack commensal bacteria had depleted IL-25 levels and elevated IL-23 expression [73]. Although IL-25 has more recently been investigated during different contexts, its role during bacterial infection remains unexplored.

Eosinophils, as a downstream effector cell of IL-25 induction, are of particular interest due to their ability to defend the host against various types of pathogens in the gut and to facilitate tissue remodeling and repair [74]. Eosinophils express numerous PRRs that recognize both PAMPS and DAMPS [74]. As a result, they can respond to both bacterial stimuli and the tissue damage. As a reaction to certain stimuli, eosinophils become activated and produce pro-inflammatory cytokines and release granules containing cationic protein which are bactericidal [74]. In addition, eosinophils have been demonstrated to release mitochondrial DNA “traps” which are toxic to extracellular bacteria [76]. Lastly, eosinopenia is associated with sepsis and increased bacterial burden in mice [77]. This provides an interesting possibility for eosinophils to control *C. difficile* directly or interact with other protective or pathogenic commensal bacteria in the gut during CDI. Interestingly, in human CDI low eosinophil numbers are a risk factor for persistent diarrhea or death and recurrent disease [75]. In contrast, eosinophils have also been shown to be pathogenic in the gut by driving IBD-like colitis through GM-CSF production and tissue damage in mice [78]. Together, this suggests that the environment and cytokine milieu present at the time of

eosinophil recruitment may dictate the activation status and function of eosinophils during varying diseases.

1.7. **The Microbiota and Immune Responses**

Crosstalk between the microbiota and the intestinal epithelium plays a role in shaping the mucosal immune response. This observation is supported by evidence that germ-free mice have under-developed innate and adaptive immune responses [79]. Therefore, alterations in the composition of the microbiota, due to antibiotic or environmental changes, can influence how immune cells react to insults and could contribute to host severity during CDI. It is well understood that both the immune response and the microbiota can influence the pathogenies of CDI. Therefore, understanding how the microbiota can influence the immune response is important to consider when discussing this infection.

Influences of the microbiota on mucosal immune responses can be attributed to distinct microbial signals recognized by cells within the intestine. Many of these microbial signals, such as flagellin and prokaryotic DNA, are recognized by Pattern Recognition Receptors (PRRs) on epithelial and immune cells [80]. These signals can alter immune responses not only by influencing the repertoire of immune cells present in the lamina propria, but also by affecting the functionality of cells. Commensal bacteria play an important role in the activation

of immune responses. Thus, a reduction in microbial abundance or diversity can lead to an unbalanced immune response in the intestine that may be harmful during CDI. This is supported by evidence that signals from the microbiota can induce a proportion of regulatory T cells (Tregs) in colonic tissue [81]. The method by which a 'healthy' microbiota induces Tregs may be through the production of short-chain fatty acids (SCFA), which are dependent on microbiota fermentation of dietary components and have been demonstrated to promote the induction of Treg cells [82]. As a result, induction of Tregs by the microbiota was associated with the reduction in Type 17 T cells (Th17) [83]. Activation of type 17 responses culminates in granulopoiesis and recruitment of neutrophils to the site of infection [84]. Therefore, the disruption of Treg/TH17 homeostasis with antibiotic treatment may play a pivotal role in disease outcome. Additionally, intestinal microbes have been suggested to play a regulatory role in the intestine by controlling the mucus barrier and gene expression of *muc2*, a major component of mucin. The induction of intestinal mucus is not only capable of reducing inflammation by creating a physical barrier between the lumen and the epithelium, but also actively skews mucosal dendritic cells to function in a regulatory capacity [85,86]. These results suggest that the microbiota plays an important role in avoiding overt inflammation by maintaining homeostasis between proinflammatory and regulatory responses in the colon. As a result, the reductions in the microbiota through antibiotic treatment and subsequent suppression of regulatory immune responses may be deleterious to the host

during CDI by promoting robust inflammation result in tissue damage increased disease severity.

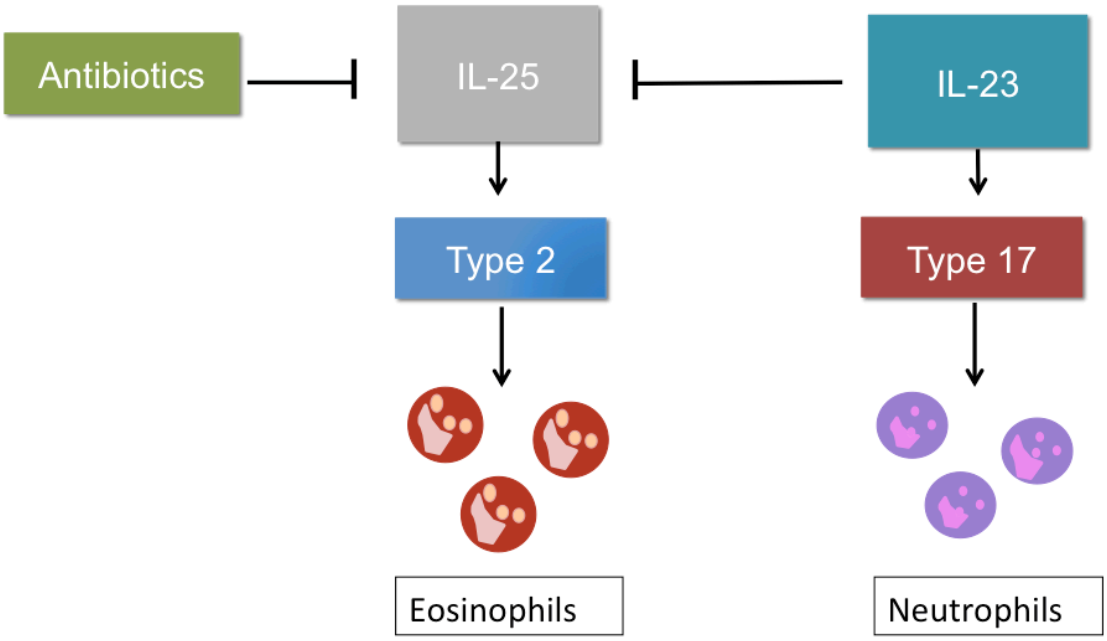
Antibiotic treatment has also been demonstrated to create a proinflammatory environment by enhancing the translocation of native commensal bacteria out of the intestine [87]. In this study antibiotic treatment was associated with enhanced CXCL1, IFNγ, and IL-17 protein in the mesenteric lymph nodes [87]. Additionally, antibiotic-induced translocation of commensals led to increased intestinal inflammation by histological analysis and worsened DSS-induced colitis [87]. In a second study, high fat diets in mice were demonstrated to facilitate commensal translocation to the blood and adipose tissues and enhanced proinflammatory cytokine TNF-α. Translocation events and resulting inflammation were reversed with probiotic *Bifidobacterium animalis* treatment, indicating that certain species of the microbiota are capable of reducing bacterial translocation [88]. This is interesting, as it has been observed that IL-22 induced complement reduces CDI-associated morbidity by limiting the translocation of commensals [42]. Therefore, in addition to establishing host susceptibility by providing a beneficial niche for *C. difficile* prior to infection, antibiotic treatment may also be pathogenic to the host throughout CDI by supporting bacterial translocation and a subsequent unbalanced immune response.

Immunoglobulin A (IgA) is the dominant immunoglobulin at mucosal sites and plays an important role in maintaining intestinal barrier integrity and protecting host tissue from pathogenic and commensal microbes [89]. The microbiota supports the induction of plasma cells and IgA responses as evidenced by decreased levels in germ-free mice [90]. Interestingly, reductions in B cells, plasma cells, and IgA responses have been observed in CDI patients and the lowest levels were observed in patients with pseudomembranous colitis and the most severe disease [91]. The importance of antibodies to protect the host from CDI is also supported by low levels of serum antibodies directed against toxins A and B observed in patients with recurrent CDI compared to controls that do not relapse [92,93,94]. Thus, the ability of the microbiota to aid in antibody responses is likely another mechanism by which the microbiota-immune response axis influences disease outcome.

As previously described, immune mediators can play either a pathogenic or protective role during CDI. An intriguing avenue to pursue is investigation into how the microbiota may affect the presence and absence of established immune mediators that are important in dictating disease outcome. Evidence supports that expression of IL-25 is dependent on the microbiota and this cytokine signals inversely of the pathogenic mediator IL-23 [72,73,95]. Germ-free and antibiotic treated mice had reduced IL-25 and enhanced IL-23 levels in the intestine [73]. The down-regulation of IL-25 associated with antibiotic treatment (a process which leads to host susceptibility) and IL-23 (an immune mediator that drives

disease severity) provides numerous hypotheses to investigate during CDI. Firstly, are cytokines which signal inversely of deleterious immune mediators, such as IL-25, protective during CDI? Secondly, does antibiotic treatment have a secondary role in contributing to pathogenesis by influencing the immune responses, like IL-25 and IL-23, to dictate disease severity and host outcome. The work of this dissertation explores the protective and pathogenic roles of IL-25 and IL-23 , respectively, during CDI.

Figure 1.4: IL-25 suppression is observed during both antibiotic treatment and increased IL-23 signaling. IL-25 promotes type 2 responses, including eosinophilia, and its signaling is inversely correlated with antibiotics, which is associated with predisposing CDI susceptibility, and IL-23 signaling, which is associated with increasing disease severity.


Antibiotics
IL-25
IL-23
Type 2
Type 17
Eosinophils
Neutrophils

Chapter Two: IL-23 and Type 17 responses during *Clostridium difficile* infection

Part of this chapter has been adapted from "The role of IL-23 during Clostridium difficile infection"

Buonomo, E.L., Madan, R., Pramoonjago, P., Li, L., Okusa, M.D., and Petri, W.A. (2013). Role of interleukin 23 signaling in Clostridium difficile colitis. J. Infect. Dis. *208*, 917–920.

2.1 Introduction

Clostridium difficile is currently one of the leading cause of nosocomial infections in the United States, with an estimated cost of $4.8 billion to the health care industry annually [6]. The incidence of infection continues to rise in health care and community-settings, in part due to the emergence of a new hyper-virulent strain, BI/NAP1 [96]. Despite therapy, strains with increased virulence have resulted in 30-day mortality in up to 15% of patients [5] and disease recurrence in 20-40% of cases [96].

The aim of this study was to evaluate the host inflammatory response to *C. difficile* infection. It has been hypothesized that the intensity of the host response and resulting inflammation may be correlated with disease severity. Understanding and targeting host-based mediators of inflammation may provide a target for more effective therapy. Interleukin-23 (IL-23) causes inflammation due to its ability to induce pro-inflammatory cytokine production from innate and immune cells, such as Interleukin-17A, F, and 21 (IL-17A, IL-17F, and IL-21), and its ability to neglect the induction and maintenance of the anti-inflammatory cytokine Interleukin-10 (IL-10) [60,97,98]. Previous studies have implicated IL-23 signaling as a precursor to colitis in models of Inflammatory Bowel Disease (IBD) [99,100]. This prompted us to examine its role in the development of *C. difficile* pathology. Colon biopsies from patients with *C. difficile* colitis demonstrated the presence of IL-23-producing infiltrating white blood cells in the lamina propria.

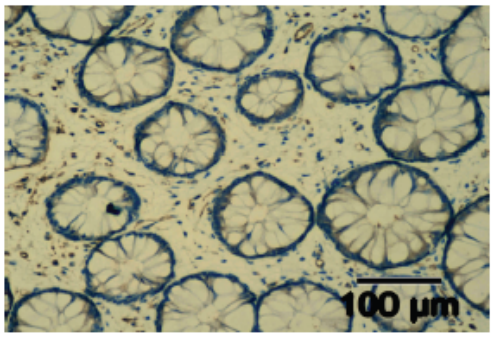
We utilized the murine model of *C. difficile* colitis to test the role of IL-23 signaling during infection. Mice lacking IL-23 signaling had decreased disease severity, as manifest by clinical scoring and survival. The implications of these studies for understanding the pathogenesis of *C. difficile* disease and potential host-targeted therapy are discussed.

2.2 Results

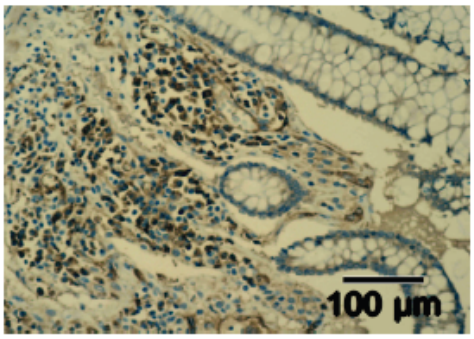
To ascertain the relevance of IL-23 in human *C. difficile* infection, human intestinal biopsies from patients with *C. difficile* colitis were stained for the presence of IL-23 positive infiltrating immune cells. Biopsies were taken from female patients that tested either positive or negative for *C. difficile* toxins. Negative tissues were taken from patients suspected of various inflammatory disease, or in remission from IBD, but had confirmed normal pathology upon biopsy. We observed increased levels of IL-23p19 staining in lamina propria cell infiltrates from patients testing positive for disease compared with negative controls (Figure 2.1). Biopsies were scored, by four independent blinded scorers, on a scale from 0-2 based on staining intensity and abundance. *C. difficile* positive tissues had an average score of 1.33 (n= 6, SEM ± 0.30) and *C. difficile* negative control tissues had an average score of 0.7 (n=9, SEM ± 0.29) ($p=0.008$) (Figure 2.1).

To evaluate the role of IL-23 in murine *C. difficile* infection we utilized both genetic knockout and monoclonal antibody neutralization techniques to disrupt signaling in mice. Survival and clinical scores of IL-23p19 gene knockout mice (IL-23p19$^{-/-}$) and wildtype C57BL6/J (WT) were compared during *C. difficile* infection. Both groups were treated with an identical regiment of antibiotics and challenged with 10^5 CFU bacteria. Mice were observed twice daily for 2 days post infection and evaluated for clinical score and survival. Clinical Scoring was based on parameters previously established [10]. IL-23p19$^{-/-}$ mice were protected from morbidity and mortality associated with infection when compared to controls (Figure 2.2a): IL-23p19$^{-/-}$ survival was 100% throughout the infection time course, whereas WT mice survival decreased to 16.7% by Day 2 of infection. Similarly, clinical scores for IL-23p19$^{-/-}$ mice were less severe than those of WT mice. On Day 2, IL-23p19$^{-/-}$ mice had a maximum score of 6.33 (n=6, SEM ±1.36) and WT mice had a significantly greater maximum score of 14.00 (n=6 ± 1.55) (p=0.0087). WT mice reached a severe clinical score (>14) on day 2 and were euthanized for ethical reasons. We also performed a second eight-day study comparing the survival of IL-23p19$^{-/-}$ and WT mice to confirm that IL-23p19$^{-/-}$ mice do not have delayed onset of disease. Mice from both groups did not exhibit a decrease in survival after Day 3, indicating that 100% survival in IL-23p19$^{-/-}$ remains constant post Day 2 of infection (Figure 2.3).

Figure 2.1 IL-23 is upregulated in the colon of patients with *C. difficile* colitis. Immunohistochemical staining for IL-23p19 in a representative colon biopsy from a patients without (left), and with *C. difficile* colitis (right) demonstrating increased lamina propria IL-23 (+) infiltrating immune cells in *C. difficile* infection



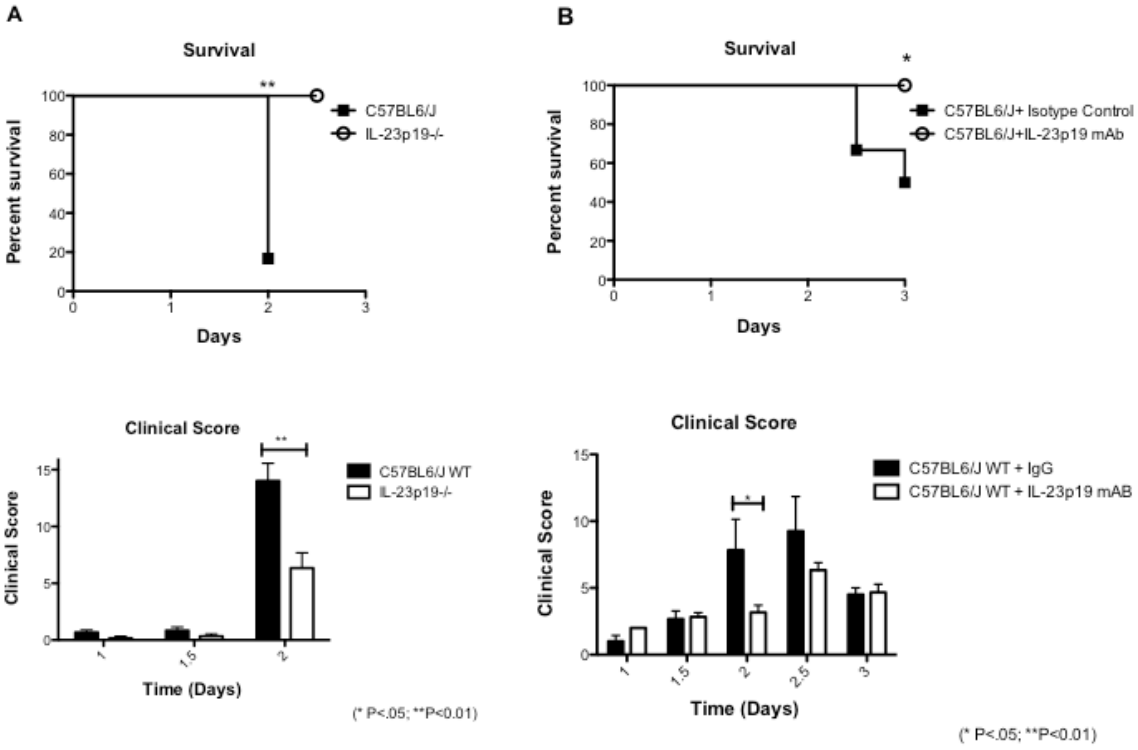
Control



Clostridium difficile positive

To further investigate the pathogenic role for IL-23 seen in gene knockout mice, we used a second murine model where IL-23p19 was neutralized using a monoclonal antibody directed toward the p19 subunit of IL-23. WT mice were given the same regiment of antibiotics described above and treated 18 hours prior to infection with either 100 µg of IgG isotype control antibody or 100 µg of anti-IL-23p19 mAb. Mice were given a subsequent dose of respective antibodies 2 days later. Mice obtained from Jackson lab were challenged with 10^4 CFU. Mice were evaluated twice daily for clinical score and survival and sacrificed on Day 3. Analogous to our previous observation, mice lacking IL-23 signaling were protected from morbidity and mortality associated with infection (Figure 2.2b). IL-23p19 neutralized mice had a 100% survival rate (n= 6), whereas isotype controls survival was 50% by Day 3 (n=6). Concurrently, isotype controls had a significantly increased clinical score compared to IL-23p19 neutralized mice at Day 2. At this time point, control mice had an average maximum score of 9.00 (SEM ± 2.00), while neutralized mice had an average maximum score of 3.17 (SEM ± 0.54) (p=0.018). The expression of cytokines downstream of IL-23 signaling was next evaluated in murine cecal whole tissue lysate. We chose to initially examine the expression of hallmark Th17-like cytokines, IL-17A and IL-22, from IL-23p19$^{-/-}$ and wildtype (WT) mice on day 2 of infection (Figure 2.4). Infected IL-23p19$^{-/-}$ mice (n=6) had significantly decreased IL-17A protein expression when compared to infected WT mice (n=9)(p=0.01). Likewise, IL-23p19$^{-/-}$ mice had significantly decreased IL-22 expression compared to WT mice ($p<0.0001$).

Figure 2.2: Mice that lack IL-23 signaling are protected from mortality and morbidity associated with *Clostridium difficile.* Mice were infected on Day 0 with 10^4-10^5 CFU *Clostridium difficile* after three days of antibiotic pretreatment. IL-23p19$^{-/-}$ knockout mice (a) and anti- IL-23p19 monoclonal neutralized mice (b) had decreased disease severity as manifest by enhanced survival and decreased clinical scores. (*=p value<0.05, **=p-value<0.01)


A
Survival
**
C57BL6/J
IL-23p19-/-
Percent survival
Days
Clinical Score
**
C57BL6/J WT
IL-23p19-/-
Clinical Score
Time (Days)
(* P<.05; **P<0.01)
B
Survival
*
C57BL6/J+ Isotype Control
C57BL6/J+IL-23p19 mAb
Percent survival
Days
Clinical Score
C57BL6/J WT + IgG
C57BL6/J WT + IL-23p19 mAB
*
Clinical Score
Time (Days)
(* P<.05; **P<0.01)

IL-23p19$^{-/-}$ infected mice did not have increased expression of either cytokine, IL-17A or IL-22, when compared to IL-23p19$^{-/-}$ sham infected mice, indicating these cytokines remained at baseline expression even during the peak of infection. In contrast, infected WT mice have significantly elevated IL-22, and an enhanced trend in IL-17A expression compared to WT sham treated mice (p=0.0004). A caveat of these data is the inability to distinguish if the decrease in IL-17A and IL-22 expression is a mechanism by which IL-23p19$^{-/-}$ mice are protected from disease, or rather a secondary effect resulting from an overall lack of inflammation seen in IL-23p19$^{-/-}$ mice. In other words, IL-22 and IL-17A may actually function in a protective capacity during *C. difficile* infection and that mechanism of protection is not needed in IL-23p19$^{-/-}$ mice because severe colitis does not develop.

To examine the effects of IL-23 signaling on immune cell recruitment, we utilized flow cytometry in WT and IL-23p19$^{-/-}$ mice on day 2 post infection. Neutrophil recruitment, the hallmark cell subset of pathology, and Ly6c^{+} monocyte cell recruitment to the lamina propria were evaluated. Colon tissue from IL-23p19$^{-/-}$ and WT mice were harvested and examined on day 2 post infection. Neutrophil (Ly6g+Ly6c+) were identified using a live+ CD45+ CD11b+ gating strategy. IL-23p19$^{-/-}$ mice had overall fewer myeloid- derived immune cells recruited to the lamina propria compared to WT mice, as evident by significantly lower CD45+ and CD11b+ live cells (data not shown). Additionally, IL-23p19$^{-/-}$ had a significantly lower average of neutrophils recruited (66.8%, n=5) compared to WT mice (82.3%, p=0.006) (Figure 2.5).

Histology of colon tissues harvested on Day 2 from infected WT and IL-23p19$^{-/-}$ mice show that infected WT mice have significantly worse colitis than IL-23p19$^{-/-}$ mice (Figure 2.6). Colitis severity scores are based on the level of inflammatory cell recruitment, thickness of mucosa, immune cells exudate, erosion in the epithelial barrier, and submucosal edema. Slides were scored by a blinded scorer and are based on a 0-3 range per parameter. Increased scores are indicative of worse pathology. Colitis severity for WT mice on Day 2 had a total average of 10.8 (n=5), while IL-23p19-/- mice scored a significantly lower averaged score of 6.75 (n=6) (p=0.03).

