Mechanisms of IL-33-mediated protection during *C. difficile* colitis.

Alyse Longtin Frisbee
Reston, VA

B.A. Biology, University of Virginia, 2011

A Dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy

Department of Microbiology, Immunology and Cancer Biology

University of Virginia
April, 2019

Thesis Abstract

Currently, *Clostridium difficile* infection (CDI) is the leading cause of hospital acquired diarrhea in the US and has been listed as one of the Centers for Disease Control (CDC)'s three most urgent infectious disease threats[1]. This is due, in part, to the emergence of hypervirulent epidemic strains of *C. difficile* (typified by strain NAP1/027) over the last decade. These strains are associated with major epidemic *C. difficile* outbreaks in both Northern America and Europe[2–5]. As a consequence, one of every five patients relapse, and 29,000 patients die yearly in the US, despite receiving antibiotic treatment[5]. Thus, it is a national priority to study novel avenues to treat and prevent hypervirulent *C. difficile* infection.

Hypervirulent strains express a third toxin, *C. difficile* Transferase (CDT), in addition to the primary virulence factors Toxins A and B (TcdA and TcdB), and are associated with higher mortality rates and rates of relapse. Our lab has identified CDT as a virulence factor capable of suppressing protective gut eosinophils and enhancing mortality during infection[6]. How the host combats the enhanced inflammation and virulence of CDT expressing hypervirulent *C. difficile* requires further investigation. To identify novel therapeutic host immune targets, we conducted a transcriptome analysis of host genes altered by CDT expressing hypervirulent infection. Through this array, we identified interleukin-33 (IL-33) as a gene upregulated in response to the increased severity of infection. Using a murine model, we show that both endogenous IL-33 and exogenous IL-33 treatment prevent mortality, weight-loss and clinical scores during hypervirulent CDI. IL-33 treatment reduced the epithelial disruption and gut leakiness characteristic of *C. difficile* toxin damage. IL-33 protected independently of *C. difficile* bacterial factors as equivalent *C. difficile* burdens and toxin levels were observed in both protected IL-33 treated mice and susceptible IL-33 receptor (ST2) knockout mice relative

to controls. IL-33 mediated protection required the action of ST2 expressing type-2 innate lymphoid cells (ILC2s), as IL-33 increased the number of activated colon-resident ST2+ ILC2s during infection. Furthermore, ILC2s were sufficient to promote survival, as adoptive transfer of sorted and purified ILC2s into immune compromised hosts prevented *C. difficile*- associated mortality and weight-loss, and increased downstream eosinophilia during infection. Thus, we identified a novel pathway of IL-33 mediated activation of ILC2s that enhances gut barrier defenses and host protection during *C. difficile* infection.

We also demonstrate that IL-33 signaling is a clinically relevant immune target during human *C. difficile* infection. IL-33 expression was increased in colon biopsies of 6 *C. difficile* infected patients relative to uninfected patients. Furthermore, dysregulated IL-33 signaling via the soluble IL-33 decoy receptor (sST2) predicted disease severity and mortality in a cohort of human patients. Lastly, we demonstrate that the host microbiome is required to regulate IL-33-mediated protection. Colonic IL-33 expression was reduced during antibiotic-depletion of the microbiome, whereas fecal microbiota transplant of mouse and a human fecal spore preparation rescued IL-33 expression. Thus, IL-33 signaling is a novel therapeutic pathway for during *C. difficile* infection which can potentially be induced with rationally designed microbial therapies.

Table of Contents

List of Figures

Chapter 1: Introduction

1.1 *C. difficile* infection- disease overview

Importance: Clostridium difficile (*C. difficile*) is a gram positive, spore-forming bacterium that colonizes the colon, causing inflammation and diarrhea[7–9]. *C. difficile* is the leading cause of hospital-acquired, antibiotic-associated diarrhea and is one of CDC's three most urgent infectious disease threats in the US. *C. difficile* incidence has tripled over the past 15 years[10] and is attributed to the emergence of hypervirulent epidemic strains of *C. difficile* (referred to as NAP1/027*)*. In a single year, *C. difficile* caused approximately 500,000 infections and $4.8 billion worth of excess health-care costs in the United States alone[11]. *C. difficile* is also attributed to high mortality rates with 29,000 *C. difficile* associated-deaths occurring in 2011[11]. Although a recent healthcare survey demonstrated an overall decline in the prevalence of health-care associated infections from 2011 to 2015, there was no reduction in the high prevalence of *C. difficile* infections (CDI) during that time. Thus, reducing *C. difficile* infections is a national priority, as emphasized in the National Action Plan for Combating Antibiotic-Resistant Bacteria issued by the White House in 2015 and the NIAID's Antimicrobial Resistance Program in 2014[12–14].

1.1.1 Epidemiology:

Over the past two decades both the incidence and severity of *C. difficile* has increased in North America and Europe. In 2005, molecular analysis led to the identification of a genetic variant strain associated with wide-spread *C. difficile* outbreaks and increased disease severity in the US, typified as North American pulsed-field gel electrophoresis type NAP1 and polymerase-chain-reaction (PCR) type 027[15]. Since identification, NAP1/027 isolates have been reported globally in the US, Canada, and Europe[11,16,17]. Their emergence, has been linked to an increase in national and global

hospital-associated outbreaks, higher mortality rates, and higher disease severity[4,15] NAP1/027 strains are more virulent than classical *C. difficile* isolates, producing higher levels of the virulence factors, toxin A (TcdA) and toxin B (TcdB), higher fluoroquinolone resistance, and the expression of a third toxin (CDT toxin)[2,18]. Because of these attributes, mortality rates are 3 times higher during NAP1/027 infection as compared to less virulent strains[19,20].

1.1.1.1 C. difficile infections in the US:

In the US, *C. difficile* is a leading cause of hospital-associated infection. Between 1995 to 2005, there was a 350% increase in *C. difficile* infections and a 400% increase in *C. difficile*-related mortalities[21,22]. More recently, the Centers for Disease Control (CDC) conducted a 2011 survey of *C. difficile* infections at 10 US sites to better understand incidence, severity, and recurrence. This study estimated 453,000 infections and 29,000 *C. difficile* associated deaths per year in the United States[11]. In addition to hospital-associated outbreaks, the incidence of community-acquired CDI has also increased in the US. Of the cases reported in the US in 2011, 46.2% were community associated infections not linked to a hospital exposure. Between 1991 and 2005, a US study reported a 5.3 fold increase in community-acquired CDI[23] and more recently, a 2011-2015 study of 25 US hospitals reported a doubling of the community-acquired CDI incidence rate[24]. Patients previously thought to be at low risk for hospital-acquired CDI are reported to be at increased risk to community-acquired CDI such as younger adults[23]. Thus, the epidemic outbreak is not solely confined to the hospital setting but extends to community-acquired exposures. These findings expand the burden of CDI to outside the hospital-setting within healthier populations and indicates possible nosocomial transmission of *C. difficile*.

1.1.1.2 C. difficile infections in Europe and Canada*:*

C. difficile outbreaks are not limited to the United States but have also occurred in Europe and Canada. In Canada, it was discovered that from 1991-2003 the rate of CDI

had increased from 65.6 to 156.3 per 100,0000 population in addition to an increase in cases with severe complications[25]. A follow up prospective study of 12 Quebec hospitals reported a 30 day mortality rate of 6.9 percent, a four-fold increase compared to the 1.5% mortality reported in a 1997 Canadian study[19,26]. In the UK, outbreaks of CDI were also reported. For example, two UK hospitals in the towns of Aylesbury and Devon, England had 565 *C. difficile* infections and at least 25 deaths in one year[27]. Additionally, in 2004, 43,672 infections were reported in the UK, a 23% increase from 2003[27]. Coinciding with the increased incidence and severity of *C. difficile* in both North America and Europe was the spread of a previously rare strain, North American Pulse-field type 1 NAP1 or polymerase chain reaction (PCR) ribotype 027 (NAP1/027)[15,18]. NAP1/027 isolates are now linked to these north American and European outbreaks and enhanced disease severity[15,18,28,29].

1.1.2 Clinical disease and pathogenesis:

Clostridium difficile is transmitted via the faecal-oral route and is especially prevalent in hospital environments. *C. difficile* spores are resistant to traditional ethanol-based disinfectants making them highly persistent and easily transmittable. Within the hospital setting, shed spores are transmitted to new hosts likely through contact with the contaminated hospital environment, other patients, or health-care providers[9,30]. Once ingested, *C. difficile* spores, can colonize the colon of susceptible hosts whose intestinal microbiota has been disrupted, most commonly through prior antibiotic exposure. Resident intestinal-commensals provide colonization resistance against *C. difficile* via indirect and direct competition[31]. After disruption of these protective communities, *C. difficile* spores can occupy the intestinal niche, germinate into vegetative cells, and express toxin within the gut lumen. Toxin expression initiates *C. difficile* disease through the killing of colonic host-epithelial cells and breakdown of the gut barrier[3,32,33]. The timing

and severity of clinical symptoms is further dependent upon the microbial and immune factors present within the intestine during infection[30,34,35].

The clinical manifestations of *C. difficile* range from mild to severe diarrhea to life-threatening pseudomembranous colitis. Patients with severe *C. difficile* infection have white blood cell counts greater than 15,000 cells/µl in addition to diarrhea, abdominal pain, and fever[36,37]. The spectrum of *C. difficile* disease manifestations is dictated by the infecting strain (ie. Toxin expression, sporulation and other genetic factors), in addition to host factors such as the quality of the inflammatory response and composition of the host microbiome (**Figure 1.1**).

Certain populations of individuals have increased risk of developing CDI and it is important to understand the multiple factors contributing CDI susceptibility. Host-related risk factors commonly associated with CDI are increased age, comorbidities such as inflammatory bowel disease, suppressed immune function and exposure to a health-care setting[11,38–41]. Increased age remains the most widely observed host risk factor, with a recent CDC study demonstrating that rates of CDI were 4–fold higher in patients aged >65 compared to adults aged 45–65 and 13–fold higher compared to adults aged 18–44[42]. Various pharmacological risk factors are also associated with CDI and include the use of antibiotics, and the use of gastric acid suppressors such as proton-pump inhibitors[1,36–38]. CDI is most commonly regarded as a disease complication of antibiotic therapy due to disruption of the microbiota. A strong statistical association between the use of antibiotics and increased risk of CDI has been reported by many studies[3,39,40,43]. Patients on antimicrobial therapy have up to a 6-fold increased risk of CDI compared to non-antibiotic exposed individuals[44]. The underlying mechanisms by which these various risk factors predispose the host to CDI are likely multifactorial and may be caused by a dysbiotic microbiota and/or a dysregulated immune response.

Figure 1.1

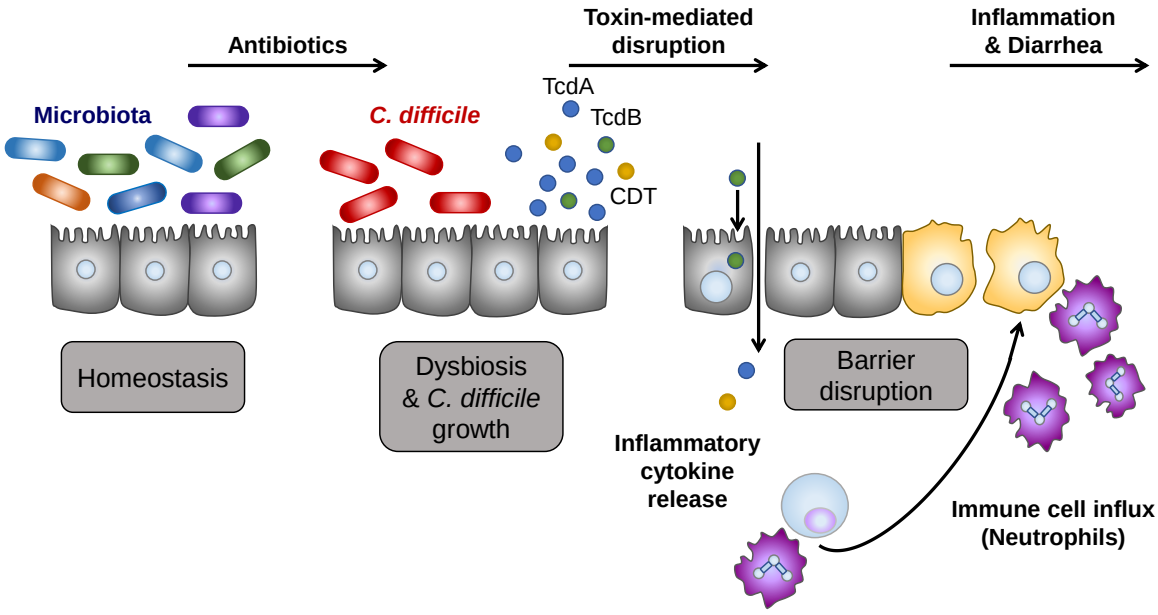
Antibiotics
Toxin-mediated disruption
Inflammation & Diarrhea
Microbiota
C. difficile
TcdA
TcdB
CDT
Homeostasis
Dysbiosis & C. difficile growth
Barrier disruption
Inflammatory cytokine release
Immune cell influx (Neutrophils)

Figure 1.1: Pathogenesis of *C. difficile* in the colon.

The pathogenesis of *C. difficile* is dictated by the microbiota, *C. difficile* virulence factors, and the host immune response. Antibiotics are the primary risk factor for acquiring CDI due to disruption of colonization resistance. In the intestine, orally digested *C. difficile* spores germinate into vegetative cells upon sensing of nutrients indicative of a favorable growth environment[45]. Expression of toxins by vegetative cells is tightly linked to disease onset. Toxins internalization leads to enzymatic disruption of epithelial cell cytoskeleton resulting in loss of barrier function and pro-inflammatory cell death. Sensing of *C. difficile* and its toxins by the immune system triggers an influx of immune cells into the colon, typically characterized by high neutrophil migration.

While the above risk factors have been assessed in the context of hospital-acquired CDI, recently, several studies have described the onset of community-acquired CDI (CA-CDI)[46–48]. In 2011, a study conducted by the CDC estimated that 46.2% of the total CDI cases were community-acquired[11]. High proportions of CA-CDI have been replicated by other studies with one recent population-based study finding that 41% of CDI is community-acquired[23]. Interestingly, CA-CDI has been reported to affect populations of individuals not traditionally at high risk for hospital-acquired CDI. This includes younger patients and individuals with no exposure to antibiotics 12 weeks prior to infection, and pregnant women[23,46,47,49]. The modes of transmission of community-acquired infection remain to be elucidated however outpatient health-care exposures and contact with asymptomatic carriers such as infants have been proposed[33,46,48]. As *C. difficile* isolates most commonly found in animals, such as the ribotype 078 isolate, are increasingly reported in hospital-acquired and community-acquired CDI, the possibility of zoonotic spread into the community has also been proposed[28,50–52]. Further studies are required to understand whether the rise in CA-CDI is caused by increased testing for CDI in the community, increased transmission outside of the hospital, increased virulence of infecting strains, or host-mediated changes in the population such as the microbiota or immune response.

1.2 *C. difficile*: the bacterium:

C. difficile is a gram-positive, spore forming bacillus and an obligate anaerobe that invades the colon. The start of its life cycle in the colon is triggered by sensing of a favorable gut environment, and subsequent signaling cascades that control germination into vegetative cells, release of toxin, and then sporulation.

1.2.1 Germination and vegetative growth:

Inside the gastrointestinal tract, *C. difficile* spores germinate into vegetative cells with the capability to express toxin depending on the genetics of the infecting strain.

Germination and growth are dictated by sensing of a favorable lower gastrointestinal (GI) environment. The environment of the lower GI tract has low oxygen content, making it a favorable site for the growth of anaerobic *C. difficile*[53]. Germination of the spore is triggered by receptor-based sensing of small molecule germinants within the intestinal lumen, most notably bile acids. This process is controlled by the cspBAC operon, a region encoding subtilisin-like proteases, CspAB and CspC, that initiate germination signaling[30,45]. The process of germination is composed of three steps: 1) sensing of bile acids via the germinant receptor, CspC, at the inner spore membrane[54]. 2) Rehydration of the spore and release of major spore constituent, calcium-dipicolinic acid (CA-DPA), which actives the cortex lytic enzyme, SleC. 3) SleC degrades the peptidoglycan cortex causing full rehydration of the spore[30,45,55]. Thus, the sequential activation of proteases triggers the full enzymatic activity necessary to degrade the *C. difficile* spore.

Bile acids are important germinants and different bile acids have varying capabilities to induce germination and growth. The conjugated primary bile acid, taurocholate, was initially demonstrated by Wilson and colleagues to induce germination of spores when added to culture media[56,57]. Subsequent studies demonstrated that all cholate derivatives, in addition to the co-germinant, glycine, can promote germination of *C. difficile*[58]. The secondary bile acid, deoxycholate, supports *C. difficile* germination but also inhibits *C. difficile* vegetative growth[56,58]. Thus, signals received from the bile acid pool of the intestine dictate the balance between germination and growth of *C. difficile* within the intestine.

1.2.2 Toxin Virulence factors:

Once *C. difficile* has germinated in the intestine, it can release its virulence factors to drive epithelial disruption, inflammation, and disease. Like germination, toxin production is also regulated by favorable environmental signals within the gastrointestinal tract. Toxin production typically occurs during stationary phase of growth under nutrient limiting

conditions. Nutrients like glucose and amino acids inhibit toxin synthesis, whereas high temperatures, nutrient starvation, and the short-chain fatty acid, butyrate, can all activate toxin synthesis[59–63]. The mechanisms by which these various nutrients modulate toxin production are not well understood. However, it appears that master regulator proteins involved in cellular metabolism, can also regulate the transcription of toxins. For example, the catabolite control protein, CcpA, a transcriptional regulator of metabolizing carbon sources, also binds to regulatory regions of TcdA and TcdB and is required for glucose-dependent repression of toxin[64]. Thus, there is a connection between toxin expression, metabolism and nutrient sensing by *C. difficile*.

The primary virulence factors expressed by *C. difficile* are TcdA and TcdB, encoded from the Pathogenicity locus (PaLoc)[65,66]. While both toxins can lead to disease, a recent study using isogenic mutants and three separate animal infection models, comprehensively demonstrated that toxin B is the major virulence factor during infection and also the major driver of inflammation[67]. In addition to the well-characterized TcdA and TcdB, epidemic Ribotype 027 isolates and non-epidemic Ribotype 078 isolates express an additional third toxin, CDT[68]. In fact, CDT toxin is detected in 15-25% of CDI strains during non-outbreak situations[69,70]. In contrast to TcdA and TcdB, CDT is encoded by a different region called the CDT locus[71,72]. The presence of CDT during infection increases the severity of weight-loss, mortality, epithelial disruption, and inflammation in a murine model of CDI[6]. Furthermore, *in vitro*, CDT can increase the *C. difficile* adherence through the induction of microtubule protrusions on host epithelial cells[73]. All three toxins (TcdA, TcdB, and CDT) produced by *C. difficile* together cause symptomatic disease by damaging colonic epithelial cells and breaching the protective gut barrier. These toxins initiate epithelial damage by binding to their cell surface receptors followed by entrance into cells, disruption of the actin cytoskeleton, and inflammatory cell death. The release

of inflammatory cytokines by toxin-damaged epithelial cells causes an influx in neutrophils, monocytes, macrophages, ILCs and eosinophils at the site of infection[6,74,75].

1.2.2.1 TcdA and TcdB:

TcdA and TcdB enter cells by clathrin-mediated endocytosis and each have four domains essential for activity: 1) a glucosyl transferase domain at the amino terminus, 2) a cysteine protease domain, 3) a pore forming domain, and 4) a CROPS domain[76]. The CROPS domain is canonically thought to be the domain important for binding and entry into the cell. The glucosyl transferase domain mediates Rho GTPase glucosylating activity in intoxicated host cells, an activity essential for cytoskeleton disruption. Adjacent to the glucosyl transferase domain is the cysteine protease domain which autocatalytically cleaves the glycosyl transferase domain, thus releasing it into the cytosol[77].

The receptors that mediate entry of TcdA and TcdB into epithelial cells have been elusive with multiple receptors being identified. TcdA receptors include: gp96, blood antigens I, X, Y[78,79] and TcdB receptors include Poliovirus Receptor-like 3 (PVRL3), Wnt receptor frizzled family (FZDs), and Chondroitin sulfate proteoglycan 4 (CSPG4)[80–82]. Likely dual receptors can mediate toxin entry within the gut epithelium. Endocytosis of the toxin/receptor complex and subsequent vesicle acidification leads to conformational changes triggering the insertion of the pore forming domain into the vesicle membrane. After pore formation, the glucosyl transferase exits the vesicle whereby autocatalytic cleavage occurs via the cysteine protease domain[76,83,84]. Once inside the cytosol, the glucosyl transferase domain mediates toxicity by glucosylating and inactivating Rho family GTPases, leading to defects in F-actin, disruption of tight junctions, and cell death (**Figure 1.2**)[77,85].

1.2.2.2 CDT Toxin:

CDT toxin is expressed by hypervirulent, epidemic Ribotype 027 strains of *C. difficile* in addition non-epidemic Ribotype 078 strains. Hospital surveys indicate that 15%-

Figure 1.2

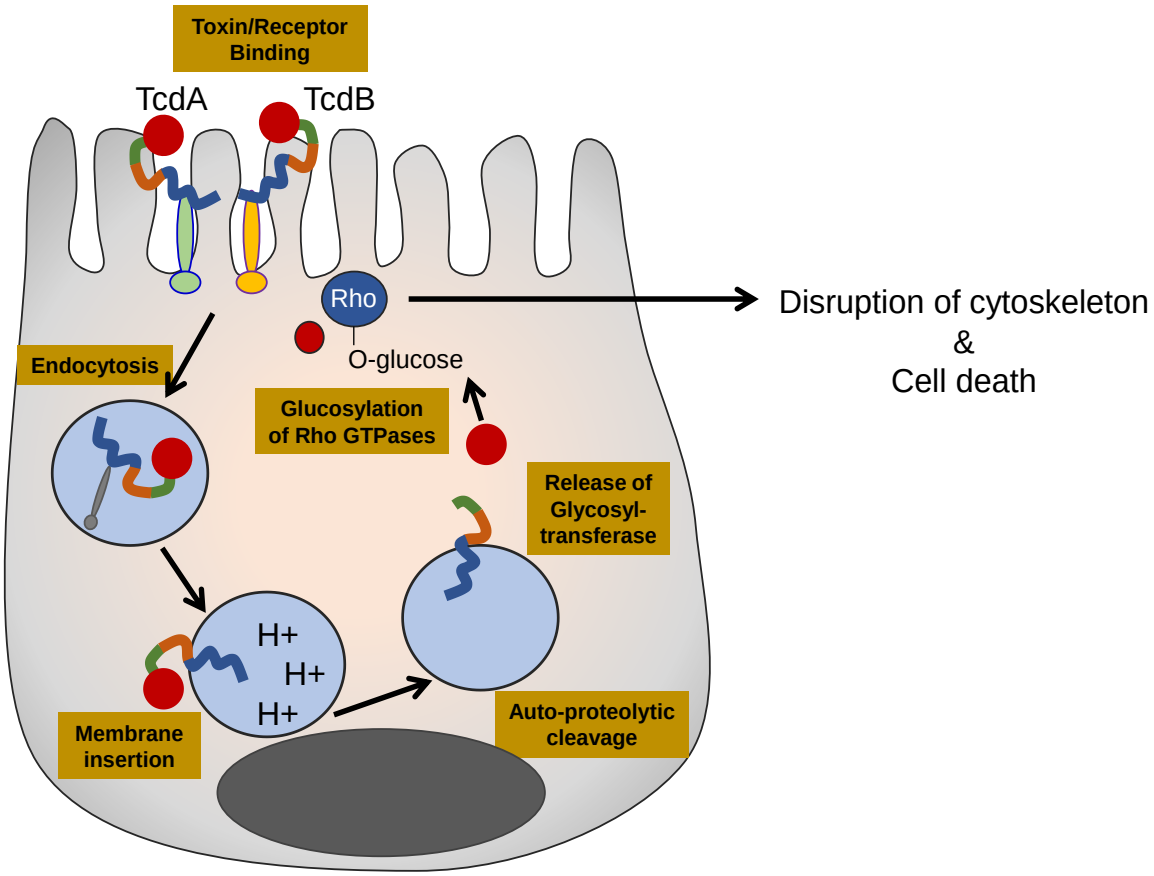
Toxin/Receptor Binding
TcdA
TcdB
Rho
O-glucose
Disruption of cytoskeleton
&
Cell death
Endocytosis
Glucosylation of Rho GTPases
Release of Glycosyl-transferase
H+
H+
H+
Membrane insertion
Auto-proteolytic cleavage

Figure 1.2: Mechanism of TcdA and TcdB intoxication of epithelial cells

TcdA and TcdB bind to their respective receptors on epithelial cells. TcdA binds to GP96 and carbohydrate structures via the CROPS domain (blue). TcdB can bind to various receptors such as CSPG4, Frizzled-proteins, and/or PVRL3 via regions of the toxin that are not within the CROPS domain and also the CROPs domain. After binding to their respective receptors both TcdA and TcdB enter target cells by clathrin-mediated endocytosis. After the toxin enters the cell via endocytosis, vesicle acidification leads to conformational changes that initiate toxin insertion into the vesicle and translocation of the enzymatic glucosyltransferase (red) into the cytosol. The glycosyltransferase domain is then cleaved off (stimulated by inositol hexakisphosphate binding) by the adjacent cysteine protease domain (green). After release into the cytosol, the glucosyl transferase domain glucosylates and inactivates Rho family GTPases at the cell membrane. This modification leads to pathogenic effect such as cytoskeleton disruption, cell death, and subsequent loss of barrier integrity.

25% of toxinogenic isolates express CDT toxin[86,87]. CDT toxin is encoded by a 6.2 kb region called the CDT locus[71] and belongs to a family of toxins called binary ADP-ribosylating toxins[71,83]. CDT is composed of two subunits: the binding subunit, CDTb, and the enzymatic subunit, CDTa. To intoxicate host epithelial cells, CDTb oligomerizes and associates with a host cell surface receptor, shown to be the lipolysis-stimulated lipoprotein receptor (LSR) in addition to the endocytic uptake mediator, CD44[88–90]. Following endocytosis of the CDTb-receptor complex, the CDTa subunit is translocated into the cytosol, where it carries out ADP-ribosylation of G-actin and disruption of the colon epithelial barrier[6,68,91]. CDT toxin also disrupts the microtubule dynamics within the cell, leading to microtubule protrusions that can increase bacterial adherence to epithelial cells (**Figure 1.3**)[73]. Additionally, our lab has demonstrated that CDT toxin increases pro-inflammatory cytokine release and mortality in a TLR2 dependent mechanism. TLR2 serves as an immune sensor for CDT and interestingly, $TLR2^{-/-}$ mice are protected from CDT-mediated toxicity[6]. Further investigation is required to determine whether TLR2 is a receptor or coreceptor for CDT toxin and additionally whether a direct interaction occurs between TLR2 and CDT. Importantly, our lab and others have demonstrated that CDT is a virulence factor *in vivo*, that enhances mortality, weight-loss, and clinical severity of infection[6,92]. In line with these animal studies, cases of diarrhea due to infection with a CDT positive strain that is negative for both TcdA and B (TcdA-TcdB-CDT+) have been reported, albeit these strains are very rare[93,94].

1.3 Microbiota resistance to CDI:

The microbiota provides the host with colonization resistance against *C. difficile.* Colonization resistance comes from both direct and indirect interactions. To directly limit *C. difficile* infection, commensals can express bacteriocins that kill *C. difficile* while

Figure 1.3

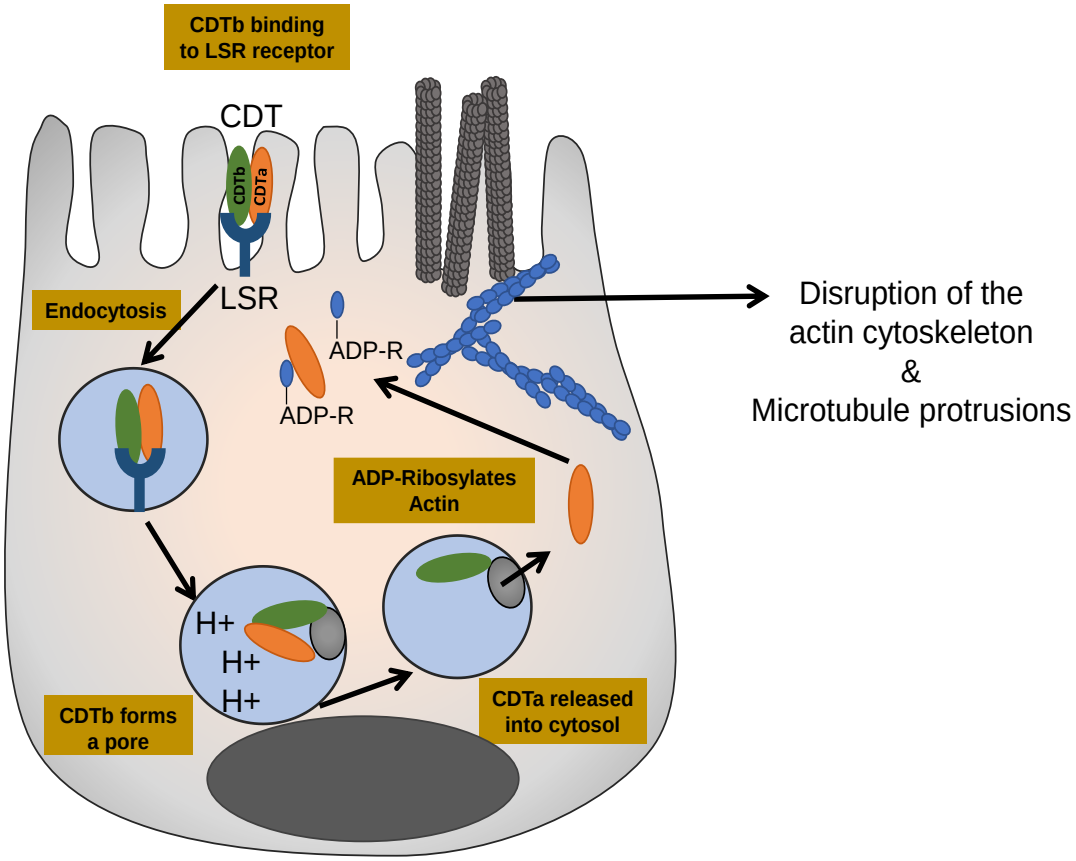
CDTb binding to LSR receptor
CDT
CDTb
CDTa
LSR
Endocytosis
ADP-R
ADP-R
Disruption of the actin cytoskeleton & Microtubule protrusions
ADP-Ribosylates Actin
H+
H+
H+
CDTb forms a pore
CDTa released into cytosol

Figure 1.3: Mechanism of CDT intoxication of epithelial cells.

CDT is a binary toxin composed of two subunits: the binding subunit, CDTb, (green) and the enzymatic subunit, CDTa (orange). CDTb forms a heptamer and binds to the receptor LSR, expressed in gut tissues[88,89]. Heptamer formation triggers binding of CDTa to the complex. The CDT-receptor complex enters epithelial cells by endocytosis. Vesicle maturation leads to reduced endosomal pH, triggering the CDT heptamer to form a pore in the vesicle membrane. Through the pore, CDTa is released into the cytosol a process aided by the chaperone proteins HSP90 and cyclophilins[95,96]. In the cytosol, CDTa then ADP-ribosylates F-actin, resulting in the depolymerization of F-actin and subsequently resulting in microtubule protrusions that increased bacterial adherence[73,88].

preserving other commensal microbes. To indirectly limit *C. difficile* growth, commensals can alter the bile acid and short-chain fatty acid pool of the gut. For example, certain commensals can convert primary acids into secondary bile acids which limit the growth of *C. difficile*. Finally, many protective host defenses are maintained by interactions with the microbiota and thus may indirectly strengthen the immune response to infection. Here we review the mechanisms by which the microbiota confers protection to *C. difficile* colitis.

1.3.1 Antibiotic induced dysbiosis confers susceptibility to CDI.

Antibiotic-mediated disruption to the intestinal microbiota is a risk factor for community and hospital acquired *C. difficile* infection[97–99]. Risk of hospital acquired and community acquired CDI is highest in individuals with prior exposure to cephalosporins, fluoroquinolones and clindamycin antibiotics[98,99]. Antibiotics can cause long-lasting alterations to the microbial community that provides colonization resistance against *C. difficile*[100]. Many animal studies have demonstrated decreased colonization resistance after antibiotics[101–106]. A single treatment with clindamycin confers murine susceptibility to infection and induces long-lasting alterations in the composition of the gut microbiome, eliminating ca. 90% of bacteria taxa for up to 28 days[102]. Similarly, in humans subjects, a 7 day clindamycin regimen caused long lasting deficiencies in the microbiota, especially a significant decline in the diversity of Bacteroides that over a span of two-years, never returned to its original composition[100]. Patients who have developed recurrent *C. difficile* have lower microbiota diversity than patients with non-recurrent disease[101]. Furthermore, using next-generation sequencing, additional studies have demonstrated decreases in the diversity and species richness of CDI patients relative to controls[107,108]. Thus, it is clear that intestinal dysbiosis is a risk factor for the development of *C. difficile* colitis. However, the mechanisms underlying commensal resistance to *C. difficile* colitis are not as well understood[109]. Here we review recent advancements regarding the interplay between *C.*

difficile, the microbiota and the host immune response and additionally discuss fecal microbiota transplantation (FMT) strategies to restore antibiotic-induced dysbiosis.

1.3.2 Mechanisms of microbiota mediated protection from CDI:

Commensal bacteria are dynamic; their nutrient acquisition, production of metabolites, expression of anti-microbial factors, and their interaction with the host immune system aid in their resistance against pathogens. Recent studies have begun to make associations between the composition and function of the microbiota in conferring resistance against *C. difficile*.

1.3.2.1 Bile acid metabolism alters C. difficile growth.

One important mechanism of commensal protection from CDI is the intestinal bile acid pool. Bile acids are produced in the liver and metabolized by the microbiota into secondary forms. The primary bile acid chenodeoxycholate in addition to the secondary bile acids deoxycholate and lithocholate are inhibitors of *C. difficile* growth whereas the primary bile acids taurocholate and cholate induce spore germination[58,110–112]. Antibiotic exposure disrupts the relative balance of primary to secondary bile acids in mice and humans[113–115]. Caecal contents from susceptible antibiotic treated mice have reduced levels of inhibitory secondary bile acids and a corresponding increase in growth-promoting primary bile acids. Additionally, compared to a healthy microbiome, extracts from an antibiotic-treated microbiome had an enhanced ability to stimulate *C. difficile* colony formation from spores in vitro[111]. Furthermore, human patients with recurrent CDI had high levels of primary bile acids yet were completely deficient in secondary bile salts prior to FMT therapy[115]. FMT therapy could restore bile-acid metabolizing microbiota, rescuing protective levels of secondary bile acids[115]. Modeling of the microbiota of susceptible mice and human patients, demonstrated an association between resistance from CDI and the commensal microbe *Clostridium scindens*, a primary to secondary bile acid converter[112].

Recolonization of mice with *C. scindens* conferred resistance to *C. difficile* infection and *C. scindens* engraftment was associated with enhanced deoxycholate abundance[112]. Thus, commensal-mediated alterations to the bile acid pool of the intestinal lumen contribute to *C. difficile's* ability to expand and infect hosts.

1.3.2.2 Direct and indirect competition between the microbiota and C. difficile:

Commensals organisms can also compete with *C. difficile* for nutrients and space within the intestine. Commensal metabolism affects the available nutrients and metabolites that can both limit and promote *C. difficile* growth. Break down of carbohydrates provides an energy source for commensals whereas antibiotic treatment alters the balance of commensal fermenters leading to reduced short-chain fatty acid production[116,117]. Lawley *et al*. demonstrated that in the presence of hypervirulent *C. difficile* infection, the microbiota of infected mice was marked by low diversity and reductions in SCFAs, with most notable reductions in acetate and butyrate[106]. Similarly, an additional study characterized the metabolome of susceptible, antibiotic-treated mice and demonstrated reduced fermenting commensal activity with increases in sugar alcohols and carbohydrates and reciprocal reductions in SCFAs[113]. Aligning with these studies, 16 S rDNA sequencing of the human flora demonstrated reductions in the families *Ruminococcaceae*, *Lachnospiraceae*, and butyrate-producing C2 to C4 anaerobic fermenters in human CDI patients[108]. Finally, a recent study demonstrated that microbiota accessible carbohydrates (MAC) diets caused outgrowth of MAC utilizing bacteria (eg. *Bacteroides*). This outgrowth was associated with increased fermentation end products acetate, proprionate, and butyrate and decreased *C. difficile* fitness *in vivo* despite also increasing virulence factor TcdB expression[60,118]. While the MAC+ diet elevated TcdB expression on a per cell basis, likely due to stress of a non-permissive environmental change, the overall toxin B abundance mirrored *C. difficile* burden and was lowered by the

protective MAC+ diet. The exact mechanism by which a MAC enriched diet inhibits *C. difficile* burden during infection remains unclear. It is possible that the resulting increase in intestinal SCFAs directly inhibit *C. difficile* growth[119,120] or indirectly by lowering the luminal pH of the colon creating unfavorable growth conditions for pH-sensitive *C. difficile*[121–124]. Thus, diets that favor MACS may be a novel avenue for treatment of CDI through their ability to alter the microbiota however, further studies deciphering the interaction between diet and CDI in humans are required. Furthermore, while it is clear that reductions in the fermenting capacity of the microbiota and the SCFA pool of the intestine are associated with CDI, further investigations into the mechanism by which SCFAs decrease the fitness of *C. difficile* are essential.

The microbiota also consumes intestinal nutrients that positively contribute to *C. difficile* growth, thus competing with *C. difficile* for these nutrients. The organic acid, succinate, is a product of bacterial fermentation, and is used as an energy source by commensals. Additionally, certain sialidase-producing commensals such as *Bacteroides thetaiotaomicron,* cleave polysaccharide sugars from host mucosal barrier, and release monosaccharides like sialic acid into the lumen[125]. Released sialic acid is exploited by other commensals that lack the scavenging sialidase enzyme. Thus, during homeostasis, the monosaccharide sialic acid and the organic acid succinate are metabolized by other commensals, depleting their availability from the gut lumen. As such, antibiotic-associated depletion of commensals frees up excess sialic acid and succinate within the intestine. *C. difficile* encodes succinate transporters and sialic acid catabolism genes, enabling it to exploit these nutrients to promote growth and disease[126,127]. In summary, competition between *C. difficile* and the microbiota for common energy sources aids in colonization resistance against infection.

In addition to nutrient competition, the microbiota can alter *C. difficile* growth more directly by the expression of bacteriocins. Bacteriocins are ribosomally synthesized anti-microbial peptides that can more narrowly target *C. difficile* than antibiotics. For example, the commensal *B. thuringiensis*, produces the bacteriocin, thuricin CD, which has anti-*C. difficile* properties *in vivo* with limited effects on bystander commensals[128,129]. Thus, commensals can offer direct anti-microbial effects against *C. difficile* through bacteriocin production which offers a more targeted approach to combating infection.

1.3.3 Interaction between the microbiota and host immune defenses.

The host immune system has a multifaceted role during CDI with some level of inflammation required to clear the infection. However, this inflammation must be tightly regulated to prevent overly robust damage to the host. Protective host defenses during CDI include, bacterial killing via antimicrobial peptides; granulocyte-mediated bacterial clearance; and tissue-repair via type-2 immunity[6,75,130–133]. Thus, the type of host immune response elicited during CDI, plays an important role in dictating disease severity in murine models and human patients[30,133–138]. Many studies have investigated how the microbiota reduces *C. difficile* colonization via bacterial competition. However, how the microbiota harnesses a protective immune response to reduce *C. difficile* disease severity is largely understudied. Given the rise of FMT therapy to treat severe CDI and efforts to define specific, purified, commensals that elicit protection from disease, it is increasingly important to understand how members of the microbiota influence the type of host-immune response generated during CDI[139,140].

1.3.3.1 The microbiota influences antimicrobial peptide expression.

While few studies have directly tested whether microbiota-immune interactions play a role in dictating the severity of CDI, many inferences can be made from other infection models. For example, host-derived anti-microbial peptides are important barrier

defenses against pathogens and are effective at killing *C. difficile*[130,131]. The expression of host-antimicrobial peptides can be regulated by the presence of the microbiota and in-turn, regulate both the composition and spatial proximity of the microbiota. For example, the expression of the anti-microbial C-type lectin, RegIIIγ, is regulated by the microbiota in a MYD88-dependent manner and germ-free mice lack RegIIIγ expression[141,142]. RegIIIγ, is expressed by epithelial cells and Paneth cells of the intestine and possesses selective antimicrobial activity against gram-positive bacteria[141]. Antibiotic-mediated down regulation of RegIIIγ leads to reduced killing of Vancomycin Resistant *Enterococcus* (VRE)[142,143]. Intestinal RegIIIγ expression is regulated by TLR5 mediated sensing of flagellin by dendritic cells of the lamina propria[144]. After sensing of flagellin, CD103+CD11b+ dendritic cells produce high amounts of the type-17 associated cytokine, IL-23, causing a burst of IL-22 production and a subsequent induction of RegIIIγ in the epithelium[145]. Interestingly, TLR5 mediated sensing of microbial flagellin protects mice from *C. difficile* infection by reducing *C. difficile* growth and toxin expression. However, whether RegIIIγ-mediated killing of *C. difficile* is downstream of commensal-activated TLR5 requires further investigation[146].

1.3.3.2 The microbiota influences granulocyte function.

Cues from the microbiota also have an important impact on granulocyte function. Prolonged antibiotic therapy impairs granulopoiesis in the bone marrow by depletion of the microbiota in a STAT1 dependent manner[147]. Additionally, the depletion of the maternal microbiota results in impaired numbers of circulating neutrophil and granulocyte-precursors in neonates[148]. Restoration of the neonatal microbiota results in a rescue of IL-17 producing type-3 innate lymphoid cells (ILC3), G-CSF and neutrophil numbers, in a TLR4 and MYD88 dependent manner[148]. In line with these results, another study demonstrated that microbiota-derived peptidoglycan enhances neutrophil killing of the

pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus*[149]. Recognition of peptidoglycan by the pattern recognition receptor, NOD1, was sufficient to restore neutrophil function[149]. Interestingly, mice lacking the pattern-recognition receptors TLR4, MYD88, and NOD-1 are more susceptible to *C. difficile* infection and have deficiencies in their granulocyte responses, however whether a lack of host/microbiota immune development contributes to disease severity has not been addressed[132,149,150].

1.3.3.3 The microbiota influences type-1 immunity.

In addition to granulocyte defects, commensal dysbiosis also impairs lymphocyte function. Type-1 immunity is typically characterized by expression of the cytokines IFN-γ, IL-12, and IL-18 which canonically have anti-viral functions. Type-1 immunity is also an important defense mechanism during *C. difficile* infection and can be altered by commensal signals[75]. For example, the commensal *Bacteroides fragilis* contributes to the maintenance of CD4+ T cell development, TH-1 differentiation, and lymphoid organogenesis through the production and subsequent immunological presentation of the zwitterionic polysaccharide PSA[151]. In addition to adaptive TH-1 cells, innate NK cell function is also impaired in the absence of the microbiota. NK cells are cytotoxic type-1 innate lymphoid cells that require priming by mononuclear phagocytes in order to function[152]. Similar to TH-1 cells, NK function is also dependent on the microbiota. Germ-free mice have defective type-1 interferon production from mononuclear phagocytes and thus lack primed NK cells[153]. This defect in NK cell priming leads to diminished IFN-γ+ NK cells during viral infection and impaired viral clearance[153]. Type-1 immunity, specifically ILC1s and IFN-γ responses, were recently demonstrated to be critical for recovery from *C. difficile* infection[75,135]. Further investigations regarding antibiotic mediated depletion of microbiota-derived signals and how they contribute to *C. difficile* susceptibility via impairment of type-1 immunity are required.

