Chapter 4

The Role of Gr-1$^+$ Cells and TNFα Signaling During *Clostridium difficile* Colitis in Mice

Portions of this chapter have been previously published

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Introduction

Gr-1$^+$ cells, including neutrophils, are recruited in large numbers to the large intestine in response to *C. difficile* infection(1-4), and protect against bacterial dissemination and mortality (1-3). TNFα promotes leukocyte recruitment and the expression of inflammatory cytokines during mucosal inflammation (5-7). Furthermore, TNFα expression is significantly increased during acute *C. difficile* colitis (4), and macrophage TNFα production is also enhanced by exposure to *C. difficile* toxins (8). However, much remains unknown about the contributions of Gr-1$^+$ cells and TNFα in promoting intestinal histopathology, leukocyte recruitment, and the expression of inflammatory cytokines during *C. difficile* colitis. In this chapter, we investigated the roles of TNFα signaling and Gr-1$^+$ cells in driving

inflammatory cytokine expression, intestinal histopathology, and myeloid cell recruitment during acute, severe *C. difficile* colitis.

Results

Effect of ceftriaxone treatment on the colonic microbiota

In order to assess the effect of ceftriaxone treatment on the diversity and membership of the colonic microbiota, animals were given ceftriaxone (0.5g/L) in their drinking water for four days and analyzed four days later (Figure 4-1). 454-pyrosequencing analysis of 16S amplicon libraries generated from colonic tissue from untreated and ceftriaxone treated mice allowed for characterization and comparisons of the colonic mucosa-associated microbiota between groups (9). This analysis revealed that four days of ceftriaxone treatment was sufficient to significantly decrease the diversity of the mucosa-associated microbiota, compared to untreated mice, at four days post-antibiotic (Figure 4-1). While untreated animals possessed a complex microbiota comprised primarily of members of the phyla Bacteroidetes and Firmicutes (Figure 4-1), ceftriaxone treatment was associated with a marked shift in the membership of the community towards a composition dominated by a single Firmicute; Enterococcaceae (Figure 4-1). These experiments demonstrated that ceftriaxone treatment was sufficient to significantly decrease the diversity and markedly alter the membership of the colonic microbiota even four days after the cessation of antibiotic treatment.

Effect of ceftriaxone treatment on colonic gene expression

To investigate the effect of ceftriaxone treatment and the associated loss of microbial diversity on immune regulation the colonic mucosa, quantitative RT-PCR was used to examine colonic cytokine expression following ceftriaxone treatment. Ceftriaxone treatment was associated with significantly increased expression of the chemokines *Ccl5* and *Cxcl9*, and significantly decreased expression of *Ccl11*, *Ccl3*, and *Cxcl2* (Figure 4-2). Additionally, *Il2*, *Il10*, *Il5*, and *Il3* expression were all significantly increased following ceftriaxone treatment, while *Il23a*, *Il17f*, *Il1b*, *Il17a*, *Tgfb*, and *Slpi* were decreased (Figure 4-2). Examination of histopathological sections from the colons of ceftriaxone treated mice revealed no evidence of cellular infiltration or inflammation in these animals (Figure 4-3). Taken together, these data demonstrate that ceftriaxone treatment alone induces significant changes in the cytokine expression pattern in the colonic mucosa but does not result in overt inflammatory histopathological changes.

***C. difficile* infection following ceftriaxone treatment**

To determine if ceftriaxone treatment was sufficient to permit colonization and infection by *C. difficile*, ceftriaxone treated mice were challenged with vegetative cells (10^5 CFU) from *C. difficile* strain VPI 10463 two days after the cessation of ceftriaxone treatment and were followed for an additional two days (Figure 4-1). Significant colonization by *C. difficile* was detected in the colonic mucosa of infected mice via qPCR by two days post challenge (Figure 4-4). Colonization did not cause a shift in the membership or diversity (Figure 4-1) of the colonic microbiota beyond

that attributable to ceftriaxone treatment alone. *C. difficile* colonization of ceftriaxone-treated mice resulted in an influx of inflammatory cells into the colon and marked epithelial damage, indicative of active infection (Figure 4-5). Taken together, these data demonstrate that ceftriaxone treatment renders mice susceptible to *C. difficile* colonization with the concomitant development of colitis.