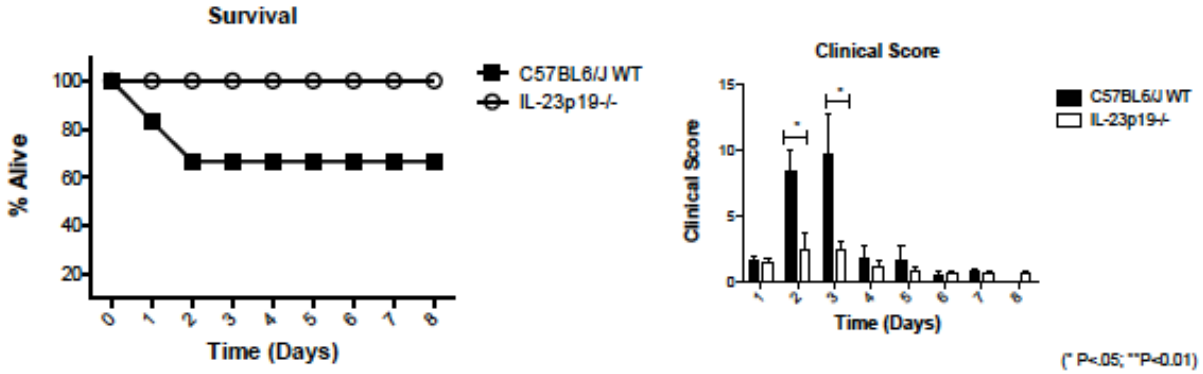
The largest differences between infected WT and IL-23p19$^{-/-}$ mice were that WT mice had increased inflammatory cell recruitment, immune cellular exudate, and submucosal edema. Histology from colons harvested from antibiotic treated uninfected WT and IL-23p19$^{-/-}$ mice have normal epithelial barrier architecture and were absent of pathology. Together, these data indicate that the immune response may dictate disease severity by influencing the intestinal barrier.

2.3 Discussion

Our present study suggests the relevance for IL-23 in human *C. difficile* associated colitis and disease. We have demonstrated that IL-23p19 protein levels are elevated in human colon biopsies from patients suffering from *C.*

difficile infection when compared with negative controls. *C. difficile* positive

Figure 2.3. Mice that lack IL-23 signaling are protected from mortality and morbidity associated with *Clostridium difficile* throughout an 8 day time course. Mice were infected on day 0 with10^6 CFU *Clostridium difficile* after three days of antibiotic pretreatment. IL-23p19-/- knockout mice had decreased disease severity as manifest by enhanced survival and decreased clinical scores. This phenotype remained 8 days post-infection(*=p value<0.05).


Survival
% Alive
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Time (Days)
C57BL6/J WT
IL-23p19-/-
Clinical Score
Clinical Score
15
10
5
0
1
2
3
4
5
6
7
8
Time (Days)
C57BL6/J WT
IL-23p19-/-
(* P<.05; **P<0.01)

biopsies have upregulated expression of IL-23p19+ cell infiltrates to the lamina propria, suggesting IL-23 may be involved in mediating the enhanced inflammation responsible for tissue injury and severe disease seen in the mouse model. IL-23 is a known pathogenic mediator in IBD-associated colitis [7]. Likewise, IL-23R mRNA levels are upregulated in lamina propria CD4+ cells from IBD patients when compared to controls [8]. Active clinical trials indicate that Ustekinumab, a monoclonal antibody directed towards IL-23 and IL-12, is effective in increasing remission rates in some IBD Crohn's disease patients [11]. Although these diseases utilize differing initiators in pathogenesis, our observations for IL-23 in *C. difficile* infection closely resemble the data described for the pathogenic role of IL-23 in IBD-colitis. The effectiveness of anti-IL-23p19 as a therapy for IBD supports the plausibility for IL-23 as a potential target in human *C. difficile* disease treatment.

Our study also demonstrates a pathogenic role for IL-23 signaling by using two distinct murine models indicating that mice deficient IL-23 signaling are protected against CDI-associated mortality and morbidity. Mice lacking IL-23 signaling through genetic knockout or monoclonal antibody neutralization had enhanced overall survival and clinical health when compared to wild type controls.

IL-23 was originally implicated as an essential driver in the differentiation of the CD4+ T cell subset categorized by the production IL-17, T helper 17 (Th17) cells. It is now understood that IL-23 does not drive differentiation, but instead

enhances proliferation of Th17 cells and the maintenance of IL-17 production in both an innate and adaptive capacity [98,101,102]. IL-17A and IL-17F are key mediators of inflammation by inducing proinflammatory cytokines (TNF, IL-1, IL-6, GM-CSF) and chemokines (CXCL1-2, CXCL5, IL-8, CCL2, CCL7), which are important in granulopoiesis and the recruitment of neutrophils to the site of infection [69,103]. In a previous study, IL-6 and TGF- β stimulation were shown to induce IL-17, and low amounts of IL-10, production from Th17 cells. Interestingly, the addition of IL-23 as a stimulus neglected IL-10 production and skewed the cell subset towards pathogenicity [60]. IL-23 has since been implicated in the neglected maintenance of IL-10 production from not only Th17 CD4+ cells, but through the prevention of Foxp3+ inducible T regulatory (iTreg) cell differentiation [4]. IL-23 signaling also has been shown to play a vital role in IL-17A, IL-17F, and IL-22 production in other T cell subsets such as Natural killer T cells (NKT) and γδ T cells, as well as in the non-T cells innate lymphoid cells (iLCs) and lymphoid tissue inducer-like (LTi) cell subsets [64,65]. We hypothesize that IL-23 leads to increased susceptibility following *C. difficile* infection by signaling to both T cells and non-T cells to produce IL-17A and/or IL-22 to enhance the recruitment of immune cells the site of infection and create a robust, deleterious neutrophilic inflammatory response (Figure 2.7). Resulting inflammation will be sustained at the site through the ability of IL-23 to maintain downstream cytokines, such as IL-17A and IL-22, and chemokines capable of continually attracting neutrophils. Additionally, recruitment of immune cells may be dysregulated by the restriction of anti-inflammatory cytokine IL-10 production.

These changes could result in enhanced intestinal injury and pseudomembranous colitis, and increased disease severity. We hypothesize that in the absence of IL-23, immune cells are still capable of responding to infection and clearing the bacterium, but acute colitis and severe disease is avoided through regulatory responses.

The significant decrease in neutrophils was expected, although we suspected the reduction would be more robust. Previous studies have demonstrated that total neutrophil blockade during infection resulted in increased mortality and morbidity. An explanation for our observation could be that a delicate balance of neutrophil recruitment is essential to confer protection through bacterial clearance, but if a slight increase in recruitment occurs the consequence is detrimental to the host. Additionally, neutrophils that have trafficked out of the lamina propria and into the lumen will not be detected through flow cytometry analysis.

A limitation of this study is that the mice were not cohoused during either antibody treatment. Given the advances in the field that focuses on the contribution of the microbiota to control and influence the pathogenesis of CDI, cohousing mice is an important control to further interpret these data. In conclusion, our study suggests a pathogenic role for IL-23 signaling during *C. difficile* infection in murine models and establishes that IL-23 expression is upregulated in human CDI. Future work is necessary to elucidate mechanisms by which IL-23 drives pathogenesis in both models and to understand whether neutralization of IL-23 may ultimately serve as a potential therapy for *C. difficile* disease

Figure 2.4: IL-23p19$^{-/-}$ mice have decreased IL-17A and IL-22 protein expression. IL-17A (left) and IL-22 (right) protein expression in day 2 cecal tissue lysates from infected and sham treated IL-23p19$^{-/-}$ and wildtype (WT) mice

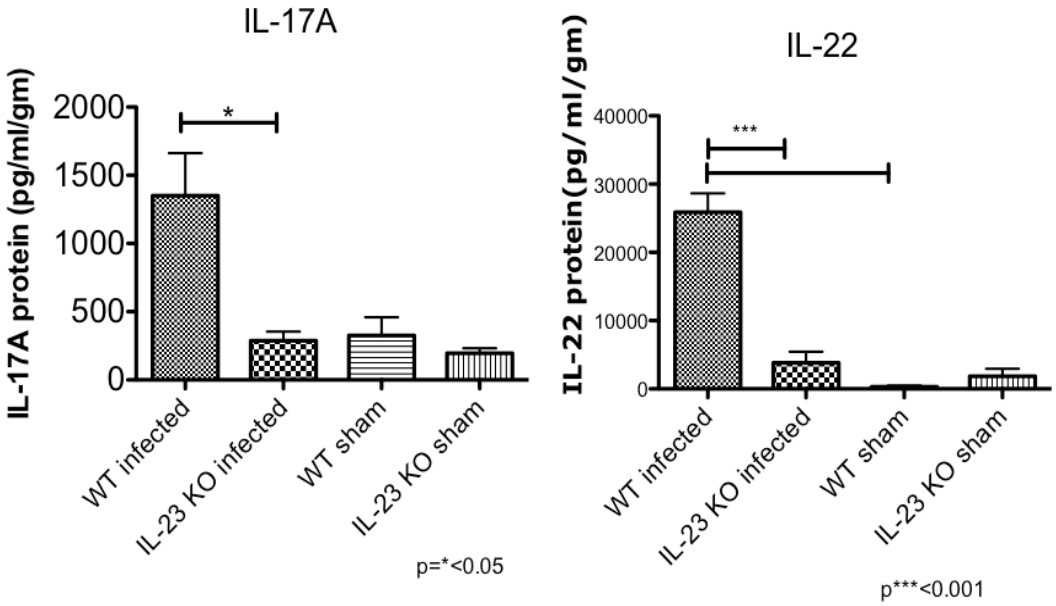

IL-17A
IL-17A protein (pg/ml/gm)
2000
1500
1000
500
0
*
WT infected
IL-23 KO infected
WT sham
IL-23 KO sham
p=*<0.05
IL-22
IL-22 protein(pg/ml/gm)
40000
30000
20000
10000
0
***
WT infected
IL-23 KO infected
WT sham
IL-23 KO sham
p***<0.001

Figure 2.5: IL-23$^{-/-}$ mice have increased neutrophil recruitment compared to WT. Plots representing neutrophil and inflammatory monocyte recruitment for individual IL-23$^{-/-}$ (left) and WT (right) mice. Quantitative analysis of IL-23$^{-/-}$ (n=5) and WT (n=9) are shown in the right panel.

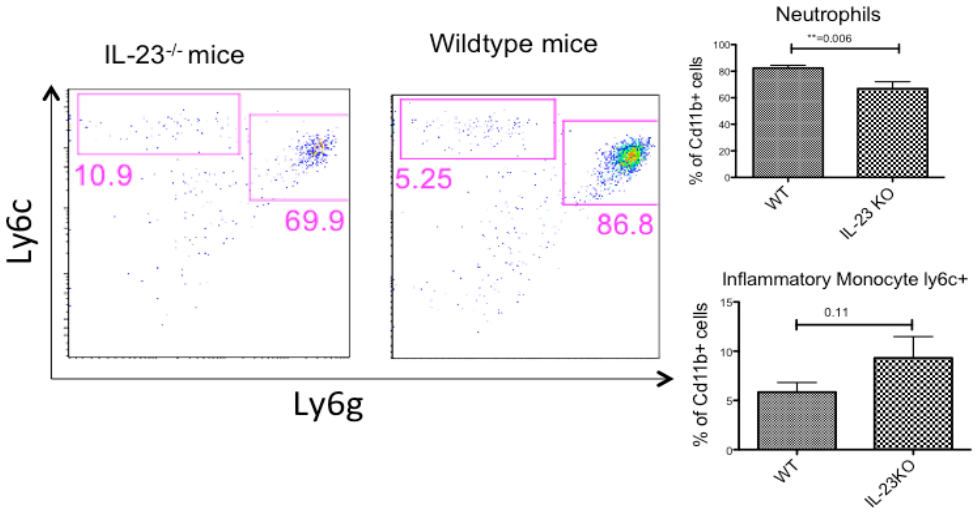
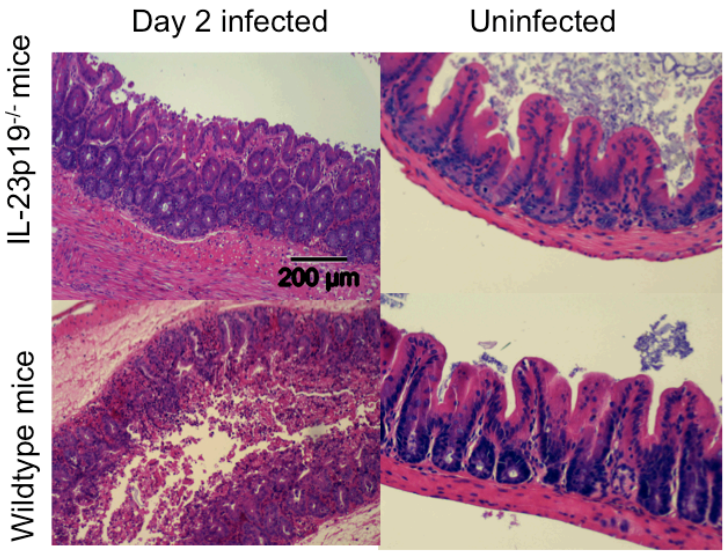

IL-23-/- mice
Wildtype mice
10.9
69.9
5.25
86.8
Ly6c
Ly6g
Neutrophils
**=0.006
% of Cd11b+ cells
WT
IL-23 KO
Inflammatory Monocyte ly6c+
0.11
% of Cd11b+ cells
WT
IL-23KO

Figure 2.6: IL-23p19$^{-/-}$ mice (top) have reduced colitis during infection when compared to WT mice (bottom). Histology is from colon tissue WT and IL-23p19$^{-/-}$ mice harvest on day 2 of infection (left) or antibiotic-treated but uninfected controls (right).


Day 2 infected
Uninfected
IL-23p19-/- mice
Wildtype mice
200 µm

1.4 Materials and Methods

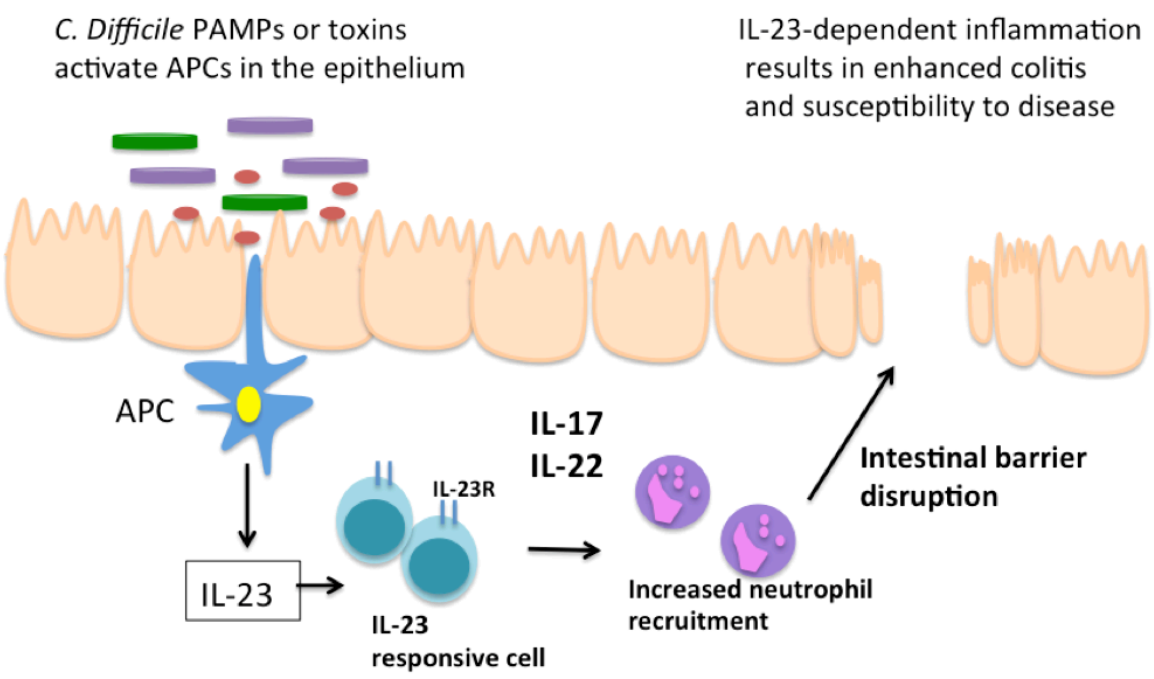
Animals

C57Bl/6 wildtype (WT) mice were purchased from Jackson laboratory (Bar Harbor, ME) for use in the IL-23p19 neutralization study. IL-23p19$^{-/-}$ mice on a C57Bl/6 background were a generous gift from Dr. Daniel Cua (Merck, Palo Alto). Mice used in the gene knockout study were bred for use in the University of Virginia vivarium. Mice were 8-16 weeks of age and housed under the same conditions with free access to food and water. Protocols were approved by the Center of Comparative Medicine at the University of Virginia and were in agreement with NIH and IACUC standards.

Bacteria Culture

Clostridium difficile strain VPI 10463 (ATCC 43255) was cultured overnight in anaerobic Chopped Meat Medium (Anaerobe Systems, Morgan Hill, CA) at 37° C. It was then sub-cultured under the same conditions for 5 hours before infection.

Figure 2.7. Hypothesis: IL-23 drives pathogenesis by activating IL-23 responsive cells to promote downstream cytokines IL-17A and/or IL-22 to increase neutrophil recruitment to the lamina propria which have off-target deleterious impacts to the integrity of the intestinal epithelial barrier.


C. Difficile PAMPs or toxins
activate APCs in the epithelium
IL-23-dependent inflammation
results in enhanced colitis
and susceptibility to disease
APC
IL-23
IL-23R
IL-23
responsive cell
IL-17
IL-22
Increased neutrophil
recruitment
Intestinal barrier
disruption

Murine Model for Clostridium difficile *challenge*

A murine *C. difficile* infection model has previously been established [104]. Briefly, mice were treated with a cocktail of four antibiotics prior to infection. Mice received antibiotic water for 3 days, followed by autoclaved, antibiotic-free water for 2 days, with a subsequent intraperitoneal injection of clindamycin (10mg/kg) one day prior to infection. IL-23p19$^{-/-}$ and C57BL/6 control mice were bred in the University of Virginia vivarium and were challenged by gavage with 10^5 colony forming units (CFU) of vegetative *C. difficile*. C57BL/6 mice used in neutralization studies obtained directly from Jackson laboratory were challenged by gavage with 10^4 CFU *C. difficile,* due to a heightened sensitivity to challenge. Mice were observed twice daily and survival and clinical scores were recorded. The scoring system for clinical score has been previously described [^{105}] and is based on weight loss, coat ruffling, ocular discharge, activity level, posture, and diarrhea severity. Each parameter was scored on a scale from 0-3, with higher clinical scores indicative of higher morbidity. Mice were euthanized if score indicated intense morbidity (score > 14) on any day of the experiment.

Protein Detection

Cecal tissue was flushed with sterile PBS and homogenized by bead beating for 1 minute in a buffer consisting of 1M HEPES and HALT protease inhibitor cocktail (Thermo-Fisher Scientific Inc., Rockford, IL) followed by a 30 minute incubation on ice and with an Triton X 100, HEPES and HALT protease inhibitor cocktail containing buffer. Cytokines levels were evaluated by ELISA (IL-17A and

IL-22(Duo-Set ,R&D systems). Cytokine protein expression was normalized to total protein concentration generated from the Pierce BCA Protein Assay (ThermoFisher).

Immunohistochemistry (IHC) staining.

Mouse biopsies were attained from the cecum of mice on Day 3 of infection. They were fixed in Bouin's solution and cut and H&E stained by the Histology Core at the Univeristy of Virginia. Human biopsies were obtained from the University of Virginia Biorepository and Tissue Research Facility. Biopsy samples were identified as positive or negative for *C. difficile* through a toxin A/B ELISA assay. Only female patients with the closest age match were chosen to keep the population single gendered. Samples were stained for IL-23 from *C. difficile* positive (n=6) and *C. difficile* negative (n=9) patients. Immunochemistry staining was performed using the DAKO Autostainer Universal System (Dako, Denmark) with a primary antibody directed against IL-23p19 (Sigma Prestige) at a 1:200 dilution. Scoring was done by four independent blinded scorers and was based on intensity and abundance of IL-23p19 stain on lamina propria cell infiltrates. The staining scale was between 0-2, with one point given for high intensity of stain and one point given for high abundance of staining. IL-23p19 stained thyroid tissue was used as a positive control.

Isolation of Lamina propria and flow cytometry

Lamina propria and epithelial cells in the colon were separated as previously described (Madan, 2013). Briefly, the colon was removed, cut longitudinally, and

rinsed in a Hank's Balanced Salt Solution (HBSS), 5% Fetal Calf Serum (FCS), 215 25mM HEPES Buffer. The tissue was incubated in pre-warmed buffer consisting of HBSS, 15mM HEPES, 5mM EDTA, 10% FCS 217 and 1mM DTT at 37°C on a shaking incubator for two 20 minute cycles in fresh media to remove the epithelial layer. The tissue was minced and incubated in prewarmed RPMI containing 0.17mg/ml liberase TL (Roche) and 30ug/ml DNase (Roche) for 40 minutes. After digestion, tissue was passed through 40µm and 100µm nylon strainers, resuspended in FACS buffer, and quantified for total cell numbers and cell viability using trypan blue cell counting. Single cell colonic lamina propria cells were plated at 10^6 live cells per sample and stained. After FC blockade (anti-mouse CD16/32 TruStain, Biolegend), cells were stained using monoclonal antibodies to markers: Live/dead (Fixable Viability Dye eFluor 506), CD11b-APC(M1/70), CD45-APC-Cy7(30-F11)), Ly6g-PeCy7(1A8), Ly6c-Fitc (HK1.4) (Biolegend, BD, eBiosciences). Data were acquired on a Becton Dickinson LSRFortessa flow cytometry BD FACSDiva version 6. software (BD Biosciences). 5x10^5-10^6 events were collected and data were analyzed using FlowJo version 9.2 software (Treestar 233 Inc., Ashland, OR). Cell populations were calculated from total cells per colon and as a percentage of live cells.

Statistical Analysis.

All data are expressed as the mean value and analysis was performed as a two tailed Student's *t* test using Excel software (Microsoft). Probability (P) values of <0.05 were considered statistically significant.

Chapter Three: Microbiota-regulated IL-25 protects during CDI via eosinophilia

Part of this chapter has been adapted from "The role of IL-23 during Clostridium difficile infection"

Erica L. Buonomo[1], Carrie A. Cowardin[1], Madeline G. Wilson[1], Mahmoud M. Saleh[1], Patcharin Pramoonjago, and William A. Petri, Jr. (2016). Microbiota regulated IL-25 protects from *Clostridium difficile* infection via Eosinophils. In revision at Cell Host Microbe

Authors Contribution

E.L.B and W.A.P designed experiments and wrote the manuscript. E.L.B performed all experiments and analyzed and interpreted data. C.A.C helped with manuscript review, tissue extraction and processing, and bacterial quantification. M.G.W aided in IL-25 protein detection and tissue processing. M.M.S aided in manuscript editing and tissue processing. P.P. conducted IL-25 histological staining on human and mouse tissues

Introduction

Clostridium difficile infection (CDI) is currently the leading cause of hospital-acquired infection and gastroenteritis-associated deaths in the United States [4]. As a result, it has been listed as one of three 'Urgent Threats' by the Center for Disease Control and Prevention. The emergence of a hypervirulent strain NAP1 has been implicated in the evolving epidemiology of CDI and an increasing number of cases are being reported both inside and outside of acute care facilities each year [4]. *C. difficile* causes an estimated 453,000 infections, 83,000 relapses, and 29,300 deaths annually [4], stressing the need for better treatment and management options. This Gram-positive, spore forming anaerobic bacterium infects the colon when the normal microbiota has been disrupted, primarily through antibiotic use. Following colonization, the chief virulence factors, toxins A and B, are released and cause epithelial cell rounding and death compromising the integrity of the intestinal barrier. Therapy involves treatment with antibiotics such as vancomycin, fidaxomicin, or metronidazole [106]. In addition to effectively targeting *C. difficile,* these antibiotics can inhibit the reestablishment of beneficial endogenous flora, which may in part explain the high numbers of relapses and deaths associated with this disease.

