Mechanisms of Neutrophil Recruitment and Immunopathology During Acute *Clostridium difficile* Colitis

by

Andrew J McDermott

A dissertation submitted in partial fulfillment
Of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
2015

Doctoral Committee:

Professor Gary B. Huffnagle, Chair
Professor Philip C. Hanna
Associate Professor John Y. Kao
Associate Professor Vincent B. Young

To Mom, Dad, and Daniel

Acknowledgments

Firstly, I must recognize the tremendous contributions of my mentor, Dr. Gary Huffnagle, towards completing this dissertation. Your mentorship, the people you have surrounded yourself and myself with, and your motivation allowed me to grow and succeed as a scientist. I am proud to have been a part of your lab for the past 4 years, and I wish you nothing but success in the future. Thank you for everything.

I would also thank my family for all the support they have given me throughout my life. Thank you for always supporting me, and for always believing in me, especially when I wasn't able to believe in myself. A son never had more devoted and loving parents. Daniel, you are a tremendous brother. Thank you for being exactly who you are, because you are the man.

I would also thank all of the students in the Microbiology and Immunology department. Each of us has faced different challenges over the past five years, both personally and professionally, and I am undoubtedly the better having shared this part of my life with all of you. You are some of the most talented and motivated individuals I have ever known, and there is no doubt in my mind that you will all be successful.

I would also acknowledge Bill and Jean Gosling. Bill and Jean, you have both shown me nothing but kindness. Thank you so much for all the dinners and lunches

and shopping trips, but especially for being so thoughtful and welcoming to a 22 year old who had just moved 1,000 miles away from home. You are both class acts, and I wish you and your family the best in the future.

I must also acknowledge the great contributions of the office staff of both the Microbiology and Immunology Department and the Pulmonary Medicine and Critical Care. Heidi, thank you for relentlessly efforts advocating for the students of the Microbiology and Immunology Department. Cat Meyer, thank you for helping to schedule my public defense. Mary Jo Sheedy Lapointe, thanks for recommending Fraser's Pub to me, and for appreciably increasing my knowledge of NHL hockey. You are all wonderful young ladies.

I must also thank my dissertation committee: Drs. Philip Hanna, John Kao, and Vincent Young. Thank you for your insight, your advice, and you constructive criticism. Your input made my project all the stronger. But most of all, thank you for your commitment to my own personal development. Your motivation and professional insight helped me to progress immensely as a scientist, and have in no small part contributed to my success.

I cannot conclude these acknowledgments without thanking the wonderful members of the Huffnagle lab, both past and present. Nicole, John, Amir, Rod, Brittan, Bob, Ryan, Katy, Zach, Dayana, Dustin, Nick, Ellie, Chinmay, and Charlie; you are all such characters. But you all have character, and having such fun and caring coworkers made coming to work a blast.

Nicole and Rod, thank you all for your help during those long days of isolating leukocytes. Under your tutelage, I went from a guy afraid to handle the mice to a

professional mouse wrangler. Amir, thank you for teaching me the ways of Immunology. I now think like an immunologist, most of the time. John, thank you for being a nerd and for forcing me to watch awesome movies. I still owe you a game of Starcraft. Charlie: thank you for all your contributions to my project. It was my privilege to mentor you and assist with your honors thesis. You will be a tremendous clinician. Brittan, keep on dancing, even if you are the only one who can hear the music. I wish all of you the very best in the future.

Huffnagle Lab Keurig: thank you for the hundreds of cups of coffee and tea you have made me over the past few years. There will never be another coffee maker like you.

Table of Contents

List of Figures

Chapter 1

Background

Portions of this chapter have been previously published

McDermott AJ, Huffnagle GB. 2014. The microbiome and regulation of mucosal immunity. Immunology **142:**24-31.

Kingdom: Eubacteria(1)
Phylum: Firmicutes(2)
Class: Clostridia(2)
Order: Clostridiales(2)
Family: Peptostreptococcaceae(2)
Genus: *Clostridium*(2)
Species: *difficile*(2)
Gram Staining: Gram positive(3)
Morphology: Bacillus(4)
Spore Formation: Yes(3, 5)
Spore Characteristics: Antibiotic-resistant(5, 6), Alcohol-resistant(3, 5, 7), Aero tolerant(5). Heat-resistant (60^0C for 24 hours)(7).
Motility: Motile(4), Flagellated(8-11). Flagellar components have been implicated in adherence and colonization in the host(8, 12, 13), but data is conflicting and inconclusive.
Aerotolerance: Obligate Anaerobe(4, 14)
Vegetative Propagation: Grows well at 37^0C in the absence of oxygen(14)
Doubling Time: 40-70 minutes(15)
Virulence Factors: Two large clostridial toxins, TcdA(308kDa) and TcdB(270kDa)(3). Binary toxin (CDT) found in a low percentage of strains as well(3, 16).
Genetic Features: Pathogenicity Locus (PaLoc). 19.6 kilobases long. Contains *tcdA* and *tcdB* genes (encoding TcdA and TcdB). Also contains *tcdE* (encodes a holin permitting toxin release), and *tcdD* and *tcdC*, positive and negative regulators of the locus respectively(3, 17, 18).
Cdt locus (CdtLoc). Encodes the two subunits of binary toxin, *cdtA* and *cdtB*, as well as *cdtR*, a putative regulator of the locus. Found in only a small percentage of *C. difficile* stains(3, 19, 20).
Genome Size: 4.29 megabases(21)
Antibiotic Resistance (Reported): Bacitracin, Daunorubicin, Nogalamycin, Erythromycin, Clindamycin, Gentamicin, Tetracycline, Ampicillin, Cefotaxime, Cefoxitin, Cefuroxime(21). Cycloserine(14).
Antibiotic Sensitivity (Reported): Teicoplanin, Vancomycin(21).
Strains Used in This Dissertation: VPI 10463: ATCC 43255 630: ATCC BAA-1382
Representative Genomes: VPI 10463: GenBank Accession Number: NZ_ABKJ00000000.2 630: GenBank Accession Number: AM180355

Table 1: Microbiological Characteristics of *Clostridium difficile*

Clinical Significance of *Clostridium difficile*

Clostridium difficile (*C. difficile*) is a Gram-positive spore-forming obligate anaerobic bacterium (Table 1) and is a causative agent of antibiotic-associated diarrhea and the major causative agent of antibiotic associated pseudomembranous colitis(22-24). Though *C. difficile* was first described in 1935 after its isolation from the feces of healthy infants(22-24), it was not until the late 1970s when a series of experiments with clindamycin-treated hamsters identified *C. difficile* as the causative agent of antibiotic-associated pseudomembranous colitis(22, 25, 26). Subsequent studies have revealed that colonization with *C. difficile* was associated with a broad array of clinical outcomes in patients: from asymptomatic colonization to diarrhea to severe outcomes including pseudomembranous colitis and fulminant colitis(23, 27, 28). Indeed, despite *C. difficile* being first described as a human pathogen in relation to pseudomembranous colitis, less than half of all patients infected with *C. difficile* actually develop pseudomembranous colitis(23, 29, 30).

The prevalence and severity of *C. difficile* infection in patients has increased considerably in recent years(23, 31, 32). The incidence of *C. difficile* infection in the US has drastically increased in recent years from less than 150,000 cases in 2000 to nearly 350,000 cases reported in 2010(31). The higher incidence of disease has also been associated with an increase in mortality attributable to *C. difficile* infection: while *C. difficile* was associated with less than 6 deaths per one million population in 1999, by 2004 that number had climbed to nearly 24 deaths per one million population(23, 32). All told, *C. difficile* infection is estimated to be responsible for at

least 14,000 deaths and $1 billion in healthcare-associated costs annually in the United States(33-35), underscoring the clinical importance of this disease.

In recent years, it has been suggested that the emergence of so-called "hypervirulent" B1/NAP1/027 strains of *C. difficile* may be at least partly responsible for the increased frequency of *C. difficile* infection(36, 37). In addition to resistance to fluoroquinolones(36, 37), preliminary studies suggested that these "hypervirulent" isolates produced much higher levels of both TcdA and TcdB as compared to other clinical isolates(37, 38). Furthermore, NAP1/027 strains were also reported to sporulate at a faster rate and produce more spores overall than other *C. difficile* strains(37, 39, 40). Additionally, a recent study by Carlson Jr. and colleagues using a large collection of ribotype 027 strains found that in aggregate, ribotype 027 strains did not produce more spores than other *C. difficile* strains *in vitro*(41). However, *C. difficile* isolates from cases of severe disease produced significantly higher numbers of spores than all other strains assayed, suggesting that spore production, independent of ribotype, may contribute to the severity of *C. difficile* infection(41).

The main virulence factors of *C. difficile* are two large clostridial toxins, named TcdA and TcdB(18, 23, 27, 42). Both of these toxins are glycosyltransferases, which glucosylate and irrevocably inactivate small GTPases including Rho, Rac, and Cdc42 within target cells(18, 42). Upon binding to a target cell, both toxins are endocytosed and undergo a conformational change within the endosome(42-45). Though the process is better understood for TcdB, the internalization toxin ultimately results in the release of the N-terminal glucosyltransferase domain of the

toxin into the host cytosol(42, 46-48). Intoxication of eukaryotic cells with *C. difficile* toxin results in cell rounding and loss of cell membrane integrity, as well as cell death(18, 49, 50).

Pathogenesis of *C. difficile*

C. difficile was first identified as a causative agent of pseudomembranous colitis in the hamster model(22, 25, 26), and many investigations into the pathogenesis of *C. difficile* have been performed using this same model(51-56). As infection of hamsters(57, 58) or mice(59) with non-toxigenic (TcdA and TcdB negative) strains of *C. difficile* does not result in disease, and indeed precolonization of hamsters with non-toxin producing strains of *C. difficile* prevents the development disease upon challenge with toxigenic *C. difficile* strains(53, 57), many studies have focused on the role of *C. difficile* toxins in disease(51, 52, 56, 58, 60). However, many of these investigations have produced conflicting results. In one study genetic deletion of TcdB, but not TcdA, resulted a drastic decrease in mortality following infection with TcdB deficient mutants in an acutely fatal hamster model in one study(52). Additionally, numerous studies have demonstrated that *C. difficile* strains producing only TcdB are still capable of producing high levels of morbidity and mortality in clindamycin treated hamsters(51, 61). A subsequent study using TcdA or TcdB deficient mutants reported near identical mortality following infection of hamsters with strains expressing each toxin alone(58). While initial studies using strains of *C. difficile* deficient in TcdA and TcdB but positive for binary toxin indicated no role for binary toxin in promoting morbidity or mortality in

hamsters(56), a recent study has reported mortality following infection of hamsters with *C. difficile* expressing binary toxin but not TcdA and TcdB (60). Thus, while disease as a result of *C. difficile* infection is most certainly toxin-mediated, the relative importance of each individual toxin in driving disease development is unclear.

Colonization Resistance, the Microbiome, and Antibiotics

The microbiome provides numerous nutritional benefits to the host, including synthesizing vitamins(62) and short chain-fatty acids (SCFAs)(63), and the presence of the microbiome is also vitally important for the development and functionality of the intestinal immune system(64, 65). Additionally, the presence of the microbiome within the gastrointestinal tract limits the ability of pathogens, including *C. difficile*, to persist within the gut and cause disease, a phenomenon known as colonization resistance(66, 67).

Alteration of the intestinal microbiome through the use of antibiotics is commonly associated with increased susceptibility to intestinal pathogens(68-70). Metronidazole treatment markedly alters the composition of the colonic microbiota and is associated with increased disease severity during *C. rodentium* colitis(70). Antibiotic treatment also permits efficient *Campylobacter jejuni* infection in mice(69), as well as the development of acute typhlocolitis following *Salmonella typhimurium* infection(68). Furthermore, disruption of the intestinal microbiota by host inflammatory responses also permits the expansion of members of the Family

Enterobacteriaceae(71), highlighting the potential for unwanted bacterial overgrowth following disruption of the normal microbiota.

The intestinal microbiome controls the susceptibility of experimental animals to *C. difficile* colonization and infection. Under normal conditions mice are refractory to *C. difficile* infection(66, 72), however, antibiotic administration permits subsequent *C. difficile* colonization and the development of intestinal disease(59, 66, 72-82). Antibiotic treatments that permit *C. difficile* infection are associated with marked alterations in the composition and diversity, but not overall bacterial density, of the intestinal microbiome(66, 70, 73, 80, 83). Alterations in the structure of the microbiome are also associated with increased levels of certain metabolites, including taurocholate, succinate, and fructose, which can be utilized by *C. difficile* for outgrowth(83, 84), suggesting a potential mechanism of colonization resistance to *C. difficile*.

Structure and Cellular Composition of The Intestines

The gastrointestinal tract is the largest environment-exposed surface area in the body, and is in direct contact with a large and varied microbial community(85). Fortunately, the gastrointestinal tract is also home to a large variety of immune cells and structures that help maintain intestinal homeostasis in the face of microbial challenge(86-88). Intestinal epithelial cells physically separate underlying tissues from the intestinal lumen(89, 90), while goblet cells maintain a mucus layer to prevent microbial contact with epithelial cells(91, 92). Leukocytes beneath the

epithelial cell layer can both promote or inhibit inflammatory responses(93-96), and are efficiently organized into effector and inductive sites(97-99). This organization largely prevents unwanted inflammation while retaining the ability to respond rapidly to a wide array of perturbations.

The **intestinal epithelium** is actually a single layer of cells, all of which are derived from multipotent stem cells located within the intestinal crypts(86, 90). Collectively, these cells are responsible for nutrient absorption, physical exclusion of luminal contents from underlying tissues, antimicrobial peptide production, and maintenance of the intestinal mucus layer(86, 90).

Columnar epithelial cells constitute the majority of cells present in the intestinal epithelium(90, 100). Enterocytes provide a physical barrier separating the luminal contents of the GI tract from underlying tissues, as well as participating in the absorption of materials from lumen(89, 90). Epithelial cells directly participate in immunological surveillance and direction of host responses in the gut. Epithelial cells can express numerous Pattern Recognition Receptors (PRRS), including TLR5(101), TLR1, TLR2, TLR3, TLR9(86), and NOD2(89), and can produce chemotactic factors for both myeloid and lymphoid cells following inflammatory stimulation(102). IL-17 stimulation of intestinal epithelial cells can drive the expression of neutrophil chemokines(103). Epithelial cells can produce anti-microbial peptides, such as CRAMP, to directly influence microbial populations in the lumen of the gut(104). Additionally, epithelial cells can interact with leukocyte populations through the expression of both MHCII(105) and MHCI(106). Therefore, enterocytes play a key role in not only preventing microbes and microbial products

from penetrating to underlying tissues, but also initiating and directing inflammatory responses.

Goblet cells are another class of specialized epithelial cells found in the intestinal epithelium(100, 107). While goblet cells can be found in both the small and large intestines, they represent approximately 15 percent of the cells found in the large intestine epithelium(100, 107). Goblet cells contain large mucus-laden vacuoles(107), and express high levels of the MUC2 gene(91). MUC2 is the major structural component of both intestinal mucus layers(108). The lower mucus layer makes direct contact with the intestinal epithelium and is rarely contaminated with bacteria, while the outer layer contacts the intestinal lumen and the intestinal microbiome(92). Goblet cells have also recently been found to produce the antimicrobial peptides Ang4, RegIIIγ and RegIIIβ(109, 110). RegIIIγ activity is especially important in preventing microbial contact with the underlying epithelium(111). Goblet cells may also transfer antigens acquired in the intestinal lumen to dendritic cells in the lamina propria(112). These studies have demonstrated a potential role for goblet cells beyond mucus production by participating directly in the uptake of antigen and influencing the development of inflammatory responses.

Within the intestinal epithelium resides a population of lymphocytes referred to as **Intraepithelial lymphocytes** (IEL)(113). Almost all IELs are T cells, with both $\alpha\beta^+$ and $\gamma\delta^+$ populations represented(113, 114). Adherence of IELs to epithelial cells is mediated by interactions between CD103 expressed on IELS, and E-cadherin expressed on epithelial cells(115). Many IELs at baseline display a mixed

phenotype, with expression of some activation markers but not others(116). However, following stimulation, IELs become activated and express effector cytokines including IFNγ and KGF(116-118). IELs can exert both protective and pathogenic roles during inflammation: while IEL-derived KGF is believed to protect the epithelium from damage during chemically induced colitis(119), IELs producing IFNγ and TNFα have been associated with the development of inflammatory bowel disease(120). The proximity of IELs to the lumen of the gut, and their ability to rapidly produce both inflammatory and epithelial-protective signals, make them key "first line" defenders in the intestinal tract.

Underlying the intestinal epithelium is the **lamina propria**, an area rich in B and T lymphocytes(86). In contrast to Peyer's patches, which are inductive sites for the priming of lymphocytes, the lamina propria is an effector site where activated lymphocytes respond to appropriate stimulation(97-99). αβ TCR^+ T-cells are the most common lymphocyte within the small intestinal lamina propria(114). In keeping with the effector function of the lamina propria, T-cells found within the lamina propria express markers indicative of activation, including high levels of CD69 and CD25 (121), as well as spontaneously secreting of IL-4 and IFNγ(122). Subsets within this population have drastically different activities: while $CD4^+$ $CD25^+$ regulatory T-cells in the lamina propria can inhibit T-cell proliferation, cytokine production and the development of colitis(94, 95), lamina propria $CD4^+$ T-cells can secrete both IL-17 and IL-22 and are associated with the development of intestinal inflammation(93, 96). Therefore, lamina propria T-cells have the ability to

rapidly react to signals received from the luminal environment and initiate both inflammatory and anti-inflammatory responses.