Host responses to *C. difficile* colitis

In order to characterize the mucosal response to *C. difficile* colitis following ceftriaxone treatment, colonic gene expression was examined. *C. difficile* infection was associated with increased expression of *Inos*, *Slpi*, *Il1b*, *Il6*, *Il17f*, *Ifng*, *Il17a*, *Il22*, *Il2*, *Il33*, and *Tnfa* (Figure 4-8 and 4-9). *Il18*, *Il23*, *Il12*, *Il3*, *Il4*, *Il5*, *Il13*, *Il10*, *Tgfb*, and *Ang4* expression were not induced in response to *C. difficile* infection (Figure 4-8 and 4-9). Consistent with the robust cellular recruitment observed in histological sections from *C. difficile* infected mice (Figure 4-5), there was increased expression of the CC chemokines *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, and *Ccl7*, but not *Ccl11* or *Ccl24* (Figure 4-7) and the expression of the CXC chemokines *Cxcl1* and *Cxcl2*, as well as the neutrophil stabilization factors *Csf2* and *Csf3* were also increased (Figure 4-7). Thus, these data indicate that mucosal response to *C. difficile* infection is characterized by increased expression of CC and CXC chemokines and inflammatory cytokines including *Il1b*, *Il6*, *Il17f*, and *Tnfa.*

Flow cytometry was utilized to further delineate the leukocyte populations recruited to the colon following *C. difficile* infection. Analysis of the side scatter (SSC) and forward scatter (FSC) parameters of $CD45^+$ leukocytes revealed a drastic

influx of FSC^{Mid} SSC^{High} leukocytes in response to *C. difficile* infection (Figure 4-6). Nearly all of the recruited FSC^{Mid} SSC^{High} cells were $CD11b^{High}$ $CD11c^{Low}$ inflammatory myeloid cells (Figure 4-6). Within the $CD11b^{High}$ $CD11c^{Low}$ population, there were two populations, the largest of which were $Ly6C^{Mid}$ $Gr\text{-}1^{High}$ neutrophils, and a smaller population of $Ly6C^{High}$ $Gr\text{-}1^{Mid}$ monocytes (Figure 4-6). Thus, these data demonstrate marked recruitment of two distinct $Gr\text{-}1^{+}$ leukocyte populations, $Ly6C^{Mid}$ $Gr\text{-}1^{High}$ neutrophils and $Ly6C^{High}$ $Gr\text{-}1^{Mid}$ monocytes, to the colon in response to *C. difficile* colitis.

The role of $Gr\text{-}1^{+}$cells during *C. difficile* colitis

In order to determine the role of $Gr\text{-}1^{+}$ cells in supporting mucosal inflammatory response to *C. difficile* infection, animals were treated with an anti-Gr-1 mAb (clone RB6-8C5) one day prior to and one day post *C. difficile* infection (Figure 4-1). There was a marked reduction in the frequency of $CD11b^{High}$ $CD11c^{Low}$ inflammatory myeloid cells, as well as a reduction in both the $Ly6C^{Mid}$ $Gr\text{-}1^{High}$ neutrophil and $Ly6C^{High}$ $Gr\text{-}1^{Mid}$ monocyte populations following anti-Gr-1 treatment (Figure 4-6). However, anti-Gr-1 treatment had no effect on *C. difficile* levels in the colonic mucosa (Figure 4-4).

Compared to *C. difficile* infection alone, *Il5*, *Il12*, and *Il22* expression was significantly reduced, and *Il3* expression significantly increased, in *C. difficile* infected anti-Gr-1 treated mice (Figure 4-8). However, expression of *Il1b*, *Il6*, *Csf2*, *Csf3*, *Tnfa*, and *Il33* was unchanged following anti-Gr-1 treatment (Figure 4-7 and 4-8). Expression of *Cxcl1*, *Cxcl2*, *Ccl2*, and *Ccl3* was also not significantly affected by

anti-Gr-1 treatment (Figure 4-7). Anti-Gr-1 treatment did not protect against the colonic histopathology that developed during the normal course of infection (Figure 4-4 and 4-5). Taken together, these data indicate that the expression of CC and CXC chemokines, the expression of inflammatory cytokines including *Tnfa*, *Il1b*, and *Il6*, and the development of severe colonic histopathology during *C. difficile* colitis are all independent of the presence of Gr-1^{+} cells within the colon.