CDI symptoms range from mild diarrhea to life threatening pseudomembranous colitis and toxic megacolon. Recent studies indicate that increased inflammatory markers, such as IL-8, are more accurate at predicting poor patient outcome then

increased bacterial burden, suggesting that the type and/or intensity of the immune response may control the severity of the disease [44,107]. In agreement, numerous studies support a dual role for the immune response to CDI. For instance, innate mediators such as MyD88 signaling, innate lymphoid cells (ILCs), leptin, and IL-22 with subsequent complement activation have each been observed to play a protective role during CDI in mice [36,37,42,108–110]. Conversely, inflammasome-driven IL-23 signaling is deleterious during CDI in mice [47,111] and depletion of proinflammatory moderators such as IL-1R, IFNγ, and neutrophils reduce toxin induced tissue damage [50,106,112,113]. Together, these studies support a multifaceted role for the immune response during CDI.
In addition to the host immune response, the status of the microbiota plays a fundamental role during CDI. The protective capabilities of a healthy microbiota to both inhibit and resolve disease is emphasized by the lack of host susceptibility to *C. difficile* in the presence of an intact microbiota and the recently demonstrated efficacy of fecal transplants in preventing relapses. Commensal bacteria protect the host from CDI through various mechanisms such as direct inhibition, competition for nutrients or space, and stimulation of host factors such as secondary bile acids and various metabolites [114,115]. Despite evidence of its central role in CDI, the beneficial influence of the microbiota to regulate host immune response during CDI has not been examined.

Crosstalk between the microbiota and the immune system is critical for shaping both the immune response and the microbial composition of the gut. One

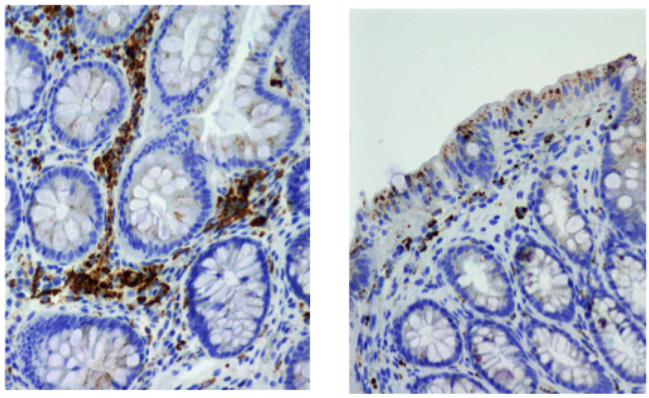
example of this relationship is the cytokine IL-25, which is dependent on the microbiota, as germ free and antibiotic treated mice show decreased IL-25 production [73]. IL-25 is an inducer of type 2 immune responses and increased levels have been demonstrated to correlate with decreased IL-23 expression [72,73]. Since IL-25 is regulated by the microbiota and is expressed inversely of the cytokine IL-23, which is deleterious during CDI, we hypothesized that IL-25 was down-regulated during CDI and repletion of its signal would reduce disease severity via influences on the immune response.

IL-25 signals to both innate and adaptive cells to promote type 2 immune responses characterized by the production of IL-4, IL-5 and IL-13, mucus, IgE, and IgA production. IL-25 is also capable of inducing eosinophil, basophil, and mast cell accumulation systemically and at local sites of inflammation [116–118]. The consequences of these effector functions are versatile and can mediate pathogenic, protective, or regulatory responses given the environmental contexts [68]. Despite evidence of communication between the microbiota and IL-25, the role of IL-25 in bacterial infections and specifically CDI has not been investigated. Eosinophils are of particular interest due to their ability to defend the host against various types of pathogens in the gut and to facilitate tissue remodeling and repair [74]. In human CDI low eosinophil numbers are a risk factor for persistent diarrhea or death and recurrent disease [75]. Eosinophils have classically been considered effective in anti-parasite protection although recent *in vivo* studies

Figure 3.1: IL-25 was suppressed during human *Clostridium difficile* infection (CDI).

(A) Representative histology of human colonic biopsies from CDI negative (-) (n=9) and CDI positive (+) (n=5) patients stained for IL-25 protein expression. (B) Histology was scored for IL-25 expression; four independent blinded scorers; *pvalue<0.05.

A. *CDI*



B.

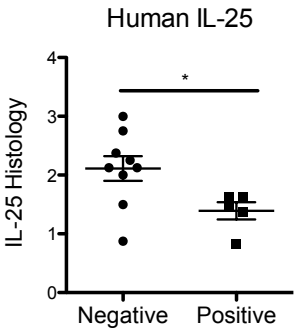


Table 3.1: Descriptive data on patients from human biopsies.

Case#	Age	Gender	*C. difficile*	Reason for biopsy
WP09-3	76	Female	positive	inflammation with C.difficle colitis
WP09-4	68	Female	positive	inflammation with C.difficle colitis
WP09-5	23	Female	positve	inflammation withC.difficle colitis
WP09-6	60	Male	negative	rectal colitis
WP09-7	35	Female	negative	Crohn's diseses
WP09-9	69	Female	positive	inflammation with C.difficle colitis
WP09-11	68	female	positive	inflammation with C.difficle colitis
WP09-12	67	female	negative	polyp
WP09-13	69	female	negative	diarrhea
WP09-14	68	Female	negative	history of Crohn's disese
WP09-15	69	Female	negative	dysplasia
WP09-16	23	Female	negative	diarrhea
WP09-17	23	Female	negative	diarrhea
WP09-18	75	Female	negative	diarrhea

have found this to be limited to a subset of parasites [119]. Although not typically thought to play a role in bacterial infections, eosinophils have been demonstrated to protect against *Pseudomonas aeruginosa* infection *in vivo* [120]. These data suggest that eosinophils may be important to combating bacterial infections and prompt the question of how they function during CDI.

Given that an intact microbiota promotes IL-25 production and protects the host from CDI, we asked whether IL-25 signaling was downregulated during CDI and if its restoration would protect against mortality. Here we show that IL-25 protein was reduced during CDI in both humans and mice. Furthermore, repletion of IL-25 led to decreased CDI-associated mortality and morbidity that was independent of *C. difficile* bacterial burden. We established a novel role for eosinophils as the effector cell in IL-25 mediated protection against CDI-associated mortality and morbidity. Thus, our results identify IL-25 as the first immune mediator that is regulated by the microbiota and enhances survival during CDI.

Results

IL-25 is regulated by the microbiota and is suppressed during human and murine *Clostridium difficile* infection (CDI)

The presence of a healthy microbiota has been shown to both protect against susceptibility to and rescue from *Clostridium difficile* infection (CDI). The

expression of the type 2 cytokine IL-25 is dependent on the microbiota as demonstrated by its diminished expression in antibiotic treated and germ free mice (Zaph et al., 2008). Therefore, we hypothesized that IL-25 protein expression is decreased during CDI. To evaluate IL-25 protein regulation during human *C. difficile* disease, we stained colon biopsies of CDI negative (-) and CDI positive (+) patients (Figure 3.1A, Table 3.1) and scored IL-25 staining (Figure 3.1B). Significant reductions in IL-25 expression were observed in human CDI biopsies when compared to controls.

In order to understand whether decreased IL-25 expression during CDI was due to antibiotic treatment or the infection itself, we compared IL-25 protein in the cecum of C57BL/6J mice that were untreated, only given antibiotics, or after day 1, 2, and 3 of *C. difficile* infection. Our infection model consisted of approximately $10^{\wedge 4}$-$10^{\wedge 5}$ CFU of vegetative *C. difficile* (strain VPI10643) after antibiotic treatment [104]. This mode of infection results in significant intestinal tissue damage and a high mortality rate in mice and therefore is representative of patients with severe CDI symptoms. Immunohistochemical staining and protein measurements for IL-25 were performed on day 3 of infection, the time with greatest CDI-associated mortality. IL-25 production in cecal tissue was suppressed by antibiotics, and further diminished by CDI (Figure 3.2A,3.2B). These data suggest that the environment created by CDI not only sustains, but further decreases IL-25 protein levels. Separation of the colonic epithelial and lamina propria layers and analysis of IL-25 protein in mice during these conditions and time points indicated that protein was primarily found in epithelial

cells (Figure 3.2C). Epithelial cell-specific IL-25 was similarly reduced on day 3 of CDI (Figure 3.2D. Interestingly, IL-25 protein was observed in both epithelial cell and cells infiltrating the

Specific components of the microbiota regulate IL-25 expression in the lamina propria

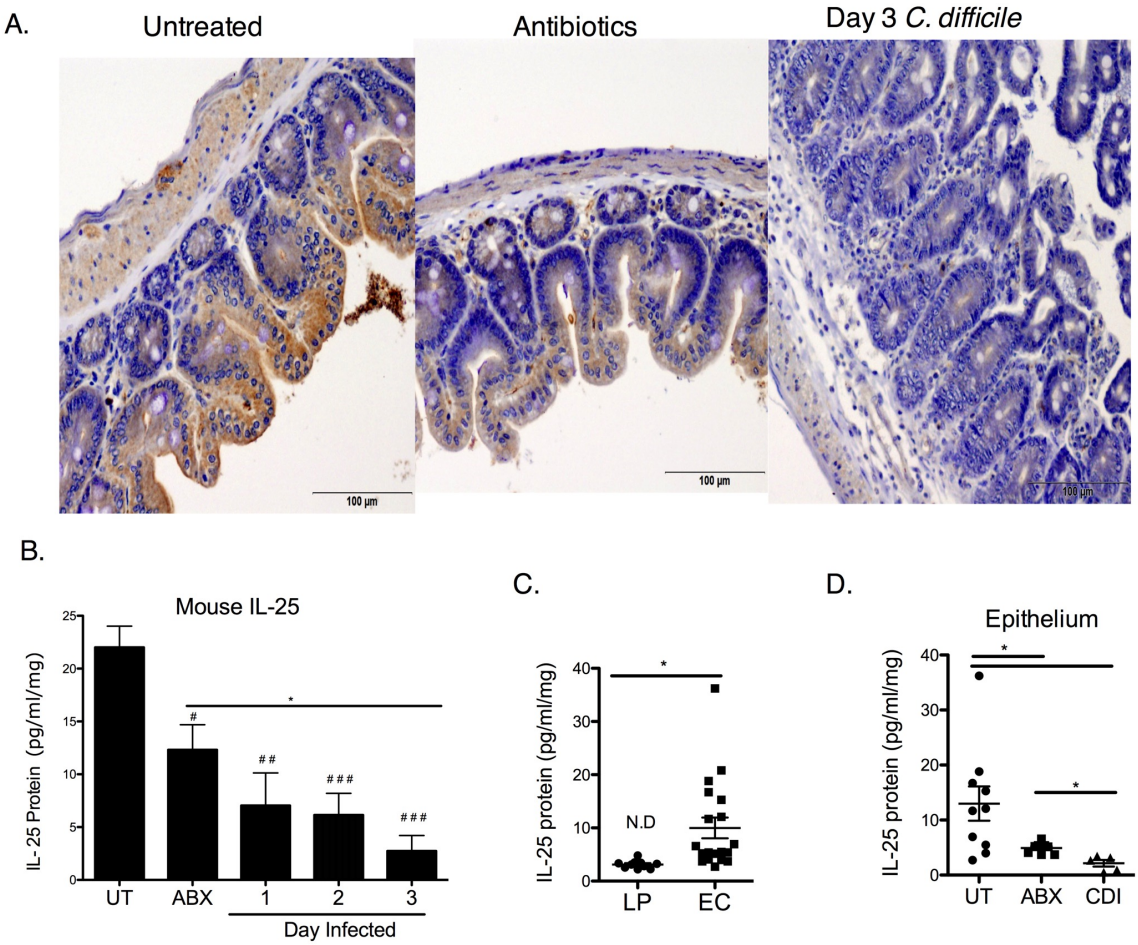
In order to begin to understand which components of the microbiota regulate IL-25 expression, we treated mice with different courses of antibiotic treatments and assessed IL-25 expression in cecal tissue (Figure 3.3). Mice were given regular water, the full antibiotic cocktail used in the CDI mouse model, or a single antibiotic treatment with clindamycin, metronidazole, gentamicin, or vancomycin. As expected, untreated mice given water had a significant increase in IL-25 expression compared to mice treated with the full cocktail of antibiotics. Treatment with clindamycin was the only antibiotic capable of independently reducing IL-25 expression to that of mice treated with the entire cocktail of antibiotics. Interestingly, mice treated with metronidazole alone had significantly enhanced IL-25 expression compared to mice given regular water. These data suggest that metronidazole specifically targets components of the microbiota that decrease IL-25 expression, while clindamycin actively targets components of the microbiota that induce IL-25.

Figure 3.2: IL-25 was suppressed during murine *Clostridium difficile* infection (CDI).

(A) Immunohistochemical staining for IL-25 in ceca of C57BL/6J mice that were untreated (UT), antibiotic treated (ABX), or infected with *C. difficile*.

(B) IL-25 protein in mouse cecal tissue measured by ELISA. Data are representative of two experiments; n= 5-8 mice per group per experiment; mean +/- SEM; p value from Abx treated *< 0.05, pvalue from untreated #<0.05, ##<0.005, ###<0.0005.

(C-D) Lamina propria (LP) and epithelial cells (EC) in the colon were separated and analyzed for IL-25 protein from untreated, antibiotic only, and day 3 post *C. difficile* infected mice. (C) Data represent IL-25 protein from combined time points (D) Data represent IL-25 protein in the epithelium alone on each time point. n=4-10 per group; mean +/- SEM; *p value < 0.05.


A.
Untreated
Antibiotics
Day 3 C. difficile
100 µm
100 µm
100 µm
B.
Mouse IL-25
IL-25 Protein (pg/ml/mg)
25
20
15
10
5
0
*
#
##
###
###
UT
ABX
1
2
3
Day Infected
C.
IL-25 protein (pg/ml/mg)
40
30
20
10
0
*
N.D
LP
EC
D.
Epithelium
IL-25 protein (pg/ml/mg)
40
30
20
10
0
*
*
UT
ABX
CDI

Restoration of IL-25 protected mice against CDI-associated mortality and morbidity

The microbiota can prevent susceptibility to CDI by outcompeting the pathogen and inducing host factors, yet potentially beneficial influences of the microbiota to modulate the immune response to regulate disease severity have not been previously examined. Our observation of decreased IL-25 protein during CDI suggested that IL-25 regulation of type 2 immunity could be a downstream mechanism of microbiota-mediated protection. To address this question, we tested if IL-25 could protect in the setting of antibiotics and active CDI. Mice were treated with a daily dose of 0.5 μg of recombinant IL-25 protein or PBS daily for five days prior to infection with $10^{\wedge 4}$-$10^{\wedge 5}$ CFU of *C. difficile* (Figure 3.4). Protection was assessed by mortality and a clinical scoring system of morbidity [105]. Restoration of IL-25 led to significant decreases in mortality (Figure 3.5A) and morbidity (Figure 3.5B) indicating that IL-25 reduced disease severity. IL-25 and PBS treated mice surprisingly had similar levels of *C. difficile* colony forming units (CFU) (Figure 3.6D) and virulence factors toxins A and B (Figure 3.6C) in the cecal contents, suggesting that IL-25 is likely not protecting the host by dampening *C. difficile* growth or toxin production. Immunohistological evaluation of the cecum at day 3 post infection showed that IL-25 significantly decreased CDI-associated tissue pathology (Figure 3.6A and 3.6B). IL-25 treated mice displayed decreased cellular exudate and inflammatory cell numbers in the lamina propria, but the most profound impact of IL-25 was observed in prevention of epithelial cell disruption at the intestinal barrier. IL-25 pretreatment was also

capable of reducing CDI-associated morbidity during both spore challenge (strain VPI10643) (Figure 3.7A and 3.7B) and using a different toxin A and B producing *C. difficile* strain (strain 630) (Figure 3.7C and 3.7D). We concluded that IL-25 reduced disease severity by protecting host tissue, rather then influencing the pathogen.

Pretreatment with IL-25 resulted in increased IL-4 and mucin production during CDI

The crosstalk between immune responses and the intestinal epithelial is critical to maintain homeostasis in the gut [121].Therefore, we hypothesized that IL-25 treatment mediated host tissue protection against CDI by influencing the immune response. To understand how IL-25 shaped the immune response to infection, we evaluated protein levels of inflammatory cytokines in cecal tissue on day 3 of infection (Figure 3.8). IL-23 has been published to have a deleterious role during CDI and has also been indicated to signal inversely of IL-25, therefore we examined whether IL-25 dampened IL-23 levels [47,72,73]. IL-25 pretreatment reduced IL-23 protein production, but had no effect on downstream Th17-like cytokines IL-17 or IL-22 (Figure 3.8). Next, we sought to evaluate how IL-25 pretreatment manipulated two canonical type 2 cytokines, IL-4 and IL-13. IL-25 has historically enhanced both cytokines, yet we only observed increased production of IL-4 during CDI (Figure 3.8. Conversely, we detected decreased IL-13 protein levels, suggesting that IL-25 selectively induced IL-4 during CDI (Figure 3.8). To test if induction of IL-13 by IL-25 occurs earlier than day 3, protein levels were also measured on days 0 and 2 without evidence of

increased protein with IL-25 treatment (Figure 3.9). Our studies were done in antibiotic treated and infected mice, which may explain the differences observed in IL-25 regulation of IL-13 in our study. Further investigation into the role of IL-13 during CDI is needed to establish the relevance of this observation. In order to evaluate the cellular source of IL-4, we utilized flow cytometry to intracellularly stain and measure lamina propria cell populations that were producing IL-4 on day 3 of infection (Figure 3.10). In agreement with protein levels, the absolute numbers of IL-4 producing cells were increased with IL-25 treatment (Figure 3.10A). The majority of IL-4 producing cells were $CD11b^+$ (Figure 3.10B). Further examination revealed that IL-4^+ cells were mainly $CD11b^+$ $SiglecF^+$, identifying eosinophils as the primary source of this cytokine during CDI (Figure 3.10C). Periodic acid-Schiff (PAS) staining (Figure 3.11A) and scoring (Figure 3.11B) of the cecal tissue on day 3 revealed that IL-25 also induced mucus production. RNA analysis of cecal tissue by qPCR confirmed increased transcripts for *muc2*, a gene which encodes a major component of mucin, in IL-25 treated mice (Figure 3.11C). These data prompted the hypothesis that IL-25 may protect the host by bolstering the physical mucus barrier lining the epithelial tissue or by inducing IL-4. We concluded that IL-25 decreased the deleterious cytokine IL-23, and increased IL-4 and mucin production during CDI.

Figure 3.3 Specific components of the microbiota regulate IL-25 expression.

C57BL/6 mice were treated with regular water, a full course of antibiotics or metronidazole, gentamicin, or vancomycin alone in their drinking water for 3 days. Mice treated with clindamycin alone were given one injection. All antibiotic treatments were discontinued one day prior to harvest. IL-25 expression was measured in cecal tissue of all groups. n=5 mice per group. *p value <0.05, **<0.005.

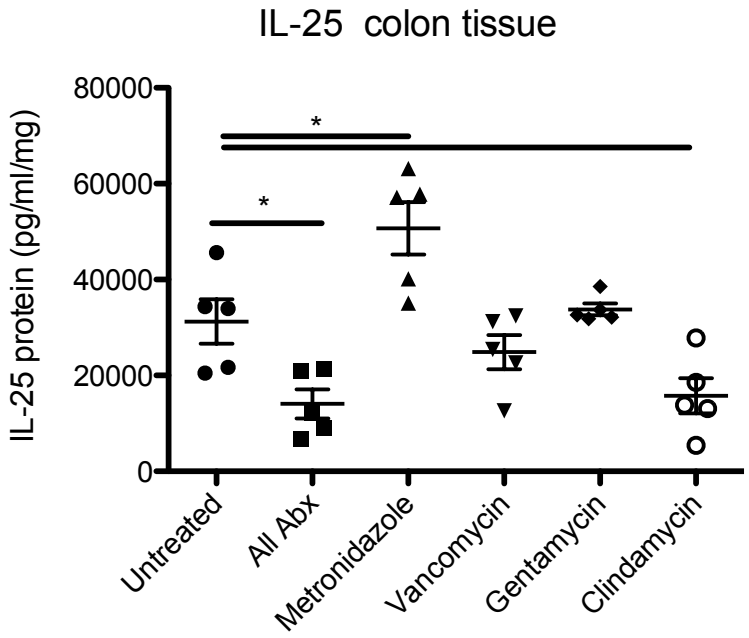

IL-25 colon tissue
IL-25 protein (pg/ml/mg)
80000
60000
40000
20000
0
*
*
Untreated
All Abx
Metronidazole
Vancomycin
Gentamycin
Clindamycin

Figure 3.4: Time course of IL-25 pretreatment and *Clostridium difficile* infection.

C57BL/6J mice received in their drinking water gentamicin (50 mg/ml), metronidazole (5 mg/ml), colistin (25 mg/ml), and vancomycin (50 mg/ml) for three days followed by two days of fresh water and a subsequent single intraperitoneal injection of clindamycin (10 mg/kg) one day prior to infection with 10^4-10^5 CFU of vegetative *C. difficile* (strain VPI10643). Mice were also given a daily dose of either 0.5 µg of recombinant IL-25 protein or PBS daily for five days prior to infection.