In contrast to the small intestine, **B-cells** are the predominant lymphocyte present in the lamina propria of the large intestine(114). Lamina propria B-cells secrete dimeric IgA, which is trancytosed through epithelial cells to the lumen of the gut through the action of the polymeric immunoglobulin receptor (pIgR)(123, 124). While antigen-specific IgA can be generated during intestinal infection(125), intestinal IgA secretion also plays a key role at baseline by inhibiting the penetration of commensal microbes through the epithelium and enhancing the uptake of luminal bacterial by M-cells(123). Intestinal IgA can also directly modulate the composition of the intestinal microbiome(126), highlighting the key role of IgA and lamina propria B-cells in shaping both the membership and location of the microbiome.

Lamina propria dendritic cells (LPDCs) play a large role in determining whether the response to a particular antigen will be inflammatory or anti-inflammatory. LPDCs capture luminal antigen by extending their processes through the epithelial cell layer, a process dependent on CX3CR1(127). There are two broad classifications of LPDCs to consider: $CD103^+$ and $CD103^-$. $CD103^+$ LPDCS promote the generation of $Foxp3^+$ regulatory T-cells through the secretion of retinoic acid and in combination with TGF-β(87, 88). In contrast, $CD103^-$ LPDCs support the development of inflammation, and increase expression of inflammatory mediators such as TNFα and IL-6 following stimulation with TLR ligands(128). The presence of $CD103^+$ LPDC is particularly important in preventing unnecessary inflammation, as

the absence of $CD103^+$ $CX3CR1^-$ LPDCs enhances epithelial damage during colitis (129).

Innate Lymphoid Cells (ILCs) are another cellular population found in the lamina propria(130, 131). ILCs morphologically resemble lymphocytes, but do not possess recombination activating gene-dependent antigen receptors(132). ILCs can be broken down into three broad groups(132). The defining characteristic of group 1 ILCs, such as NK cells, is the production of IFNγ(132). Many group 1 ILCs are also T-bet^+(132, 133), and group 1 ILCs can be found at sites of mucosal inflammation(133). In contrast, generation of group 2 ILCs requires GATA3 and RORα(132), and IL-5 and IL-13 are the signature cytokines of this group(132). Group 2 ILCs are important in responding to nematode infections(132).

Particularly relevant to the intestinal tract are **group 3 ILCs**, which are primarily defined by their ability to produce IL-22 and IL-17(132). Additionally, the generation and activity of group 3 ILCs is dependent on RORγt(132). Recent evidence has strongly suggested that IL-17 positive group 3 ILCs drive colonic inflammation during *Helicobacter hepaticus* infection(131). In contrast, during *Citrobacter rodentium* (*C. rodentium*) colitis group 3 ILCs are known to produce IL-22(130). IL-22 drives antimicrobial peptide expression and is required to prevent severe intestinal pathology and mortality during *C. rodentium* colitis(134). Thus, group 3 ILCs are important intestinal sources of IL-17 and IL-22, and can both promote and protect against intestinal pathology during insult(131, 132, 134).

Innate Inflammation in the Large Bowel

The host response to a wide range of bacterial and chemical perturbations within the large intestine is characterized by conserved immunological phenomena. Inflammatory cytokines, including IL-23, IFNγ, and TNFα are induced locally in response to the challenge(82, 135-137). Leukocyte recruiting chemokines, including CXCL1, CXCL2, and CCL2 are also induced, and are associated with an influx of monocytes and neutrophils into the mucosa(78, 80, 82, 135, 138, 139). Finally, intestinal histopathology characterized by epithelial damage and/or edema develops, as a result of either the perturbing agent directly or by the resulting immune response(49, 72, 80, 134, 138, 140).

Neutrophils, defined by high levels of Gr-1 (Ly6G) and CD11b expression, are myeloid cells that are rapidly recruited to the colonic epithelium during colitis(78, 80, 82, 139). Neutrophil recruitment is often beneficial to the host, as neutrophils are capable of phagocytosing pathogens(141, 142), and interference with neutrophil recruitment is associated with decreased host survival during acute large bowel infections(74, 76, 78, 143). Neutrophils are also capable of supporting inflammatory responses directly by producing inflammatory cytokines directly including IL-1β, TNFα, and IL-8(141, 144). However, neutrophil recruitment is commonly associated with the development of colonic histopathology(82, 138), and neutrophil elastase is partially responsible for intestinal histopathology during DSS colitis(140). Thus, neutrophil recruitment can both reduce pathogen burden and protect against mortality as well as drive tissue damage during innate colitis.

IL-23 is a critical driver of the host response during acute inflammation at mucosal sites, including in the large bowel(135, 138, 145-147). IL-23 promotes neutrophil recruitment during acute pulmonary inflammation(145-147), and the recruitment of neutrophils is largely dependent on IL-23 in both infectious and chemically induced models of large bowel inflammation(135, 138). The expression of the neutrophil chemotactic factor KC (CXCL1) is also driven by IL-23 during *S. typhimurium* typhlocolitis(135). Additionally, expression of the antimicrobial peptide RegIIIγ is almost entirely dependent on IL-23 during *S. typhimurium* typhlocolitis as well as DSS colitis(135, 138).

IL-22 is a pleiotropic cytokine that is induced in response to mucosal inflammation, including innate colitis(80, 82, 135, 138, 148). The induction of IL-22 at mucosal sites is driven by IL-23(134, 135, 138, 146), and several studies have demonstrated an epithelial-protective role for IL-22 during mucosal inflammation(134, 149). IL-22 limits the severity of intestinal histopathology during infectious and chemically induced colitis(134, 149). Furthermore, IL-22 both promotes RegIIIγ induction and limits mortality during *C. rodentium* colits(134). However, IL-22 also promotes neutrophil recruitment during chemically induced pulmonary inflammation(148), and stimulation of colonic epithelial cells and colonic subepithelial myofibroblasts with IL-22 results in the production of neutrophil chemotactic factors(150, 151). As such, IL-22 can potentiate a wide array of host responses at mucosal sites.

IL-17 is a proinflammatory cytokine that is commonly and rapidly induced at sites of mucosal inflammation(80, 135, 136, 138, 146, 148). IL-17 promotes

neutrophil recruitment in models of pulmonary inflammation including *K. pneumoniae* infections(152) and bleomycin challenge(148), as well as intestinal inflammation models including *S. typhimurium* infection(153) and TNBS(154) and DSS(155) colitis. IL-17 also contributes to epithelial damage during colonic inflammation as well(154, 155). Recent studies have demonstrated that IL-23 can drive the expression of IL-17 during mucosal inflammation(135, 138).

Previous studies have reported a role for **TNFα** signaling in promoting myeloid cell recruitment during mucosal inflammation (156, 157). TNFα can enhance the expression of CCL3 during chemically-induced pulmonary inflammation (157), and interference with TNFα signaling reduces neutrophil recruitment in response to acute allergic airway inflammation (156). TNFα can contribute to inflammatory cytokine expression and tissue damage during mucosal inflammation(158, 159). Directly applicable to gastrointestinal inflammation, during 2,4,6-Trinitrobenzenesulfonic acid (TNBS) colitis, TNFα signaling promotes both IL-18 and TNFα expression as well as the development of intestinal histopathology (159). Furthermore, TNFα also protects against immunopathology in response to *C. rodentium* colitis(137), highlighting the pleiotropic nature of TNFα signaling during mucosal inflammation.

Likewise, **GM-CSF** is a potent driver of mucosal inflammation in numerous settings, including the intestinal tract(160-162). GM-CSF can play a role in neutrophil recruitment during acute pulmonary inflammation (both chemical and microbial)(163-166) and drive maximal production of TNFα and CXCL2 in response to pulmonary LPS challenge(163). Colonic IL-6 production during chemically-

induced colitis has also been shown to be GM-CSF-dependent(167). However, GM-CSF signaling also serves to protect the epithelium from damage during mucosal inflammation(167-170). Ablation of GM-CSF signaling can result in a significant increase in colonic histopathology, including colonic ulceration, during dextran sulfate sodium (DSS)-induced colitis(167, 169). Furthermore, treatment of afflicted animals with exogenous GM-CSF is capable of reducing colonic ulceration in the same model(168). Thus, the host response to innate colitis is characterized by the induction of pleiotropic cytokines, capable of potentiating inflammatory responses such as neutrophil recruitment as well as epithelial protective responses(134).

Innate Inflammatory Responses to *C. difficile* TcdA and TcdB

Many studies have focused on the innate inflammatory responses of cultured myeloid cell populations to *C. difficile* TcdA and TcdB; the two best characterized virulence factors in *C. difficile*. Stimulation of human monocytes with TcdA or TcdB induces high levels of IL-8 production, and TcdA stimulation alone is sufficient to elicit significant production of both IL-1β and TNFα(171). A more recent study has demonstrated that IL-1β production in THP-1 monocyte-like cells in response to TcdA and TcdB is associated with caspase 1 activation, and that IL-1β production was drastically reduced in peritoneal macrophages isolated from ASC-deficient mice(172). Dendritic cells also react rapidly to *C. difficile* stimulation: treatment of bone marrow derived immature dendritic cells with TcdA results in maturation,

characterized by increased expression of CD80, CD86, and MHCII, within 24 hours of stimulation(173). Dendritic cells under these conditions also express high levels of CXCL2 transcript within 12 hours of treatment with TcdA(173). Bone marrow derived dendritic cell maturation and activation can also be driven by stimulation with *C. difficile* surface layer proteins in a TLR4-dependent manner(174). Additionally, mast cells release high levels of TNFα following treatment with TcdA(175).

Though intoxication of intestinal epithelial cells with *C. difficile* toxins results in cell rounding and death(49, 176, 177), these cells also mount innate inflammatory responses following exposure to *C. difficile* toxins(176, 178-180). One of the most commonly reported responses of epithelial cells *in vitro* is increased expression of IL-8, which has been reported from both colonocyte and goblet cell-like cell lines(176, 178, 180). The secretion of these chemokines also appears to be directed, as treatment of polarized epithelial cells with TcdA *in vitro* results in primarily basolateral secretion of IL-8(180). Furthermore, epithelial cells isolated from *in vivo* ileal loops injected with TcdA show increased expression of CXCL2(179).

As mice are refractory to *C. difficile* colonization without antibiotic pretreatment(72), many early animal studies were performed by delivering purified *C. difficile* toxins directly to the gastrointestinal tract(172, 181-183). *in vitro* experiments using excised rabbit ileum revealed that treatment with *C. difficile* toxins promotes intestinal fluid secretion(184). Treatment of intestinal epithelial cell monolayers *in vitro* with purified TcdA results in rapid loss of transepithelial resistance and enhances tight junction permeability(185), suggesting the fluid

secretion seen in toxin-treated animals was due to loss of epithelial integrity caused by TcdA intoxication. Subsequent studies utilizing ileal loops instilled with *C. difficile* toxin also demonstrated high levels of fluid secretion in response to toxins, as well as marked disruption of intestinal architecture and neutrophil recruitment (172, 182, 183, 186, 187).

The ileal loop model has also been used to investigate the immunological drivers of inflammation in response to *C. difficile* toxins, especially those mechanisms underlying neutrophil recruitment(172, 179, 181, 186, 188). Studies using the ileal loop model reported decreased CXCL1 expression and decreased MPO levels in the absence of IFNγ(188). The depletion of mast cells also impairs neutrophil recruitment in response to TcdA(187), as does the chemical inhibition p38 MAP kinase activation(189). Rat ileum epithelial cells have also a cellular source of CXCL2 following TcdA administration to the rat ileum(179). Furthermore, neutrophils have also been suggested to promote intestinal histopathology in response to *C. difficile* toxins, as interference with neutrophil recruitment using an anti-CD18 antibody is also associated with decreased fluid secretion and epithelial damage in the rabbit ileum(186). Finally, Ng and colleagues demonstrated that IL-1β, IL-18, and CXCL1 production as well as intestinal histopathology, in the ileum in response to TcdA and TcdB was largely dependent upon the NLRP3 inflammasome(172). Many of these results, including the requirement for NLRP3 inflammasome activity for IL-1β production and neutrophil recruitment (as assessed by MPO activity), were later confirmed in the colon by administering *C. difficile* toxin intrarectally(181).

Mouse Modeling of *Clostridium difficile* Colitis

While the work by Hirota and colleagues and others demonstrate that toxin-based models can provide mechanistic insight into the initial inflammatory response to *C. difficile* toxins(172, 179, 181, 186, 188), such models are not without limitations. In addition to potential differences in the host response to purified bacterial toxins as opposed to a metabolically active infectious organism, toxin based models often offer a very limited timeframe before which samples must be collected (often four hours or less)(172, 179, 181, 188).

In recent years numerous murine models of *C. difficile* infection, relying on antibiotic pretreatment to permit infection, have been developed. The first published model, developed by Chen at colleagues, relied on the administration of a cocktail of kanamycin, gentamicin, colistin, metronidazole, and vancomycin in the drinking water as well as a subsequent intraperitoneal injection of clindamycin to permit *C. difficile* colonization and infection(72). This particular model has been utilized in several studies to infect mice with *C. difficile* and investigate the host response to infection(66, 77, 82, 190). Subsequent studies have utilized modified versions of the aforementioned protocol with the addition of ampicillin to the cocktail(74-76), while other groups have developed models using solely intraperitoneal clindamycin injections(73, 78), or the use of broad-spectrum third-generation cephalosporins in drinking water(59, 79-81, 83, 191, 192) to permit *C. difficile* colonization.

Two strains of *C. difficile*, VPI 10463 and 630, are commonly used in mouse models of *C. difficile* colitis(59, 66, 74-76, 78-83). Both VPI 10463(17, 193) and

630(21) contain the pathogenicity locus, which encodes genes for TcdA and TcdB production, release, and regulation(18). However, *in vivo* infection with *C. difficile* strain 630 is associated with less cytotoxicity (per gram of cecal content) than VPI 10463 infection(59). Consistently, 630 infection is associated with less mortality and less severe intestinal histopathology as compared to VPI 10463 infection(59). However, infection of susceptible animals with either strain results in a conserved host response characterized by inflammatory cell recruitment, the induction of inflammatory cytokines and chemokines, and the development of intestinal histopathology in the large bowel (59, 66, 74-76, 78-82). Thus, while the overall severity of disease differs between the two strains of *C. difficile*, the host inflammatory responses to both strains are conserved, and infection with either strain serves as an appropriate context in which the investigate the mechanisms underlying inflammatory responses during *C. difficile* colitis.

The Host Response to *Clostridium difficile* Colitis *in vivo*

Utilizing the mouse models mentioned above, numerous groups have begun to investigate the mechanisms underlying inflammation, microbial recognition, and immunopathology during *C. difficile* infection(74-82, 174, 190, 191, 194). Initial studies, using vegetative *C. difficile* as the infectious challenge, reported marked myeloid cell recruitment to the large bowel(59, 72, 74, 76, 80, 82, 195). For example, Sadighi Akha and colleagues reported significant neutrophil recruitment to the colon in association with marked intestinal histopathology and increased expression

of inflammatory cytokines including TNFα, GM-CSF, and CXCL1(82). Subsequent studies inoculating with *C. difficile* spores likewise reported marked neutrophil recruitment to the large bowel associated with the activation of numerous inflammatory pathways(78, 79, 81, 191, 194). Collectively, these studies demonstrate that infection with viable *C. difficile*, either vegetative cells or *C. difficile* spores, results in a conserved host response highlighted by robust neutrophil recruitment, marked intestinal histopathology, and the induction of inflammatory cytokines and chemokines including CXCL1(59, 72, 74-82, 191, 194).

Recent studies have focused on the host signals required for full recruitment of neutrophils in response to infection(74-76, 78, 79, 81). For example, Hasegawa and colleagues demonstrated a key role for NOD1 in supporting CXCL1 expression and neutrophil recruitment to the large bowel following *C. difficile* infection(76). Furthermore, Jarchum and colleagues also reported decreased neutrophil recruitment and CXCL1 expression in MyD88-deficient mice(78). Additional studies have suggested roles for ASC(74) and the combined signaling of IL-22 and CD160(81) in driving neutrophil recruitment to the large intestine in response to *C. difficile* infection.

These investigations into the host mechanisms promoting neutrophil recruitment have also revealed an association between reduced neutrophil recruitment and increased mortality during *C. difficile* infection(74, 76, 78). For example, in the study mentioned above by Hasegawa et al, the deficiency in neutrophil recruitment seen in NOD1-deficient mice was also associated with a significant increase in mortality following *C. difficile* challenge(76). Furthermore,

elimination of neutrophils through the use of a depleting mAb was also associated with decreased survival(78), seeming to suggest a protective role for neutrophil recruitment in response to *C. difficile* infection.

Other studies, however, have reported interventions that reduce neutrophil recruitment without increased mortality(79-81). For example, Sadighi Akha and colleagues reported decreased CXCL1 expression and reduced neutrophil recruitment following treatment with a combination of anti-IL-22 and anti-CD160 mAb, without any increase in mortality(81). Additionally, while El-Zaatari et al reported increased neutrophil recruitment in association with decreased *C. difficile* colonization in IDO-deficient mice, the increase in neutrophil recruitment was also associated with more severe intestinal histopathology(191). Thus, the host inflammatory signals that drive neutrophil recruitment to the colon, as well as the role of neutrophils themselves in promoting inflammatory responses in the colon, in response to *C. difficile* infection is poorly understood.

