

Chapter 5

The Role of IL-23, IL-22, and IL-17a During *Clostridium difficile* Colitis in Mice

Introduction

Marked neutrophil recruitment is one of the most prominent host responses to *C. difficile* infection in antibiotic pretreated mice(1-6). IL-23 is a heterodimeric cytokine comprised of a p19 and a p40 subunit(7), which promotes innate inflammatory responses and neutrophil recruitment during mucosal inflammation at numerous sites(8-12). Additionally, IL-23 can drive the induction of both IL-22 and IL-17 (8, 10, 12-14), two cytokines known to promote neutrophil recruitment to mucosal surfaces (15-21). However, the role of IL-23, IL-22, and IL-17a in promoting inflammatory responses during *C. difficile* colitis remains poorly understood. In this chapter, we investigated the roles of IL-23, IL-22, and IL-17 in driving neutrophil recruitment, intestinal histopathology, and inflammatory cytokine expression in the colon in response to *C. difficile* infection.

Results

Effect of IL-23 deficiency on colonic neutrophil recruitment

In order to investigate the role of IL-23 in promoting innate inflammation during *C. difficile* colitis, WT and p19^(-/-) (IL-23KO) mice were given cefoperazone (0.5g/L) in their drinking water for five days as described previously(6, 22). Following a two day recovery period on regular water, mice were challenged with $5.70 \pm 0.25 \log_{10}$ *C. difficile* spores (strain VPI 10463). Animals were followed for an additional 2 days, and all samples were collected at 2 days post infection. All infected groups had a mean *C. difficile* colonization level of $\geq 10^5$ CFU/g host tissue by qPCR (data not shown).

In order to determine the role of IL-23 in driving neutrophil recruitment in response to *C. difficile* colitis, flow cytometry was utilized to identify recruited leukocytes. Analysis of colonic leukocytes isolated from WT animals revealed a drastic influx of CD11b^{High} Ly6G^{High} neutrophils following *C. difficile* infection (Figure 5-1). In contrast, the frequency of the CD11b^{High} Ly6G^{High} neutrophil population was markedly reduced in IL-23KO animals (Figure 5-1). Further quantification of the total number of CD11b^{High} Ly6G^{High} neutrophils revealed a statistically significant reduction in the total number of neutrophils recruited to the colons of IL-23KO animals as compared to WT (Figure 5-1). These data demonstrate a significant reduction in neutrophil recruitment to the colon in response to *C. difficile* colitis in the absence of IL-23.

Inflammatory chemokine expression in the absence of IL-23

To investigate the effect of IL-23 in driving chemokine expression during *C. difficile* colitis, quantitative RT-PCR was used to examine colonic cytokine expression at two days post infection. *C. difficile* infection was associated with increased expression of the neutrophil chemokines *Cxcl1*, *Cxcl2*, and *Ccl3*, as well as the neutrophil stabilization factor *Csf3* within the colonic mucosa (Figure 5-1). Consistent with the reduced neutrophilic influx observed response to *C. difficile* infection in IL-23KO mice (Figure 5-1), *Cxcl1*, *Cxcl2*, *Ccl3*, and *Csf3* expression levels were significantly reduced in IL-23KO animals as compared WT (Figure 5-1). There was no defect in the expression of the eosinophil chemokines *Ccl11* and *Ccl24*, or the T-cell chemokines *Cxcl9* and *Cxcl10* in IL-23 deficient animals (Figure 5-2). Taken together, these data demonstrate that IL-23 deficiency is associated with significant defects in both the recruitment of neutrophils to the colon and the expression of neutrophil chemoattractants within the colonic mucosa during *C. difficile* colitis.

Effect of IL-23 deficiency on colonic inflammatory cytokine expression

RT-PCR analysis was also utilized to determine the role of IL-23 in promoting inflammatory cytokine expression in response to *C. difficile* infection. Expression of the antimicrobial C-type lectin *RegIIIg* was significantly increased in response to *C. difficile* infection, and this induction was significantly reduced in IL-23KO mice

(Figure 5-2). IL-23 deficient animals displayed no reduction in *Il1b* or *Ifng* expression levels in response to *C. difficile* infection (Figure 5-2). However, expression levels of the inflammatory cytokines *Il6*, *Il33*, and *Tnf* were all significantly reduced in the absence of IL-23 (Figure 5-2). Additionally, the increased expression of *Il17a* and *Il22* seen in WT animals was completely abrogated in IL-23KO mice (Figure 5-2). Thus, these data indicate that IL-23 promotes the induction of numerous inflammatory cytokines including *Il6*, *Il17a*, and *Tnf*, as well as the pleiotropic cytokine *Il22*, in response to *C. difficile* colitis.

In order to assess the contribution of IL-23 signaling towards the development of intestinal inflammation and epithelial destruction during *C. difficile* colitis, sections of the colonic mucosa were examined for histopathological evidence of severe inflammation. In addition to significant neutrophilic influx (Figure 5-1), *C. difficile* infection was associated with significant epithelial damage and edema, indicative of severe intestinal inflammation (Figure 5-3). The absence of IL-23 had no impact on the development of colonic epithelial damage (Figure 5-3), and the level of edema was not significantly reduced in IL-23KO mice (Figure 5-3).

The role of IL-17 during *C. difficile* colitis

To investigate the contribution of IL-17 in supporting neutrophil recruitment and mucosal inflammatory responses during *C. difficile* colitis, IL-17a^(-/-) (IL-17KO) mice were infected with *C. difficile*. As in previous experiments, all samples were collected at two days post infection.

In order to determine the role of IL-17 in promoting neutrophil recruitment during *C. difficile* colitis, the expression of neutrophil chemokines in *C. difficile*-infected IL-17KO mice was assessed. Compared to *C. difficile* infection in WT animals, there was no reduction in expression of the neutrophil chemokines *Cxcl1*, *Cxcl2*, and *Ccl3* within the colonic mucosa of IL-17KO mice (Figure 5-4). Consistently, cellular infiltrates were apparent in colonic sections from IL-17KO mice (Figure 5-5) and the levels of colonic neutrophilic inflammation were equivalent between IL-17KO and WT animals infected with *C. difficile* (Figure 5-5). These data suggest that IL-17 signaling is not required for the expression of neutrophil chemokines or the development of neutrophilic inflammation in response to *C. difficile* colitis.

RT-PCR analysis was also utilized to investigate the role of IL-17 in promoting inflammatory cytokine expression in response to *C. difficile* infection. Interestingly, the absence of IL-17 was not associated with any reduction in inflammatory cytokines, including *Ifng*, *Il1b*, *Il6*, *Il33*, and *Tnfa* (Figure 5-4). Additionally, *Ccl24*, *Cxcl9*, and *Cxcl10* expression levels were unchanged in IL-17KO mice (Figure 5-4). Consistent with the unaltered induction of inflammatory cytokines seen in these animals, IL-17KO mice were not protected against the development of significant colonic epithelial damage and edema during *C. difficile* infection (Figure 5-5). Taken together, these data support that hypothesis that neutrophil recruitment, inflammatory cytokine expression, and the development of colonic histopathology during *C. difficile* colitis are independent of IL-17 signaling.

The role of IL-22 during *C. difficile* colitis

In order to determine the role of IL-22 in supporting mucosal inflammatory responses to *C. difficile* infection, mice were treated with an anti-IL-22 mAb (clone 8E11) one day prior to and one day post *C. difficile* infection. Animals were followed for two days post infection at which point all samples were collected.