The Role of TNFα during *C. difficile* colitis

In order to examine the role of TNFα in promoting inflammatory myeloid cell recruitment and epithelial damage in response to *C. difficile* infection, mice were treated with a TNFα neutralizing monoclonal antibody (clone MP6-XT3) one day prior to infection (Figure 4-1). *C. difficile* colonization within the colonic mucosa was equivalent between anti-TNFα treated and *C. difficile* infected animals (Figure 4-4). There was near identical recruitment of CD11b^{High} CD11c^{Low} inflammatory myeloid cells following anti-TNFα treatment compared to that seen in response to *C. difficile* infection alone (Figure 4-6). Ly6C^{Mid} Gr-1^{High} neutrophil and Ly6C^{High} Gr-1^{Mid} monocyte frequencies were also unchanged following anti-TNFα treatment (Figure 4-6). Consistent with this observation, *Cxcl1*, *Cxcl2*, *Ccl2*, and *Ccl3* expression were not reduced in these mice (Figure 4-7). While the expression of *Il33*, *Ifng*, and *Il17f* were not significantly reduced by anti-TNFα treatment, expression of *Mif*, *Il1b,* and *Il6* were all significantly increased following anti-TNFα treatment (Figure 4-7 and 4-8). Furthermore, expression of *Ccl5*, *Il5*, and *Il22* were all significantly lower in anti-TNFα treated mice (Figure 4-7 and 4-8).

Epithelial damage was prominent in anti-TNFα treated mice (Figure 4-5) and anti-TNFα treatment was associated with more severe colonic histopathology (Figure 4-4). Anti-TNFα treatment was also associated with a significant decrease in the expression levels of the colonic goblet cell gene *Ang4*, the free fatty acid receptors *Ffar2* and *Ffar3*, epithelium-derived effector and signal molecules encoded by *Tslp*, *Tff*, *Defb3,* and *Lyz1*, in addition to a trend toward higher expression levels of *Slpi* compared to *C. difficile* infection alone (Figure 4-9). Additionally, there was a trend towards decreased expression of the goblet cell-specific gene *Muc2* after anti-TNFα treatment (Figure 4-9). Taken together, these data indicate that anti-TNFα treatment was associated with enhanced colonic epithelial dysfunction/destruction and can augment the epithelial inflammatory milieu during *C. difficile* infection.