Central Hypothesis:

***C. difficile* infection results in induction of interleukin-23, interleukin-17, and interleukin-22, which drives increased chemokine and inflammatory cytokine production, neutrophil recruitment, and immunopathology in the colon**

Aims:

1. **To determine the role of GM-CSF in promoting neutrophil recruitment and inflammatory cytokine expression during acute *C. difficile* colitis.**

Induction of GM-CSF in response to *C. difficile* infection has been previously reported(79, 82), and GM-CSF is known to promote neutrophil recruitment and inflammatory cytokine expression during inflammation at numerous mucosal sites(160-167). However, the role of GM-CSF is driving neutrophil recruitment and the induction of inflammatory cytokines during *C. difficile* infection has never been directly investigated.

2. **To determine the role of neutrophils in driving intestinal histopathology and inflammatory cytokine expression, and the role of TNFα in promoting neutrophil recruitment and inflammatory cytokine expression, in response to *C. difficile* infection.**

Neutrophil recruitment, in association with inflammatory cytokine expression including TNFα, is one of the most prominent host responses to *C. difficile* infection(74, 76, 78-82, 191). Neutrophils are known to produce inflammatory cytokines(141, 144) and their recruitment is often associated with host tissue damage during inflammation(82, 138, 140). However, the role of neutrophils in driving epithelial damage and inflammatory cytokine expression has not been extensively studied. Additionally, TNFα promotes neutrophil recruitment in numerous models of mucosal inflammation(156, 157), and is induced in response to *C. difficile* infection(80, 82), yet the role of TNFα in supporting neutrophil recruitment and inflammatory cytokine expression during *C. difficile* colitis is unknown.

3. **To determine the roles of IL-23, IL-22, and IL-17a in driving CXC chemokine expression and neutrophil recruitment during *C. difficile* colitis.**

IL-23 promotes neutrophil recruitment and neutrophil-recruiting chemokine expression in numerous models of mucosal inflammation in both the lungs and the intestinal tract(135, 138, 145-147). Furthermore, IL-23 controls the expression of both IL-22 and IL-17a, two cytokines capable of controlling neutrophil recruitment(148, 150-154), during inflammation at mucosal sites(134, 135, 138). Though a recent study has suggested a role for IL-23 in driving morbidity and mortality during *C. difficile* infection(190), the role of IL-23 in promoting neutrophil influx during *C. difficile* colitis has not been explicitly examined.

Works Cited

1. **Schleifer K-H.** 2009. Phylum XIII.Firmicutes Gibbons and Murray 1978, 5 (Firmacutes [sic] Gibbons and Murray 1978, 5), p. 19-1317. *In* De Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F, Schleifer K-H, Whitman W (ed.), Bergey,Äôs Manual¬Æ of Systematic Bacteriology. Springer New York.
2. **Ludwig W, Schleifer K-H, Whitman W.** 2009. Revised road map to the phylum Firmicutes, p. 1-13. *In* De Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F, Schleifer K-H, Whitman W (ed.), Bergey,Äôs Manual¬Æ of Systematic Bacteriology. Springer New York.
3. **Carroll KC, Bartlett JG.** 2011. Biology of Clostridium difficile: implications for epidemiology and diagnosis. Annu Rev Microbiol **65:**501-521.
4. **Hall IC, O'toole E.** 1935. Intestinal flora in new-borin infants - With a description of a new pathogenic anaerobe, Bacillus difficilis. Am J Dis Child **49:**390-402.
5. **Paredes-Sabja D, Shen A, Sorg JA.** 2014. Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol **22:**406-416.
6. **Baines SD, O'Connor R, Saxton K, Freeman J, Wilcox MH.** 2009. Activity of vancomycin against epidemic Clostridium difficile strains in a human gut model. J Antimicrob Chemother **63:**520-525.
7. **Lawley TD, Croucher NJ, Yu L, Clare S, Sebaihia M, Goulding D, Pickard DJ, Parkhill J, Choudhary J, Dougan G.** 2009. Proteomic and genomic characterization of highly infectious Clostridium difficile 630 spores. J Bacteriol **191:**5377-5386.
8. **Tasteyre A, Barc MC, Collignon A, Boureau H, Karjalainen T.** 2001. Role of FliC and FliD flagellar proteins of Clostridium difficile in adherence and gut colonization. Infect Immun **69:**7937-7940.
9. **Tasteyre A, Karjalainen T, Avesani V, Delmee M, Collignon A, Bourlioux P, Barc MC.** 2001. Molecular characterization of fliD gene encoding flagellar cap and its expression among Clostridium difficile isolates from different serogroups. J Clin Microbiol **39:**1178-1183.
10. **Tasteyre A, Barc MC, Karjalainen T, Dodson P, Hyde S, Bourlioux P, Borriello P.** 2000. A Clostridium difficile gene encoding flagellin. Microbiology **146 (Pt 4):**957-966.
11. **Tasteyre A, Karjalainen T, Avesani V, Delmee M, Collignon A, Bourlioux P, Barc MC.** 2000. Phenotypic and genotypic diversity of the flagellin gene (fliC) among Clostridium difficile isolates from different serogroups. J Clin Microbiol **38:**3179-3186.
12. **Dingle TC, Mulvey GL, Armstrong GD.** 2011. Mutagenic analysis of the Clostridium difficile flagellar proteins, FliC and FliD, and their contribution to virulence in hamsters. Infect Immun **79:**4061-4067.
13. **Baban ST, Kuehne SA, Barketi-Klai A, Cartman ST, Kelly ML, Hardie KR, Kansau I, Collignon A, Minton NP.** 2013. The role of

flagella in Clostridium difficile pathogenesis: comparison between a non-epidemic and an epidemic strain. PLoS One **8:**e73026.
14. **Sorg JA, Dineen SS.** 2009. Laboratory maintenance of Clostridium difficile. Curr Protoc Microbiol **Chapter 9:**Unit9A 1.
15. **Curry S.** 2010. Clostridium difficile. Clin Lab Med **30:**329-342.
16. **Goncalves C, Decre D, Barbut F, Burghoffer B, Petit JC.** 2004. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from Clostridium difficile. J Clin Microbiol **42:**1933-1939.
17. **Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M, von Eichel-Streiber C.** 1997. Transcription analysis of the genes tcdA-E of the pathogenicity locus of Clostridium difficile. Eur J Biochem **244:**735-742.
18. **Voth DE, Ballard JD.** 2005. Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev **18:**247-263.
19. **Carter GP, Lyras D, Allen DL, Mackin KE, Howarth PM, O'Connor JR, Rood JI.** 2007. Binary toxin production in Clostridium difficile is regulated by CdtR, a LytTR family response regulator. J Bacteriol **189:**7290-7301.
20. **Perelle S, Gibert M, Bourlioux P, Corthier G, Popoff MR.** 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by Clostridium difficile CD196. Infect Immun **65:**1402-1407.
21. **Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeno-Tarraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Dougan G, Barrell B, Parkhill J.** 2006. The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat Genet **38:**779-786.
22. **Bartlett JG.** 1994. Clostridium difficile: history of its role as an enteric pathogen and the current state of knowledge about the organism. Clin Infect Dis **18 Suppl 4:**S265-272.
23. **Dubberke ER, Haslam DB, Lanzas C, Bobo LD, Burnham CA, Grohn YT, Tarr PI.** 2011. The ecology and pathobiology of Clostridium difficile infections: an interdisciplinary challenge. Zoonoses Public Health **58:**4-20.
24. **Rupnik M, Wilcox MH, Gerding DN.** 2009. Clostridium difficile infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **7:**526-536.
25. **Bartlett JG, Onderdonk AB, Cisneros RL, Kasper DL.** 1977. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. J Infect Dis **136:**701-705.

26. **Bartlett JG, Chang TW, Gurwith M, Gorbach SL, Onderdonk AB.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N Engl J Med **298:**531-534.
27. **Lyerly DM, Krivan HC, Wilkins TD.** 1988. Clostridium difficile: its disease and toxins. Clin Microbiol Rev **1:**1-18.
28. **Vaishnavi C.** 2010. Clinical spectrum & pathogenesis of Clostridium difficile associated diseases. Indian J Med Res **131:**487-499.
29. **Bouza E, Munoz P, Alonso R.** 2005. Clinical manifestations, treatment and control of infections caused by Clostridium difficile. Clin Microbiol Infect **11 Suppl 4:**57-64.
30. **Olson MM, Shanholtzer CJ, Lee JT, Jr., Gerding DN.** 1994. Ten years of prospective Clostridium difficile-associated disease surveillance and treatment at the Minneapolis VA Medical Center, 1982-1991. Infect Control Hosp Epidemiol **15:**371-381.
31. **Honda H, Dubberke ER.** 2014. The changing epidemiology of Clostridium difficile infection. Curr Opin Gastroenterol **30:**54-62.
32. **Redelings MD, Sorvillo F, Mascola L.** 2007. Increase in Clostridium difficile-related mortality rates, United States, 1999-2004. Emerg Infect Dis **13:**1417-1419.
33. **Dubberke ER, Carling P, Carrico R, Donskey CJ, Loo VG, McDonald LC, Maragakis LL, Sandora TJ, Weber DJ, Yokoe DS, Gerding DN.** 2014. Strategies to prevent Clostridium difficile infections in acute care hospitals: 2014 update. Infect Control Hosp Epidemiol **35 Suppl 2:**S48-65.
34. **Dubberke ER, Olsen MA.** 2012. Burden of Clostridium difficile on the healthcare system. Clin Infect Dis **55 Suppl 2:**S88-92.
35. **Hall AJ, Curns AT, McDonald LC, Parashar UD, Lopman BA.** 2012. The roles of Clostridium difficile and norovirus among gastroenteritis-associated deaths in the United States, 1999-2007. Clin Infect Dis **55:**216-223.
36. **McDonald LC, Killgore GE, Thompson A, Owens RC, Jr., Kazakova SV, Sambol SP, Johnson S, Gerding DN.** 2005. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med **353:**2433-2441.
37. **O'Connor JR, Johnson S, Gerding DN.** 2009. Clostridium difficile infection caused by the epidemic BI/NAP1/027 strain. Gastroenterology **136:**1913-1924.
38. **Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, Frost E, McDonald LC.** 2005. Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. Lancet **366:**1079-1084.
39. **Akerlund T, Persson I, Unemo M, Noren T, Svenungsson B, Wullt M, Burman LG.** 2008. Increased sporulation rate of epidemic Clostridium difficile Type 027/NAP1. J Clin Microbiol **46:**1530-1533.
40. **Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, Johnson S, Gerding DN, Vedantam G.** 2010. Human hypervirulent

Clostridium difficile strains exhibit increased sporulation as well as robust toxin production. J Bacteriol **192:**4904-4911.

41. **Carlson PE, Jr., Walk ST, Bourgis AE, Liu MW, Kopliku F, Lo E, Young VB, Aronoff DM, Hanna PC.** 2013. The relationship between phenotype, ribotype, and clinical disease in human Clostridium difficile isolates. Anaerobe **24:**109-116.
42. **Jank T, Giesemann T, Aktories K.** 2007. Rho-glucosylating Clostridium difficile toxins A and B: new insights into structure and function. Glycobiology **17:**15R-22R.
43. **Florin I, Thelestam M.** 1983. Internalization of Clostridium difficile cytotoxin into cultured human lung fibroblasts. Biochim Biophys Acta **763:**383-392.
44. **Henriques B, Florin I, Thelestam M.** 1987. Cellular internalisation of Clostridium difficile toxin A. Microb Pathog **2:**455-463.
45. **Barth H, Pfeifer G, Hofmann F, Maier E, Benz R, Aktories K.** 2001. Low pH-induced formation of ion channels by clostridium difficile toxin B in target cells. J Biol Chem **276:**10670-10676.
46. **Pfeifer G, Schirmer J, Leemhuis J, Busch C, Meyer DK, Aktories K, Barth H.** 2003. Cellular uptake of Clostridium difficile toxin B. Translocation of the N-terminal catalytic domain into the cytosol of eukaryotic cells. J Biol Chem **278:**44535-44541.
47. **Rupnik M, Pabst S, von Eichel-Streiber C, Urlaub H, Soling HD.** 2005. Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of Clostridium difficile toxin B (TcdB) by host cells. Microbiology **151:**199-208.
48. **Giesemann T, Egerer M, Jank T, Aktories K.** 2008. Processing of Clostridium difficile toxins. J Med Microbiol **57:**690-696.
49. **Chumbler NM, Farrow MA, Lapierre LA, Franklin JL, Haslam DB, Goldenring JR, Lacy DB.** 2012. Clostridium difficile Toxin B causes epithelial cell necrosis through an autoprocessing-independent mechanism. PLoS Pathog **8:**e1003072.
50. **Chang TW, Lin PS, Gorbach SL, Bartlett JG.** 1979. Ultrastructural changes of cultured human amnion cells by Clostridiu difficile toxin. Infect Immun **23:**795-798.
51. **Siddiqui F, O'Connor JR, Nagaro K, Cheknis A, Sambol SP, Vedantam G, Gerding DN, Johnson S.** 2012. Vaccination with parenteral toxoid B protects hamsters against lethal challenge with toxin A-negative, toxin B-positive clostridium difficile but does not prevent colonization. J Infect Dis **205:**128-133.
52. **Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S, Gerding DN, Rood JI.** 2009. Toxin B is essential for virulence of Clostridium difficile. Nature **458:**1176-1179.
53. **Nagaro KJ, Phillips ST, Cheknis AK, Sambol SP, Zukowski WE, Johnson S, Gerding DN.** 2013. Nontoxigenic Clostridium difficile protects hamsters against challenge with historic and epidemic

strains of toxigenic BI/NAP1/027 C. difficile. Antimicrob Agents Chemother **57:**5266-5270.

54. **Razaq N, Sambol S, Nagaro K, Zukowski W, Cheknis A, Johnson S, Gerding DN.** 2007. Infection of hamsters with historical and epidemic BI types of Clostridium difficile. J Infect Dis **196:**1813-1819.
55. **Sambol SP, Tang JK, Merrigan MM, Johnson S, Gerding DN.** 2001. Infection of hamsters with epidemiologically important strains of Clostridium difficile. J Infect Dis **183:**1760-1766.
56. **Geric B, Carman RJ, Rupnik M, Genheimer CW, Sambol SP, Lyerly DM, Gerding DN, Johnson S.** 2006. Binary toxin-producing, large clostridial toxin-negative Clostridium difficile strains are enterotoxic but do not cause disease in hamsters. J Infect Dis **193:**1143-1150.
57. **Sambol SP, Merrigan MM, Tang JK, Johnson S, Gerding DN.** 2002. Colonization for the prevention of Clostridium difficile disease in hamsters. J Infect Dis **186:**1781-1789.
58. **Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP.** 2010. The role of toxin A and toxin B in Clostridium difficile infection. Nature **467:**711-713.
59. **Theriot CM, Koumpouras CC, Carlson PE, Bergin, II, Aronoff DM, Young VB.** 2011. Cefoperazone-treated mice as an experimental platform to assess differential virulence of Clostridium difficile strains. Gut Microbes **2:**326-334.
60. **Kuehne SA, Collery MM, Kelly ML, Cartman ST, Cockayne A, Minton NP.** 2014. Importance of toxin A, toxin B, and CDT in virulence of an epidemic Clostridium difficile strain. J Infect Dis **209:**83-86.
61. **Borriello SP, Wren BW, Hyde S, Seddon SV, Sibbons P, Krishna MM, Tabaqchali S, Manek S, Price AB.** 1992. Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of Clostridium difficile. Infect Immun **60:**4192-4199.
62. **Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI.** 2011. Human nutrition, the gut microbiome and the immune system. Nature **474:**327-336.
63. **Topping DL, Clifton PM.** 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev **81:**1031-1064.
64. **Hooper LV.** 2004. Bacterial contributions to mammalian gut development. Trends Microbiol **12:**129-134.
65. **Pollard M, Sharon N.** 1970. Responses of the Peyer's Patches in Germ-Free Mice to Antigenic Stimulation. Infect Immun **2:**96-100.
66. **Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB.** 2011. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut Microbes **2:**145-158.

67. **van der Waaij D, Berghuis-de Vries JM, Lekkerkerk L-v.** 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg (Lond) **69:**405-411.
68. **Barthel M, Hapfelmeier S, Quintanilla-Martinez L, Kremer M, Rohde M, Hogardt M, Pfeffer K, Russmann H, Hardt WD.** 2003. Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun **71:**2839-2858.
69. **Stahl M, Ries J, Vermeulen J, Yang H, Sham HP, Crowley SM, Badayeva Y, Turvey SE, Gaynor EC, Li X, Vallance BA.** 2014. A novel mouse model of Campylobacter jejuni gastroenteritis reveals key pro-inflammatory and tissue protective roles for Toll-like receptor signaling during infection. PLoS Pathog **10:**e1004264.
70. **Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, Russell SL, Vallance BA, Finlay BB.** 2011. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infect Immun **79:**1536-1545.
71. **Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB.** 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe **2:**119-129.
72. **Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP.** 2008. A mouse model of Clostridium difficile-associated disease. Gastroenterology **135:**1984-1992.
73. **Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG.** 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun **80:**62-73.
74. **Hasegawa M, Kamada N, Jiao Y, Liu MZ, Nunez G, Inohara N.** 2012. Protective role of commensals against Clostridium difficile infection via an IL-1beta-mediated positive-feedback loop. J Immunol **189:**3085-3091.
75. **Hasegawa M, Yada S, Liu MZ, Kamada N, Munoz-Planillo R, Do N, Nunez G, Inohara N.** 2014. Interleukin-22 regulates the complement system to promote resistance against pathobionts after pathogen-induced intestinal damage. Immunity **41:**620-632.
76. **Hasegawa M, Yamazaki T, Kamada N, Tawaratsumida K, Kim YG, Nunez G, Inohara N.** 2011. Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen. J Immunol **186:**4872-4880.
77. **Jarchum I, Liu M, Lipuma L, Pamer EG.** 2011. Toll-like receptor 5 stimulation protects mice from acute Clostridium difficile colitis. Infect Immun **79:**1498-1503.