Colonic sections from anti-IL-22 treated mice were examined for signs of marked histopathology. Anti-IL-22 treatment was associated with no reduction in epithelial damage or edema as compared to WT *C. difficile* infected animals (Figure 5-5). In agreement with these findings, colonic expression of numerous proinflammatory cytokine including *Il1b*, *Il6*, and *Il33* were unchanged in anti-IL-22 treated mice (Figure 5-4). Interestingly, the expression levels of other inflammatory cytokines, most notably *Tnfa* and *Ifng* as well as *Cxcl10*, were significantly increased following anti-IL-22 treatment, while *RegIIIg* was significantly reduced (Figure 5-4). These data indicate that IL-22 does not promote the development of severe intestinal histopathology or the expression of inflammatory cytokines during *C. difficile* colitis.

In order to investigate the role of IL-22 in supporting neutrophil recruitment in response to *C. difficile* colitis, colonic sections from anti-IL-22 treated mice were scored for neutrophilic inflammation. Anti-IL-22 treatment was not associated with any reduction in neutrophilic inflammation (Figure 5-5), and consistently, the expression levels of *Cxcl1*, *Cxcl2*, and *Ccl3* were unchanged following anti IL-22

treatment (Figure 5-4). Taken together, these data suggest that IL-22 does not promote neutrophil recruitment during response to *C. difficile* colitis.

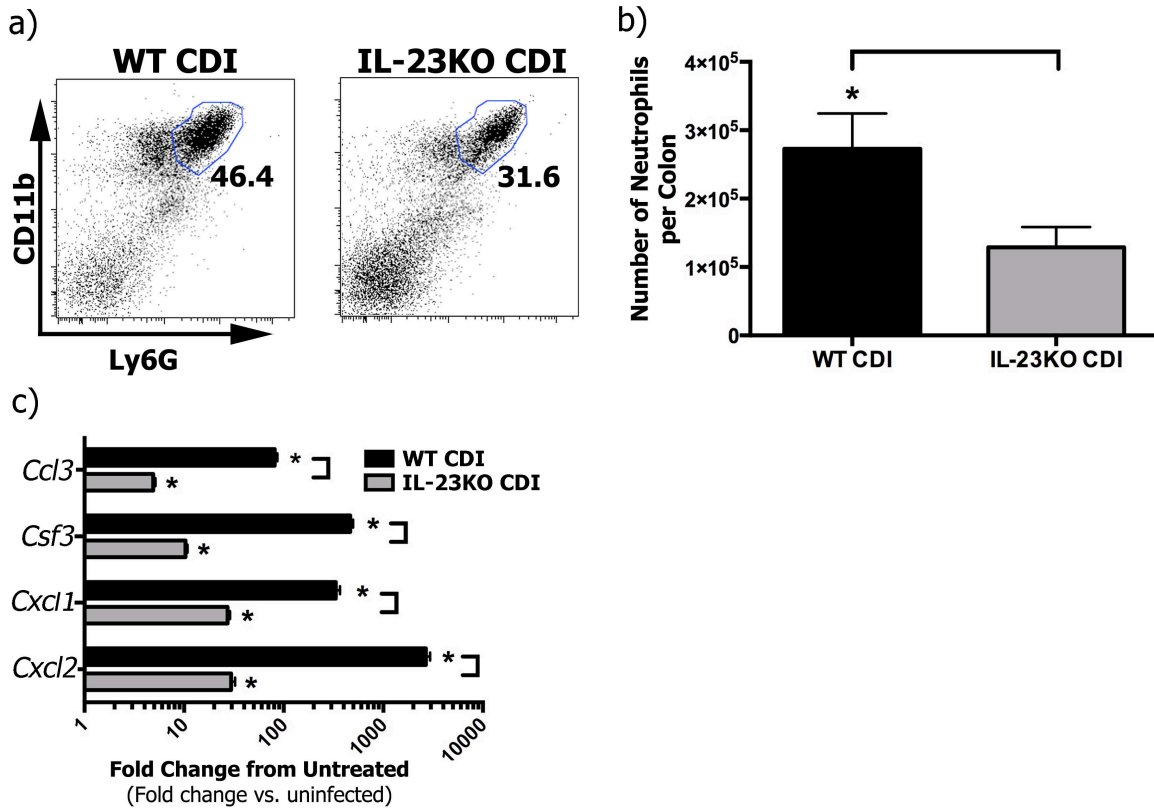


Figure 5-1: Colonic neutrophil recruitment and neutrophil chemokine expression in response to *C. difficile* infection in the absence of IL-23 signaling. (a) Analysis of CD11b and Ly6G expression profiles of CD45⁺ colonic leukocytes. The bolded number represents the percentage of total CD45⁺ leukocytes contained within the indicated gate. (b) Total number of recruited CD11b^{High} Ly6G^{High} neutrophils as defined in panel (a). Bars represent mean ± SEM number of recruited neutrophils for the indicated group. n = 8 per group. (c) Colonic gene expression was assessed via qPCR as outlined in the methods. n ≥ 6 per group. Data are shown as mean ± SEM fold change gene expression of WT *C. difficile* infected (black bars) and IL-23KO *C. difficile* infected (gray bars) animals compared to untreated WT mice. CDI = *C. difficile* infected. For all analyses, * p<0.05 as compared to untreated WT animals and] Brackets indicate p<0.05 for the differences between indicated groups.