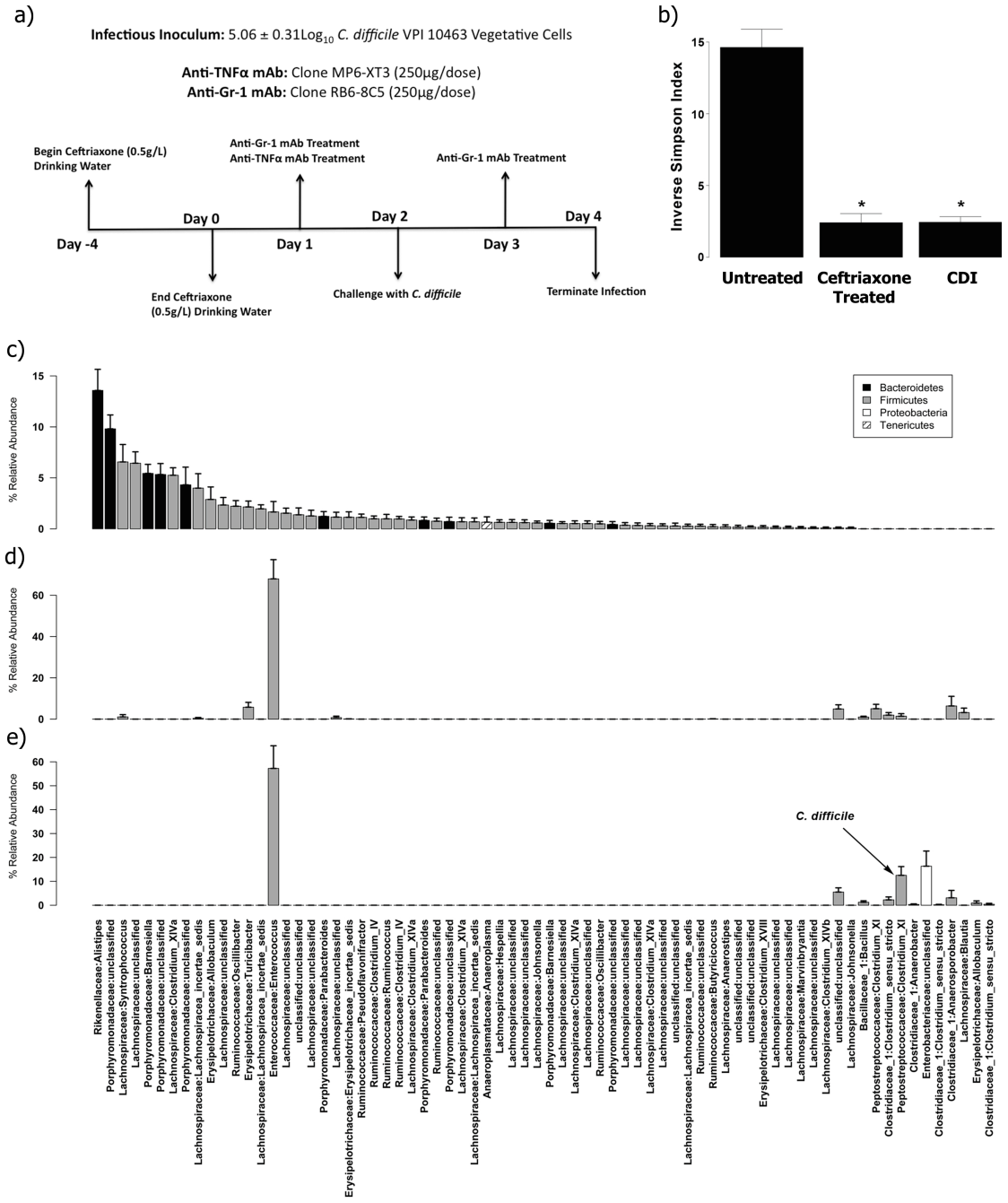

Figure 4-1: Effect of Ceftriaxone Treatment and *C. difficile* Infection on the colonic Microbiota.

(a) Experimental approach and timeline. (b) Diversity (Inverse Simpson Index) of the mucosa-associated bacterial communities of untreated, ceftriaxone treated, or ceftriaxone treated and *C. difficile* infected mice. (c-e) Relative abundance of the bacterial communities from panel b. OTUs were ordered by decreasing abundance of genera in untreated mice. Data are shown as mean ± SEM relative abundance. The Family and Genus-level taxonomies are displayed along the x-axis and the Phylum-level taxonomy is identified in the shading of individual bars.