78. **Jarchum I, Liu M, Shi C, Equinda M, Pamer EG.** 2012. Critical role for MyD88-mediated neutrophil recruitment during Clostridium difficile colitis. Infect Immun **80:**2989-2996.
79. **McDermott AJ, Frank CR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB.** 2014. Role of GM-CSF in the inflammatory cytokine network that regulates neutrophil influx into the colonic mucosa during Clostridium difficile infection in mice. Gut Microbes **5:**476-484.
80. **McDermott AJ, Higdon KE, Muraglia R, Erb-Downward JR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB.** 2014. The Role of Gr-1 Cells and TNFalpha Signaling During Clostridium difficile Colitis in Mice. Immunology.
81. **Sadighi Akha AA, McDermott AJ, Theriot CM, Carlson PE, Jr., Frank CR, McDonald RA, Falkowski NR, Bergin IL, Young VB, Huffnagle GB.** 2014. IL22 and CD160 play additive roles in the host mucosal response to Clostridium difficile infection in mice. Immunology.
82. **Sadighi Akha AA, Theriot CM, Erb-Downward JR, McDermott AJ, Falkowski NR, Tyra HM, Rutkowski DT, Young VB, Huffnagle GB.** 2013. Acute infection of mice with Clostridium difficile leads to eIF2alpha phosphorylation and pro-survival signalling as part of the mucosal inflammatory response. Immunology **140:**111-122.
83. **Theriot CM, Koenigsknecht MJ, Carlson PE, Jr., Hatton GE, Nelson AM, Li B, Huffnagle GB, J ZL, Young VB.** 2014. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. Nat Commun **5:**3114.
84. **Ferreyra JA, Wu KJ, Hryckowian AJ, Bouley DM, Weimer BC, Sonnenburg JL.** 2014. Gut microbiota-produced succinate promotes C. difficile infection after antibiotic treatment or motility disturbance. Cell Host Microbe **16:**770-777.
85. **Vereecke L, Beyaert R, van Loo G.** 2011. Enterocyte death and intestinal barrier maintenance in homeostasis and disease. Trends Mol Med **17:**584-593.
86. **Abreu MT.** 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol **10:**131-144.
87. **del Rio ML, Bernhardt G, Rodriguez-Barbosa JI, Forster R.** 2010. Development and functional specialization of CD103+ dendritic cells. Immunol Rev **234:**268-281.
88. **Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y.** 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med **204:**1775-1785.
89. **Artis D.** 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol **8:**411-420.

90. **Yen TH, Wright NA.** 2006. The gastrointestinal tract stem cell niche. Stem Cell Rev **2:**203-212.
91. **Audie JP, Janin A, Porchet N, Copin MC, Gosselin B, Aubert JP.** 1993. Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by in situ hybridization. J Histochem Cytochem **41:**1479-1485.
92. **Matsuo K, Ota H, Akamatsu T, Sugiyama A, Katsuyama T.** 1997. Histochemistry of the surface mucous gel layer of the human colon. Gut **40:**782-789.
93. **Kleinschek MA, Boniface K, Sadekova S, Grein J, Murphy EE, Turner SP, Raskin L, Desai B, Faubion WA, de Waal Malefyt R, Pierce RH, McClanahan T, Kastelein RA.** 2009. Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. J Exp Med **206:**525-534.
94. **Makita S, Kanai T, Nemoto Y, Totsuka T, Okamoto R, Tsuchiya K, Yamamoto M, Kiyono H, Watanabe M.** 2007. Intestinal lamina propria retaining CD4+CD25+ regulatory T cells is a suppressive site of intestinal inflammation. J Immunol **178:**4937-4946.
95. **Makita S, Kanai T, Oshima S, Uraushihara K, Totsuka T, Sawada T, Nakamura T, Koganei K, Fukushima T, Watanabe M.** 2004. CD4+CD25bright T cells in human intestinal lamina propria as regulatory cells. J Immunol **173:**3119-3130.
96. **Munoz M, Heimesaat MM, Danker K, Struck D, Lohmann U, Plickert R, Bereswill S, Fischer A, Dunay IR, Wolk K, Loddenkemper C, Krell HW, Libert C, Lund LR, Frey O, Holscher C, Iwakura Y, Ghilardi N, Ouyang W, Kamradt T, Sabat R, Liesenfeld O.** 2009. Interleukin (IL)-23 mediates Toxoplasma gondii-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. J Exp Med **206:**3047-3059.
97. **Bailey M, Plunkett FJ, Rothkotter HJ, Vega-Lopez MA, Haverson K, Stokes CR.** 2001. Regulation of mucosal immune responses in effector sites. Proc Nutr Soc **60:**427-435.
98. **Brandtzaeg P, Pabst R.** 2004. Let's go mucosal: communication on slippery ground. Trends Immunol **25:**570-577.
99. **Lycke NY, Bemark M.** 2012. The role of Peyer's patches in synchronizing gut IgA responses. Front Immunol **3:**329.
100. **Chang WW, Leblond CP.** 1971. Renewal of the epithelium in the descending colon of the mouse. I. Presence of three cell populations: vacuolated-columnar, mucous and argentaffin. Am J Anat **131:**73-99.
101. **Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL.** 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol **167:**1882-1885.
102. **Yang SK, Eckmann L, Panja A, Kagnoff MF.** 1997. Differential and regulated expression of C-X-C, C-C, and C-chemokines by human colon epithelial cells. Gastroenterology **113:**1214-1223.

103. **Awane M, Andres PG, Li DJ, Reinecker HC.** 1999. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. J Immunol **162:**5337-5344.
104. **Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF.** 2005. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. J Immunol **174:**4901-4907.
105. **Mayer L, Shlien R.** 1987. Evidence for function of Ia molecules on gut epithelial cells in man. J Exp Med **166:**1471-1483.
106. **Parr EL, Kirby WN.** 1979. An immunoferritin labeling study of H-2 antigens on dissociated epithelial cells. J Histochem Cytochem **27:**1327-1336.
107. **Karam SM.** 1999. Lineage commitment and maturation of epithelial cells in the gut. Front Biosci **4:**D286-298.
108. **Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC.** 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A **105:**15064-15069.
109. **Forman RA, deSchoolmeester ML, Hurst RJ, Wright SH, Pemberton AD, Else KJ.** 2012. The goblet cell is the cellular source of the anti-microbial angiogenin 4 in the large intestine post Trichuris muris infection. PLoS One **7:**e42248.
110. **Burger-van Paassen N, Loonen LM, Witte-Bouma J, Korteland-van Male AM, de Bruijn AC, van der Sluis M, Lu P, Van Goudoever JB, Wells JM, Dekker J, Van Seuningen I, Renes IB.** 2012. Mucin Muc2 deficiency and weaning influences the expression of the innate defense genes Reg3beta, Reg3gamma and angiogenin-4. PLoS One **7:**e38798.
111. **Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, Ley R, Wakeland EK, Hooper LV.** 2011. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science **334:**255-258.
112. **McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, Newberry RD, Miller MJ.** 2012. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature **483:**345-349.
113. **Cheroutre H, Lambolez F, Mucida D.** 2011. The light and dark sides of intestinal intraepithelial lymphocytes. Nat Rev Immunol **11:**445-456.
114. **Resendiz-Albor AA, Esquivel R, Lopez-Revilla R, Verdin L, Moreno-Fierros L.** 2005. Striking phenotypic and functional differences in lamina propria lymphocytes from the large and small intestine of mice. Life Sci **76:**2783-2803.
115. **Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB.** 1994. Adhesion between epithelial cells and T

lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature **372:**190-193.
116. **Wang HC, Zhou Q, Dragoo J, Klein JR.** 2002. Most murine CD8+ intestinal intraepithelial lymphocytes are partially but not fully activated T cells. J Immunol **169:**4717-4722.
117. **Moretto M, Weiss LM, Khan IA.** 2004. Induction of a rapid and strong antigen-specific intraepithelial lymphocyte response during oral Encephalitozoon cuniculi infection. J Immunol **172:**4402-4409.
118. **Boismenu R, Havran WL.** 1994. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. Science **266:**1253-1255.
119. **Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R.** 2002. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A **99:**14338-14343.
120. **Simpson SJ, Hollander GA, Mizoguchi E, Allen D, Bhan AK, Wang B, Terhorst C.** 1997. Expression of pro-inflammatory cytokines by TCR alpha beta+ and TCR gamma delta+ T cells in an experimental model of colitis. Eur J Immunol **27:**17-25.
121. **MacDonald TT, Pender SL.** 1998. Lamina propria T cells. Chem Immunol **71:**103-117.
122. **Carol M, Lambrechts A, Van Gossum A, Libin M, Goldman M, Mascart-Lemone F.** 1998. Spontaneous secretion of interferon gamma and interleukin 4 by human intraepithelial and lamina propria gut lymphocytes. Gut **42:**643-649.
123. **Macpherson AJ, Slack E.** 2007. The functional interactions of commensal bacteria with intestinal secretory IgA. Curr Opin Gastroenterol **23:**673-678.
124. **Cerutti A.** 2008. Location, location, location: B-cell differentiation in the gut lamina propria. Mucosal Immunol **1:**8-10.
125. **Frankel G, Phillips AD, Novakova M, Field H, Candy DC, Schauer DB, Douce G, Dougan G.** 1996. Intimin from enteropathogenic Escherichia coli restores murine virulence to a Citrobacter rodentium eaeA mutant: induction of an immunoglobulin A response to intimin and EspB. Infect Immun **64:**5315-5325.
126. **Suzuki K, Meek B, Doi Y, Muramatsu M, Chiba T, Honjo T, Fagarasan S.** 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A **101:**1981-1986.
127. **Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC.** 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science **307:**254-258.
128. **del Rio ML, Rodriguez-Barbosa JI, Bolter J, Ballmaier M, Dittrich-Breiholz O, Kracht M, Jung S, Forster R.** 2008. CX3CR1+ c-kit+ bone marrow cells give rise to CD103+ and CD103- dendritic cells with distinct functional properties. J Immunol **181:**6178-6188.
129. **Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, Fehling HJ, Hardt WD, Shakhar G, Jung S.** 2009. Intestinal lamina

propria dendritic cell subsets have different origin and functions. Immunity **31:**502-512.
130. **Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, Mention JJ, Thiam K, Cerf-Bensussan N, Mandelboim O, Eberl G, Di Santo JP.** 2008. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity **29:**958-970.
131. **Buonocore S, Ahern PP, Uhlig HH, Ivanov, II, Littman DR, Maloy KJ, Powrie F.** 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature **464:**1371-1375.
132. **Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie AN, Mebius RE, Powrie F, Vivier E.** 2013. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol **13:**145-149.
133. **Bernink JH, Peters CP, Munneke M, te Velde AA, Meijer SL, Weijer K, Hreggvidsdottir HS, Heinsbroek SE, Legrand N, Buskens CJ, Bemelman WA, Mjosberg JM, Spits H.** 2013. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. Nat Immunol **14:**221-229.
134. **Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W.** 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med **14:**282-289.
135. **Godinez I, Raffatellu M, Chu H, Paixao TA, Haneda T, Santos RL, Bevins CL, Tsolis RM, Baumler AJ.** 2009. Interleukin-23 orchestrates mucosal responses to Salmonella enterica serotype Typhimurium in the intestine. Infect Immun **77:**387-398.
136. **Godinez I, Haneda T, Raffatellu M, George MD, Paixao TA, Rolan HG, Santos RL, Dandekar S, Tsolis RM, Baumler AJ.** 2008. T cells help to amplify inflammatory responses induced by Salmonella enterica serotype Typhimurium in the intestinal mucosa. Infect Immun **76:**2008-2017.
137. **Goncalves NS, Ghaem-Maghami M, Monteleone G, Frankel G, Dougan G, Lewis DJ, Simmons CP, MacDonald TT.** 2001. Critical role for tumor necrosis factor alpha in controlling the number of lumenal pathogenic bacteria and immunopathology in infectious colitis. Infect Immun **69:**6651-6659.
138. **Cox JH, Kljavin NM, Ota N, Leonard J, Roose-Girma M, Diehl L, Ouyang W, Ghilardi N.** 2012. Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. Mucosal Immunol **5:**99-109.
139. **Chami B, Yeung AW, van Vreden C, King NJ, Bao S.** 2014. The role of CXCR3 in DSS-induced colitis. PLoS One **9:**e101622.
140. **Morohoshi Y, Matsuoka K, Chinen H, Kamada N, Sato T, Hisamatsu T, Okamoto S, Inoue N, Takaishi H, Ogata H, Iwao Y, Hibi T.** 2006. Inhibition of neutrophil elastase prevents the

development of murine dextran sulfate sodium-induced colitis. J Gastroenterol **41:**318-324.
141. **Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA.** 2000. The neutrophil as a cellular source of chemokines. Immunol Rev **177:**195-203.
142. **Lee WL, Harrison RE, Grinstein S.** 2003. Phagocytosis by neutrophils. Microbes Infect **5:**1299-1306.
143. **Lebeis SL, Bommarius B, Parkos CA, Sherman MA, Kalman D.** 2007. TLR signaling mediated by MyD88 is required for a protective innate immune response by neutrophils to Citrobacter rodentium. J Immunol **179:**566-577.
144. **Takahashi GW, Andrews DF, 3rd, Lilly MB, Singer JW, Alderson MR.** 1993. Effect of granulocyte-macrophage colony-stimulating factor and interleukin-3 on interleukin-8 production by human neutrophils and monocytes. Blood **81:**357-364.
145. **Dubin PJ, Kolls JK.** 2007. IL-23 mediates inflammatory responses to mucoid Pseudomonas aeruginosa lung infection in mice. Am J Physiol Lung Cell Mol Physiol **292:**L519-528.
146. **Dubin PJ, Martz A, Eisenstatt JR, Fox MD, Logar A, Kolls JK.** 2012. Interleukin-23-mediated inflammation in Pseudomonas aeruginosa pulmonary infection. Infect Immun **80:**398-409.
147. **Gasse P, Riteau N, Vacher R, Michel ML, Fautrel A, di Padova F, Fick L, Charron S, Lagente V, Eberl G, Le Bert M, Quesniaux VF, Huaux F, Leite-de-Moraes M, Ryffel B, Couillin I.** 2011. IL-1 and IL-23 mediate early IL-17A production in pulmonary inflammation leading to late fibrosis. PLoS One **6:**e23185.
148. **Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D.** 2010. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. J Exp Med **207:**1293-1305.
149. **Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, Lehr HA, Hirth S, Weigmann B, Wirtz S, Ouyang W, Neurath MF, Becker C.** 2009. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med **206:**1465-1472.
150. **Andoh A, Zhang Z, Inatomi O, Fujino S, Deguchi Y, Araki Y, Tsujikawa T, Kitoh K, Kim-Mitsuyama S, Takayanagi A, Shimizu N, Fujiyama Y.** 2005. Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. Gastroenterology **129:**969-984.
151. **Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkuhn T, Goke B, Auernhammer CJ, Dambacher J.** 2006. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol **290:**G827-838.
152. **Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito**

JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med **194:**519-527.

153. **Keestra AM, Godinez I, Xavier MN, Winter MG, Winter SE, Tsolis RM, Baumler AJ.** 2011. Early MyD88-dependent induction of interleukin-17A expression during Salmonella colitis. Infect Immun **79:**3131-3140.
154. **Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK.** 2006. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. Inflamm Bowel Dis **12:**382-388.
155. **Ito R, Kita M, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, Imanishi J, Iwakura Y, Okanoue T, Yoshikawa T, Kataoka K, Mazda O.** 2008. Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. Biochem Biophys Res Commun **377:**12-16.
156. **Lukacs NW, Strieter RM, Chensue SW, Widmer M, Kunkel SL.** 1995. TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation. J Immunol **154:**5411-5417.
157. **Smith RE, Strieter RM, Phan SH, Lukacs N, Kunkel SL.** 1998. TNF and IL-6 mediate MIP-1alpha expression in bleomycin-induced lung injury. J Leukoc Biol **64:**528-536.
158. **Parameswaran N, Patial S.** 2010. Tumor necrosis factor-alpha signaling in macrophages. Crit Rev Eukaryot Gene Expr **20:**87-103.
159. **Shen C, de Hertogh G, Bullens DM, Van Assche G, Geboes K, Rutgeerts P, Ceuppens JL.** 2007. Remission-inducing effect of anti-TNF monoclonal antibody in TNBS colitis: mechanisms beyond neutralization? Inflamm Bowel Dis **13:**308-316.
160. **Dabritz J.** 2014. Granulocyte macrophage colony-stimulating factor and the intestinal innate immune cell homeostasis in Crohn's disease. Am J Physiol Gastrointest Liver Physiol **306:**G455-465.
161. **Hamilton JA.** 2008. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol **8:**533-544.
162. **Lambrecht BN, Hammad H.** 2013. Asthma: the importance of dysregulated barrier immunity. Eur J Immunol **43:**3125-3137.
163. **Puljic R, Benediktus E, Plater-Zyberk C, Baeuerle PA, Szelenyi S, Brune K, Pahl A.** 2007. Lipopolysaccharide-induced lung inflammation is inhibited by neutralization of GM-CSF. Eur J Pharmacol **557:**230-235.
164. **Vlahos R, Bozinovski S, Chan SP, Ivanov S, Linden A, Hamilton JA, Anderson GP.** 2010. Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. Am J Respir Crit Care Med **182:**34-40.
165. **Bozinovski S, Jones J, Beavitt SJ, Cook AD, Hamilton JA, Anderson GP.** 2004. Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. Am J Physiol Lung Cell Mol Physiol **286:**L877-885.