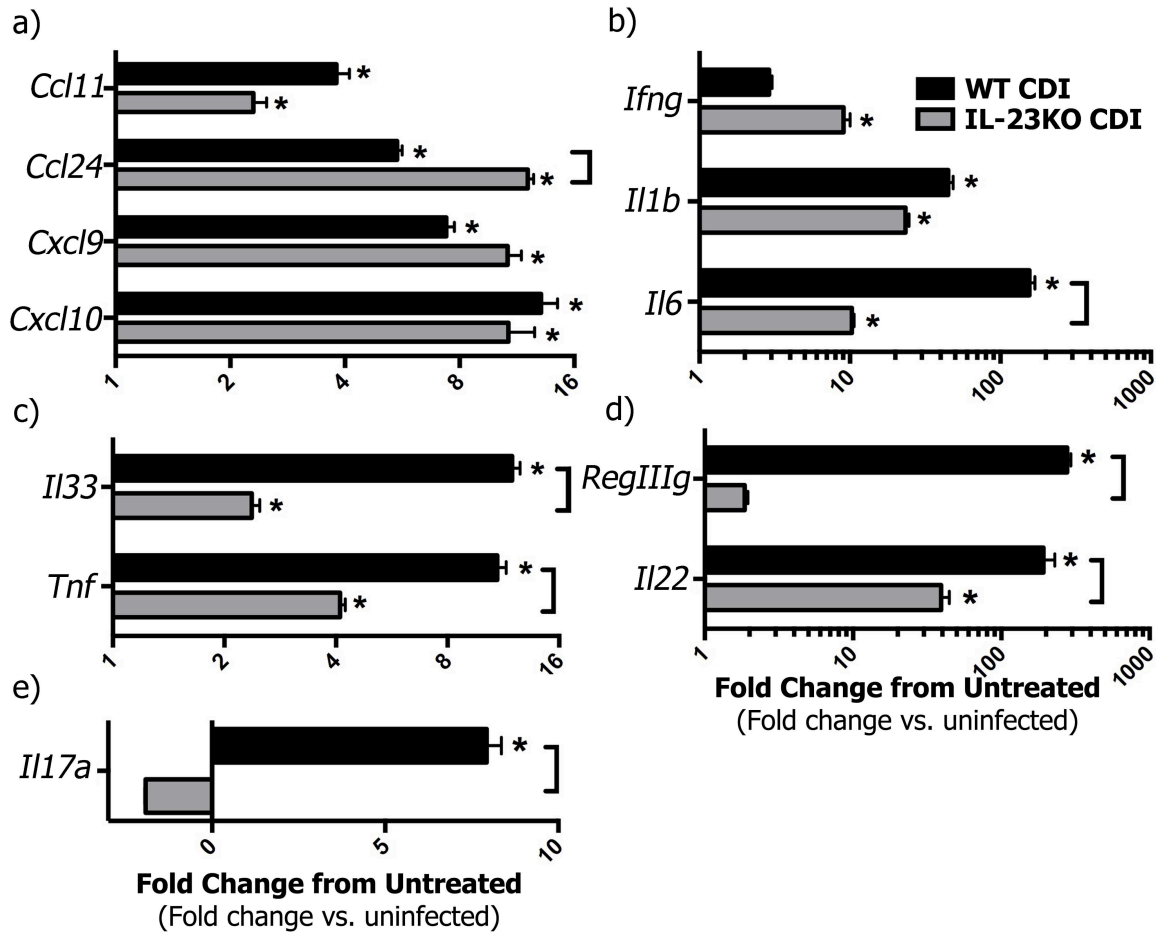
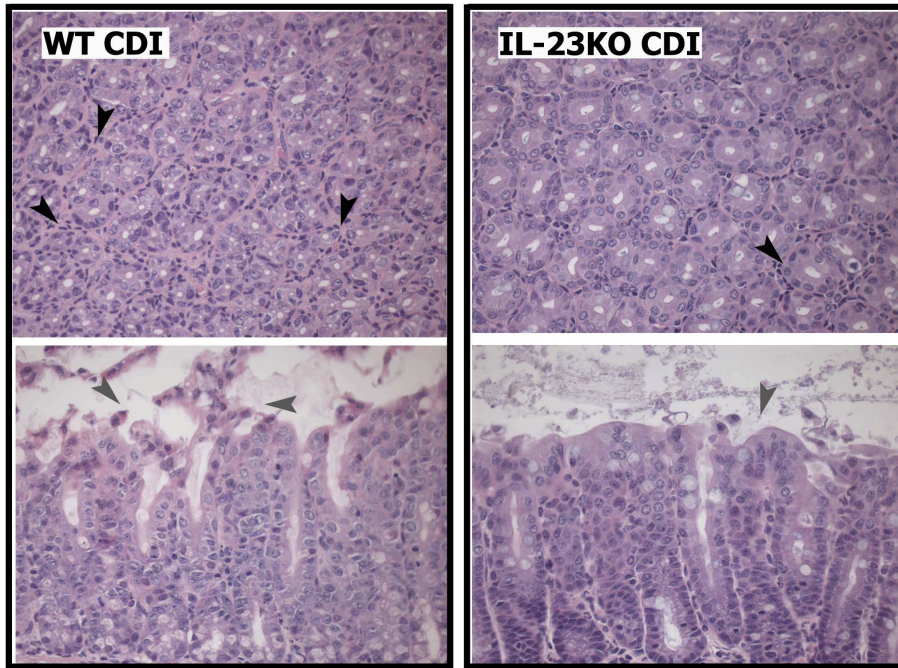


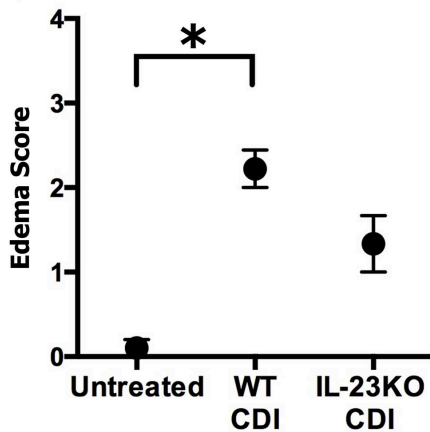
Figure 5-2: Effect of IL-23 deficiency on colonic inflammatory cytokine and chemokine expression during *C. difficile* colitis.

Host gene expression was measured as outlined in the methods. $n \geq 6$ per group. Data are shown as mean \pm SEM fold change gene expression of WT *C. difficile* infected (black bars) and IL-23KO *C. difficile* infected (gray bars) animals compared to untreated WT mice. CDI = *C. difficile* infected. * $p < 0.05$ as compared to untreated WT animals.] Brackets indicate $p < 0.05$ for the differences between indicated groups.

a)



b)



c)

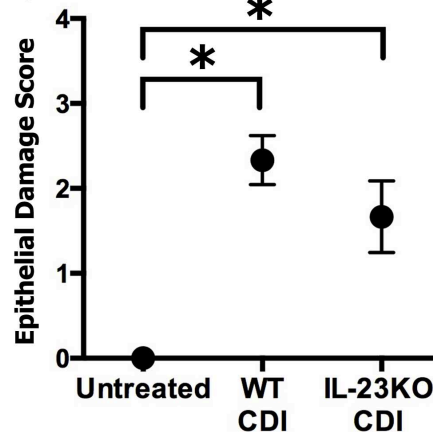


Figure 5-3: Colonic histopathology during *C. difficile* infection in the absence of IL-23.

(a) Representative photomicrographs of H&E-stained colonic sections from WT *C. difficile* infected and IL-23KO *C. difficile* infected animals. Cross-sections of colonic crypts (upper images) and longitudinal sections of the epithelial-luminal interface (lower images) are shown for each genotype. Black arrowheads highlight cellular infiltrate, while gray arrowheads highlight epithelial damage. Total magnification for all images is 400X. (b-c) Histopathological scoring of colonic sections from Untreated, WT CDI, and IL-23KO CDI mice. Slides were scored for edema (b) and epithelial damage (c) as described in the methods. Data are shown as mean \pm SEM. $n \geq 6$ per group. CDI = *C. difficile* infected.] Brackets indicate $p < 0.05$ for the differences between indicated groups.