1.3.3.4 The microbiota influences type-17 immunity.

Another flavor of immune defense, type-17 associated immunity, is critically intertwined with commensal derived signals. Type-17 immunity is associated with the expression of the pro-inflammatory cytokines, IL-23, IL-22, IL-17, and IL-21, and canonically drives recovery during extracellular bacterial infection[154,155]. Interestingly, during CDI, type-17 immunity can be both pathogenic and protective to host recovery. IL-23 and type-17 (TH-17) T cells increase mortality and tissue pathology during infection while IL-22 increases survival by promoting bacterial clearance[132,137,138,156,157]. Furthermore, antibiotic-mediated inhibition of neutrophil recruitment reduces tissue pathology during CDI while complete depletion of neutrophils causes severe mortality[132,158]. These findings indicate a dual-role for type-17 immunity during CDI – contributing to both pathogen clearance and host tissue damage. Signals from the microbiota seem to be important in maintaining effective type-17 defenses. For example, adaptive type-17 T-cell (TH-17) differentiation is impaired in the small intestine of germ-free mice and rescued with fecal transplant[159]. Additionally, the commensal organism segmented filamentous bacteria (SFB) drives TH-17 differentiation in the gut and can aggravate colitis and other auto-immune diseases[160–164]. SFB makes direct contact with intestinal epithelial cells, activating a IL-22/IL-23 dependent circuit between ILC3s, epithelial cells, and TH-17 cells[165,166]. Specifically, SFB colonization induces the expression of serum amyloid A proteins 1 and 2 (SAA1/2) by epithelial cells and subsequent IL-17A production by RoryT+ TH-17 cells[166]. The majority of intestinal TH-17 cells have TCR specificity for SFB derived antigens, demonstrating the striking immunodominance elicited to this commensal microbe[167]. In addition to adaptive TH-17 cells, the microbiota can additionally shape the function of type-3 innate lymphoid cells (ILC3s). Microbiota - induced TNF-like ligand 1A (TL1A) released from CX3CR1+ mononuclear phagocytes can increase the production of IL-22 by ILC3s[168]. This response

is important for mucosal healing and protection from acute DSS colitis and infectious *C. rodentium* mediated colitis[168]. Furthermore, the specific commensal, segmented filamentous bacteria, can induce IL-22 and IL-17 production by ILC3s, aiding in the expansion of TH-17 cells via serum amyloid A[165,166]. Thus, the microbiota is an important signal for maintaining type-17 immunity in mucosal tissue.

In addition to maintaining type-17 immune defenses, the microbiota also controls type-17 immune responses indirectly, via the induction of type-2 polarizing cytokines, IL-25, TSLP, and IL-33. Microbiota-regulated IL-25 expression by intestinal epithelial cells leads to the suppression of IL-22 production by ILC3s[169]. In a similar fashion, expression of colonic thymic stromal lymphopoietin (TSLP) is also dependent on the microbiota and bacterial-induced TLSP suppresses excessive TH-17 cell activation[170]. Lastly, during small intestinal inflammation, expression of microbiota-regulated IL-33 by intestinal epithelial cells causes TH-17 cells to acquire immunosuppressive properties such as IL-10 production[171,172]. These studies suggest that the microbiota is an important signal for controlling type-17 immunity via maintaining expression of IL-33, IL-25 and TSLP by intestinal epithelial cells.

1.3.3.5 The microbiota influences type-2 immunity.

Interestingly, microbiota-regulated IL-25 and IL-33 are important activators of type-2 innate lymphoid cells (ILC2s) and TSLP is important for ILC2 survival[173–177]. ILC2s express the transcription factor, GATA3, and make large amounts of type-2 cytokines, IL-4, IL-5, and IL-13 upon activation with type-2 polarizing cytokines IL-25, IL-33, and TSLP. Interestingly, while these polarizing cytokines are regulated by the microbiota[133,169,170,172], ILC2 seeding of both the lung[178] and intestine[179] occurs independently of the microbiota as germ-free mice have comparable ILC2s numbers. Furthermore, germ-free mice also have comparable expression of ILC2 activation markers IL-17RB and KLRG1 relative to SPF controls[179]. While the microbiota may not play a role in the seeding or abundance of

intestinal ILC2s, it may have an important role in promoting ILC2 function. In line with this idea, human ILC2s express toll-like receptors and can directly sense microbial PAMPs resulting in increased expression of IL-5 and IL-13[180]. Furthermore, during a model of intestinal colitis, germ-free mice had reduced expression of type-2 cytokines, IL-4, IL-5, IL-13, and IL-33 in addition to lack of eosinophils[181]. These studies indicate that the microbiota may have some influence on type-2 immune function. Given recent findings that IL-25 elicited eosinophils are protective during *C. difficile* infection[133], further studies are required to understand the connection between susceptibility to *C. difficile*, and possible antibiotic impairment of type-2 immune function.

1.3.3.6 The microbiota influences immune suppression.

In addition to mounting anti-microbial immunity, certain microbes of the commensal community are also essential for mounting immune suppression. One of the prime examples of this is the induction of regulatory Foxp3+ T cells (T-regs) by *B. fragilis* and Clostridia commensals. In addition to impairing TH-1 differentiation, *B. fragilis* derived PSA inhibits IL-17 production in the intestine by enhancing the production of anti-inflammatory IL-10 and T-regs to protect from experimental colitis[182]. Commensal Clostridia also contribute to IL-10+ T-reg accumulation in the gut by increasing the expression of TGF-β from intestinal epithelial cells[183]. Additionally, a consortia of 17 Clostridia strains, falling within clusters IV, XIVa and XVIII, were rationally identified from a human faecal sample based on their T-reg inducing activity, and could attenuate colitis upon administration to mice[184]. Finally, short-chain fatty acids, metabolites derived from commensal fermentation of carbohydrates, are important regulators of T-reg homeostasis and protect against colitis[185–187]. Therefore, sensing of the microbiota additionally maintains homeostasis through immune suppression, preventing an overly robust immune response to host-proximal commensals.

1.3.4 FMT strategies to treat C. difficile infection:

Our understanding and utilization of Fecal microbiota transplantation (FMT) has jump-started over the past two decades. FMT therapy is a procedure whereby a healthy faecal microbiota is introduced into a diseased gut to restore recipient microbial density and diversity and most importantly, health. Recent technological advancements in sequencing and metabolomics have allowed for better characterization of our intestinal microbial counterparts, triggering a surge of excitement in the fields of mucosal immunology and microbiology. This excitement is well-founded, as evidenced by 90% relapse free-cure rates in FMT treatment for recurrent CDI.

1.3.4.1 FMT treatment of recurrent C. difficile:

Fecal Microbiota Transplantation (FMT) therapy has had great success in the treatment of recurrent CDI. Standard of care treatments for CDI include treatment with antibiotics such as vancomycin and fidaxomicin. However, high rates of recurrence are problematic and occur in up to 30% of patients due to sustained antibiotic-associated dysbiosis[10,32]. While *C. difficile* is susceptible to antibiotic therapy, the antibiotics used to treat the infection further disrupt the microbiota that provides colonization resistance and immune-mediated protection against CDI. Thus, while killing *C. difficile*, antibiotics also cause greater host susceptible to reinfection and recurrent disease[188,189]. FMT combats this cyclical diseased state, by restoring a diverse community of microbes in the colon that restore colonization resistance.

FMT was first introduced into modern medicine in 1958 by Eisenmen *et al.* to treat pseudomembranous colitis with fecal enema[190]. Since then, multiple studies have demonstrated the effectiveness of FMT to treat recurrent CDI. However, the first randomized controlled trial was not conducted until 2013 in which 94% relapse-free *C. difficile* cure rates were reported, surpassing conventional vancomycin treatment by 3-

fold[140]. How FMT therapy alters the microbiota community structure is beginning to be understood. Patients with recurrent CDI who were successfully treated with FMT had increased diversity in their fecal microbiota with increased relative abundance of *Bacteroidetes* and decreased relative abundance of *Proteobacteria*[191].

The idea that a defined mixture of purified commensals within healthy stool are sufficient to transfer FMT-mediated protection was initially demonstrated by Tvede & Rask-Madsen in 1989. This initial human study demonstrated that the administration of 10 bacterial strains into 5 patients caused a loss of *C. difficile* and toxin levels within the infected bowel while replenishing *Bacteroides* species[192]. After this study, bacteriotherapy for CDI was limited and primarily focused on probiotic drinks in combination with standard antibiotic care that had some success[193,194]. A seminal study by Lawley et al, demonstrated that a defined consortium of six bacteria (S*taphylococcus, Enterococcus, Lactobacillus, Anaerostipes, Bacteroidetes*, and *Enterorhabdus)* could suppress hypervirulent *C. difficile* 027/BI and promote recovery from antibiotic associated dysbiosis[106]. Another group identified the family of bacteria, *Lachnospiraceae*, as a potential therapeutic target for bacteriotherapy[195]. Mice with mild *C. difficile* disease had microbiota communities dominated by *Lachnospiraceae* relative to mice with severe disease. A follow up study, demonstrated that pre-colonization of germ-free mice with *Lachnospiraceae*, leads to a dramatic reduction in *C. difficile* colonization and mortality after infection[196]. Additionally administration of *Clostridium scindens*, an intestinal bacteria whose depletion is associated with more severe disease in humans and mice, was able to restore colonization resistance against *C. difficile* infection in mice and protection is associated with secondary bile acid synthesis[112]. In addition to these studies, a phase II clinical trial demonstrated that 86.7% of patients were absent of *C. difficile* positive

diarrhea for 8 weeks after treatment with a cocktail of 50 species of Firmicutes spores derived from healthy donor stool[139].

Thus, in the past few years, great advancements have been made to improve our understanding of how defined species of bacteria can combat *C. difficile* infection. Likely, there is not just one commensal or one community architecture that contributes to colonization resistance, but multiple community structures exist that can transfer protection from *C. difficile* colitis. Given the vast complexity of the microbiota, its expansive influence on the metabolic state of the gut, and its numerous interactions with the mucosal interface and the host immune response, it is likely that not one magic bullet exists to combat CDI. This idea is supported by a recent study demonstrating that multiple CDI-resistant states of the microbiota exist with differing community structures however their functional outputs are similar[113]. Further studies defining mechanistic and functional outputs of commensals, such as their metabolomes and their interactions with the host, are key to the advancement of purified, next-generation bacteriotherapy to treat both *C. difficile* and other diarrheal diseases.

While it is clear the microbiota shapes the immune response, how host immunity alters the effectiveness of FMT therapy for CDI is still not well understood. Adaptive immunity does not play a critical role in primary *C. difficile* infection[75,197], however it does have a role in recurrent *C. difficile*[197–199] and thus it may contribute to the effectiveness of FMT for recurrent, severe, CDI. In line with this thinking, patients with recurrent CDI have increased pro-inflammatory IL-17+ or IFNY+ CD4+ T cells circulating in their peripheral blood[200]. Additionally, TH-17 cells are both necessary and sufficient to enhance the severity of *C. difficile* infection in settings of prior gut insult like IBD colitis[157]. Recently, it was also demonstrated that FMT therapeutically controls gut inflammation and colitis via the induction of IL-10 and TGF-β, cytokines critical for T-reg accumulation in the

intestine[201,202]. These studies indicate FMT-induced IL-10 + T-regs may be involved in the inhibition of pathogenic TH-17 cells during recurrent CDI. Thus, further investigation regarding the involvement of the immune system in FMT mediated control of recurrent *C. difficile* colitis is essential.

1.4 Host immune response to primary *C. difficile* infection:

While *C. difficile* toxins drive epithelial disruption and disease during CDI, our lab and others have demonstrated that the type of host immune response generated against *C. difficile* also dictates the severity of symptoms during infection independent of bacterial factors[6,74,203,204]. Both protective and pathogenic associations have been made between immune defenses and *C. difficile* severity. Human clinical studies have demonstrated a detrimental role for an over-active immune response during CDI as IL-8 and CXCL5 levels positively predict disease severity, whereas *C. difficile* bacterial burden does not [134,203]. In contrast to this, high eosinophil levels, a type-2 immune cell canonically important for parasite expulsion from infected mucosal tissue[205], are associated with protection during human and mouse studies of CDI[6,133,136]. These studies indicate that in addition to toxin epithelial damage, the type of immune response generated during CDI dictates disease severity. Here we will review the balance between anti-microbial vs. tissue regulatory immunity during *C. difficil*e infection.

1.4.1 Immune recognition of C. difficile:

After toxin mediated damage to the epithelium, pro-inflammatory cytokines and chemokines are quickly released both locally and systemically to orchestrate an acute immune response that prevents bacterial dissemination. TcdA, TcdB, and CDT activate the transcription factors NF-kB and AP-1 leading to expression of various proinflammatory cytokines such as IL-1β, IL-8, IL-6 IFN-γ, and IL-23 [74,135,206–211]. These cytokines are important orchestrators of neutrophil recruitment, ILC activation, and monocyte activation that aid in bacterial clearance[30,75,132,212,213]. Interestingly, IL-1β release is regulated by

toxin activation of the pyrin inflammasome sensor[214,215]. Pyrin interacts with the adapter protein, apoptosis-associated speck-like protein (ASC), to orchestrate caspase-1 cleavage of IL-1β and IL-18. Our lab has discovered that CDT toxin serves as an inflammasome priming signal, increasing pro-IL-1β transcripts and NF-kB activation in a TLR2-dependent fashion[6]. *C. difficile* toxins TcdA and TcdB serve as inflammasome activation signals, triggering oligomerization of the inflammasome sensor via their Rho-GTPase glucosylating activity[214,215]. Thus, all three *C. difficile* toxins (CDT, TcdA and TcdB) together are sufficient to prime and activate the inflammasome during infection and mediate IL-1β maturation and release. The inflammasome is essential for recovery from acute CDI as complete abolishment of the inflammasome complex in $ASC^{-/-}$ mice, causes severe mortality during infection and impaired CXCL1 mediated neutrophil chemotaxis[216].

In addition to immune recognition of *C. difficile* toxins, other *C. difficile* derived PAMPs are also recognized by pattern-recognition receptors to elicit an inflammatory response during infection. Nucleotide-binding oligomerization domain 1 (Nod-1), important for recognition of bacterial peptidoglycan, recognizes *C. difficile* and aids in bacterial clearance and neutrophil recruitment during infection[217]. TLR4 recognizes *C. difficile* surface layer proteins (SLPs), important for DC maturation and the development of antibody responses to SLPs during infection[150]. Finally, *C. difficile* flagellin can stimulate TLR5 leading to NF-kB and p38 MAPK activation[144,146,218]. Flagellin stimulation leads to increased production of IL-8 and CCL20 by epithelial cells that can be augmented by pre-treatment with TcdB[219]. This immune pathway may have less impact *in vivo* as $TLR5^{-/-}$ mice have equivalent disease severity during infection[146]. These studies indicate that immune recognition of *C. difficile* toxins or other *C. difficile* derived PAMPS aids are key drivers of host immunity during infection to aid in bacterial clearance.

1.4.2 Cellular immunity during primary CDI:

After the release of cytokines, chemokines, and alarmins, a swift cellular response is mounted to control *C. difficile* and pathobiont semination. A balance between anti-microbial vs. tissue regulatory immune responses is essential for host protection. Here we review the innate immune subsets important for driving resolution of *C. difficile* infection. While adaptive immunity does not play a central role in acute primary *C. difficile* infection[75,197], innate lymphoid cells and myeloid cells are essential for CDI recovery and restoration of homeostasis.

*1.4.2.1 Type-17 immunity, a double-edged sword during CDI***:**

Type-17 associated cytokines, IL-1β, IL-23, IL-17a, IL-22 and IL-6 are upregulated in the gut in response to *C. difficile* infection[74,135]. Both pathogenic and protective outcomes are associated with type-17 immunity during *C. difficile* colitis[74,75,138,156,220,221]. IL-23 signaling is pathogenic during CDI, contributing to increased mortality and tissue pathology[74,156] whereas IL-22 contributes to survival and systemic pathobiont elimination through the complement system[138]. The type-17 cytokine network is a key driver of neutrophil production and accumulation during mucosal infections. In accordance, neutrophils are characteristically recruited at high levels into the colon during *C. difficile* colitis via MyD88 signaling[132,222]. Neutrophils have important anti-microbial functions during infection through their phagocytic activity, granule formation, and production of antimicrobial proteases, peptides, and reactive oxygen species[222]. Dual roles for neutrophils have been reported during *C. difficile* colitis. Complete depletion of neutrophils with anti-GR1 antibody, increases CDI- associated mortality however inhibition of neutrophil recruitment through blockage of CD18 or MIP-2 or inhibition of IL-23 reduces tissue pathology and mortality during infection[74,132,212,223,224]. Similarly in human patients, high neutrophil counts are associated with more severe disease whereas neutropenia is a risk factor during infection[225,226]. Thus, type-17 associated immunity is essential for

pathogen elimination however if left unregulated, can contribute to profound tissue damage during infection.

1.4.2.2 Type-1 immunity promotes recovery during CDI:

Recently an important role for type-1 immune responses has been demonstrated during *C. difficile* infection[75,135,211]. Type-1 associated cytokines, IFN-γ and IL-12, are upregulated in response to *C. difficile* infection in humans and mice[75,211,227]. Innate lymphoid cells are lymphocytes that lack antigen specific receptors however can rapidly respond to inflammatory signals by producing large amounts of cytokine. ILCs, similar to their T cells counterparts, come in three flavors identified by the transcription factors they express in addition to the cytokines they make: T-bet expressing ILC1s, GATA3 expressing ILC2s, and RorγT expressing ILC3s. Mice lacking ILCs have increased mortality during *C. difficile* infection and lack the ability to upregulate IFN-γ and IL-22[75,228]. Further investigation revealed that IFN-γ+ ILC1s were central to survival during CDI with a very limited role for IL-22+ ILC3s[75]. This important role for IFN-γ+ ILC1s was corroborated in humans demonstrating that high levels of IFN-γ in the serum of CDI-patients is associated with less severe infection[135]. How IFN-γ protects during CDI requires further investigation however it may act by increasing phagocytic functions during infection[229].

1.4.2.3 Type 2 immunity contributes to immune protection during CDI.

Recent studies from our lab and others demonstrate that type-2 immune responses play an important role in recovery and survival during CDI[6,133,136]. Eosinophils, a type-2 associated myeloid subset, have protective roles in both human and murine studies of *C. difficile* colitis[6,133,136]. CDT toxin expressed by epidemic NAP1/027 isolates can kill protective eosinophils during infection contributing to their heightened virulence[6]. Thus, promoting type-2 immunity during infection presents a new avenue for treatment during hypervirulent CDI. IL-25 and IL-5 are type 2 cytokines that contribute to eosinophil

accumulation in the intestine[230], and are associated with survival and less severe disease during CDI[133,135]. Eosinophil survival and maintenance within mucosal tissue depends on the action of type-2 innate lymphoid cells (ILC2s). ILC2s constitutively express the eosinophil survival cytokine, IL-5, resulting in eotaxin production and eosinophil accumulation in the circulation and peripheral tissues[230]. Central to our current studies, we have identified a novel protective role for the type-2 cytokine, interleukin-33 (IL-33) during murine and human CDI studies via the action of type-2 innate lymphoid cells (ILC2s). We will discuss the importance of IL-33 in greater detail below.

1.5 IL-33: an alarmin and type-2 cytokine that maintains intestinal homeostasis

The IL-33 receptor, ST2, was first reported on in 1989[231] and is a member of the IL-1 receptor family. The ligand for ST2 remained unknown until the discovery of IL-33 in 2005 through a computational sequence database search of the IL-1 cytokine family[232]. This seminal study also described IL-33's ability to induce type-2 cytokine expression, eosinophilia, goblet cell hyperplasia, and serum IgE and IgA at mucosal surfaces[232]. Since its discovery, many studies have described a role for IL-33 in various diseases including asthma, anaphylaxis, and atherosclerosis. Within the gut, IL-33 is host-protective against helminth infection while both protective and pathogenic roles have been described in IBD colitis. Here we will describe the biology of IL-33, how it regulates mucosal immunity, and its involvement in infectious disease.

1.5.1 IL-33 biology:

IL-33 is a member of the IL-1 family of cytokines and functions both as an alarmin, released from cells upon death, and as a cytokine capable of inducing type-2 immunity in the colon[233,234]. IL-33 is produced by intestinal pericryptal myofibroblasts, in addition to epithelial, endothelial, and adventitial stromal cells[173,235–238]. Full-length IL-33 is biologically active through its carboxy-terminal IL-1-like domain. IL-33 can be cleaved by

caspases 3 and 7 during apoptosis[239]. Unlike the IL-1 family member, IL-1β, the caspase cleavage site of IL-33 is located within the IL-1-like domain, thus rendering its cleavage products biologically inactive and limiting inflammation during programmed cell death[239]. IL-33 lacks a signal sequence[240] thus release of full-length active IL-33 most likely occurs after inflammatory cell death such as necrosis or active necroptosis[241,242]. After release, IL-33 binds to its receptor complex ST2 and the accessory receptor, IL-1R accessory protein (IL-1RAP). The ST2 receptor complex induces downstream signaling via the TIR domain of IL-1RAP through the recruitment of the adapter proteins, MyD88, IRAK1, and IRAK4 and activation of NF-kB and MAPK signaling pathways[232]. ST2 is expressed on a variety of innate and adaptive cells, including type-2 innate lymphoid cells (ILC2s), eosinophils, regulatory T- cells (T-regs), and T helper-2 cells[243]. IL-33 can also act directly on the intestinal epithelium as LGR5+ stem cells also express ST2 and their activation with IL-33 leads to differentiation into secretory-cell lineages[235]. Thus, IL-33 is abundantly expressed at mucosal surfaces and is released after cell damage to alert and activate an immune response.

1.5.2 IL-33 barrier and immune regulation in the intestine:

Within the intestine, IL-33 is constitutively expressed by pericryptal myofibroblasts located at the base of intestinal crypts and it's expression is increased in response to systemic infection and sepsis[235]. Additionally, IL-33 was recently demonstrated to be expressed by adventitial stromal cells, a fibroblast-like cell type that line the perivascular cuffs of blood vessels[238]. Additionally, during inflammation IL-33 can be expressed by additional cell type such as epithelial cells within intestinal crypts[171]. Interestingly, a recent study demonstrated that IL-33 can act directly on the ST2+ LGR5+ intestinal stem cells to reprogram them towards secretory lineages, increasing goblet cell, Paneth cell, and enteroendocrine cell numbers per crypt[235]. This resulted in enhanced mucin production

by the epithelium, a key barrier defense that limits the interaction between the epithelium and invading pathogens of the intestine[244]. Thus, IL-33 can stimulate mucosal barrier defenses by directly reprogramming the epithelium.

In addition to maintaining epithelial barrier defenses, IL-33 also activates both innate and adaptive immune cells in the intestinal lamina propria. Many innate and adaptive cell types express the ST2 receptor and respond to IL-33 such as type-2 innate lymphoid cells, eosinophils, mast cells, macrophages, type-2 T cells, regulatory T-cells, and B cells. Upon release, IL-33 elicits a strong type-2 inflammatory response in the intestine, characterized by IL-4, IL-5, and IL-13 cytokine production[232,245]. This type-2 immune response is essential for dispelling parasites in the intestine. Additionally, IL-33 elicits important tissue-repair pathways such as stimulation of the epithelial growth factor, amphiregulin, by intestinal ILC2s[175]. Amphiregulin acts on the epithelium to increase regeneration and tight-junction formation[178]. IL-33 also increases tissue-reparative M2 macrophages to promote wound healing after tissue damage[246]. Thus, IL-33 has essential functions in maintaining intestinal homeostasis (**Figure 1.4**) and we will review these functions in the context of intestinal disease below.

1.5.3 IL-33 during colitis:

During intestinal inflammatory diseases such as irritable bowel disease (IBD), IL-33 has been reported to act in both a protective manner by increasing tissue-repair or in a pathogenic manner by enhancing inflammation. A handful of studies have demonstrated that IL-33 signaling exacerbates colitis in murine models. Specifically, three studies have demonstrated that IL-33 signaling increases inflammation during a dextran sulphate sodium (DSS) chemical model of IBD colitis, which closely resembles human ulcerative colitis (UC).

Figure 1.4

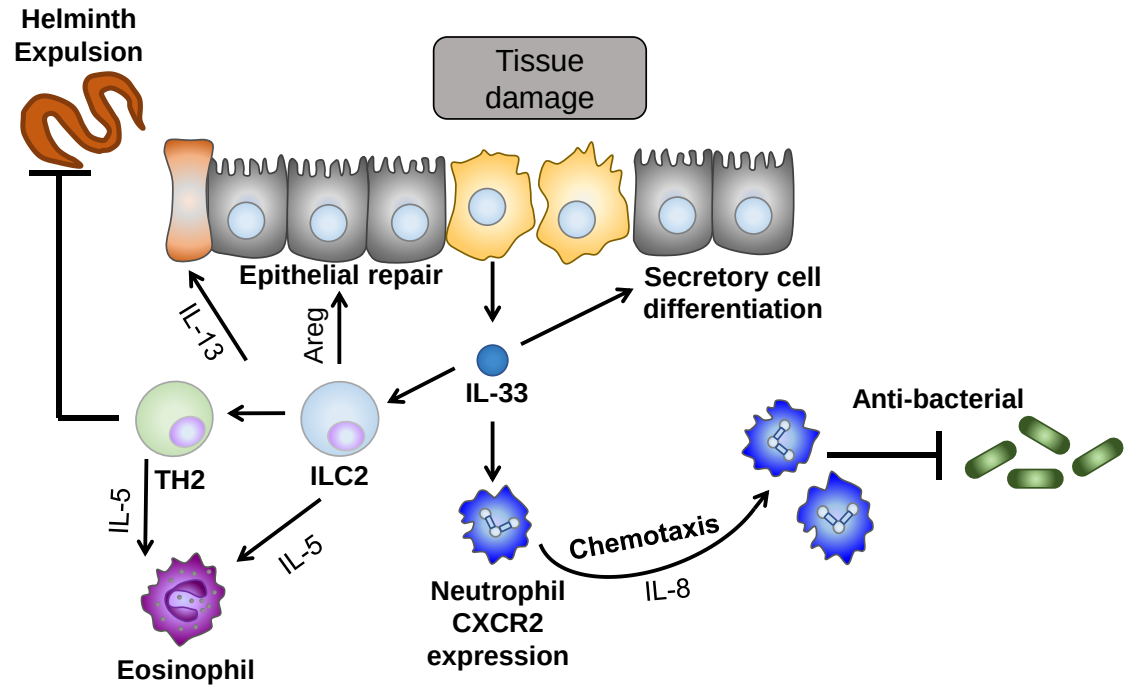
Helminth Expulsion
Tissue damage
Epithelial repair
Secretory cell differentiation
IL-13
Areg
IL-33
TH2
ILC2
IL-5
IL-5
Eosinophil
Neutrophil CXCR2 expression
Chemotaxis
IL-8
Anti-bacterial

Figure 1.4: Mechanism of IL-33 mediated protection of mucosal tissue.

IL-33 is released from mucosal barrier cells, such as pericryptal myofibroblasts, endothelial cells and epithelial cells, upon inflammatory cell death[235,245]. During bacterial infections, IL-33 can act in an anti-microbial manner, increasing neutrophil chemotaxis through inhibition of TLR4 signaling and preventing the downregulation of chemokine receptor, CXCR2, on the neutrophil surface[247]. Additionally, IL-33 can act directly on epithelial LGR5+ ST2+ intestinal stem cells to increase secretory lineage differentiation (Paneth cells, goblet cells, and enteroendocrine cells)[235]. This direct interaction between the epithelium and IL-33 increases anti-microbial peptide production, aiding in bacterial clearance. During helminth infection, IL-33 can polarize type-2 immune responses by the activation of ST2+ ILC2s and ST2+ TH-2 cells[233,245]. Activation ILC2s and TH-2 cells elicit downstream eosinophil accumulation via their IL-5 expression[230,232,248]. Furthermore, IL-33 mediated activation of ILC2s and TH-2 cells enhances their expression of IL-13, which increases goblet cell hyperplasia and mucin responses which aid in parasite expulsion[249–251]. IL-33 activated ILC2s can also promote epithelial cell regeneration via their expression of the growth factor, amphiregulin (AREG)[175,178].

One study demonstrated that blockade of ST2 decreased histopathology, inflammation and disease-activity index during colitis induced by DSS or trinitrobenzene sulfonic acid (TNBS)[252]. Furthermore, IL-33 treatment of 0.5µg on days 1,3, and 5 of DSS-treated mice, increased the disease-activity index at disease onset[252]. A second study demonstrated that a higher dosage of IL-33, 1µg/mouse daily for 19 days during DSS administration, exacerbated DSS- mediated diarrheal scores and colon shortening via IL-4 mediated inflammation[253].

Additionally, $ST2^{-/-}$ had delayed diarrhea development during DSS. However, weight-loss was never assessed between $ST2^{-/-}$, IL-33+ or controls[253]. Weight-loss is an indicator of epithelial disruption, water-loss, and inflammation during colitis and its measurement helps assess IL-33's influence at different phases of disease (ie. acute disease vs. recovery). Along these lines, a third study demonstrated that deficiency of the *IL-33* gene causes amelioration of weight-loss and inflammation early on during DSS-colitis but impairs weight-gain during the recovery phase of DSS[254]. This data indicates that IL-33 may have dual roles during DSS colitis by enhancing inflammation at disease onset yet subsequently being required for recovery and weight-gain.

IL-33 has also been demonstrated to play a pathogenic role in a SAMP1/YitFc (SAMP) murine model of spontaneous Crohns-like ileitis[181,255]. Neutralization of ST2 in transgenic SAMP mice, possessing a mixed TH-1/TH-2 immunological profile, results in less severe intestinal inflammation, specifically eosinophilic infiltration, however weight-loss, and colon length were not assessed[181]. Additionally IL-33 was upregulated in SAMP mice and correlated with histopathology disease severity[255]. As neither of these studies measured weight-loss or the severity of diarrhea, it is difficult to assess the role of IL-33 on morbidity and recovery. It is possible that IL-33 exacerbates inflammation at acute phases of colitis, however it may have a differential role during later phases of colitis in promoting recovery and weight-gain that were not assessed. However, in summary of the above findings, IL-

33 can have a pathogenic role during murine IBD colitis models by exacerbating intestinal inflammation at acute phases of disease.

In contrary to these findings, other investigations have demonstrated that IL-33 is protective from colitis-associated disease via the enhancement of anti-inflammatory and tissue repair pathways[175,256]. One investigation of the pathogenesis of DSS-induced colitis demonstrated that treatment of mice with 1 µg of IL-33 for 5 days post-DSS reduced weight-loss and increased colon length[246]. The same study also demonstrated that IL-33 treatment prevented weight-loss very early on during TNBS-induced colitis, a Th1-mediated CD-like colitis model. The authors demonstrate that IL-33 protection was mediated via the enhancement of wound-healing, M2 macrophages. Specifically, transfer of IL-33 treated M2 macrophages were sufficient to protect from weight-loss and tissue pathology.

Similar to the above study, a second study also has demonstrated that IL-33 acts in a protective manner during DSS-associated colitis via innate immunity. Treatment with IL-33 for 7 days during DSS-administration, activates amphiregulin expressing ILC2s and limits tissue damage and intestinal disease[175]. Amphiregulin is an epidermal growth factor (EGR)-like protein that promotes epithelial regeneration and tight-junction formation[257]. Adoptive transfer of ILC2s into amphiregulin deficient mice is sufficient to limit tissue pathology, enhance mucin production, and decrease weight-loss associated with colitis[175]. Thus, both the above two studies have demonstrated that IL-33 can protect from IBD-colitis models by enhancing innate-like tissue repair pathways.

Adaptive immunity has also been demonstrated to play an important role in IL-33-mediated protection from IBD-like colitis. IL-33-activated T-regs (ST2+ T-regs) have innate-like behavior; producing high levels of IL-10 and TGF-β in the absence of TCR stimulation[258,259]. Adoptive transfer of ST2(+) T-regs, but not ST2(-) T-regs protects mice from experimental T-cell mediated colitis models[260]. Furthermore, an additional study

demonstrated that IL-33 treatment reduced weight-loss and histopathology during TNBS-induced colitis in a T-reg dependent manner [261]. Thus, IL-33 can limit colitis severity via the action of anti-inflammatory, ST2+ T-regs.

It is clear that IL-33 signaling is dysregulated in human IBD. Human patients with IBD had decreased levels of circulating IL-33 in their serum[246]. Additionally, multiple studies have demonstrated that IL-33 is significantly upregulated in human biopsies from Ulcerative colitis patients[252,255,262,263]. However, the exact role of IL-33 in the pathogenesis of IBD requires further investigation. Both beneficial and pathogenic roles for IL-33 have been identified using murine colitis models making it difficult to assess whether IL-33 upregulation in human IBD is a driver of disease or a mechanism of repair. Discrepancies in the role of IL-33 may be attributed to differences in the immunological profiles between experimental colitis models, the timing and dosage of IL-33, or the phase at which disease severity is assessed. It appears that the timing of IL-33 signaling has an important role in the development of disease as IL-33$^{-/-}$ mice have enhanced disease early on yet reduced ability to recover from weight-loss[254]. These findings may explain the discrepancies between animal models with variations in treatment timing. However, based on the studies reviewed here it is currently not clear whether IL-33 treatment during disease onset vs. recovery is pathogenic or protective. Thus, further assessment of the timing and dosage of IL-33 in the context of IBD is required to evaluate therapeutic applications in the future.

1.5.4 IL-33 in intestinal helminth disease.

Given IL-33's central location at barrier surfaces, it is an important danger signal released upon pathogen infection. Upon IL-33 activation, ILC2s and TH-2 cells promote eosinophilia, goblet cell hyperplasia, mucin production, and alternatively activated macrophage activation within the mucosal tissue of the intestine[175,249], skin[176,264] and lung[248,265,266]. These responses are characteristic of the 'weep and sweep' immunity

required to expel helminth parasites from host mucosal tissue[267,268]. For example, administration of recombinant IL-33 early during infection with the intestinal nematode, *Trichuris muris,* leads to enhanced TH-2 responses and increased worm expulsion[250]. IL-33 mediated protection from *T. muris* was dependent on TH-2 cells as it was absent in SCID mice. Recently IL-33 activated ILC2s were also demonstrated to also play a critical role during intestinal nematode infection. Mice lacking IL-33 signaling have impaired expulsion of *Nippostrongylus brasilensis* due to their lack of IL-13 production from ILC2s in addition to TH-2 cells[251,269]. Adoptive transfer of ILC2s was sufficient to decrease worm burdens. ILC2 mediated protection was dependent on IL-13 expression as IL-13 deficient ILC2s failed to expel *Nippostrongylus brasilensis*[269]*.* In summary, the induction of type-2 immune cells by IL-33 plays a protective role in expelling nematodes from mucosal tissue.

1.5.5 IL-33 in bacterial infections:

IL-33 signaling via the ST2 receptor has also been shown to protect during bacterial infections via both anti-microbial and anti-inflammatory mechanisms. For example, during *S. aureus* infection, IL-33 improved antimicrobial iNOS production by macrophages in the skin, thus inhibiting *S. aureus* growth[270]. Another study demonstrated that IL-33 treatment inhibited *S. aureus* colonization, enhancing neutrophil trafficking and tissue healing within the *S. aureus* infected wound[271]. IL-33 similarly protected via anti-microbial mechanisms during intestinal *Salmonella Typhimurium* challenge*.* IL-33 knockout mice have increased *Salmonella* burden, decreased Paneth cells and anti-microbial peptides, and increased tissue disruption[235]. Thus, during these infections, IL-33 elicits anti-microbial responses to aid bacterial expulsion.

IL-33 can also protect via anti-inflammatory mechanisms during bacterial infections. During *P. aeruginosa* infection of the cornea, IL-33 protects from disease pathology. Treatment of mice with IL-33 reduced *P. aeruginosa* bacterial burden and

corneal disease by increasing anti-inflammatory macrophage polarization and IL-10 levels while decreasing pro-inflammatory cytokines and neutrophil influx[272]. In *M. tuberculosis* infection of the lung, IL-33 also protects by promoting anti-inflammatory immunity. In this model, IL-33 treatment ameliorates *M. tuberculosis* infection, reducing neutrophils into the lung and increasing protective M2 macrophage tissue-repair[273]. Thus, during bacterial infections of mucosal tissue, IL-33 can act in both anti-inflammatory and anti-microbial ways to mediate protection.

IL-33 also plays an important role during bacterial sepsis. Sepsis is a systemic inflammatory state caused by bacterial infection that has high rates of mortality. During human sepsis, sST2, the soluble decoy receptor for IL-33, is a marker of mortality and severity, demonstrating that dysregulation of IL-33 is clinically relevant[274,275]. Building off of this, IL-33 protected from a murine model of bacterial sepsis, by increasing neutrophil chemotaxis into the peritoneal cavity by preventing neutrophil downregulation of the chemokine receptor CXCR2[274]. A similar anti-microbial effect via IL-33 induction of neutrophils was also seen in *S. aureas* caused peritonitis in addition to enhanced wound-healing[276]. Another study, demonstrated that IL-33 protected from *Staphylococcus aureus* bacteremia-induced mortality via an ILC2 dependent mechanism[277]. IL-33 signaling to ILC2s and eosinophils balanced dysregulated and overly robust septic inflammatory responses. Furthermore, patients who survived *Staphylococcus aureus* bacteremia had increased circulating TH-2 cells and reduced TH-17 cells[277]. Thus, similar to bacterial infection of mucosal tissues, IL-33 protects from systemic bacteremia by maintaining anti-microbial and tissue-regulatory immune responses to promote bacterial clearance and homeostasis.

Finally, IL-33 is also essential for the development of endotoxin tolerance, a phenomenon whereby repeated exposure to endotoxin leads to immune paralysis,

protecting against bacterial septic shock. Investigations demonstrated that IL-33 signaling through the ST2 receptor competes with TLR4 activation in a MYD88 dependent manner therefor limiting endotoxin exposure and protecting from a subsequent lethal challenge of endotoxin[278]. Similarly mice lacking the IL-33 receptor, showed decreased tolerance and increased mortality against a subsequent lethal bacterial lipoprotein (BLP) challenge[279]. In contrast to these studies, IL-33 mediated immune-suppression, induced by CLP sepsis challenge, was pathogenic and contributed to mortality during a subsequent infection with *Legionella pneumophila*[280]. In this study, IL-33 signaling contributed to long-term immune suppression after sepsis by induction of M2 macrophages and ST2+ regulatory T cells[280]. Thus, IL-33 signaling is important for the development of endotoxin tolerance after survival from initial septic challenge. ST2 mediated tolerance is a double-edged sword as it can protect against multiple septic challenges while also contributing long term immune-suppression and risk of not clearing a subsequent infection.

In summary, these studies demonstrate a critical role for IL-33 in bacterial infections through its ability to re-establish homeostasis. IL-33 can harness type-2 immunity, tissue-repair, and activate anti-microbial pathways during bacterial infections. We establish here an additionally important role for IL-33 curing *C. difficile* infection via ILC2s and demonstrate an important interaction between IL-33 and the microbiota.

1.6 Significance and Overview

The CDC has listed *C. difficile* among the top three urgent antimicrobial resistant health threats in the United States[1]. *C. difficile* incidence has tripled over the past 15 years and is attributed to the emergence of hypervirulent epidemic strains of *C. difficile* (referred to as NAP1/027*)*. Hypervirulent epidemic strains of *C. difficile* express an additional virulence factor, binary toxin (CDT), and are associated with more severe disease.

While it is clear that *C. difficile* toxins cause damaging colonic inflammation during infection, the immune mechanisms protecting the host from tissue damage require further investigation. Emerging evidence indicates gut immunity to *C. difficile* is a delicate balance between protection and pathology. Based on our current understanding, the balance and intensity of the host immune response can dictate disease severity during infection. Specifically, type-2 associated eosinophils can promote recovery while an overly robust TH-17 response can exacerbate *C. difficile* severity. The goal of my research was to identify novel therapeutic host immune targets that are dysregulated during hypervirulent CDT expressing *C. difficile* infection.

In this dissertation I: 1) demonstrate that IL-33 protects the gut barrier and prevents mortality during *C. difficile* infection via the action of type-2 innate lymphoid cells; a cell type previously not known to play a role during *C. difficile* recovery and 2) identify IL-33 signaling as a predictor of human *C. difficile* mortality, demonstrating that IL-33 is regulated by the microbiota and rescued with FMT therapy. In summary, IL-33 signaling is a novel therapeutic pathway for severe CDI which can potentially be targeted with rationally designed microbial therapies.

Chapter Two: IL-33 drives group 2 innate lymphoid cell-mediated protection during *Clostridium difficile* infection.

This chapter has been adapted from "Microbiota-regulated IL-33 drives group 2 innate lymphoid cell-mediated protection during *Clostridium difficile* infection." *Nature Communications* (2019).

Alyse L. Frisbee, Mahmoud M. Saleh[1], Mary K. Young[1], Morgan E. Simpson[1], Carrie A. Cowardin[3], Stephen D. Turner[3], Erica L. Buonomo[2], and William A. Petri, Jr.

[1] M.M.S, M.K.Y., M.E.S, helped with tissue extraction and processing, bacterial quantification, and invaluable advice.

[2] E.L.B. aided with colon flow protocol development, tissue extraction and processing, and invaluable experimental guidance.

[3] S.D.T., and C.A.C. aided with microarray sample collection, processing and analysis.

2.1 Introduction

C. difficile is an anaerobic, spore forming bacterium that is a growing public health threat. The emergence of NAP1/027 isolates over the past decade have caused worldwide epidemics with high mortality rates[10,281]. In the US alone, an estimated half a million *C. difficile* cases and 29,000 deaths occurred in 2011[11]. Disruption of the intestinal microbiota, most often through antibiotic exposure, greatly increases the risk of *C. difficile* infection of the colon. Hypervirulent NAP1/027 clones, such as the epidemic R20291 strain used in this study, are associated with higher mortality rates, express higher amounts of the classical *C. difficile* exotoxins, TcdA and TcdB, and also produce a third toxin called *C. difficile* transferase (CDT)[5,8,68,281]. CDT is a binary toxin composed of a binding domain, CDTb, and an enzymatic domain, CDTa, which together mediate intoxication of colonic epithelial cells via the lipolysis stimulated lipoprotein (LSR) and result in cytoskeleton disruption[88,89,282]. Our lab and others have recently identified CDT as a virulence factor that increases toll-like receptor 2 (TLR2)-mediated inflammation during infection[6,68,283]. All three toxins can together cause symptomatic disease by damaging colonic epithelial cells and eliciting pro-inflammatory, inflammasome-dependent IL-1β release[6,214,284]. Development of a murine model of *C. difficile* colitis has allowed for better understanding of disease onset which is characterized by an acute 2-5 day inflammatory response with high neutrophil influx, diarrhea, and weight-loss followed by a resolution phase with weight-gain[105,132,285,286].

The murine model of *C. difficile* colitis has led to new insights into the relationship between the host immune response and disease development. Recent studies in both mice and humans have demonstrated an important yet multifaceted role for the host immune system during *C. difficile* infection. Early acute cellular responses such as MYD88-mediated neutrophilia, IL-25-responsive eosinophils, and IFN-γ+ producing type-

1 innate lymphoid cells, provide protection during *C. difficile* infection. However, many pro-inflammatory pathways such as Th-17 associated IL-23, IL-17 and toll-like receptor 2 (TLR2) signaling exacerbate *C. difficile*-associated mortality[6,75,132,133,156,221,287]. Several of these murine studies have been corroborated in human patients with IFN-γ, IL-5 and peripheral eosinophil counts being associated with less severe infection yet pro-inflammatory cytokines such as IL-8, IL-2, and IL-6 being associated with poor prognosis[134–136,204]. These studies emphasize the complexity of the immune network elicited during CDI. Likely, a robust anti-microbial response is vital for bacterial clearance, however, this circuit must be tightly regulated to prevent chronic intestinal damage. Thus, further investigation is required to discover novel immune avenues that control CDI-associated tissue damage and improve patient survival.

In the present study, we use whole-tissue transcriptomics to identify potential therapeutic immune pathways that aid in recovery during infection with the hypervirulent CDT expressing strain, R20291. We identify the IL-1 cytokine family member, IL-33, as an important guardian of the gut barrier during *C. difficile* colitis that prevents CDI-associated mortality via IL-33 responsive type-2 innate lymphoid cells (ILC2s). IL-33 signaling protects the gut barrier, decreasing toxin-mediated epithelial disruption and required ST2+ ILC2s to protect. Adoptive transfer of ILC2s is sufficient to transfer protection from *C. difficile*-associated weight-loss and mortality. These findings identify a novel pathway essential for host-recovery and tissue-repair during *C. difficile* colitis.

2.2 Results

2.2.1 CDT toxin drives more severe disease during CDI.

To study host-derived gene expression changes during *C. difficile* infection (CDI), we infected mice with the CDT expressing epidemic PCR-ribotype 027 strain, R20291, or with an isogenic mutant lacking the binding region of CDT (R20291_*cdtb*)[283]. As we

previously reported, the *CDTb* mutant strain caused a less virulent infection with a reduction in mortality, weight-loss and clinical score severity (**Fig 2.1A-C**), confirming CDT's role as a virulence factor[6]. Loss of CDT caused a 55% decrease in mortality during infection. Peak weight-loss and clinical scores occurred around day 3 post infection with loss of CDT causing 13% less weight-loss relative to R20291 infection. Our previous findings demonstrate that CDT toxin has immune-modulatory activity; enhancing inflammasome activation and increasing pro- IL-1β transcripts[6]. Given the high mortality and weight-loss during R20291 infection, we wondered how the host immune response changed in response to more severe CDT expressing infection.

2.2.2 Host gene expression changes in response to CDT toxin in wildtype mice.

To find possible therapeutic immune targets relevant during severe infection, we compared whole caecal tissue transcriptomes of R20291 vs. R20291_*cdtb* infected mice via Affymetrix microarray. 484 genes were differentially expressed in response the severe CDT+ infection [logfc ≥ 0.5, $P < 0.05$]. The top 50 significantly upregulated (**Table 2.1**) or downregulated genes (**Table 2.2**) are listed.