166. **Balloy V, Chignard M.** 2009. The innate immune response to Aspergillus fumigatus. Microbes Infect **11:**919-927.
167. **Xu Y, Hunt NH, Bao S.** 2008. The role of granulocyte macrophage-colony-stimulating factor in acute intestinal inflammation. Cell Res **18:**1220-1229.
168. **Bernasconi E, Favre L, Maillard MH, Bachmann D, Pythoud C, Bouzourene H, Croze E, Velichko S, Parkinson J, Michetti P, Velin D.** 2010. Granulocyte-macrophage colony-stimulating factor elicits bone marrow-derived cells that promote efficient colonic mucosal healing. Inflamm Bowel Dis **16:**428-441.
169. **Egea L, McAllister CS, Lakhdari O, Minev I, Shenouda S, Kagnoff MF.** 2013. GM-CSF produced by nonhematopoietic cells is required for early epithelial cell proliferation and repair of injured colonic mucosa. J Immunol **190:**1702-1713.
170. **Cakarova L, Marsh LM, Wilhelm J, Mayer K, Grimminger F, Seeger W, Lohmeyer J, Herold S.** 2009. Macrophage tumor necrosis factor-alpha induces epithelial expression of granulocyte-macrophage colony-stimulating factor: impact on alveolar epithelial repair. Am J Respir Crit Care Med **180:**521-532.
171. **Linevsky JK, Pothoulakis C, Keates S, Warny M, Keates AC, Lamont JT, Kelly CP.** 1997. IL-8 release and neutrophil activation by Clostridium difficile toxin-exposed human monocytes. Am J Physiol **273:**G1333-1340.
172. **Ng J, Hirota SA, Gross O, Li Y, Ulke-Lemee A, Potentier MS, Schenck LP, Vilaysane A, Seamone ME, Feng H, Armstrong GD, Tschopp J, Macdonald JA, Muruve DA, Beck PL.** 2010. Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome. Gastroenterology **139:**542-552, 552 e541-543.
173. **Lee JY, Kim H, Cha MY, Park HG, Kim YJ, Kim IY, Kim JM.** 2009. Clostridium difficile toxin A promotes dendritic cell maturation and chemokine CXCL2 expression through p38, IKK, and the NF-kappaB signaling pathway. J Mol Med (Berl) **87:**169-180.
174. **Ryan A, Lynch M, Smith SM, Amu S, Nel HJ, McCoy CE, Dowling JK, Draper E, O'Reilly V, McCarthy C, O'Brien J, Ni Eidhin D, O'Connell MJ, Keogh B, Morton CO, Rogers TR, Fallon PG, O'Neill LA, Kelleher D, Loscher CE.** 2011. A role for TLR4 in Clostridium difficile infection and the recognition of surface layer proteins. PLoS Pathog **7:**e1002076.
175. **Calderon GM, Torres-Lopez J, Lin TJ, Chavez B, Hernandez M, Munoz O, Befus AD, Enciso JA.** 1998. Effects of toxin A from Clostridium difficile on mast cell activation and survival. Infect Immun **66:**2755-2761.
176. **Mahida YR, Makh S, Hyde S, Gray T, Borriello SP.** 1996. Effect of Clostridium difficile toxin A on human intestinal epithelial cells:

induction of interleukin 8 production and apoptosis after cell detachment. Gut **38:**337-347.

177. **Pothoulakis C, Barone LM, Ely R, Faris B, Clark ME, Franzblau C, LaMont JT.** 1986. Purification and properties of Clostridium difficile cytotoxin B. J Biol Chem **261:**1316-1321.
178. **Branka JE, Vallette G, Jarry A, Bou-Hanna C, Lemarre P, Van PN, Laboisse CL.** 1997. Early functional effects of Clostridium difficile toxin A on human colonocytes. Gastroenterology **112:**1887-1894.
179. **Castagliuolo I, Keates AC, Wang CC, Pasha A, Valenick L, Kelly CP, Nikulasson ST, LaMont JT, Pothoulakis C.** 1998. Clostridium difficile toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. J Immunol **160:**6039-6045.
180. **Kim JM, Kim JS, Jun HC, Oh YK, Song IS, Kim CY.** 2002. Differential expression and polarized secretion of CXC and CC chemokines by human intestinal epithelial cancer cell lines in response to Clostridium difficile toxin A. Microbiol Immunol **46:**333-342.
181. **Hirota SA, Iablokov V, Tulk SE, Schenck LP, Becker H, Nguyen J, Al Bashir S, Dingle TC, Laing A, Liu J, Li Y, Bolstad J, Mulvey GL, Armstrong GD, MacNaughton WK, Muruve DA, MacDonald JA, Beck PL.** 2012. Intrarectal instillation of Clostridium difficile toxin A triggers colonic inflammation and tissue damage: development of a novel and efficient mouse model of Clostridium difficile toxin exposure. Infect Immun **80:**4474-4484.
182. **Lonnroth I, Lange S.** 1983. Toxin A of Clostridium difficile: production, purification and effect in mouse intestine. Acta Pathol Microbiol Immunol Scand B **91:**395-400.
183. **Triadafilopoulos G, Pothoulakis C, O'Brien MJ, LaMont JT.** 1987. Differential effects of Clostridium difficile toxins A and B on rabbit ileum. Gastroenterology **93:**273-279.
184. **Hughes S, Warhurst G, Turnberg LA, Higgs NB, Giugliano LG, Drasar BS.** 1983. Clostridium difficile toxin-induced intestinal secretion in rabbit ileum in vitro. Gut **24:**94-98.
185. **Hecht G, Pothoulakis C, LaMont JT, Madara JL.** 1988. Clostridium difficile toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J Clin Invest **82:**1516-1524.
186. **Kelly CP, Becker S, Linevsky JK, Joshi MA, O'Keane JC, Dickey BF, LaMont JT, Pothoulakis C.** 1994. Neutrophil recruitment in Clostridium difficile toxin A enteritis in the rabbit. J Clin Invest **93:**1257-1265.
187. **Wershil BK, Castagliuolo I, Pothoulakis C.** 1998. Direct evidence of mast cell involvement in Clostridium difficile toxin A-induced enteritis in mice. Gastroenterology **114:**956-964.
188. **Ishida Y, Maegawa T, Kondo T, Kimura A, Iwakura Y, Nakamura S, Mukaida N.** 2004. Essential involvement of IFN-gamma in

Clostridium difficile toxin A-induced enteritis. J Immunol **172:**3018-3025.

189. **Warny M, Keates AC, Keates S, Castagliuolo I, Zacks JK, Aboudola S, Qamar A, Pothoulakis C, LaMont JT, Kelly CP.** 2000. p38 MAP kinase activation by Clostridium difficile toxin A mediates monocyte necrosis, IL-8 production, and enteritis. J Clin Invest **105:**1147-1156.
190. **Buonomo EL, Madan R, Pramoonjago P, Li L, Okusa MD, Petri WA, Jr.** 2013. Role of interleukin 23 signaling in Clostridium difficile colitis. J Infect Dis **208:**917-920.
191. **El-Zaatari M, Chang YM, Zhang M, Franz M, Shreiner A, McDermott AJ, van der Sluijs KF, Lutter R, Grasberger H, Kamada N, Young VB, Huffnagle GB, Kao JY.** 2014. Tryptophan catabolism restricts IFN-gamma-expressing neutrophils and Clostridium difficile immunopathology. J Immunol **193:**807-816.
192. **Koenigsknecht MJ, Theriot CM, Bergin IL, Schumacher CA, Schloss PD, Young VB.** 2014. Dynamics and Establishment of Clostridium difficile Infection in the Murine Gastrointestinal Tract. Infect Immun.
193. **Hammond GA, Johnson JL.** 1995. The toxigenic element of Clostridium difficile strain VPI 10463. Microb Pathog **19:**203-213.
194. **Trindade BC, Theriot CM, Leslie JL, Carlson PE, Jr., Bergin IL, Peters-Golden M, Young VB, Aronoff DM.** 2014. Clostridium difficile-induced colitis in mice is independent of leukotrienes. Anaerobe **30:**90-98.
195. **Madan R, Guo X, Naylor C, Buonomo EL, Mackay D, Noor Z, Concannon P, Scully KW, Pramoonjago P, Kolling GL, Warren CA, Duggal P, Petri WA, Jr.** 2014. Role of leptin-mediated colonic inflammation in defense against Clostridium difficile colitis. Infect Immun **82:**341-349.

Chapter 2

Materials and Methods

Animals and Housing:

Male C57BL/6, mice aged 5-11 weeks were used in all studies. All mice were either obtained directly from Jackson labs, or acquired from a breeding colony maintained at the University of Michigan founded by Jackson breeders.

Chapter 5: Male and female IL-17$^{-/-}$ (IL-17KO) and p19$^{-/-}$ (IL-23KO) mice aged 5-14 weeks were used as well. IL-17KO and IL-23KO mice on a C57BL/6 background were likewise obtained from breeding colonies maintained at the University of Michigan. IL-23KO breeders were a kind gift from Dr. Benjamin Segal, University of Michigan.

All mice were maintained under specific pathogen-free conditions, and autoclaved food, water, and bedding was provided *ad libitum*. All animal manipulations were carried out in laminar flow hood. All experiments were conducted in accordance with a protocol approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

***Clostridium difficile* Spore Preparation**:

C. difficile spore stocks, both VPI 10463 and 630, were generated by plating an earlier spore preparation on Taurocholate Cefoxitin Cycloserine Fructose Agar (TCCFA) plates anaerobically. Single colonies were isolated, and grown overnight in Columbia broth. 2ml of the overnight culture was inoculated into 40ml of Clospore broth(1), and the culture was allowed to grow for seven days. Spores were collected by centrifugation, and washed to remove vegetative cell debris. All spore stocks were stored in water at 4^0C until used.

Clostridium difficile Vegetative Cell Preparation:

C. difficile was prepared for infection as described previously(2, 3). Briefly, an overnight culture of *C. difficile* strain VPI 10463 (ATCC 43255) was back-diluted 1:10 in fresh brain-heart infusion (BHIS) broth and grown for 4-6 hours. The culture was then collected, washed in deoxygenated PBS (x3), and diluted to the desired dose. Mice were challenged via oral gavage. The inoculum was serially diluted and plated on BHIS plates to confirm dosage. *C. difficile* was grown and prepared for gavage in a Coy anaerobic chamber (Coy Industries).

***Clostridium difficile* Infection**

Chapter 3: Mice were treated with cefoperazone (0.5g/L) in their drinking water for five days in order to permit *C. difficile* infection. Following a 2-day recovery period, animals were challenged with 4.73 ± 0.59 $\log_{10}$ 630 spores as described previously(2). Following challenge, the inoculum was serially diluted and

plated on Taurocholate Cefoxitin Cycloserine Fructose Agar (TCCFA) plates anaerobically in order to confirm the dosage. All animals were monitored for weight change during the course of the experiment, and were monitored for signs of severe *C. difficile* infection (lethargy, hunched posture, >20% weight loss) and were euthanized if meeting any of these criteria. Uninfected animals received neither antibiotic treatment, nor *C. difficile* challenge. Samples were collected at four days post-infection.

Chapter 4: For ceftriaxone and *C. difficile* infection studies, mice were treated with ceftriaxone (0.5g/L) (Sigma) given *ad libitum* in their drinking water for 4 days. Antibiotic water was replaced every other day. Mice were then given a two-day recovery period on drinking water without antibiotic prior to infection with *C. difficile* as described previously(2, 3). Ceftriaxone treated mice were given the antibiotic regimen only, and untreated animals were not manipulated at all.

For *C. difficile* infection studies, mice received 5.06 ± 0.31 Log_{10} CFU vegetative *C. difficile* via oral gavage on. *C. difficile*-infected animals were monitored for signs of severe disease (hunched posture, lethargy, weight loss exceeding 20% of baseline body weight) and were humanely euthanized if moribund. All surviving animals were euthanized two days post infection for subsequent analysis.

Chapter 5: Mice were given a 5 day course of cefoperazone (0.5g/L) in their drinking water in order to permit *C. difficile* infection as described previously(2, 4). After a two-day recovery period, mice were challenged with 5.70±0.25log_{10} *C. difficile* spores from strain VPI 10463. Animals were followed for an additional two days, and all samples were collected at two days post infection. Inoculum dosage

was confirmed by serially diluting and plating the inoculum on TCCFA plates anaerobically. Animals were monitored following infection for signs of severe disease, including lethargy, hunched posture, >20% weight loss, and any animals found moribund were humanely euthanized. Untreated animals did not receive antibiotics or *C. difficile* challenge.

Neutralizing Antibody Treatments:

Chapter 3 Anti-GM-CSF Treatment: Animals were given three intraperitoneal injections of anti-GM-CSF mAb (clone MP1-22E9). Each mouse received 250μg per injection, and injections were given every 48 hours beginning 24 hours prior to infection(5, 6).

Chapter 4 Anti-Gr-1 and anti-TNFα Treatment: Mice were given intraperitoneal injections of 250μg of anti-TNFα mAb (clone MP6-XT3) one day prior to infection with *C. difficile* or injections of 250μg of anti-Gr-1 mAb (clone RB6-8C5) one-day prior and one-day post infection. Mouse serum (Sigma) injections were administered to control mice.

Chapter 5 Anti-IL-22 Treatment: Animals were given two intraperitoneal injections of anti-IL-22 mAb (clone 8E11). Each mouse received 150μg antibody one day prior and one day post infection(7). The anti-IL-22 mAB was a kind gift from Dr. Wenjun Ouyang(7).

Histology:

Colonic tissue was fixed in 10% formalin for at least 24 hours, and then transferred to 70% ethanol. Tissue was processed, paraffin embedded, sectioned, and used to prepare haematoxylin and eosin stained slides by McClinchey Histology Labs Inc. Representative images were acquired using an Olympus BX40 light microscope (Olympus Corporation) and a QImaging MicroPublisher RTV 5.0 5 megapixel camera. All images were acquired at a total magnification of 400X. Panels were assembled in Adobe Photoshop CS5, version 12.0. Image processing was restricted to global adjustments of brightness, contrast, and image size.

Histological Scoring:

Chapter 4: Histological sections were coded, randomized, and scored in a blinded manner. The slides were first scored categorically on a 0-5 scale for epithelium damage and for inflammation, using defined criteria. Epithelium damage was scored as follows: 0, intact epithelium; 1, minimal, scattered goblet cell loss with no significant epithelium destruction and no histologically defined loss of surface integrity; 2, widespread moderate goblet cell loss with no significant epithelium destruction and no histologically defined loss of surface integrity; 3, moderate to extensive widespread goblet cell loss with scattered epithelium destruction and histologically defined loss of surface integrity; 4, extensive epithelium and goblet cell destruction with histologically defined loss of surface integrity; 5, severe epithelium destruction and goblet cell destruction with widespread histologically apparent loss of surface integrity. Inflammation was

scored as follows: 0, no inflammation; 1, minimal multifocal leukocytic infiltrates; 2, moderate multifocal leukocytic infiltrates and low level edema (greater submucosal involvement); 3, significant multifocal leukocytic infiltrates, edema, submucosal involvement; 4, extensive multifocal leukocytic infiltrates, edema, extensive submucosal involvement; 5, severe multifocal leukocytic infiltrates, extensive edema and submucosal involvement with luminal involvement and/or abscess formation. The slides were then assigned an overall score using a rank-order scoring system. The total of both categorical scores (epithelium damage and inflammation) were used to rank all the slides in the study in order of increasing severity of histopathological changes (1=least, 16=most). This method offers significant advantages over straight categorical scoring systems for comparing histological changes between groups and has been previously reported for scoring *C. difficile*-induced intestinal pathology(2, 8, 9).

Chapter 5: Light microscopic evaluation of H&E stained colonic sections was performed by a board-certified veterinary pathologist (ILB). The pathologist was blinded to experimental groupings at the time of the evaluation, and sections were scored using a previously established system(2). Edema: 0 no edema, 1 mild, focal or multifocal edema with minimal submucosal expansion (<2X), 2 moderate multifocal edema with moderate submucosal expansion (2-3X), 3 severe multifocal to coalescing edema with severe submucosal expansion (>3X), 4 same as 3 with diffuse submucosal expansion. Inflammation: 0 no inflammation, 1 minimal, multifocal neutrophilic infiltration, 2 moderate, multifocal neutrophilic infiltration (greater submucosal involvement), 3 severe multifocal to coalescing neutrophilic

infiltration (greater submucosal +/- mural involvement), 4 same as 3 with abscesses or extensive transmural involvement. Epithelial damage: 0 no epithelial damage, 1 mild multifocal, superficial damage (vacuolation, increased apoptosis, villus tip attenuation/necrosis), 2 moderate, multifocal superficial damage (same qualitative changes as above), 3 severe multifocal to coalescing mucosal damage +/- pseudomembrane formation (intraluminal aggregate of neutrophils and sloughed epithelium in a fibrinous matrix covering eroded or ulcerated mucosa), 4 same as 3 with extensive pseudomembrane or ulcer formation.