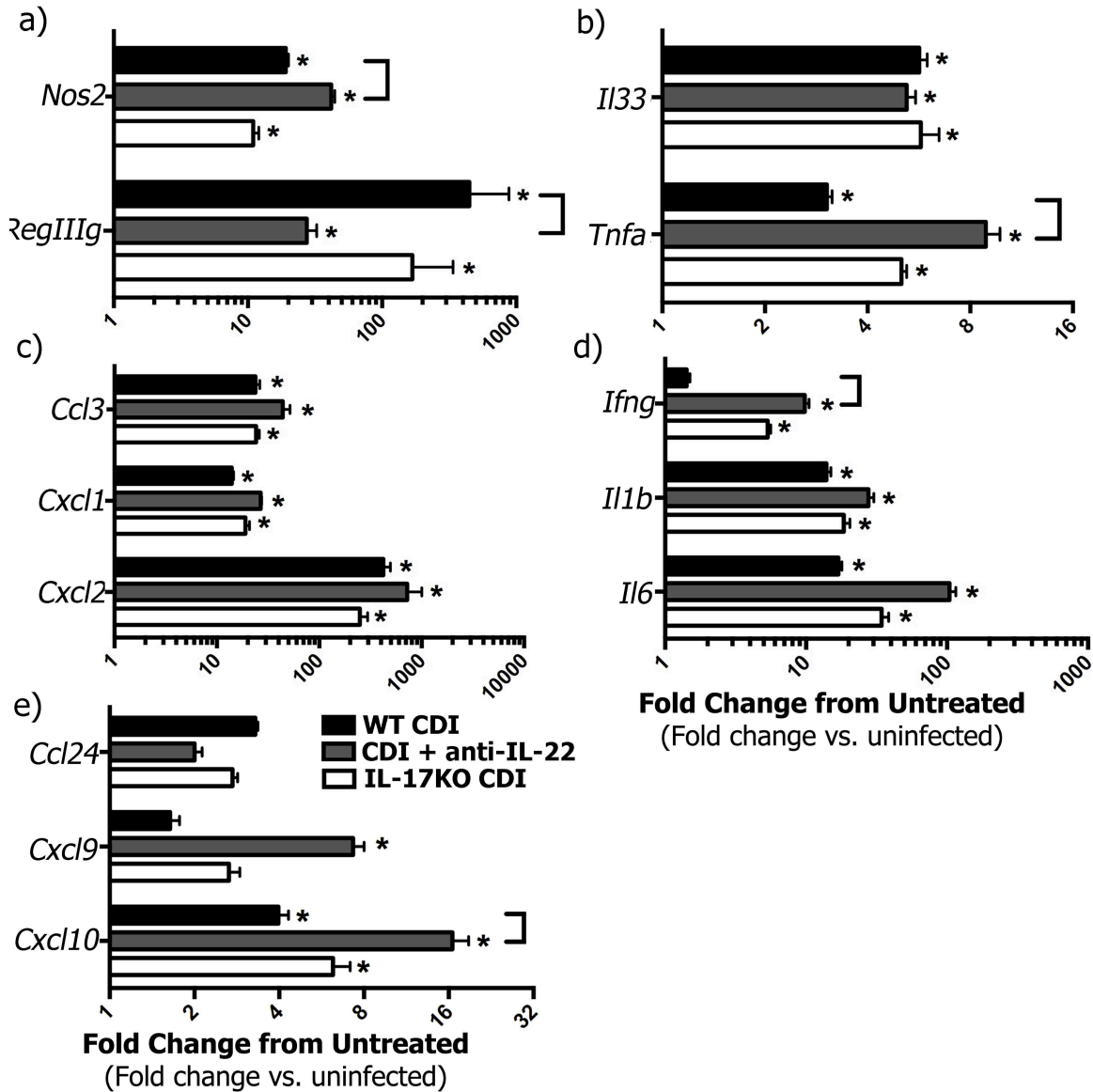


Figure 5-4: Effect of anti-IL-22 treatment or IL-17 deficiency on colonic inflammatory cytokine and chemokine expression during *C. difficile* colitis. Colonic gene expression was assessed via qPCR as outlined in the methods. $n \geq 6$ per group. Data are shown as mean \pm SEM fold change gene expression of WT *C. difficile* infected (black bars), *C. difficile* infected and anti-IL-22 treated (gray bars), and IL-17KO *C. difficile* infected (white bars) animals compared to untreated WT mice. CDI = *C. difficile* infected. * $p < 0.05$ as compared to untreated WT animals.] Brackets indicate $p < 0.05$ for the differences in expression levels between indicated groups.

a)

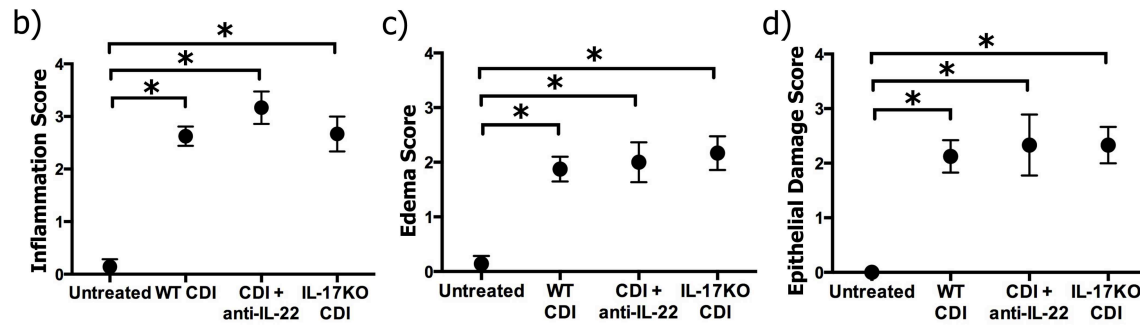
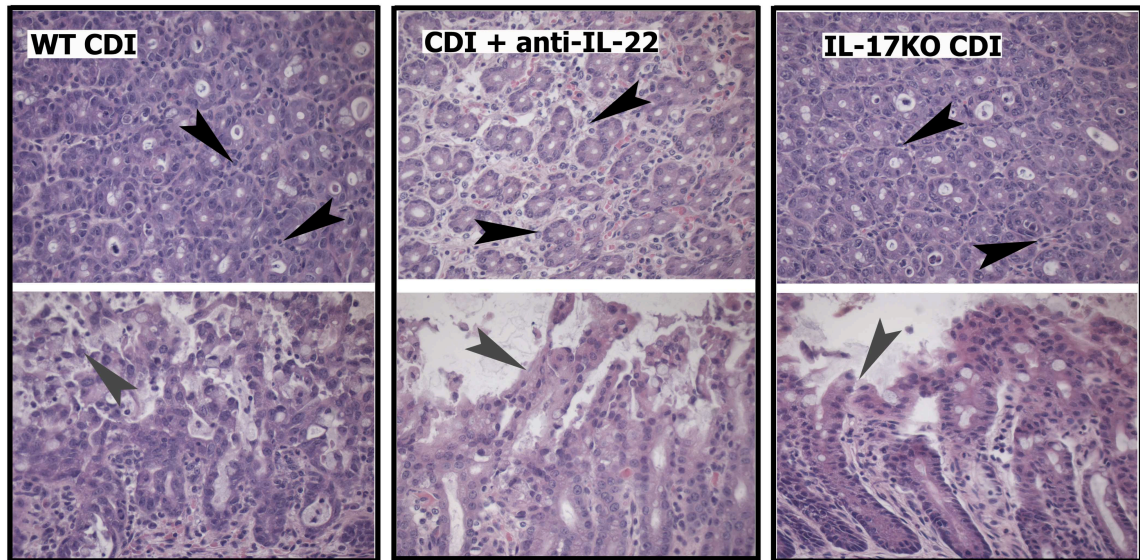


Figure 5-5: Colonic histopathology during *C. difficile* infection in the absence of IL-17 or following anti-IL-22 treatment.

Representative photomicrographs of H&E-stained colonic sections from WT *C. difficile* infected, *C. difficile* infected and anti-IL-22 treated, and IL-17KO *C. difficile* infected animals. Cross-sections of colonic crypts (upper images) and longitudinal sections of the epithelial-luminal interface (lower images) are shown for each genotype. Black arrowheads highlight leukocytic infiltrate, and gray arrowheads highlight areas of epithelial damage. Total magnification for all images is 400X. (b-d) Histopathological scoring of colonic sections from Untreated, WT CDI, CDI+ anti-IL-22, and IL-17KO CDI mice. Slides were scored for neutrophilic inflammation (b), edema (c), and epithelial damage (d) as described in the methods. Data are shown as mean \pm SEM. $n \geq 6$ per group. CDI = *C. difficile* infected.] Brackets indicate $p < 0.05$ for the differences between indicated groups.