2.2.2.1 Identification of IL-33 as a potential immune target:

The microarray analysis revealed a 1.8-fold increase in the alarmin and type-2 cytokine, interleukin-33 (IL-33) (**Fig 2.2 A-B**). To assess the relevance of IL-33 during *C. difficile* infection, we quantified IL-33 protein and RNA during R20291 and R20291_*cdtb*

Figure 2.1

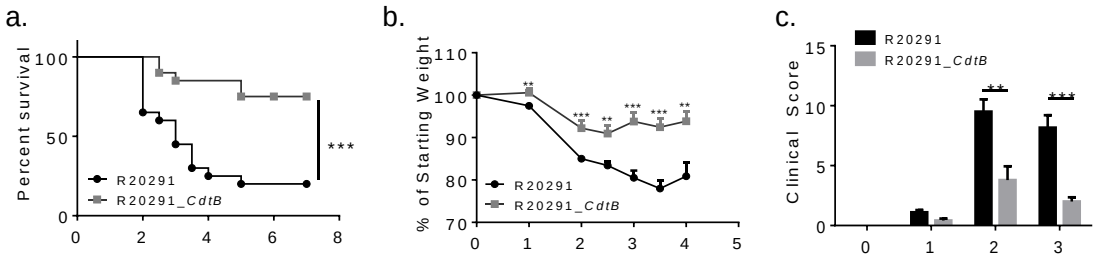
a.
Percent survival
100
50
0
0
2
4
6
8
R20291
R20291_CdtB
***
b.
% of Starting Weight
110
100
90
80
70
0
1
2
3
4
5
R20291
R20291_CdtB
c.
Clinical Score
15
10
5
0
0
1
2
3
R20291
R20291_CdtB
**
***

Figure 2.1: CDT expressing R20291 has increased virulence relative to attenuated, R20291_*cdtB*.

(A-C) Mice were infected with R20291 (CDT+) or R20291_*cdtb* (CDT-) and (A) survival, (B) body-weight and (C) clinical scores were assessed on each day post infection. (A–C) n = 10 per experiments and combined from 2 independent infections (A) Comparison made by log rank test. (B-C) Comparison made by student t test. *P < 0.05, **P < 0.01, ***P < 0.001.

Table 2.1 Host immune changes upregulated in response to CDT expressing R20291.

AffyID	Entrez	SYMBOL	logFC	AveExpr	T	P.Value	adj.P.Val	Fold Change
17408897	12655	CHIL3	2.431	7.473	4.18	0.0004	0.051	5.392
17278321	20717	SERPINA3M	2.140	6.320	4.23	0.0004	0.049	4.407
17284505	380809	IGHV10-3	1.962	8.624	3.04	0.0062	0.136	3.896
17343628	57875	ANGPTL4	1.594	6.141	3.56	0.0018	0.083	3.020
17514541	17384	MMP10	1.405	8.372	4.27	0.0003	0.047	2.648
17383892	16819	LCN2	1.371	8.241	3.14	0.0049	0.122	2.587
17298775	11752	ANXA8	1.258	8.300	3.96	0.0007	0.056	2.391
17212174	16178	IL1R2	1.233	6.171	3.20	0.0043	0.115	2.351
17514482	17386	MMP13	1.206	6.751	3.25	0.0037	0.109	2.307
17312219	546644	LY6G	1.177	8.344	2.21	0.0384	0.308	2.261
17211305	94227	PI15	1.177	5.385	3.38	0.0028	0.095	2.260
17217399	12654	CHIL1	1.149	8.564	3.38	0.0028	0.096	2.217
17514515	17392	MMP3	1.125	6.362	2.35	0.0282	0.268	2.181
17318089	17067	LY6C1	1.123	4.480	3.25	0.0038	0.109	2.177
17255023	72381	2210409E12RIK	1.111	5.173	4.11	0.0005	0.053	2.159
17492947	80982	CEMIP	1.107	6.446	3.59	0.0017	0.080	2.153
17514553	17394	MMP8	1.100	4.950	3.26	0.0037	0.107	2.143
17348492	16774	LAMA3	1.076	8.325	4.46	0.0002	0.044	2.108
17329759	11815	APOD	1.069	6.527	2.74	0.0121	0.184	2.097
17461205	12661	CHL1	1.051	5.906	3.62	0.0016	0.078	2.073
17362973	68774	MS4A6D	1.048	6.919	3.68	0.0014	0.072	2.068
17491941	56847	ALDH1A3	1.040	7.902	4.52	0.0002	0.041	2.057
17330359	85031	PLA1A	1.024	5.827	4.83	0.0001	0.034	2.034
17222800	227059	SLC39A10	1.016	7.509	5.29	0.0000	0.029	2.022
17228136	16782	LAMC2	1.011	9.044	4.93	0.0001	0.032	2.015
17230045	15951	IFI204	1.007	5.101	2.65	0.0150	0.203	2.009
17414802	18491	PAPPA	0.996	7.517	3.25	0.0038	0.109	1.994
17221401	277328	TRPA1	0.980	5.843	4.30	0.0003	0.047	1.973
17403205	229898	GBP5	0.966	6.040	2.96	0.0073	0.146	1.954
17249036	14584	GFPT2	0.963	6.339	2.52	0.0197	0.231	1.949
17267617	217066	GM15698	0.935	6.337	2.67	0.0141	0.197	1.912
17535774	104368	SNORA70	0.928	5.494	2.82	0.0101	0.169	1.903
17349552	436583	SNORA74A	0.914	7.206	4.27	0.0003	0.047	1.884
17358598	77125	IL33	0.907	7.838	2.96	0.0074	0.148	1.875
17388999	228413	PRRG4	0.903	7.530	4.94	0.0001	0.032	1.870
17533713	21857	TIMP1	0.896	6.613	2.89	0.0087	0.159	1.861
17391554	16175	IL1A	0.886	5.051	3.35	0.0030	0.099	1.848
17470627	56619	CLEC4E	0.884	4.608	4.16	0.0004	0.051	1.846
17526707	235320	ZBTB16	0.875	6.493	3.04	0.0061	0.135	1.834
17449718	15945	CXCL10	0.873	6.862	2.46	0.0223	0.243	1.832
17218845	20344	SELP	0.865	6.874	2.57	0.0177	0.220	1.822
17268066	217122	GM11545	0.864	9.800	4.84	0.0001	0.034	1.820
17455507	15505	HSPH1	0.860	8.839	3.48	0.0022	0.087	1.815
17399823	20201	S100A8	0.858	5.204	3.54	0.0019	0.084	1.813
17333731	14289	FPR2	0.854	3.991	3.11	0.0053	0.127	1.807
17391709	269356	SLC4A11	0.849	5.979	6.14	0.0000	0.020	1.802
17354589	225594	GM4841	0.847	4.781	2.52	0.0197	0.231	1.799
17464654	27273	PDK4	0.847	6.137	2.49	0.0210	0.238	1.799
17508850	20288	MSR1	0.84	6.38	3.35	0.0030	0.099	1.80

Table 2.1: Host immune changes upregulated in response to CDT expressing R20291.

Top significantly upregulated genes in response to CDT expressing R20291 relative to R20291_*cdtb*. Wildtype C57BL6 mice were infected with R20291 or R20291_*cdtb* and 3 days post infection mouse caecal tissue was harvested, washed with PBS, and RNA was extracted. Affymetrix microarray revealed relative gene expression changes in R20291 vs. R20291_*cdtb* infected mice. n = 5-6 mice per condition.

Table 2.2 Host immune changes downregulated in response to CDT expressing R20291.

AffyID	Entrez	SYMBOL	logFC	AveExpr	t	P.Value	adj.P.Val	Fold Change
17525578	56857	SLC37A2	-2.573	7.721	-4.60	0.0002	0.037	-5.951
17467529	16019	IGHM	-2.073	6.880	-2.49	0.0213	0.238	-4.207
17459383	243469	IGK	-1.861	9.221	-2.96	0.0074	0.148	-3.632
17284919	105387	AKR1C14	-1.751	7.242	-4.96	0.0001	0.032	-3.366
17482897	11833	AQP8	-1.750	8.411	-2.76	0.0115	0.180	-3.364
17290263	105349	AKR1C18	-1.656	5.994	-5.02	0.0001	0.032	-3.151
17410251	13809	ENPEP	-1.628	7.755	-4.80	0.0001	0.034	-3.091
17336526	100038862	BTNL1	-1.575	6.982	-4.12	0.0005	0.053	-2.980
17507308	20494	SLC10A2	-1.548	7.579	-3.05	0.0060	0.134	-2.925
17268884	217166	NR1D1	-1.417	8.006	-6.32	0.0000	0.020	-2.670
17467696	330350	9130221F21RIK	-1.399	5.447	-3.01	0.0066	0.139	-2.638
17273447	58210	SECTM1B	-1.392	9.731	-4.05	0.0006	0.054	-2.625
17408360	56185	HAO2	-1.310	8.361	-2.54	0.0191	0.228	-2.479
17224015	74591	ABCA12	-1.257	8.537	-3.48	0.0022	0.087	-2.390
17378685	383766	TLDC2	-1.246	8.476	-6.64	0.0000	0.020	-2.371
17491205	20208	SAA1	-1.221	10.607	-2.63	0.0155	0.206	-2.331
17284577	780931	IGHV1-53	-1.184	10.209	-3.56	0.0018	0.083	-2.273
17284507	102642252	LOC102642252	-1.183	8.584	-2.25	0.0354	0.297	-2.271
17343488	64385	CYP4F14	-1.175	8.479	-3.82	0.0010	0.064	-2.258
17408336	15494	HSD3B3	-1.171	8.452	-3.78	0.0011	0.066	-2.252
17329847	106407	SLC51A	-1.161	7.913	-3.35	0.0030	0.099	-2.236
17545899	12309	S100G	-1.152	5.524	-3.74	0.0012	0.068	-2.222
17319707	76279	CYP2D26	-1.150	8.068	-2.35	0.0285	0.270	-2.219
17400162	20341	SELENBP1	-1.150	10.394	-4.37	0.0003	0.044	-2.218
17365098	20249	SCD1	-1.135	8.248	-2.70	0.0132	0.191	-2.196
17313583	13105	CYP2D9	-1.125	4.727	-4.58	0.0002	0.038	-2.181
17274813	13487	SLC26A3	-1.114	10.866	-3.10	0.0054	0.128	-2.165
17484477	22264	PRAP1	-1.095	6.891	-4.98	0.0001	0.032	-2.137
17348860	17288	MEP1B	-1.094	9.497	-4.13	0.0005	0.053	-2.135
17510772	15245	HHIP	-1.059	7.444	-3.99	0.0007	0.055	-2.084
17400124	20342	SELENBP2	-1.041	8.243	-4.19	0.0004	0.051	-2.058
17516997	434402	GM5617	-1.014	6.314	-2.60	0.0165	0.214	-2.019
17266436	20500	SLC13A2	-1.003	8.577	-4.45	0.0002	0.044	-2.004
17246602	237625	PLA2G3	-0.996	6.308	-5.96	0.0000	0.023	-1.994
17362839	69826	MS4A10	-0.985	6.326	-3.17	0.0046	0.118	-1.980
17477169	72431	CEACAM18	-0.984	5.707	-5.94	0.0000	0.023	-1.978
17300717	17224	MCPT1	-0.969	7.060	-2.43	0.0240	0.251	-1.958
17330799	433023	GM5485	-0.959	7.660	-3.78	0.0011	0.065	-1.944
17284519	16061	IGH-VJ558	-0.954	7.479	-2.53	0.0195	0.230	-1.937
17408323	15493	HSD3B2	-0.954	5.820	-3.94	0.0007	0.057	-1.937
17346401	171168	ACER1	-0.949	7.136	-3.28	0.0035	0.106	-1.931
17225993	320311	RNF152	-0.942	6.639	-3.26	0.0037	0.108	-1.921
17496354	20887	SULT1A1	-0.933	8.344	-2.93	0.0080	0.152	-1.909
17409130	14864	GSTM3	-0.928	7.776	-3.39	0.0028	0.095	-1.902
17459696	100038714	GM15401	-0.919	6.110	-4.28	0.0003	0.047	-1.891
17322750	268860	ABAT	-0.919	8.230	-5.30	0.0000	0.029	-1.891
17427928	13370	DIO1	-0.918	6.859	-4.58	0.0002	0.038	-1.890
17449406	53315	SULT1D1	-0.902	9.604	-3.25	0.0038	0.109	-1.869
17345066	17287	MEP1A	-0.898	9.431	-4.17	0.0004	0.051	-1.863

Table 2.2: Host immune changes downregulated in response to CDT expressing R20291.

Top significantly downregulated genes in response to CDT expressing R20291 relative to R20291_*cdtb*. Wildtype C57BL6 mice were infected with R20291 or R20291_*cdtb* and 3 days post infection mouse caecal tissue was harvested, washed with PBS, and RNA was extracted. Affymetrix microarray revealed relative gene expression changes in R20291 vs. R20291_*cdtb* infected mice. n = 5-6 mice per condition.

Figure 2.2

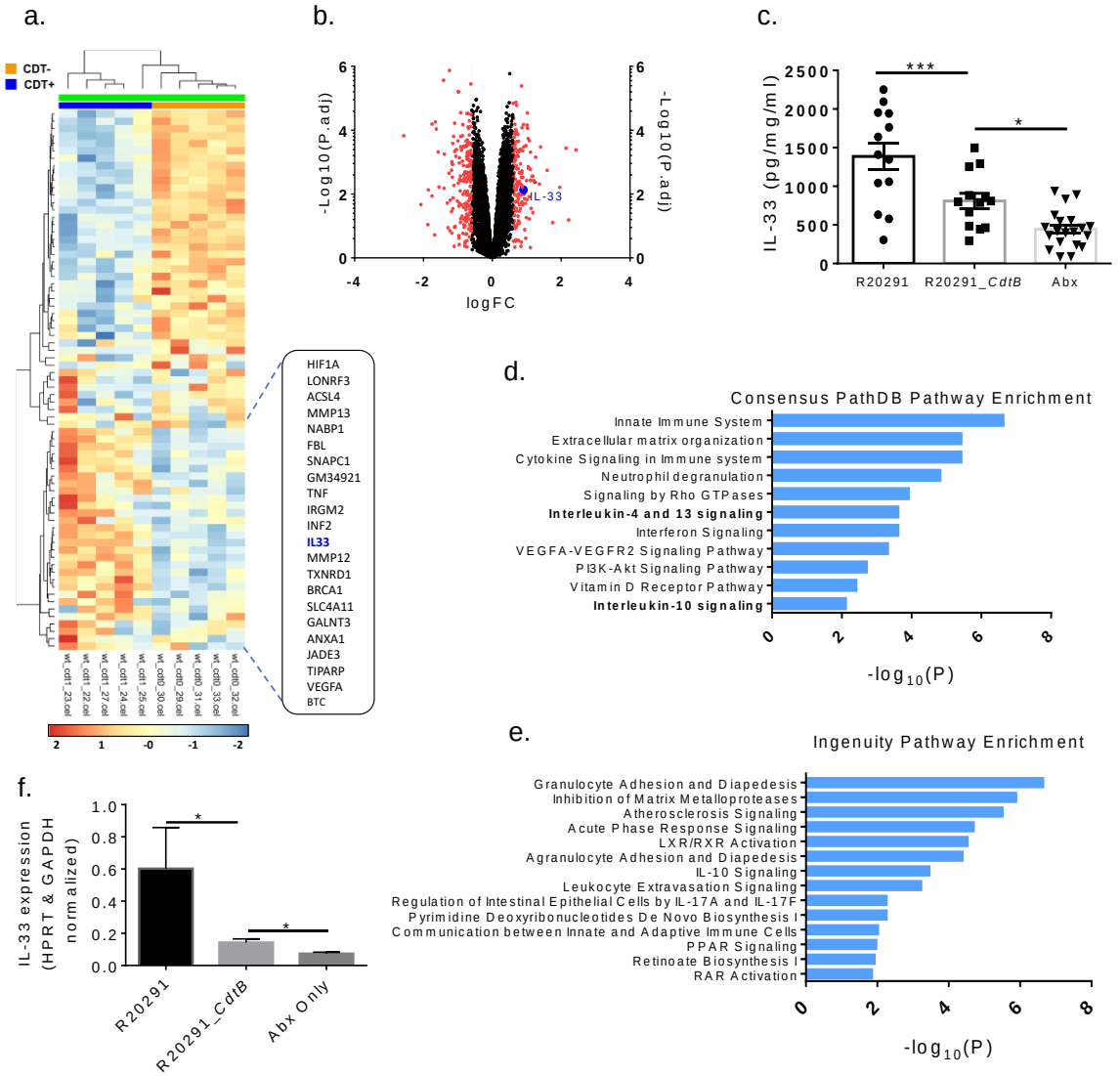
a.
b.
c.
CDT-
CDT+
HIF1A
LONRF3
ACSL4
MMP13
NABP1
FBL
SNAPC1
GM34921
TNF
IRGM2
INF2
IL33
MMP12
TXNRD1
BRCA1
SLC4A11
GALNT3
ANXA1
JADE3
TIPARP
VEGFA
BTC
-Log10(P.adj)
logFC
IL-33
IL-33 (pg/mg/ml)
R20291
R20291_CdtB
Abx
d.
Consensus PathDB Pathway Enrichment
Innate Immune System
Extracellular matrix organization
Cytokine Signaling in Immune system
Neutrophil degranulation
Signaling by Rho GTPases
Interleukin-4 and 13 signaling
Interferon Signaling
VEGFA-VEGFR2 Signaling Pathway
PI3K-Akt Signaling Pathway
Vitamin D Receptor Pathway
Interleukin-10 signaling
-log10(P)
e.
Ingenuity Pathway Enrichment
Granulocyte Adhesion and Diapedesis
Inhibition of Matrix Metalloproteases
Atherosclerosis Signaling
Acute Phase Response Signaling
LXR/RXR Activation
Agranulocyte Adhesion and Diapedesis
IL-10 Signaling
Leukocyte Extravasation Signaling
Regulation of Intestinal Epithelial Cells by IL-17A and IL-17F
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I
Communication between Innate and Adaptive Immune Cells
PPAR Signaling
Retinoate Biosynthesis I
RAR Activation
-log10(P)
f.
IL-33 expression (HPRT & GAPDH normalized)
R20291
R20291_CdtB
Abx Only

Figure 2.2: IL-33 is upregulated by the host in response to *C. difficile* infection

Mice were infected with R20291 (CDT+) or avirulent R20291_*cdtb (CDT-)* and whole-caecal tissue transcriptomic analyses was performed on Day 3 post infection. (*A*) Heat map of genes upregulated (red) or downregulated (blue) in response to CDT toxin. (*B*) Volcano plot highlighting IL-33 (blue) among genes altered (> 0.5 logFC = red). (*C*) ELISA of IL-33 protein within the caecal tissue (Day 3). (*D*) Enriched pathways of the top upregulated transcripts (log FC > 0.5; p<0.05) listed by significance (-log10(p)) and created using the ConsensusPathDP database. (E) Enriched pathways of the top upregulated transcripts (log FC > 0.5) listed by significance (-log10(p)) and created using the Ingenuity Database. (F) IL-33 transcript abundance in infected caecal tissue was measured (Day 3) by Taqman qPCR normalized to housekeeping genes. Data are presented as means ± SEM. (*A*-*B*), *n* = 5 mice (*C*) *n* = 7-10 per experiment combined from 2-3 infections. (F) n = 3-6 per group. (*C&F*) Comparison made by ANOVA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

infections and in uninfected controls. Both qPCR and ELISA analyses indicated upregulation of IL-33 in response to both hypervirulent R20291 and attenuated R20291_*cdtb* infection relative to uninfected controls (**Fig 2.2C, 2.2F**). Mice infected with the hypervirulent strain, had a 1.71-fold increase in IL-33 protein relative to R20291_*cdtb* infected mice and R20291_*cdtb* infected mice had a 1.82-fold increase in IL-33 protein relative to uninfected controls (**Fig 2.2C, 2.2F**). This data indicates, that IL-33 expression is increased in response to increasing severity of *C. difficile* infection.

2.2.2.2 Pathways significantly enriched in response to CDT toxin.

In order to understand higher level functional changes within the transcriptome of CDT+ vs. CDT- infected mice, we conducted pathway analysis of the significantly altered genes within our data set. This analysis of the microarray data set using Ingenuity[288], and Consensus Path DB[289] pathway tools revealed enrichment of many immune-related pathways in response to the CDT expressing strain, including many tissue-regulatory pathways including IL-10R, IL-4, and IL-13 (**Fig 2.2D-E**). These pathways have been associated with tissue repair and can counter pro-inflammatory responses to promote healing[268,290–294]. Given increased IL-33 expression in response to CDI, in addition to its established role in promoting IL-10, IL-4, and IL-13 tissue regulatory pathways[243,295–298], we hypothesized IL-33 may be an important upstream regulator of gut barrier defense during *C. difficile* infection.

2.2.3 IL-33 protects from mortality and intestinal damage during epidemic and classical C. difficile infection.

In order to determine if IL-33 mediates protection during infection, we used a mouse model of CDI to ascertain the effect of IL-33 on disease severity, tissue pathology and mortality[6,74,105,133]. First, we asked whether increasing IL-33 levels in the gut alters disease severity during infection. IL-33 was administered in 5 doses by intraperitoneal

injection (0.75µg/mouse) prior to infection (**Fig 2.3A**). Our IL-33 treatment regimen caused a 5-fold increase in IL-33 protein levels within the colon prior to infection (**Fig 2.3B**). Notably, this IL-33 treatment regimen reduced mortality following infection with R20291 as evidenced by the increased survival [70% with IL-33 treatment vs. 30% without) (**Fig 2.4A**). Additionally, IL-33 treated mice had reduced weight-loss, and less severe clinical scores (a measurement system of weight loss, piloerection, ocular discharge, activity, posture, and diarrhea) (**Fig 2.4B**-**C**). We also noticed that IL-33 treatment reduced epithelial barrier disruption during infection. Blinded histology scoring of the epithelial barrier by H&E staining revealed a significant reduction in epithelial damage and submucosal edema (**Fig 2.4D-E**). Accordingly, IL-33 treated mice had reduced gut permeability as quantified by FITC-dextran gut-barrier permeability assay (**Fig 2.3C**).

Despite the reduction in epithelial damage, IL-33-mediated protection was not due to a reduction in *C. difficile* bacterial burden as IL-33 treated mice were colonized to equivalent levels as vehicle controls (**Fig 2.4F**). IL-33-mediated protection was also not caused by a reduction in TcdA and B or CDT as IL-33 treatment mice had equivalent toxin levels over the course of acute infection when peak disease occurred (**Fig 2.5A-B**). Furthermore, IL-33 treatment did not prevent translocation of gut commensals as IL-33 treated mice had equivalent dissemination of commensal bacteria into their livers likely caused by gut leakiness still present in IL-33 treated mice as demonstrated by FITC-dextran within their serum. (**Fig 2.3C-D**).

Since the microbiota plays a critical role in the pathogenesis of CDI[104,299], we assessed whether IL-33 treatment altered the microbiota prior to infection via analysis of the V4 region 16 S rRNA gene amplified from caecal contents of IL-33 vs. vehicle treated mice. We found no significant differences in the alpha diversity or beta diversity between

Figure 2.3

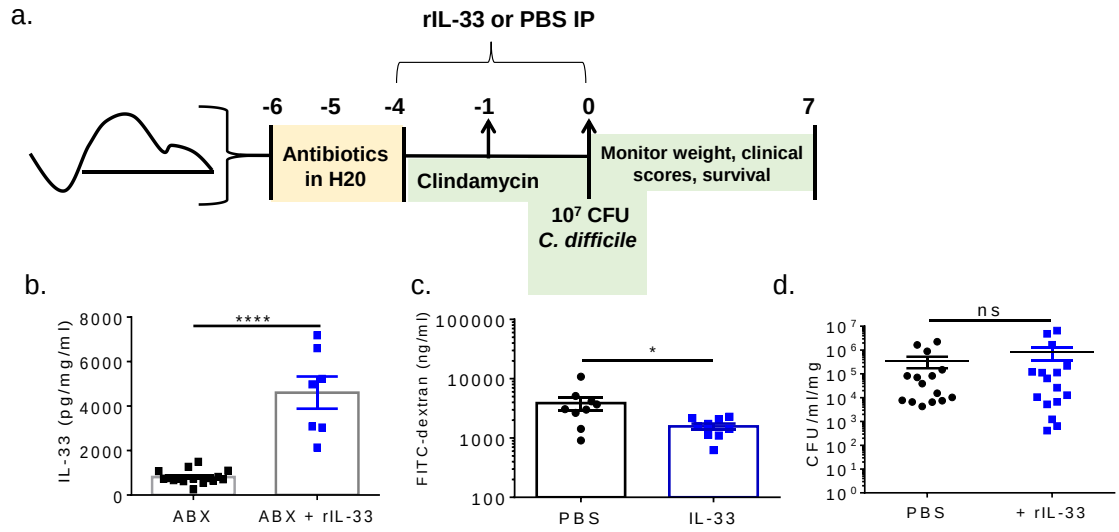
a.
rIL-33 or PBS IP
-6
-5
-4
-1
0
7
Antibiotics in H20
Clindamycin
Monitor weight, clinical scores, survival
10^7 CFU
C. difficile
b.
IL-33 (pg/mg/ml)
8000
6000
4000
2000
0
****
ABX
ABX + rIL-33
c.
FITC-dextran (ng/ml)
100000
10000
1000
100
*
PBS
IL-33
d.
CFU/ml/mg
ns
PBS
+ rIL-33

Figure 2.3. IL-33 treatment protects the gut epithelial barrier during infection but does not decrease translocating commensals into the liver. *(A)* IL-33 treatment experimental design. *(B)* Total IL-33 protein present in caecal tissue with and without IL-33 treatment measured by ELISA. *(C)* FITC-dextran concentration present in serum after gut permeability assay during infection (Day 2). (*D*) IL-33 and PBS treated mice were infected with R20291 *C. difficile* liver tissue was harvested and colony forming units were counted on BHI agar plates (Day 2). Data are presented as means ± SEM *(B-C)* n = 7–12 per group *(C)* n = 7-10 per group combined from two infections. *(B)* Comparison made by student t test. *(C)* Comparisons for commensal burden were made by student t test and Mann–Whitney U tests. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2.4

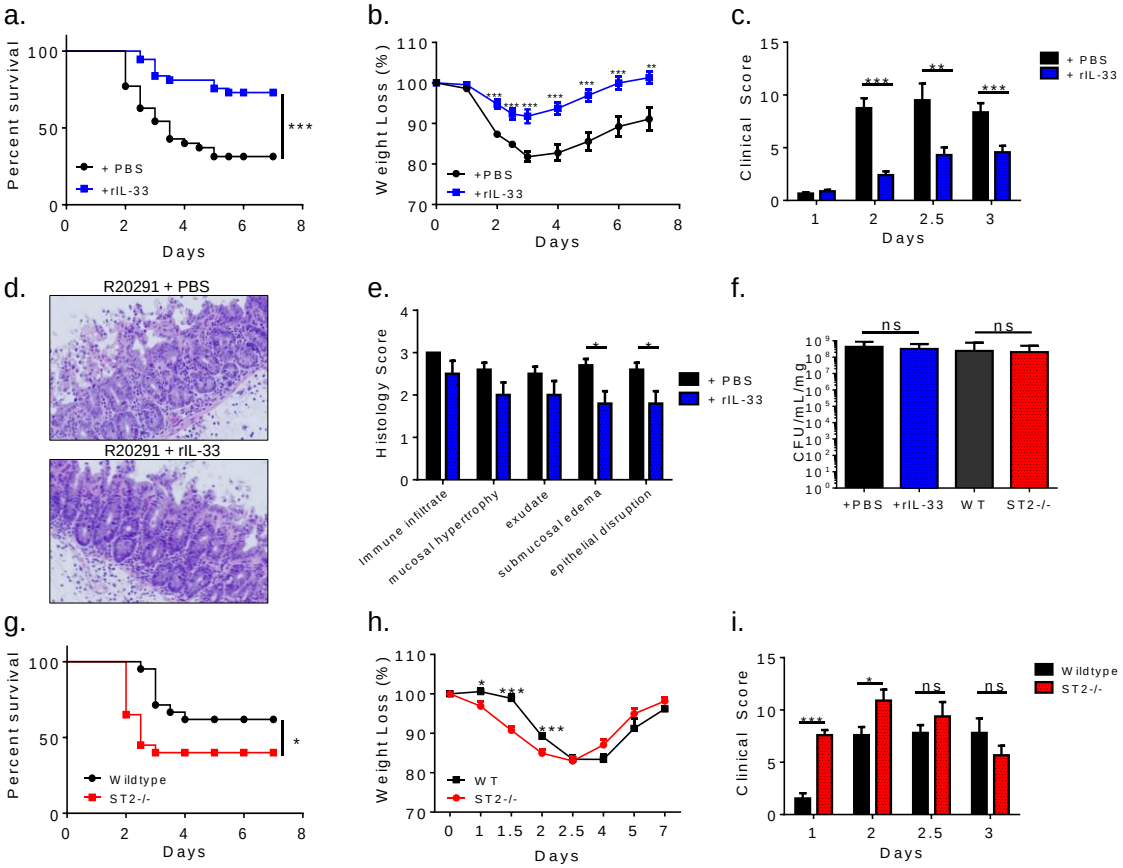
a.
Percent survival
100
50
0
0 2 4 6 8
Days
+ PBS
+rIL-33
***
b.
Weight Loss (%)
110
100
90
80
70
0 2 4 6 8
Days
+PBS
+rIL-33
c.
Clinical Score
15
10
5
0
1 2 2.5 3
Days
+ PBS
+ rIL-33
***
**
***
d.
R20291 + PBS
R20291 + rIL-33
e.
Histology Score
4
3
2
1
0
+ PBS
+ rIL-33
*
*
Immune infiltrate
mucosal hypertrophy
exudate
submucosal edema
epithelial disruption
f.
CFU/mL/mg
ns
ns
+PBS +rIL-33 WT ST2-/-
g.
Percent survival
100
50
0
0 2 4 6 8
Days
Wildtype
ST2-/-
*
h.
Weight Loss (%)
110
100
90
80
70
0 1 1.5 2 2.5 4 5 7
Days
WT
ST2-/-
*
***
***
i.
Clinical Score
15
10
5
0
1 2 2.5 3
Days
Wildtype
ST2-/-
***
*
ns
ns

Figure 2.4. IL-33 protects from mortality and epithelial barrier disruption during *C. difficile* infection. C57BL6 mice (*A-F*) or ST2$^{-/-}$ mice (*F-I*) were treated with an antibiotic cocktail prior to infection with R20291 and treated by IP injection with recombinant IL-33 (rIL-33) or vehicle control (PBS) for 5 days prior to infection. (*A*) Survival curves (*B*), weight-loss (*C*), and clinical scores after infection and treatment. (*D-E*) Infected caecal tissue was examined with IL-33 treatment and without (Day 3). (*D*) Representative epithelial damage (H&E) of treatment groups assessed by (E) blinded scoring of infected tissue (H&E). (*F*) Peak *C. difficile* bacterial burden in caecal contents after infection (Day 2) of IL-33 treated mice or ST2$^{-/-}$ mice compared to vehicle or wildtype controls. (*G-I)* ST2$^{-/-}$ on C57BL6 background and C57BL6 controls were cohoused for 3 weeks prior to R20291 infection. (*G*) Survival curves (*H*) weight-loss and (*I*) clinical scores were assessed. Data are presented as means ± SEM (*A*–*F*) n = 10 per experiment combined from 2 to 4 infections and (*D*) Images at 10X magnification. (*G*–*I*) n = 7-10 per experiment combined from 2-4 infections. (*A and G*) Comparison made by log rank test (*B-E and H-I*) Comparison made by student *t* test. (*F*) Comparisons for *C. difficile* burden were made by Mann–Whitney *U* tests. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2.5

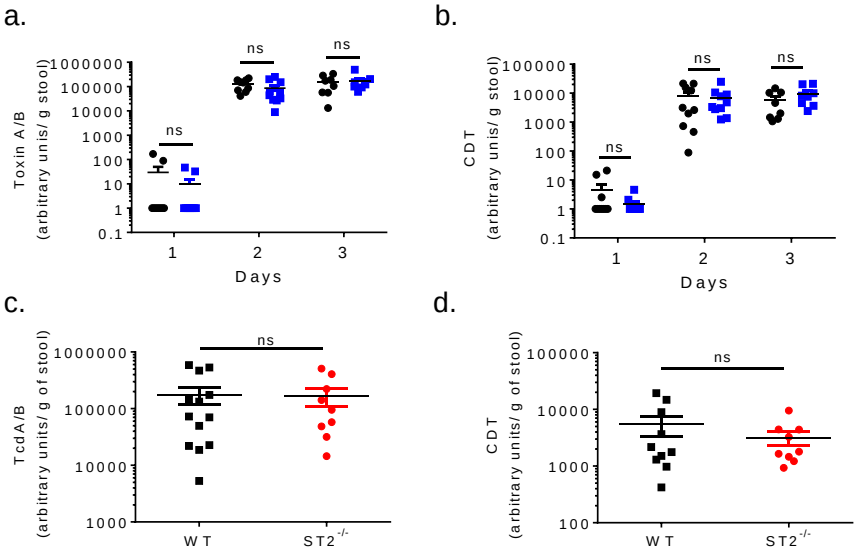
a.
b.
c.
d.
Toxin A/B
(arbitrary unis/ g stool)
CDT
(arbitrary unis/ g stool)
TcdA/B
(arbitrary units/ g of stool)
CDT
(arbitrary units/ g of stool)
ns
Days
WT
ST2-/-

Figure 2.5. Exogenous and endogenous IL-33 do not alter *C. difficile* toxin levels during infection. *(A-B)* IL-33 treated (blue square) vs vehicle treated (black circles) mice were sacrificed on day 1, 2 and 3 post infection and (*A*) Tcd A and TcdB in the caecal contents were assessed by ELISA and *(B)* CDT in the caecal contents was assessed by ELISA. *(C-D)* $ST2^{-/-}$ vs. WT mice were sacrificed on day 2 post infection and (*C*) TcdA and TcdB in the caecal contents were assessed by ELISA and *(B)* CDT in the caecal contents was assessed by ELISA. Data are presented as means ± SEM *(A-C)* n = 7-12 per group representative of two infections. Comparisons for toxins were made by student t test and Mann–Whitney U tests. *P < 0.05, **P < 0.01, ***P < 0.001.

the treatment groups (**Fig 2.6A-C**). Thus, IL-33 treatment did not significantly alter the microbiota composition prior to infection with *C. difficile*.

Given the striking protection elicited by exogenous IL-33 treatment, we additionally asked whether endogenous IL-33 signaling contributes to survival from CDI using transgenic mice lacking the IL-33 receptor, $ST2^{-/-}$. Complementing the IL-33 treatment data, $ST2^{-/-}$ mice had higher mortality rates compared to cohoused wildtype controls (**Fig 2.4G**). Markedly, $ST2^{-/-}$ mice lost more weight and developed clinical signs by Day 1 of infection when wildtype mice lacked signs of disease (**Fig 2.4H-I**). Similar to the IL-33 treatment data, $ST2^{-/-}$ mice did not have more severe disease due to increased *C. difficile* bacterial burden or increased toxin levels as there were no significant differences in CDI colonization or toxins TcdA and TcdB, or CDT levels between $ST2^{-/-}$ vs. controls on Day 2 post infection when disease was most severe. (**Fig 2.4F, Fig 2.5C-D**). Together these results indicate that IL-33 signaling protects from mortality during CDI independent of *C. difficile* bacterial burden and toxin expression.

2.2.3.1 IL-33 mediated protection is not restricted to CDT expressing NAPI/027 infection

As IL-33 was identified to be highly upregulated in response to CDT toxin, we wondered whether IL-33 mediated protection was specific to CDT toxin expressing *C. difficile*. Thus, we applied our IL-33 treatment regimen to mice infected with the isogenic CDTb mutant (R20291_*cdtb*) in addition to the non-ribotype 027 strain, VPI 10463. IL-33 mediated protection was not restricted to hypervirulent R20291 infection as IL-33 treatment robustly protected against the non-ribotype 027 *C. difficile* strain VPI 10463 (**Fig 2.7A-C**), and the attenuated R20291_*cdtb* mutant strain (**Fig 2.7D-F**).

Figure 2.6

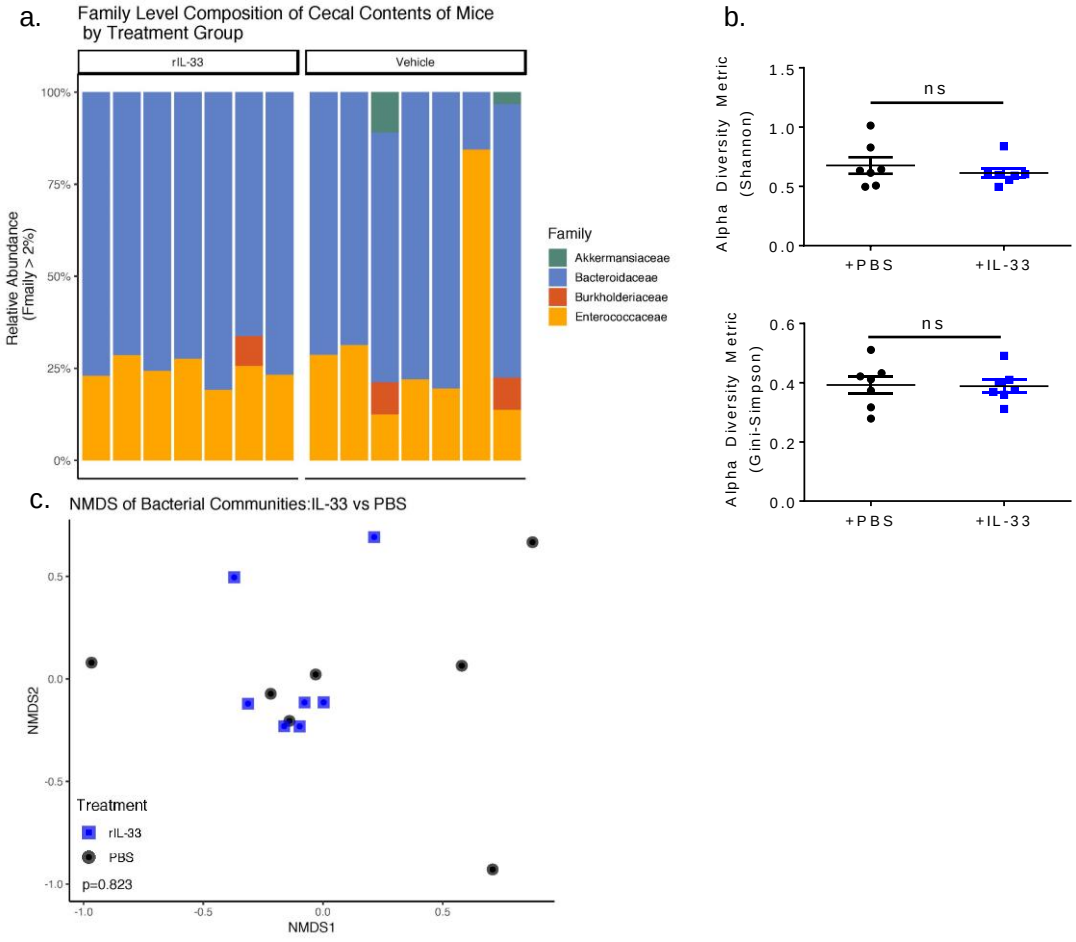
a.
Family Level Composition of Cecal Contents of Mice by Treatment Group
rIL-33
Vehicle
100%
75%
50%
25%
0%
Relative Abundance (Fmaily > 2%)
Family
Akkermansiaceae
Bacteroidaceae
Burkholderiaceae
Enterococcaceae
b.
Alpha Diversity Metric (Shannon)
1.5
1.0
0.5
0.0
ns
+PBS
+IL-33
Alpha Diversity Metric (Gini-Simpson)
0.6
0.4
0.2
0.0
ns
+PBS
+IL-33
c.
NMDS of Bacterial Communities:IL-33 vs PBS
NMDS2
0.5
0.0
-0.5
-1.0
NMDS1
-1.0
-0.5
0.0
0.5
Treatment
rIL-33
PBS
p=0.823

Figure 2.6. IL-33 treatment does not significantly alter the microbiota community prior to infection. Mice were treated with IL-33 or vehicle control, in addition to broad-spectrum antibiotic cocktail used in infection model. (*A*) Bar plot of relative phylum abundance of IL-33 vs. control groups prior to infection with *C. difficile.* (*B*) Microbiota diversity in caecal contents of untreated donors and antibiotic treated FMT recipients using Shannon (upper) and Simpson index (lower). (*C*) NMDS plot of Bray-Curtis dissimilarity index from caecal samples of IL-33 and vehicle mice post antibiotic treatment and before *C. difficile* infection. *(B)* Comparison made by ANOVA test. (*C*) comparisons made using permutational multivariate analysis of variance (PERMANOVA) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 2.7

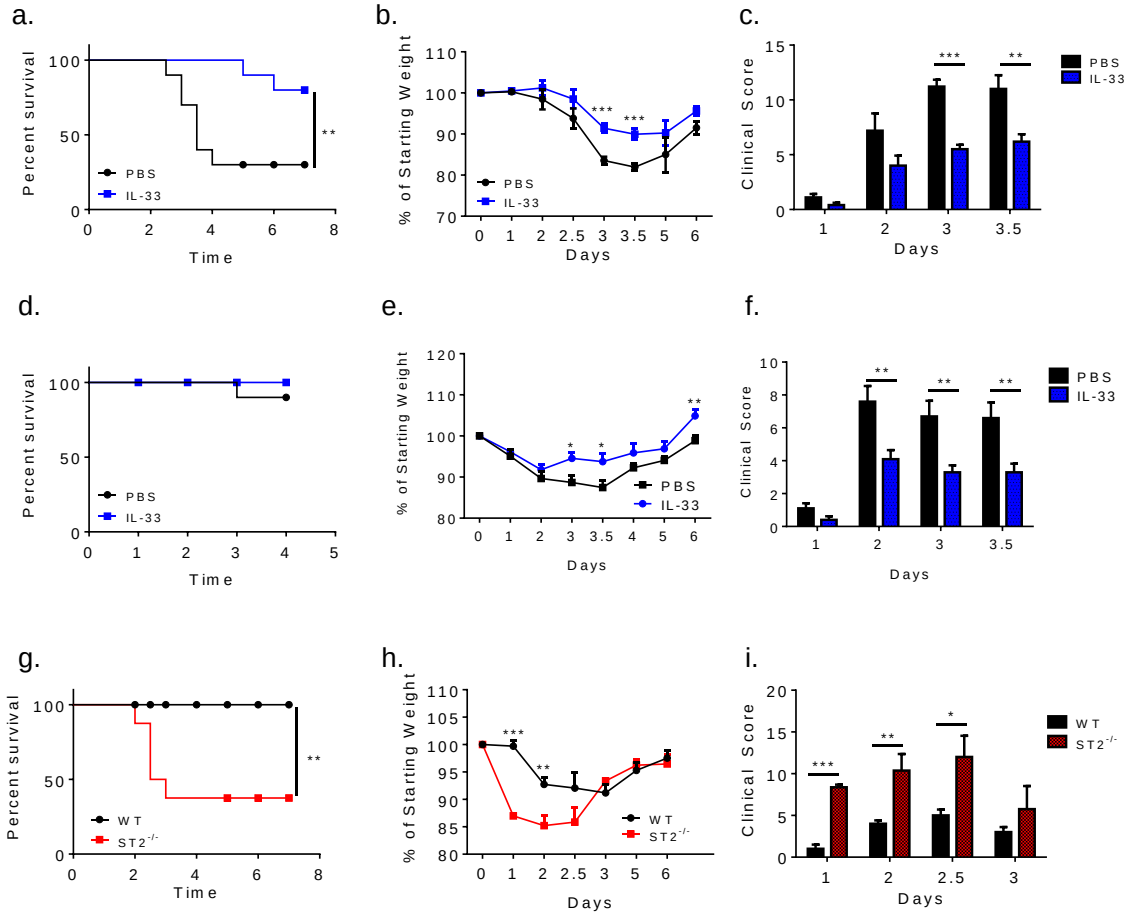
a.
Percent survival
Time
PBS
IL-33
**
b.
% of Starting Weight
Days
PBS
IL-33
***
***
c.
Clinical Score
Days
PBS
IL-33
***
**
d.
Percent survival
Time
PBS
IL-33
e.
% of Starting Weight
Days
PBS
IL-33
*
*
**
f.
Clinical Score
Days
PBS
IL-33
**
**
**
g.
Percent survival
Time
WT
ST2-/-
**
h.
% of Starting Weight
Days
WT
ST2-/-
***
**
i.
Clinical Score
Days
WT
ST2-/-
***
**
*

Figure 2.7. IL-33 protects from mortality, weight-loss and clinical scores in non-027 (VPI 10643) and non-CDT, R20291_*cdtb* infection. (*A-F*) Wildtype mice treated with IL-33 or with vehicle control were infected with (*A-C*) VPI 10643, or (*D-F*) attenuated mutant, R20291_*cdtb (CDT-)* and (*A,D*) survival, (*B,E*) body-weight and (*C,F*) clinical scores were assessed on each day post infection. (*G-I*) ST2$^{-/-}$ mice and WT controls were infected with the avirulent mutant, R20291_*cdtb* and (*G*) survival, (*H*) body-weight and (*I*) clinical scores were assessed on each day post infection. Data are presented as means ± SEM (*A*–*I*) representative experiment of *n* = 10-12 mice per group and (*A,D,G*) comparison made by log rank test. (*B-C,E-F,H-I)* Comparison made by student *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Similar to the IL-33 treatment model, endogenous IL-33 protection was not restricted to binary toxin expressing strains as only 40% of ST2$^{-/-}$ mice survived attenuated R20291_*cdtb* infection, compared to 100% of wildtype mice (**Fig 2.7G-I**). These results indicate that IL-33 signaling protects from mortality independently of CDT status.