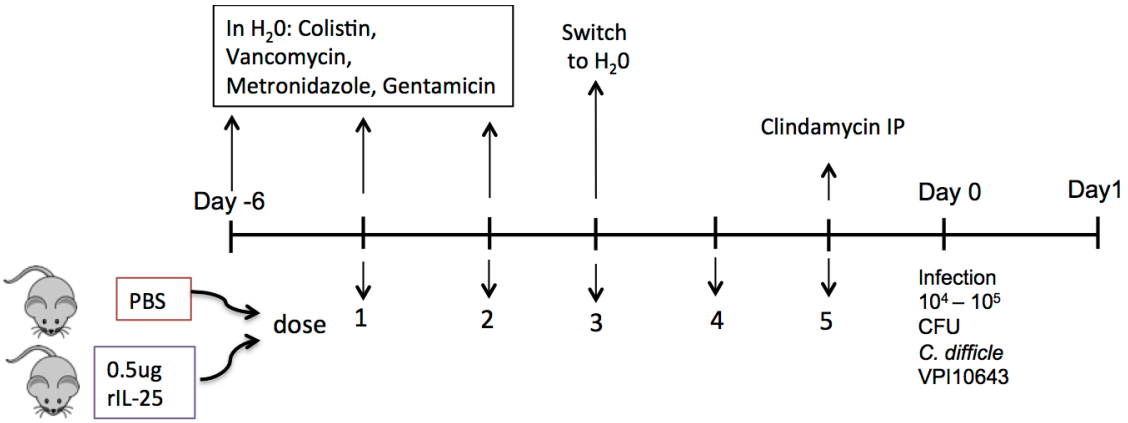
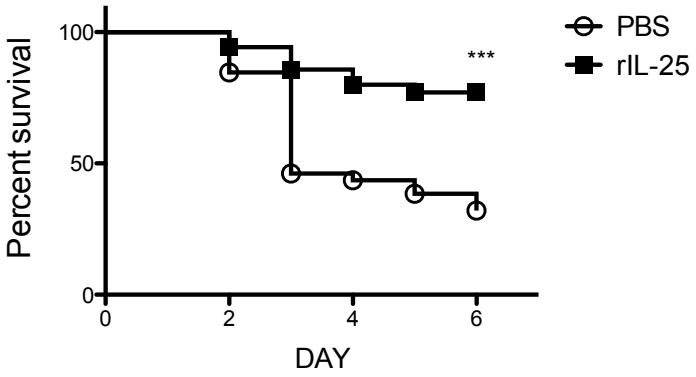
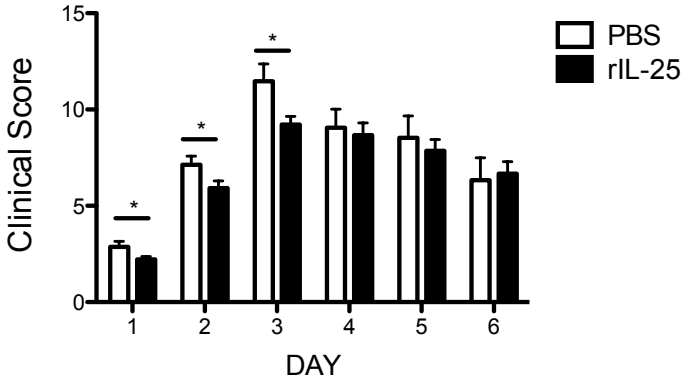

In H_20: Colistin,
Vancomycin,
Metronidazole, Gentamicin
Switch
to H_20
Clindamycin IP
Day -6
Day 0
Day1
PBS
0.5ug
rIL-25
dose
1
2
3
4
5
Infection
$10^4 – 10^5$
CFU
C. difficle
VPI10643

Figure 3.5: Recombinant IL-25 pretreatment protected against CDI-associated mortality and morbidity.

C57BL/6J mice were treated with a daily dose of either 0.5 µg of recombinant IL-25 protein or PBS daily for five days prior to infection with *C. difficile*.

(A) Survival and (B) clinical scores over the initial 6 days of infection. Data representative of four experiments; n=6-10 mice per group per experiment; mean +/- SEM; * <0.05, ***<0.0005.


Percent survival
100
50
0
0
2
4
6
DAY
***
PBS
rIL-25


Clinical Score
15
10
5
0
1
2
3
4
5
6
DAY
*
*
*
PBS
rIL-25

Accumulation of eosinophils, but not neutrophils in the lamina propria was observed with IL-25 treatment

Neutrophils are considered the hallmark innate effector cell of human *C. difficile* infection, while IL-25 signaling is primarily associated with eosinophilia [116]. Therefore, we sought to identify the ability of IL-25 to modulate neutrophils, eosinophils, and Ly6c^{hi} monocytes in lamina propria of the colon on day 3 of CDI. Eosinophils (Figure 3.12A) and neutrophils (Figure 3.12B) were increased during infection compared to antibiotic treated mice, indicating that both granulocyte subsets were recruited to the lamina propria during infection. Neutrophils (Figure 3.12B) and Ly6c^{hi} monocytes (Figure S4) were unchanged by IL-25 during infection in terms of absolute cell numbers and percentage of live cells. Absolute cell numbers and percentages of eosinophils were enhanced by IL-25 during CDI (Figure 3.12A). Increased eosinophilia by both measurements correlated with decreased clinical scores (Figures 3.13) implying that eosinophilia may play a role in dampening CDI severity. These data demonstrated that IL-25 pretreatment selectively enhanced eosinophil, but not neutrophil, accumulation during CDI and prompted the hypothesis that eosinophils facilitate IL-25 mediated protection.

Figure 3.6: Recombinant IL-25 pretreatment protected against CDI-associated mortality and morbidity without changing *C. difficile* CFU or toxin.

(A) Representative H&E stained cecal sections of mice on day 3 after infection with *C. difficile* (Scale = 100 μM) and (B) pathology scores. (C) Toxin A/B levels and (D) *C. difficile* bacterial burden in cecal contents on day 3 post *C. difficile* infection. Data are representative of two experiments; n= 4-7 mice per group per experiment; mean +/- SEM; *<0.05, **<0.005.

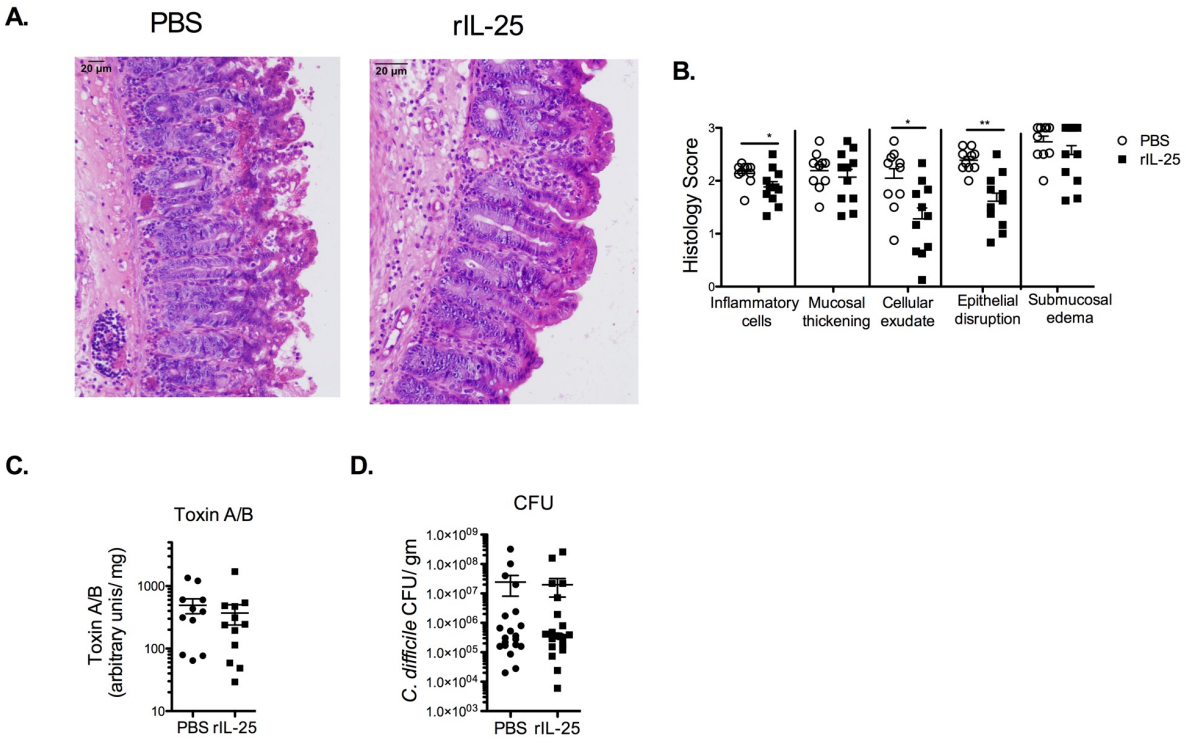
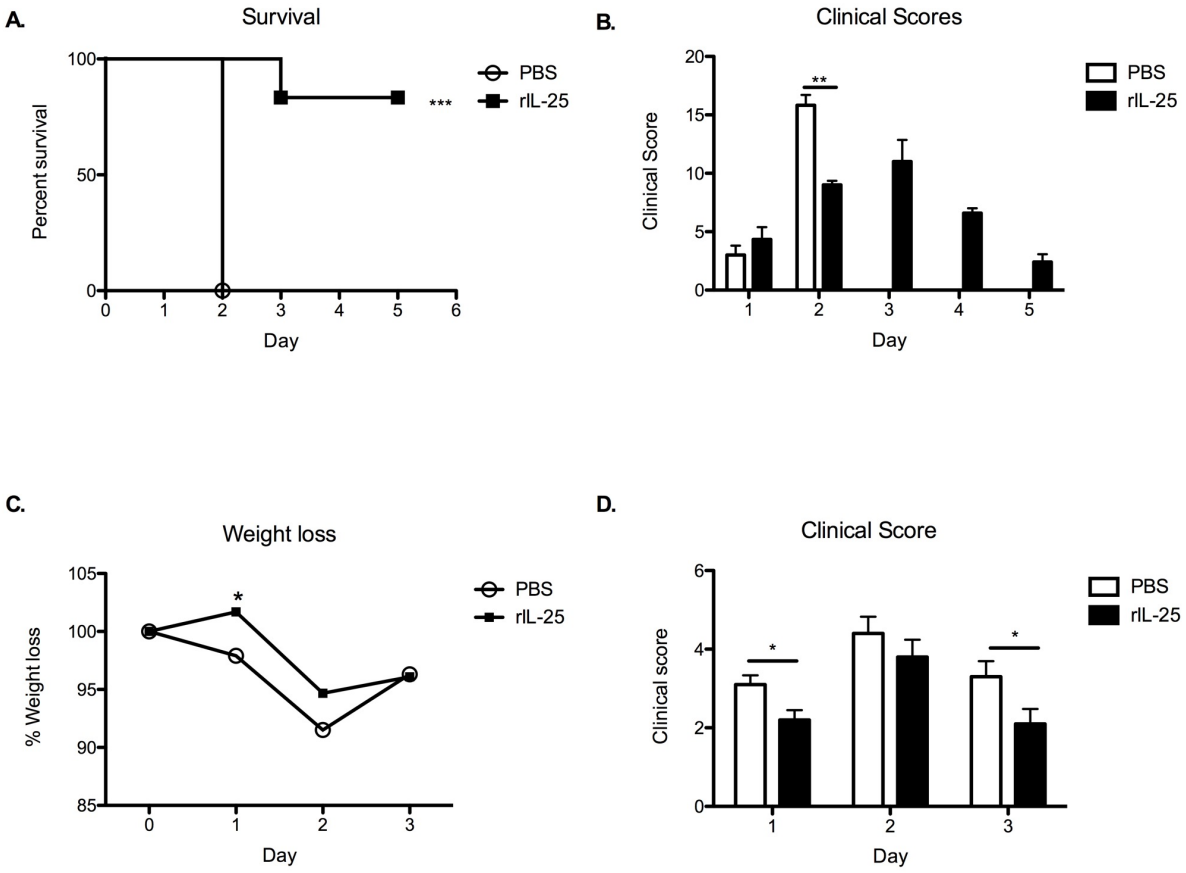

A.
PBS
rIL-25
20 µm
20 µm
B.
Histology Score
0
1
2
3
*
*
**
PBS
rIL-25
Inflammatory cells
Mucosal thickening
Cellular exudate
Epithelial disruption
Submucosal edema
C.
Toxin A/B
Toxin A/B (arbitrary unis/ mg)
1000
100
10
PBS rIL-25
D.
CFU
C. difficile CFU/ gm
1.0×10^09
1.0×10^08
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
PBS rIL-25

Figure 3.7: IL-25 also protects against *C. difficile* strain 630 vegetative bacterial challenge and a strain VPI 10643 spore challenge.

(A-D) C57BL/6J mice were treated with a daily dose of either 0.5 µg of recombinant IL-25 protein or PBS prior to CDI.

(A)Survival and (B) clinical scores were evaluated during the first 5 days of infection with 10^5 spores (strain VPI10643). n=6 mice per group; mean +/- SEM; *<0.05, ***<0.001

(C) Weight loss and (D) clinical scores were evaluated during the first 3 days of infection with 10^6 CFU of C. difficile (strain 630). N=10 mice per group; mean +/- SEM; *<0.05


A.
Survival
Percent survival
100
50
0
0 1 2 3 4 5 6
Day
***
PBS
rIL-25
B.
Clinical Scores
Clinical Score
20
15
10
5
0
1 2 3 4 5
Day
**
PBS
rIL-25
C.
Weight loss
% Weight loss
105
100
95
90
85
0 1 2 3
Day
*
PBS
rIL-25
D.
Clinical Score
Clinical score
6
4
2
0
1 2 3
Day
*
*
PBS
rIL-25

Eosinophils are essential for IL-25 mediated protection against CDI severity

IL-25 pretreatment decreased mortality and induced robust eosinophilia during CDI. Furthermore, increased eosinophils in the colon correlated with less severe clinical scores. This led us to hypothesize that eosinophils may be downstream of IL-25 mediated protection. Two distinct models where mice lacked eosinophils were utilized to test the hypothesis. First, PBS and IL-25 treated mice were treated with either an eosinophil depleting anti-SiglecF monoclonal antibody or an IgG isotype antibody. Anti-SiglecF treatment selectively depleted eosinophils, as demonstrated by a significant decrease in eosinophils (Figure 3.15A), but not neutrophils (Figure 3.15B), in the lamina propria of the colon [78,122]. Similarly, total IL-4 (Figure 3.15C) and $CD11b^{+}$ $IL\text{-}4^{+}$ (Figure 3.15D) expressing cells were significantly reduced with anti-SiglecF treatment. This was expected since eosinophils are the primary producers of IL-4 during CDI. Mice lacking eosinophils with anti-SiglecF depletion experienced increased mortality (Figure 3.14A) and morbidity (Figure 3.14B) despite IL-25 treatment demonstrating that eosinophils were essential for IL-25 mediated protection. Secondly, PHIL mice, transgenic mice that genetically lack eosinophils, and wild type littermate controls were treated with PBS or IL-25 and assessed for survival rates (Figure 3.14C) and clinical scores (Figure 3.14D) during infection [70]. Mice were treated with a sub-lethal dose of $10^{\wedge 3}$ CFU of *C. difficile* in order to delineate differences between genotypes. In addition to a lower dose of *C. difficile*, reduced mortality observed in PBS treated mice may also be a result of differences in initial microbiota as mice used in PHIL experiments were the only mice bred at the

University of Virginia vivarium. In agreement with antibody-mediated depletion of eosinophils, PHIL mice could not be rescued from severe disease with IL-25 pretreatment. Enhanced disease severity in PHIL mice, regardless of IL-25 treatment, compared to wildtype controls stresses the importance of eosinophils to reduce disease severity during CDI. This experiment independently demonstrated that eosinophils are an essential component to IL-25 mediated protection during CDI. We concluded that eosinophils were the cellular mechanism by which IL-25 protects against CDI mortality and morbidity.

Eosinophils did not protect by inducing IL-4 or mucin production

IL-25 treatment led to enhanced IL-4 production. Since eosinophils were the primary source of IL-4 during CDI and were necessary for IL-25 mediated reduction in mortality, we speculated that IL-4 production may be the mechanism by which eosinophils reduce disease severity. To test this, we compared survival (Figure 3.16A) and morbidity (Figure 3.16B) in PBS control, IL-25 treated, and IL-25+anti-IL4 monoclonal antibody treated mice. Neutralization of IL-4 did not influence mortality (Figure 3.16A) but did slow disease resolution (Figure 3.16B). These data suggest that IL-4 does not play a role increasing survival during initial disease, but may be important in disease resolution.

Increased mucin was also observed with IL-25 treatment during CDI. Mucus plays a fundamental role in providing a second barrier between the epithelium and commensal bacteria, therefore we hypothesized that enhancement of this barrier may be beneficial to protect against *C. difficile.*

Figure 3.8: IL-25 pretreatment increased IL-4 and reduced IL-23 expression. (A) ELISA analysis of protein expression of type 17 and type 2 cytokines in the colon of C57BL/6J mice on day 3 of *C. difficile* infection. Data are representative of two experiments; n=6-8 mice per group per experiment; mean +/- SEM; *<0.05, **<0.005.

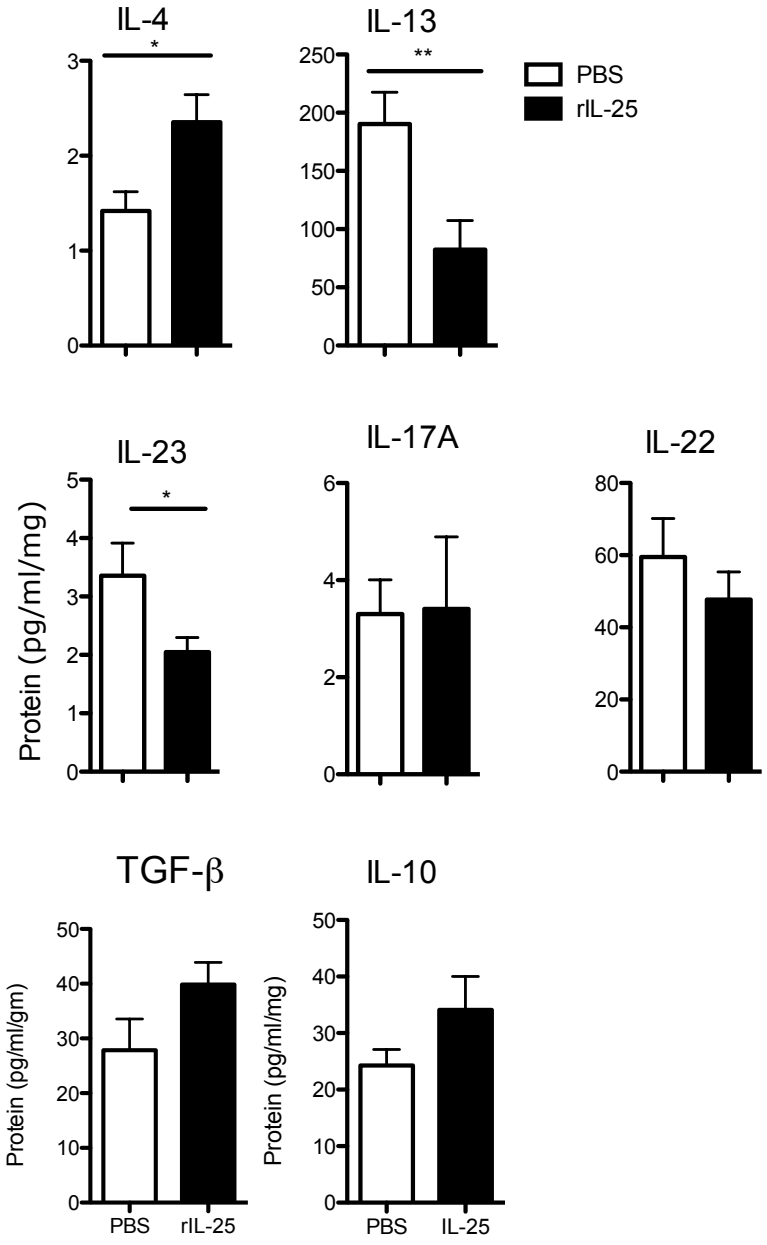

IL-4
IL-13
PBS
rIL-25
IL-23
IL-17A
IL-22
Protein (pg/ml/mg)
TGF-β
IL-10
Protein (pg/ml/gm)
Protein (pg/ml/mg)
PBS
rIL-25
PBS
IL-25

Figure 3.9: IL-13 protein was not influenced at day 0 and day 2, but suppressed on day 3 of CDI infection with IL-25 treatment.

ELISA analysis of protein expression of IL-13 in the colon of C57BL/6J mice on Day 0, 2 and 3 of *C.difficile* infection +/- IL-25 treatment. Data are representative of two experiments; n=6-8 mice per group per experiment.; mean +/- SEM; **<0.005.

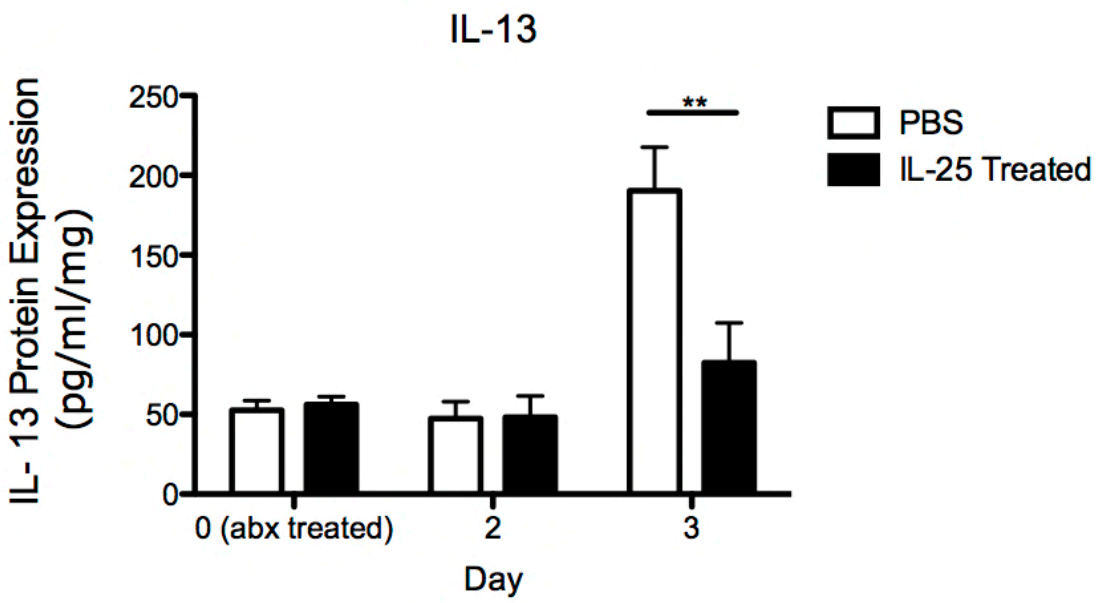

IL-13
IL- 13 Protein Expression (pg/ml/mg)
250
200
150
100
50
0
**
PBS
IL-25 Treated
0 (abx treated)
2
3
Day

Figure 3.10: Eosinophils were the main producers of IL-4 during CDI.

(A-D) Cells from the lamina propria of the colon of untreated or Day 3 infected C57BL/6J mice +/- rIL-25 mice were analyzed by flow cytometry. Gating strategy of Live^{+} singlets^{+} CD45^{+} was used for all populations.

Representative FACs plots and absolute cell count for: (A) total IL-4^{+} cells; (B) CD11b^{+} IL-4^{+} and CD11b^{-} IL-4^{+} cells; and (C) CD11b^{+} CD11c^{mid} Ly6g^{-} SiglecF^{+} IL-4^{+} cells. Data are representative of two experiments; n= 4-6 mice per group per experiment; mean +/- SEM; **<0.005.