Discussion

In the current study, we reported decreased neutrophil recruitment in IL-23 deficient animals in response to *C. difficile* colitis. This decrease in neutrophil recruitment was associated with decreases in *Cxcl1* and *Cxcl2* expression, as well as reduced expression of *Il-17a* and *Il-22*. However, neither *Cxcl1* and *Cxcl2* expression nor neutrophilic inflammation was reduced in either IL-17-deficient mice or mice treated with a depleting anti-IL-22 mAb. Thus, our data strongly suggest that IL-23, independent of IL-17 or IL-22, drives neutrophil recruitment and innate inflammatory responses during *C. difficile* colitis.

In the absence of IL-23, neutrophil recruitment was significantly reduced in response to *C. difficile* colitis. Recent studies have demonstrated increased levels of IL-23 in colonic biopsies from *C. difficile* infected patients(23), as well as increased levels of IL-23 production from myeloid cells stimulated with *C. difficile* toxins *in vitro*(24). However, the role of IL-23 in supporting innate inflammatory responses, including neutrophil recruitment, remains poorly understood. In the current study, we observed reduced neutrophil recruitment in association with decreased expression of neutrophil chemotactic factors in the absence of IL-23. Previous studies have reported a role for IL-23 in supporting neutrophil recruitment and neutrophil chemokine production in other models of mucosal inflammation(8-12, 25, 26). IL-23 is required for the full recruitment of neutrophils to the large intestine in response to both *S. typhimurium* typhlocolitis(12), as well as dextran sodium sulfate (DSS) colitis(8). Additionally, neutrophil recruitment during pulmonary inflammation in response to both chemical(11) and microbial challenges(9, 10, 26)

is supported by IL-23. We observed a significant defect in the recruitment of CD11b^{High} Ly6G^{High} neutrophils, as well as the induction of the neutrophil chemokines *Cxcl1* and *Cxcl2*, in IL-23 deficient mice infected with *C. difficile*. Taken together, these data strongly suggest that IL-23 promotes neutrophil chemokine expression and neutrophil recruitment to the colon during *C. difficile* colitis.

Despite the robust increase in *Il22* expression within the colonic mucosa during *C. difficile* colitis, we observed no decrease in inflammatory cytokine and chemokine expression following anti-IL-22 treatment. IL-22 promotes CXCL1 production from mouse tracheal epithelial cells(27), and also supports neutrophil recruitment in response to chemical pulmonary challenge(15). Additionally, IL-22 is capable of stimulating neutrophil chemokine expression from both colonic epithelial cells(21) and subepithelial myofibroblasts(20) *in vitro*. However, in agreement with a recent study by Hasegawa et al(28), we observed no decrease in expression of the neutrophil chemokines *Cxcl1* or *Cxcl2*, or reduced expression of the inflammatory cytokines *Il1b*, *Il6*, or *Il33* following anti-IL-22 treatment. Neutrophilic inflammation, edema, and epithelial damage levels were also unchanged following the administration of anti-IL-22. Furthermore, anti-IL-22 treatment was associated with increased expression of *Cxcl10*, *Ifng*, and *Tnfa*. Thus, these data strongly suggest that IL-22 is not a major driver of neutrophil chemokine or inflammatory cytokine expression in response to *C. difficile* colitis.

Likewise, we observed no significant change in the severity of intestinal histopathology following anti-IL-22 treatment. The role of IL-22 during mucosal inflammation is pleiotropic: while IL-22 is protective against severe colonic

histopathology and mortality during *Citrobacter rodentium* infection(14), IL-22 drives severe intestinal histopathology and necrosis during *Toxoplasma gondii* infection(13). Recent studies have reported no change in the severity of intestinal histopathology in IL-22KO animals(28) or following anti-IL-22 treatment(6) during *C. difficile* infection. Consistently, we found that anti-IL-22 treatment was not associated with a significant increase or amelioration of neutrophilic inflammation, colonic epithelial damage or edema during *C. difficile* colitis. Thus, while IL-22 may support other host responses, the data presented here indicate that the development of colonic histopathology is independent of IL-22.

IL-17 deficiency was not associated with any reduction in expression of inflammatory cytokines or neutrophil-attracting chemokines, including *Cxcl1* and *Cxcl2* within the colonic mucosal following *C. difficile* infection. IL-17 has a well-documented role supporting neutrophil recruitment during inflammatory responses at mucosal sites(15-19, 29). Specific to the gut, IL-17 promotes CXCL1 expression in response to *Salmonella typhimurium* typhlocolitis(16), as well as supporting neutrophil recruitment during both DSS(18) and TNBS(19) colitis. However, we observed no reduction in expression of the neutrophil chemoattractants *Cxcl1*, *Cxcl2*, or *Ccl3* in the absence of IL-17. In agreement with the unaltered levels of chemokine expression, as well as the unaltered expression of the inflammatory cytokines *Il1b*, *Il6*, *Il33*, and *Tnfa*, IL-17 deficiency was not associated with a reduction in the severity of colonic histopathology. Taken together, our data support the hypothesis that IL-17 is dispensable for the recruitment of neutrophils, the induction of

inflammatory cytokines, and the development of intestinal histopathology during *C. difficile* colitis.

In addition to decreased neutrophil recruitment, IL-23 deficiency was associated with decreased expression of the inflammatory cytokines *Il6*, *Il33*, and *Tnfa* as well as a trend towards decreased colonic edema. IL-23 contributes to IL-6 production in response to *Pseudomonas aeruginosa* pulmonary infection(9), and IL-6 and IL-1 β expression in response to *Toxoplasma gondii* ileitis is partially dependent upon IL-23(13). Furthermore, interference with IL-23 signaling has been shown to reduce the severity of intestinal histopathology in both infectious(13) and chemical(8) models of gastrointestinal inflammation. In the current study, we report decreased colonic edema in association with reduced inflammatory cytokine expression in IL-23 deficient animals. These data suggest that IL-23 contributes to the development of severe intestinal histopathology and drives the induction of inflammatory cytokine including *Il6* and *Il33* during *C. difficile* colitis.

The data presented in the current study suggests a clear role for IL-23 in supporting neutrophil recruitment, the induction of inflammatory cytokines, and the development of severe colonic histopathology during *C. difficile* infection. One possible model that could explain these phenomena is that neutrophils, recruited in part by IL-23 signaling, contribute to the development of colonic histopathology and severe disease outcomes. Indeed, a recent study has demonstrated reduced morbidity and mortality in IL-23 deficient mice infected with *C. difficile*(23). However, numerous previous studies have reported increased mortality during *C. difficile* infection following interventions that reduced neutrophilic influx(1-3),

suggesting a protective role for neutrophil recruitment. Furthermore, a recent study from our laboratory found no reduction in the severity of colonic histopathology during *C. difficile* colitis following anti-Gr-1 treatment(4). Taken together these studies suggest that IL-23 signaling may ultimately play a dual role during *C. difficile* colitis by both promoting neutrophil recruitment as well as other innate responses that contribute to morbidity and intestinal histopathology during infection.