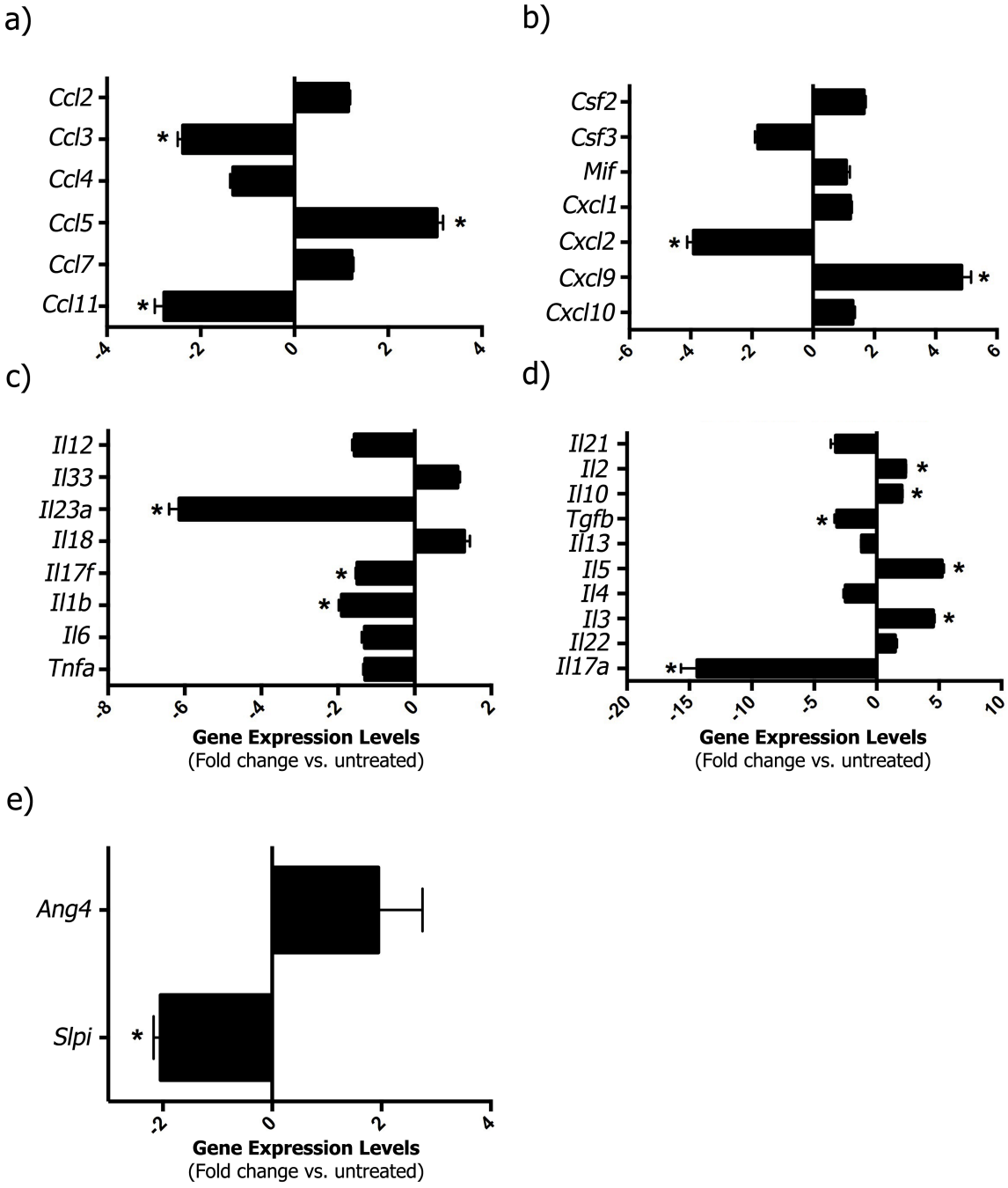

Figure 4-2: Effect of Ceftriaxone Treatment on Colonic Cytokine and Chemokine Expression.

Mice were treated as outlined in Figure 4-1. Host gene expression was measured by qPCR as described in the methods. n ≥ 9 per group. Data are shown as mean ± SEM fold change gene expression of ceftriaxone treated animals as compared to untreated mice. * $p<0.05$ as compared to untreated animals.

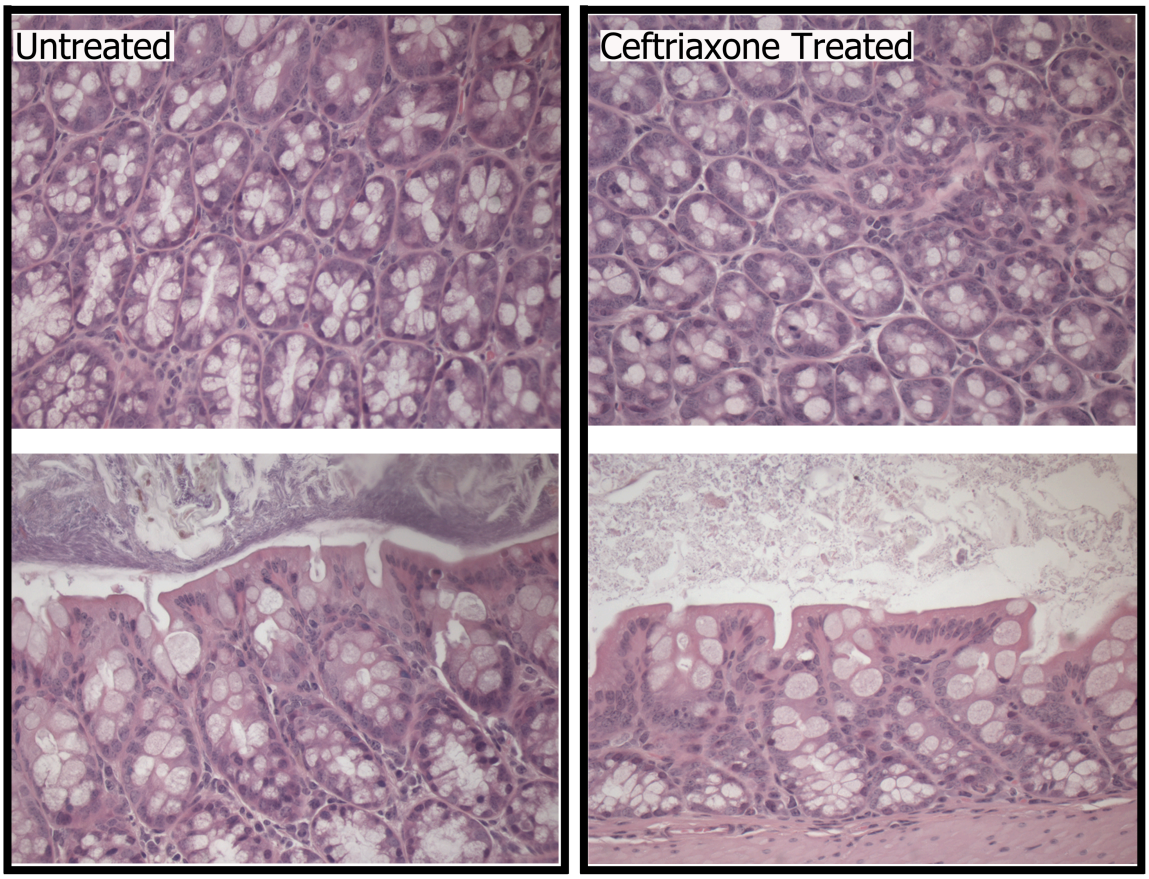

Figure 4-3: Effect of Ceftriaxone Treatment of Colonic Histopathology. Photomicrographs of representative Hematoxylin and Eosin (H&E) stained colonic sections from both untreated and ceftriaxone treated mice (Day 4). Both a cross-section and longitudinal section of the colonic crypts (top row) depicting the epithelial-luminal interface (bottom row) are shown for each group. All mice were treated as described in Figure 4-1. All images have a total magnification of 400X.