2.2.4 IL-33 regulates the balance between Type-17 and Type-2 immunity during CDI.

In addition to the important role of *C. difficile* toxins in driving epithelial disruption and disease[77,92,300], the type of immune response generated during CDI is an important predictor of disease severity[133–135,301]. Colonic neutrophil invasion and Type-17 inflammation are hallmarks of CDI whereas Type-2 associated eosinophilia is protective in both human and murine CDI[74,133,136,156,222,301]. Given these findings, we wanted to determine whether IL-33 modulates the local intestinal immune response during infection to prevent mortality and epithelial disruption. Indeed, flow cytometry revealed that IL-33 treatment caused a dramatic switch in the myeloid cells of the colon by day 2 post infection, decreasing pro-inflammatory neutrophil and monocyte frequencies and numbers (**Fig 2.8A-B**, **Fig 2.9A, 2.9C-D**). Instead of the canonical neutrophilic response seen in wildtype infection, IL-33 treatment shifted the myeloid compartment towards dominant eosinophilia by Day 2 post infection [57% with IL-33 vs. 20% without] and a trend toward increased numbers of eosinophils (**Fig 2.8A-B; Fig 2.9A-B**). This shift in myeloid cells occurred only after infection as there were no differences in the frequency or number of neutrophils, monocytes, or eosinophils in uninfected controls (**Fig 2.8B, Fig 2.9B-D**). In alignment with the IL-33 treatment model, ST2$^{-/-}$ mice, which lack IL-33 signaling, had a reduction in colonic eosinophils both by frequency and number compared to wildtype controls indicating that endogenous IL-33 signaling contributes to eosinophil accumulation within the colon during infection (**Fig 2.9F-G**).

Figure 2.8

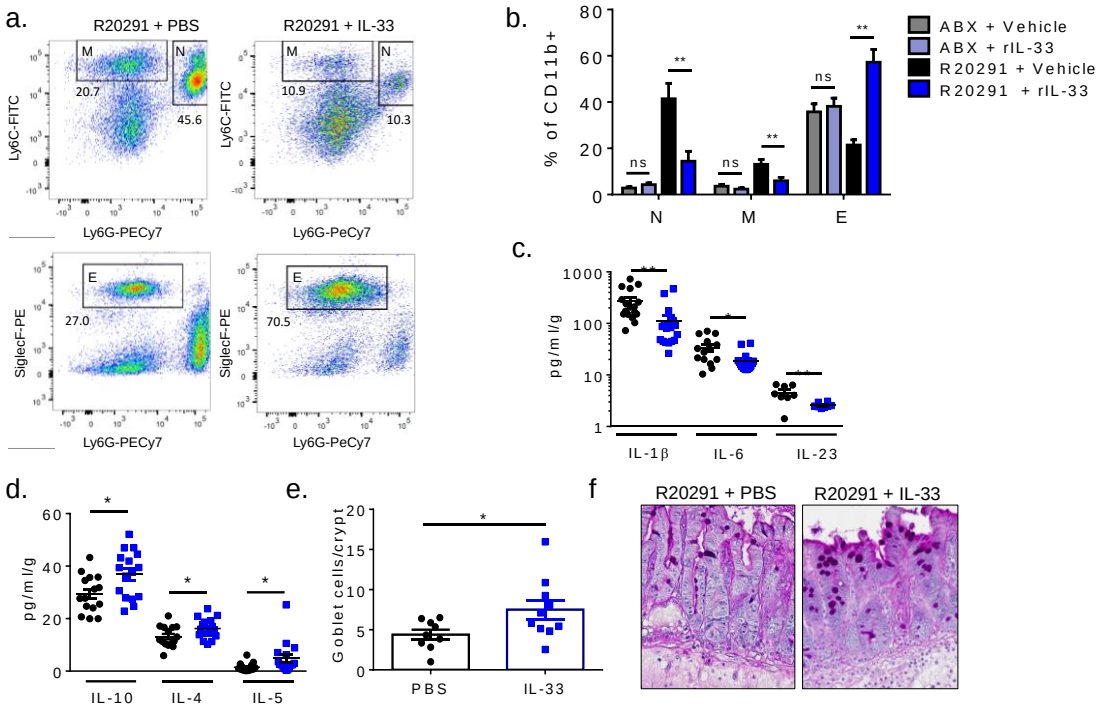
a.
R20291 + PBS
R20291 + IL-33
M
N
20.7
45.6
10.9
10.3
Ly6C-FITC
Ly6G-PECy7
E
27.0
70.5
SiglecF-PE
b.
% of CD11b+
ABX + Vehicle
ABX + rIL-33
R20291 + Vehicle
R20291 + rIL-33
ns
N
M
E
c.
pg/ml/g
IL-1β
IL-6
IL-23
d.
IL-10
IL-4
IL-5
e.
Goblet cells/crypt
PBS
IL-33
f
R20291 + PBS
R20291 + IL-33

Figure 2.8. IL-33 dampens inflammation and skews gut type-2 immunity during CDI. (*A-B*) Immune profiling of myeloid cells within the colon of IL-33 treated or untreated mice during R20291 infection. $CD45^+$ $CD11b^+$ $Ly6g^+$ $Ly6c^+$ neutrophils; $CD45^+$ $CD11b^+$ $Ly6g^-$ $Ly6c^+$ monocytes, and $CD45^+$ $CD11b^+$ $SiglecF^+$ $Ly6g^-$ eosinophils were quantified by flow cytometry. (*A*) Representative dot plots of eosinophils, monocytes and neutrophils within the colon of IL-33 treated (right plots) vs. untreated (left plots) mice (Day 2). (*B*) Quantification of the frequency of neutrophils (N), monocytes (M), and eosinophils in the colon of uninfected mice or during infection (Day 2). (*C-D*) Cytokine protein expression in caecal tissue during *C. difficile* infection (Day 2). (*C*) Pro-inflammatory IL-1β, IL-6, and IL-23 expression and (*D*) Anti-inflammatory IL-10, IL-4, and IL-5 expression with and without IL-33 treatment. (*E-F*) Enumeration of mucin+ goblet cells with and without IL-33 treatment by microscopy. (*E*) Blinded quantification of number of PAS+ goblet cells per colon crypt during R20291 infection (Day 2). (*F*) Representative PAS+ goblet cell staining of IL-33 or vehicle treated mice (20x magnification). Data are presented as means ± SEM (*B*) n = 8-10 per group combined from 2 infections and (*C–D*) n = 4-10 per group combined from 2-3 infections. (*E*) n = 4-5 per group combined from 2 infections. (*B-E*) Comparison made by two-tailed student t test or Mann Whitney Test as described in methods. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 2.9

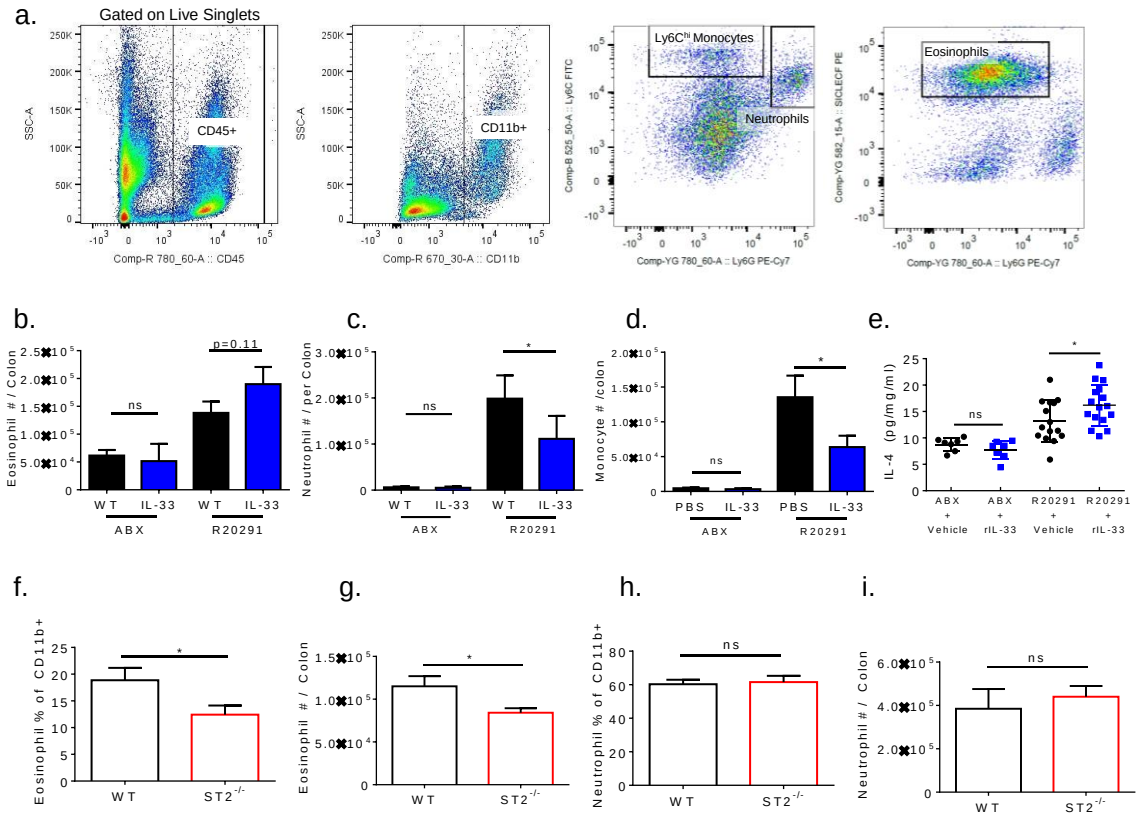
a.
Gated on Live Singlets
SSC-A
CD45+
Comp-R 780_60-A :: CD45
CD11b+
Comp-R 670_30-A :: CD11b
Ly6C^hi Monocytes
Neutrophils
Comp-YG 780_60-A :: Ly6G PE-Cy7
Eosinophils
b.
Eosinophil # / Colon
p=0.11
ns
WT
IL-33
ABX
R20291
c.
Neutrophil # / per Colon
d.
Monocyte # /colon
PBS
e.
IL-4 (pg/mg/ml)
ABX + Vehicle
ABX + rIL-33
R20291 + Vehicle
R20291 + rIL-33
f.
Eosinophil % of CD11b+
ST2-/-
g.
Eosinophil # / Colon
h.
Neutrophil % of CD11b+
i.
Neutrophil # / Colon

Figure 2.9. IL-33 regulates type-17 vs. type-2 immunity during CDI. (*A*) Gating strategy for flow cytometry characterization of eosinophils, neutrophils, and Ly6C^{hi} monocytes in the colon. Representative dot plot from an IL-33 treated mouse during R20291 infection (Day 2). (*B-D*) Total number of (*B*) Eosinophils, (*C*) Neutrophils (*D*) Ly6C^{hi} Monocytes in IL-33 vs. vehicle treated mice during infection or in uninfected mice. (*E*) IL-4 expression in gut caecal tissue with and without IL-33 treatment in infected and uninfected mice. (*F-I*) ST2$^{-/-}$ mice and wildtype mice were infected with R20291 and (*F*) eosinophil frequency (% of CD11b+), (*G*) eosinophil number, (*H*) neutrophil frequency and (*I*) neutrophil number were assessed by flow cytometry (Day 2). Representative experiment from 2 independent infections n = 7-10 mice per group. Comparisons between groups made by two-tailed student *t* test or Mann-Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Unlike the IL-33 treatment model, ST2$^{-/-}$ knockout mice had no change in colon neutrophilia during CDI indicating endogenous IL-33 dominantly regulates colonic eosinophil recruitment during infection.

Given the IL-33 mediated switch toward gut eosinophilia, we questioned whether IL-33 treatment also altered the cytokine milieu during CDI. We found that IL-33 treatment reduced pro-inflammatory IL-1β, IL-6, and IL-23 and increased anti-inflammatory IL-4, IL-5 and IL-10 during CDI (**Fig 2.8C**-**D**). In accordance with the myeloid cell data, the increase in the Type-2 associated cytokine, IL-4, occurred only after infection as uninfected IL-33 treated mice had equivalent levels of IL-4 relative to controls (**Fig 2.9E**). In addition to eosinophilia, Type-2 associated mucosal immunity is linked to goblet cell expansion to aid in parasite expulsion and is regulated by IL-13 production from type-2 innate lymphoid cells in the gut[173,302]. CDI causes a loss of goblet cells and mucin production at the epithelial barrier of the colon[303,304]. Thus, we explored the effects of IL-33 treatment on goblet cell mucin responses by Periodic Acid Schiff (PAS+) goblet cell mucin staining of infected caecal sections. IL-33 treated mice had significantly more goblet cells per colonic crypt during CDI infection compared to untreated controls (**Fig 2.8E**-**F**). Taken together, these results indicate that IL-33 is an important regulator of the balance between Type-17 vs. Type-2 associated mucosal immunity during CDI.

2.2.5 IL-33 responsive ILC2s protect from CDI- associated mortality.

IL-33 has been shown to reduce intestinal damage during IBD colitis via the action of type-2 innate lymphoid cells (ILC2s) or ST2+ regulatory T- cells[175,256,260]. Furthermore, B cells have also been implicated in IL-33 mediated protection from IBD colitis via IgA or IL-10 production[297,305]. We wondered whether innate lymphoid cells (ILCs) or adaptive T and B cells were involved in the therapeutic capacity of IL-33 to protect from hypervirulent *C. difficile* colitis. To address this question, we treated Rag2$^{-/-}$ (lacking T and B cells) and

Rag2$^{-/-}$ γc$^{-/-}$ (additionally lacking ILCs) mice with IL-33 or the vehicle control. In line with Abt *et al*, Rag2$^{-/-}$ γc$^{-/-}$ mice had significantly higher mortality compared to Rag2$^{-/-}$ mice [75% Rag2$^{-/-}$γc$^{-/-}$ vs. 24% Rag2$^{-/-}$] confirming the importance of ILCs in recovery from *C. difficile*[75]. IL-33 treatment of Rag2$^{-/-}$ mice led to a significant increase in survival and reduction in weight-loss indicating T and B cells are dispensable for IL-33 mediated protection during CDI (**Fig 2.10A**,**B**).

In contrast, IL-33 mediated protection from mortality and weight-loss was lost in Rag2$^{-/-}$γc$^{-/-}$ mice indicating ILCs are necessary for IL-33's therapeutic effects (**Fig 2.10A, C**). We found that during CDI, colonic GATA3+ ILC2s co-expressed the IL-33 receptor, ST2, leading us to believe that ILC2s were downstream of IL-33 mediated protection (**Fig 2.11A-B**). Indeed, protective IL-33 treatment led to a significant increase in ST2+ GATA3+ ILC2s within the colon lamina propria during infection (**Fig 2.10D-E, 2.11C**). Furthermore, IL-33 treated Rag2$^{-/-}$ mice had an equivalent increase in the number of colonic ILC2s indicating that IL-33-mediated ILC2 induction was independent of the adaptive immune system (**Fig 2.10E**). We also noticed IL-33 treatment led to a significant reduction in the frequency of ILC1s, which canonically drive recovery during CDI[75], and a reduction in ILC3s (**Fig 2.10D, 2.11C**). The increase in ILC2s and corresponding decrease in ILC1s and ILC3s may be due to ILC plasticity within the intestinal lamina propria[306–309]. GATA3+ ILC2s isolated directly *ex vivo* from the colon during *C. difficile* infection were activated, as evidenced by their increased capacity to produce IL-13 compared to uninfected controls without further stimulation (**Fig 2.12A-B**). ILC2s isolated from IL-33 treated mice had 2.5-fold increase in IL-13+ ILC2s during infection compared to vehicle controls (**Fig 2.12A-B**). While ST2$^{-/-}$ mice had normal numbers of ILC2s in their colons, their ILC2s were significantly dysfunctional as evidenced by a reduced ability to make IL-13 by frequency

Figure 2.10

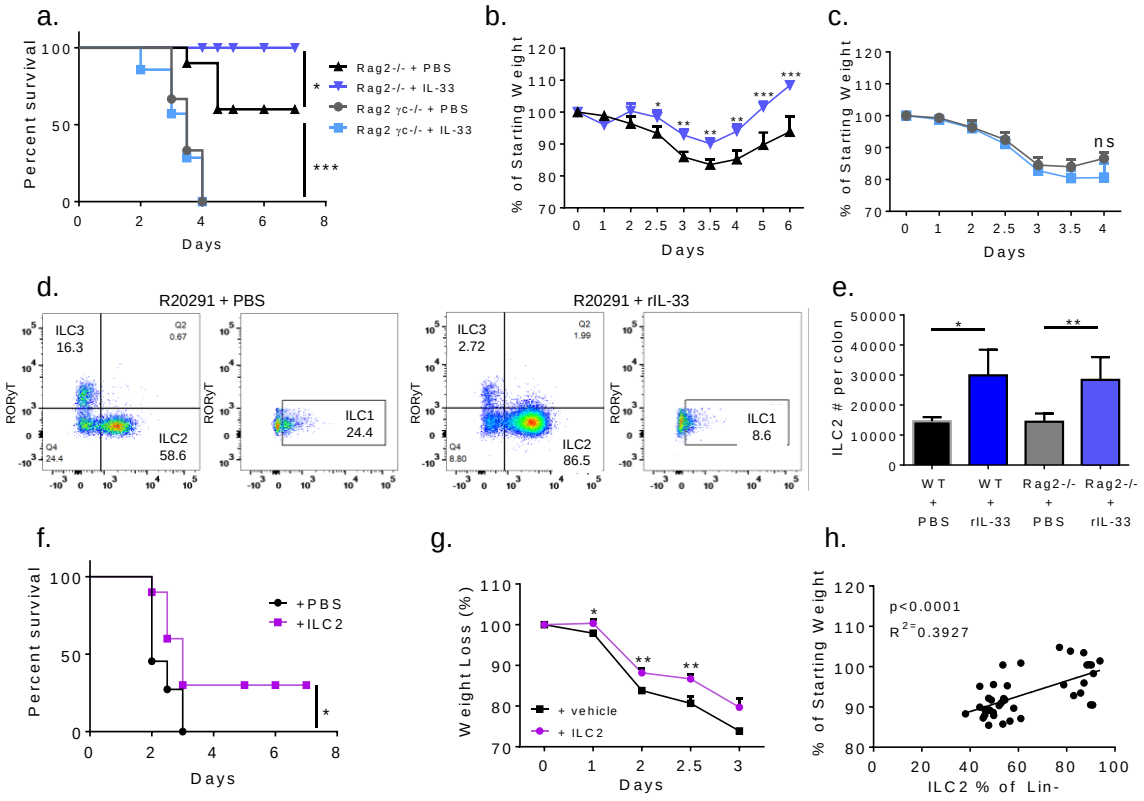
a.
Percent survival
Days
Rag2-/- + PBS
Rag2-/- + IL-33
Rag2 γc-/- + PBS
Rag2 γc-/- + IL-33
b.
% of Starting Weight
Days
c.
% of Starting Weight
Days
ns
d.
R20291 + PBS
R20291 + rIL-33
ILC3
16.3
ILC2
58.6
ILC1
24.4
ILC3
2.72
ILC2
86.5
ILC1
8.6
RORγT
e.
ILC2 # per colon
WT + PBS
WT + rIL-33
Rag2-/- + PBS
Rag2-/- + rIL-33
f.
Percent survival
Days
+PBS
+ILC2
g.
Weight Loss (%)
Days
+ vehicle
+ ILC2
h.
% of Starting Weight
p<0.0001
R2=0.3927
ILC2 % of Lin-

Figure 2.10. IL-33-ILC2 crosstalk is protective during *C. difficile* infection. (*A-C*) Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ were infected with R20291 and treated with or without IL-33. (*A*) Survival curves, (*B*) Rag2$^{-/-}$ weight-loss, and (*C*) Rag2$^{-/-}$γc$^{-/-}$ weight-loss after IL-33 treatment and infection. (*D-E*) Immune cell profiling of ILCs within the colon of IL-33 treated or untreated mice during R20291 infection. Lin- CD45^{+} CD90^{+} CD127^{+} ILCs were subsetted into ST2^{+} GATA^{+} ILC2s; Tbet^{+} ILC1s, and RORγT^{+} ILC3s by flow cytometry. (*D*) Representative dot plots of ILC subsets within the colon of PBS controls or IL-33 treated mice during infection (Day 2). (*E*) Enumeration of the number of ILC2s in the colon of IL-33 treated C57BL6 or Rag2$^{-/-}$ mice during R20291 infection. (*F-G*) Adoptive transfer of ex vivo expanded and sorted purified ILC2s from uninfected IL-33 treated mice into naïve Rag2$^{-/-}$γc$^{-/-}$ mice. (*F*) Survival curves and (*G*) weight-loss after ILC2 transfer and R20291 infection. (*H*) Pearson correlation between the frequency of ILC2s and the percent weight-loss during R20291 infection. Data are presented as means ± SEM (*A*-C) *n* = 6-10 mice per group representative of 2 infections and (E) *n* = 4-10 mice per group representative of 2 independent infections. (*F*) *n* = 10 mice per group representative of from 2 adoptive transfers. (*G*) n = 10 per group representative of 2 independent adoptive transfers (*A and F*) Comparison made by log rank test (*B-C, E & G)* Comparison made by two-tailed student t test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 2.11

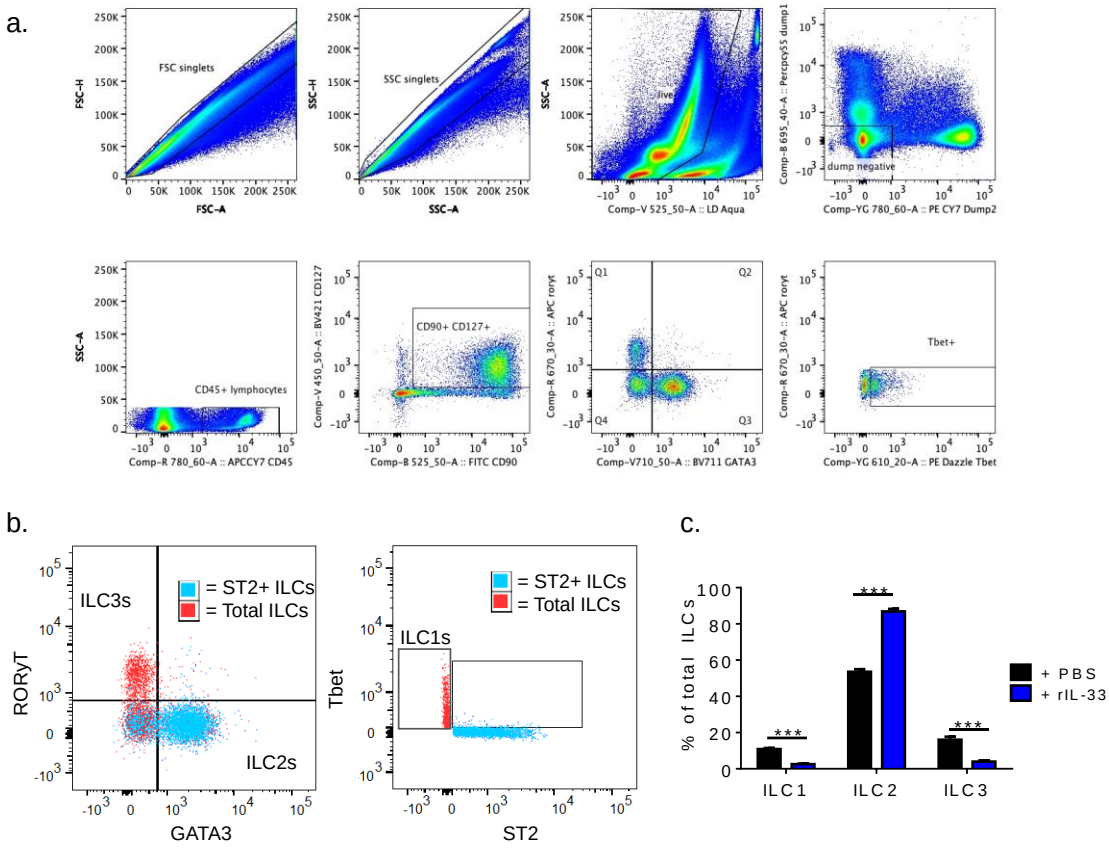
a.
FSC singlets
FSC-H
FSC-A
SSC singlets
SSC-H
SSC-A
live
Comp-V 525_50-A :: LD Aqua
dump negative
Comp-YG 780_60-A :: PE CY7 Dump2
CD45+ lymphocytes
Comp-R 780_60-A :: APCCY7 CD45
CD90+ CD127+
Comp-B 525_50-A :: FITC CD90
Q1
Q2
Q3
Q4
Comp-V710_50-A :: BV711 GATA3
Tbet+
Comp-YG 610_20-A :: PE Dazzle Tbet
b.
ILC3s
ILC2s
= ST2+ ILCs
= Total ILCs
RORγT
GATA3
ILC1s
Tbet
ST2
c.
% of total ILCs
+ PBS
+ rIL-33
***
ILC1
ILC2
ILC3

Figure 2.11. IL-33 regulates type-17 vs. type-2 immunity during CDI. (*A*) Gating strategy for flow cytometry characterization of eosinophils, neutrophils, and Ly6C^{hi} monocytes in the colon. Representative dot plot from an IL-33 treated mouse during R20291 infection (Day 2). (*B-D*) Total number of (*B*) Eosinophils, (*C*) Neutrophils (*D*) Ly6C^{hi} Monocytes in IL-33 vs. vehicle treated mice during infection or in uninfected mice. (*E*) IL-4 expression in gut caecal tissue with and without IL-33 treatment in infected and uninfected mice. (*F-I*) ST2$^{-/-}$ mice and wildtype mice were infected with R20291 and (*F*) eosinophil frequency (% of CD11b+), (*G*) eosinophil number, (*H*) neutrophil frequency and (*I*) neutrophil number were assessed by flow cytometry (Day 2). Representative experiment from 2 independent infections n = 7-10 mice per group. Comparisons between groups made by two-tailed student t test or Mann-Whitney test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 2.12

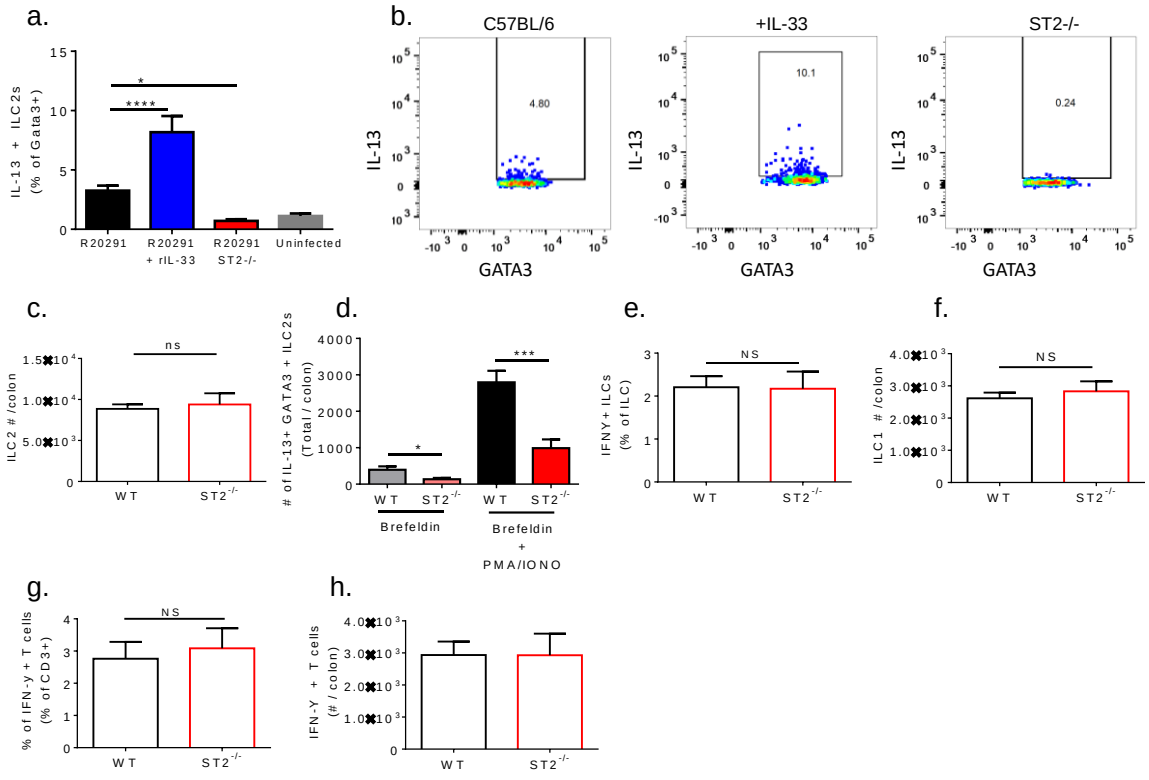
a.
IL-13 + ILC2s (% of Gata3+)
R20291
R20291 + rIL-33
R20291 ST2-/-
Uninfected
b.
C57BL/6
+IL-33
ST2-/-
IL-13
GATA3
4.80
10.1
0.24
c.
ILC2 # /colon
ns
WT
ST2-/-
d.
# of IL-13+ GATA3 + ILC2s (Total / colon)
Brefeldin
Brefeldin + PMA/IONO
e.
IFNγ+ ILCs (% of ILC)
NS
f.
ILC1 # /colon
NS
g.
% of IFN-γ + T cells (% of CD3+)
NS
h.
IFN-γ + T cells (# / colon)

Figure 2.12. Activated IL-13+ ILC2s are dysfunctional during ST2$^{-/-}$ *C. difficile* infection. (*A*-*D*) Immune profiling of IL-13+ GATA3+ ILC2s within the colon of infected IL-33 treated, ST2$^{-/-}$, WT, or uninfected mice by flow cytometry. (*A*) % of IL-13 + ILC2s in *C. difficile* infected or uninfected mice (Day 2). (*B*) Representative flow plots of IL-13 expression during R20291 infection. (*C*) Total number of ILC2s and (*D*) IL-13+ ILC2s in the colon during infection. *(E-H*) Immune profiling type-1 immunity during infection. *(E)* Frequency of IFN-γ+ ILC1s and (*F*) Number of T-bet+ ILC1s in the colon during ST2$^{-/-}$ vs. WT infection. (*G*) Frequency of IFN-γ+ CD3+ T cells and (*H*) Number of IFN-γ+ T cells in the colon during infection. (*A*) Groups compared with ANOVA. (*E-H*) Groups compared by student-t tailed t test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

and total cell number, indicating ST2$^{-/-}$ mice have limited functional ILC2s during infection (**Fig 2.12A-D**). ST2$^{-/-}$ mice have deficiencies in Type-1 CD4+ and CD8+ T cell responses during viral infection[310,311], however we did not see decreased frequencies or numbers of IFN-γ+ T cells during infection likely due do the limited role for adaptive immunity during acute primary *C. difficile* infection (**Fig 2.12G-E**)[30,75,312]. Furthermore, we did not see deficiencies in type-1 Tbet+ ILC1 numbers or alterations in their ability to produce IFN-γ in infected ST2$^{-/-}$ mice, together indicating that type-1 immunity is intact during ST2$^{-/-}$ infection (**Fig 2.12E-F**).

2.2.5.1 ILC2s are sufficient to transfer protection during C. difficile infection.

To determine whether ILC2s are sufficient to transfer protection during CDI, we adoptively transferred ILC2s from the mesenteric lymph nodes and colon of IL-33 treated mice and *ex vivo* expanded them in the presence of IL-33, IL-2 and IL-7 as described previously[313–315]. We then sort purified Lin- CD45+ CD901.1+ CD127+ ST2+ CD25+ ILC2s and transferred them into naïve Rag2$^{-/-}$γc$^{-/-}$ mice prior to infection (1 x 10^6 ILC2s per mouse). Rag2$^{-/-}$γc$^{-/-}$ mice displayed 100% mortality during infection, however, ILC2 recipients had a 30% rescue in survival (**Fig 2.10F**). Furthermore, ILC2 recipients were also protected from weight-loss during their infection (**Fig 2.10G**). Direct *ex vivo* transfer of ILC2s (1 x 10^5) from IL-33 treated mice into naïve Rag2$^{-/-}$γc$^{-/-}$ mice also conferred protection from CDI associated mortality (**Fig 2.13A**). We confirmed the presence of congenitally marked donor ILC2s within the colon of recipient Rag2$^{-/-}$γc$^{-/-}$ mice (**Fig 2.13B**). Intestinal ILC2s contribute to eosinophil accumulation and homeostasis within the gut[230,316]. Accordingly, ILC2 transfer enhanced colonic eosinophilia during *C. difficile* infection demonstrating that transferred ILC2s were functional after trafficking into the colon (**Fig 2.13C**).

Figure 2.13

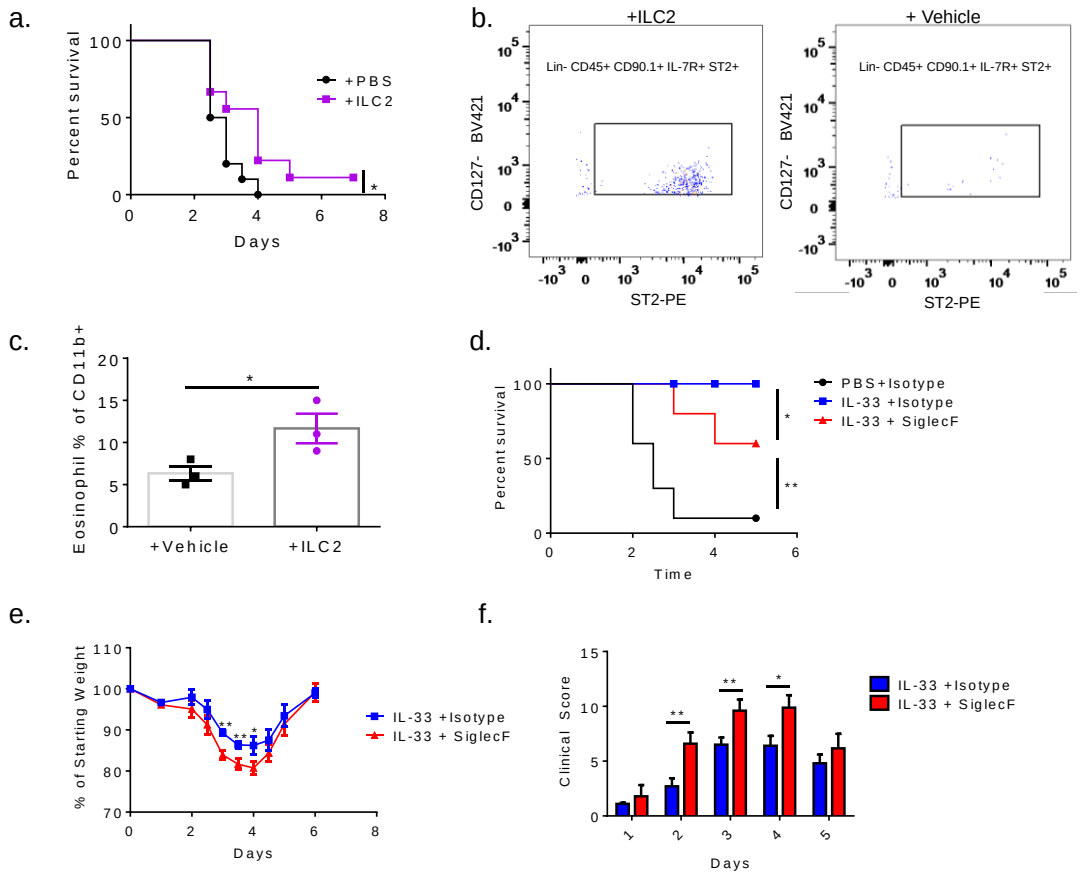
a.
Percent survival
100
50
0
0
2
4
6
8
Days
+PBS
+ILC2
*
b.
+ILC2
+ Vehicle
Lin- CD45+ CD90.1+ IL-7R+ ST2+
CD127- BV421
ST2-PE
c.
Eosinophil % of CD11b+
20
15
10
5
0
*
+Vehicle
+ILC2
d.
Percent survival
100
50
0
0
2
4
6
Time
PBS+Isotype
IL-33 +Isotype
IL-33 + SiglecF
*
**
e.
% of Starting Weight
110
100
90
80
70
0
2
4
6
8
Days
IL-33 +Isotype
IL-33 + SiglecF
f.
Clinical Score
15
10
5
0
1
2
3
4
5
Days
IL-33 +Isotype
IL-33 + SiglecF
**
**
*

Figure 2.13. Direct *ex-vivo* transfer of IL-33 activated ILC2s protects from CDI-associated mortality and increases protective colon eosinophilia during CDI. (*A*) Survival of direct *ex vivo* adoptive transfer study where sort purified ILC2s were isolated from the MLN, colon, and spleen of IL-33 treated mice (CD90.1) and, without expansion, were directly transferred into $Rag2^{-/-}\gamma c^{-/-}$ mice followed by R20291 infection. (*B*) Representative flow plot of donor Lin- CD45+ CD90.1+ CD127+ ST2+ ILC2s within the colon of $Rag2^{-/-}\gamma c^{-/-}$ ILC2 recipients vs. control recipients and (*C*) frequency of colon eosinophils within ILC2 vs. control recipients during infection (Day 4). (*D-F*) C57BL6 Mice +/-rIL-33 treatment were given 20 µg of anti-SiglecF or isotype control one day prior and one day after infection with R20291 *C. difficile* and were assessed for (*D*) Survival, (*E*) Weight-loss and (*F*) Clinical scores. (*A,D*) Comparison made by log rank test *(C,E,F)* Two groups compared by student T test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Lastly, ILC2 frequency in the colon inversely correlated with weight-loss during *C. difficile* colitis. (**Fig 2.10H**). Eosinophil induction is in part necessary for IL-33 activated ILC2s to protect as depletion of eosinophils with an anti-Siglec-F antibody partially abrogated IL-33 mediated protection from mortality, weight-loss, and clinical severity (**Fig 2.13D-F)**. Collectively, these experiments reveal a previously unrecognized mechanism of protection from *C. difficile* colitis via IL-33 responsive ILC2s.

2.3 Discussion

In this study, we investigated the host-derived mechanisms of immune protection during severe *C. difficile* via unbiased gut-derived transcriptomics. Here we demonstrate that IL-33 signaling is a critical mechanism of intestinal protection during *C. difficile* colitis via the action of ILC2s. IL-33 mediated protection is broad and protects from disease in multiple strains: severe CDT expressing R20291, the attenuated R20291_*cdtb* mutant, and the non-027 strain, VPI. We see that IL-33 activates ILC2s in the large intestine and induces type-2 repair pathways that protect from toxin-mediated epithelial damage. ST2 signaling on ILC2s is essential for their activation and functioning during *C. difficile* infection. Our findings have important relevance to intestinal health and infectious disease. The activity of IL-33 has centered around its contribution to protective 'weep and sweep' defenses during helminth infection, however, this work expands IL-33's relevance to gut bacterial immunity[250,317]. Importantly, this work is the first evidence that IL-33 signaling to ILC2s is a therapeutically important pathway in CDI.

Innate lymphoid cells have an important role in shaping gut immunity at homeostasis and during pathogen insult. While these cells lack antigen-specific receptors, they are tissue-resident at the mucosal interface, enabling a quick and robust cytokine response. Recently, ILC1s were demonstrated to have a critical role in host defense during CDI while ILC3 involvement is limited[75]. ILC1s likely aid in localized anti-microbial defenses during

CDI via IFN-γ production[75]. Our findings build off this work, demonstrating that IL-33 responsive ILC2s are also of high-importance during CDI. ILC2s have tissue protective functions during murine IBD colitis and lung influenza, and function by protecting the epithelial barrier via the epidermal growth factor, amphiregulin[175]. Intestinal ILC2s also express the cytokine IL-13 which recently was demonstrated to act directly on the epithelium to drive goblet cell expansion and tuft cell hyperplasia aiding in parasite expulsion[173,302]. In line with this work, we saw that IL-33 activates ILC2s during *C. difficile* infection, increasing IL-13 production, increasing goblet mucin responses and improving epithelial barrier function.

Additionally, intestinal ILC2s constitutively express the eosinophil-stimulating cytokine IL-5 and have an essential role in eosinophil survival in the intestine[175,230,318–320]. In accordance with these findings, we see IL-33 treatment increases IL-5 levels during *C. difficile* infection and adoptive transfer of ILC2s caused increased eosinophilia in the colon during *C. difficile* infection in addition to preventing CDI-associated weight-loss and mortality. Furthermore, depletion of eosinophils partially abrogated the ability of IL-33 to protect, indicating these cells are an important downstream mechanism of IL-33 protection. Our previous studies have demonstrated an important role for eosinophils during infection with both hypervirulent and classical strains of *C. difficile*[6,133]. Specifically, IL-25 elicited eosinophils prevent CDI-associated mortality and eosinophil adoptive transfer is sufficient to promote survival[6,133]. Thus, ILC2s may act as upstream regulators of eosinophil function and barrier protection during CDI by integrating signals from the epithelium, such as IL-33 or IL-25, to promote repair. In line with this idea, recent studies have demonstrated a pivotal role for both IL-5 and eosinophils in human *C. difficile* infection with IL-5 being negatively correlated with disease severity and low peripheral eosinophil counts predicting poor CDI prognosis[135,136]. Thus, ILC2s may have a key

regulatory role in countering *C. difficile* toxin-mediated epithelial damage and an overly robust anti-microbial response through their promotion of type-2 associated immunity.

IL-33 is considered an alarmin protein, transcriptionally regulated by PAMPS, and held within the nucleus until it is released upon cell death to activate rapid immunity[233,321]. The acute nature of IL-33 signaling likely explains its importance very early on during *C. difficile* colitis in our murine studies. Our work demonstrates a new role for IL-33 responsive ILC2s in protection from *Clostridium difficile* colitis.

Chapter 3: IL-33 signaling is dysregulated during human *C. difficile* infection and is targetable with bacteriotherapy

This chapter has been adapted from "Microbiota-regulated IL-33 drives group 2 innate lymphoid cell-mediated protection during *Clostridium difficile* infection." *Nature Communications* (2019).

Alyse L. Frisbee, Mahmoud M. Saleh[1], Mary K. Young[1], Jhansi L. Leslie[2], Morgan E. Simpson[1], Mayuresh M. Abhyankar[3], Jennie Z. Ma[3], Patcharin Pramoonjago[3], Alice P. Liou[5], and William A. Petri, Jr.

[1] M.M.S, M.K.Y., M.E.S, helped with tissue extraction and processing, bacterial quantification, and invaluable advice.

[2] J.L.L. aided with analyzing and interpreting 16S rRNA Sequencing data and writing 16S rRNA sequencing methods section.

[3] M.M.A., J.Z.M., and P.P., aided with human studies including sample collection, processing, quantification, and statistical analysis.

[4] A.P.L. assisted with human spore cocktail preparation and experimental advice.

3.1 Introduction

We demonstrated an important role for IL-33 activation of ILC2s during a murine model of *C. difficile* infection. IL-33 is upregulated in response to the severity of *C. difficile* infection and can protect from both hypervirulent CDT expressing strains and also non-CDT expressing classical isolates. IL-33 drives recovery in an ILC2 dependent manner, promoting tissue repair and eosinophilia in the gut during CDI. While it is clear that both endogenous and exogenous IL-33 are important during infection, we wondered whether this pathway had relevance in human disease. Thus, we decided to evaluate the IL-33 signaling pathway in human CDI+ biopsies and serum.