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Chapter 6

Summary and Model, Critical Analysis, Future Direction, and Significance

Summary and Model

In this study, we tested the following 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

The current study examined numerous host cell types and inflammatory signals in order to identify host-derived drivers of inflammatory cytokine expression, neutrophil recruitment, and colonic histopathology during *C. difficile* colitis. This study revealed that both IL-23 and GM-CSF promote the expression of inflammatory cytokines, including IL-1 β and IL-6, in response to *C. difficile* infection. GM-CSF and IL-23 also drive the expression of the neutrophil attractant chemokines CXCL1 and CXCL2 in response to acute *C. difficile* colitis. Furthermore, IL-23 is required for full recruitment of neutrophils in response to *C. difficile* colitis. While IL-17 was dispensable for both the induction of inflammatory cytokines and neutrophil recruitment, both IL-22 and TNF α signaling restrained the inflammatory cytokine expression, and in the case of TNF α , prevented the development of severe

colonic histopathology. IL-23-dependent IL-22 signaling is also required for induction of the antimicrobial lectin RegIII γ . Despite the marked neutrophil recruitment in response to *C. difficile* colitis, neutrophils were not responsible for the development of intestinal histopathology or the induction of inflammatory cytokines or neutrophil-recruiting chemokines within the colon.

Taken together, these data support a model where IL-23 and GM-CSF independently initiate inflammatory cascades that promote neutrophil chemokine expression, neutrophil recruitment, and inflammatory cytokine expression within the colon following *C. difficile* infection. Activation of these cascades results in the induction of other inflammatory cytokines, including TNF α , IL-22, and IL-17, whose individual contributions to the development of inflammation are minimal, potentially due to the induction of numerous inflammatory cytokines with redundant functions as the cascades progress. Ultimately, these cascades result in robust neutrophil recruitment, which is ultimately dispensable for the development of colonic histopathology and inflammatory cytokine expression in response to acute, severe *C. difficile* colitis (Figure 6-1).

Critical Analysis

While the results from the experiments described above provide support for our conclusions, alternative experimental methodologies or interpretations are possible. This section will address some of the potential technical and conceptual limitations of these studies.

- The intestinal microbiota of knockout mice used in the current study, specifically the IL-23^{-/-} (p19^{-/-}) mice, may have influenced the progression of disease in these animals. The composition of the intestinal microbiota influences susceptibility to *C. difficile* infection(1-5), and previous studies have demonstrated that mouse colonies maintained separately can develop distinct intestinal microbiota in the presence or absence of an immune deficiency (6-9). However, the antibiotic pretreatment required to permit *C. difficile* infection drastically reduces the diversity of the intestinal microbiota(2, 10, 11), likely reducing the potential impact of any initial differences in the colonic microbiota of knockout animals on the outcome of infection. However, in order to explicitly control for this possibility, subsequent experiments should either cohoused knockout and wild-type mice to permit “equilibration” of their microbiota, or eliminate knockout mice completely and utilize depleting anti-IL-23 antibody in wild-type mice to preclude differences in the microbiota.
- The anti-Gr-1 treatment used in the current study depleted numerous cell types beyond neutrophils. While Gr-1 is expressed at high levels on neutrophils(12, 13), other cells including monocytes and plasmacytoid dendritic cells also express high levels of Gr-1(13-15). We cannot exclude depletion of these other cell types as a confounding factor in our experiments, though our experiments suggest that no Gr-1⁺ cell type, including neutrophils, drives the development of inflammation during *C. difficile* colitis.

More precise experiments could be performed with the anti-Ly6G depleting mAb (clone IA8), which selectively depletes neutrophils while sparing monocytes(16, 17). Additionally, CXCR2-deficient mice, which exhibit a drastic defect in neutrophil recruitment in response to chemically induced colitis(18), would also be a viable platform to investigate the role of neutrophils in this model.

- The difference in disease severity following infection with the two *C. difficile* strains VPI 10463 (hereafter referred to as VPI) and 630 may also influence the resulting host response. While infection with both strains results in robust neutrophil recruitment, VPI infection is associated with more severe intestinal histopathology and weight loss than that seen following 630 infection(5, 10-12, 19-21). Therefore, it is likely that host inflammatory pathways are more robustly activated following VPI infection than during 630 infection, complicating interpretation of host response data.

Inflammatory signals found to promote inflammation in response to VPI infection, such as IL-23, are likely central mediators of inflammation.

However, it is possible that signals that appear to have no role in promoting inflammation in response to VPI infection may in fact have pro-inflammatory functions that are redundant with other pathways active in response to infection. Therefore, investigations using 630 as the infectious challenge may reveal roles for inflammatory signals that are not apparent in the more immunologically “complicated” environment following VPI infection.

- Another potential confounding factor in the current study is the use of both vegetative *C. difficile* cells and *C. difficile* spores as the infectious challenge. Though early *in vivo* investigations of the host response to *C. difficile* infection utilized vegetative cells as the infectious inoculum(3, 11, 12, 22), the development of reliable techniques to generate *C. difficile* spores(23, 24) has allowed subsequent investigations to infect with spores(10, 19, 20, 25, 26). However, studies challenging with either the vegetative or spore form of the *C. difficile* reference strain VPI 10463 have reported a similar host response to infection, characterized by neutrophil recruitment, induction of CXC chemokines, and marked intestinal histopathology(11, 12, 19, 21, 22, 26). The current study also supports this observation, with similar neutrophil recruitment and induction of IL-22 and CXCL1 and CXCL2 following challenge with VPI vegetative cells (Chapter 4) or spores (Chapter 5). Taken together, these studies suggest that infection with the same strain of *C. difficile*, irrespective of the form of the infectious inoculum, initiates a conserved host response, minimizing the potential impact of the form of the infectious agent on interpretation of the results.

Future Directions:

While the data presented in this dissertation have advanced our understanding of the host mechanisms promoting inflammation in response to *C. difficile* colitis, numerous questions remain regarding the host response to *C. difficile*

infection. In this section, we will propose additional lines of investigation that would supplement the studies described in this dissertation.

- Colonic epithelial cells represent a potential source of inflammatory cytokines, especially CXC chemokines during *C. difficile* colitis. The present study has identified numerous host signals that support inflammatory cytokine and CXC chemokine expression in the colon during *C. difficile* infection, yet the cellular source of these cytokines remains unknown. Castagliuolo and colleagues reported increased expression of CXCL2 from rat intestinal epithelial cells (IECs) following administration of toxin A *in vivo*(27). Deficiencies in MyD88 and NOD1, two components of bacterial detection systems expressed in intestinal epithelial cells(28, 29), have also been associated with decreased expression of CXCL1 in response to *C. difficile* infection(22, 26). Fluorescence Assisted Cell Sorting (FACS) would allow for enrichment of colonic epithelial cells (using EpCAM as a marker for colonic epithelial cells(30)) from *C. difficile* infected animals for subsequent expression analysis. If colonic epithelial cells were identified as a source of inflammatory cytokines, subsequent experiments with intestinal epithelial cell (IEC)-specific knockout animals, such as the IEC-MyD88^{-/-} mouse described by Frantz and colleagues(29), could be used to identify of the signaling cascades driving inflammatory responses in these cells.
- The role of resident intraepithelial lymphocytes (IEL) in amplifying inflammatory and epithelial-protective responses to *C. difficile* infection

remains poorly understood. As mentioned above, the cellular subset(s) responsible for producing inflammatory cytokines during *C. difficile* colitis are not known. T-cells are the predominant IEL(31), and IL-23 has been shown to promote inflammatory cytokine production from IEL isolated from inflammatory bowel disease patients(32). Additionally, defects in $\gamma\delta$ IEL function is associated with increased colonic ulceration during DSS colitis, underscoring the pleiotropic role of IEL during colonic inflammation(33). *Ex vivo* experiments using IEL isolated from *C. difficile* infected mice would help characterize how these cells respond in the face of *C. difficile* infection, as well as the host and microbial signals that promote their activation. Specifically, experiments should examine the ability of isolated IEL from untreated and *C. difficile* infected mice to respond to inflammatory cytokines, including IL-23 and GM-CSF. The responses assessed would include the production of inflammatory cytokines, including IL-6, but also the production of epithelial protective factors such as IL-22 and keratinocyte growth factor 2 (KGF-2). Should these experiments suggest a role for particular subset(s) of IEL in driving inflammatory or protective responses during *C. difficile* colitis, such as $\gamma\delta$ IEL, follow up studies could be performed using mice deficient in the cell-subset in question, such as $\gamma\delta$ T-cell deficient *Trd*^{-/-} mice(34).