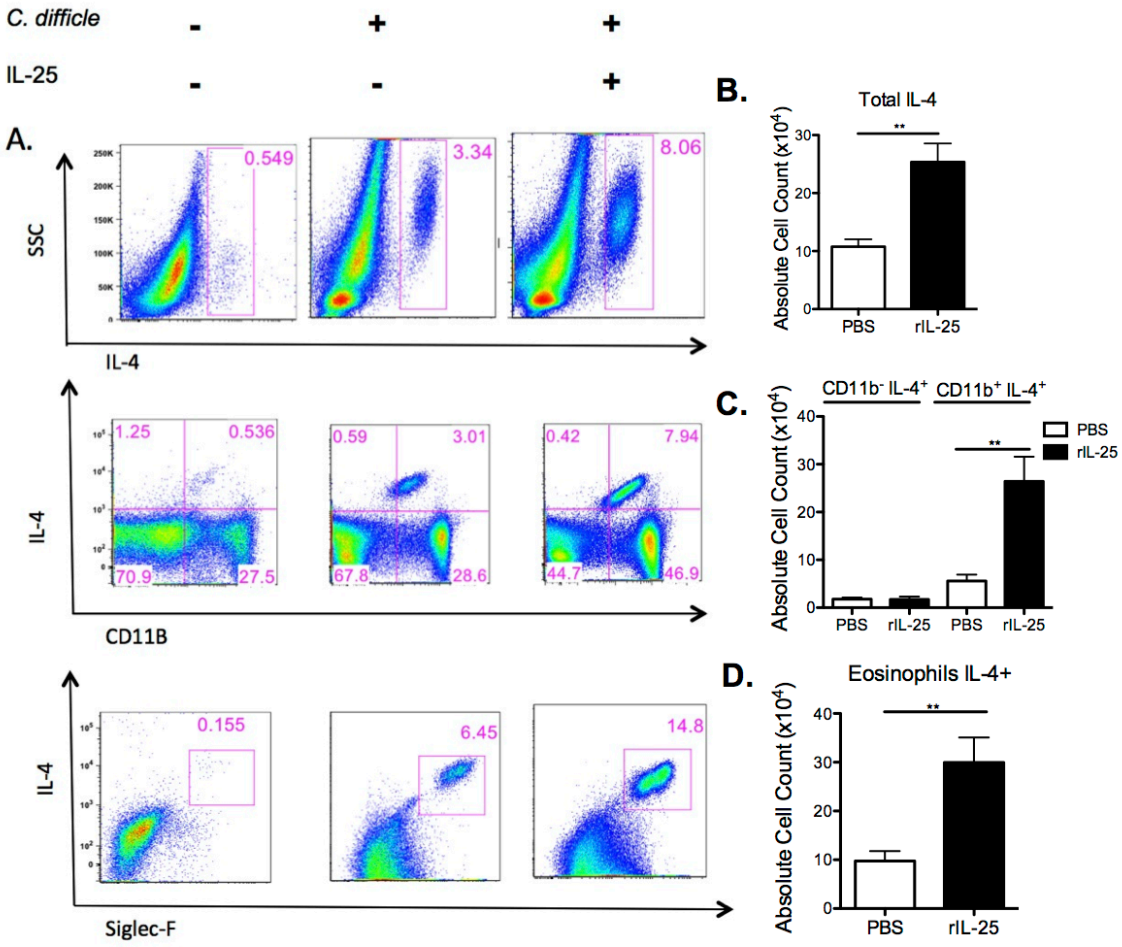
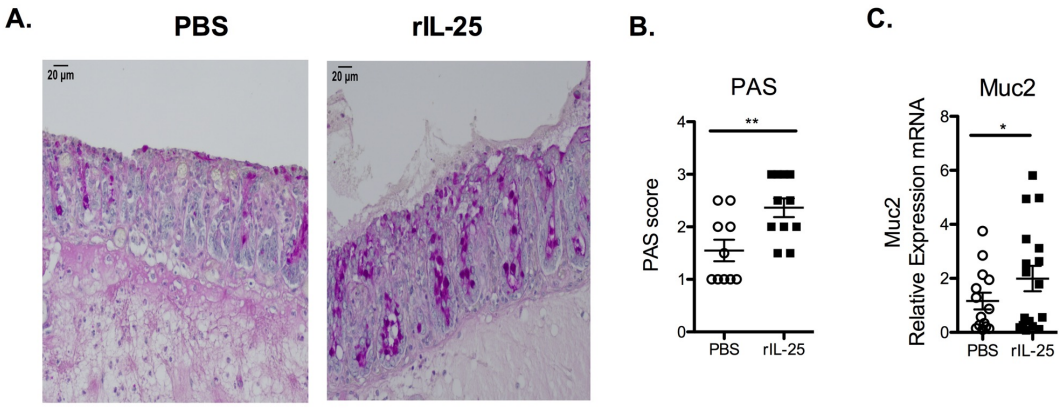

C. difficile - + +
IL-25 - - +
A.
SSC
IL-4
0.549
3.34
8.06
IL-4
CD11B
1.25
0.536
70.9
27.5
0.59
3.01
67.8
28.6
0.42
7.94
44.7
46.9
IL-4
Siglec-F
0.155
6.45
14.8
B.
Total IL-4
Absolute Cell Count (x10⁴)
PBS
rIL-25
C.
CD11b⁻ IL-4⁺
CD11b⁺ IL-4⁺
PBS
rIL-25
Absolute Cell Count (x10⁴)
PBS rIL-25 PBS rIL-25
D.
Eosinophils IL-4+
Absolute Cell Count (x10⁴)
PBS
rIL-25

Figure 3.11: IL-25 pretreatment increased mucin expression during CDI.

(B) Periodic acid-Schiff (PAS) staining of mucins in control and IL-25 treated cecal sections (Scale=100 μM) and (C) scoring on day 3 of *C. difficile* infection. Data are representative of two experiments; n=4-6 mice per group per experiment; mean +/- SEM; **p<0.005.

(D) Fold change of *muc2* RNA in cecal tissue by qPCR relative to *gapdh* and *actin*. Data are representative of three experiments; n=4-6 mice per group per experiment; mean +/- SEM; *<0.05.


A.
PBS
rIL-25
20 µm
20 µm
B.
PAS
**
PAS score
0
1
2
3
4
PBS
rIL-25
C.
Muc2
*
Muc2
Relative Expression mRNA
0
2
4
6
8
PBS
rIL-25

In order to test this, we compared *muc2* gene expression (Figure 3.16C) and mucin by PAS histological analysis (Figure 3.16D) in PBS control, IL-25 treated, and IL-25 treated, eosinophil depleted mice on day 3 of infection. We did not observe differences in mucin production regardless of the absence of eosinophils. We concluded that mucin is not likely to contribute to the protective capabilities of eosinophils.

Lastly, due to mounting evidence supporting a role for eosinophils in promoting and maintaining IgA responses, we tested whether eosinophils may be protective via antibody responses in the gut [122]. We measured total IgA (Figure 3.16E) and IgG (Figure 3.16F) levels in the cecal contents of PBS controls, IL-25 treated, and IL-25 treated, eosinophil depleted mice on day 3 post infection. IgG production was comparable in all groups of mice demonstrating that total IgG levels did not correlate with disease outcome. Although we did observe increased IgA levels with IL-25 treatment, we did not see a difference in the absence of eosinophils, indicating that they did not influence total IgA production during the initial 3 days of CDI infection. Together these data signify that IL-4, mucin, IgA, and IgG responses were likely not responsible for the ability of eosinophils to reduce mortality during CDI.

Eosinophils protected the intestinal epithelial barrier during CDI

In order to understand how eosinophils may be protecting against CDI-associated mortality, we investigated the impact of eosinophil depletion on the colonic intestinal epithelial barrier. Immunohistochemical staining (Figure 3.17A)

and scoring (Figure 3.17B) of cecal tissue was analyzed in PBS controls, IL-25 treated, and IL-25 treated, eosinophil depleted mice on day 3 post infection. In the absence of eosinophils, IL-25 was unable to reduce intestinal epithelium pathology as evidenced by increased epithelial destruction and cellular exudate. All infected groups of mice had raised albumin levels in the cecal contents compared to untreated or antibiotic-only treated mice indicating barrier disruption during CDI (Figure 3.18A). Mice lacking eosinophils were the only group to have significantly elevated luminal albumin compared to protected IL-25 treated mice demonstrating that these mice had the most severe barrier disruption (Figure 3.18A). In line with these findings, IL-25 treated mice lacking eosinophils and PBS control mice had significantly shorter colon length, a measure of more severe colitis, when compared to IL-25 treated mice (Figure 3.18B). These data suggest that the loss of eosinophils permitted the most drastic intestinal tissue damage, even in the presence of IL-25. In order to rule out the possibility that eosinophils protected mice from mortality by a direct bactericidal function against *C. difficile*, we quantified *C. difficile* toxins A and B (Figure 3.18C) and bacterial burden (Figure 3.18D) in the cecal contents on day 3 of infection. We found comparable levels in all groups of mice, indicating that eosinophils do not alter the ability of *C. difficile* to colonize and produce toxins in the colon. Together, these data signify that eosinophils contribute to IL-25 mediated protection during CDI by protecting host tissue, rather than reducing the capabilities of the pathogen.

Figure 3.12: IL-25 increased lamina propria eosinophils during CDI.

(A-C) C57BLl/6J mice were infected with 10^4 CFU of *C. difficile* +/- rIL-25 and colon were isolated on Day 3.

(A) $CD45^+$ $CD11b^+$ $CD11c^{mid}$ $SiglecF^+$ $Ly6g^-$ eosinophils and (B) $CD45^+$ $CD11b^+$ $Ly6g^+$ $Ly6c^+$ neutrophils were isolated from the colonic lamina propria and quantified by flow cytometry for absolute numbers and percentage of live cells on day 3 of CDI. Representative flow plot shows percentage eosinophils from $CD11b^+$ $CD11c^{mid}$ parent population. Data are representative of three experiments; n= 4-6 mice per group per experiment; mean +/- SEM; **<0.005. #<0.05 from uninfected PBS treated group.

(C) $CD45^+$ $CD11b^+$ $CD11c^-$ $Ly6g^-$ $Ly6c^{hi}$ monocytes were isolated and quantified by flow cytometry for absolute numbers and percentage of live cells in the lamina propria of the entire colon on day 3 of CDI. Data are representative of two experiments; n= 4-6 mice per group per experiment; mean +/- SEM

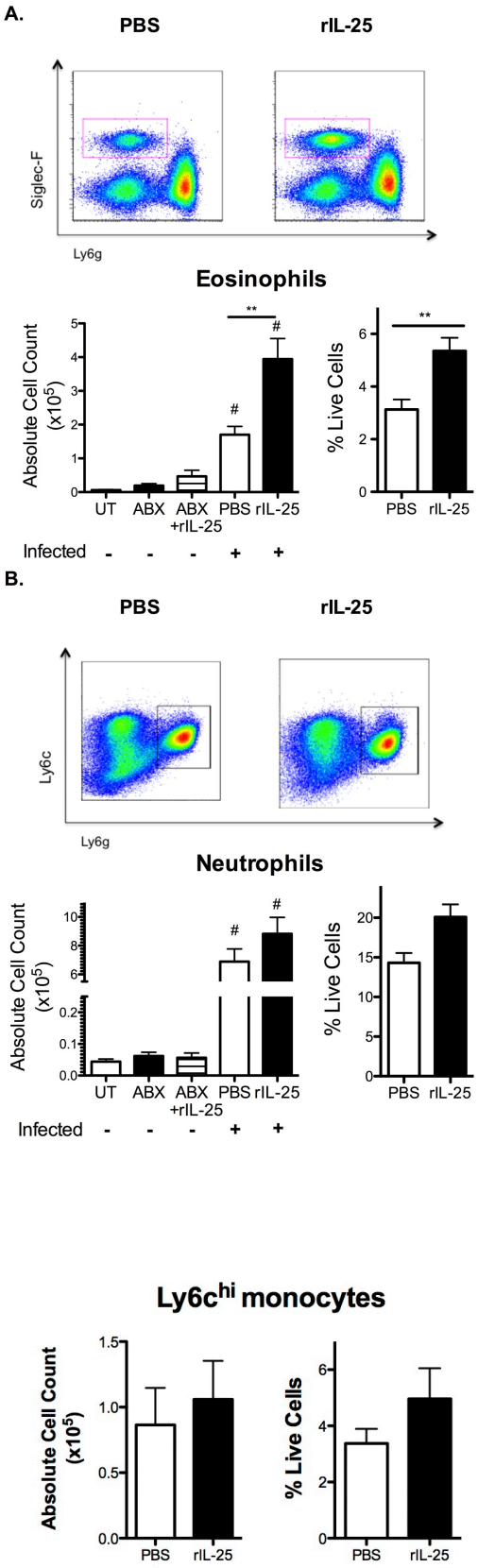
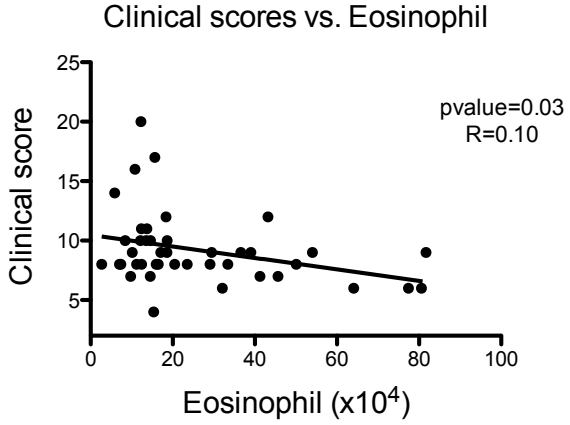

A.
PBS
rIL-25
Siglec-F
Ly6g
Eosinophils
Absolute Cell Count (x10^5)
% Live Cells
UT
ABX
ABX +rIL-25
PBS
rIL-25
Infected
B.
PBS
rIL-25
Ly6c
Ly6g
Neutrophils
Absolute Cell Count (x10^5)
% Live Cells
Ly6c^hi monocytes
Absolute Cell Count (x10^5)
% Live Cells

Figure 3.13: Eosinophilia correlated with reduced clinical scores during CDI.

Absolute number (top) and percentage of live eosinophils (bottom) in the lamina propria of PBS and IL-25 treated mice plotted against clinical scores on day 3 of CDI. Data are representative of three experiments; n= 4-6 mice per group per experiment; mean +/- SEM


Clinical scores vs. Eosinophil
pvalue=0.03
R=0.10
Clinical score
Eosinophil (x10^4)
0
20
40
60
80
100
5
10
15
20
25

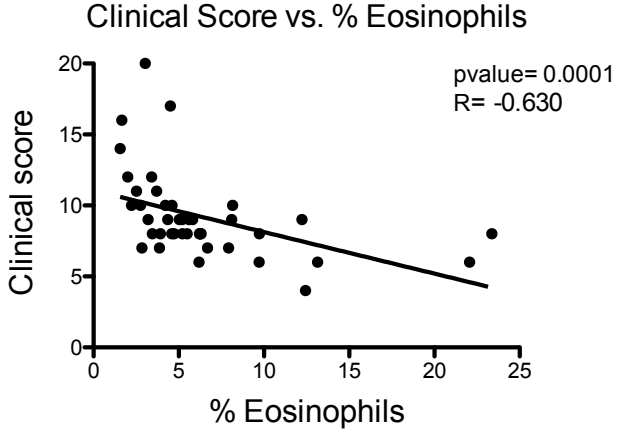

Clinical Score vs. % Eosinophils
pvalue= 0.0001
R= -0.630
Clinical score
% Eosinophils
0
5
10
15
20
25

Figure 3.14: IL-25 protected from CDI through an eosinophil-dependent mechanism.

(A-B) C57BL/6J mice +/- IL-25 treatment were given with 20 µg of anti-SiglecF or isotype control one day prior and one day after infection with *C. difficile* and assessed for (A) survival and (B) clinical scores during infection. Data are representative of two experiments; n=10 mice per group per experiment; mean +/- SEM; *<0.05.

(C-D) C57BL/6J or PHIL mice +/- IL-25 treatment and infected with a sub-lethal dose of $10^{\wedge 3}$ CFU of *C. difficile* were assessed for (C) survival and (D) clinical scores. n=5-7 mice per group; mean +/- SEM; p value +< 0.05 compared to C57BL/6+PBS infected mice.

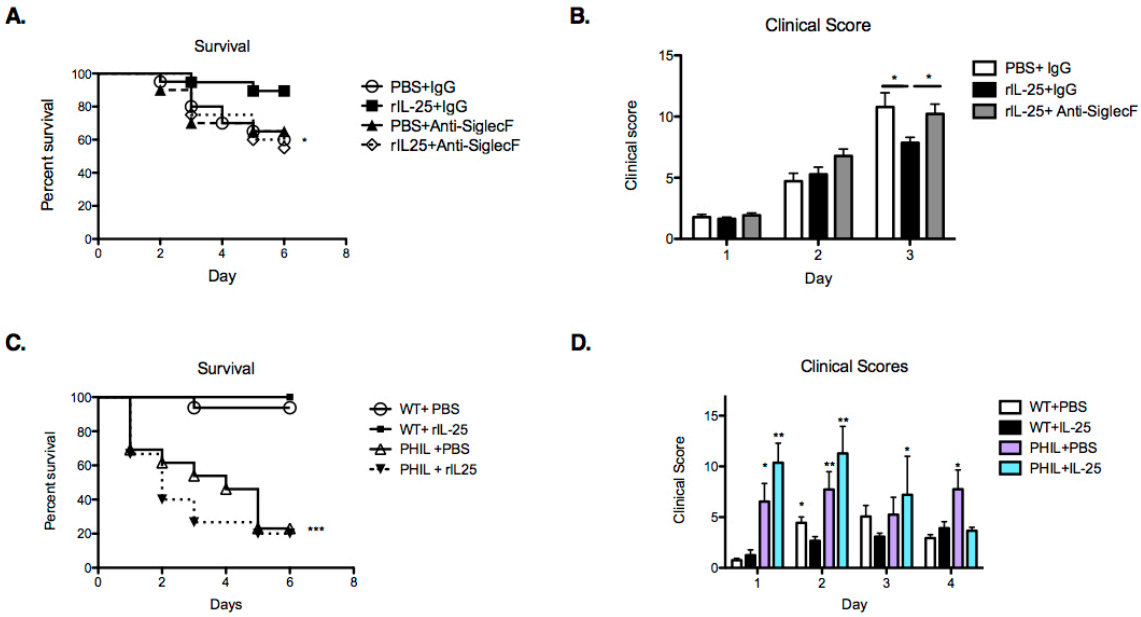

A.
Survival
Percent survival
Day
PBS+IgG
rIL-25+IgG
PBS+Anti-SiglecF
rIL25+Anti-SiglecF
B.
Clinical Score
Clinical score
Day
PBS+ IgG
rIL-25+IgG
rIL-25+ Anti-SiglecF
C.
Survival
Percent survival
Days
WT+ PBS
WT+ rIL-25
PHIL +PBS
PHIL + rIL25
D.
Clinical Scores
Clinical Score
Day
WT+PBS
WT+IL-25
PHIL+PBS
PHIL+IL-25

Figure 3.15: Anti-SiglecF treatment depleted eosinophils and IL-4 protein but did not influence neutrophil numbers.

C57BL/6J mice were treated with a daily dose of either 0.5 µg of recombinant IL-25 protein or PBS +/- 20 µg of monoclonal anti-SiglecF one day prior and one day after infection with 10^4 CFU of *C. difficile*. (A) Eosinophil ($CD45^+$ $CD11b^+$ $CD11c^{mid}$ $SiglecF^+$ $Ly6g^-$), (B) neutrophils ($CD45^+$ $CD11b^+$ $Ly6g^+$ $Ly6c^+$), (C) $CD45^+$ total $IL\text{-}4^+$ cells, and (D) $CD45^+$ $CD11b^+$ $IL\text{-}4^+$ cells were quantified from the lamina propria of the colon on Day 3 of CDI. Data are representative of two experiments; n= 4-6 mice per group per experiment; mean +/- SEM; **<0.005.