The microbiota provides colonization resistance against pathogen infection such as *C. difficile* in addition to its important role in influencing and priming immune system development. Disturbances to the microbiota, such as antibiotic therapy, is the primary risk factor for acquiring CDI. Although antibiotic exposure is the foremost risk factor, broad-spectrum antibiotic therapy is also used to treat *C. difficile* infection. The antibiotics vancomycin, fidaxomicin, and metronidazole are the current standard of care, however, recurrence occurs in up to 35% of patients[37,322–324]. High rates of relapse may be attributed to further disruption of a patient's microbiota and the lack of reestablishment of a diverse flora. Thus, bacteriotherapy to treat *C. difficile* has gained great interest over the past decade. Microbial strategies to combat severe *C. difficile* colitis through fecal microbiota transplantation (FMT) have had great advancements in recent years with the first randomized clinical trial demonstrating ~90% efficacy[106,109,112,113,191,285,325]. However, how FMT strategies alter mucosal immune defenses and how the host immune system contributes to *C. difficile* disease pathogenesis are not fully understood. Given IL-33's robust protection in our murine model of *C. difficile* colitis, we also wondered whether antibiotic-associated dysbiosis or FMT therapy modulate IL-33 levels in the colon.

In this study, we demonstrate that IL-33 is regulated by the microbiota and is a therapeutically relevant pathway during human *C. difficile* infection and FMT therapy. Specifically, IL-33 expression is increased in human biopsies from *C. difficile* infected patients and high levels of the soluble decoy receptor for IL-33, sST2, is a risk factor for patient mortality. Furthermore, we demonstrate a connection between the microbiota and IL-33 expression with antibiotics causing a reduction in IL-33 levels within the intestine. Both FMT treatment and a purified human spore prep can rescue IL-33 levels after antibiotic-mediated depletion. This data indicates a potentially targetable approach for increasing colonic IL-33 with rationally designed, next-generation microbial cocktails.

3.2 Results

3.2.1 IL-33 signaling is clinically relevant during human CDI

Given that IL-33 is upregulated in the murine colon during CDI, we wondered whether IL-33 signaling is similarly dysregulated in human patients infected with *C. difficile*. To understand changes in IL-33 in response to CDI in the human intestine, we conducted immunohistochemistry staining of IL-33 protein within colon biopsies of patients infected with *C. difficile*. IL-33 immunohistochemistry staining revealed abundant anti-IL-33 staining of colon tissue biopsies from 6 human CDI patients (**Fig 3.1A-B**). Blinded quantification of IL-33 staining in 6 CDI+ vs. 6 CDI- colon biopsies revealed a trend toward increased IL-33 expression within the colon of patients with *C. difficile* (**Fig 3.1C**). This complements our murine data that colonic IL-33 is upregulated in response to *C. difficile* (**Fig 2.2C**).

Figure 3.1

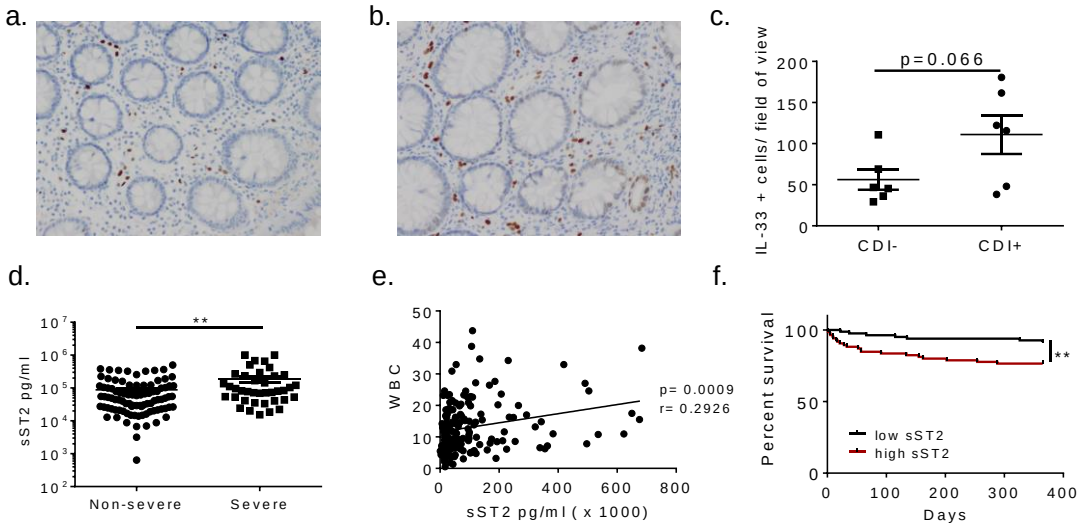
a.
b.
c.
p=0.066
IL-33 + cells/ field of view
200
150
100
50
0
CDI-
CDI+
d.
e.
f.
**
sST2 pg/ml
Non-severe
Severe
WBC
p= 0.0009
r= 0.2926
sST2 pg/ml (x 1000)
Percent survival
low sST2
high sST2
Days

Figure 3.1: IL-33 signaling is dysregulated during human CDI.

(*A*-*C*) Immunohistochemistry staining of IL-33 from colon tissue biopsies of 6 CDI- patients and 6 CDI+ patients. (*A*) Representative image from a CDI negative patient biopsy (10x mag). (*B*) Representative image from a CDI+ patient biopsy (10x mag). (*C*) Quantification of IL-33-positive cells per field view taken as an average of 5 blinded images from each patient biopsy and quantified on Image-J. (*D*-*F*) Analysis of systemic sST2 (IL-33 decoy receptor) in the serum of 167 patients with CDI by ELISA. (*D*) Patients were stratified based on their WBC into severe vs. non-severe CDI and sST2 was assessed between the two groups. (*E*) Spearman correlation between WBC and sST2 concentration. (*F*) Survival curves of CDI+ patients stratified into high (sST2 > 55,000 pg/ml) or low sST2 (sST2 < 55,000 pg/ml). (*C*) n= 6 CDI+ patients and 6 CDI- patients (5 image replicates per patient biopsy) (*D*-*H*) *n* = 167 patients (*C*-*D*) Comparison made by Mann-Whitney Test. (*F*) Comparison made by log rank test **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

3.2.1.1 Soluble decoy receptor for IL-33 is associated with more severe disease.

We also detected the soluble IL-33 decoy receptor (sST2) at high levels in human serum by ELISA within a cohort of 160 CDI+ patients. sST2 neutralizes the biological activity of IL-33 and in our murine CDI model, administration of the soluble IL-33 decoy receptor (sST2-FC) also increased weight-loss and clinical symptoms, although no mortality occurred in either treatment or controls groups likely due to daily saline IPs during infection (**Fig 3.2A-C**). To determine whether sST2 expression is a biomarker of *C. difficile* disease severity in human patients, we stratified our CDI cohort into severe and non-severe disease based on their white blood cell count. Patients with severe CDI were defined by a white blood cell count greater than or equal to 15,000 as we and others have defined previously[135,326]. We found that serum sST2 levels were higher in patients with severe CDI than non-severe CDI patients and there was a significant correlation between sST2 expression and high-WBC count (**Fig 3.1D**-**E**). A Kaplan-Myer curve and log-rank test revealed that patients with high sST2 expression (> median) had significantly increased mortality compared to patients with low sST2 expression (≤ median) (**Fig 3.1F**). Expression of these results as hazard ratios through a Cox regression model adjusting for age, gender, race and Charson comorbidity score revealed that high sST2 expression was an independent prognosis factor for poor survival from *C. difficile* infection with a hazard ratio of 3.19 (**Table 3.1**). Together, this data indicates that the decoy receptor for IL-33, sST2, is a novel poor prognosis factor during human CDI.

3.2.1.2 CDT toxin is associated with more severe disease in human CDI.

We previously identified CDT as a virulence factor in murine studies. To try and understand the role of CDT during human CDI, we measured CDT positivity in patient

Figure 3.2

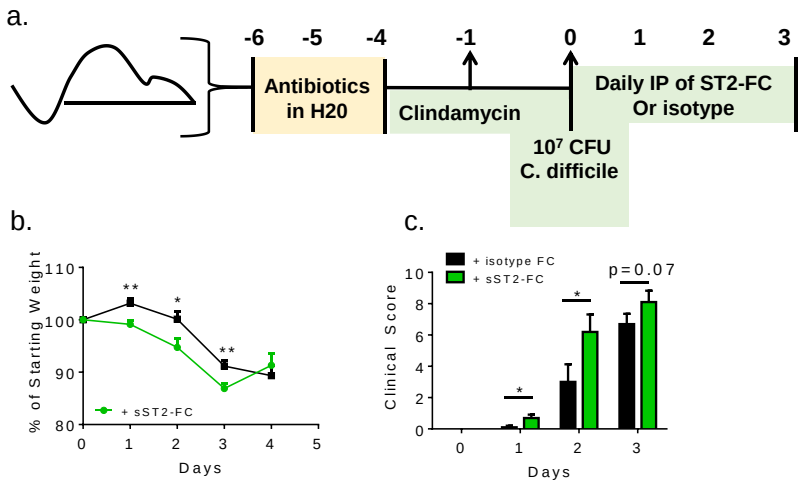
a.
-6
-5
-4
-1
0
1
2
3
Antibiotics in H20
Clindamycin
Daily IP of ST2-FC Or isotype
10^7 CFU C. difficile
b.
% of Starting Weight
110
100
90
80
**
*
**
+ sST2-FC
0
1
2
3
4
5
Days
c.
+ isotype FC
+ sST2-FC
p=0.07
Clinical Score
10
8
6
4
2
0
*
*
0
1
2
3
Days

Figure 3.2. Soluble ST2 increases *C. difficile* disease severity in mice.

(*A*-*C*) C57BL6 mice were infected with R20291 and subsequently given 4 injection of sST2-FC or isotype control by IP (D0-3). *(A)* Experimental design of sST2 treatment and *C. difficile* infection model. (*B*) Weight-loss and (*C*) clinical scores were assessed after treatment and infection. (*B*-*C*) n= 10 representative of 2 independent experiments and comparison made by student t test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Table 3.1 Mortality risk of CDI + human patients based on sST2 levels.

Parameter		Standard Error	Chi-Square	Pr > ChiSq	**Hazard Ratio (Mortality Risk)**	95% Hazard Ratio Confidence Limits	
sST2	>50%tile	0.44357	6.8323	**0.0090**	**3.188**	1.336	7.605
Age		0.01573	9.8886	**0.0017**	**1.051**	1.019	1.084
Female		0.40698	0.7917	0.3736	1.436	0.647	3.189
African American		0.48369	0.0001	0.9921	1.005	0.389	2.593
Charlson Index		0.05366	0.8791	0.3484	1.052	0.947	1.168

[1] Correlation between ST2 levels in serum and survival assessed through Cox regression analysis.

[2] Mortality risk (hazard ratio) adjusted for age, sex, race, charlson comorbidity index.

stool within our *C. difficile* cohort. Using an ELISA for the CDTb subunit, we measured whether CDTb protein was detectable in-patient stool from the day of CDI diagnosis. We found that 16% of patients were CDT positive by ELISA; indicating not only were these patients infected with an epidemic isolate that possesses the CDT gene, but CDTb toxin is also being actively expressed by the infecting strain. Strikingly, patients infected with a CDT expressing strain had higher mortality and higher weight-blood cell counts indicating more severe *C. difficile* disease (**Fig 3.3A-B**). This data indicates that CDT toxin is a virulence factor in human *C. difficile* colitis in aligns with our previous murine studies identifying CDT as a virulence factor[6].

3.2.1.3 sST2 is a marker for *C. difficile* severity independent of CDT status.

Given previous findings that CDT depletes protective type-2 eosinophil during infection, we wondered whether the association between sST2 and clinical severity was specific for CDT expressing strains or whether sST2's relevance expanded to non-CDT expressing strains of *C. difficile*. Thus, we excluded patient infected with a CDT expressing strain from the analysis and measured the correlation between sST2 and clinical severity. We saw that patients with high sST2 had increased mortality during infection and increased white blood cell counts, indicating that sST2 is a biomarker of *C. difficile* severity regardless of CDT status (**Fig 3.3 C-D**). To further determine whether there was an association between CDT status and IL-33 signaling, we assessed if there was an interaction between CDT positivity and sST2. Through a cox-regression analysis we saw no significant interactive effect between CDT status and high sST2. Thus, we subsequently considered sST2 and CDT as independent predictors of mortality in a cox-regression analysis. This analysis demonstrated that sST2 > 50% and positive CDT are each independently associated with higher risk of mortality after also adjusting for age, gender, race, and Charlson score (**Table 3.2**). In summary, this data indicates that the soluble decoy receptor for IL-33, sST2, is an independent prognosis factor during human

Figure 3.3

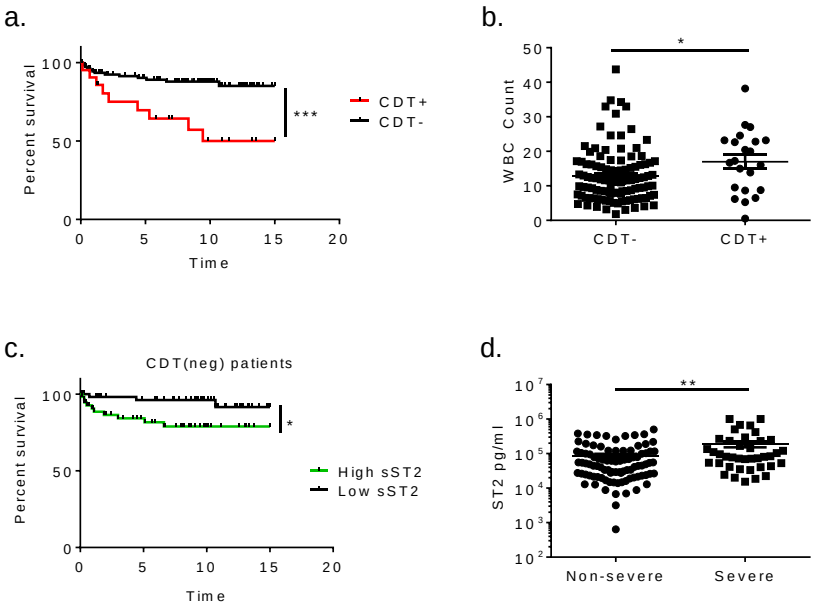
a.
Percent survival
100
50
0
0
5
10
15
20
Time
***
CDT+
CDT-
b.
WBC Count
50
40
30
20
10
0
*
CDT-
CDT+
c.
CDT(neg) patients
Percent survival
100
50
0
0
5
10
15
20
Time
*
High sST2
Low sST2
d.
ST2 pg/ml
10^7
10^6
10^5
10^4
10^3
10^2
**
Non-severe
Severe

Figure 3.3: CDT is associated with increased disease severity during human CDI and the pathogenicity of sST2 is not restricted to CDT expressing strains.

(*A-B*) Analysis of *C. difficile* disease severity in association with CDT toxin positivity in human CDI+ stool on the day of diagnosis. (*A*) Survival curves of CDI+ patients stratified into CDT(+) or CDT(-) based on CDTb ELISA. *B*) Patients were stratified based on their WBC into severe vs. non-severe CDI and CDT status was assessed between the two groups. (*C-D*) Analysis of systemic sST2 (IL-33 decoy receptor) in the serum of 115 patients with CDT (-) stool by ELISA. (*C*) Survival curves of CDI+ patients stratified into high (sST2 > 55,000 pg/ml) or low sST2 (sST2 < 55,000 pg/ml). (*D*) Patients were stratified based on their WBC into severe vs. non-severe CDI and sST2 was assessed between the two groups. (*A-B*) n= 137 CDI+ patients (*C-D*) n= 115 CDI+ patients that are CDT(-) (*A,C*) Comparison made by log rank test (*B,D*) Comparison made by Mann-Whitney Test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Table 3.2 Mortality risk of CDI + human patients based on CDT status and sST2 levels.

Parameter		Standard Error	Chi-Square	Pr > ChiSq	**Hazard Ratio (Mortality Risk)**	95% Hazard Ratio Confidence Limits	
sST2_grp	>50%tile	0.51849	4.6105	0.0318	**3.044**	1.102	8.411
cdt_value	1	0.45775	6.4768	0.0109	**3.206**	1.307	7.863
Age		0.01726	8.6624	0.0032	1.052	1.017	1.088
Female		0.45666	0.5079	0.4760	1.385	0.566	3.389
Black		0.64634	0.1647	0.6849	0.769	0.217	2.731
Charlson		0.06655	0.1198	0.7292	1.023	0.898	1.166

[1] Correlation between CDT status in stool and ST2 levels in serum and survival assessed through Cox regression analysis. ST2 and CDT status are considered as independent variables as the interaction of sST2 (sST2 >50%tile or not) and CDT indicator is not significant.

[2] Mortality risk (hazard ratio) adjusted for age, sex, race, charlson comorbidity index.

infection and is therapeutically relevant in both CDT expressing and non-CDT expressing *C. difficile* infection.

3.2.2 FMT therapy rescues colonic IL-33 expression after antibiotics.

As FMT therapy is a highly effective treatment for severe relapsing CDI in humans, we wondered whether microbial therapy alters IL-33 abundance in the colon. We treated mice with a broad spectrum cocktail of antibiotics to disrupt their protective microbial communities making them susceptible to CDI[105]. 24-hours after antibiotic treatment, mice were orally gavaged with a mouse FMT isolated from 10 age and sex matched wildtype donors (**Fig 3.4A**). We found that mice treated with antibiotics had a significant decrease in their total colonic IL-33 protein expression (**Fig 3.4B-C**). Oral gavage with a mouse FMT rescued IL-33 levels up to ten days post treatment (**Fig 3.4B**). Blinded immunohistochemistry scoring of IL-33 staining intensity within the colon aligned with our ELISA data, showing higher IL-33 staining within the epithelium, muscularis mucosa and lamina propria of sections from FMT treated mice (**Figure 3.4D-E)**.

Our murine FMT increased the diversity of antibiotic-treated recipients, making their communities more similar to donor non-antibiotic treated controls with an expansion in the firmicutes phylum (**Fig 3.5A-B**). More specifically, the treatment with the murine FMT caused an expansion in the relative abundance of *Lachnospiraceae, Lactobacillaceae, Muribaculaceae, Ruminococcaceae* and reductions in the relative abundance of *Akkermansiaceae, Bacteroidaceae, and Enterococcaceae* (**Fig 3.5C)**. Additionally, oral gavage with a human-derived fecal spore preparation (HSP) also significantly rescued IL-33 levels, indicating that a commensal or commensal-derived product is capable of regulating colonic IL-33 expression and is conserved between mouse and human microbial therapy (**Fig 3.4C**).

Figure 3.4

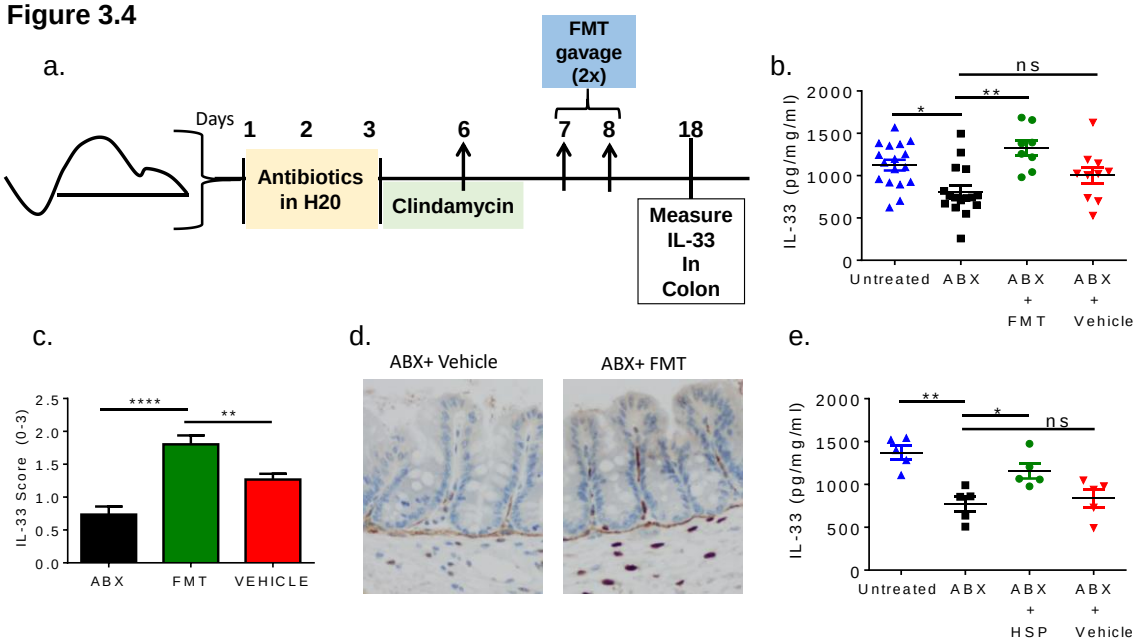
a.
FMT gavage (2x)
Days
1
2
3
6
7
8
18
Antibiotics in H20
Clindamycin
Measure IL-33 In Colon
b.
ns
**
*
IL-33 (pg/mg/ml)
2000
1500
1000
500
0
Untreated
ABX
ABX + FMT
ABX + Vehicle
c.
****
**
IL-33 Score (0-3)
2.5
2.0
1.5
1.0
0.5
0.0
ABX
FMT
VEHICLE
d.
ABX+ Vehicle
ABX+ FMT
e.
**
*
ns
IL-33 (pg/mg/ml)
2000
1500
1000
500
0
Untreated
ABX
ABX + HSP
ABX + Vehicle

Figure 3.4: IL-33 signaling in the intestine is targetable with FMT or HSP therapy. (*A-C*) Mice treated with a broad-spectrum antibiotic cocktail were orally gavaged twice with a murine FMT isolated from 10 untreated C57BL6 donors. (*B*) IL-33 protein within the caecal tissue was measured by ELISA 10 days post treatment. (*C-D*) The average intensity of IL-33 immunohistochemistry staining of caecal sections from FMT vs. vehicle recipients was assessed. *D)* Images of IL-33 staining from two representative mice. A score of 0-3 (with 3 being highest IL-33 staining and 0 being no IL-33 staining) was given by 3 independent scorers and averaged for 10 mice. *E)* Mice were orally gavaged with a spore prep derived from a normal human donor and total caecal IL-33 levels were assessed by ELISA 24 hours after treatment. Data are presented as means ± SEM. (B-C) n = 9-16 mice combined from 2 independent experiments. (*E*) n = 5 per group representative of 2 independent experiments. (*B,C,E*) Comparison made by Mann-Whitney Test. (*F*) Comparison made by log rank test (*G-H*) Comparison made by ANOVA for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 3.5

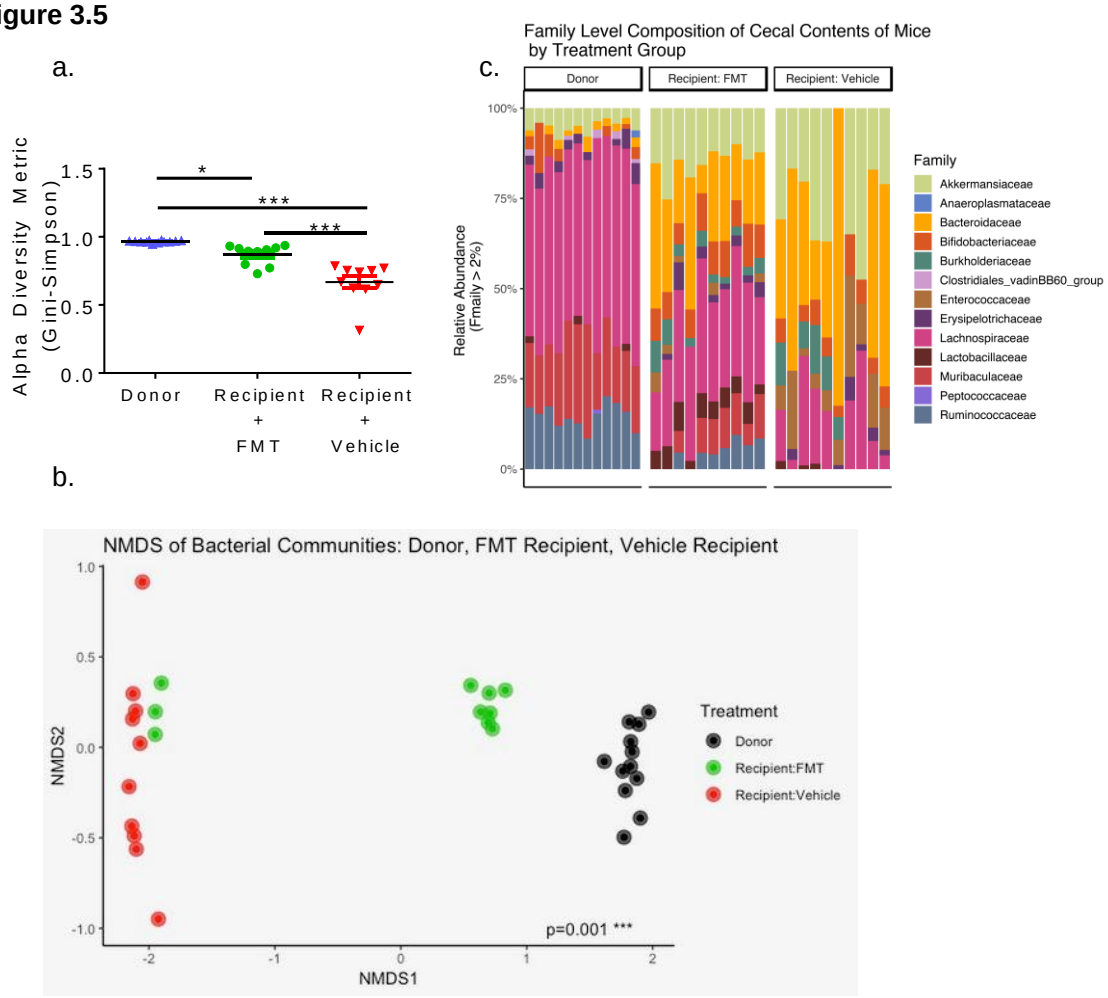
a.
Alpha Diversity Metric (Gini-Simpson)
1.5
1.0
0.5
0.0
*
***
***
Donor
Recipient + FMT
Recipient + Vehicle
b.
c.
Family Level Composition of Cecal Contents of Mice by Treatment Group
Donor
Recipient: FMT
Recipient: Vehicle
Relative Abundance (Fmaily > 2%)
100%
75%
50%
25%
0%
Family
Akkermansiaceae
Anaeroplasmataceae
Bacteroidaceae
Bifidobacteriaceae
Burkholderiaceae
Clostridiales_vadinBB60_group
Enterococcaceae
Erysipelotrichaceae
Lachnospiraceae
Lactobacillaceae
Muribaculaceae
Peptococcaceae
Ruminococcaceae
NMDS of Bacterial Communities: Donor, FMT Recipient, Vehicle Recipient
NMDS2
NMDS1
1.0
0.5
0.0
-0.5
-1.0
-2
-1
0
1
2
p=0.001 ***
Treatment
Donor
Recipient:FMT
Recipient:Vehicle

Figure 3.5. FMT alters the gut microbiota after treatment. Mice were treated with FMT therapy after broad-spectrum antibiotic cocktail used in infection model. (*A*) Microbiota diversity in caecal contents of untreated donors and antibiotic treated FMT recipients (Simpson index y axis). (*B*) NMDS plot of Bray-Curtis dissimilarity index from caecal samples of Donors vs. FMT and Vehicle recipient mice. (*C*) Bar plot of relative family abundance of Donors vs. FMT recipients vs. vehicle controls. Data are presented as means ± SEM. (*A*) comparisons made by ANOVA. (*B*) comparisons made using permutational multivariate analysis of variance (PERMANOVA) $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Taken together this data indicates that IL-33 is a novel therapeutic pathway relevant in human CDI that is regulated by the microbiota and may be targetable with next-generation bacterial preparations.

3.3 Discussion

The key positioning of IL-33 at the epithelial interface enables it to integrate signals between the microbiota, pathogens, and the immune system. Our work highlights this sensing function as colonic IL-33 was amplified in response to mouse and human *C. difficile* bacterial infection in addition to murine FMT or a human spore preparation. Thus, although IL-33 is constitutively expressed within the nucleus[237,327–329], its homeostatic expression is also regulated by microbial signals received from the gut lumen. Thus IL-33 is a novel therapeutic pathway for *C. difficile* that may be targeted with next-generation, rationally designed bacterial preparations.

Disturbances of the gut microbiota have long been associated with risk of developing *C. difficile* colitis[109,330]. Gut microbial diversity provides colonization resistance against *C. difficile* through direct and indirect mechanisms of competition, production of metabolites, and induction of antimicrobial peptides[30,104,109,325]. Restoration of microbial diversity through FMT therapy is a successful treatment strategy in patients that fail to resolve their infection with traditional antibiotic therapy[140]. Previous studies have demonstrated a close relationship between IL-33 and the microbiota during murine IBD models with germ-free mice having reduced IL-33 levels in the small intestinal ilea during chronic ileitis[172,305]. Here we show that murine FMT and a human-derived spore-based fecal preparation can rescue antibiotic-mediated depletion of colonic IL-33. The bacterial species or microbiota-derived product regulating colonic IL-33 expression requires further investigation. IL-33 transcription can be regulated in inflammatory settings by pattern recognition receptors (PRRs), TLR4, TLR3, and NOD-1, sensing of pathogen associated molecular patterns

(PAMPs)[321,331,332]. Thus, PRR sensing of commensal derived antigens may contribute to steady-state maintenance of IL-33 in the presence of the microbiota, similar to the MYD88-dependent antimicrobial peptide, RegIIIγ, which aids in resistance to gram positive-bacteria[141,142,333,334]. Commensal derived metabolites may also regulate colonic IL-33 expression in a similar fashion to epithelial- derived interleukin-25 (IL-25), a cytokine expressed by intestinal tuft cells and regulated by the organic acid succinate[133,179,267,335]. *Thus alterations to the microbiota in after FMT may be sensed by IL-33 producing cells, possibly via TLR signaling*[321]*, or metabolite sensing*[179,336]*, to increase IL-33 expression in the intestine.*

Like IL-33, IL-25 can protect the gut barrier during *C. difficile* colitis indicating a shared role of barrier defense between these two epithelial-derived cytokines, possibly through ILC2s[133]. While IL-25 expression by tuft cells is regulated by intestinal succinate, it is unknown whether microbial-derived metabolites can also regulate IL-33 expression. After murine FMT we see an expansion in Firmicutes within the large intestine in addition to increased microbial diversity. Furthermore, our human spore cocktail was enriched in members of the Firmicutes phylum including the families Clostridiaceae, Erysipelotrichaeceae, Eubacteriaceae, Lachnospiraceae, and Ruminococcaceae. The majority of these families are members of the Clostridiales order which has associated with CDI resistance through direct and indirect bacterial competition and metabolic alterations including secondary bile acid production[108,112,337–341]. *Thus, in addition to bacterial competition, these microbial communities may also alter host immunity during CDI via regulation of IL-33.*

Here our murine studies were validated in humans where we showed that high serum levels of the soluble IL-33 decoy receptor, sST2, were associated with more severe *C. difficile* colitis (defined by white blood cell count[35, 79]) and mortality. Previous findings

have identified sST2 as a biomarker for mortality during human sepsis and interestingly murine sepsis and bacteremia models have identified a protective role for IL-33 via anti-microbial neutrophil chemotaxis and type-2 associated ILC2s[247,275,277,342]. A common theme may be that a robust inflammatory anti-microbial response is essential to control infection yet this response must be tightly regulated by tissue-regulatory pathways to prevent tissue damage to the host.

Furthermore, the infecting *C. difficile* strain dictates human clinical disease severity[28,343,344] based on toxin status[345,346], and also changes to the host immune response[226,301,346,347]. We previously demonstrated a detrimental role for CDT toxin expressed by NAP1/027 strains via its ability to kill eosinophils during infection[6]. Based on these previous findings and given IL-33's role in eosinophil survival, we evaluated whether sST2 is a predictor of *C. difficile* severity only in CDT expressing infection. Interestingly, we saw that sST2 was a risk factor for mortality in patients independent of CDT status. This aligns with our previous mouse work demonstrating that IL-33 signaling can protect from both CDT+ NAP1/027 infection and non-CDT expressing classical infection. Thus, IL-33 is general mechanism of gut protection independent of CDT status. This finding broadens the impact of IL-33 as a potential therapy to treat CDI, as only 15-20% of clinical isolates express CDT toxin. Whether IL-33 can rescue CDT-mediated killing of eosinophils is an important question. Further assessment stratifying patient risk with CDT status, IL-33 expression, and eosinophil counts during human *C. difficile* infection will be important in future clinical studies.

This work highlights that IL-33 is an important potential therapeutic target in human CDI. The ability to manipulate colonic IL-33 with spore-forming commensals may allow for more targeted approaches for treatment of at-risk patients with rationally designed next-generation microbial therapy. This work advances our fundamental understanding of

the interplay between FMT therapy and the immune system and also identifies a novel therapeutic pathway of IL-33 signaling in human CDI patients.

Chapter 4: Perspectives

A recent point-prevalence survey conducted by the Emerging Infections Program demonstrated that the overall incidence of hospital acquired infections in the US has declined from 4% of hospitalized patients in 2011 to 3.2 % of hospitalized patients in 2015. In contrast, there was no reduction in the prevalence of *C. difficile* infections. *C. difficile* remained the most common gastrointestinal infection with an incidence of 0.54 percent of hospitalized patients[348]. Thus, reducing *C. difficile* infection remains a high priority. While *C. difficile* disease is driven by toxin mediated disruption of the epithelial barrier, we now have a better understanding of other factors that contribute to the severity of infection including the type of host immune response. Recent studies have demonstrated that an overly robust type-17 inflammatory response (e.g. the IL-23 signaling axis) can drive increased tissue pathology and higher mortality while type-2 associated immune pathways, such as IL-25 elicited eosinophils, can drive survival[6,74,133,136,156]. Toxin expression by the infecting strain can also drive more severe disease through alterations to the host immune response. For example, TcdA and TcdB increase type-17 immunity via induction of IL-1β, IL-6, and IL-23 expression while CDT toxin kills protective type-2 associated eosinophils[67,214,301]. Given the importance of host immunity in dictating disease severity, manipulation of the immune response may be a promising therapeutic strategy for severe or relapsing *C. difficile* infection.

In this thesis we asked the broad question of how the host immune system changes in response to hypervirulent, CDT expressing strains of *C. difficile*. Specifically, we were searching for new immune candidates dysregulated during hypervirulent CDI with the goal of targeting these pathways with follow-up interventional studies. Thus, we conducted a transcriptomics array on murine caecal tissue infected with either the hypervirulent CDT expressing strain, R20291, or an attenuated, isogenic mutant R20291_*cdtb,* lacking CDT expression.

Our transcriptomics array revealed many tissue-repair pathways were enriched during severe CDT+ infection including IL-10, IL-4 and IL-13 signaling. Additionally, the cytokine, IL-33, which is a known upstream regulator of mucosal barrier repair during nematode infection and colitis models was also upregulated. Thus, we hypothesized that IL-33 may have protective barrier function during hypervirulent CDT+ *C. difficile* infection. To elucidate the role of IL-33 during CDI, we treated mice with IL-33, increasing gut IL-33 levels by 5-fold prior to infection. This IL-33 treatment regimen reduced mortality, weight-loss, and clinical disease over the course of infection and also reduced toxin-mediated epithelial disruption and gut leakiness. IL-33 treated mice had a dominant type-2 associated cytokine milieu with increased numbers of ILC2s and eosinophils during to infection. Furthermore, IL-33 treated mice had reductions in canonical type-17 associated immunity such as reduced neutrophils, ILC3s, and IL-23 during infection. Importantly, IL-33 mediated protection required the action of ST2+ ILC2s, an immune cell not previously known to be involved in recovery from CDI. Adoptive transfer of ILC2s was sufficient to prevent mortality, weight-loss and increase downstream eosinophilia in highly susceptible immunocompromised $Rag2^{-/-}\gamma c^{-/-}$ mice. Importantly, IL-33 mediated protection was independent of microbial factors as protected IL-33 treated mice had equivalent *C. difficile* burdens, toxins TcdA/B and CDT levels, and translocating commensals during infection. While many studies have described important anti-microbial immune pathways that aid in *C. difficile* clearance, here we describe a new pathway of barrier protection that instead, acts in a tissue-regulatory manner via ILC2s.

In addition to the protection elicited by IL-33 treatment, we also describe an important role for endogenous IL-33 during CDI. As such, $ST2^{-/-}$ mice are highly susceptible to CDI-associated mortality and weight-loss relative to wildtype controls. Susceptible $ST2^{-/-}$ mice lack activated ILC2s in the colon lamina propria, having reduced numbers of IL-13+ ILC2s and diminished eosinophil numbers during infection. Thus, IL-

33 signaling to ILC2s is an important mechanism of endogenous host protection and eosinophil maintenance during CDI. Likely, IL-33 signaling to ILC2s is a mechanism to limit an overly robust type-17 immune response during infection and promote recovery via tissue-repair. How ILC2s protect from *C. difficile*-associated mortality requires further investigation which we will outline in greater detail during this discussion.

Our studies also reveal an important role for IL-33 signaling during human *C. difficile* colitis. We demonstrate that IL-33 is upregulated in colon biopsies from human *C. difficile* patients. Furthermore, the soluble decoy receptor for IL-33, sST2, is expressed at high levels in human serum and predicts mortality risk. Interestingly, the microbiota influences the expression of IL-33 in the intestine as antibiotic therapy depletes colonic IL-33 levels. Treatment of antibiotic-depleted mice with a murine FMT or a purified human spore prep, rescued antibiotic-mediated depletion of IL-33. This data indicates that IL-33 signaling is a protective immune pathway during human CDI that may be targetable with next-generation purified bacteriotherapy.

Importantly, while we identified IL-33 as a candidate immune target by screening genes altered in response to CDT + infection, we demonstrate that IL-33 mediated protection is not restricted to CDT expressing isolates of *C. difficile*. In our murine model of infection, endogenous and exogenous IL-33 can protect from the non-CDT expressing strains, classical VPI or the isogenic mutant strain R20291_*cdtb*. Furthermore, the soluble decoy receptor, sST2, is associated with increased mortality and severe CDI, independent of CDT status in human patients. Thus, protection from IL-33 signaling has broad applicability to both CDT and non-CDT expressing *C. difficile* infection.

This work expands our fundamental understanding of how the host immune system can alter *C. difficile* severity and highlights a new immune pathway of IL-33 signaling to ILC2s that influences disease pathogenesis. Our study introduces a mechanism by which microbiota regulated IL-33 implements tissue-repair and type-2

immunity via ILC2s to promote recovery from *C. difficile* colitis. This work opens up additional questions and areas of research. 1) First and foremost, it is important to understand the connection between the microbiota and IL-33 in the intestine. While we demonstrate that IL-33 is regulated by the microbiota, further studies are essential to understand which cells sense the presence of the microbiota in the colon, and which commensal species or commensal derived signal triggers IL-33 expression. 2) In addition to understanding the upstream mechanisms of how IL-33 is regulated, our work opens up further exploration regarding the downstream mechanism of IL-33 protection via ILC2s. Specifically, what are the pathways ILC2s activate to promote recovery? 3) Finally, it is important for future studies to explore the therapeutic relevance of IL-33 mediated protection, the pathogenic soluble decoy receptor, and the potential to target these proteins for human treatment.

4.1 How is IL-33 regulated by the microbiota?

Previous studies have demonstrated that IL-33, is constitutively expressed at homeostasis, and is transcriptionally increased by pathogen derived PAMPS and inflammatory cytokines during the context of inflammation[321,331,332]. However, our work indicates that constitutive expression of IL-33 may in fact be dependent on the presence of a diverse commensal flora. Whether commensal derived PAMPS or metabolic products contribute to baseline IL-33 expression is a question opened up by our studies.

4.1.1 What cell types are producing IL-33 in response to FMT?

Our immunohistochemistry staining of IL-33 within human and murine colon sections demonstrates pronounced subepithelial IL-33 staining beneath colon crypts. These IL-33+ cells are localized to the area where pericryptal myofibroblasts have previously been identified to express IL-33, at the base of the crypt beneath LGR5+ stem cells[235]. Additionally, we see IL-33+ staining in the lamina propria of tissue sections, localized to cells surrounding the perimeter of blood vessels, suggestive of IL-33+

endothelial cells or adventitial stromal cells (ASCs) as described by others[237,238,327]. This microscopy data is in alignment with seminal work describing IL-33 as a cytokine expressed by barrier, radioresistant cells[311]. During stress and inflammation, macrophages and monocytes can also upregulate IL-33 expression. Thus, it is possible that during *C. difficile* colitis, infiltrating myeloid cells may also be cellular sources of IL-33[321,349]. As such, we see abundant IL-33 expression within the lamina propria of colon sections after antibiotics and FMT therapy, indicating it is also possible that myeloid cells express IL-33 after commensal sensing. It is important for future studies to determine the exact cellular source of IL-33 in the context of antibiotics, FMT therapy, and *C. difficile* infection.

Recently, we acquired IL-33-GFP reporter mice which will enable us to study the co-localization of IL-33 with other cell specific markers after FMT and during *C. difficile* infection. Using these reporter mice, we can measure co-localization of IL-33-GFP with epithelial, endothelial, adventitial stromal cell, fibroblast and myeloid cell markers. For example, immunohistochemistry staining of myofibroblasts using the marker α-smooth muscle actin (α-SMA); epithelial cells using the marker epithelial Cell Adhesion Molecule (Ep-CAM); endothelial cells using the marker VE-cadherin; adventitial stromal cells using the marker platelet-derived growth factor receptor (PDGFR) and myeloid cells using the leukocyte adhesion marker CD11b will allow us to quantify which cells co-localized with IL-33-GFP expression. We can use this immunohistochemistry staining protocol to measure IL-33 colocalization after antibiotics, after FMT therapy, and after *C. difficile* infection relative to untreated controls. Thus, IL-33-GFP reporter mice will be a useful tool to begin to understanding the cellular source of IL-33 in response to *C. difficile* colitis and in response to colonization with commensals. It is possible that there are multiple cellular sources of IL-33 in the context of homeostasis vs. colitis. For example, *C. difficile* infection may increase the IL-33 expression from immune cells due to their influx after toxin-

mediated damage. This may differ in the context of FMT therapy when the epithelial barrier is intact and myeloid cells are limited. After conducting immunohistochemistry to identify candidate cellular populations that express IL-33 in the context of FMT therapy or *C. difficile* infection, we can go on to more specifically define these populations using flow cytometry and cell sorting. Thus, we can measure the ability of these cellular populations to increase IL-33 with FMT or CDI by quantifying the abundance of IL-33+ cells via flow or quantifying IL-33 transcript abundance of the sorted subset.

4.1.2 How is the microbiota inducing IL-33 expression?

We demonstrate that there is an interaction between the microbiota and IL-33 expression within the colon. Antibiotic-depletion of the microbiota leads to a corresponding significant decrease in IL-33 levels. Furthermore, IL-33 can be rescued by replenishment of the microbiota using FMT therapy. These findings open up additional questions and areas of further research. It is important to conduct additional experimentation to understand: 1) which commensals are capable of inducing IL-33, and 2) what commensal-derived signals are sensed by the host to regulate IL-33 expression.

4.1.2.1 What members of the microbiota induce IL-33?

Our research points to an immunomodulatory microbe which upon colonization can influence IL-33 expression. 48 hours and up to 10 days after antibiotics, IL-33 expression is reduced in the murine colon. Additionally, 48 hrs and 10 days post FMT therapy, IL-33 levels can be restored by recolonization with commensals. Our 16S V4 sequencing data revealed that FMT recipients had increased commensal diversity 48 hrs after FMT and additionally had commensal communities more similar to FMT donors. More specifically, murine FMT therapy caused an expansion in the relative abundance of commensal families *Lachnospiraceae, Lactobacillaceae, Muribaculaceae, Ruminococcaceae* and reductions in the relative abundance of *Akkermansiaceae, Bacteroidaceae*, and *Enterococcaceae*. Some of these families are also enriched in our

human spore prep (HSP) which, similar to the crude murine FMT, also increased colonic IL-33 after oral gavage. The HSP innoculum was enriched in the families, *Clostridiaceae, Erysipelotrichaeceae, Eubacteriaceae, Lachnospiraceae,* and *Ruminococcaceae.* Both *Lachnospiraceae*, and *Ruminococcaceae* are two bacteria families conserved between the protective HSP inoculum and engrafted murine FMT, both of which induced IL-33 expression. Thus, bacteria from the Clostridiales order and more specifically *Lachnospiraceae*, and *Ruminococcaceae* may be promising candidates for inducing IL-33 in the colon and thus aiding in immune protection during CDI. In line with this thinking, pre-colonization of germ-free mice with *Lachnospiraceae* protects from *C. difficile* associated mortality[196]. Additionally, a study of human recipients of allogeneic hematopoietic stem cell transplantation who are at major risk of *C. difficile* infection, demonstrated that the presence *of Lachnospiraceae, Ruminococcaceae* and *Bacteroidetes*, was associated with a 60% lower risk for acquiring *C. difficile*[350]. Thus, *Lachnospiraceae* and *Ruminococcaceae* are already associated with protection from CDI in humans and mice, however whether this protection also involves induction of IL-33 is an exciting area of future study.