- The role of IL-6 signaling in driving inflammatory and epithelial-protective responses during *C. difficile* colitis remains largely unknown. While the data presented above demonstrate a correlation between reduced IL-6 gene

expression and reduced inflammatory cytokine expression and neutrophil recruitment in IL-23-deficient mice, the role of IL-6 during *C. difficile* colitis has not been specifically investigated. IL-6 promotes neutrophil recruitment(35) and CCL3 production(36) during bleomycin-mediated pulmonary inflammation. However, IL-6 also protects the colonic epithelium from ulceration during both *C. rodentium* and DSS colitis(37). While this study and others have reported significantly increased expression of IL-6 in response to *C. difficile* infection(10-12, 20), the role of IL-6 during *C. difficile* colitis has never been specifically investigated. The functions of IL-6 during *C. difficile* colitis could be directly investigated by infecting IL-6-deficient mice(37, 38), following cohousing with WT mice to normalize their colonic microbiota, and assess inflammatory responses such as neutrophil recruitment as well as monitoring colonic histopathology in order to assess epithelial protective. If these studies do suggest a role for IL-6 during *C. difficile* infection, subsequent studies utilizing Immunofluorescence microscopy (IFM) could identify IL-6-producing and IL-6 receptor expressing cell subsets within the colon for further investigation.

- Colonic dendritic cells represent a potential source of IL-23 during *C. difficile* colitis. The current study suggests a critical role for IL-23 in promoting neutrophil recruitment and inflammatory cytokine expression in response to *C. difficile* colitis, yet the source of this cytokine in the colon remains unknown. A recent study identified robust IL-23 expression in small

intestinal dendritic cells following stimulation with flagellin(39). Additionally, Cowardin and colleagues recently reported that IL-23 expression in bone marrow and monocyte-derived dendritic cells in response to TLR ligands was markedly increased by co-treatment with *C. difficile* toxins A and B(40), strongly implicating dendritic cells as a potential source of IL-23 during *C. difficile* colitis. Immunofluorescence microscopy could be performed on colonic sections from *C. difficile* infected mice to directly determine if colonic dendritic cell subsets produce IL-23 under those conditions. Additionally, intracellular flow cytometry could also be used to examine the potential for IL-23 expression by several colonic dendritic cell subsets during *C. difficile* colitis. Should either or both of these approaches identify candidate DC subsets, additional experiments could use various depletion methods, such as CD11c(41) or CD11b(42) DTR mice, could be used to specifically depleted the cell subset(s) in question.

- The role of RegIII γ in restraining inflammatory responses during *C. difficile* colitis could be further investigated. Though the data presented in this dissertation clearly demonstrate that IL-23-dependent IL-22 signaling drives RegIII γ expression in the colon, the function of RegIII γ during *C. difficile* colitis has not been thoroughly investigated. RegIII γ is an antimicrobial peptide that prevents bacterial contact with the intestinal epithelium(43), and protects against mortality during *C. rodentium* infection(44). The induction of RegIII γ in the colon during *C. rodentium* colitis is dependent

upon IL-22(44). However, the *C. rodentium* burden in IL-22-deficient, and therefore RegIII γ insufficient, mice is equivalent to that seen in WT animals(44), suggesting that the direct killing of pathogens during colonic infections is not the main function of RegIII γ . Interestingly, mice deficient in RegIII γ show evidence of increased intestinal inflammatory responses in the absence of infection(43). Consistently, in the current study we observed increased induction of inflammatory cytokines in association with reduced RegIII γ expression in anti-IL-22 treated animals. RegIII γ knockout mice(43) could be used to explicitly assess the role of RegIII γ in restraining inflammatory responses in the colon in response to *C. difficile* infection. However, the altered inflammatory state of the gastrointestinal tract of these animals at baseline would make the interpretation of any such experiments difficult.

While all of these future directions are important avenues of research regarding the host response to *C. difficile* infection, the host mechanisms capable of protecting the epithelium from damage during infection are particularly poorly understood. To that end, future investigations should focus on the epithelial-protective roles of IL-6 signaling, as well as epithelial-protective responses initiated by resident IEL in response to *C. difficile* infection. Identification of the cellular sources of inflammatory cytokines and chemokines during *C. difficile* infection, though important questions, remain secondary to these lines of investigation.

Significance

In this dissertation, we tested the following 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.

The results of the research detailed above have yielded the following contributions to the field.

- **Evidence that colon-resident cells, and not recruited inflammatory cells, are the major drivers of inflammation and epithelial damage during acute and severe *C. difficile* colitis.**

Robust neutrophil recruitment in *C. difficile* infection is well documented(11, 12, 19, 20, 22, 26, 45, 46). Neutrophils are a known source of inflammatory cytokines, including IL-1 β and TNF α (47), and neutrophil recruitment is known to be associated with tissue damage during mucosal inflammation(19, 34, 48, 49). Additionally, Morohoshi and colleagues demonstrated that inhibitor of neutrophil elastase was associated with decreased intestinal histopathology during chemically induced colitis(50). However, in the current study the depletion of Ly6C^{Mid} Gr-1^{High} neutrophils was not associated with any reduction in colonic histopathology or the expression of major inflammatory cytokines including CXCL1, IL-1 β , and IL-6, suggesting that this cell type does not represent a major source of inflammatory cytokines. As such this data strongly suggests that, at least during acute and severe disease,

that colon-resident Gr-1^{Low} cells, such as T-cells or colonic epithelial cells, are the main sources of inflammatory cytokine expression and immunopathology during *C. difficile* colitis.

- **Implicated IL-23-responsive cell types as critical drivers of inflammation during *C. difficile* colitis.**

Recent studies have begun to identify innate lymphoid cells that respond to IL-23 and can promote both inflammatory and epithelial-protective responses during large bowel inflammation(49, 51, 52). Thy1^{High} SCA^{High} cells produce IL-22 in an IL-23-dependent manner during DSS colitis in Rag2-deficient mice, and anti-Thy1 treatment is associated with more severe intestinal histopathology in this model as well(49). During innate colitis following anti-CD40 treatment of Rag^{-/-} mice, IL-23R deficiency was also associated with drastically reduced expression of IL-22(52). However, in this model IL-22 was shown to promote neutrophil recruitment (52), highlighting the inflammatory potential of IL-23 signaling during mucosal inflammation. Additionally, Buonocore and colleagues recently reported that Thy1^{High} SCA^{High} cells from the colons of *Helicobacter hepaticus* infected mice respond to IL-23 by producing IL-22, IFN γ , and IL-17a(51). Furthermore, anti-Thy1 treatment was associated with decreased colonic histopathology in this model and a loss of IL-23 dependent production of the inflammatory cytokines IL-17 and IFN γ from the lamina propria (51). In this study, we describe reduced inflammatory cytokine expression and neutrophil

recruitment during *C. difficile* colitis in IL-23-deficient mice. As such, this data strongly suggests that IL-23-responsive cell subsets, including IL-23-responsive ILCs, may be important drivers of inflammatory and/or host-protective responses during *C. difficile* colitis.