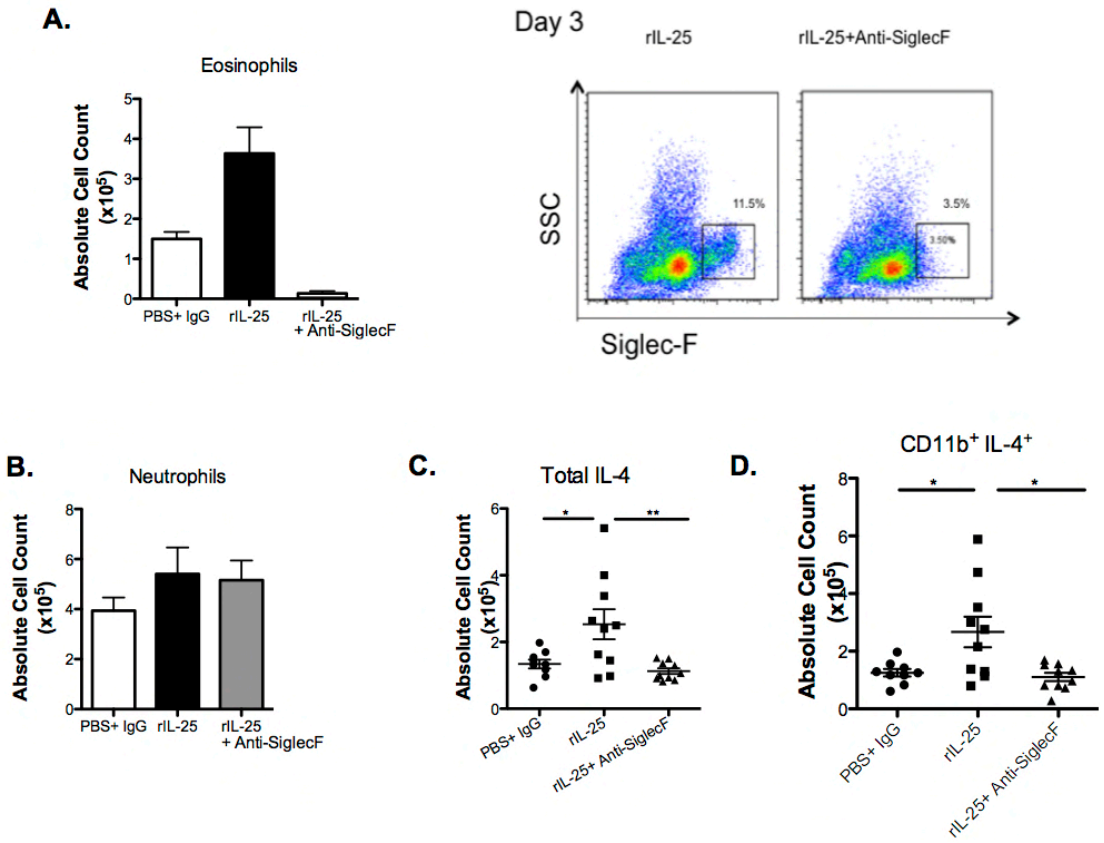

A.
Eosinophils
Absolute Cell Count (x10^5)
PBS+ IgG
rIL-25
rIL-25 + Anti-SiglecF
Day 3
rIL-25
rIL-25+Anti-SiglecF
SSC
Siglec-F
11.5%
3.5%
B.
Neutrophils
Absolute Cell Count (x10^5)
PBS+ IgG
rIL-25
rIL-25 + Anti-SiglecF
C.
Total IL-4
Absolute Cell Count (x10^5)
PBS+ IgG
rIL-25
rIL-25+ Anti-SiglecF
D.
CD11b+ IL-4+
Absolute Cell Count (x10^5)
PBS+ IgG
rIL-25
rIL-25+ Anti-SiglecF

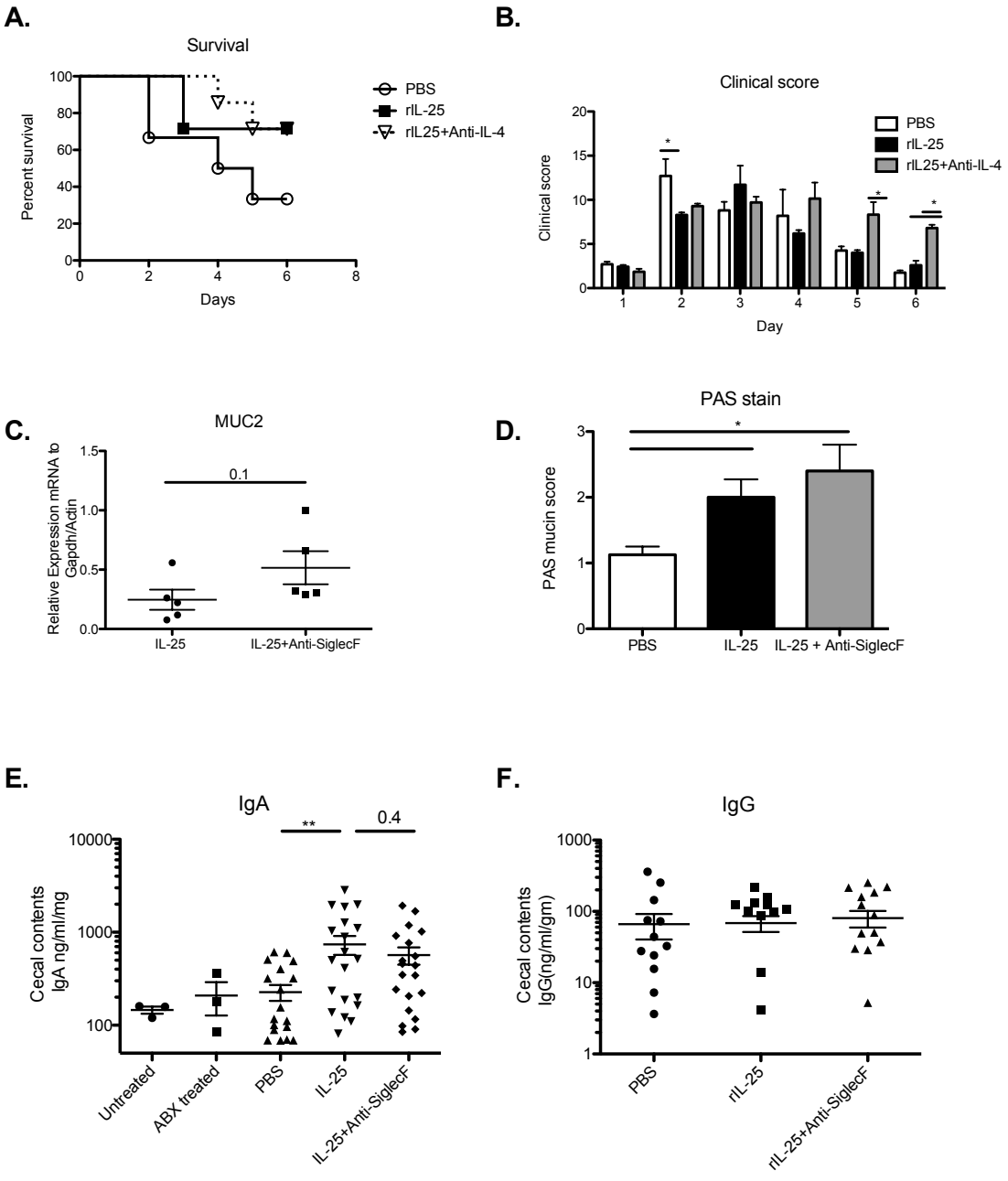
Figure 3.16: IL-25 pretreatment did not protect by inducing IL-4, mucin, IgG or IgA responses during CDI.

(A-B) C57BL/6J mice were treated with a daily dose of either 0.5 µg of recombinant IL-25 protein or PBS +/- 1mg of Anti-IL-4 one day prior and one day after infection with *C difficile.* (A) Survival and (B) clinical scores were evaluated during the first 6 days of infection. n=7 mice per group; mean +/- SEM; *<0.05.

(C-F) C57BL/6J mice were treated with a daily dose of either 0.5 µg of recombinant IL-25 protein or PBS +/- 20µg of monoclonal anti-SiglecF one day prior and one day after infection with *C difficile.*

Fold change of *muc2* RNA in cecal tissue by (C) qPCR relative to *gapdh* and *actin.* (D) PAS scoring by a blinded scorer from mice on Day 3 of *C.difficile* infection. n=5 mice per group; mean +/- SEM; *<0.05.

(E) IgA and (F) IgG levels were quantified in the cecal contents on Day 3 of infection. Data are representative of two-three experiments; n= 4-6 mice per group per experiment; mean +/- SEM; **<0.005.


A.
Survival
Percent survival
Days
PBS
rIL-25
rIL25+Anti-IL-4
B.
Clinical score
Clinical score
Day
PBS
rIL-25
rIL25+Anti-IL-4
C.
MUC2
Relative Expression mRNA to Gapdh/Actin
0.1
IL-25
IL-25+Anti-SiglecF
D.
PAS stain
PAS mucin score
PBS
IL-25
IL-25 + Anti-SiglecF
E.
IgA
Cecal contents IgA ng/ml/mg
0.4
Untreated
ABX treated
PBS
IL-25
IL-25+Anti-SiglecF
F.
IgG
Cecal contents IgG(ng/ml/gm)
PBS
rIL-25
rIL-25+Anti-SiglecF

Eosinophils may combat commensal bacteria locally or systemically

To investigate the interactions between eosinophils and the microbiota or *C. difficile* during active CDI, we analyzed the translocation of bacteria by measuring the bacterial burden of aerobic bacteria, *C. difficile*, and toxins A/B in the liver of PBS, rIL-25, and rIL-25+anti-siglecF treated mice on day 3 post infection (Figure 3.19). *C. difficile* did not translocate to the liver in any of the groups (Figure 3.19B). Although toxins were detectable in the liver, they translocated equivalently despite treatment groups suggesting they did not play a role in disease outcome (Figure 3.19C). Interestingly, IL-25 treatment significantly reduced bacterial translocation of aerobic bacteria to the liver, and eosinophil depletion negated this phenomenon (Figure 3.19A). Upon bacterial culture and sequencing, *Enterobacter cloacae* and *Enterococcus faecalis* were the main species observed migrating to the liver in the absence of IL-25 mediated eosinophilia. Both *E. cloacae* and *E. faecalis* are documented pathobionts and have been associated with CDI. For instance, *E. cloacae* was identified as driving CDI-associated morbidity in the mouse model and *E. faecalis* is associated with susceptibility to CDI in humans [31,42].

In an attempt to determine whether translocation of *E. cloacae* and *E. faecalis* contributed to increased mortality in IL-25+anti-siglecF treated mice, we compared IL-25 mediated eosinophilia depleted mice with or without ampicillin treatment (the goal of ampicillin was to target both *E. facaelis* and *E. cloacae* but not *C. difficile)*. Eosinophil-depleted mice treated with intraperitoneal ampicillin

Figure 3.17: Eosinophils were necessary for IL-25-mediated maintenance of the intestinal epithelial barrier during CDI.

(A) H&E staining and (B) tissue pathology scores of cecal tissue from C57BL/6J mice treated with PBS, rIL-25, or rIL-25+anti-Siglecf on day 3 of CDI (Scale=100μm). Data are representative of two experiments; n=4-7 mice per group per experiment; mean +/- SEM; *<0.05, **<0.005.

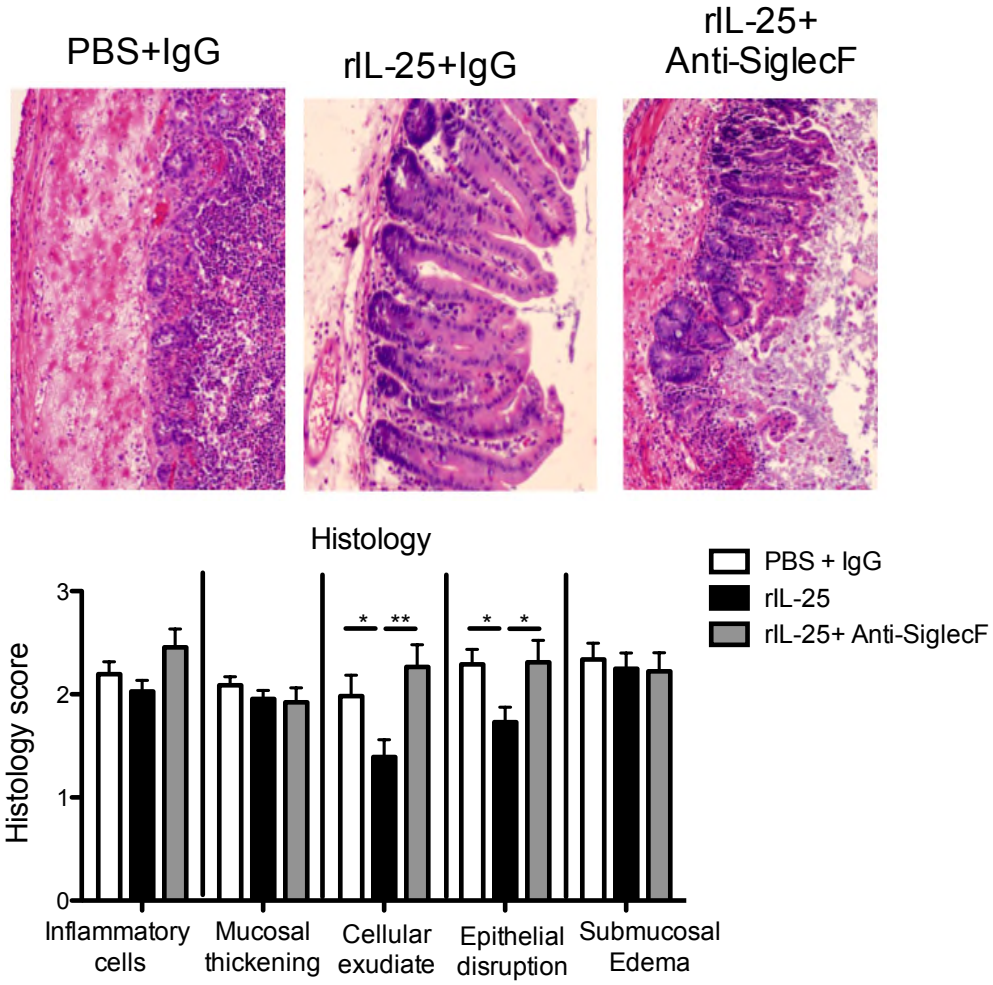

PBS+IgG
rIL-25+IgG
rIL-25+
Anti-SiglecF
Histology
PBS + IgG
rIL-25
rIL-25+ Anti-SiglecF
Histology score
3
2
1
0
*
**
*
*
Inflammatory cells
Mucosal thickening
Cellular exudiate
Epithelial disruption
Submucosal Edema

Figure 3.18: Eosinophils were necessary for IL-25-mediated maintenance of the intestinal epithelial independently of bacterial burden.

(A) Albumin concentration in the cecal contents on day 3 of CDI. Data are from three combined experiments; n=2-5 mice per infected groups per experiment; mean +/- SEM; *<0.05, **<0.005.

(B) Colon length and (C) toxin A/B level and (D) *C. difficile* bacterial burden in cecal contents on day 3 of CDI. Data are from two combined experiments; n=4-7 mice per group per experiment; mean +/- SEM; *<0.05, **<0.005.

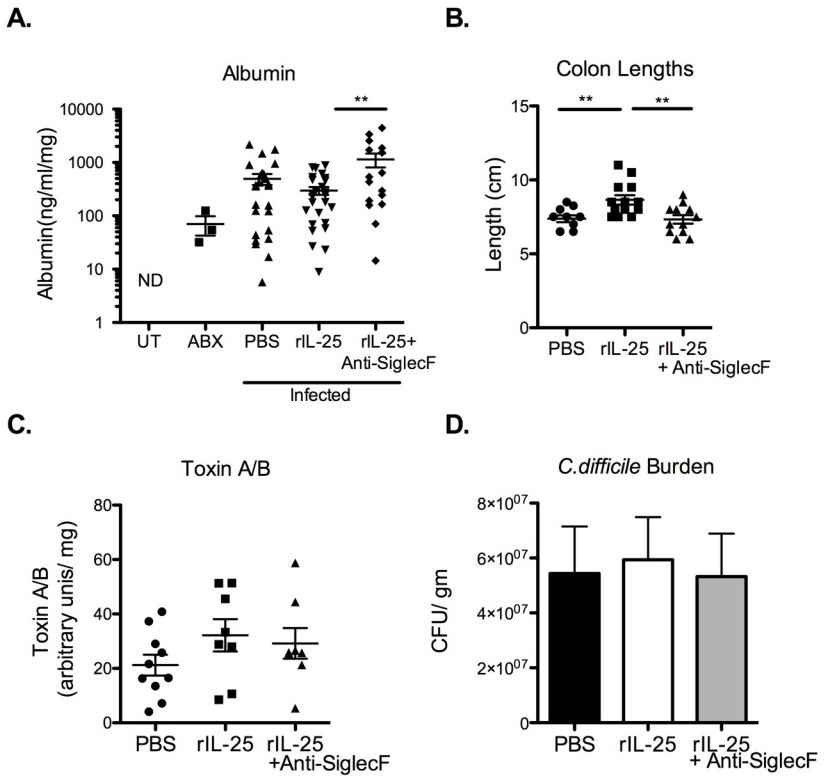

A.
Albumin
Albumin(ng/ml/mg)
10000
1000
100
10
1
ND
**
UT
ABX
PBS
rIL-25
rIL-25+
Anti-SiglecF
Infected
B.
Colon Lengths
Length (cm)
15
10
5
0
**
**
PBS
rIL-25
rIL-25
+ Anti-SiglecF
C.
Toxin A/B
Toxin A/B
(arbitrary unis/ mg)
80
60
40
20
0
PBS
rIL-25
rIL-25
+Anti-SiglecF
D.
C.difficile Burden
CFU/ gm
8×10^07
6×10^07
4×10^07
2×10^07
0
PBS
rIL-25
rIL-25
+ Anti-SiglecF

Figure 3.19 Eosinophils interact with components of the microbiota during CDI

(A) Colony forming units (CFU) of aerobic bacteria in the liver on day 3 of CDI. Data are from two combined experiments; n=4-7 mice per infected groups per experiment; mean +/- SEM; *<0.05

(B) CFU of C. difficile in the cecal contents and the liver from anaerobic cultures on day 3 of CDI. n=4-7 mice per infected groups per experiment; mean +/- SEM

(C) Toxins A//B in the liver of mice on day 3 of CDI. n=4-7 mice per infected groups per experiment; mean +/- SEM

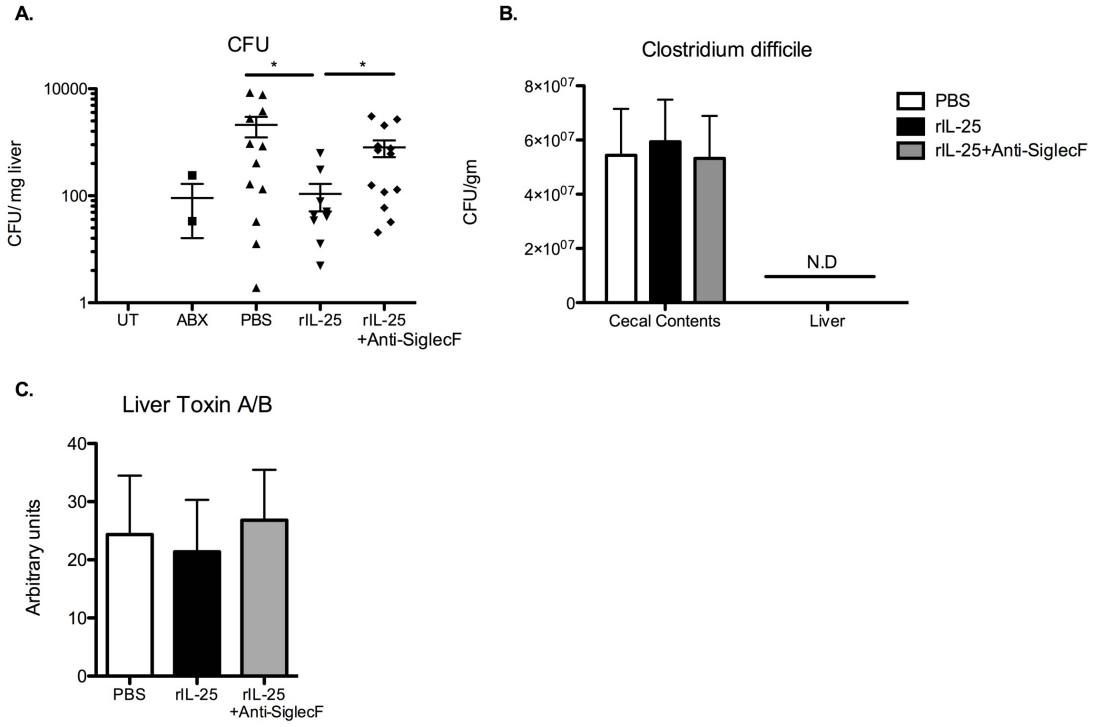

A.
CFU
CFU/ mg liver
10000
100
1
UT
ABX
PBS
rIL-25
rIL-25 +Anti-SiglecF
B.
Clostridium difficile
CFU/gm
8×10^07
6×10^07
4×10^07
2×10^07
0
N.D
Cecal Contents
Liver
PBS
rIL-25
rIL-25+Anti-SiglecF
C.
Liver Toxin A/B
Arbitrary units
40
30
20
10
0
PBS
rIL-25
rIL-25 +Anti-SiglecF

Figure 3.20 Ampicilin treatment reduces mortality and unidentified anaerobic bacteria in eosinophil depleted mice.

(A-F) C57BL/6J mice were treated with a daily dose of either 0.5 µg rIL-25+ 20ug anti-siglecF +/- 0.08mg/g ampicillin one day prior and one day after infection with *C difficile.*

(A) Clinical scores and (B) survival during five days of CDI infection. n=8 mice per group; mean +/- SEM; *<0.05

(C) Liver and (D) spleen anaerobic bacteria colony forming units on day 3 post infection

(E) C. difficile and (F) unidentified anaerobic bacteria colony forming units in the cecal contents on day 3 post infection

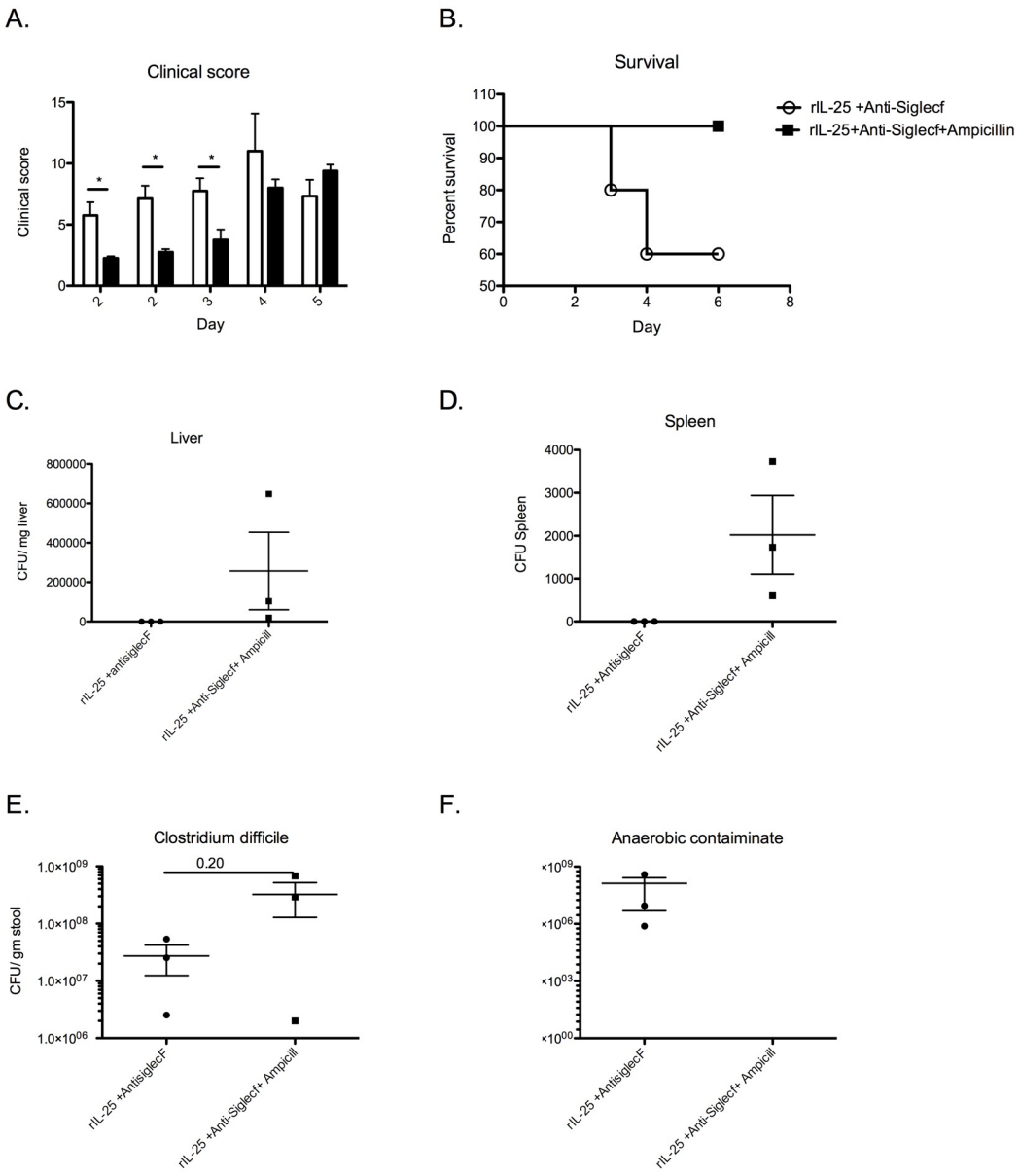
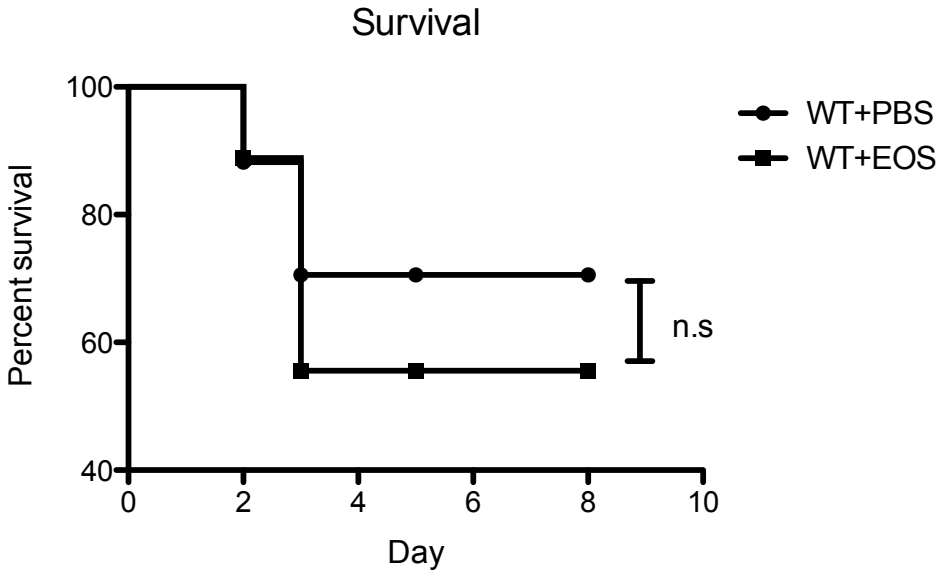

A.
Clinical score
Clinical score
Day
B.
Survival
Percent survival
Day
rIL-25 +Anti-Siglecf
rIL-25+Anti-Siglecf+Ampicillin
C.
Liver
CFU/ mg liver
rIL-25 +antisiglecF
rIL-25 +Anti-Siglecf+ Ampicill
D.
Spleen
CFU Spleen
rIL-25 +AntisiglecF
rIL-25 +Anti-Siglecf+ Ampicill
E.
Clostridium difficile
0.20
CFU/ gm stool
rIL-25 +AntisiglecF
rIL-25 +Anti-Siglecf+ Ampicill
F.
Anaerobic contaiminate
rIL-25 +AntisiglecF
rIL-25 +Anti-Siglecf+ Ampicill

Figure 3. 21 Adoptive transfer of bone marrow derived eosinophils do not protect against CDI.