Quantification of *C. difficile* colonization

Mucosal *C. difficile* colonization was determined using a species-specific qPCR of DNA isolated from colonic tissue. The *C. difficile*-specific qPCR was performed as described previously(3, 10). All reactions were carried out in a total volume of 10μl. Each reaction contained 2μl of template primers, 6.25pmol forward and reverse tcdB primers, and 1pmol tcdB probe. The cycling conditions and probe and primer sequences are identical to those used previously(3). Raw Ct values were normalized to signal from a single-copy host internal control gene to generate dCt values(3, 11). dCt values were then converted to "*C. difficile* genomes/g tissue" using a standard curve generated with known amounts of vegetative *C. difficile* and colonic tissue(4, 12).

Microbiome Analysis

Chapter 3: The DNeasy blood and tissue kit (Qiagen, Valencia, CA) was used to extract genomic DNA from colonic tissue samples. The extraction was performed according to the manufacturer's instructions except for the following modifications: adding a bead-beating step using UltraClean fecal DNA bead tubes (Mo Bio Laboratories, Carlsbad, CA); doubling the amount of ATL buffer and the proteinase K used in the protocol; and decreasing by half the amount of the AE buffer used to elute the DNA. Subsequently, the V3, V4 and V5 hyper-variable regions of the 16S ribosomal RNA gene in each of the samples were targeted for amplification with the 357F and 929R primer sets(13). Amplicons were purified with the Agencourt AMPure XP PCR purification system (Beckman Coulter, Indianapolis, IN), and quantified with the Quant-iT PicoGreen dsDNA kit (Life Technologies, Grand Island, NY) to obtain an equal pool for pyrosequencing. They were then sequenced on a Roche 454 GS Junior Titanium platform according to the manufacturer's specifications. Bacterial 16S sequences were first processed using the microbial ecology software suite *mothur*(14) to generate operational taxonomic units (OTUs) at a 3%, i.e. species level of difference. These data, in the form of the *.shared* file, were then imported into the R software and analyzed using the R-package vegan. The inverse Simpson diversity measure was calculated using the function *diversity()*.

Chapter 4: All procedures and analyses were performed as previously described(15). Briefly, DNA was isolated from rinsed colonic tissue and V3-V5 16S ribosomal RNA gene amplicon libraries generated. They were then sequenced on a Roche 454 GS Junior Titanium platform according to the manufacturer's

specifications. Bacterial 16S rRNA gene sequences were processed using the microbial ecology software suite *mothur*(14) to generate operational taxonomic units (OTUs) at a 3% level of difference (approximating species-level differences). These data were then imported into the software package R and analyzed using the R add on-package vegan(16). Rank abundance plots were generated by selecting for the OTUs that contributed to >0.5% of the population. The content of each treated tissue was ordered according to the average rank order of its untreated counterpart. Taxonomic classification of an OTU was assigned within *mothur* by identifying the consensus sequence of the OTU and assigning taxonomy using a Bayesian classifier trained on an RDP training set (classify.otu).

RNA Isolation and Expression Analysis:

Colonic tissue snips ($1cm^2$) were collected from the center of the colon and stored in RNAlater (Ambion). RNA isolation and purification from colonic tissue was performed as described previously(4, 12, 15, 17). Tissue was homogenized in TRIzol reagent (Life Technologies) and the resulting RNA was purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The concentration of the purified RNA was determined using a Nanodrop instrument (Thermo Fisher). cDNA synthesis, using the purified RNA as a template, was performed using the RT^2 First Strand kit (Qiagen), and colonic gene expression was assessed using RT^2 Profiler PCR arrays (Qiagen). All reactions were run on a Roche Lightcycler 480. In order to correct for variation between RT^2 Profiler PCR arrays, cross card normalization was performed as described previously(15, 18). ΔCt (dCt) values

were calculated by subtracting the geometric mean of two internal control genes from the Ct value of the gene of interest.(19) The 2^{-ddCt} method was utilized to calculate fold change gene expression in treatment groups as compared to untreated animals for all comparisons(11).

Leukocyte Isolation:

Leukocytes were isolated from colonic tissue as described previously(12). Isolated colonic tissue was minced with serrated scissors to physically disrupt the tissue, and was subsequently incubated in 20ml Hank's Balanced Salt Solution (HBSS) supplemented with 2.5% fetal bovine serum, 5mM EDTA, and 1 mM DTT for 20 minutes at 37^0C. Tissue was then incubated in 20ml of a digest solution consisting of HBSS supplemented with 2.5% fetal bovine serum, 400U/ml collagenase type 3 (Worthington Biochemical) and 0.5mg/ml DNAse I (Roche) for 60 minutes at 37^0C. Samples were then resuspended in 20% Percoll (Sigma) in PBS, and centrifuged at 900f for 30 minutes at room temperature without brake. The resulting single cell suspensions were stained for flow cytometric analysis.

Flow Staining and Analysis:

Single cell suspensions were plated at a concentration of approximately 10^6 cells per well in a 96 well plate. Cells were blocked with unlabeled FC RIII/II, and then stained fluorescently labeled antibodies for 30 minutes. Cells were washed to remove excess antibody, and were resuspended in stabilizing fixative (BD

Biosciences). Data was collected on a three-laser Canto II using FACSDiva software (BD biosciences). All data analysis was performed in FlowJo (Treestar).

Chapter 4: The following antibodies were used for flow cytometric analysis of intestinal leukocytes. CD11c (clone HL3), CD45 (clone 30-F11), CD11b (clone M1/70), Gr-1 (clone RB6-8C5), and Ly6C (clone AL-21) as well as Fc RIII/II (clone 2.4G2). All antibodies were purchased from BD Biosciences (San Diego, CA) and Biolegend (San Diego, CA).

Chapter 5: Isolated colonic cells were stained with the following antibodies: CD45 (clone 30-F11), CD11b (clone M1/70) and Ly6G (clone IA8) as well as Fc RIII/II (clone 2.4G2). All antibodies were purchased from eBioscience, BD Pharmingen, and Biolegend.

Total number of neutrophils per colon was calculated by multiplying the frequency of $CD45^{High}$ $CD11b^{High}$ $Ly6G^{High}$ neutrophils as defined by flow cytometry by the total number of cells in the colon in question.

Statistical Analysis:

Chapter 3: For the initial assessment of GM-CSF expression during *C. difficile* colitis, statistical significance was determined via an unpaired two-tailed t-test comparing dCt values from uninfected and *C. difficile* infected mice. For all other analyses of colonic gene expression, datasets were first checked by outlier analysis and then statistically significant changes were identified using a One-Way ANOVA with a Tukey post hoc test comparing normalized dCt values from uninfected, *C. difficile* infected, and anti-GM-CSF treated and *C. difficile* infected animals.

Statistically significant changes in the Inverse Simpson Index were also determined using One-Way ANOVA with a Tukey post hoc test. Significance was set at $p<0.05$ in all analyses.

Chapter 4: Unpaired two-tailed t-tests were used to identify statistically significant differences in gene expression between untreated and ceftriaxone-treated mice. For all other analyses, statistically significant changes were identified using a One-Way ANOVA with Tukey's *post hoc* test for multiple comparisons. For all qPCR data (colonic gene expression and *C. difficile* colonization), statistical analysis was performed on normalized dCt values. Significance was set at $p\leq0.05$ in all analyses.

Chapter 5: Statistically significant differences in gene expression were determined using a One-Way ANOVA with Tukey's *post hoc* test for multiple comparisons. For all qPCR data, statistical tests were performed on normalize dCt values(4, 12). A One-Way ANOVA with Tukey's *post hoc* test was also used to identify significant differences in the number of neutrophils per colon. Significant differences in histopathological scoring were determined using the Kruskal-Wallis test with followed by Dunn's multiple comparisons test. For all analyses, significance was set at $p\leq0.05$.

Works Cited

1. **Perez J, Springthorpe VS, Sattar SA.** 2011. Clospore: a liquid medium for producing high titers of semi-purified spores of Clostridium difficile. J AOAC Int **94:**618-626.
2. **Theriot CM, Koumpouras CC, Carlson PE, Bergin, II, Aronoff DM, Young VB.** 2011. Cefoperazone-treated mice as an experimental platform to assess differential virulence of Clostridium difficile strains. Gut Microbes **2:**326-334.
3. **Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB.** 2011. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut Microbes **2:**145-158.
4. **McDermott AJ, Frank CR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB.** 2014. Role of GM-CSF in the inflammatory cytokine network that regulates neutrophil influx into the colonic mucosa during Clostridium difficile infection in mice. Gut Microbes **5:**476-484.
5. **Cook AD, Pobjoy J, Steidl S, Durr M, Braine EL, Turner AL, Lacey DC, Hamilton JA.** 2012. Granulocyte-macrophage colony-stimulating factor is a key mediator in experimental osteoarthritis pain and disease development. Arthritis Res Ther **14:**R199.
6. **Wang Y, Han G, Wang K, Liu G, Wang R, Xiao H, Li X, Hou C, Shen B, Guo R, Li Y, Chen G.** 2014. Tumor-derived GM-CSF promotes inflammatory colon carcinogenesis via stimulating epithelial release of VEGF. Cancer Res **74:**716-726.
7. **Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, Abbas AR, Modrusan Z, Ghilardi N, de Sauvage FJ, Ouyang W.** 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med **14:**282-289.
8. **Holland T.** 2001. A survey of discriminant methods used in toxicological histopathology. Toxicol Pathol **29:**269-273.
9. **Holland T.** 2005. The comparative power of the discriminant methods used in toxicological pathology. Toxicol Pathol **33:**490-494.
10. **Luna RA, Boyanton BL, Jr., Mehta S, Courtney EM, Webb CR, Revell PA, Versalovic J.** 2011. Rapid stool-based diagnosis of Clostridium difficile infection by real-time PCR in a children's hospital. J Clin Microbiol **49:**851-857.
11. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc **3:**1101-1108.
12. **McDermott AJ, Higdon KE, Muraglia R, Erb-Downward JR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB.** 2014. The Role of Gr-1 Cells and TNFalpha Signaling During Clostridium difficile Colitis in Mice. Immunology.
13. **Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, Young VB, LiPuma JJ.** 2012. Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A **109:**5809-5814.
14. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B,**

Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol **75:**7537-7541.

15. **Sadighi Akha AA, Theriot CM, Erb-Downward JR, McDermott AJ, Falkowski NR, Tyra HM, Rutkowski DT, Young VB, Huffnagle GB.** 2013. Acute infection of mice with Clostridium difficile leads to eIF2alpha phosphorylation and pro-survival signalling as part of the mucosal inflammatory response. Immunology **140:**111-122.
16. **Oksanen J, Blanchet, F. G., Kindt, R., Legendre, P., O'Hara, R. B., Simpson, G. L., Solymos, P., Henry, M., Stevens, H. and Wagner, H.** 2010. vegan: Community Ecology Package. R Package version 1.17-3,1.17-3 Edn. 2010.
17. **Sadighi Akha AA, McDermott AJ, Theriot CM, Carlson PE, Jr., Frank CR, McDonald RA, Falkowski NR, Bergin IL, Young VB, Huffnagle GB.** 2014. IL22 and CD160 play additive roles in the host mucosal response to Clostridium difficile infection in mice. Immunology.
18. **Sadighi Akha AA, Harper JM, Salmon AB, Schroeder BA, Tyra HM, Rutkowski DT, Miller RA.** 2011. Heightened induction of proapoptotic signals in response to endoplasmic reticulum stress in primary fibroblasts from a mouse model of longevity. J Biol Chem **286:**30344-30351.
19. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3:**RESEARCH0034.

Chapter 3

Role of GM-CSF in the Inflammatory Cytokine Network that Regulates Neutrophil Influx into the Colonic Mucosa during *Clostridium difficile* Infection in Mice

Portions of this chapter have been previously published

McDermott AJ, Frank CR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB. 2014. Role of GM-CSF in the inflammatory cytokine network that regulates neutrophil influx into the colonic mucosa during Clostridium difficile infection in mice. Gut Microbes **5:**476-484.

Introduction

Clostridium difficile infection is associated with robust neutrophil recruitment, increased expression of inflammatory cytokines and chemokines, and marked damage to the intestinal epithelium(1-7). GM-CSF promotes myeloid cell recruitment(8-12) and inflammatory cytokine production during mucosal inflammation(10, 13), as well protecting against epithelial damage during colonic inflammation(13-15). While recent studies in our lab have reported increased expression of GM-CSF in response to *C. difficile* infection(5), the role of GM-CSF during *C. difficile* colitis has not been directly investigated. In this chapter, we assessed the role of GM-CSF in promoting inflammatory cytokine expression, neutrophil recruitment, and epithelial damage during *C. difficile* colitis.

Results

Expression of GM-CSF during *C. difficile* infection

We used a *C. difficile* infection model adapted from a previously described mouse model of acute *C. difficile* infection.(6) Briefly, mice received the broad-spectrum antibiotic cefoperazone in their drinking water for 5 days, were infected with spores from *C. difficile* strain 630 by oral gavage 2 days after the cessation of antibiotics and then followed for 4 days (Figure 3-1). *C. difficile* 630 infection causes relatively mild disease, and this strain was chosen to permit investigation of both proinflammatory and epithelial-protective functions of GM-CSF. Cefoperazone treatment and *C. difficile* challenge resulted in a significant decrease in total bacterial diversity in the colon that persisted for at least one week post-antibiotic treatment (Figure 3-2) and the establishment of *C. difficile* colonization in the colon (Figure 3-2). Beginning at one-day post-infection, *C. difficile*-infected mice began to lose body weight (Figure 3-2). Additionally, there was a statistically significant increase in GM-CSF expression in the colon of *C. difficile*-infected mice compared to uninfected mice (Figure 3-2), which was not seen in mice treated only with cefoperazone (data not shown).

Effect of anti-GM-CSF treatment on *C. difficile* infection and the intestinal epithelium

To begin to investigate the role of GM-CSF in the pathogenesis of *C. difficile* infection, mice were treated with a neutralizing anti-GM-CSF monoclonal antibody (MP1-22E9), every other day beginning one day prior to infection (Figure 3-1). This treatment did not affect the low bacterial diversity in these mice (Figure 3-2), nor did it significantly alter the composition of the bacterial microbiome (data not shown). Although not statistically significant, there was a trend toward lower *C. difficile* colonization levels and more modest weight loss during the course of disease in mice treated with anti-GM-CSF mAb (Figure 3-2). *C. difficile* infection induced robust expression of the IL-22 pathway in the colonic mucosa, including induction of RegIIIγ (Figure 3-3). There was a trend toward lower IL-22 expression levels in anti-GM-CSF treated mice, but neither IL-22 nor RegIIIγ expression was significantly lower in these mice (Figure 3-3). Thus, treatment with anti-GM-CSF did not exacerbate disease; rather, *C. difficile* colonization levels, weight loss and induction of the IL-22 pathway remained the same, if not slightly improved.

One of the other salient features of *C. difficile* infection in the colon is the destruction of goblet cells in the epithelium. This loss was observed in both levels angiogenin-4 expression (Figure 3-3) and histologically evident changes (Figure 3-4). In the colon, angiogenin-4 is expressed solely by goblet cells in the crypts(41), and the expression of angiogenin-4 was significantly reduced in *C. difficile*-infected mice. Loss of goblet cells from colonic crypts was evident in histology sections, with a marked reduction in these cells (denoted by their vacuoles) observed in both transverse and oblique sections (Figure 3-4). Treatment with anti-GM-CSF mAb did

not significantly protect against loss of angiogenin-4 expression (Figure 3-3) or goblet cells (Figure 3-4) that occurred during *C. difficile* infection.

Effect of anti-GM-CSF treatment on inflammation and neutrophil recruitment during *C. difficile* infection

However, investigation into some of the specific inflammatory pathways associated with *C. difficile* infection revealed a role for GM-CSF in driving inflammatory cell recruitment, most notably neutrophils. *C. difficile* 630 infection induced significant expression of the inflammatory cytokines IFNγ, IL-1β, IL-6 and TNFα in the colon mucosa at four days post-infection (Figure 3-5). Treatment of *C. difficile* infected mice with anti-GM-CSF mAb resulted in significantly lower expression of IL-1β and TNFα (Figure 3-5). There was no significant induction of IL-12, IL-23, IL-17, IL-10 or TGF-β in either group of infected mice (data not shown). Consistent with the leukocytic infiltrates evident in histological sections of *C. difficile* 630 infected mice (Figure 3-4), there was a significant increase in expression of the CC chemokines CCL2 (MCP-1) and CCL4 (MIP-1α) and the IFNγ-inducible non-ELR CXC chemokines CXCL9 (MIG) and CXCL10 (IP10) (Figure 3-6). Anti-GM-CSF treatment had no effect on expression of these chemokines. In contrast to CCL2, CCL4, CXCL9 and CXCL10, anti-GM-CSF treatment significantly reduced expression of the ELR+ CXC chemokines CXCL1 (KC) and CXCL2 (MIP-2) (Figure 3-6). ELR+ CXC chemokines are predominantly neutrophil chemotactic factors and contain a conserved amino acid sequence motif (glutamic acid-leucine-arginine, i.e. E-L-R)

that immediately precedes the first cysteine residue near the amino-terminal end and confers binding specificity to specific CXC chemokine receptors. Expression of both of these ELR+ CXC chemokines in the colon following *C. difficile* infection was also associated with the influx of neutrophils into the parenchyma (Figure 3-4). Consistent with the lower, but still significant, expression of CXCL1 and CXCL2 in anti-GM-CSF treated mice, neutrophils were still evident in colonic mucosal sections, but their numbers were reduced (Figure 3-4).