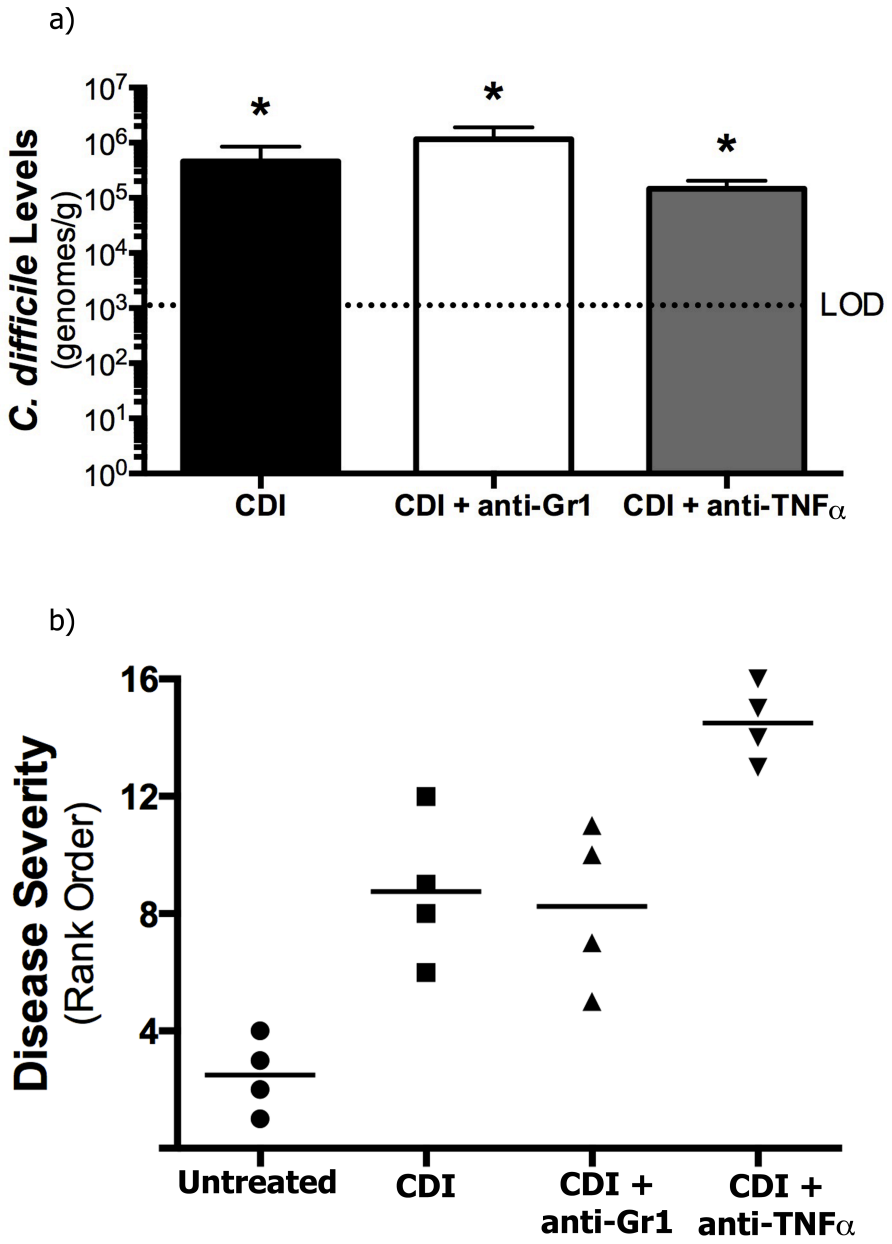

Figure 4-4: Colonic *C. difficile* Colonization and Colonic Histopathology Following anti-Gr-1 or anti-TNFα Treatment.
(a) Mucosal *C. difficile* colonization as determined by species-specific qPCR (Day 4). n ≥ 9 mice per group. Data are shown as mean ± SEM. CDI = *C. difficile* infected. LOD = Limit of Detection. * $p<0.05$ as compared to untreated animals. (b) Rank order analysis of colonic histopathological sections from Untreated, *C. difficile* infected, *C. difficile* infected and anti-Gr-1 treated, *C. difficile* infected and anti-TNFα treated animals (Day 4). Slides were ordered on the basis of disease severity, with 16 having the most severe histopathology. n = 4 mice per group.