Future studies are required that more specifically define commensals capable of upregulating IL-33 in the intestine. Specifically, we can colonize germ-free or antibiotic-treated mice with commensal candidates that were enriched after FMT engraftment, such as family members *Lachnospiraceae* and *Ruminococcaceae*. If *Lachnospiraceae* and *Ruminococcaceae* are sufficient to rescue IL-33, mice colonized with these defined microbial cocktails will have significantly elevated IL-33 levels relative to germ-free or antibiotic only controls.

To more specifically define IL-33 inducing commensals at the species levels, we can conduct deep-sequencing of caecal contents from mice pre and post FMT treatment and correlate OTUs with IL-33 levels using a random-forest analysis. This analysis may

aid in defining a specific bacterial species that predicts high IL-33 levels. Follow-up validation studies could then be conducted through mono-colonization of germ-free mice, demonstrating a predicted bacterial species is sufficient to increase IL-33 expression in the colon. Likely, multiple species are capable of increasing IL-33 as microbial-derived signals tend to be conserved between commensals and sensed by the host immune system.

4.1.2.2 What signals are being received by the microbiota to regulate IL-33?

Given that that IL-33 is regulated by the microbiota, it is important to decipher which microbiota-derived signals are sensed by the host to subsequently increase intestinal IL-33. One possibility is that microbiota-derived PAMPS are directly sensed by pattern-recognition receptors (PRRs) to induce IL-33 expression. Another possibility is that upon colonization, microbiota-derived changes to the metabolome, such as the production of short chain-fatty acids, lead to increased IL-33 expression within the intestine. Both of these possibilities are discussed below.

4.1.2.2.1 PAMP signals

IL-33 can be transcriptionally regulated during pathogen infection by sensing of PAMPs via pattern recognition receptors. LPS administration and sepsis *in vivo* increases IL-33 expression on intestinal pericryptal myofibroblasts[235]. Additionally, *in vitro*, LPS sensing by TLR4 increases IL-33 mRNA expression by macrophages[321]. Within murine mucosal-associated dendritic cells and additionally human corneal epithelial cells, stimulation of TLR5 with microbial derived flagellin also induced expression of IL-33 mRNA[351,352]. Another immune sensor, NOD1, which recognizes small peptides derived from peptidoglycan (PGN), is necessary for upregulation of IL-33 mRNA expression by gastric epithelial cells during *Helicobacter pylori* (*H. pylori*) infection[331,353]. Thus, IL-33 expression is transcriptionally regulated by PRR sensing of microbial-derived PAMPS.

The expression of PRRs such as TLRs and NOD receptors and their subsequent sensing of microbial-derived patterns is not restricted to pathogen encounter. In contrast, PRRs are expressed in the intestinal epithelium at steady-state and are thus uniquely positioned to sense and interact with the luminal intestinal microbiota[354]. In fact, the interaction between PRRs and commensal-derived signals is essential for maintaining intestinal symbiosis[355,356]. For example, sensing of commensal-derived signals by intestinal TLR-MyD88 signaling, protects mice from colitis[355,356]. Given that TLR4, TLR5 and NOD-1 can sense commensal associated molecular patterns in addition to their ability to regulate IL-33 transcription, future studies should test the necessity of these PRRs to stimulate IL-33 after FMT.[355–358]. Interestingly, TLR4, TLR5, and NOD-1 signaling have all been associated with protection from *C. difficile* colitis; however, whether a lack of intestinal IL-33 expression contributes to these phenotypes requires further investigation[217,304,359,360]. Given that all TLRs, except TLR3, require MyD88 to signal, MyD88$^{-/-}$ mice can be used to broadly ask whether TLR signaling is required for rescue of IL-33 after FMT engraftment[361]. Lack of IL-33 upregulation in MyD88$^{-/-}$ mice after FMT therapy indicates that TLR signaling is required for commensal-derived IL-33 expression. If MYD88 is not required for FMT-mediated induction of IL-33, then additional PRR knockout mice can be used, such as Nod-1 and Nod-2 null mice, to decipher the involvement of other PRRs. These *in vivo* studies can be informed by additional *in vitro* screens of the ability of various PAMPS to induce IL-33 expression after co-culture with intestinal organoids. These future studies will aid in our fundamental understanding of how intestinal IL-33 levels are rescued after FMT therapy and which receptors and PAMPS are required for homeostatic IL-33 expression.

4.1.2.2.2 Metabolite signals

The microbiota can also alter the expression of host cytokines through the production of metabolites. For example the intestinal pool of short chain fatty-acids (SCFAs) is largely

dependent on commensal fermentation of carbohydrates[362]. SCFAs such as butyrate, propionate, and acetate are sensed by epithelial cells and immune cells via G-protein coupled receptors, GPR41 and GPR43; maintaining the transcription of many cytokine and chemokines within the intestine[363]. Within this thesis, we show that a human spore preparation derived of spore-forming Firmicute commensals such as family members, *Clostridiaceae, Erysipelotrichaeceae, Eubacteriaceae, Lachnospiraceae*, and *Ruminococcaceae* is suffient to rescue IL-33 levels after antibiotics. Interestingly, many of these Firmicute strains, such as *Lachnospiraceae*, and *Ruminococcaceae*, are major butyrate producers and are very abundant members of the commensal flora[364]. Additionally, these commensals and the SCFA derived from their fermentation are associated with protection from *C. difficile*[118,196,365]. Whether SCFAs also regulate protective IL-33 expression in the intestine is a new question opened up by this thesis. To answer this question, we can more broadly screen metabolites present in the caecal contents of antibiotic treated and FMT treated mice using metabolomics. For these same mice we can quantifying the corresponding amount of intestinal IL-33 and associate certain metabolites or metabolic signatures with high IL-33 expression. This screening technique will help us generate a metabolic profile that correlates with IL-33 expression which can then be validated using both *in vitro* and *in vivo* systems.

Alternatively, we can take a more candidate-driven approach, directly testing the role of SCFA's in activating IL-33 signaling, given their strong association with CDI protection and their ability to regulate other gut cytokines. Using this method, we can add SCFAs to the drinking water of antibiotic treated mice or alternatively orally gavage mice with SCFAs[118,179] and measure intestinal IL-33 expression relative to vehicle controls. An upregulation of IL-33 in SCFA treated mice would indicate these metabolites are involved in regulating gut IL-33 expression.

4.2 What protective immunological processes are downstream of IL-33 and ILC2s during CDI?

In this thesis, we establish a new role for ILC2s in recovery from *C. difficile* colitis. We demonstrate that IL-33 elicited ILC2s are sufficient to protect from mortality and weight-loss during *C. difficile* colitis. Lack of ILCs completely abrogates IL-33 mediated protection. Adoptive transfer of IL-33 activated ILC2s is also sufficient to increase colonic eosinophilia, demonstrating ILC2s have an important role in maintaining protective eosinophils during *C. difficile* colitis. IL-33 elicited eosinophils are partially required for protection as depletion of eosinophils only partially abrogated IL-33 mediated protection. Thus, in addition to eosinophils, other protective mechanisms downstream of ILC2s must exist. Here we discuss how IL-33 signaling to ILC2s may promote recovery from *C. difficile* colitis via several mechanisms which are discussed in detail below. These mechanisms include 1) induction of eosinophils via IL-5 expression; 2) protection of the epithelium by amphiregulin expression; 3) increased mucin barrier via IL-13 expression; 4) increased tissue repair via IL-4 signaling to alternatively activated macrophages and; 5) inhibition of pathogenic type-17 immunity. In this section, we explore each of these downstream pathways and suggest future experiments to test their involvement in IL-33/ILC2-mediated protection.

4.2.1 IL-33 mediated protection via IL-5 and eosinophils and its relevance during CDT(+) vs. CDT(-) isolate infection.

Our findings demonstrate that IL-33 requires ILC2s and eosinophils to protect, and additionally that IL-33 elicited ILC2s are important for colonic eosinophil accumulation during CDI. The cytokine IL-5 may have a role in ILC2-mediated maintenance of eosinophils as previous studies have demonstrated that at steady-state ILC2s from the intestine constitutively express IL-5 and are essential for eosinophil homeostasis[175,366]. Eosinophil accumulation in the intestine is independent of the microbiota and is also

regulated by the chemokine, eotaxin[367]. IL-13 and IL-5 expression by ILC2s contribute to the constitutive expression of eotaxin, maintaining eosinophil abundance in the gut[318,366,368]. IL-5 is particularly important for eosinophil differentiation and trafficking to the intestine in addition to their responsiveness to eotaxin[369]. As such, IL-5 and IL-5R knockout mice are deficient in eosinophils[370,371].

In these studies, we demonstrate that eosinophils are in part, necessary for IL-33 mediated protection and that IL-33 is important for IL-5 expression and eosinophil accumulation during *C. difficile* infection. Whether IL-5 is the intermediate between IL-33, ILC2s and eosinophil accumulation during *C. difficile* colitis requires further investigation. To begin to understand this, we can neutralize IL-5 during our IL-33 treatment model of infection. If IL-5 is necessary for IL-33 mediated protection and eosinophil accumulation, IL-5 neutralized mice should have reduced survival and eosinophilia after IL-33 treatment relative to IL-5 sufficient controls. It is also important to understand the source of IL-5 during *C. difficile* infection and whether it is ILC2s as we predict from previous literature[230]. To do this, we can quantify IL-5+ ILC2s using intracellular cytokine staining and flow cytometry of gut ILC2s during infection. Furthermore, we can compare the number and frequency of IL-5+ ILC2s in $ST2^{-/-}$ vs. Wildtype mice. If IL-33 contributes to IL-5 expression in ILC2s during CDI, then $ST2^{-/-}$ mice will possess significantly less IL-5+ ILC2s during *C. difficile* colitis. Furthermore, adoptive transfer of IL-5+ ILC2s should protect $ST2^{-/-}$ mice from mortality relative to mice receiving IL-5(-) ILC2s. High IL-5 and eosinophils have been previously associated with less severe *C. difficile* associated disease in human patients, however these future studies will aid in our understanding of whether IL-33 mediates eosinophilic protection via the action of IL-5 + ILC2s[135,136].

4.2.1.1 Does IL-33 signaling protect from CDT mediated killing of eosinophils?

We have demonstrated in this thesis that CDT toxin expression at the day of CDI diagnosis is a predictor of mortality in human patients. Our previous studies have

demonstrated that CDT toxin increases virulence by killing protective eosinophils in a TLR2 dependent manner[6]. Specifically, mice infected with a CDT expressing strain have reduced numbers of colonic eosinophils during infection and increased apoptotic eosinophils in their peripheral circulation during infection[6]. Interestingly, CDT expression does not reduce eotaxin, or IL-5 levels during infection and furthermore co-culture of eosinophils with CDT does not directly induce eosinophil death[6]. Thus, the mechanism of CDT-mediated eosinophil apoptosis is not currently known. Our findings show that IL-33 increases eosinophils during CDT + hypervirulent CDI. This brings up the interesting question regarding whether IL-33 mediated protection is more effective during CDT+ infection by rescuing CDT-mediated eosinophil loss. While we know endogenous and exogenous IL-33 can protect from non-CDT expressing infection, it is still unknow whether IL-33 reduces the CDT-mediated eosinophil deficiency by preventing eosinophil apoptosis. To test this idea, we can quantify the number of apoptotic eosinophils in the periphery of IL-33 treated mice vs. vehicle controls, infected with CDT expressing R20291 or the isogenic mutant R20291_*cdtb*. If IL-33 prevents CDT-mediated eosinophil apoptosis, then R20291 infected mice treated with IL-33 will have diminished circulating apoptotic eosinophils, comparable levels to R20291_*cdtb* infected mice.

Our human data indicates that IL-33 signaling is protective independent of CDT status of the infecting strain. Specifically, sST2 is pathogenic in non-CDT expressing human infection and, after correcting for CDT status, sST2 is an independent predictor of mortality. However, the relationship between circulating eosinophils, sST2, and CDT requires further investigation in human patients. Specifically, whether patients infected with a CDT expressing strain have less eosinophils or more sST2 still needs to be determined. Furthermore, whether patients with high sST2 have reductions in peripheral eosinophil counts also requires further investigation. These studies will aid in our

understanding of IL-33 signaling in human CDI and its relationship to the CDT status of the infecting isolate.

4.2.2 Amphiregulin-mediated protection of epithelial barrier

The majority of IL-33 activated ILC2s in the colon express the epidermal like growth factor (EGF)-like molecule, amphiregulin, in addition to canonical type-2 cytokines IL-5, IL13, and IL-4[175]. Amphiregulin expression by ILC2s is an important mechanism of epithelial restoration after damage that protects mice from colitis associated tissue pathology and weight-loss[175]. Given our findings that IL-33 treated mice have reduced epithelial disruption and gut permeability during CDI, this makes amphiregulin expression by IL-33 activated ILC2s a potential mechanism of improved intestinal integrity during CDI. To test this idea, we can determine if the capacity of IL-33 to increase survival during CDI is blocked by a neutralizing antibody against amphiregulin[372] or blocked in amphiregulin knockout mice[373]. If amphiregulin is downstream of IL-33, mice lacking amphiregulin will have significantly reduced survival and increased epithelial disruption after IL-33 treatment relative to amphiregulin sufficient controls. To determine whether amphiregulin expression specifically by ILC2s is sufficient to protect, adoptive transfer studies must be conducted. If AREG+ILC2s are sufficient to protect, adoptive transfer of AREG(+) ILC2s should have increased survival, weight-loss, and/or less epithelial disruption relative AREG(-) ILC2 recipients. If AREG depletion does not reduce IL-33 mediated protection or AREG+ ILC2s recipients are not protected relative to AREG(-) ILC2 recipients, this would indicate that amphiregulin is not downstream of IL-33-ILC2 recovery during *C. difficile*. Given that IL-33 activated ILC2s have multiple effector functions in the gut, this data would indicate that another ILC2 derived cytokine contributes to tissue protection such as IL-4 or IL-13 discussed in greater detail below.

4.2.3 Mucin responses via IL-13

Our findings point to a potential role of the cytokine IL-13 in protection from *C. difficile*-associated disease. IL-13 is TH-2 family cytokine that can induce goblet cell hyperplasia and mucus hypersecretion in the epithelium of mucosal tissue[173,249,374,375]. ILC2s express IL-13 in response to epithelial derived polarizing signals, IL-25, IL-33, and TSLP[173,175,376,377]. ILC2 expression of IL-13 is necessary for helminth expulsion through the activation of mucin responses and tissue-repair[269,378]. In our studies, we demonstrate that IL-33 treated mice have increased IL-13+ ILC2s during CDI in addition to enhanced goblet cells responses. Furthermore, susceptible $ST2^{-/-}$ mice completely lack IL-13 expressing ILC2s indicating IL-33 activation of ILC2s is necessary for their expression of IL-13 during *C. difficile* colitis.

Whether IL-13 is necessary for IL-33 mediated protection requires additional investigation and is an important future question from this thesis. In order to answer this question, additionally studies can be performed neutralizing IL-13 with an anti-IL-13 antibody during our IL-33 treatment model of *C. difficile* colitis[379,380]. If IL-33 mediated protection is abrogated in anti-IL-13 treated mice, then IL-13 is downstream of protective IL-33 signaling. Additionally, goblet cell numbers should be quantified in these studies to determine of IL-33 elicited goblet cell hyperplasia requires IL-13 expression during CDI. A reduction in goblet cells numbers in IL-33 treated mice that lack IL-13 signaling would indicate that IL-13 is downstream of IL-33 and aids in goblet cell barrier defenses during CDI. Furthermore, additional studies are required to understand whether the defect in IL-13 expression by ILC2s in $ST2^{-/-}$ mice contributes to their enhanced disease severity. Adoptive transfer studies of IL-13+ ILC2s into $ST2^{-/-}$ mice will be required to address this point. If adoptive transfer of IL-13+ ILC2s into $ST2^{-/-}$ mice rescues their survival or weight-loss relative to IL-13(-) ILC2s, this would indicate that IL-13 expression by ILC2s is sufficient to rescue IL-33 deficient mice.

The additional studies we have outlined are of great importance to increase our understanding of the role of IL-33 elicited IL-13 during CDI. Interestingly, *C. difficile* reduces goblet cell numbers and MUC2 expression during infection however little is known about the mechanism by which goblet cell depletion occurs[303,381]. It is possible that *C. difficile* either indirectly alters goblet cell differentiation or directly kills goblet cells via toxin production. Regardless of the mechanism of depletion, enhancement of IL-13+ ILC2s by IL-33 may be an important mechanism to combat *C. difficile*- mediated mucin barrier loss and an exciting future area of study.

4.2.4 Alternatively-activated macrophage activation via IL-4Rα

IL-13 shares 25% sequence homology with the structurally related cytokine, IL-4. IL-13 and IL-4 both signal through a shared receptor comprised of IL-4 receptor alpha (IL-4R*α*) and IL-13 receptor alpha 1 (IL-13R*α*1)[382,383]. IL-4 and IL-13 are transcriptionally coordinated in their expression via the transcription factors STAT6 and GATA3[384–386]. Many studies have demonstrated that IL-13 and IL-4 signaling via their shared IL-4R induces alternative activation of macrophages (AAMs)[230,387–389]. AAMs are characterized by arginase-1 (Arg-1), chitinase-like-3 (YM-1), and matrix metallopeptidase expression and are important in tissue repair, remodeling, and dampening of inflammation[390,391]. During, lung infection with the nematode, *Litomosoides sigmodontis,* signaling through the IL-33 receptor was required for IL-4Rα activation of alternatively activated macrophages[392]. Importantly, IL-33 activated AAMs inhibit the severity of colitis upon adoptive transfer[246,393]. As IL-33 protects the gut barrier during *C. difficile* colitis, the possibility that AAMs are downstream of IL-33 should be tested. Future studies can quantify and characterize the macrophage population during *C. difficile* colitis as little is known about the relative abundance of classical macrophages vs. AAMs during infection. Flow cytometry can be used to measure changes in tissue resident AAMs within the infected colon using the AAM markers, CD206 and F4/80. If AAMs are increased in

protected IL-33 treated mice, or alternatively, if they are depleted in susceptible $ST2^{-/-}$ mice, this data will point towards a possible role for these cells in IL-33 mediated recovery from CDI. Adoptive transfer studies of IL-33 activated AAMs will be required to conclusively demonstrate that these cells can prevent clinical features of *C. difficile* disease. Furthermore, the contribution of ILC2s in AAM macrophage development should also be assessed. As IL-33 activated ILC2s produce IL-4[175], it is possible that IL-4+ ILC2s are essential for AAM development during *C. difficile* colitis. We can quantify AAM abundance after adoptive transfer or depletion of ILC2s and thus assess whether ILC2s contribute to AAM development. Furthermore, we can assess the requirement of IL-4 in IL-33 mediated protection through antibody-mediated depletion of IL-4 during CDI. A significant reduction IL-33 elicited survival or weight-loss would indicate IL-4 is at least in part necessary for IL-33 mediated protection.

4.2.5 Does the reduction type-17 inflammation contribute to IL-33 mediated protection?

We have discussed in detail many possible mechanisms by which IL-33 activated ILC2s can protect through expression of type-2 cytokines, IL-4, IL-5, and IL-13. However, our studies also point to a possible protective anti-inflammatory role of IL-33 during infection. In addition to increased type-2 cytokines, IL-33 treated mice also have elevated levels of the anti-inflammatory cytokine, IL-10. Additionally, these protected mice have reduced Type-17 associated neutrophils, inflammatory monocytes, ILC3s, and cytokines within their lamina propria during infection. Type-17 immunity has been demonstrated in multiple studies to cause more severe disease during CDI. For example, IL-23 knockout mice have increased survival, reduced neutrophil influx, and reduced tissue pathology[137,212]. Furthermore adoptive transfer of TH-17 cells is sufficient to enhance mortality and TH-17 polarizing cytokines, IL-6 and IL-23 are associated with more severe

disease in human patients[157]. Given that Type-17 immune responses are associated with more severe CDI, this data bodes the question of whether IL-33 mediated protection is also acting through inhibition of type-17 immunity. Specifically, does suppression of pathogenic IL-23 signaling or TH-17 cells by IL-33 or IL-33 activated ILC2s also contribute to protection?

To test whether a reduction in type-17 immunity is downstream of IL-33, we can first characterize type-17 immune responses in highly susceptible $ST2^{-/-}$ mice. While $ST2^{-/-}$ mice have reductions in protective activated ILC2s, we currently do not know if they have amplified Type-17 associated immunity. If IL-33 signaling inhibits pathogenic type-17 immunity, we expect that $ST2^{-/-}$ mice would have amplified type-17 immune responses during infection. Thus, future studies will be required to measure IL-23, IL-6, ILC3s, TH-17 cells and neutrophils in $ST2^{-/-}$ mice infected with *C. difficile*. Increased numbers of ILC3s, TH-17 cells, neutrophils, or IL-23 levels in $ST2^{-/-}$ mice relative to wildtype controls opens up the possibility that IL-33 also protects by dampening of type-17 immunity. To further assess this point, we can block IL-23 signaling (IL-23 KO or anti-IL-23 neutralized) in $ST2^{-/-}$ mice. If amplification of type-17 immunity is contributing to the increased disease severity of $ST2^{-/-}$ mice, we expect that IL-23 deficiency will prevent the increased mortality of $ST2^{-/-}$ mice. Recent studies from our lab have demonstrated that adoptive transfer of activated TH-17 cells can increase mortality during CDI[157]. Interestingly, it was recently demonstrated that TH-17 cells in the small intestine express the ST2 receptor and in response to IL-33, these cells acquire an anti-inflammatory properties such as IL-10 expression[171]. Thus, the connection between IL-33 and TH-17 cells should be further assessed during CDI. Specifically, whether TH-17 cells express the IL-33 receptor in the colon during CDI and additionally whether IL-33 treatment inhibits the activation of CDI-activated TH-17 cells or converts them to a more immunosuppressive phenotype.

These additional characterizations of the mechanisms downstream of IL-33 and ILC2s will greatly aid in our understanding of the signals required for barrier defense and survival during *C. difficile* infection. From the work we have demonstrated in this thesis, we now know that IL-33 elicited ILC2s are sufficient to protect and increase eosinophils in the gut. Additionally, IL-33 mediated protection is in part due to eosinophil induction. However, given that depletion of eosinophils only partially inhibits IL-33 mediated survival, additional mechanisms downstream of IL-33 signaling to ILC2s must exist. Given the increase in epithelial repair and goblet cell numbers, IL-33 may additionally protect via tissue-regulatory pathways such as amphiregulin-mediated epithelial repair, IL-4Rα-dependent AAM polarization, or IL-13 mediated goblet cell regeneration. Additionally, given the reduction in type-17 immunity, IL-33 may also act by inhibiting detrimental inflammation during CDI. The studies we have outlined in this section address additional experiments that can be conducted to test each of these possibilities. It is possible that not one sole pathway is contributing to IL-33 mediated protection, but a combination of enhancing tissue-repair and eosinophils while suppressing detrimental inflammation are simultaneously required to restore homeostasis.

4.3 What is the therapeutic relevance of IL-33 signaling to ILC2s during human CDI?

Given that IL-33 activated ILC2s can transfer protection from mortality and weight-loss in our murine model of *C. difficile* colitis and additionally that IL-33 is abundantly expressed in human CDI biopsies, this opens up the question of whether ILC2s are also relevant to human CDI. Here we will summarize findings from other studies about the localization, function, and abundance of human ILC2s and propose ways to characterize these cells in human *C. difficile* infection.

4.3.1 Are human ILC2s localized in the gut and can they sense IL-33?

A recent study used mass cytometry to characterize the abundance of human ILC subsets within mucosal and non-mucosal tissue at homeostasis[394]. In contrast to our

murine studies whereby ILC2s are the dominant ILC population found within the colon, in humans, ILC2s comprise only 3.2% of the colon resident ILCs. 37% of colonic ILCs are ILC3s and 60% are NK or ILC1-like cells[394]. Thus, at steady-state, human ILC2s are a minority subset of the total tissue-resident ILCs. In contrast to this human study, our ILC quantification was conducted only after murine antibiotic treatment. Given that several studies have demonstrated that ILC3s are dependent on the microbiota[169,395,396], the skewing towards an ILC2 dominant population in our murine studies may be a consequence of antibiotic-mediated depletion of ILC3s. Thus, it is possible that in the context of antibiotics, or *C. difficile* infection, human ILC2s constitute a more dominant subset. Additionally, ILC2s may expand in the context of human CDI, similar to our murine studies whereby infection caused a 3-fold expansion of ILC2 numbers in the colon. Along these same lines, IL-33 which can activate and expand ILC2s is highly expressed in human CDI biopsies and is increased relative to CDI (-) patients. Human ILC2s, respond to IL-33 treatment and become activated by expressing the cytokines IL-4, IL-5, IL-9, and IL-13[394]. Thus, IL-33 may be a key signal for ILC2 activation and expansion during human CDI and further studies should quantify ILC2s in human CDI biopsies and also circulating levels in the periphery.

Our work opens up many questions about the importance of ILC2s in human CDI. Are ILC2s present within CDI infected gut tissue and do they expand after infection? Are these ILC2s activated? Do they colocalize with IL-33? To begin to assess these questions, we will need to use flow cytometry to quantify human ILC2s and their activation state in the periphery and gut tissue of patients with *C. difficile* infection.

4.3.2 How can you target IL-33 as a treatment for human patients?

Our findings demonstrate that IL-33 signaling is a relevant therapeutic target to treat human *C. difficile* infection. Thus, increasing IL-33 signaling in human patients may be of value in the clinic. Here we discuss three potential avenues that could be used to

bolster IL-33 signaling during human *C. difficile* colitis. These proposed strategies to bolster IL-33 include: 1) Increasing endogenous intestinal IL-33 using defined cocktails of commensal bacteria; 2) Administration of purified recombinant IL-33; and 3) Inhibition of the soluble decoy receptor, sST2. Future studies in both murine models and humans will be required to further explore these possible treatment avenues.

4.3.2.1 Bacteriotherapy

FMT therapy has emerged as a very successful treatment for recurrent *C. difficile* infection[140]. Recently, randomized controlled trials are increasingly reported and demonstrate high FMT success rates, approximately 70-90% cure rates[140,397–400]. Additionally, recent advancements have been made to improve the accessibility of FMT. Firstly, a randomized controlled study demonstrated that frozen FMT material was non-inferior to fresh FMT material[398]. Freezing of FMT material enables many advances such as pre-screening, banking, and re-testing of donor stool in addition to the possibility of delivery to other hospitals. An additional advance in making FMT therapy more accessible is the demonstration that oral FMT capsules are an effective way to administer donor material relative to other procedures such as enemas[401,402]. These studies advance our refinement of the approach and application of FMT therapy in the clinic.

The ideal, gold-standard FMT treatment would be the identification of a defined, purified, microbial cocktail that is sufficient to transfer FMT protection. Defining a purified microbial therapeutic has proven to be more challenging. The feces-derived spore preparation, SER-109, initially demonstrated success in the treatment of recurrent CDI in 30 patients[139] however the subsequent phase II clinical trial demonstrated no prevention of recurrent infection relative to the placebo[403]. Phase II results may however be confounded by overdiagnosis of *C. difficile* through the usage of highly sensitive PCR to detect cytotoxin genes rather than testing for toxin protein expression. Thus, more studies are required to test the ability of defined cocktails of stool-derived microbes in the

treatment of recurrent infection in humans. We demonstrate that oral administration of a crude murine FMT and a spore-prep from a normal human donor elevate IL-33 levels in the intestine. This FMT rescued IL-33 levels that were depleted by the antibiotic treatment necessary for *C. difficile* susceptibility. This indicates that certain spore-forming microbes can be orally administered, colonize the intestine, and subsequently increase intestinal IL-33 expression. Thus, in defining a consortium of microbes that protect during CDI, the ability to increase IL-33 should be assessed. High throughput screening of a microbe's ability to induce IL-33 may be conducted with in vitro co-culture with fibroblasts or colonic epithelial cells.

Towards the goal of refining FMT therapy, and defining a consortium of commensals that can be produced under good-manufacturing practice, multiple factors should be taken into consideration: limited antibiotic-resistance; durability in passing through the gastrointestinal tract; engraftment stability, and anti-*C. difficile* competition (e.g. secondary bile acid production). While reducing *C. difficile* growth via competition is of key importance, the work outlined in this thesis indicates that *the additional ability of microbes to enhance IL-33 should also be considered.* Thus, work to identify microbes that increase IL-33 signaling within the intestine will add an important layer of host-protection into next-generation bacteriotherapy in the face of *C. difficile* colitis.

4.3.2.2 IL-33 treatment

Another possible avenue of treatment is the use of purified recombinant IL-33 in patients with severe CDI. In our studies we use IL-33 prophylactically which may be relevant for high risk patients such as immunocompromised individuals on antibiotics or patients with low peripheral eosinophil counts at hospital admission[43,136]. The use of IL-33 after the onset of disease requires further investigation. Specifically, we need to conduct additional experiments that assess the effectiveness of IL-33 treatment at several doses and several timepoints *after* infection and after the onset of disease. As time-

dependent differences in IL-33 mediated protection during colitis models (e.g. during recovery vs. disease onset) have been proposed[404], it is important to determine if IL-33 is also therapeutic when administered post *C. difficile* infection.

Recombinant cytokines have been used as treatments for other diseases. For example, Avonex and Rebif are recombinant forms of IFNβ and are initial treatments for recurrent multiple sclerosis (MS), a neurological disease driven by overly robust inflammation and neurodegeneration. IFNβ treatment is well-tolerated and can be self-administered by subcutaneous injections every other day that decrease the severity and frequency of MS attacks[405,406]. Thus, precedent has been set for the therapeutic use of recombinant cytokine to treat human patients and further investigation is required into the use of recombinant IL-33 for *C. difficile* colitis.

Interestingly, a fusion cytokine of IL-33 to the lymphocyte growth factor, IL-2, has been generated that ameliorates renal injury and termed IL233[407]. Treatment with this novel fusion cytokine, containing the activity of IL-33 and IL-2 in one molecule, has superior protection from renal ischemia-reperfusion injury (IRI) than using IL-2 and IL-33 as a mixture. Furthermore, IL233 was superior at increasing circulating and kidney-resident ILC2s and T-regs and similar to our *C. difficile* model of colitis, adoptive transfer of IL233 activated ILC2s protected from IRI[407]. This data indicates that an IL-2/IL-33 fusion protein may have superior therapeutic action during *C. difficile* colitis than IL-33 alone. Thus, future studies testing the dosage, timing, and route of exogenous IL-33 as a therapeutic for CDI should also include treatment with the IL233 fusion protein.

4.3.2.3 Inhibitors of sST2

Within our human studies, we found that the soluble decoy receptor, sST2 is highly abundant within the serum of human patients with CDI. Patients with high white blood cells counts have higher levels of circulating soluble ST2 in their serum, indicating that sST2 expression may be a consequence of inflammation during infection. sST2 predicted

C. difficile severity and mortality within our cohort after correcting for age, race, gender, comorbidities, and CDT status. Furthermore, treatment with sST2 exacerbates weight-loss and clinical severity in our murine model. This data indicates that blocking of sST2 may be a novel therapeutic strategy in the treatment of CDI. However, how sST2 expression is regulated and which cells are the dominant source of sST2 during CDI requires further investigation.

4.3.2.3.1 How is sST2 expression regulated?

The ST2 subunit of the IL-33 signaling receptor is encoded by the IL1RL1 gene. Two major transcriptional variants of IL1RL1 have been reported: 1) A full length form of ST2 and 2) A soluble form lacking the transmembrane domain[232,242,404,408]. Full-length ST2 (STL) is membrane bound form and requires association the accessory subunit, IL-1RAcP to induce MyD88-dependent signaling in cells[409]. In contrast, in soluble form, sST2 binds to free IL-33, acting as a natural decoy receptor and inhibiting IL-33's biological activity. Sequestration of IL-33 by sST2 attenuates type-2 immune responses leading to less severe disease during airway inflammation however in our model of *C. difficile* colitis, this attenuation of type-2 immunity is pathogenic[410].

sST2 expression can be enhanced by pro-inflammatory cytokines such as IL-1β and TNF-α *in vitro* by human lung epithelial cells and cardiac myocytes[411,412]. Interestingly, regulation of sST2 expression is also genetically mediated. High circulating sST2 is a marker of worse prognosis in patients with heart failure[413]. A genome-wide association study of participants in the Framingham cardiovascular cohort, demonstrated that SNPs within *IL1RL1* were associated with high systemic sST2 levels[414]. Additionally, this study demonstrated that approximately 40% of the variability in sST2 concentrations between individuals was attributed to genetic factors. Interestingly, 5 of the *IL1RL1* missense variants associated with elevated sST2 were located within the TIR signaling domain membrane-bound ST2. Upon *in vitro* expression, these missense variants increased

soluble sST2 expression by enhancing IL-33/ST2L signaling via MyD88 and NFkB[414]. This data indicates that IL-33 signaling through the membrane bound ST2 receptor enhances the transcription of its own natural decoy receptor, likely a negative feedback loop to limit overly robust IL-33 signaling. Furthermore, these studies demonstrate that sST2 expression is regulated by both pro-inflammatory signals and also genetic factors. Given our findings that high sST2 is associated with increased mortality during CDI, future studies are required to assess if genetic variants within the *IL1RL1* locus are associated with increased risk for *C. difficile* colitis.

Soluble sST2 can be secreted by many different cell types depending on the cell stimuli including barrier cell types, such as epithelial and fibroblast cells and also immune cell types, such as Th-1 $CD4^+$ and $CD8^+$ T cells and mast cells[415–418]. Interestingly, during a murine model of graft versus host disease (GVHD), intestinal stromal cells and TH-17 cells were major sources of sST2[415]. The cellular source of sST2 during *C. difficile* infection requires further investigation. However, it is possible that intestinal epithelial cells and mononuclear cells of the lamina propria which are major sources of sST2 in colitis models, can release sST2 upon *C. difficile* toxin-induced cell death[172,235]. Very interestingly, our lab has recently identified a pathogenic role for TH-17 cells in enhancing mortality during *C. difficile* colitis. Whether TH-17 cells are a major source of sST2 during *C. difficile* colitis and whether they exacerbate disease through sST2 production is an exciting topic of future studies.

4.3.2.3.2 How can sST2 be targeted therapeutically during CDI?

Blocking sST2 is a possible therapeutic strategy to ameliorate disease during *C. difficile* colitis. As IL-33 is sequestered by the soluble form of ST2, releasing IL-33 from sST2 may augment IL-33 signaling through membrane bound ST2 and subsequently enhance the activation of ILC2s to treat *C. difficile* disease. Like *C. difficile*, IL-33 is also protective during GVHD and similar to our studies, high sST2 is a risk factor for

GVHD[415,419–421]. Recent advancements have been made in the treatment of GVHD with IL-33 in murine models and a similar strategy of neutralizing sST2 has been proposed. Blockade of sST2 with a monoclonal antibody led to reductions sST2 producing cells while maintaining protective membrane bound signaling[415]. sST2 blockade caused increased levels of free IL-33 in the serum, reduced TH-17 associated inflammation, and increased survival during a murine model of GVHD. Additionally, small molecular inhibitors of sST2 have been discovered and are also effective at promoting IL-33 signaling. Treatment with inhibitors caused reductions in plasma levels of sST2, improved survival, reductions in TH-17 cells, and maintenance of protective TH-1 and T-reg immunity during GVHD murine models[415,419]. An additional study also discovered small molecule inhibitors of sST2 that can decrease plasma levels of sST2 and enhance T-regs to protect from mortality during GVHD[421].

These studies propose that small molecule inhibition of soluble ST2 is effective by preventing decoy receptor binding to IL-33 or releasing IL-33 from sST2. Investigations studying the efficacy of these small molecular inhibitors of sST2 during *C. difficile* colitis will be required with additional evaluation of protective ILC2s and eosinophils and pathogenic TH-17 cells after treatment. As small molecule inhibitors of sST2 are also capable of inhibiting membrane bound ST2 signaling, there is a potential for these compounds to also be pathogenic during CDI. Drug delivery methods that target highly abundant sST2 in the serum, while limiting mucosal tissue delivery, may be useful to promote systemic IL-33 release from sST2 while preventing small-molecule inhibition of protective ST2 signaling in the intestine. In summary, inhibition of highly abundant and pathogenic sST2 may be a novel therapeutic avenue for reducing the severity of *C. difficile* colitis. Thus, additional studies should assess the efficacy of sST2 inhibitors during animal models of *C. difficile* colitis with the goal of releasing IL-33 from sST2 while preserving protective IL-33 signaling on ST2+ ILC2s.

4.4 *Closing Statements*

Clostridium difficile is the leading cause of hospital acquired antibiotic-associated diarrhea with worldwide distribution. The increased prevalence and severity of circulating hypervirulent strains of *C. difficile* poses a significant health threat to health-care facilities. In addition to the two primary virulence factors, toxins TcdA and TcdB, hypervirulent epidemic strains of *C. difficile*, referred to as NAP1/027, express an additional toxin, binary toxin (CDT). NAP1/027 strains are associated with mortality rates three times higher than those of less virulent ribotypes[19]. While the genetics of the infecting strain dictates *C. difficile* clinical severity, the type of intestinal immune response generated also contributes to disease pathogenesis[134,225].

We conducted a transcriptome analysis of host genes altered by NAP1/027 infection and identified interleukin-33 (IL-33) as a gene upregulated in response to CDT expressing, hypervirulent infection. In chapter 2, using a murine model, we show that both endogenous IL-33 and exogenous IL-33 treatment protect from the mortality, weight-loss and tissue pathology during infection with both hypervirulent and classical strains of *C. difficile*. IL-33 mediated protection required type-2 innate lymphoid cells (ILC2s) and adoptive transfer of purified ILC2s was sufficient to mitigate CDI- associated mortality and weight-loss and increase eosinophilia. In chapter 3 we demonstrate the clinical relevance of IL-33 signaling in human *C. difficile* colitis. Specifically, dysregulated IL-33 signaling via the soluble IL-33 decoy receptor (sST2) predicted disease severity and mortality in human patients. The protective relevance of IL-33 was broad, predicting disease severity in patients infected with epidemic, CDT expressing strains or non-CDT expressing strains. Lastly, we show that colonic IL-33 expression is regulated by the microbiota as antibiotic-associated depletion of IL-33 was rescued with mouse fecal microbiota transplant (FMT) or a human fecal spore preparation (HSP). Thus, IL-33 signaling is a novel therapeutic

pathway for severe CDI which can potentially be targeted with rationally designed microbial therapies.

In summary, within this thesis we have identified a novel mechanism of intestinal barrier defense during *C. difficile* colitis through the activation of ILC2s by IL-33. This pathway is important during human *C. difficile* infection and can possibly be therapeutically upregulated to treat *C. difficile* with bacteriotherapy, inhibitors of sST2, and/or recombinant IL-33 treatment. Further studies investigating the mechanisms by which ILC2s protect, their relevance to human CDI and the microbial signals regulating intestinal IL-33 are required. This work illustrates a novel pathway of IL-33 activation of ILC2s that protects the intestinal barrier during CDI. Furthermore, this work expands our knowledge regarding how microbial communities strengthen host immunity and may provide protection against *C. difficile* via regulation of IL-33.

Chapter 5: Material and Methods

5.1 Mice and *Clostridium difficile* infection

Experiments were carried out using sex matched 8–12 week old C57BL6, ST2$^{-/-}$, Rag2$^{-/-}$, and Rag2$^{-/-}$γc$^{-/-}$ mice. C57BL6 were purchased from Jackson Laboratory and ST2$^{-/-}$ mice[422] were obtained from Dr. Andrew McKenzie (Laboratory of Molecular Biology, Cambridge University, Cambridge, United Kingdom). Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice were purchased from Taconic Biosciences with an excluded flora. All animals were housed under specific pathogen free conditions at the University of Virginia's animal facility and procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia (IACUC). Bedding exchange every two days between ST2$^{-/-}$ and C57BL6 mice, or Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice, for a minimum of three weeks was conducted to equilibrate microbiota between strains. Mice were infected using a previously published murine model for CDI[6,133,137]. 3 days prior to infection, mice were given an antibiotic cocktail in drinking water consisting of 45 mg/L Vancomycin (Mylan), 35 mg/L Colistin (Sigma), 35 mg/L Gentamicin (Sigma), 215 mg/L Metronidazole (Hospira). Mice were then switched to regular drinking water and given a single IP injection (0.016 mg/g) of Clindamycin (Hospira) on Day −1. On Day 0, mice were orally gavaged with 1x10^8 *CFU/ml of C. difficile.* Mice were monitored twice daily over the course of infection and evaluated according to clinical scoring parameters[423]. Scores were based on weight loss, coat condition, activity level, diarrhea, posture and eye condition for a cumulative clinical score between 1 and 20. Weight loss and activity levels were scored between 0-4 with 4 being greater than or equal to 25% loss in weight. Coat condition, diarrhea, posture and eye condition were scored between 0-3. Diarrhea scores were: 1 for soft or yellow stool, 2 for wet tail, and 3 for liquid or no stool. Mice were euthanized if severe illness developed based on a clinical score ≥14.

5.2 Bacterial Strains and Culture

Isogenic *C. difficile* strain R20291_*CdtB* was generated using the ClosTron system of insertional mutagenesis and inactivation CDTb was confirmed by Western blot by us and others[6,283]. To prepare the *C. difficile*, strains were plated onto BHI agar from frozen stocks and incubated at 37°C overnight in an anaerobic work station (Shel Labs). A single colony was inoculated into BHI medium and grown anaerobically overnight at 37°C. The next day, cultures were spun for 1 minute at 6,000 × *g* and washed twice in anaerobic PBS and the optical density was measured. For the strain VPI10643 (ATCC 43244), 100µl of overnight culture was subcultured for 5 hrs prior to optical density measurement. The culture density was adjusted in anaerobic PBS to $1x10^8$ CFU/mL (R20291 strains) and $1 x 10^5$ CFU/ml (VPI strain) and loaded into syringes. Each mouse received 100 µl ($1x10^7$ CFU for R20291 and $1x10^4$ CFU for VPI) of inoculum by oral gavage. *C. difficile* burden was quantified from caecal contents at Day 2 of infection. Briefly, caecal contents were resuspended by weight in anaerobic PBS. Serial dilutions of caecal contents were plated on BHI Agar supplemented with 1% Sodium Taurocholate, 1 mg/mL D-cycloserine and 0.032 mg/mL cefoxitin (Sigma) and anaerobically incubated at 37°C overnight followed by colony counts in triplicate. Toxins TcdA/B and CDT were quantified using the ELISA *C. difficile* TOX A/B II kit from Techlab and CDT ELISA (CDTb subunit detected) generously gifted from TechLab according to the manufacturer's instructions and normalized to stool weight.

5.3 FITC Dextran Gut Permeability Assay:

Mice were gavaged with 44mg/100g body weight of Fluorescein isothiocyanate (FITC)– dextran solution (Sigma). 4 hours after gavage, mice were sacrificed, and serum was collected. FITC dextran within the serum was detected on a spectrophotometer at 485/530 nm.

5.4 Transcriptome Microarray

Mice were infected with R20291 (CDT+) or R20291_*cdtb (CDT-)* and whole-caecal tissue transcriptomic analysis was performed on Day 3 post infection. R20291 and R20291_*Cdtb* RNA samples were processed by the Affymetrix Gene Chip® WT PLUS Reagent Kit and hybridized to the Affymetrix Mouse Gene 2.0 ST GeneChip®. The Affymetrix Mouse Transcriptome .CEL files were analyzed by the UVA Bioinformatics core. All preprocessing and analysis was done using R. Expression intensities were summarized, normalized, and transformed using Robust Multiarray Average algorithm[424]. Probesets not mapping to an Entrez gene were excluded. For examining differential gene expression, a linear model was fit with empirical-Bayes moderated standard errors using the limma package in R. The microarray analysis datasets are included in Table S1 comparing R20291 vs. R20291_*Cdtb*. Enriched pathways of the top upregulated transcripts (log FC > 0.5; $p<0.05$) were created using the ConsensusPathDP database[289]. Enriched pathways of the top upregulated transcripts (log FC > 0.5) were also created using the Ingenuity Database[288].

5.5 Tissue protein and transcript analysis

IL-33, IL-4, IL-10, IL-1β, IL-6, and IL-23 were detected in caecal tissue lysates using the Mouse Duoset Sandwich ELISA kits (R & D) according to manufacturer's instructions. Total caecal lysate was generated by removing the ceca and rinsing gently with 1x PBS. Tissue was bead beaten for 1 minute and resuspended in 400 µl of Lysis Buffer I: 1× HALT Protease Inhibitor (Pierce), 5 mM HEPES. Following mechanical tissue disruption, 400 µl of Lysis Buffer II was added: 1× HALT Protease Inhibitor (Pierce), 5 mM HEPES, 2% Triton X-100. Tissue samples were incubated on ice for 30 minutes after gently mixing. Lysed samples were pelleted to remove tissue debris in a 5 minute spin at 13,000 × *g* at 4°C. Supernatant was collected and total protein concentration was measured by BCA assay according to manufacturer's instructions (Pierce). Cytokine concentration was normalized to total protein concentration. For IL-33 mRNA transcript

analysis, caecal tissue was flushed with sterile PBS and immediately stored in RNA-later at -80º C. Tissue was later processed using the RNeasy mini kit (Qiagen) + DNAse digestion. RNA was reverse transcribed with Tetro cDNA synthesis kit (Bioline) and IL-33 was amplified using the commercially available Taqman IL-33 primer/probe set (Applied Biosciences: Mm00505403_m1). Gene expression was normalized to HPRT and GAPDH housekeeping genes.