Inducible nitric oxide synthase (iNOS) is often associated with host antimicrobial responses, and can be expresed by a variety of cells including neutrophils(42, 43) and intestinal epithelial cells.(44) iNOS was expressed in colonic tissue following *C. difficile* infection concomitant with the development of inflammation and neutrophil influx (Figure 3-4). Anti-GM-CSF treatment also resulted in a significant decrease in iNOS expression (Figure 3-6).

Secretory leukocyte protease inhibitor (SLPI) is a well-documented inhibitor of neutrophil elastase, and other serine proteases, is produced by epithelial cells and can protect against neutrophil-mediated protease damage.(45-49) Following *C. difficile* infection, SLPI expression is significantly upregulated in the colonic mucosa (Figure 3-6). However, treatment with anti-GM-CSF significantly reduced expression of SLPI, concomitant with the reduced neutrophil influx in these mice (Figure 3-6).

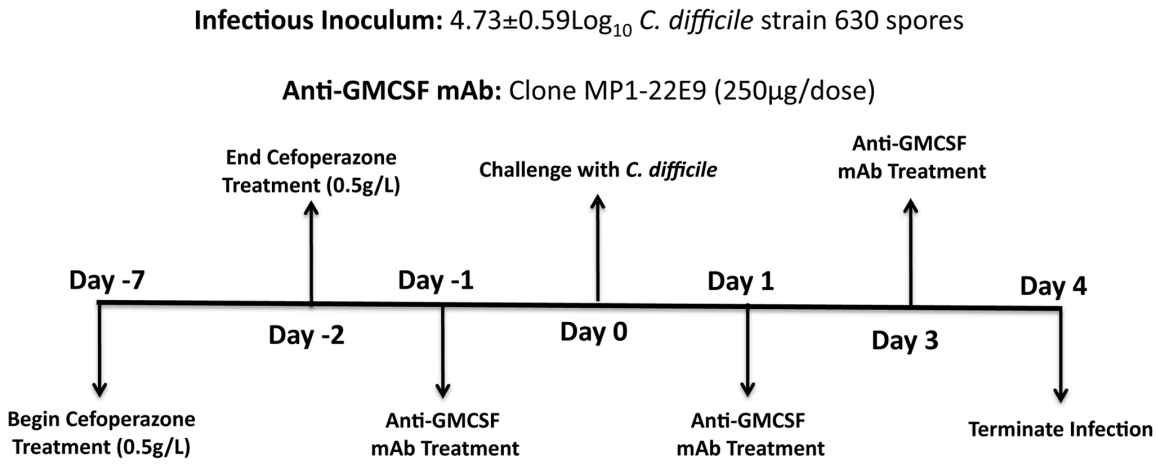

Figure 3-1. Experimental Timeline for Investigating the Role of GM-CSF During *C. difficile* Colitis.

Experimental approach and timeline. Briefly, mice were treated with cefoperazone (0.5g/L) in their drinking water for five days in order to permit *C. difficile* infection. Following a 2-day recovery period, animals were challenged with 4.73±0.59 $\log_{10}$ 630 spores. All samples were collected four days post infection.

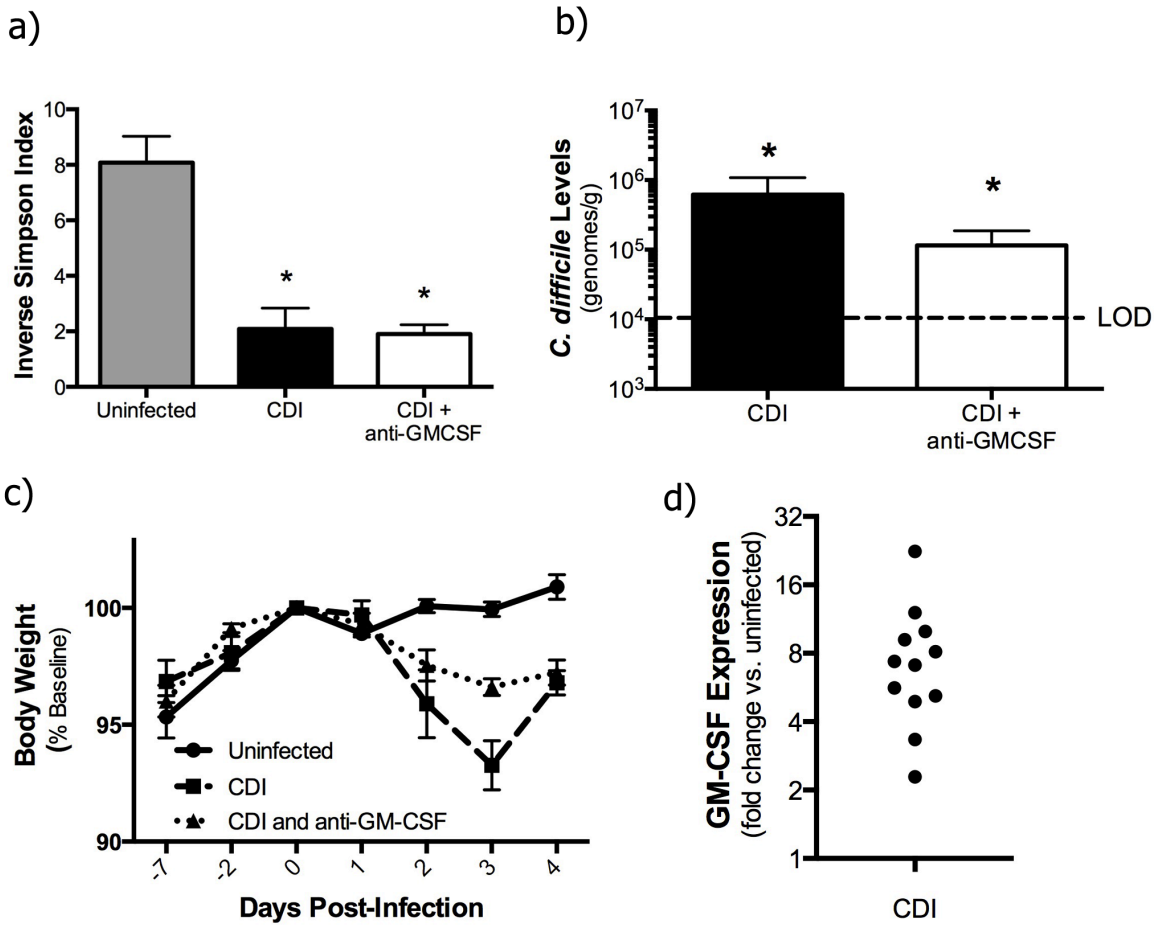

Figure 3-2. Impact of Anti-GM-CSF Treatment on *Clostridium difficile*-Induced Disease.

(a) Colonic microbiota diversity during *C. difficile* 630 infection (Day 4). (b) *C. difficile* 630 colonization of the colonic mucosa, as determined by *C. difficile*-specific qPCR (Day 4). LOD = Limit of Detection (c) Change in body weight during *C. difficile* infection, expressed as percent of baseline body weight at start of experiment. (d) Change in expression of GM-CSF following *C. difficile* 630 infection (Day 4) compared to uninfected mice. (A-C) Mice were treated as outlined in Figure 3-1. CDI= *C. difficile* infected. n= 8 mice per group. Data are the mean ± SEM. *$p<0.05$ compared to uninfected. (d) Mice were treated as outlined in Figure 3-1. n=12 per group (infected & uninfected). $p<0.05$ for dCt values of infected vs. uninfected.

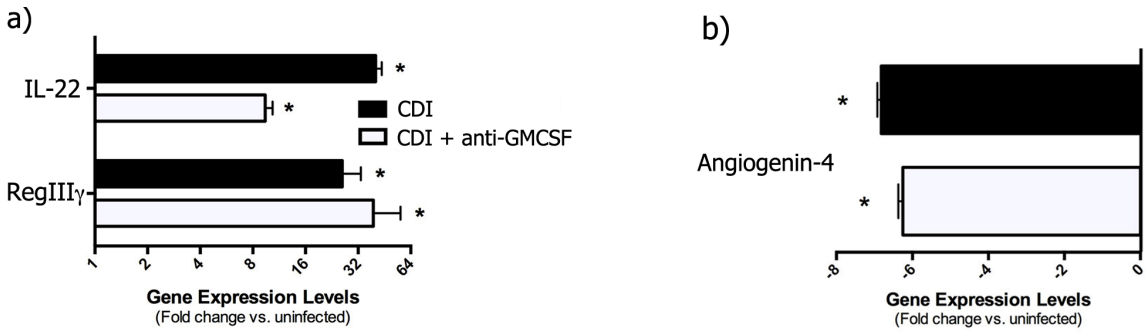

Figure 3-3. Effect of anti-GM-CSF Treatment on IL-22, RegIIIγ, and Angiogenin-4 Expression in the Colonic Mucosa.
Mice were treated as outlined in Figure 3-1. CDI= *C. difficile* infected. Expression was measured by qPCR as outlined in the methods. n ≥ 8 mice per group. Data are the mean ± SEM. *$p<0.05$ compared to uninfected.

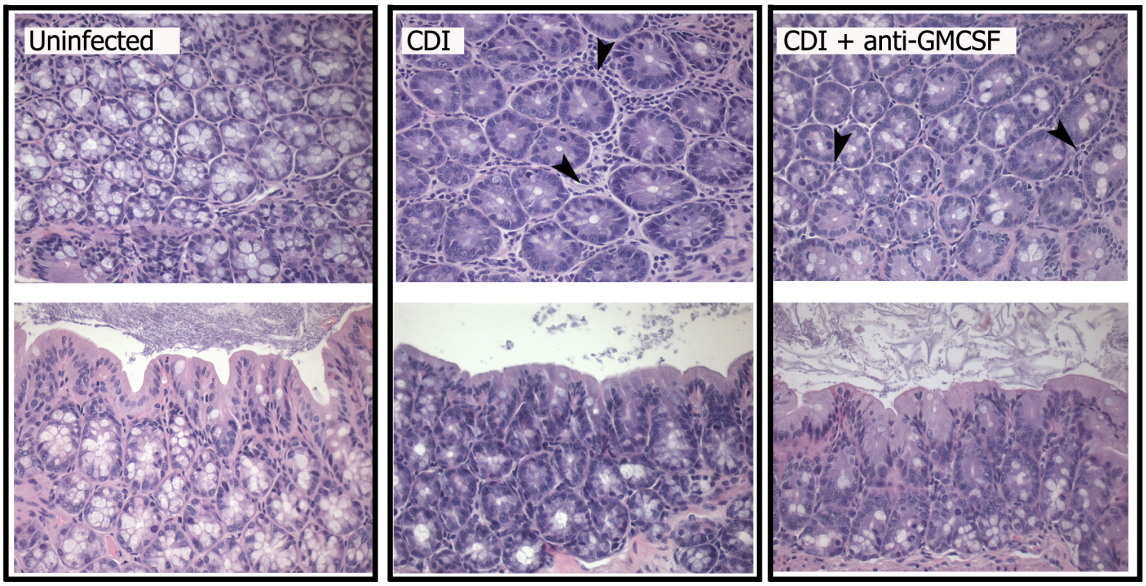

Figure 3-4. Colonic Histopathology Following anti-GM-CSF Treatment.
Photomicrographs of representative H&E-stained oblique and transverse sections of colonic crypts from uninfected (untreated), *C. difficile* 630 infected (Day 4) and *C. difficile* 630 infected, anti-GM-CSF treated (Day 4) mice. CDI= *C. difficile* infected. Mice were treated as outlined in Figure 3-1. Black arrows indicate infiltrating neutrophils. 400X.

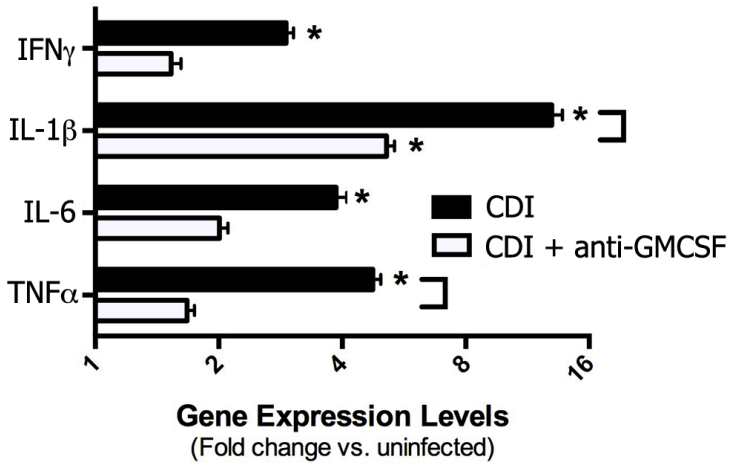

Figure 3-5. Inflammatory Cytokine Expression in the Colonic Mucsoa Following anti-GM-CSF Treatment.
Mice were treated as outlined in Figure 3-1. Expression was measured by qPCR as outlined in the methods. CDI= *C. difficile* infected. n= 8 mice per group. Data are the mean ± SEM. *p<0.05 compared to uninfected.]Brackets p<0.05 in comparing CDI vs. CDI+anti-GMCSF.

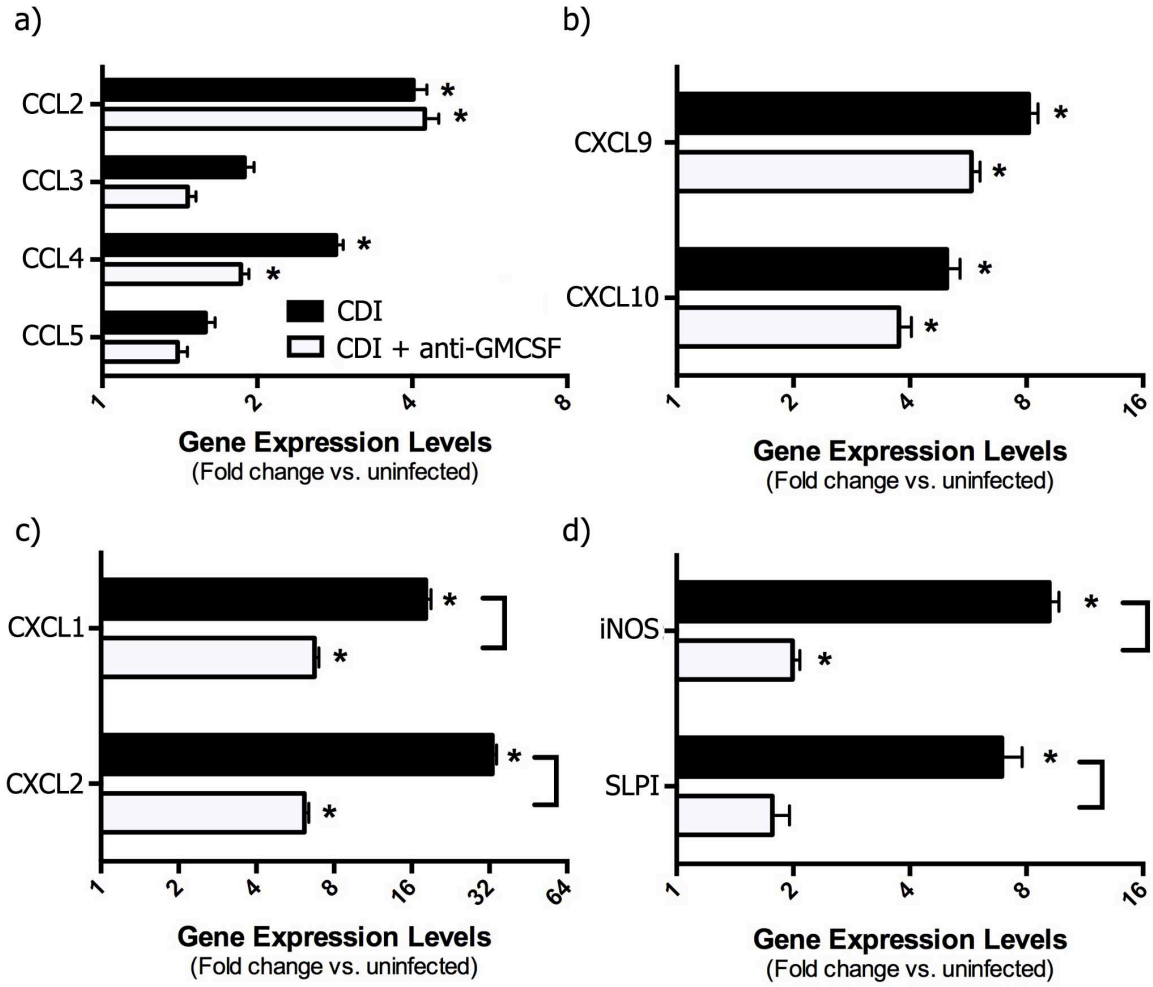

Figure 3-6. Effect of anti-GM-CSF Treatment on Colonic Chemokine Expression. (a-c) Effect of anti-GM-CSF treatment on chemokine expression in the colonic mucosa during *C. difficile* infection (Day 4). (d) Effect of anti-GM-CSF treatment on iNOS and SLPI expression in the colonic mucosa during *C. difficile* infection (Day 4). (A-D) Mice were treated as outlined in Figure 3-1. Expression was measured by qPCR as outlined in the methods. CDI= *C. difficile* infected. n= 8 mice per group. Data are the mean ± SEM. *p<0.05 compared to uninfected.]Brackets p<0.05 in comparing CDI vs. CDI+anti-GMCSF.