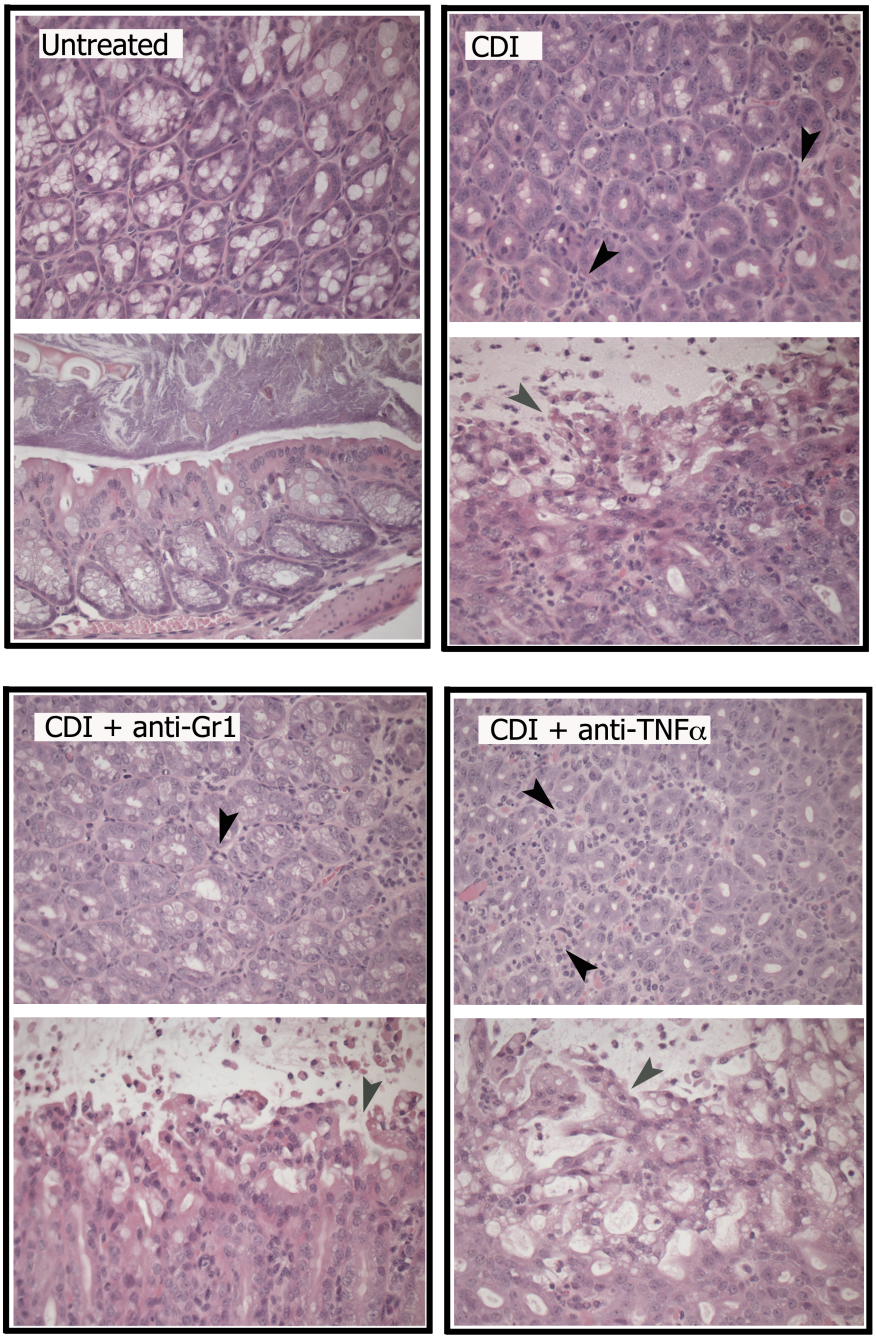

Figure 4-5: Effect of anti-Gr-1 or anti-TNFα Treatment on Colonic Histopathology.
Photomicrographs of representative H&E-stained colonic sections from Untreated, *C. difficile* infected, *C. difficile* infected and anti-Gr-1 treated, *C. difficile* infected and anti-TNFα treated animals (Day 4). Cross-sections of colonic crypts (upper images) and longitudinal sections of the epithelial-luminal interface (lower images) are shown for each treatment. Black arrowheads indicate infiltrating inflammatory cells, while gray arrowheads indicate areas of epithelial damage. Total magnification for all images is 400X. CDI = *C. difficile* infected