Survival was assessed in C57BL/6J mice treated with PBS or 750,000 BM-derived eosinophils on day -1,0, and 1 of infection with *C difficile.*


Survival
100
80
60
40
Percent survival
0
2
4
6
8
10
Day
WT+PBS
WT+EOS
n.s

injections had decreased CDI-associated morbidity and mortality (Figure 3.20A, 3.20B). Surprisingly, this did not correlate with aerobic bacterial translocation of *E. cloacae* or *E. faecalis* to the liver or spleen. Eosinophil depleted mice did not have culturable bacteria in the liver of spleen, yet ampicillin treatment had the opposite influence as expected and functioned to increase translocation of bacteria. These data suggest that the translocation of pathobionts to the liver in the absence of eosinophils is likely not a component driving mortality and morbidity. Both treatment groups had equivalent levels of *C. difficile* in the gut (Figure 3.20E). Interestingly, eosinophil-depleted mice had significantly elevated levels of an unidentified anaerobic bacteria in the cecal contents when compared with protected ampicillin treated, eosinophil depleted mice (Figure 3.20F). These data suggest that the bacteria present locally at the site of infection may influence disease outcome more accurately than bacteria that translocate systemically.

Bone marrow-derived eosinophils do not protect from CDI

In order to establish whether general eosinophilia could protect during CDI, we adoptively transferred non-IL-25 treated bone marrow derived eosinophils and assessed their ability to enhance survival. We derived eosinophils from bone marrow based on the work done in previous studies [123]. Bone marrow-derived eosinophils (BM-eosinophils) have been demonstrated to have similar surface markers and functions to naturally derived eosinophils [123]. After differentiation, 750,000 live BM-eosinophils were adoptively transferred one day prior to and

after infection. Survival was not significantly different between mice who received BM-eosinophils and those that did not (Figure 3.21). These data demonstrate that eosinophils that are differentiated *ex vivo* are not sufficient to rescue mice from CDI-mortality and suggests that *in vivo* differentiated or IL-25 primed eosinophils are necessary to protect mice.

Discussion

This work demonstrates that repletion of IL-25 protected from CDI-associated mortality and morbidity via induction of gut eosinophils. We discovered that IL-25, a cytokine induced by the microbiota, was repressed in the colon of humans and mice with CDI to levels lower than antibiotic treatment alone. Restoration of IL-25 reduced disease severity despite the presence of equivalent levels of *C. difficile* and toxins in the gut lumen. IL-25 treatment reduced mortality and morbidity and enhanced integrity of gut epithelial barrier through the actions of eosinophils. Therefore, this work demonstrates a novel mechanism of innate immunity driven by a microbiota-regulated cytokine to protect the host epithelium during CDI and identifies a previously unrecognized role of eosinophils in gut defense.

Our results suggest a new and unexpected role for the microbiota in CDI, one that occurs after *C. difficile* colonization and that does not act by decreasing the burden of *C. difficile* infection or intoxication. Antibiotic treatment reduces microbial diversity and leads to host susceptibility to CDI [31,124]. The

mechanism by which gut commensal bacteria protect has historically been linked to the ability of a healthy microbiota to outcompete *C. difficile* for space and nutrients [27,114,125]. Disruption of the microbiota has also been shown to alter primary and secondary bile acids, resulting in enhanced germination of spores and subsequent outgrowth of *C. difficile* [31,114,115]. Therefore, prior studies support the paradigm that the microbiota provides resistance to CDI by acting to block host susceptibility to *C. difficile*. In contrast, our study demonstrates that restoration of IL-25, a cytokine induced by the microbiota and reduced during CDI, prevented death and disease from CDI without reducing *C. difficile* bacterial burden or toxin production. Thus IL-25 functions to reduce mortality in the face of *C. difficile* toxin production, and does so despite active *C. difficile* colonization. These findings indicate that there are several mechanisms by which the microbiota protects, and understanding the importance of microbiota regulation of innate immune responses may provide insight to the development of bacterial cocktails used for transplant and probiotic treatments.

It is unclear how IL-25 is regulated during homeostasis, antibiotic treatment, and active CDI. However, it is possible that the immune response to CDI may directly contribute to reducing IL-25 levels. CDI has been associated with the induction of the proinflammatory cytokines IL-23 and IL-1β [111]. Both cytokines correlate with lower IL-25 levels and their increased production during CDI may explain the significant drop in IL-25 during infection [73,126]. Alternatively, CDI has been shown to sustain microbial dysbiosis in the intestine after antibiotics [127–129]. Thus, persistent decreases in microbial diversity combined with the

outgrowth of *C. difficile* during active infection may abolish signals from commensal organisms that induce IL-25 expression. Investigation into the bacterial components of the microbiota that regulate IL-25 expression is required to better understand these relationships.

Eosinophils were identified as the cellular mechanism by which IL-25 signaling protected against CDI-associated mortality. While previously shown to be protective against gut helminth infection, the role of eosinophils in CDI was unanticipated. In humans peripheral eosinophils have been associated with protection from persistent diarrhea and death, which supports our finding in mice of their protective role [75]. Currently, eosinophils remain heavily examined in the context of allergy, asthma, and parasitic infection while our understanding of their role in the broader context of bacterial infections remains incomplete. While there has been evidence of eosinophils having antibacterial capabilities *in vitro*, *in vivo* correlatives of their role in bacterial infections are limited [74,120]. Since eosinophils did not reduce the burden of the pathogen, it is likely that their action occurred downstream and involved maintaining the intestinal barrier.

The method by which eosinophils protect against mortality and host tissue destruction without altering bacterial burden and toxin level remains an active area of investigation in our lab. Neutrophils have long been classified as the most critical cells involved in the immune response to CDI, yet our finding that the restoration of IL-25 signaling reduces disease severity through an eosinophil dependent mechanism challenges this viewpoint. Eosinophils have several effector functions that may be beneficial to protecting host tissue and reducing

disease severity during CDI, such as regulating immune responses and promoting tissue remodeling and repair. In a model of DSS-induced colitis, eosinophils reduced intestinal pathology by dampening inflammatory mediators in the colon via the lipid mediator protectin D1 [130]. Likewise, recent reports indicate that eosinophils specific to the lamina propria are capable of inducing the development of regulatory T cells (Treg) and play an important role in maintaining gut homeostasis by promoting IgA responses [131,122]. In our model, it is possible that the environment created by enhanced eosinophils may influence other immune mediators and result in a balanced immune response capable of combating *C. difficile* while limiting off-target deleterious effects to the host tissue. This hypothesis is supported by our results that IL-25 can selectively reduce deleterious IL-23 but does not influence downstream cytokine IL-22, which has been demonstrated to have a protective role during CDI [42,47]. Numerous studies outlining both protective and pathogenic roles for the immune response during CDI reinforces the notion of a balanced immune response contributing to successful disease outcome [106].

In addition to regulating the immune response, eosinophils may also protect host tissues through their well-documented ability to remodel and repair host tissue. It is possible that rapid tissue remodeling limits pathogen entry into tissue and the impact of virulence factors resulting in reduced damage to the epithelial barrier. Likewise, eosinophils may facilitate rapid wound healing responses after disruptions by the pathogen occurs [119]. It is possible that neutralization of IL-4 slows resolution of CDI by hampering tissue repair

pathways initiated by IL-25. Therefore, it is plausible that IL-25 and subsequent eosinophilia may activate tissue remodeling pathways during earlier stages of disease to reduce mortality. Furthermore, eosinophils may provide protection through interactions with commensal bacteria such as *e. facaelis*, *e. cloacae,* or the unidentified anaerobic bacteria.

Our data indicate that eosinophils play a role in inhibiting the translocation of commensal bacteria to systemic tissues, such as the liver. Although these data do not consistently associate with host mortality, these actions of these pathobionts to spread systemically may still be involved in disease. The ability of ampicillin to mediate survival and lower clinical scores while simultaneously inhibiting an unidentified anaerobic bacterium presents an interesting possibility of this bacteria to drive disease. Further experiments are needed to identify this bacteria, examine its potential interactions with eosinophils, and investigate whether it correlates to disease severity. Thus, IL-25 mediated eosinophilia may protect against CDI-associated mortality by creating a balance between proinflammatory and tolerogenic immune responses, interacting with commensal bacteria locally or systemically, and/or by inducing tissue remodeling and repair pathways to strengthen the epithelial barrier.

While our study indicates that eosinophils are necessary for IL-25 mediated protection it is unknown whether this is a direct or indirect relationship. IL-25 receptor (IL-25RB) expression has been observed on human eosinophils supplying evidence that a direct relationship is possible [132–134]. However, IL-25RB is also found on type 2 innate lymphoid cells (ILC2), macrophages, mast,

and T cells, prompting the question of their involvement in IL-25-eosinophilia mediated protection from CDI severity [68]. Furthermore, it remains unclear whether the ability of eosinophils to reduce mortality is specific to the IL-25 signal, or if other cytokines and chemokines that promote eosinophilia are capable of protecting the host. Our data demonstrate that in wild type infection where IL-25 signal is diminished, depletion of eosinophils does not influence host mortality, suggesting that the eosinophils recruited during CDI in wildtype mice are not sufficient to reduce the severity of disease. It is possible that robust eosinophilia to levels higher then those seen in wild type infection is necessary to reduce mortality and that any eosinophilia- promoting cytokine is capable of protecting the host. Alternatively, it is conceivable that IL-25 not only supports eosinophilia in the gut, but directly or indirectly primes eosinophils to function in a manner that is protective towards host tissue. Our current data indicate that ex vivo derived BM-eosinophils were not sufficient to protect the host from CDI, suggesting general eosinophilia may not be protective and other signals, such as IL-25, may be important in dictating the function of these cells. Further examination is required to understand the cellular and molecular components of the IL-25-eosinophil pathway in the context of CDI.

There are numerous mouse models of *Clostridium difficile* infection that contain many variables that influence disease outcome such as antibiotic cocktail pretreatment, spore vs. vegetative bacterial challenge, bacterial dose, virulence of strain, and the initial composition of the microbiota. Our model utilizes vegetative and spore challenge with a *C. difficile* strain that produces high levels

of toxins A and B, resulting in significant tissue damage and mortality rates. This model was used to recapitulate the most severe patient symptoms seen clinically. Investigation into the ability of IL-25 and resulting eosinophilia to protect across various models of infection will delineate the robustness of this pathway to reduce mortality and to be targeted to treat various stages and settings of CDI.

Overall, our study identifies IL-25 as the first component of the immune response that regulated by a healthy microbiota and reduces pathology associated with CDI. We identified a novel role for eosinophils as being essential to this process(Figure 3.22). Enhanced mortality and relapse rates in patients and increased prevalence of disease in the United States stress the need for better therapies and management strategies for CDI. Antibiotic therapy to treat disease is associated with high relapse rates and although emerging fecal transplantation (FMT) treatment results in resolution of recurrent CDI in most patients, the long-term risks of FMT have not been thoroughly investigated. Our research on modulating the innate immune response to reduce CDI-associated pathology may offer advantages to currently inadequate antibiotic therapies, and by acting downstream of the microbiota may complement transplants and probiotic development.

Experimental Procedures

Mice

C57BL/6J mice were purchased from Jackson Laboratory and PHIL mice were a provided by J. Lee (Mayo Clinic, Scottsdale, AZ). Mice were between 8-10 weeks of age and given access to autoclaved food and water at the animal facility at the University of Virginia. Sex and aged-matched controls were used in all experiments. All procedures were approved by the Animal Care and Use Committee at the University of Virginia.

Clostridium difficile infection

Mice were received from Jackson Laboratories and started immediately on antibiotic treatment. PHIL mice were littermates and bred at the UVA vivarium. Antibiotics consisted of gentamycin (Sigma) (50 mg/ml), metronidazole (Hopsra) (5 mg/ml), colistin (Sigma) (25 mg/ml) , and vancomycin (Hopsra)(50 mg/ml) treatment in the drinking water for three days followed by two days of fresh water and a subsequent single intraperitoneal injection of clindamycin (Hopsra)((10 mg/kg) one day prior to infection with $10^{\wedge 3}$-$10^{\wedge 5}$ CFU of vegetative *C. difficile* (strain VPI10643 ATCC# 43255) via oral gavage. Vegetative *C.difficile* was obtained by overnight culture of a plated single colony of *C. difficile* in anaerobic Chopped Meat broth (Anerobic Systems) followed by a subculture of 100ul in the same media for 5 hours. 1mL of *C. difficile* in broth was pelleted, washed, quantified by spectrophotometer, and resuspended to desired concentration in sterile PBS and given orally by gavage. Quantification of *C. difficile* inoculum was verified by counting CFUs on anaerobic BHI agar plates (BD) supplemented with

taurocholate (Sigma) (BHI-T). Mice were treated with a daily dose of 0.5 µg of recombinant IL-25 protein (Biolegend or R&D systems) daily for five days prior to infection. In eosinophil depleting experiments, mice received 20ug of monoclonal anti-SiglecF (clone 238047, R&D Systems) or IgG isotype (clone 54447, R&D systems) on day -1 and day 1 of infection. For IL-4 neutralization experiments, mice received 1mg of anti-IL4 (Clone 11B11, University of Virginia, Lymphocyte Culture Center) monoclonal antibody or isotype control on day -1 and day 1 of infection. For ampicillin experiments, mice were given 0.08mg/g ampicillin on day -1 and day 1 of CDI. Post-infected mice were assessed for mortality rates and morbidity based clinical scores (weight loss, hair ruffling, ocular discharge, activity, posture, and diarrhea severity) determined by scorer blinded to experimental conditions [105].

***Clostridium difficile* quantification**

Cecal contents were suspended in sterile, anaerobic PBS and serially diluted. Bacterial burden was determined by quantification of colony forming units (CFU) grown anaerobically on BHI-T and 2x *C. difficile* supplement (Sigma) (BHIS-T) agar plates. The same procedure was performed to detect the anaerobic contaminate bacteria. Toxins A/B were quantified using the ELISA *C. difficile* TOX A/B II kit (Techlab, Blacksburg, VA). Both CFU and toxin levels were normalized to stool weight.

Isolation of Lamina propria and flow cytometry

Lamina propria and epithelial cells in the colon were separated as previously described [14]. Briefly, the colon was removed, cut longitudinally, and rinsed in a Hank's Balanced Salt Solution (HBSS), 5% Fetal Calf Serum (FCS), 215 25mM HEPES Buffer. The tissue was incubated in pre-warmed buffer consisting of HBSS, 15mM HEPES, 5mM EDTA, 10% FCS 217 and 1mM DTT at 37°C on a shaking incubator for two 20 minute cycles in fresh media to remove the epithelial layer. The tissue was minced and incubated in prewarmed RPMI containing 0.17mg/ml liberase TL (Roche) and 30ug/ml DNase (Roche) for 40 minutes. After digestion, tissue was passed through 40μm and 100μm nylon strainers, resuspended in FACS buffer, and quantified for total cell numbers and cell viability using trypan blue cell counting. Single cell colonic lamina propria cells were plated at 10^6 live cells per sample and stained. After FC blockade (anti-mouse CD16/32 TruStain, Biolegend), cells were stained using monoclonal antibodies to markers: Live/dead (Fixable Viability Dye eFluor 506), CD11b-APC(M1/70), CD45-APC-Cy7(30-F11), CD11c-BV421 (N418), Siglecf-PE(E50-2440), Ly6g-PeCy7(1A8), Ly6c-Fitc (HK1.4) (Biolegend, BD, eBiosciences). For *ex vivo* intracellular analysis cells were incubated without stimulation for 3 hours with (Golgiplug, eBioscience) in IMDM + 5% FBS at 37°C. Following incubation, cells were stained with clones above and IL-4 (11B11, BD Biosciences) using the Fixation/Permeabilization Solution Kit (BD Biosciences). Data were acquired on a Becton Dickinson LSRFortessa flow cytometry BD FACSDiva version 6. software (BD biosciences). 5x10^5-10^6 events were collected and data were analyzed using FlowJo version 9.2 software (Treestar 233 Inc., Ashland, OR). Cell

populations were calculated from total cells per colon and as a percentage of live cells.

Cytokine and *muc2* analysis

Cecal tissue was flushed with sterile PBS and homogenized by bead beating for 1 minute in a buffer consisting of 1M HEPES and HALT protease inhibitor cocktail (Thermo-Fisher Scientific Inc., Rockford, IL) followed by a 30 minute incubation on ice and with an Triton X 100, HEPES and HALT protease inhibitor cocktail containing buffer. Cytokines levels were evaluated by ELISA (IL-23,IL-25, IL-22, IL-17A, IL-4, IL-13 Duo-Set ,R&D systems). Cytokine protein expression was normalized to total protein concentration generated from the Pierce BCA Protein Assay (ThermoFisher). For epithelial cell and lamina propria extraction, the above protocol was used. For *muc2* analysis, cecal tissue was flushed with sterile PBS and processed using the RNeasy mini kit (Qiagen) and Turbo DNA-free kit (Ambion). RNA was reverse transcribed with Tetro cDNA synthesis kit (Bioline). Amplification of *muc2* was done using the Sensifast SYBR and fluorescein mix (Bioline), and Forward (TGCCCAGAGAGTTTGGAGAG) and Reverse (CCTCACATGTGGTCTGGTTG) primers. Gene expression was normalized to β-actin and GAPDH.

Human and mice histology

Human biopsies were obtained from the University of Virginia Biorepository and Tissue Research Facility. Only patients with the closest age match were chosen. Positivity of CDI was based on the presence of **C.** *difficile* toxins in stool samples of patients. CDI negative tissues were derived from patients suspected of various

other intestinal diseases, but confirmed negative for tissue pathology upon biopsy analysis. Immunochemistry staining was performed using the DAKO Autostainer Universal System (Dako, Denmark) with a primary antibody directed against IL-25 (R&D Systems). Scoring was done by four independent blinded scorers and was based on intensity and abundance of IL-25 staining in lamina propria cell infiltrates. The staining scale was between 0-3. Mouse cecal tissue was extracted and fixed for 24 hours in Bouin's solution (or Corony's fixative for PAS stain), washed, and stored in 70% ethanol. Tissue was processed and hematoxylin and eosin (H&E) and Periodic acid–Schiff (PAS) stained by the University of Virginia Research Histology core. Mouse IL-25 (US biological life sciences) staining was performed by the University of Virginia Biorepository and Tissue Research Facility. Two independent blinded scorers graded tissues based on 5 parameters (immune infiltrates, cellular exudate, mucosal thickening, epithelial disruption, edema) with individual scales of 0-3 per parameter.

Figure 3.22: Hypothesis model for IL-25-eosinophil mediated protection

During homeostasis IL-25 levels are high, but antibiotic or CDI associated dysbiosis suppresses IL-25 levels and severe morbidity and mortality is observed with CDI. When IL-25 is restored, eosinophils are recruited and reduce mortality and morbidity. Eosinophilia mediated protection may be through the ability to enhance the integrity of the intestinal tissue and barrier and/or by interactions with components of the microbiota.