- **Insight into the differential host responses to toxin producing bacterial pathogens in the colon as compared to epithelial-adherent pathogens.**

Investigations into the host response to epithelial-adherent bacteria in the colon have focused on *Citrobacter rodentium* (*C. rodentium*)(44, 53-56), a mouse specific pathogen that forms attaching and effacing lesions on colonic epithelial cells(55). While similar immune signals appear to have critical roles during the host response to both *C. difficile* and *C. rodentium* colitis(10, 20, 25, 44, 46, 53, 56), their functions appear to vary greatly between infections. GM-CSF deficiency is associated with increased evidence of inflammatory myeloid cell infiltration and induction of inflammatory cytokines including CXCL1 and TNF α in response to *C. rodentium* colitis(56), while this study demonstrates that GM-CSF is required for full induction of inflammatory cytokines and neutrophil-recruiting chemokines in response to *C. difficile* colitis. Additionally, during *C. rodentium* colitis IL-23 is a critical driver of IL-22 production, which in turn protects against colonic histopathology (44). While IL-23 was also found to drive IL-22 expression during *C. difficile* colitis, the major function of IL-23 was to promote neutrophil recruitment, and in agreement with recent studies(20, 46) there

was no increase in colonic histopathology following ablation of IL-22. Other recent studies have reported differential host responses to *C. difficile* and *C. rodentium*: while MyD88 deficiency is predominantly associated with increased epithelial damage and decreased epithelial repair during *C. rodentium* infection(53), there was no increase in epithelial cell loss during *C. difficile* colitis in MyD88-deficient mice(26). Taken together, these data strongly suggest that the host responses to toxin-producing bacterial pathogens are distinct from those seen in response to epithelial-adherent bacterial pathogens within the colon.

- **Insight into the functions of neutrophils in the colon following *C. difficile* infection.**

Numerous studies have demonstrated significant increases in mortality following interventions that reduce neutrophil recruitment during *C. difficile* infection(22, 26, 45). Furthermore, many of the studies have reported increased bacterial burden in distal organs, including the mesenteric lymph nodes and liver, following anti-Gr-1 treatment(22, 26), suggesting a role for neutrophils in preventing the bacterial dissemination following *C. difficile*-driven GI epithelial damage. In the current study, we reported no reduction the expression of major inflammatory cytokines, including IL-1 β and IL-6, or reduction in the severity of intestinal histopathology following the depletion of Ly6C^{Mid} Gr-1^{High} neutrophils by anti-Gr-1 mAb treatment. Furthermore, we reported no change in *C. difficile* burden in the colon following anti-Gr-1

treatment, in agreement with previous studies by Jarchum and colleagues(26). Taken together, the data presented in the current study and that found in the literature strongly suggest an important role for recruited neutrophils in preventing bacterial dissemination and mortality, but not driving inflammatory responses or reducing pathogen burden in the large bowel, during *C. difficile* colitis.

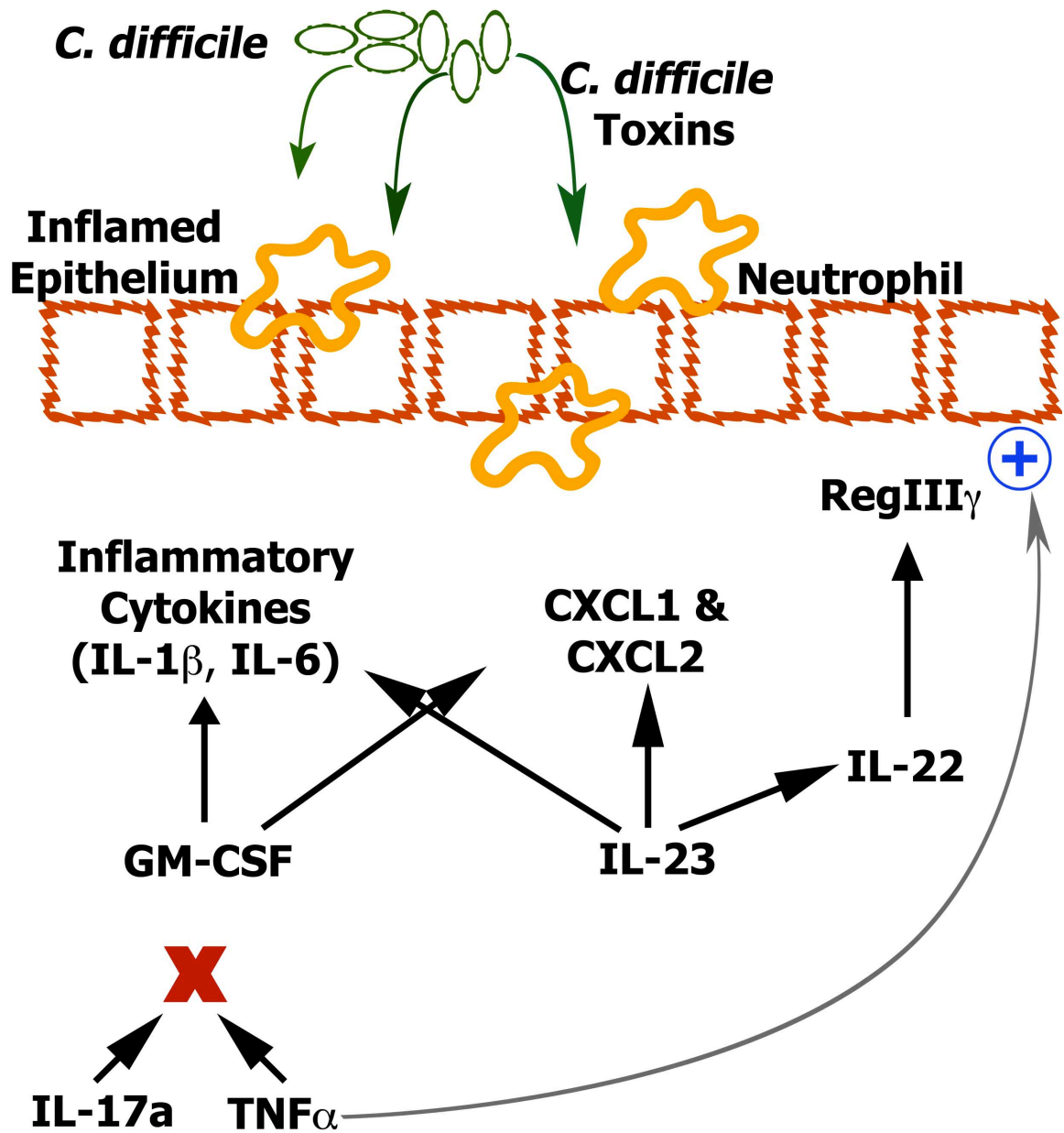


Figure 6-1: Mechanisms of neutrophil recruitment and immunopathology during acute *Clostridium difficile* colitis.

C. difficile infection and toxin production results in colonic epithelial damage and the activation of numerous inflammatory pathways. IL-23 and GM-CSF promote inflammatory cytokine induction and CXC chemokine expression, resulting in neutrophil recruitment to the colon. IL-23 also drives IL-22 expression, which does not influence inflammatory responses or epithelial damage, but does drive RegIII γ expression. Neither IL-17a nor TNF α promotes inflammatory cytokine expression, though TNF α protects against colonic epithelial damage.

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