5.6 Flow Cytometry

Colons were dissected longitudinally and rinsed in HBSS supplemented with 25mM HEPES and 5% FBS. Epithelial cells were separated from the lamina propria via a 40 min incubation with gentle agitation in dissociation buffer (HBSS with 15 mM HEPES, 5 mM EDTA, 10% FBS and 1 mM DTT) at 37°C. Next, the lamina propria tissue was manually diced using scissors and further digested in RPMI 1640 containing 0.17 mg/mL Liberase TL (Roche) and 30 µg/mL DNase (Sigma). Samples were digested for 40 minutes at 37°C with gentle shaking. Single cell suspensions were generated by passaging samples through a 100 µM cell strainer followed by a 40 uM cell strainer (both Fisher Scientific). For intracellular cytokine staining, 1×10^6 cells/ sample were incubated in complete media with Golgi block (RPMI Meida 1640, 629 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5mM 2-β-630 mercaptoethanol, BD GolgiPlug containing Brefeldin A) for 4 hours prior to surface staining procedure. For surface staining, 1×10^6 cells/ sample were Fc-blocked with TruStain fcX (anti-mouse CD16/32 antibody, BioLegend) for ten minutes at room temperature followed by LIVE/DEAD Fixable Aqua (Life Technologies) for 30 minutes at 4ºC. Cells were washed twice in FACS buffer (PBS+ 2% FBS) and stained with fluorochrome conjugated antibodies for 30 minutes at 4ºC. Cells were washed and resuspended in Foxp3 Fix/Perm Working Solution (Ebiosciences) and incubated overnight at 4ºC. Cells were washed twice with Permeabilization buffer and stained for GATA3, RORγT, T-bet, and/or IL-13 and IFN-γ for 45 minutes at room

temperature. Cells were washed twice and resuspended in FACs buffer. Flow cytometry was performed on an LSR Fortessa cytometer (BD Biosciences) and all data analysis performed via FlowJo (Tree Star Inc.).

5.7 IL-33 Treatment and ILC2 Adoptive Transfer Studies

For IL-33 treatment, mice were intraperitoneally injected for 5 days prior to infection with 7.5µg/ml (100µl dose) of carrier-free, recombinant mouse IL-33 (Biolegend; Catalog #: 580504). For sST2 studies, mice were injected with 5µg/mouse of recombinant ST2-FC fusion or FC control (R&D systems, Catalog # 1004-MR-050). For ILC2 adoptive transfer studies, the colons, mesenteric lymph nodes, and spleens of CD90.1+ IL-33 treated mice were harvested into single cell suspension as described above. Bulk ILCs were isolated by magnetic bead purification of lineage + cells (Miltenyi Lineage Cell Depletion Kit: 130-110-470). Cells were surface stained and sorted on the Influx Cell Sorter (BD Biosciences) based on Lin- ST2+ CD25+ CD127+ CD45+ CD90.1+ expression. Sorted cells were immediately injected intraperitoneally into recipient CD90.2+ mice. For ILC2 expansion in vitro, ILCs purified from MLN and colon of IL-33 treated mice were cultured in complete medium (RPMI - 1640 + 10% FBS +2 mM glutamine, 100 U/ml penicillin, streptomycin 100 µg/ml) in the presence of IL-33 (50 ng/ml), IL-2 and IL-7 (10ng/ml) for 4 days and sort purified by Lin- CD25+ CD127+ CD45+ CD90+ ST2+expression.

5.8 FMT Treatment

Fecal pellets 10 age and sex matched C57BL6 mice were collected and immediately homogenized by vortexing in 1.5mls of sterile anaerobic PBS[425]. 100 µl of the supernatant was orally gavaged 24 hours and 48 hours post clindamycin injection. Human Spore Prep (HSP) is a research-grade product produced and provided by Seres Therapeutics and is comprised of Firmicutes spores fractionated from a stool specimen obtained from a healthy donor[139]. Spore content was determined by measuring spore colony forming units

per mL (sCFU/mL). 200µl of the human spore prep or vehicle control was administered 24 hours post clindamycin IP into mice by oral gastric gavage.

5.9 DNA extraction and 16S rRNA gene sequencing of murine FMT

Generation of 16S rRNA gene amplicons from mouse cecal content was performed as previously described[425]. Briefly, DNA from cecal content was extracted using the Qiagen MagAttract PowerMicrobiome kit with the Eppedorf EpMotion liquid handling system. The V4 region of the 16S rRNA gene was amplified from each sample using a dual indexing sequencing strategy[426]. Samples were sequenced on the Illumina MiSeq platform using the MiSeq Reagent Kit V2 500 cycles (Illumina cat# MS102-2003) according to manufacture instructions with modifications found in the Schloss Wet Lab SOP (https://github.com/SchlossLab/MiSeq_WetLab_SOP).

5.10 16S Sequence curation and analysis

Raw sequence files have been deposited in the Sequence Read Archive database under the project PRJNA521980. All analysis was performed using R version 3.5.1. Sequences were analyzed using the R package DADA2 version 1.10.1, following the DADA2 pipeline v1.8 tutorial[427]. Taxonomy was assigned to amplicon sequence variants (ASVs) using the SLIVA rRNA database project, release 128[428]. The package phyloseq v1.26.1 was used to calculate both alpha and beta diversity as well as generate the NMDS plots[429]. The package vegan was used to calculate the permutational multivariate analysis of variance (PERMANOVA)[430].

5.11 Human Spore Prep Sequencing and Characterization

Human Spore Prep was characterized by using 16S rRNA sequencing, sequenced to a depth of 22 500 operational taxonomic units (OTUs), at which point the rarefaction curves begin to plateau. The following families were detected by 16S sequencing: Clostridiaceae, Erysipelotrichaeceae, Eubacteriaceae, Lachnospiraceae, and Ruminococcaceae.

5.12 Mouse and Human Histology and Immunohistochemistry

Mouse caecal tissue sections were fixed in Bouin's solution and transferred to 70% ethanol after 24 hours. Sections were embedded in paraffin and stained with either haematoxylin and eosin (H & E) or Periodic Acid Schiff (PAS) by the University of Virginia Research Histology Core. Scoring was conducted by two blinded observers. H&E tissue pathology scoring was conducted using a scale from 0-3 for multiple parameters: epithelial disruption, submucosal edema, inflammatory infiltrate, mucosal thickening and luminal exudates[431]. PAS+ goblet cells were counted and normalized to the number of crypts. For human anti-IL-33 staining, CDI positivity was assessed based on the presence of *C. difficile* toxins TcdA/B in patient stool by ELISA. The University of Virginia Biorepository Core stained the human colon biopsy sections using a primary antibody directed against IL-33 (R & D). For human colon biopsies 5 representative images were taken blindly of each tissue biopsy and IL-33 positive cells were counted using the Image-J software particle analysis function.

5.13 Detection of Human sST2 in Serum

Serum samples were collected at the time of *C. difficile* diagnosis from 167 patients at the University of Virginia. Serum samples were stored at -80ºC until the time of use. Samples were thawed on ice and sST2 was quantified using R & D System Human ST2(IL-33R) Quantikine ELISA (Cat # DST200). The study was approved by the University of Virginia Institutional Review Board for Health Sciences Research.

5.14 Statistical Analysis

For murine work, survival curves were estimated using Kaplan-Meier method and the survival difference between groups were tested for statistical significance using Log-rank test. Comparisons between two groups were conducted using a 2-tailed T test or a Mann-Whitney test when data were not normally distributed. For CDI human patient, comparisons between two groups were conducted using the two-sample t-test or Mann-

Whitney test as appropriate for continuous patient characteristics and using Chi-square test for categorical variables. 7 observations with invalid time, censoring, or strata values were excluded from survival and cox-regression. Based on the distribution of sST2, patients were classified into low and high 50 percentiles of sST2 or tertiles of sST2. Survival differences between sST2 groups were assessed using Log-rank test, and further evaluated in Cox regression adjusting for age, gender, race, Charlson comorbidity index. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) and SAS 9.4 (SAS Institute, Cary, NC).

Chapter 6: References

1. CDC. Biggest Threats | Antibiotic/Antimicrobial Resistance | CDC. *CDC* (2016). Available at: http://www.cdc.gov/drugresistance/biggest_threats.html.

2. O'Connor, J. R., Johnson, S. & Gerding, D. N. Clostridium difficile Infection Caused by the Epidemic BI/NAP1/027 Strain. *Gastroenterology* (2009). doi:10.1053/j.gastro.2009.02.073

3. Heinlen, L. & Ballard, J. D. Clostridium difficile infection. *Am. J. Med. Sci.* **340**, 247–252 (2010).

4. Freeman, J. *et al.* The changing epidemiology of Clostridium difficile infections. *Clin. Microbiol. Rev.* **23**, 529–549 (2010).

5. Hookman, P. & Barkin, J. S. Clostridium difficile associated infection, diarrhea and colitis. *World Journal of Gastroenterology* **15**, 1554–1580 (2009).

6. Cowardin, C. A. *et al.* The binary toxin CDT enhances Clostridium difficile virulence by suppressing protective colonic eosinophilia. *Nat. Microbiol.* (2016). doi:10.1038/nmicrobiol.2016.108

7. Aslam, S., Hamill, R. J. & Musher, D. M. Treatment of Clostridium difficile-associated disease: old therapies and new strategies. *Lancet Infect. Dis.* **5**, 549–57 (2005).

8. Bartlett, J. G. Annals of Internal Medicine Review Narrative Review : The New Epidemic of Clostridium difficile –. *An. Intern. Med.* 758–764 (2006).

9. Rupnik, M., Wilcox, M. H. & Gerding, D. N. Clostridium difficile infection: new developments in epidemiology and pathogenesis. *Nat. Rev. Microbiol.* **7**, 526–36 (2009).

10. Kelly, C. P. & LaMont, J. T. Clostridium difficile--more difficult than ever. *N. Engl. J. Med.* **359**, 1932–1940 (2008).

11. Lessa, F. C. *et al.* Burden of *Clostridium difficile* Infection in the United States. *N. Engl. J. Med.* (2015). doi:10.1056/NEJMoa1408913

12. The White House Administration. *National Action Plan for Combating Antibiotic-Resistant Bacteria*. *Open Government National Action Plans* (2015).

13. Infectious National Institute of Allergy and Diseases. NIAID ' s Antibacterial Resistance Program : Current Status and Future Directions. (2014).

14. National Institute of Allergy and Infectious Diseases. NIAID Emerging Infectious Diseases/Pathogens. *National Institute of Health* 1–5 (2015). Available at: https://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx.

15. McDonald, L. C. *et al.* An Epidemic, Toxin Gene–Variant Strain of *Clostridium difficile*. *N. Engl. J. Med.* (2005). doi:10.1056/NEJMoa051590

16. Hubert, B. *et al.* A Portrait of the Geographic Dissemination of the Clostridium difficile North American Pulsed-Field Type 1 Strain and the Epidemiology of C. difficile-Associated Disease in Quebec. *Clin. Infect. Dis.* (2007). doi:10.1086/510391

17. Kuijper, E. J. *et al.* Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. *Euro Surveill.* (2008). doi:18942 [pii]

18. Warny, M. *et al.* Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. *Lancet* (2005). doi:10.1016/S0140-6736(05)67420-X

19. Loo, V. G. *et al.* A Predominantly Clonal Multi-Institutional Outbreak of *Clostridium difficile* –Associated Diarrhea with High Morbidity and Mortality. *N. Engl. J. Med.* (2005). doi:10.1056/NEJMoa051639

20. Leffler, D. A. & Lamont, J. T. *Clostridium difficile* Infection. *N. Engl. J. Med.* (2015). doi:10.1056/NEJMra1403772

21. Redelings, M. D., Sorvillo, F. & Mascola, L. Increase in Clostridium difficile-related mortality rates, United States, 1999-2004. *Emerg. Infect. Dis.* (2007). doi:10.3201/eid1309.061116

22. Elixhauser, A. & Jhung, M. Clostridium difficile-*Associated Disease in U.S. Hospitals, 1993-2005: HCUP Statistical Brief #50*. *Agency for Healthcare Research and Quality* (2008). doi:10.3390/s16040439

23. Khanna, S. *et al.* The epidemiology of community-acquired clostridium difficile infection: A population-based study. *Am. J. Gastroenterol.* (2012). doi:10.1038/ajg.2011.398

24. Lewis, S. S., Moehring, R. W., Anderson, D. J., Sexton, D. & Baker, A. W. Increasing Incidence of Community-Acquired Clostridium difficile Infections Among Hospitalized Patients. *Open Forum Infect. Dis.* **3**, (2016).

25. Pépin, J. *et al.* Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: A changing pattern of disease severity. *CMAJ* (2004). doi:10.1503/cmaj.1041104

26. Hyland, M. *et al.* N-CDAD in Canada: Results of the Canadian nosocomial infection surveillance program 1997 N-CDAD prevalence surveillance project. *Can. J. Infect. Dis.* (2001). doi:10.1155/2001/304098

27. Katikireddi, V. UK launches inquiry into Clostridium difficile outbreak. *CMAJ* (2005). doi:10.1503/cmaj.050771

28. Goorhuis, A. *et al.* Emergence of *Clostridium difficile* Infection Due to a New Hypervirulent Strain, Polymerase Chain Reaction Ribotype 078. *Clin. Infect. Dis.* (2008). doi:10.1086/592257

29. Pépin, J., Valiquette, L. & Cossette, B. Mortality attributable to nosocomial Clostridium difficile-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* (2005). doi:10.1503/cmaj.050978

30. Abt, M. C., McKenney, P. T. & Pamer, E. G. Clostridium difficile colitis: pathogenesis and host defence. *Nat Rev Microbiol* (2016). doi:10.1038/nrmicro.2016.108

31. Theriot, C. M. & Young, V. B. Interactions Between the Gastrointestinal Microbiome and *Clostridium difficile*. *Annu. Rev. Microbiol.* (2015). doi:10.1146/annurev-micro-091014-104115

32. Shields, K., Araujo-Castillo, R. V., Theethira, T. G., Alonso, C. D. & Kelly, C. P. Recurrent clostridium difficile infection: From colonization to cure. *Anaerobe* (2015). doi:10.1016/j.anaerobe.2015.04.012

33. Rupnik, M., Wilcox, M. H. & Gerding, D. N. Clostridium difficile infection: New developments in epidemiology and pathogenesis. *Nature Reviews Microbiology* (2009). doi:10.1038/nrmicro2164

34. Buonomo, E. L. & Petri, W. A. The microbiota and immune response during Clostridium difficile infection. *Anaerobe* (2016). doi:10.1016/j.anaerobe.2016.05.009

35. Cowardin, C. A. & Petri, W. A. Host recognition of Clostridium difficile and the innate immune response. *Anaerobe* (2014). doi:10.1016/j.anaerobe.2014.08.014

36. Surawicz, C. M. *et al.* Guidelines for diagnosis, treatment, and prevention of clostridium difficile infections. *American Journal of Gastroenterology* (2013). doi:10.1038/ajg.2013.4

37. McDonald, L. C. *et al.* Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clinical Infectious Diseases* (2018). doi:10.1093/cid/ciy149

38. Eze, P., Balsells, E., Kyaw, M. H. & Nair, H. Risk factors for Clostridium difficile infections – an overview of the evidence base and challenges in data synthesis. *J. Glob. Health* (2017). doi:10.7189/jogh.07.010417

39. Deshpande, A. *et al.* Risk Factors for Recurrent Clostridium difficile Infection: A Systematic Review and Meta-Analysis. *Infect. Control Hosp. Epidemiol.* (2015). doi:10.1017/ice.2014.88

40. Vardakas, K. Z., Konstantelias, A. A., Loizidis, G., Rafailidis, P. I. & Falagas, M. E. Risk factors for development of Clostridium difficile infection due to BI/NAP1/027 strain: A meta-analysis. *International Journal of Infectious Diseases* (2012). doi:10.1016/j.ijid.2012.07.010

41. Revolinski, S. L. & Munoz-Price, L. S. Clostridium difficile in Immunocompromised Hosts: A Review of Epidemiology, Risk Factors, Treatment, and Prevention. *Clin. Infect. Dis.* (2018). doi:10.1093/cid/ciy845

42. Banaei, N., Anikst, V. & Schroeder, L. F. Burden of Clostridium difficile infection in the United States. *N. Engl. J. Med.* (2015). doi:10.1056/NEJMc1505190

43. Johnson, S. Recurrent Clostridium difficile infection: A review of risk factors, treatments, and outcomes. *Journal of Infection* **58**, 403–410 (2009).

44. Bignardi, G. E. Risk factors for Clostridium difficile infection. *J. Hosp. Infect.* **40**, 1–15 (1998).

45. Gil, F., Lagos-Moraga, S., Calderón-Romero, P., Pizarro-Guajardo, M. & Paredes-Sabja, D. Updates on Clostridium difficile spore biology. *Anaerobe* (2017). doi:10.1016/j.anaerobe.2017.02.018

46. Gupta, A. & Khanna, S. Community-acquired clostridium difficile infection: An increasing public health threat. *Infection and Drug Resistance* (2014). doi:10.2147/IDR.S46780

47. Chitnis, A. S. *et al.* Epidemiology of community-associated Clostridium difficile infection, 2009 through 2011. *JAMA Intern. Med.* (2013). doi:10.1001/jamainternmed.2013.7056

48. Ofori, E. *et al.* Community-acquired Clostridium difficile: epidemiology, ribotype, risk factors, hospital and intensive care unit outcomes, and current and emerging therapies. *Journal of Hospital Infection* (2018). doi:10.1016/j.jhin.2018.01.015

49. Centers for Disease Control and Prevention (CDC). Severe Clostridium difficile-associated disease in populations previously at low risk--four states, 2005. *MMWR. Morb. Mortal. Wkly. Rep.* (2005).

50. Debast, S. B. *et al.* Clostridium difficile PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. *Environ. Microbiol.* (2009). doi:10.1111/j.1462-2920.2008.01790.x

51. Rupnik, M., Widmer, A., Zimmermann, O., Eckert, C. & Barbut, F. Clostridium difficile toxinotype V, ribotype 078, in animals and humans. *Journal of Clinical Microbiology* (2008). doi:10.1128/JCM.00598-08

52. Weese, J. S. Clostridium difficile in food - innocent bystander or serious threat? *Clinical Microbiology and Infection* (2010). doi:10.1111/j.1469-0691.2009.03108.x

53. Koenigsknecht, M. J. *et al.* Dynamics and establishment of Clostridium difficile infection in the murine gastrointestinal tract. *Infect. Immun.* (2015). doi:10.1128/IAI.02768-14

54. Francis, M. B., Allen, C. A., Shrestha, R. & Sorg, J. A. Bile Acid Recognition by the Clostridium difficile Germinant Receptor, CspC, Is Important for Establishing Infection. *PLoS Pathog.* (2013). doi:10.1371/journal.ppat.1003356

55. Burns, D. A., Heap, J. T. & Minton, N. P. SleC is essential for germination of Clostridium difficile spores in nutrient-rich medium supplemented with the bile salt taurocholate. *J. Bacteriol.* (2010). doi:10.1128/JB.01209-09

56. Wilson, K. H. Efficiency of various bile salt preparations for stimulation of Clostridium difficile spore germination. *J. Clin. Microbiol.* (1983).

57. Wilson, K. H., Kennedy, M. J. & Fekety, F. R. Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile. *J. Clin. Microbiol.* (1982).

58. Sorg, J. A. & Sonenshein, A. L. Bile salts and glycine as cogerminants for Clostridium difficile spores. *J. Bacteriol.* (2008). doi:10.1128/JB.01765-07

59. Bouillaut, L., Dubois, T., Sonenshein, A. L. & Dupuy, B. Integration of metabolism and virulence in Clostridium difficile. *Res. Microbiol.* (2015). doi:10.1016/j.resmic.2014.10.002

60. Karlsson, S., Lindberg, A., Norin, E., Burman, L. G. & Akerlund, T. Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in Clostridium difficile. *Infect. Immun.* (2000). doi:10.1128/IAI.68.10.5881-5888.2000

61. Karlsson, S., Burman, L. G. & Åkerlund, T. Suppression of toxin production in Clostridium difficile VPI 10463 by amino acids. *Microbiology* (1999). doi:10.1099/13500872-145-7-1683

62. Yamakawa, K., Karasawa, T., Ikoma, S. & Nakamura, S. Enhancement of Clostridium difficile toxin production in biotin-limited conditions. *J. Med. Microbiol.* (1996). doi:10.1099/00222615-44-2-111

63. Dupuy, B. & Sonenshein, A. L. Regulated transcription of Clostridium difficile toxin genes. *Mol. Microbiol.* (1998). doi:10.1046/j.1365-2958.1998.00663.x

64. Antunes, A., Martin-Verstraete, I. & Dupuy, B. CcpA-mediated repression of Clostridium difficile toxin gene expression. *Mol. Microbiol.* (2011). doi:10.1111/j.1365-2958.2010.07495.x

65. Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M. & Von Eichel-Streiber, C. Definition of the single integration site of the pathogenicity locus in Clostridium difficile. *Gene* (1996). doi:10.1016/S0378-1119(96)00398-8

66. Rupnik, M. *et al.* Revised nomenclature of Clostridium difficile toxins and associated genes. in *Journal of Medical Microbiology* (2005). doi:10.1099/jmm.0.45810-0

67. Carter, G. P. *et al.* Defining the roles of TcdA and TcdB in localized gastrointestinal disease, systemic organ damage, and the host response during Clostridium difficile infections. *MBio* (2015). doi:10.1128/mBio.00551-15

68. Gerding, D. N., Johnson, S., Rupnik, M. & Aktories, K. Clostridium difficile binary toxin CDT: mechanism, epidemiology, and potential clinical importance. *Gut microbes* **5**, 15–27 (2014).

69. Bauer, M. P. *et al.* Clostridium difficile infection in Europe: A hospital-based survey. *Lancet* (2011). doi:10.1016/S0140-6736(10)61266-4

70. Eckert, C. *et al.* Clinical and microbiological features of Clostridium difficile infections in France: The ICD-RAISIN 2009 national survey. *Med. Mal. Infect.* (2013). doi:10.1016/j.medmal.2013.01.004

71. Carter, G. P. *et al.* Binary toxin production in Clostridium difficile is regulated by CdtR, a LytTR family response regulator. *J. Bacteriol.* (2007). doi:10.1128/JB.00731-07

72. Perelle, S., Gibert, M., Bourlioux, P., Corthier, G. & Popoff, M. R. Production of a complete binary toxin (actin-specific ADP- ribosyltransferase) by Clostridium difficile CD196. *Infect. Immun.* (1997).

73. Schwan, C. *et al. Clostridium difficile* toxin CDT hijacks microtubule organization and reroutes vesicle traffic to increase pathogen adherence. *Proc. Natl. Acad. Sci.* (2014). doi:10.1073/pnas.1311589111

74. Buonomo, E. L. *et al.* Role of IL-23 signaling in Clostridium difficile Colitis. *J. Infect. Dis.* jit277 (2013). doi:10.1093/infdis/jit277

75. Abt, M. C. *et al.* Innate immune defenses mediated by two ilc subsets are critical for protection against acute clostridium difficile infection. *Cell Host Microbe* **18**, 27–37 (2015).

76. Kuehne, S. A. *et al.* The role of toxin A and toxin B in Clostridium difficile infection. *Nature* (2010). doi:10.1038/nature09397

77. Chandrasekaran, R. & Lacy, D. B. The role of toxins in clostridium difficile infection. *FEMS Microbiology Reviews* (2017). doi:10.1093/femsre/fux048

78. Na, X., Kim, H., Moyer, M. P., Pothoulakis, C. & LaMont, J. T. gp96 is a human colonocyte plasma membrane binding protein for Clostridium difficile toxin A. *Infect. Immun.* (2008). doi:10.1128/IAI.00326-08

79. Tucker, K. D. & Wilkins, T. D. Toxin A of Clostridium difficile binds to the human carbohydrate antigens I, X, and Y. *Infect. Immun.* (1991).

80. LaFrance, M. E. *et al.* Identification of an epithelial cell receptor responsible for *Clostridium difficile* TcdB-induced cytotoxicity. *Proc. Natl. Acad. Sci.* (2015). doi:10.1073/pnas.1500791112

81. Tao, L. *et al.* Frizzled proteins are colonic epithelial receptors for C. difficile toxin B. *Nature* (2016). doi:10.1038/nature19799

82. Yuan, P. *et al.* Chondroitin sulfate proteoglycan 4 functions as the cellular receptor for Clostridium difficile toxin B. *Cell Res.* (2015). doi:10.1038/cr.2014.169

83. Aktories, K., Schwan, C. & Jank, T. Clostridium difficile Toxin Biology . *Annu. Rev. Microbiol.* (2017). doi:10.1146/annurev-micro-090816-093458

84. Di Bella, S., Ascenzi, P., Siarakas, S., Petrosillo, N. & di Masi, A. Clostridium difficile toxins A and B: Insights into pathogenic properties and extraintestinal effects. *Toxins* (2016). doi:10.3390/toxins8050134

85. Hunt, J. J. & Ballard, J. D. Variations in Virulence and Molecular Biology among Emerging Strains of Clostridium difficile. *Microbiol. Mol. Biol. Rev.* (2013). doi:10.1128/mmbr.00017-13

86. Barbut, F. *et al.* Prospective study of clostridium difficile infections in Europe with phenotypic and genotypic characterisation of the isolates. *Clin. Microbiol. Infect.* (2007). doi:10.1111/j.1469-0691.2007.01824.x

87. Bauer, M. P. *et al.* First results of the European Clostridium difficile infection survey (ECDIS). *Clin. Microbiol. Infect.* (2009).

88. Papatheodorou, P. *et al.* Lipolysis-stimulated lipoprotein receptor (LSR) is the host receptor for the binary toxin Clostridium difficile transferase (CDT). *Proc. Natl. Acad. Sci. U. S. A.* **108**, 16422–7 (2011).

89. Hemmasi, S. *et al.* Interaction of the Clostridium difficile binary toxin CDT and its host cell receptor, lipolysis-stimulated lipoprotein receptor (LSR). *J. Biol. Chem.* **290**, 14031–14044 (2015).

90. Wigelsworth, D. J. *et al.* CD44 Promotes Intoxication by the Clostridial Iota-Family Toxins. *PLoS One* **7**, (2012).

91. Gülke, I. *et al.* Characterization of the enzymatic component of the ADP-ribosyltransferase toxin CDTa from Clostridium difficile. *Infect. Immun.* (2001). doi:10.1128/IAI.69.10.6004-6011.2001

92. Kuehne, S. A. *et al.* Importance of toxin a, toxin b, and cdt in virulence of an epidemic clostridium difficile strain. *J. Infect. Dis.* (2014). doi:10.1093/infdis/jit426

93. Geric, B., Johnson, S., Gerding, D. N., Grabnar, M. & Rupnik, M. Frequency of Binary Toxin Genes among Clostridium difficile Strains that Do Not Produce Large Clostridial Toxins. *J. Clin. Microbiol.* (2003). doi:10.1128/JCM.41.11.5227-5232.2003

94. Eckert, C. *et al.* Prevalence and pathogenicity of binary toxin-positive Clostridium difficile strains that do not produce toxins A and B. *New Microbes New Infect.* (2015). doi:10.1016/j.nmni.2014.10.003

95. Ernst, K. *et al.* Cyclophilin-facilitated membrane translocation as pharmacological target to prevent intoxication of mammalian cells by binary clostridial actin ADP-ribosylated toxins. *J. Mol. Biol.* (2015). doi:10.1016/j.jmb.2014.07.013

96. Haug, G. *et al.* The host cell chaperone Hsp90 is essential for translocation of the binary Clostridium botulinum C2 toxin into the cytosol. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M303980200

97. Sullivan, Å., Edlund, C. & Nord, C. E. Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect. Dis.* (2002). doi:10.1016/s1473-3099(01)00066-4

98. Deshpande, A. *et al.* Community-associated clostridium difficile infection antibiotics: A meta-analysis. *Journal of Antimicrobial Chemotherapy* (2013). doi:10.1093/jac/dkt129

99. Slimings, C. & Riley, T. V. Antibiotics and hospital-acquired Clostridium difficile infection: Update of systematic review and meta-analysis. *Journal of Antimicrobial Chemotherapy* (2014). doi:10.1093/jac/dkt477

100. Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* (2010). doi:10.1099/mic.0.040618-0

101. Chang, J. Y. *et al.* Decreased diversity of the fecal Microbiome in recurrent Clostridium difficile-associated diarrhea. *J. Infect. Dis.* (2008). doi:10.1086/525047

102. Buffie, C. G. *et al.* Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. *Infect. Immun.* (2012). doi:10.1128/IAI.05496-11

103. Bassis, C. M., Theriot, C. M. & Young, V. B. Alteration of the murine gastrointestinal microbiota by tigecycline leads to increased susceptibility to clostridium difficile infection. *Antimicrob. Agents Chemother.* (2014). doi:10.1128/AAC.02262-13

104. Theriot, C. M. & Young, V. B. Microbial and metabolic interactions between the gastrointestinal tract and Clostridium difficile infection. *Gut Microbes* (2014). doi:10.4161/gmic.27131

105. Chen, X. *et al.* A Mouse Model of Clostridium difficile-Associated Disease. *Gastroenterology* (2008). doi:10.1053/j.gastro.2008.09.002

106. Lawley, T. D. *et al.* Targeted Restoration of the Intestinal Microbiota with a Simple, Defined Bacteriotherapy Resolves Relapsing Clostridium difficile Disease in Mice. *PLoS Pathog.* (2012). doi:10.1371/journal.ppat.1002995

107. Gu, S. *et al.* Identification of key taxa that favor intestinal colonization of Clostridium difficile in an adult Chinese population. *Microbes Infect.* (2016). doi:10.1016/j.micinf.2015.09.008

108. Antharam, V. C. *et al.* Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea. *J. Clin. Microbiol.* (2013). doi:10.1128/JCM.00845-13

109. Britton, R. A. & Young, V. B. Role of the intestinal microbiota in resistance to colonization by Clostridium difficile. *Gastroenterology* (2014). doi:10.1053/j.gastro.2014.01.059

110. Sorg, J. A. & Sonenshein, A. L. Chenodeoxycholate is an inhibitor of Clostridium difficile spore germination. *J. Bacteriol.* (2009). doi:10.1128/JB.01260-08

111. Giel, J. L., Sorg, J. A., Sonenshein, A. L. & Zhu, J. Metabolism of bile salts in mice influences spore germination in clostridium difficile. *PLoS One* (2010). doi:10.1371/journal.pone.0008740

112. Buffie, C. G. *et al.* Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. *Nature* (2015). doi:10.1038/nature13828

113. Theriot, C. M. *et al.* Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. *Nat. Commun.* (2014). doi:10.1038/ncomms4114

114. Antunes, L. C. M. *et al.* Effect of antibiotic treatment on the intestinal metabolome. *Antimicrob. Agents Chemother.* (2011). doi:10.1128/AAC.01664-10

115. Weingarden, A. R. *et al.* Microbiota transplantation restores normal fecal bile acid composition in recurrent Clostridium difficile infection. *Am. J. Physiol. Liver Physiol.* (2014). doi:10.1152/ajpgi.00282.2013

116. Hove, H., Tvede, M. & Mortensen, P. B. Antibiotic-associated diarrhoea, Clostridium difficile, and short-chain fatty acids. *Scand. J. Gastroenterol.* (1996). doi:10.3109/00365529609009151

117. Høverstad, T. *et al.* Influence of ampicillin, clindamycin, and metronidazole on faecal excretion of short-chain fatty acids in healthy subjects. *Scand. J. Gastroenterol.* (1986). doi:10.3109/00365528609003109

118. Hryckowian, A. J. *et al.* Microbiota-Accessible carbohydrates suppress Clostridium difficile infection in a murine model. *Nat. Microbiol.* (2018). doi:10.1038/s41564-018-0150-6

119. Rolfe, R. D. Role of volatile fatty acids in colonization resistance to Clostridium difficile. *Infect. Immun.* (1984).

120. May, T., Mackie, R. I., Fahey, G. C., Cremin, J. C. & Garleb, K. A. Effect of fiber source on short-chain fatty acid production and on the growth and toxin production by clostridium difficile. *Scand. J. Gastroenterol.* (1994). doi:10.3109/00365529409094863

121. Ward, F. W. & Coates, M. E. Gastrointestinal pH measurement in rats: Influence of the microbial flora, diet and fasting. *Lab. Anim.* (1987). doi:10.1258/002367787781268693

122. Duncan, S. H., Louis, P., Thomson, J. M. & Flint, H. J. The role of pH in determining the species composition of the human colonic microbiota. *Environ. Microbiol.* (2009). doi:10.1111/j.1462-2920.2009.01931.x

123. Cummings, J. H., Pomare, E. W., Branch, H. W. J., Naylor, C. P. E. & MacFarlane, G. T. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* (1987). doi:10.1136/gut.28.10.1221

124. Gupta, P. *et al.* Does Alkaline Colonic pH Predispose to Clostridium difficile Infection? *South. Med. J.* (2016). doi:10.14423/smj.0000000000000414

125. Sonnenburg, J. L. *et al.* Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science (80-.).* (2005). doi:10.1126/science.1109051

126. Ng, K. M. *et al.* Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature* (2013). doi:10.1038/nature12503

127. Ferreyra, J. A. *et al.* Gut microbiota-produced succinate promotes C. Difficile infection after antibiotic treatment or motility disturbance. *Cell Host Microbe* (2014). doi:10.1016/j.chom.2014.11.003

128. Ross, R. P. *et al.* Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.1001224107

129. Vederas, J. C. *et al.* Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.0913554107

130. Hing, T. C. *et al.* The antimicrobial peptide cathelicidin modulates Clostridium difficile-associated colitis and toxin A-mediated enteritis in mice. *Gut* (2013). doi:10.1136/gutjnl-2012-302180

131. Nuding, S., Frasch, T., Schaller, M., Stange, E. F. & Zabel, L. T. Synergistic Effects of Antimicrobial Peptides and Antibiotics against Clostridium difficile. *Antimicrob. Agents Chemother.* (2014). doi:10.1128/aac.02542-14

132. Jarchum, I., Liu, M., Shi, C., Equinda, M. & Pamer, E. G. Critical role for myd88-Mediated Neutrophil recruitment during Clostridium difficile colitis. *Infect. Immun.* **80**, 2989–2996 (2012).

133. Buonomo, E. L. *et al.* Microbiota-Regulated IL-25 Increases Eosinophil Number to Provide Protection during Clostridium difficile Infection. *Cell Rep.* 1–12 (2016). doi:10.1016/j.celrep.2016.06.007

134. El Feghaly, R. E. *et al.* Markers of intestinal inflammation, not bacterial burden, correlate with clinical outcomes in clostridium difficile infection. *Clin. Infect. Dis.* **56**, 1713–1721 (2013).

135. Yu, H. *et al.* Cytokines are markers of the Clostridium difficile-induced inflammatory response and predict disease severity. *Clin. Vaccine Immunol.* (2017). doi:10.1128/CVI.00037-17

136. AS, K., EL, B., KW, S. & al, et. Development and validation of a prediction model for mortality and adverse outcomes among patients with peripheral eosinopenia on admission for clostridium difficile infection. *JAMA Surg.* (2018).

137. Buonomo, E. L. *et al.* Role of interleukin 23 signaling in clostridium difficile colitis. in *Journal of Infectious Diseases* **208**, 917–920 (2013).

138. Hasegawa, M. *et al.* Interleukin-22 Regulates the Complement System to Promote Resistance against Pathobionts after Pathogen-Induced Intestinal Damage. *Immunity* (2014). doi:10.1016/j.immuni.2014.09.010

139. Khanna, S. *et al.* A Novel Microbiome Therapeutic Increases Gut Microbial Diversity and Prevents Recurrent Clostridium difficile Infection. *J. Infect. Dis.* (2016). doi:10.1093/infdis/jiv766

140. van Nood, E. *et al.* Duodenal Infusion of Donor Feces for Recurrent *Clostridium difficile*. *N. Engl. J. Med.* (2013). doi:10.1056/NEJMoa1205037

141. Cash, H. L., Whitham, C. V., Behrendt, C. L. & Hooper, L. V. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science (80-.).* (2006). doi:10.1126/science.1127119

142. Brandl, K., Plitas, G., Schnabl, B., DeMatteo, R. P. & Pamer, E. G. MyD88-mediated signals induce the bactericidal lectin RegIIIγ and protect mice against intestinal *Listeria monocytogenes* infection. *J. Exp. Med.* (2007). doi:10.1084/jem.20070563

143. Brandl, K. *et al.* Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* (2008). doi:10.1038/nature07250

144. Kinnebrew, M. A. *et al.* Bacterial flagellin stimulates TLR5-dependent defense against vancomycin-resistant Enterococcus infection. *J. Infect. Dis.* (2011). doi:10.1086/650203.Bacterial

145. Kinnebrew, M. A. *et al.* Interleukin 23 Production by Intestinal CD103 +CD11b + Dendritic Cells in Response to Bacterial Flagellin Enhances Mucosal Innate Immune Defense. *Immunity* (2012). doi:10.1016/j.immuni.2011.12.011

146. Jarchum, I., Liu, M., Lipuma, L. & Pamer, E. G. Toll-like receptor 5 stimulation protects mice from acute Clostridium difficile colitis. *Infect. Immun.* (2011). doi:10.1128/IAI.01196-10

147. Josefsdottir, K. S., Baldridge, M. T., Kadmon, C. S. & King, K. Y. Antibiotics impair murine hematopoiesis by depleting the intestinal microbiota. *Blood* (2017). doi:10.1182/blood-2016-03-708594

148. Deshmukh, H. S. *et al.* The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nat. Med.* (2014). doi:10.1038/nm.3542

149. Clarke, T. B. *et al.* Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* (2010). doi:10.1038/nm.2087

150. Ryan, A. *et al.* A role for TLR4 in clostridium difficile infection and the recognition of surface layer proteins. *PLoS Pathog.* **7**, (2011).

151. Mazmanian, S. K., Cui, H. L., Tzianabos, A. O. & Kasper, D. L. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* (2005). doi:10.1016/j.cell.2005.05.007

152. Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. Dendritic Cells Prime Natural Killer Cells by trans-Presenting Interleukin 15. *Immunity* (2007). doi:10.1016/j.immuni.2007.03.006

153. Ganal, S. C. *et al.* Priming of Natural Killer Cells by Nonmucosal Mononuclear Phagocytes Requires Instructive Signals from Commensal Microbiota. *Immunity* (2012). doi:10.1016/j.immuni.2012.05.020

154. Jin, W. & Dong, C. IL-17 cytokines in immunity and inflammation. *Emerging Microbes and Infections* (2013). doi:10.1038/emi.2013.58

155. Klose, C. S. N. & Artis, D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat. Immunol.* (2016). doi:10.1038/ni.3489

156. McDermott, A. J. *et al.* Interleukin-23 (IL-23), independent of IL-17 and IL-22, drives neutrophil recruitment and innate inflammation during Clostridium difficile colitis in mice. *Immunology* **147**, 114–124 (2016).

157. Saleh, M. M., Frisbee, A. L., Leslie, J. L., Buonomo, E. L., Cowardin, C. A., Ma J. Z., Simpson, M. E., Scully, K. W., Abhyankar, M. M. and Petri, W. A. J. Colitis-induced Th17 cells increase the risk for severe subsequent Clostridium difficile infection. *Cell Host Microbe*

158. Kelly, C. P. *et al.* Neutrophil recruitment in Clostridium difficile toxin A enteritis in the rabbit. *J. Clin. Invest.* **93**, 1257–1265 (1994).

159. Ivanov, I. I. *et al.* Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. *Cell Host Microbe* (2008). doi:10.1016/j.chom.2008.09.009

160. Ivanov, I. I. *et al.* Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* (2009). doi:10.1016/j.cell.2009.09.033

161. Stepankova, R. *et al.* Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. *Inflamm. Bowel Dis.* (2007). doi:10.1002/ibd.20221

162. Gaboriau-Routhiau, V. *et al.* The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses. *Immunity* (2009). doi:10.1016/j.immuni.2009.08.020

163. Lee, Y. K., Menezes, J. S., Umesaki, Y. & Mazmanian, S. K. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.1000082107

164. Wu, H. J. *et al.* Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. *Immunity* (2010). doi:10.1016/j.immuni.2010.06.001

165. Atarashi, K. *et al.* Th17 Cell Induction by Adhesion of Microbes to Intestinal Epithelial Cells. *Cell* (2015). doi:10.1016/j.cell.2015.08.058

166. Sano, T. *et al.* An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell* (2015). doi:10.1016/j.cell.2015.08.061

167. Yang, Y. *et al.* Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature* (2014). doi:10.1038/nature13279

168. Castellanos, J. G. *et al.* Microbiota-Induced TNF-like Ligand 1A Drives Group 3 Innate Lymphoid Cell-Mediated Barrier Protection and Intestinal T Cell Activation during Colitis. *Immunity* (2018). doi:10.1016/j.immuni.2018.10.014

169. Sawa, S. *et al.* RORγt+ innate lymphoid cells regulate intestinal homeostasis by integrating negative signals from the symbiotic microbiota. *Nat. Immunol.* (2011). doi:10.1038/ni.2002

170. Mosconi, I. *et al.* Intestinal bacteria induce TSLP to promote mutualistic T-cell responses. *Mucosal Immunol.* (2013). doi:10.1038/mi.2013.12

171. Pascual-Reguant, A. *et al.* T H 17 cells express ST2 and are controlled by the alarmin IL-33 in the small intestine. *Mucosal Immunol.* (2017). doi:10.1038/mi.2017.5

172. De Salvo, C. *et al.* IL-33 drives eosinophil infiltration and pathogenic type 2 helper T-cell immune responses leading to chronic experimental ileitis. *Am. J. Pathol.* **186**, 885–898 (2016).

173. von Moltke, J., Ji, M., Liang, H.-E. & Locksley, R. M. Tuft-cell-derived IL-25 regulates an intestinal ILC2–epithelial response circuit. *Nature* **529**, 221–225 (2015).

174. Camelo, A. *et al.* IL-33, IL-25, and TSLP induce a distinct phenotypic and activation profile in human type 2 innate lymphoid cells. *Blood Adv.* (2017). doi:10.1182/bloodadvances.2016002352

175. Monticelli, L. A. *et al.* IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin–EGFR interactions. *Proc. Natl. Acad. Sci.* **112**, 10762–10767 (2015).

176. Salimi, M. *et al.* A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J. Exp. Med.* **210**, 2939–2950 (2013).

177. Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–70 (2010).

178. Monticelli, L. A. *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat. Immunol.* (2011). doi:10.1038/ni.2131

179. Schneider, C. *et al.* A Metabolite-Triggered Tuft Cell-ILC2 Circuit Drives Small Intestinal Remodeling. *Cell* (2018). doi:10.1016/j.cell.2018.05.014

180. Maggi, L. *et al.* Human circulating group 2 innate lymphoid cells can express CD154 and promote IgE production. *J. Allergy Clin. Immunol.* (2017). doi:10.1016/j.jaci.2016.06.032

181. De Salvo, C. *et al.* IL-33 drives eosinophil infiltration and pathogenic type 2 helper T-cell immune responses leading to chronic experimental ileitis. *Am. J. Pathol.* (2016). doi:10.1016/j.ajpath.2015.11.028

182. Mazmanian, S. K., Round, J. L. & Kasper, D. L. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* (2008). doi:10.1038/nature07008

183. Atarashi, K. *et al.* Induction of colonic regulatory T cells by indigenous Clostridium species. *Science (80-.).* (2011). doi:10.1126/science.1198469

184. Atarashi, K. *et al.* Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* (2013). doi:10.1038/nature12331

185. Arpaia, N. *et al.* Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013). doi:10.1038/nature12726

186. Furusawa, Y. *et al.* Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature* (2013). doi:10.1038/nature12721

187. Colonic, T. *et al.* The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic Treg Cell Homeostasis. *Science (80-.).* (2013). doi:10.1126/science.1241165

188. Pamer, E. G. Resurrecting the intestinal microbiota to combat antibiotic-resistant pathogens. *Science* (2016). doi:10.1126/science.aad9382

189. Buffie, C. G. & Pamer, E. G. Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology* (2013). doi:10.1038/nri3535

190. Eisenman B, Silen W, Bascom GS, K. A. Fecal enema as an adjunct in the treatment of pseudomembranous. *Surgery* (1958). doi:10.1067/S0039-6060(03)00474-4

191. Seekatz, A. M. *et al.* Recovery of the gut microbiome following fecal microbiota transplantation. *MBio* (2014). doi:10.1128/mBio.00893-14

192. Tvede, M. & Rask-Madsen, J. BACTERIOTHERAPY FOR CHRONIC RELAPSING CLOSTRIDIUM DIFFICILE DIARRHOEA IN SIX PATIENTS. *Lancet* (1989). doi:10.1016/S0140-6736(89)92749-9

193. Surawicz, C. M. *et al.* The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with Saccharomyces boulardii. *Clin. Infect. Dis.* (2000). doi:10.1086/318130

194. McFarland, L. V. Evidence-based review of probiotics for antibiotic-associated diarrhea and Clostridium difficile infections. *Anaerobe* (2009). doi:10.1016/j.anaerobe.2009.09.002

195. Reeves, A. E. *et al.* The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile infection. *Gut Microbes* (2011). doi:10.4161/gmic.2.3.16333

196. Reeves, A. E., Koenigsknecht, M. J., Bergin, I. L. & Young, V. B. Suppression of Clostridium difficile in the Gastrointestinal Tracts of Germfree Mice Inoculated with a Murine Isolate from the Family Lachnospiraceae. *Infect. Immun.* (2012). doi:10.1128/iai.00647-12

197. Johnston, P. F., Gerding, D. N. & Knight, K. L. Protection from clostridium difficile infection in CD4 T cell- and polymeric immunoglobulin receptor-deficient mice. *Infect. Immun.* (2014). doi:10.1128/IAI.01273-13

198. Cook, L. *et al.* CD4+ T Cells Specific for C. difficile Toxins are a Marker of Patients with Active Relapsing Disease. *Open Forum Infect. Dis.* **4**, S383–S383 (2017).