Discussion

This is the first reported investigation of the role of GM-CSF in the cytokine network that regulates inflammation following *C. difficile* infection. In the current study, we observed reduced ELR+ CXC chemokine expression in addition to evidence of decreased neutrophil recruitment following anti-GM-CSF treatment. GM-CSF is a potent driver of mucosal inflammation in other disease settings, including the intestinal tract(25-27), but has not been investigated for the pathogenesis of *C. difficile*, a toxin-producing bacteria with distinct virulence mechanisms from attaching and effacing enteric bacteria. GM-CSF has been reported to promote neutrophil recruitment during acute pulmonary inflammation(8-11) and is also required for full recruitment of neutrophils and production of CXCL1 (KC) in an experimental model of otitis media(50). Within the gut, GM-CSF has also been shown to promote neutrophil recruitment during 2,4,6-trinitrobenzenesulfonic acid (TNBS)-colitis(12). Additionally, one study suggests that GM-CSF can function directly as a chemoattractant for neutrophils(12). While we have demonstrated that GM-CSF is expressed in the colon, and previous studies have demonstrated colon-localized effects following intraperitoneal injection of the anti-GM-CSF mAb used in the current study(51), we have no evidence in this study that the site of activity of our anti-GM-CSF mAb is the colon itself. GM-CSF is a critical factor for the survival of infiltrating neutrophils in a tissue, protecting them against apoptosis, but it also plays a role in the differentiation of neutrophils from bone marrow-derived precursors. Thus, an important site of GM-CSF activity during *C. difficile* infection may be the bone marrow. Altogether, our data strongly support the concept that

GM-CSF can regulate neutrophil chemokine expression and neutrophil recruitment during *C. difficile* colitis although its activity may include extra-intestinal regulation of neutrophil biology.

In addition to reduced ELR+ CXC chemokine expression, we also observed decreased expression of inflammatory cytokines, most notably TNFα, during *C. difficile* colitis following treatment with anti-GM-CSF. TNFα expression has been demonstrated to be largely dependent upon GM-CSF signaling in chemical(11) and microbial(10) models of acute pulmonary inflammation. GM-CSF also promotes TNFα production during middle-ear inflammation(50). Inflammatory cytokine expression during chemically-induced colitis is also dependent upon GM-CSF(13). Consistent with other reports, our data strongly suggest that GM-CSF promotes inflammatory cytokine expression during *C. difficile* colitis.

Numerous studies have reported an epithelial-protective function for GM-CSF during mucosal inflammation(13-15, 28, 29). In the absence of GM-CSF, colonic ulceration and overall intestinal pathology in response to DSS is significantly increased(13, 14). Additionally, during *Citrobacter rodentium* infection, GM-CSF serves to protect against the development of severe intestinal pathology(15). In the current study, however, we did not observe increased epithelial damage during *C. difficile* infection following anti-GM-CSF treatment or further reduction in expression of the goblet cell-associated antimicrobial peptide angiogenin-4(41), suggesting colonic goblet cells were not under additional stress following anti-GM-CSF treatment. Altogether, this may reflect a difference in the nature of the primary

source of epithelial damage during infection, *C. difficile* toxins(21-23), compared to other mechanisms of epithelial damage.

One model that could explain the association between reduced neutrophil recruitment and reduced cytokine and chemokine expression seen following anti-GM-CSF treatment is that neutrophils may also be a cellular source of inflammatory cytokines during *C. difficile* infection. Neutrophil recruitment into the colonic mucosa and production of a "storm" of inflammatory cytokines are key features of the pathogenesis of the disease(1-7, 24, 30). *C. difficile* toxins TcdA and TcdB, as well as other factors from *C. difficile,* can elicit IL-1β, TNFα, CC and CXC chemokine production from macrophages and epithelial cells in vitro, but neutrophils have not been investigated as a cellular source(31-37). Neutrophils are capable of producing IL-1β and TNFα, as well as chemokines(52, 53). Additionally, GM-CSF not only serves to prevent neutrophil apoptosis, but also modifies neutrophil behavior and can promote the production of neutrophil chemokines(52, 54). However, other studies in our laboratory, using anti-Gr1 to deplete Gr1+ cells during *C. difficile* VPI 10463 infection in mice, found no change in the levels of IL-1β, TNFα or CXCL1 expression following anti-Gr-1 treatment(55). One caveat of this observation is that this strain of *C. difficile* produces higher levels of toxins, resulting in a more severe disease with more extensive epithelial damage(6, 7, 24). Thus, additional cellular pathways of IL-1β, TNFα or CXCL1 expression may be induced when toxin levels are higher.

Works Cited

1. **Hasegawa M, Kamada N, Jiao Y, Liu MZ, Nunez G, Inohara N.** 2012. Protective role of commensals against Clostridium difficile infection via an IL-1beta-mediated positive-feedback loop. J Immunol **189:**3085-3091.
2. **Hasegawa M, Yamazaki T, Kamada N, Tawaratsumida K, Kim YG, Nunez G, Inohara N.** 2011. Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen. J Immunol **186:**4872-4880.
3. **Jarchum I, Liu M, Lipuma L, Pamer EG.** 2011. Toll-like receptor 5 stimulation protects mice from acute Clostridium difficile colitis. Infect Immun **79:**1498-1503.
4. **Jarchum I, Liu M, Shi C, Equinda M, Pamer EG.** 2012. Critical role for MyD88-mediated neutrophil recruitment during Clostridium difficile colitis. Infect Immun **80:**2989-2996.
5. **Sadighi Akha AA, Theriot CM, Erb-Downward JR, McDermott AJ, Falkowski NR, Tyra HM, Rutkowski DT, Young VB, Huffnagle GB.** 2013. Acute infection of mice with Clostridium difficile leads to eIF2alpha phosphorylation and pro-survival signalling as part of the mucosal inflammatory response. Immunology **140:**111-122.
6. **Theriot CM, Koumpouras CC, Carlson PE, Bergin, II, Aronoff DM, Young VB.** 2011. Cefoperazone-treated mice as an experimental platform to assess differential virulence of Clostridium difficile strains. Gut Microbes **2:**326-334.
7. **Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP.** 2008. A mouse model of Clostridium difficile-associated disease. Gastroenterology **135:**1984-1992.
8. **Balloy V, Chignard M.** 2009. The innate immune response to Aspergillus fumigatus. Microbes Infect **11:**919-927.
9. **Bozinovski S, Jones J, Beavitt SJ, Cook AD, Hamilton JA, Anderson GP.** 2004. Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. Am J Physiol Lung Cell Mol Physiol **286:**L877-885.
10. **Puljic R, Benediktus E, Plater-Zyberk C, Baeuerle PA, Szelenyi S, Brune K, Pahl A.** 2007. Lipopolysaccharide-induced lung inflammation is inhibited by neutralization of GM-CSF. Eur J Pharmacol **557:**230-235.
11. **Vlahos R, Bozinovski S, Chan SP, Ivanov S, Linden A, Hamilton JA, Anderson GP.** 2010. Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. Am J Respir Crit Care Med **182:**34-40.
12. **Khajah M, Millen B, Cara DC, Waterhouse C, McCafferty DM.** 2011. Granulocyte-macrophage colony-stimulating factor (GM-CSF): a chemoattractive agent for murine leukocytes in vivo. J Leukoc Biol **89:**945-953.
13. **Xu Y, Hunt NH, Bao S.** 2008. The role of granulocyte macrophage-colony-stimulating factor in acute intestinal inflammation. Cell Res **18:**1220-1229.

14. **Egea L, McAllister CS, Lakhdari O, Minev I, Shenouda S, Kagnoff MF.** 2013. GM-CSF produced by nonhematopoietic cells is required for early epithelial cell proliferation and repair of injured colonic mucosa. J Immunol **190:**1702-1713.
15. **Hirata Y, Egea L, Dann SM, Eckmann L, Kagnoff MF.** 2010. GM-CSF-facilitated dendritic cell recruitment and survival govern the intestinal mucosal response to a mouse enteric bacterial pathogen. Cell Host Microbe **7:**151-163.
16. **Buffie CG, Pamer EG.** 2013. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol **13:**790-801.
17. **Sonnenberg GF, Fouser LA, Artis D.** 2011. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol **12:**383-390.
18. **Szabady RL, McCormick BA.** 2013. Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products. Front Immunol **4:**220.
19. **Brazil JC, Louis NA, Parkos CA.** 2013. The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease. Inflamm Bowel Dis **19:**1556-1565.
20. **Fournier BM, Parkos CA.** 2012. The role of neutrophils during intestinal inflammation. Mucosal Immunol **5:**354-366.
21. **Britton RA, Young VB.** 2012. Interaction between the intestinal microbiota and host in Clostridium difficile colonization resistance. Trends Microbiol **20:**313-319.
22. **Rupnik M, Wilcox MH, Gerding DN.** 2009. Clostridium difficile infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **7:**526-536.
23. **Voth DE, Ballard JD.** 2005. Clostridium difficile toxins: mechanism of action and role in disease. Clin Microbiol Rev **18:**247-263.
24. **Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB.** 2011. The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut Microbes **2:**145-158.
25. **Dabritz J.** 2014. Granulocyte macrophage colony-stimulating factor and the intestinal innate immune cell homeostasis in Crohn's disease. Am J Physiol Gastrointest Liver Physiol **306:**G455-465.
26. **Hamilton JA.** 2008. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol **8:**533-544.
27. **Lambrecht BN, Hammad H.** 2013. Asthma: the importance of dysregulated barrier immunity. Eur J Immunol **43:**3125-3137.
28. **Bernasconi E, Favre L, Maillard MH, Bachmann D, Pythoud C, Bouzourene H, Croze E, Velichko S, Parkinson J, Michetti P, Velin D.** 2010. Granulocyte-macrophage colony-stimulating factor elicits bone marrow-derived cells that promote efficient colonic mucosal healing. Inflamm Bowel Dis **16:**428-441.
29. **Cakarova L, Marsh LM, Wilhelm J, Mayer K, Grimminger F, Seeger W, Lohmeyer J, Herold S.** 2009. Macrophage tumor necrosis factor-alpha induces epithelial expression of granulocyte-macrophage colony-stimulating

factor: impact on alveolar epithelial repair. Am J Respir Crit Care Med **180:**521-532.

30. **Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG.** 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun **80:**62-73.
31. **Ng J, Hirota SA, Gross O, Li Y, Ulke-Lemee A, Potentier MS, Schenck LP, Vilaysane A, Seamone ME, Feng H, Armstrong GD, Tschopp J, Macdonald JA, Muruve DA, Beck PL.** 2010. Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome. Gastroenterology **139:**542-552, 552 e541-543.
32. **Sun X, Wang H, Zhang Y, Chen K, Davis B, Feng H.** 2011. Mouse relapse model of Clostridium difficile infection. Infect Immun **79:**2856-2864.
33. **Sun X, He X, Tzipori S, Gerhard R, Feng H.** 2009. Essential role of the glucosyltransferase activity in Clostridium difficile toxin-induced secretion of TNF-alpha by macrophages. Microb Pathog **46:**298-305.
34. **Kim JM, Kim JS, Jun HC, Oh YK, Song IS, Kim CY.** 2002. Differential expression and polarized secretion of CXC and CC chemokines by human intestinal epithelial cancer cell lines in response to Clostridium difficile toxin A. Microbiol Immunol **46:**333-342.
35. **Castagliuolo I, Keates AC, Wang CC, Pasha A, Valenick L, Kelly CP, Nikulasson ST, LaMont JT, Pothoulakis C.** 1998. Clostridium difficile toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. J Immunol **160:**6039-6045.
36. **Castagliuolo I, Kelly CP, Qiu BS, Nikulasson ST, LaMont JT, Pothoulakis C.** 1997. IL-11 inhibits Clostridium difficile toxin A enterotoxicity in rat ileum. Am J Physiol **273:**G333-341.
37. **Ryan A, Lynch M, Smith SM, Amu S, Nel HJ, McCoy CE, Dowling JK, Draper E, O'Reilly V, McCarthy C, O'Brien J, Ni Eidhin D, O'Connell MJ, Keogh B, Morton CO, Rogers TR, Fallon PG, O'Neill LA, Kelleher D, Loscher CE.** 2011. A role for TLR4 in Clostridium difficile infection and the recognition of surface layer proteins. PLoS Pathog **7:**e1002076.
38. **Wuthrich M, Filutowicz HI, Warner T, Deepe GS, Jr., Klein BS.** 2003. Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to CD8+ T cells: implications for vaccine development in immune-deficient hosts. J Exp Med **197:**1405-1416.
39. **Deepe GS, Jr., Gibbons R, Woodward E.** 1999. Neutralization of endogenous granulocyte-macrophage colony-stimulating factor subverts the protective immune response to Histoplasma capsulatum. J Immunol **163:**4985-4993.
40. **Cook AD, Pobjoy J, Steidl S, Durr M, Braine EL, Turner AL, Lacey DC, Hamilton JA.** 2012. Granulocyte-macrophage colony-stimulating factor is a key mediator in experimental osteoarthritis pain and disease development. Arthritis Res Ther **14:**R199.
41. **Forman RA, deSchoolmeester ML, Hurst RJ, Wright SH, Pemberton AD, Else KJ.** 2012. The goblet cell is the cellular source of the anti-microbial

angiogenin 4 in the large intestine post Trichuris muris infection. PLoS One **7:**e42248.

42. **Virtuoso LP, Harden JL, Sotomayor P, Sigurdson WJ, Yoshimura F, Egilmez NK, Minev B, Kilinc MO.** 2012. Characterization of iNOS(+) Neutrophil-like ring cell in tumor-bearing mice. J Transl Med **10:**152.
43. **Kolpen M, Bjarnsholt T, Moser C, Hansen CR, Rickelt LF, Kuhl M, Hempel C, Pressler T, Hoiby N, Jensen PO.** 2014. Nitric Oxide Production by Polymorphonuclear Leukocytes in Infected Cystic Fibrosis Sputum Consumes Oxygen. Clin Exp Immunol.
44. **Vallance BA, Deng W, De Grado M, Chan C, Jacobson K, Finlay BB.** 2002. Modulation of inducible nitric oxide synthase expression by the attaching and effacing bacterial pathogen citrobacter rodentium in infected mice. Infect Immun **70:**6424-6435.
45. **Si-Tahar M, Merlin D, Sitaraman S, Madara JL.** 2000. Constitutive and regulated secretion of secretory leukocyte proteinase inhibitor by human intestinal epithelial cells. Gastroenterology **118:**1061-1071.
46. **Reardon C, Lechmann M, Brustle A, Gareau MG, Shuman N, Philpott D, Ziegler SF, Mak TW.** 2011. Thymic stromal lymphopoetin-induced expression of the endogenous inhibitory enzyme SLPI mediates recovery from colonic inflammation. Immunity **35:**223-235.
47. **Taggart CC, Cryan SA, Weldon S, Gibbons A, Greene CM, Kelly E, Low TB, O'Neill S J, McElvaney NG.** 2005. Secretory leucoprotease inhibitor binds to NF-kappaB binding sites in monocytes and inhibits p65 binding. J Exp Med **202:**1659-1668.
48. **Mizoguchi E, Xavier RJ, Reinecker HC, Uchino H, Bhan AK, Podolsky DK, Mizoguchi A.** 2003. Colonic epithelial functional phenotype varies with type and phase of experimental colitis. Gastroenterology **125:**148-161.
49. **Nukiwa T, Suzuki T, Fukuhara T, Kikuchi T.** 2008. Secretory leukocyte peptidase inhibitor and lung cancer. Cancer Sci **99:**849-855.
50. **Kariya S, Okano M, Higaki T, Makihara S, Haruna T, Eguchi M, Nishizaki K.** 2013. Neutralizing antibody against granulocyte/macrophage colony-stimulating factor inhibits inflammatory response in experimental otitis media. Laryngoscope **123:**1514-1518.
51. **Wang Y, Han G, Wang K, Liu G, Wang R, Xiao H, Li X, Hou C, Shen B, Guo R, Li Y, Chen G.** 2014. Tumor-derived GM-CSF promotes inflammatory colon carcinogenesis via stimulating epithelial release of VEGF. Cancer Res **74:**716-726.
52. **Takahashi GW, Andrews DF, 3rd, Lilly MB, Singer JW, Alderson MR.** 1993. Effect of granulocyte-macrophage colony-stimulating factor and interleukin-3 on interleukin-8 production by human neutrophils and monocytes. Blood **81:**357-364.
53. **Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA.** 2000. The neutrophil as a cellular source of chemokines. Immunol Rev **177:**195-203.
54. **Brach MA, deVos S, Gruss HJ, Herrmann F.** 1992. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage

colony-stimulating factor is caused by inhibition of programmed cell death. Blood **80:**2920-2924.

55. **McDermott AJ, Higdon KE, Muraglia R, Erb-Downward JR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB.** 2014. The Role of Gr-1 Cells and TNFalpha Signaling During Clostridium difficile Colitis in Mice. Immunology.