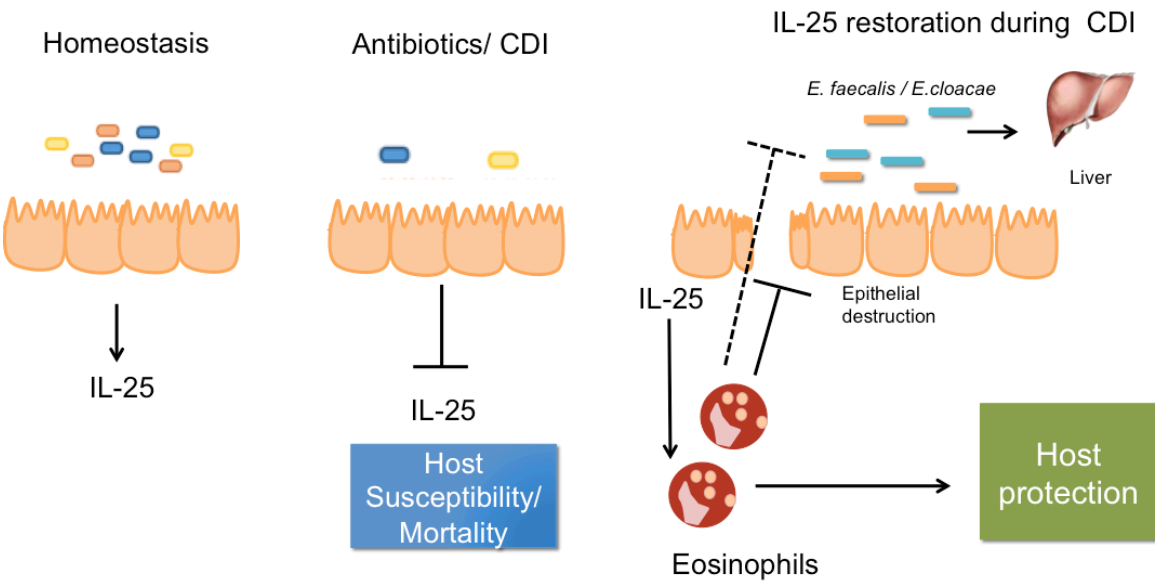
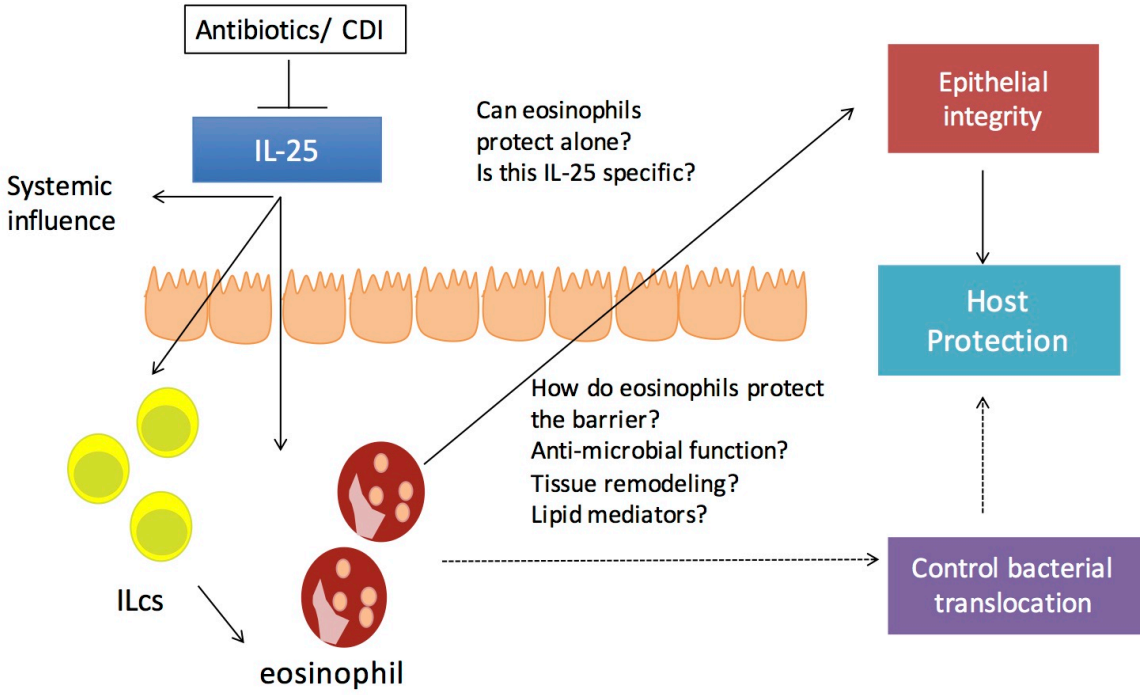

Homeostasis
IL-25
Antibiotics/ CDI
IL-25
Host Susceptibility/ Mortality
IL-25 restoration during CDI
E. faecalis / E.cloacae
Liver
IL-25
Epithelial destruction
Host protection
Eosinophils

Figure 3.23: Future directions for IL-25-eosinophil mediated protection

This work provides many avenues for additional investigation. The major questions remaining are (1) How do eosinophils reduce mortality and protect the intestinal barrier?; (1a) Do eosinophils protect the barrier by interacting with deleterious pathobioant bacteria locally or systemically?; (1b) Do eosinophils promote tissue regenerative properties to protect the tissue?; (2) Is the ability of eosinophils to protect specific to IL-25?; (2a) Can any cytokine or chemokine, such as IL-33, IL-5, or TSLP, that promotes eosinophilia provide protection?; (2b) Does IL-25 prime eosinophils to be functionally different then eosinophils induced during wildtype infection?; (3) How does IL-25 communicate with eosinophils?; (3a) Does IL-25 signal directly to eosinophils, or indirectly through an intermediary cell such as ILCs?; (4) Which components of the microbiota are responsible for inducing IL-25?; (4a) Can these bacteria be used as a targeted microbial-based therapy?; (5) How does CDI suppress IL-25 levels?; (5a) Is this through epithelial (Tuft cell) death or by manipulation of immune responses?


Antibiotics/ CDI
IL-25
Systemic influence
Can eosinophils protect alone?
Is this IL-25 specific?
Epithelial integrity
Host Protection
How do eosinophils protect the barrier?
Anti-microbial function?
Tissue remodeling?
Lipid mediators?
Control bacterial translocation
ILcs
eosinophil

Aerobic bacteria culture

Tissue (liver or spleen) was weighed and placed into tube containing sterile PBS. Tissue was bead beated for 1 minute, serially diluted, and dilutions were plated on BHI agar. Colony forming units (CFUs) were counted and normalized to tissue weight. DNA from isolated bacterial colonies was amplified and sequenced using Genewiz.

Adoptive transfer of bone marrow derived eosinophils

Bone marrow eosinophils were derived as previously described. Briefly, bone marrow was isolated and cultured in RPMI 1640(Invitrogen) with 20% FBS, 100IU/mL penicillin, 10ug/mL streptomycin, 2mM glutamine, 25mM Hepes, 50uM beta-mercaptoethanol (Sigma) and supplemented with 100ng/ml stem cell factor (SCF; PeproTech) and 100ng/mL FLT3-ligand (Peprotech) for 4 days, On day 4 media was replaced with new media containing 10ng/mL recombinant mouse interleukin-5(IL-5;Biolegend) in place of FLT-3L and SCF. On day 10 cells were spun down, washed, and counted. 750,000 live cells were injected into mice intraperitoneally on day -1, 0, and day 1 of infection.

Statistical analysis

Survival rates between groups were assessed using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. An analysis of variance (ANOVA) was used for differences among multiple groups. Student's T test (2-tailed) or Mann-Whitney test was used to compare two groups. A p-value below 0.05 was considered significant. All statistical tests were done using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Chapter 4. Conclusions and Future Directions

Part of this chapter has been adapted from "The microbiota and immune responses during *Clostridium difficile* infections"
Submitted at Anaerobe

4.1 IL-23 and IL-25 influence the severity of *Clostridium difficile* infection

The immune response plays a multifaceted role during *C. difficile* infection (CDI) as different immune components have been demonstrated to protect against or perpetuate disease in the host. CDI occurs when the microbiome is disrupted, most often through the use of antibiotics. Previous studies have demonstrated that increased inflammatory markers, such as IL-8, are better at predicting poor patient outcome than enhanced bacterial burden [44,107]. This finding, coupled with the neutrophilia observed in CDI patients, supports the hypothesis that the severity of disease may be correlated with intensity of host response [96]. It is plausible that a balanced immune response is necessary to combat CDI, but secondary off target effects can lead to epithelial cell death and host pathology. This is supported by studies that demonstrated that ablation of neutrophils and other immune mediators lead to increased mortality, whereas intervention of distinct immune mediators can lead to host protection (Figure 1.3). Thus, the goal of this work was to elucidate mediators in the immune response which may regulate a healthy or pathogenic immune response to CDI.

We have demonstrated a pathogenic role for IL-23 in CDI. A significant increase in IL-23-producing infiltrating cells in the lamina propria in human colon biopsies from patients with *C. difficile* colitis. Additionally, two individual murine models of *C. difficile* infection were used to test the role of IL-23 signaling during infection [47]. Abrogation of IL-23 signaling through genetic knock-out and by anti-IL-23 p19 monoclonal antibody prevented death due to CDI. Interestingly, the IL-23 downstream mediators IL-17 and IL-22 were significantly decreased in

IL-23p19$^{-/-}$ mice compared to WT mice, although future work is necessary to elucidate their exact contribution in disease development. Lastly, we observed a subtle, yet significant, decrease in neutrophils infiltrating into the lamina propria on Day 2 of infection in IL-23p19$^{-/-}$ mice compared to WT mice, suggesting that a balance in neutrophil recruitment is necessary to avoid off target deleterious effects to host tissue. These results of this work present several questions for the future. Primarily the influence of the microbiota in these models is unknown. During studies involving IL-23-p19$^{-/-}$ mice, mice were not cohoused therefore the contribution of the microbiota to influence neutrophilia, IL-17A, or IL-22 remains to be determined. Additionally, the contribution of IL-17A, IL-22, and neutrophils to drive IL-23 mediated pathogenesis remains to be explored.

Moving forward, the goal was to determine immune mediators that regulate beneficial inflammation during CDI. Previous studies have demonstrated an inverse relationship in the signaling of cytokines IL-23 and IL-25 [72,73]. Thus, we hypothesized that IL-25 was protective during infection. We have shown that antibiotics decrease IL-25 expression in the cecum and expression is further reduced during infection. Similarly, colons from human biopsies testing positive for CDI have lower levels of IL-25 protein compared to negative patients. We observed that restoration of IL-25 signaling by pre-treating mice with recombinant protein significantly increased survival during CDI, implicating its role in protection. Interestingly, IL-25 treatment did not change *C. difficile* burden or toxin levels, but decreased epithelial disruption at the intestinal barrier. IL-25 mice displayed decreased IL-23 expression but maintained comparable levels of

neutrophil recruitment between groups suggesting alternate properties of IL-25 may be more important in protection than its role in dampening neutrophilic inflammation. Treatment with IL-25 enhanced the recruitment of eosinophils to the gut. Depletion of eosinophils resulted in significantly reduced survival rates in IL-25 treated mice, providing an essential role for these cells in IL-25-dependent protection from CDI. Our data provide a novel role for eosinophils in immune-mediated protection against an intestinal bacterial infection. We propose that IL-25, and the associated type 2-like responses, are pivotal in protecting the integrity of the epithelial barrier by activating unique signaling pathways to counteract pathogenic inflammation normally observed during CDI. These experiments prompt many future hypotheses, including how IL-25 is regulated by the microbiota and CDI, the mechanisms by which eosinophils protect the intestinal barrier, and how IL-25 functions to recruit eosinophils during CDI.

4.2 The microbiota and CDI regulate IL-25 expression in the colon

Antibiotic treatment has been demonstrated to reduce microbial diversity and lead to host susceptibility to CDI [31,124]. The mechanism by which this occurs has historically linked to the ability of a healthy microbiota to outcompete *C. difficile* for space and nutrients, thus inhibiting the expansion of the pathogen in the gut [25,30,125,135]. Disruption of the microbiota has also been shown to alter primary and reducing secondary bile acids, resulting in enhanced germination of spores and subsequent outgrowth of *C. difficile* [31,114,136].Thus

prior studies support the paradigm that the microbiota provides resistance to CDI by acting to block host susceptibility to *C. difficile*.

Our results provide a novel role for the microbiota to protect the host during active disease, rather then prior to infection. IL-25, a cytokine that is regulated by the microbiota, reduces disease severity. Here, we show that IL-25, is suppressed in the intestine during CDI in humans and mice. Administration of IL-25 protected mice from CDI-associated mortality and enhanced the integrity of the epithelial tissue in spite of equivalent quantities of *C. difficile* bacteria and toxin in the gut.

IL-25 expression was dampened during CDI in both human and murine models. IL-25 is produced from epithelial cells in mice as a response to signals from the microbiota, antigens, and allergens [73,121]. In agreement with the literature, we find that IL-25 protein is primarily expressed in intestinal epithelial cells in mice. Interestingly, IL-25 protein was observed in both epithelial cell and cells infiltrating the lamina propria in human biopsies. Thus, future investigation into the characterization of cells expressing IL-25 in the human intestine is of importance as the distinction may be beneficial in developing IL-25-based therapies.

IL-25 protein was suppressed during active *C. difficile* infection to levels lower then those seen with antibiotic treatment alone, indicating that there were additive effects of antibiotic-mediated dysbiosis and CDI on IL-25 production. It is unclear how IL-25 expression is regulated, yet it has been demonstrated to be reliant on signals from the microbiota, as antibiotic treated and germfree mice

have low levels in the intestine [73].The ability of CDI to further reduce its expression from levels seen during antibiotic treatment may be a result of several processes. First, it is possible that the immune response to CDI may directly contribute to reducing IL-25 levels below those observed with antibiotic treatment alone. CDI induces a proinflammatory response with observed enhancement of IL-23 and IL-1β production [111,137]. IL-23 and IL-1β have been associated with decreased IL-25 expression in either homeostatic or infectious circumstances, respectively [73,126]. Therefore, it is possible that type 1/type 17 proinflammatory responses elicited by CDI may skew the immune response further from a type 2 IL-25 response then antibiotic treatment alone. Although, the inverse relationship between IL-25 and IL-1β and IL-23 is correlative, rather then mechanistic, thus requiring further investigation into their role in IL-25 suppression during CDI is necessary.

Another possibility that CDI adds to reduced IL-25 expression by perpetuating suppressed microbial diversity initiated by antibiotic treatment. CDI has been shown to sustain microbial dysbiosis in the intestine after antibiotic treatment [127,128,138]. Maintained suppression of microbial diversity combined with the outgrowth of *C. difficile* during active infection may abolish signals from commensal organisms that are beneficial for IL-25 induction. Investigation into the bacterial components of the microbiota that induce IL-25 expression is required to understand how regulation of this cytokine occurs. Our data indicate that individual treatment with either metronidazole or clindamycin results in differential IL-25 expression, suggesting that specific microbial signals are

important in IL-25 regulation. An interesting caveat of this experiment is that metronidazole not only targets bacteria, but can also combat parasite infections such as *Entamoeba histolytica,* thus providing the possibility that parasites play a role in the regulation of IL-25. Regardless, this observation supports the hypothesis that sustained intestinal dysbiosis and the associated reduction in signals from the microbiota during CDI may attribute to significantly dampened IL-25 levels from antibiotic only treated mice. Therefore, it is possible that IL-25 suppression during CDI may be a result of immune responses or maintained microbial dysbiosis.

4.3 Eosinophils provide protection from CDI-associated mortality and morbidity

Neutrophils have long been classified as the hallmark cells involved in the immune response to CDI, yet our finding that the restoration of IL-25 signaling reduces disease severity through an eosinophil dependent mechanism challenges this viewpoint. Interestingly, pretreatment with IL-25 did not influence the accumulation of neutrophils and cell numbers are comparable to those found in control mice suffering from severe morbidity. The ability of IL-25 treatment to protect by selectively manipulating eosinophils, and not neutrophils, argues that neutrophils alone do not dictate the outcome of disease severity and that other cellular factors contribute to the health of the host tissue during CDI. These results provide a novel role for eosinophils during *C. difficile* and suggest that the ratio or type of immune cells recruited, rather then the level of neutrophils alone, is important in influencing disease severity.

The mechanism by which eosinophils aid to protect the host from CDI-associated mortality and morbidity remains an exciting area to explore. In our model, eosinophils do not influence the overall number of neutrophils in the colon, but it is possible that the environment created by enhanced eosinophils may influence other immune mediators and result in a balanced immune response. An enhanced eosinophilia response may be capable of combating *C. difficile* while limiting off target deleterious effects to the host tissue. The notion of a balanced immune response contributing to successful disease outcome is supported by evidence that enhanced proinflammatory mediators, such as IL-8, correlate to a worse prognosis in humans independently of the bacterial burden of *C. difficile.* Meanwhile total ablation of inflammatory cells such as neutrophils result in more severe pathology and death in mice [36,139]. In fact, our studies indicate that in the presence of IL-25 mediated eosinophila there is a reduction in IL-23, a cytokine that drives disease severity, but no influence on its downstream mediator IL-22, which is protective during CDI. Therefore, it is plausible that IL-25 promotion of eosinophilia selectively facilitates a more balanced response between inflammatory and tolerogenic signals that is favorable to disease outcome.

Although eosinophils do not function to reduce *C. difficile* bacterial burden directly, interactions with components of the microbiota may contribute to the ability of eosinophils to reduce morbidity during CDI. We observe eosinophis ability to reduce bacterial translocation to the liver during CDI. Eosinophils contain cytotoxic granules such as major basic protein, eosinophil peroxidase,

eosinophil-derived neurotoxin, and eosinophil cationic protein [74]. These granules contribute to asthma and allergy pathologies, but have also been demonstrated to have bactericidal properties [120,140,141]. In addition, eosinophils release extracellular DNA traps which bind and kill bacteria leading to enhanced intestinal barrier function and reduced incidence of sepsis [76]. Similarly, an *ex vivo* study documents a phagocytic role for human eosinophils against bacteria [142]. Preliminary data from this dissertation suggest that eosinophils may reduce the ability of *E. cloacae* and *E. faecalis* to translocate to the liver during CDI. Translocation of *E. cloacae* systemically has been implicated to drive morbidity during CDI and abundance of *E. faecelis* correlates with increased host susceptibility [31,42]. Likewise, we observe enhanced survival in mice treated with ampicillin. Ampicillin treatment did not reduce translocation of bacteria, but did influence the bacterial communities in the cecal contents. We have found that an unidentified anaerobe is found at high levels in the cecal contents of mice lacking eosinophils, but is ablated in mice lacking eosinophils which have been ampicillin treated. These results are preliminary, and we have yet to establish whether eosinophils play a role in controlling the outgrowth of this microbe in the cecum or if this effect is independently ampicillin mediated. These results demonstrate that IL-25 primed eosinophils influence the components of the microbiota, but it remains to be determined whether translocation of bacteria systemically or microbial species present locally at the site of infection contribute to disease severity during CDI.

Finally, eosinophils have been described to aid in tissue repair which could potentially explain why IL-25-induced eosinophilia treated mice have enhanced intestinal barrier integrity and survival rates during CDI. Eosinophils secrete mediators such as TGF-β, basic fibroblast growth factor (bFGF), metalloproteinase, and type 2 cytokines such as IL-4 and IL-13 which have all been observed to influence connective tissue, epithelial cells, and endothelial cells to maintain the integrity of the epithelial barrier [141]. In the context of CDI, it may be possible that eosinophils activate these tissue repair pathways to quickly heal or inhibit toxin initiated tissue damage and thus reduce mortality rates in mice.

Two central hypotheses arise from observation that IL-25 protects via an eosinophil dependent mechanism. First, the IL-25 signal creates a specific direct or indirect signal to eosinophils which primes them to behave in a protective manner. In this scenario both IL-25 and enhanced eosinophil function are necessary to reduce disease severity. This hypothesis proposes that an intermediate link between the microbiota and eosinophils is necessary to enable eosinophils to reduce mortality to CDI. Microbiota derived IL-25 is a plausible intermediate that provides a specific signal that educates eosinophils to function in a protective manner. This hypothesis is favorable because it supports the observation that eosinophils recruited during wildtype infection, in the absence of IL-25, are insufficient to reduce mortality to CDI. Griseri et al demonstrate an opposing deleterious role for eosinophils in chronic colitis when primed with IL-23 and GM-CSF signal outlining the importance of specific signals in dictating the

effector function of eosinophils [78]. Together, these data support the heterogeneity of eosinophil function given the context in which they are elicited and present an interesting avenue of investigation.

A second hypothesis is that enhanced eosinophilia alone can protect against CDI pathology. In this scenario, IL-25 pretreatment is one of many mechanisms by which eosinophilia can occur. Thus, promotion of eosinophilia to levels above those seen during wildtype infection is what is important to mediate protection and is not necessarily dependent on IL-25. Although we observe increased eosinophils in infected wild type mice compared to uninfected mice, the level of cells recruited in the absence of an eosinophil recruiting cytokine such as IL-25, or chemokine is not sufficient to reduce mortality. Investigation into whether other pro-eosinophil molecules such as IL-5, TSLP, IL-33, and eotaxins may also reduce CDI-associated mortality is necessary to elucidate this hypothesis. Our data also support that IL-25 is capable of enhancing the mucus layer lining the epithelium during CDI. This layer serves to provide a secondary barrier between the lamina propria and both *C. difficile* and commensal bacteria. It is unclear whether eosinophils are sufficient reduce host mortality independently of these alternate affects of IL-25 restoration and examination into eosinophils dependency on IL-25 signaling to protect during CDI is necessary.

4.4 Impact

Clostridium difficile infection is one of the most challenging hospital-acquired infections in the US, costing an estimated $4.8 billion annually in acute

care facilities [6]. The opportunistic pathogen's ability to colonize the gut of patients with a disrupted microbiome further complicates prescription of antimicrobial treatments in the hospital. Despite current treatment options, infection is associated with high mortality and relapse rates with disease incidence increasing annually. Better therapies and methods to control spreading disease are badly needed.

Current therapy for CDI involves stopping the offending antibiotic that rendered the patient susceptible and beginning vancomycin, fidaxomicin, or metronidazole treatment. This treatment remains inadequate, as is made apparent by high rates of relapse and mortality in CDI patients. Fecal transplantation is being utilized more frequently for cases of CDI relapse [144]. FMT is effective at preventing relapses in 90% of cases; however, it is of unknown effectiveness for primary CDI. Despite effective inhibition of relapse, the understudied possibility of long-term risks associated with recolonization of unknown bacteria remains a disadvantage of FMT. A powerful therapy for CDI is the development of defined bacterial cocktails that effectively displace *C. difficile* while limiting long-term deleterious side effects. Emerging evidence supporting the role of specific immune mediators to shape the outcome of disease provides an interesting area to consider in future therapeutic interventions. Furthermore, facilitating targeted bacterial cocktails strategies to reconstitute commensal bacteria based on their ability promote protective immune responses while suppressing deleterious immune mediators may provide a powerful strategy to treat both primary and secondary CDI. Therefore, understanding which mediators

of both the microbiota and the immune response regulate a balanced and beneficial host response to favor eradication of *C. difficile* burden while preventing tissue destruction will provide new and innovative therapeutic approaches.

In addition to direct therapeutic applications, IL-25 and/or eosinophilia may be utilized as a diagnostic during CDI. Measurement of eosinophil or IL-25 levels in the stool or serum of CDI patients may provide a diagnostic to predict disease severity in patients. We are currently investigating whether regulation of IL-25 or eosinophil in the stool or serum correlates with patient outcome. The results of these experiments may identify a novel biomarker and be used to dictate the course of treatment for a patient. For example, if high levels of IL-25 or eosinophilia in the stool correlate with mild disease, a physician may be less likely to prescribe a rigorous course of antibiotics as the patient is more likely to recover without significant intervention. On the contrary, if very low eosinophil counts and IL-25 expression associate with severe infection and high risks of disease relapse, a physician may suggest that these patients have fecal transplant therapy. These experiments are fascinating, as we have yet to establish whether eosinophil detection in the stool will be indicative of protection or more severe disease as this would require eosinophils to migrate out of the lamina propria and into lumen.

The identification of IL-25 as a microbiota-regulated mediator that protects from death, and acts after infection has been established, may offer new advantages to complement or guide the development of bacterial cocktails used

for transplant and probiotic treatments. Currently, pharmaceutical companies are focusing on harnessing the power of the microbiota to combat diseases, primarily recurrent CDI, by isolating and developing cocktails of bacterial spores that can be used as treatment in high-risk populations prior to infection or after primary or recurrent infection. The possibility of screening spore cocktails for their effectiveness to induce IL-25 or eosinophilia may provide a powerful tool for drug development moving forward. Our data indicating that species of the microbiota targeted by clindamycin or metronidazole have different effects on IL-25 expression may be a way to determine which species are responsible for its induction and suppression in the gut. Additionally, IL-25 may provide benefits to current inadequate antibiotic therapy by acting downstream of the microbiota to aid in host survival even in the absence of microbial restoration and during active dysbiosis in the intestine. Thus, immunotherapy, using recombinant protein, that directly enhances IL-25 or subsequent eosinophilia may be a beneficial therapy to treat active CDI. Together, there are multiple opportunities for the work of this dissertation to directly contribute to the care of patients suffering from CDI.

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