199. Rees, W. D. & Steiner, T. S. Adaptive immune response to Clostridium difficile infection: A perspective for prevention and therapy. *European Journal of Immunology* (2018). doi:10.1002/eji.201747295

200. Yacyshyn, M. B. *et al.* Clostridium difficile recurrence is characterized by pro-inflammatory peripheral blood mononuclear cell (PBMC) phenotype. *J. Med. Microbiol.* (2014). doi:10.1099/jmm.0.075382-0

201. Burrello, C. *et al.* Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. *Nat. Commun.* (2018). doi:10.1038/s41467-018-07359-8

202. Wei, Y. L. *et al.* Fecal microbiota transplantation ameliorates experimentally induced colitis in mice by upregulating AhR. *Front. Microbiol.* (2018). doi:10.3389/fmicb.2018.01921

203. Steiner, T. S., Flores, C. A., Pizarro, T. T. & Guerrant, R. L. Fecal lactoferrin, interleukin-1beta, and interleukin-8 are elevated in patients with severe Clostridium difficile colitis. *Clin. Diagn. Lab. Immunol.* **4**, 719–22 (1997).

204. Jiang, Z. D. *et al.* A common polymorphism in the interleukin 8 gene promoter is associated with Clostridium difficile diarrhea. *Am. J. Gastroenterol.* **101**, 1112–1116 (2006).

205. Huang, L. & Appleton, J. A. Eosinophils in Helminth Infection: Defenders and Dupes. *Trends in Parasitology* (2016). doi:10.1016/j.pt.2016.05.004

206. El Feghaly, R. E. *et al.* Markers of intestinal inflammation, not bacterial burden, correlate with clinical outcomes in clostridium difficile infection. *Clin. Infect. Dis.* (2013). doi:10.1093/cid/cit147

207. Lee, J. Y. *et al.* Clostridium difficile toxin A promotes dendritic cell maturation and chemokine CXCL2 expression through p38, IKK, and the NF-κB signaling pathway. *J. Mol. Med.* (2009). doi:10.1007/s00109-008-0415-2

208. Jefferson, K. K., Smith, M. F. & Bobak, D. A. Roles of Intracellular Calcium and NF-□B in the Clostridium difficile Toxin A-Induced Up-Regulation and Secretion of IL-8 from Human Monocytes. *J. Immunol.* (1999). doi:ji_v163n10p5183 [pii]

209. Cowardin, C. A. *et al.* Inflammasome activation contributes to interleukin-23 production in response to Clostridium difficile. *MBio* (2015). doi:10.1128/mBio.02386-14

210. Linevsky, J. K. *et al.* IL-8 release and neutrophil activation by Clostridium difficile toxin-exposed human monocytes. *Am. J. Physiol.* (1997). doi:10.1016/j.jcomdis.2010.05.001

211. Ishida, Y. *et al.* Essential Involvement of IFN- in Clostridium difficile Toxin A-Induced Enteritis. *J. Immunol.* (2004). doi:10.4049/jimmunol.172.5.3018

212. McDermott, A. J. *et al.* Interleukin-23 (IL-23), independent of IL-17 and IL-22, drives neutrophil recruitment and innate inflammation during Clostridium difficile colitis in mice. *Immunology* (2016). doi:10.1111/imm.12545

213. Hasegawa, M. *et al.* Protective Role of Commensals against Clostridium difficile Infection via an IL-1beta-Mediated Positive-Feedback Loop. *J. Immunol.* **189**, 3085–3091 (2012).

214. Xu, H. *et al.* Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. *Nature* **513**, 237–41 (2014).

215. Van Gorp, H. *et al.* Familial Mediterranean fever mutations lift the obligatory requirement for microtubules in Pyrin inflammasome activation. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 14384–14389 (2016).

216. Miller, L. S. *et al.* Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against Staphylococcus aureus in vivo. *J. Immunol.* **179**, 6933–6942 (2007).

217. Hasegawa, M. *et al.* Nucleotide-Binding Oligomerization Domain 1 Mediates Recognition of Clostridium difficile and Induces Neutrophil Recruitment and Protection against the Pathogen. *J. Immunol.* (2011). doi:10.4049/jimmunol.1003761

218. Tasteyre, A. *et al.* A Clostridium difficile gene encoding flagellin. *Microbiology* (2000). doi:10.1099/00221287-146-4-957

219. Yoshino, Y. *et al.* Clostridium difficile flagellin stimulates toll-like receptor 5, and toxin B promotes flagellin-induced chemokine production via TLR5. *Life Sci.* (2013). doi:10.1016/j.lfs.2012.11.017

220. Darkoh, C. *et al.* Colonic immunopathogenesis of Clostridium difficile infections. *Clin. Vaccine Immunol.* (2014). doi:10.1128/CVI.00770-13

221. Nakagawa, T. *et al.* Endogenous il-17 as a factor determining the severity of Clostridium difficile infection in mice. *J. Med. Microbiol.* (2016). doi:10.1099/jmm.0.000273

222. Jose, S. & Madan, R. Neutrophil-mediated inflammation in the pathogenesis of Clostridium difficile infections. *Anaerobe* (2016). doi:10.1016/j.anaerobe.2016.04.001

223. Castagliuolo, I. *et al.* Clostridium difficile toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. *J. Immunol.* (1998). doi:10.4049/jimmunol.172.5.3018

224. Kelly, C. P. *et al.* Neutrophil recruitment in Clostridium difficile toxin A enteritis in the rabbit. *J. Clin. Invest.* (1994). doi:10.1172/JCI117080

225. Yoon, Y. K. *et al.* Predictors of mortality attributable to Clostridium difficile infection in patients with underlying malignancy. *Support. Care Cancer* (2014). doi:10.1007/s00520-014-2174-7

226. Solomon, K. *et al.* Mortality in patients with Clostridium difficile infection correlates with host pro-inflammatory and humoral immune responses. *J. Med. Microbiol.* (2013). doi:10.1099/jmm.0.058479-0

227. Jafari, N. V. *et al.* Clostridium difficile Modulates Host Innate Immunity via Toxin-Independent and Dependent Mechanism(s). *PLoS One* (2013). doi:10.1371/journal.pone.0069846

228. Xu, W. *et al.* NFIL3 orchestrates the emergence of common helper innate lymphoid cell precursors. *Cell Rep.* **10**, 2043–2054 (2015).

229. Greenlee-Wacker, M. C. & Nauseef, W. M. IFN-γ targets macrophage-mediated immune responses toward Staphylococcus aureus . *J. Leukoc. Biol.* (2016). doi:10.1189/jlb.4a1215-565rr

230. Nussbaum, J. C. *et al.* Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* **502**, 245–248 (2013).

231. Tominaga, S. ichi. A putative protein of a growth specific cDNA from BALB/C-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett.* (1989). doi:10.1016/0014-5793(89)81679-5

232. Schmitz, J. *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**, 479–490 (2005).

233. Haraldsen, G., Balogh, J., Pollheimer, J., Sponheim, J. & Küchler, A. M. Interleukin-33 - cytokine of dual function or novel alarmin? *Trends in Immunology* **30**, 227–233 (2009).

234. Schiering, C. *et al.* The Alarmin IL-33 Promotes Regulatory T Cell Function in the Intestine. *Nature* **513**, 564–568 (2014).

235. Mahapatro, M. *et al.* Programming of Intestinal Epithelial Differentiation by IL-33 Derived from Pericryptal Fibroblasts in Response to Systemic Infection. *Cell Rep.* (2016). doi:10.1016/j.celrep.2016.04.049

236. Howitt, M.R., Lavoie, S., Michaud, M., Blum, A.M., Tran, S.V., Weinstock, J.V., Gallini, C.A., Redding, K., Margolskee, R.F., Osborne, L.C. and Artis, D. Tuft cells, taste-chemosensory cells,orchestrate parasite type 2 immunityin the gut. *Science (80-.).* **351**, 1329–1333. (2016).

237. Küchler, A. M. *et al.* Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *Am. J. Pathol.* (2008). doi:10.2353/ajpath.2008.080014

238. Dahlgren, M. W. *et al.* Adventitial Stromal Cells Define Group 2 Innate Lymphoid Cell Tissue Niches. *Immunity* (2019). doi:10.1016/j.immuni.2019.02.002

239. Lüthi, A. U. *et al.* Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases. *Immunity* (2009). doi:10.1016/j.immuni.2009.05.007

240. Garlanda, C., Dinarello, C. A. & Mantovani, A. The Interleukin-1 Family: Back to the Future. *Immunity* (2013). doi:10.1016/j.immuni.2013.11.010

241. Kaczmarek, A., Vandenabeele, P. & Krysko, D. V. Necroptosis: The Release of Damage-Associated Molecular Patterns and Its Physiological Relevance. *Immunity* (2013). doi:10.1016/j.immuni.2013.02.003

242. Cayrol, C. & Girard, J. P. Interleukin-33 (IL-33): A nuclear cytokine from the IL-1 family. *Immunological Reviews* (2018). doi:10.1111/imr.12619

243. Schmitz, J. *et al.* IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**, 479–490 (2005).

244. Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nature Reviews Immunology* (2009). doi:10.1038/nri2653

245. Martin, N. T. & Martin, M. U. Interleukin 33 is a guardian of barriers and a local alarmin. *Nature Immunology* (2016). doi:10.1038/ni.3370

246. Seo, D. H. *et al.* Interleukin-33 regulates intestinal inflammation by modulating macrophages in inflammatory bowel disease. *Sci. Rep.* (2017). doi:10.1038/s41598-017-00840-2

247. Alves-Filho, J. C. *et al.* Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nat. Med.* (2010). doi:10.1038/nm.2156

248. Allen, J. E. & Sutherland, T. E. Host protective roles of type 2 immunity: Parasite killing and tissue repair, flip sides of the same coin. *Seminars in Immunology* (2014). doi:10.1016/j.smim.2014.06.003

249. Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–1370 (2010).

250. Liew, Y. *et al.* IL-33, a Potent Inducer of Adaptive Immunity to Intestinal Nematodes. *J Immunol Ref. J. Immunol.* **180**, 2443–2449 (2008).

251. Hung, L.-Y. *et al.* IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. *Proc. Natl. Acad. Sci.* **110**, 282–287 (2013).

252. Sedhom, M. A. K. *et al.* Neutralisation of the interleukin-33/ST2 pathway ameliorates experimental colitis through enhancement of mucosal healing In mice. *Gut* (2013). doi:10.1136/gutjnl-2011-301785

253. Pushparaj, P. N. *et al.* Interleukin-33 exacerbates acute colitis via interleukin-4 in mice. *Immunology* (2013). doi:10.1111/imm.12111

254. Kiyonari, H. *et al.* IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.1003059107

255. Balzola, F., Bernstein, C., Ho, G. T. & Lees, C. Epithelial-derived IL-33 and its receptor ST2 are dysregulated in ulcerative colitis and in experimental TH1/TH2 driven enteritis: Commentary. *Inflammatory Bowel Disease Monitor* (2010).

256. Liu, Q. *et al.* IL-33-Driven Innate Tissue-Protective Function of ST2+ Treg Cells. *J. Immunol.* **196**, 51.7--51.7 (2016).

257. Zaiss, D. M. W., Gause, W. C., Osborne, L. C. & Artis, D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity* (2015). doi:10.1016/j.immuni.2015.01.020

258. Liu, Q. *et al.* IL-33-Driven Innate Tissue-Protective Function of ST2+ T-reg Cells. *J. Immunol.* **196**, 51.7 LP-51.7 (2016).

259. Siede, J. *et al.* IL-33 receptor-expressing regulatory t cells are highly activated, Th2 biased and suppress CD4 T Cell proliferation through IL-10 and TGFβ Release. *PLoS One* **11**, (2016).

260. Schiering, C. *et al.* The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* **513**, 564–8 (2014).

261. Duan, L. & Chen, J. Interleukin-33 Ameliorates Experimental Colitis through Promoting Th2/Foxp3+ Regulatory T-Cell Responses in Mice. *Mol. Med.* (2012). doi:10.2119/molmed.2011.00428

262. Seidelin, J. B. *et al.* IL-33 is upregulated in colonocytes of ulcerative colitis. *Immunol. Lett.* (2010). doi:10.1016/j.imlet.2009.11.001

263. Beltrán, C. J. *et al.* Characterization of the novel ST2/IL-33 system in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* (2010). doi:10.1002/ibd.21175

264. Yamanishi, K. *et al.* Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc. Natl. Acad. Sci.* (2013). doi:10.1073/pnas.1307321110

265. Barlow, J. L. *et al.* Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J. Allergy Clin. Immunol.* (2012). doi:10.1016/j.jaci.2011.09.041

266. Russo, R. C. *et al.* IL-33 Induces Antigen-Specific IL-5+ T Cells and Promotes Allergic-Induced Airway Inflammation Independent of IL-4. *J. Immunol.* (2014). doi:10.4049/jimmunol.181.7.4780

267. Nadjsombati, M. S. *et al.* Detection of Succinate by Intestinal Tuft Cells Triggers a Type 2 Innate Immune Circuit. *Immunity* (2018). doi:10.1016/j.immuni.2018.06.016

268. Gieseck, R. L., Wilson, M. S. & Wynn, T. A. Type 2 immunity in tissue repair and fibrosis. *Nature Reviews Immunology* (2018). doi:10.1038/nri.2017.90

269. Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367–70 (2010).

270. Li, C. *et al.* Interleukin-33 Increases Antibacterial Defense by Activation of Inducible Nitric Oxide Synthase in Skin. *PLoS Pathog.* (2014). doi:10.1371/journal.ppat.1003918

271. Yin, H. *et al.* IL-33 promotes Staphylococcus aureus-infected wound healing in mice. *Int. Immunopharmacol.* **17**, 432–438 (2013).

272. Hazlett, L. D. *et al.* IL-33 shifts macrophage polarization, promoting resistance against Pseudomonas aeruginosa keratitis. *Investig. Ophthalmol. Vis. Sci.* **51**, 1524–1532 (2010).

273. Piñeros, A. R. *et al.* M2 macrophages or IL-33 treatment attenuate ongoing Mycobacterium tuberculosis infection. *Sci. Rep.* (2017). doi:10.1038/srep41240

274. Alves-Filho, J. C. *et al.* Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. *Nat. Med.* **16**, 708–712 (2010).

275. Hoogerwerf, J. J. *et al.* Soluble ST2 plasma concentrations predict mortality in severe sepsis. *Intensive Care Med.* (2010). doi:10.1007/s00134-010-1773-0

276. Lan, F. *et al.* Interleukin-33 facilitates neutrophil recruitment and bacterial clearance in S. aureus-caused peritonitis. *Mol. Immunol.* (2016). doi:10.1016/j.molimm.2016.03.004

277. Krishack, P. A. *et al.* Protection against Staphylococcus aureus bacteremia-induced mortality depends on ILC2s and eosinophils. *JCI Insight* (2019). doi:10.1172/jci.insight.124168

278. Brint, E. K. *et al.* ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat. Immunol.* **5**, 373–9 (2004).

279. Liu, J., Buckley, J. M., Redmond, H. P. & Wang, J. H. ST2 negatively regulates TLR2 signaling, but is not required for bacterial lipoprotein-induced tolerance. *J. Immunol.* **184**, 5802–8 (2010).

280. Nascimento, D. C. *et al.* IL-33 contributes to sepsis-induced long-term immunosuppression by expanding the regulatory T cell population. *Nat. Commun.* **8**, 14919 (2017).

281. He, M. *et al.* Emergence and global spread of epidemic healthcare-associated Clostridium difficile. *Nat. Genet.* (2013). doi:10.1038/ng.2478

282. Schwan, C. *et al.* Clostridium difficile toxin CDT induces formation of microtubule-based protrusions and increases adherence of bacteria. *PLoS Pathog.* (2009). doi:10.1371/journal.ppat.1000626

283. Kuehne, S. A. *et al.* Importance of toxin a, toxin b, and cdt in virulence of an epidemic clostridium difficile strain. *J. Infect. Dis.* **209**, 83–86 (2014).

284. Ng, J. *et al.* Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome. *Gastroenterology* **139**, (2010).

285. Lawley, T. D. *et al.* Antibiotic treatment of Clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. *Infect. Immun.* (2009). doi:10.1128/IAI.00558-09

286. Lawley, T. D. & Young, V. B. Murine models to study Clostridium difficile infection and transmission. *Anaerobe* (2013). doi:10.1016/j.anaerobe.2013.09.008

287. Buonomo, E. L. *et al.* Role of interleukin 23 signaling in clostridium difficile colitis. in *Journal of Infectious Diseases* (2013). doi:10.1093/infdis/jit277

288. Krämer, A., Green, J., Pollard, J. & Tugendreich, S. Causal analysis approaches in ingenuity pathway analysis. *Bioinformatics* (2014). doi:10.1093/bioinformatics/btt703

289. Kamburov, A., Stelzl, U., Lehrach, H. & Herwig, R. The ConsensusPathDB interaction database: 2013 Update. *Nucleic Acids Res.* (2013). doi:10.1093/nar/gks1055

290. Mantovani, A. & Marchesi, F. IL-10 and macrophages orchestrate gut homeostasis. *Immunity* (2014). doi:10.1016/j.immuni.2014.04.015

291. Bosurgi, L. *et al.* Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells. *Science (80-.).* (2017). doi:10.1126/science.aai8132

292. Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. & Müller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* (1993). doi:10.1016/0092-8674(93)80068-P

293. Newcomb, D. C. *et al.* IL-13 Regulates Th17 Secretion of IL-17A in an IL-10-Dependent Manner. *J. Immunol.* (2012). doi:10.4049/jimmunol.1102216

294. Matsukawa, A. *et al.* Expression and Contribution of Endogenous IL-13 in an Experimental Model of Sepsis. *J. Immunol.* (2000). doi:10.4049/jimmunol.164.5.2738

295. Zhang, H.-F. *et al.* IL-33 promotes IL-10 production in macrophages: a role for IL-33 in macrophage foam cell formation. *Exp. Mol. Med.* **49**, e388 (2017).

296. Rankin, A. L. *et al.* IL-33 Induces IL-13-Dependent Cutaneous Fibrosis. *J. Immunol.* **184**, 1526–1535 (2010).

297. Sattler, S. *et al.* IL-10-producing regulatory B cells induced by IL-33 (BregIL-33) effectively attenuate mucosal inflammatory responses in the gut. *J. Autoimmun.* **50**, 107–122 (2014).

298. Groß, P., Doser, K., Falk, W., Obermeier, F. & Hofmann, C. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflamm. Bowel Dis.* **18**, 1900–1909 (2012).

299. Jenior, M. L., Leslie, J. L., Young, V. B. & Schloss, P. D. *Clostridium difficile* Colonizes Alternative Nutrient Niches during Infection across Distinct Murine Gut Microbiomes. *mSystems* (2017). doi:10.1128/mSystems.00063-17

300. Kuehne, S. A. *et al.* The role of toxin A and toxin B in Clostridium difficile infection. *Nature* (2010). doi:10.1038/nature09397

301. Cowardin, C. A. *et al.* Inflammasome activation contributes to interleukin-23 production in response to Clostridium difficile. *MBio* **6**, 1–9 (2015).

302. Howitt, M. R. *et al.* Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. *Science (80-.).* (2016). doi:10.1126/science.aaf1648

303. Engevik, M. *et al.* Human Clostridium difficile infection: altered mucus production and composition. *Am. J. Physiol. - Gastrointest. Liver Physiol.* (2015). doi:10.1152/ajpgi.00091.2014

304. Batah, J. *et al.* Clostridium difficile flagella induce a pro-inflammatory response in intestinal epithelium of mice in cooperation with toxins. *Sci. Rep.* (2017). doi:10.1038/s41598-017-03621-z

305. Malik, A. *et al.* IL-33 regulates the IgA-microbiota axis to restrain IL-1a-dependent colitis and tumorigenesis. *J. Clin. Invest.* (2016). doi:10.1172/JCI88625

306. Colonna, M. Innate Lymphoid Cells: Diversity, Plasticity, and Unique Functions in Immunity. *Immunity* (2018). doi:10.1016/j.immuni.2018.05.013

307. Ohne, Y. *et al.* IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat. Immunol.* (2016). doi:10.1038/ni.3447

308. Belz, G. T. ILC2s masquerade as ILC1s to drive chronic disease. *Nature Immunology* (2016). doi:10.1038/ni.3467

309. Bernink, J. H. *et al.* Interleukin-12 and -23 Control Plasticity Of Cd127+Group 1 And Group 3 Innate Lymphoid Cells In The Intestinal Lamina Propria. *Immunity* (2015). doi:10.1016/j.immuni.2015.06.019

310. Baumann, C. *et al.* T-bet– and STAT4–dependent IL-33 receptor expression directly promotes antiviral Th1 cell responses. *Proc. Natl. Acad. Sci.* (2015). doi:10.1073/pnas.1418549112

311. Bonilla, W. V. *et al.* The alarmin interleukin-33 drives protective antiviral CD8+T cell responses. *Science (80-.).* (2012). doi:10.1126/science.1215418

312. Johnston, P. F., Gerding, D. N. & Knight, K. L. Protection from clostridium difficile infection in CD4 T cell- and polymeric immunoglobulin receptor-deficient mice. *Infect. Immun.* **82**, 522–531 (2014).

313. Cao, Q. *et al.* Potentiating Tissue-Resident Type 2 Innate Lymphoid Cells by IL-33 to Prevent Renal Ischemia-Reperfusion Injury. *J. Am. Soc. Nephrol.* (2018). doi:10.1681/ASN.2017070774

314. Duerr, C. U. *et al.* Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat. Immunol.* (2015). doi:10.1038/ni.3308

315. Newland, S. A. *et al.* Type-2 innate lymphoid cells control the development of atherosclerosis in mice. *Nat. Commun.* **8**, 15781 (2017).

316. Carlens, J. *et al.* Common -Chain-Dependent Signals Confer Selective Survival of Eosinophils in the Murine Small Intestine. *J. Immunol.* (2009). doi:10.4049/jimmunol.0801581

317. Hung, L.-Y. *et al.* IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 282–7 (2013).

318. Carlens, J. *et al.* Common gamma-chain-dependent signals confer selective survival of eosinophils in the murine small intestine. *J. Immunol.* **183**, 5600–7 (2009).

319. Moro, K. *et al.* Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Sca-1+lymphoid cells. *Nature* (2010). doi:10.1038/nature08636

320. Mishra, a, Hogan, S. P., Brandt, E. B. & Rothenberg, M. E. Peyer's patch eosinophils: identification, characterization, and regulation by mucosal allergen exposure, interleukin-5, and eotaxin. *Blood* (2000).

321. Polumuri, S. K. *et al.* Transcriptional Regulation of Murine IL-33 by TLR and Non-TLR Agonists. *J. Immunol.* (2012). doi:10.4049/jimmunol.1003554

322. Cornely, O. A., Miller, M. A., Louie, T. J., Crook, D. W. & Gorbach, S. L. Treatment of first recurrence of clostridium difficile infection: Fidaxomicin versus vancomycin. *Clin. Infect. Dis.* (2012). doi:10.1093/cid/cis462

323. Lowy, I. *et al.* Treatment with monoclonal antibodies against Clostridium difficile toxins. *N. Engl. J. Med.* (2010). doi:10.1056/NEJMoa0907635

324. Louie, T. J. *et al.* Fidaxomicin versus vancomycin for Clostridium difficile infection. *N. Engl. J. Med.* (2011). doi:10.1056/NEJMoa0910812

325. Buffie, C. G. & Pamer, E. G. Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology* (2013). doi:10.1038/nri3535

326. Shivashankar, R. *et al.* Clinical factors associated with development of severe-complicated Clostridium difficile infection. *Clin. Gastroenterol. Hepatol.* **11**, 1466–1471 (2013).

327. Moussion, C., Ortega, N. & Girard, J. P. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: A novel 'Alarmin'? *PLoS One* (2008). doi:10.1371/journal.pone.0003331

328. Pichery, M. *et al.* Endogenous IL-33 Is Highly Expressed in Mouse Epithelial Barrier Tissues, Lymphoid Organs, Brain, Embryos, and Inflamed Tissues: In Situ Analysis Using a Novel Il-33-LacZ Gene Trap Reporter Strain. *J. Immunol.* (2012). doi:10.4049/jimmunol.1101977

329. Gadani, S. P., Walsh, J. T., Smirnov, I., Zheng, J. & Kipnis, J. The Glia-Derived Alarmin IL-33 Orchestrates the Immune Response and Promotes Recovery following CNS Injury. *Neuron* (2015). doi:10.1016/j.neuron.2015.01.013

330. Bartlett, J. G., Moon, N., Chang, T. W., Taylor, N. & Onderdonk, A. B. Role of Clostridium difficile in antibiotic-associated pseudomembranous colitis. *Gastroenterology* (1978). doi:10.5555/URI:PII:0016508578904572

331. Tran, L. S. *et al.* NOD1 is required for Helicobacter pylori induction of IL-33 responses in gastric epithelial cells. *Cell. Microbiol.* (2018). doi:10.1111/cmi.12826

332. Li, J. *et al.* Pollen/TLR4 Innate Immunity Signaling Initiates IL-33/ST2/Th2 Pathways in Allergic Inflammation. *Sci. Rep.* (2016). doi:10.1038/srep36150

333. Chu, H. & Mazmanian, S. K. Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nature Immunology* (2013). doi:10.1038/ni.2635

334. Vaishnava, S. *et al.* The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. *Science (80-.).* (2011). doi:10.1126/science.1209791

335. Lei, W. *et al.* Activation of intestinal tuft cell-expressed Sucnr1 triggers type 2 immunity in the mouse small intestine. *Proc. Natl. Acad. Sci.* (2018). doi:10.1073/pnas.1720758115

336. Calvén, J., Akbarshahi, H., Menzel, M., Idzko, M. & Uller, L. Involvement of purine metabolites in dsRNA-induced IL-33 expression in human bronchial smooth muscle cells. *Eur. Respir. J.* **44**, 1718 (2014).

337. Seekatz, A. M. & Young, V. B. Clostridium difficile and the microbiota. *Journal of Clinical Investigation* (2014). doi:10.1172/JCI72336

338. Fuentes, S. *et al.* Reset of a critically disturbed microbial ecosystem: Faecal transplant in recurrent Clostridium difficile infection. *ISME J.* (2014). doi:10.1038/ismej.2014.13

339. Manges, A. R. *et al.* Comparative Metagenomic Study of Alterations to the Intestinal Microbiota and Risk of Nosocomial *Clostridum difficile* –Associated Disease. *J. Infect. Dis.* (2010). doi:10.1086/657319

340. Daquigan, N., Seekatz, A. M., Greathouse, K. L., Young, V. B. & White, J. R. High-resolution profiling of the gut microbiome reveals the extent of Clostridium difficile burden. *npj Biofilms Microbiomes* (2017). doi:10.1038/s41522-017-0043-0

341. Pérez-Cobas, A. E. *et al.* Structural and functional changes in the gut microbiota associated to Clostridium difficile infection. *Front. Microbiol.* (2014). doi:10.3389/fmicb.2014.00335

342. Brunner, M. *et al.* Increased levels of soluble ST2 protein and IgG1production in patients with sepsis and trauma. *Intensive Care Med.* (2004). doi:10.1007/s00134-004-2184-x

343. Petrella, L. A. *et al.* Decreased cure and increased recurrence rates for clostridium difficile infection caused by the Epidemic C. difficile BI strain. *Clin. Infect. Dis.* (2012). doi:10.1093/cid/cis430

344. Seekatz, A. M. *et al.* Presence of multiple Clostridium difficile strains at primary infection is associated with development of recurrent disease. *Anaerobe* (2018).

345. Bacci, S., Mølbak, K., Kjeldsen, M. K. & Olsen, K. E. P. Binary toxin and death after clostridium difficile infection. *Emerg. Infect. Dis.* **17**, 976–982 (2011).

346. Rao, K. *et al.* Clostridium difficile Ribotype 027: Relationship to Age, Detectability of Toxins A or B in Stool with Rapid Testing, Severe Infection, and Mortality. *Clin. Infect. Dis.* (2015). doi:10.1093/cid/civ254

347. Bianco, M. *et al.* Immunomodulatory activities of surface-layer proteins obtained from epidemic and hypervirulent Clostridium difficile strains. *J. Med. Microbiol.* (2011). doi:10.1099/jmm.0.029694-0

348. Magill, S. S. *et al.* Changes in Prevalence of Health Care–Associated Infections in U.S. Hospitals. *N. Engl. J. Med.* (2018). doi:10.1056/NEJMoa1801550

349. Furukawa, S. *et al.* Interleukin-33 produced by M2 macrophages and other immune cells contributes to Th2 immune reaction of IgG4-related disease. *Sci. Rep.* (2017). doi:10.1038/srep42413

350. Lee, Y. J. *et al.* Protective factors in the intestinal microbiome against clostridium difficile infection in recipients of allogeneic hematopoietic stem cell transplantation. *J. Infect. Dis.* (2017). doi:10.1093/infdis/jix011

351. Zhang, L., Lu, R., Zhao, G., Pflugfelder, S. C. & Li, D. Q. TLR-mediated induction of pro-allergic cytokine IL-33 in ocular mucosal epithelium. *Int. J. Biochem. Cell Biol.* (2011). doi:10.1016/j.biocel.2011.06.003

352. Su, Z. *et al.* Potential autocrine regulation of interleukin-33/ST2 signaling of dendritic cells in allergic inflammation. *Mucosal Immunol.* (2013). doi:10.1038/mi.2012.130

353. Viala, J. *et al.* Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat. Immunol.* (2004). doi:10.1038/ni1131

354. Peterson, L. W. & Artis, D. Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. *Nature Reviews Immunology* (2014). doi:10.1038/nri3608

355. Abreu, M. T. Toll-like receptor signalling in the intestinal epithelium: How bacterial recognition shapes intestinal function. *Nature Reviews Immunology* (2010). doi:10.1038/nri2707

356. Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. & Medzhitov, R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* (2004). doi:10.1016/j.cell.2004.07.002

357. Tsuji, Y. *et al.* Sensing of commensal organisms by the intracellular sensor nod1 mediates experimental pancreatitis. *Immunity* (2012). doi:10.1016/j.immuni.2012.05.024

358. Rutkowski, M. R. *et al.* Microbially driven TLR5-dependent signaling governs distal malignant progression through tumor-promoting inflammation. *Cancer Cell* (2015). doi:10.1016/j.ccell.2014.11.009

359. Jarchum, I., Liu, M., Lipuma, L. & Pamer, E. G. Toll-Like Receptor 5 Stimulation Protects Mice from Acute Clostridium difficile Colitis . *Infect. Immun.* (2011). doi:10.1128/iai.01196-10

360. Ryan, A. *et al.* A role for TLR4 in clostridium difficile infection and the recognition of surface layer proteins. *PLoS Pathog.* (2011). doi:10.1371/journal.ppat.1002076

361. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nature Reviews Immunology* (2004). doi:10.1038/nri1391

362. Morrison, D. J. & Preston, T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* (2016). doi:10.1080/19490976.2015.1134082

363. Kim, M. H., Kang, S. G., Park, J. H., Yanagisawa, M. & Kim, C. H. Short-chain fatty acids activate GPR41 and GPR43 on intestinal epithelial cells to promote inflammatory responses in mice. *Gastroenterology* (2013). doi:10.1053/j.gastro.2013.04.056

364. Duncan, S. H., Louis, P. & Flint, H. J. Cultivable bacterial diversity from the human colon. *Letters in Applied Microbiology* (2007). doi:10.1111/j.1472-765X.2007.02129.x

365. Antharam, V. C. *et al.* Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea. *J. Clin. Microbiol.* (2013). doi:10.1128/JCM.00845-13

366. Nussbaum, J. C. *et al.* Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* **502**, 245–248 (2013).

367. Mishra, A., Hogan, S. P., Lee, J. J., Foster, P. S. & Rothenberg, M. E. Fundamental signals that regulate eosinophil homing to the gastrointestinal tract. *J. Clin. Invest.* (1999). doi:10.1172/JCI6560

368. Jones, D. G. The Eosinophil. *Journal of Comparative Pathology* (1993). doi:10.1016/S0021-9975(08)80204-6

369. Mould, A. W., Matthaei, K. I., Young, I. G. & Foster, P. S. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J. Clin. Invest.* (1997). doi:10.1172/JCI119234

370. Yoshida, T. *et al.* Defective B-1 cell development and impaired immunity against angiostrongylus cantonensis in IL-5Rα-deficient mice. *Immunity* (1996). doi:10.1016/S1074-7613(00)80414-8

371. Kopf, M. *et al.* IL-5-Deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* (1996). doi:10.1016/S1074-7613(00)80294-0

372. Fujiu, K. *et al.* A heart-brain-kidney network controls adaptation to cardiac stress through tissue macrophage activation. *Nat. Med.* (2017). doi:10.1038/nm.4326

373. Luetteke, N. C. *et al.* Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* (1999).

374. Zhu, Z. *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* (1999). doi:10.1172/JCI5909

375. Tanabe, T. *et al.* Modulation of mucus production by interleukin-13 receptor α2 in the human airway epithelium. *Clin. Exp. Allergy* (2008). doi:10.1111/j.1365-2222.2007.02871.x

376. Liang, H.-E. *et al.* Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.1003988107

377. Kim, B. S. *et al.* TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci. Transl. Med.* (2013). doi:10.1126/scitranslmed.3005374

378. Urban, J. F. *et al.* IL-13, IL-4Rα, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. *Immunity* (1998). doi:10.1016/S1074-7613(00)80477-X

379. Zhao, J. *et al.* Sphingosine-1-phosphate receptor-2 facilitates pulmonary fibrosis through potentiating IL-13 pathway in macrophages. *PLoS One* (2018). doi:10.1371/journal.pone.0197604

380. Daniel, C. *et al.* FTY720 ameliorates oxazolone colitis in mice by directly affecting T helper type 2 functions. *Mol. Immunol.* (2007). doi:10.1016/j.molimm.2007.02.026

381. Hung, Y. P. *et al.* Proton-Pump Inhibitor Exposure Aggravates Clostridium difficile-Associated Colitis: Evidence from a Mouse Model. *J. Infect. Dis.* (2015). doi:10.1093/infdis/jiv184

382. Moy, F. J., Diblasio, E., Wilhelm, J. & Powers, R. Solution structure of human IL-13 and implication for receptor binding. *J. Mol. Biol.* (2001). doi:10.1006/jmbi.2001.4764

383. Zurawski, S. M., Vega, F., Huyghe, B. & Zurawski, G. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. *EMBO J.* (2018). doi:10.1002/j.1460-2075.1993.tb05927.x

384. Goenka, S. & Kaplan, M. H. Transcriptional regulation by STAT6. *Immunologic Research* (2011). doi:10.1007/s12026-011-8205-2

385. Zhu, J. *et al.* Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses. *Nat. Immunol.* (2004). doi:10.1038/ni1128

386. Kaplan, M. H., Schindler, U., Smiley, S. T. & Grusby, M. J. Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity* (1996). doi:10.1016/S1074-7613(00)80439-2

387. Kurowska-Stolarska, M. *et al.* IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J. Immunol.* **183**, 6469–77 (2009).

388. Li, D. *et al.* IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. *J. Allergy Clin. Immunol.* **134**, 1422–1432 (2014).

389. Borthwick, L. a *et al.* Macrophages are critical to the maintenance of IL-13-dependent lung inflammation and fibrosis. *Mucosal Immunol.* **9**, 1–18 (2015).

390. Herbert, D. R. *et al.* Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis. *J. Immunol.* **184**, 6438–6446 (2010).

391. Martinez, F. O., Helming, L. & Gordon, S. Alternative Activation of Macrophages: An Immunologic Functional Perspective. *Annu. Rev. Immunol.* **27**, 451–483 (2009).

392. Jackson-Jones, L. H. *et al.* IL-33 delivery induces serous cavity macrophage proliferation independent of interleukin-4 receptor alpha. *Eur. J. Immunol.* (2016). doi:10.1002/eji.201646442

393. Tu, L. *et al.* IL-33-induced alternatively activated macrophage attenuates the development of TNBS-induced colitis. *Oncotarget* (2017). doi:10.18632/oncotarget.15984

394. Simoni, Y. *et al.* Human Innate Lymphoid Cell Subsets Possess Tissue-Type Based Heterogeneity in Phenotype and Frequency. *Immunity* (2017). doi:10.1016/j.immuni.2016.11.005

395. Klose, C. S. N. *et al.* A T-bet gradient controls the fate and function of CCR6-RORγt+innate lymphoid cells. *Nature* (2013). doi:10.1038/nature11813

396. Satoh-Takayama, N. *et al.* Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46+ Cells that Provide Innate Mucosal Immune Defense. *Immunity* (2008). doi:10.1016/j.immuni.2008.11.001

397. Youngster, I. *et al.* Fecal microbiota transplant for relapsing clostridium difficile infection using a frozen inoculum from unrelated donors: A randomized, open-label, controlled pilot study. *Clin. Infect. Dis.* (2014). doi:10.1093/cid/ciu135

398. Lee, C. H. *et al.* Frozen vs fresh fecal microbiota transplantation and clinical resolution of diarrhea in patients with recurrent clostridium difficile infection a randomized clinical trial. *JAMA - J. Am. Med. Assoc.* (2016). doi:10.1001/jama.2015.18098

399. Hota, S. S. *et al.* Oral vancomycin followed by fecal transplantation versus tapering oral vancomycin treatment for recurrent clostridium difficile infection: An open-label, randomized controlled trial. *Clin. Infect. Dis.* (2017). doi:10.1093/cid/ciw731

400. Kelly, C. R. *et al.* Effect of fecal microbiota transplantation on recurrence in multiply recurrent clostridium difficile infection a randomized trial. *Ann. Intern. Med.* (2016). doi:10.7326/M16-0271

401. Youngster, I. *et al.* Oral, Capsulized, Frozen Fecal Microbiota Transplantation for Relapsing Clostridium difficile Infection. *JAMA - J. Am. Med. Assoc.* (2014). doi:10.1001/jama.2014.13875

402. Youngster, I. *et al.* Oral, frozen fecal microbiota transplant (FMT) capsules for recurrent Clostridium difficile infection. *BMC Med.* (2016). doi:10.1186/s12916-016-0680-9

403. NCT02437487. SER-109 Versus Placebo to Prevent Recurrent Clostridium Difficile Infection (RCDI). *Https://clinicaltrials.gov/show/nct02437487* (2015).

404. Chen, W.-Y., Tsai, T.-H., Yang, J.-L. & Li, L.-C. Therapeutic Strategies for Targeting IL-33/ST2 Signalling for the Treatment of Inflammatory Diseases. *Cell. Physiol. Biochem.* **49**, 349–358 (2018).

405. Kappos, L. *et al.* Treatment with interferon beta-1b delays conversion to clinically definite and McDonald MS in patients with clinically isolated syndromes. *Neurology* (2006). doi:10.1212/01.wnl.0000237641.33768.8d

406. Paty, D. W. & Li, D. K. B. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis: II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. *Neurology* (2012). doi:10.1212/wnl.43.4.662

407. Stremska, M. E. *et al.* IL233, A Novel IL-2 and IL-33 Hybrid Cytokine, Ameliorates Renal injury. *J. Am. Soc. Nephrol.* (2017). doi:10.1681/ASN.2016121272

408. Cayrol, C. & Girard, J.-P. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. *Curr. Opin. Immunol.* (2014). doi:10.1016/j.coi.2014.09.004

409. Liew, F. Y., Girard, J. P. & Turnquist, H. R. Interleukin-33 in health and disease. *Nature Reviews Immunology* (2016). doi:10.1038/nri.2016.95

410. Hayakawa, H., Hayakawa, M., Kume, A. & Tominaga, S. I. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J. Biol. Chem.* (2007). doi:10.1074/jbc.M704916200

411. Weinberg, E. O. *et al.* Expression and regulation of ST2, an interleukin-1 receptor family member, in cardiomyocytes and myocardial infarction. *Circulation* (2002). doi:10.1161/01.CIR.0000038705.69871.D9

412. Kumar, S., Tzimas, M. N., Griswold, D. E. & Young, P. R. Expression of ST2, an interleukin-1 receptor homologue, is induced by proinflammatory stimuli. *Biochem. Biophys. Res. Commun.* (1997). doi:10.1006/bbrc.1997.6810

413. Weinberg, E. O. *et al.* Identification of serum soluble ST2 receptor as a novel heart failure biomarker. *Circulation* (2003). doi:10.1161/01.CIR.0000047274.66749.FE

414. Ho, J. E. *et al.* Common genetic variation at the IL1RL1 locus regulates IL-33/ST2 signaling. *J. Clin. Invest.* (2013). doi:10.1172/JCI67119

415. Zhang, J. *et al.* ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease. *Sci. Transl. Med.* (2015). doi:10.1126/scitranslmed.aab0166

416. Bandara, G., Beaven, M. A., Olivera, A., Gilfillan, A. M. & Metcalfe, D. D. Activated mast cells synthesize and release soluble ST2-a decoy receptor for IL-33. *Eur. J. Immunol.* (2015). doi:10.1002/eji.201545501

417. Zhao, J. *et al.* Lysophosphatidic acid increases soluble ST2 expression in mouse lung and human bronchial epithelial cells. *Cell. Signal.* (2012). doi:10.1016/j.cellsig.2011.08.004

418. Demyanets, S. *et al.* Components of the interleukin-33/ST2 system are differentially expressed and regulated in human cardiac cells and in cells of the cardiac vasculature. *J. Mol. Cell. Cardiol.* **60**, 16–26 (2013).

419. Ramadan, A. M. *et al.* From proteomics to discovery of first-in-class ST2 inhibitors active in vivo. *JCI Insight* **3**, (2018).

420. Reichenbach, D. K. *et al.* The IL-33/ST2 axis augments effector T-cell responses during acute GVHD. *Blood* (2015). doi:10.1182/blood-2014-10-606830

421. C.-Y., Y. *et al.* Small molecule ST2 inhibitors cause reduction of soluble ST2 and improve GVHD and survival in vivo. *Blood* (2016).

422. Townsend, M. J., Fallon, P. G., Matthews, D. J., Jolin, H. E. & McKenzie, a N. T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J. Exp. Med.* **191**, 1069–1076 (2000).

423. Warren, C. A. *et al.* Amixicile, a Novel Inhibitor of Pyruvate:Ferredoxin Oxidoreductase, Shows Efficacy against Clostridium difficile in a Mouse Infection Model. *Antimicrob. Agents Chemother.* **56**, 4103–4111 (2012).

424. Irizarry, R. A. *et al.* Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* (2003). doi:10.1093/nar/gng015

425. Seekatz, A. M. *et al.* Fecal microbiota transplantation eliminates Clostridium difficile in a murine model of relapsing disease. *Infect. Immun.* (2015). doi:10.1128/IAI.00459-15

426. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* (2013). doi:10.1128/AEM.01043-13

427. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* (2016). doi:10.1038/nmeth.3869

428. Pruesse, E. *et al.* SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* (2007). doi:10.1093/nar/gkm864

429. McMurdie, P. J. & Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* (2013). doi:10.1371/journal.pone.0061217

430. Oksanen, A. J. *et al.* Vegan: Community Ecology Package. *https://github.com/vegandevs/vegan* (2016). doi:10.4135/9781412971874.n145

431. Pawlowski, S. W. *et al.* Murine Model of *Clostridium difficile* Infection with Aged Gnotobiotic C57BL/6 Mice and a BI/NAP1 Strain. *J. Infect. Dis.* (2010). doi:10.1086/657086