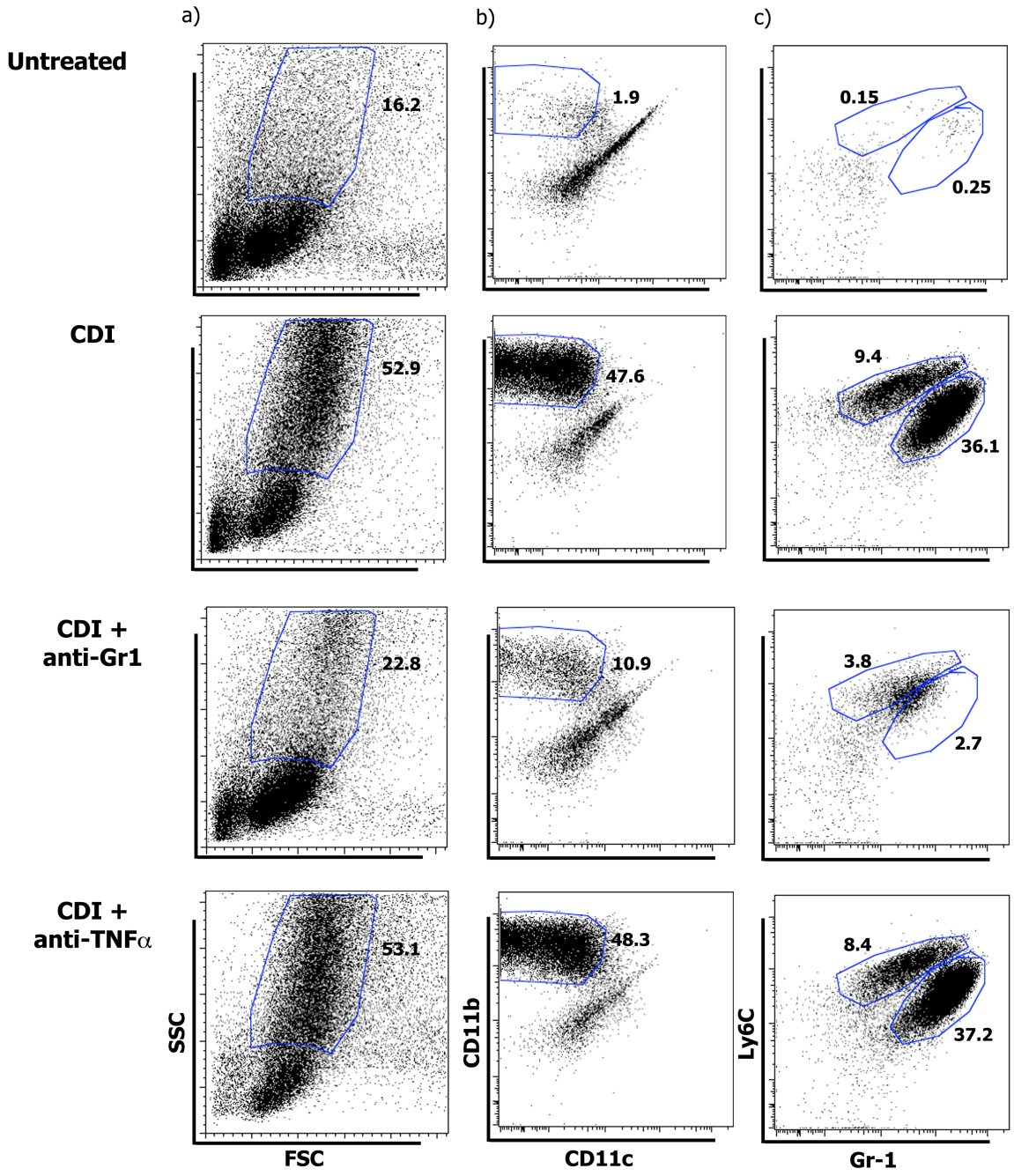

Figure 4-6: Flow cytometric Analysis of Colonic Leukocytes from Untreated, *C. difficile* Infected, *C. difficile* Infected and anti-Gr-1 treated, *C. difficile* Infected and anti-TNFα Treated Animals.

(a) Analysis of forward-scatter (FSC) and side-scatter (SSC) properties of total colonic $CD45^+$ leukocytes. (b) Analysis of CD11b and CD11c expression profiles of SSC^{High} FSC^{Low} $CD45^+$ leukocytes as defined in panel (a). (c) Analysis of Ly6C and Gr-1 expression profiles of the $CD11b^{High}$ $CD11c^{Low}$ population defined in panel (b). CDI = *C. difficile* infected. The bolded number indicates the percentage of total $CD45^+$ leukocytes contained within the indicated gate.

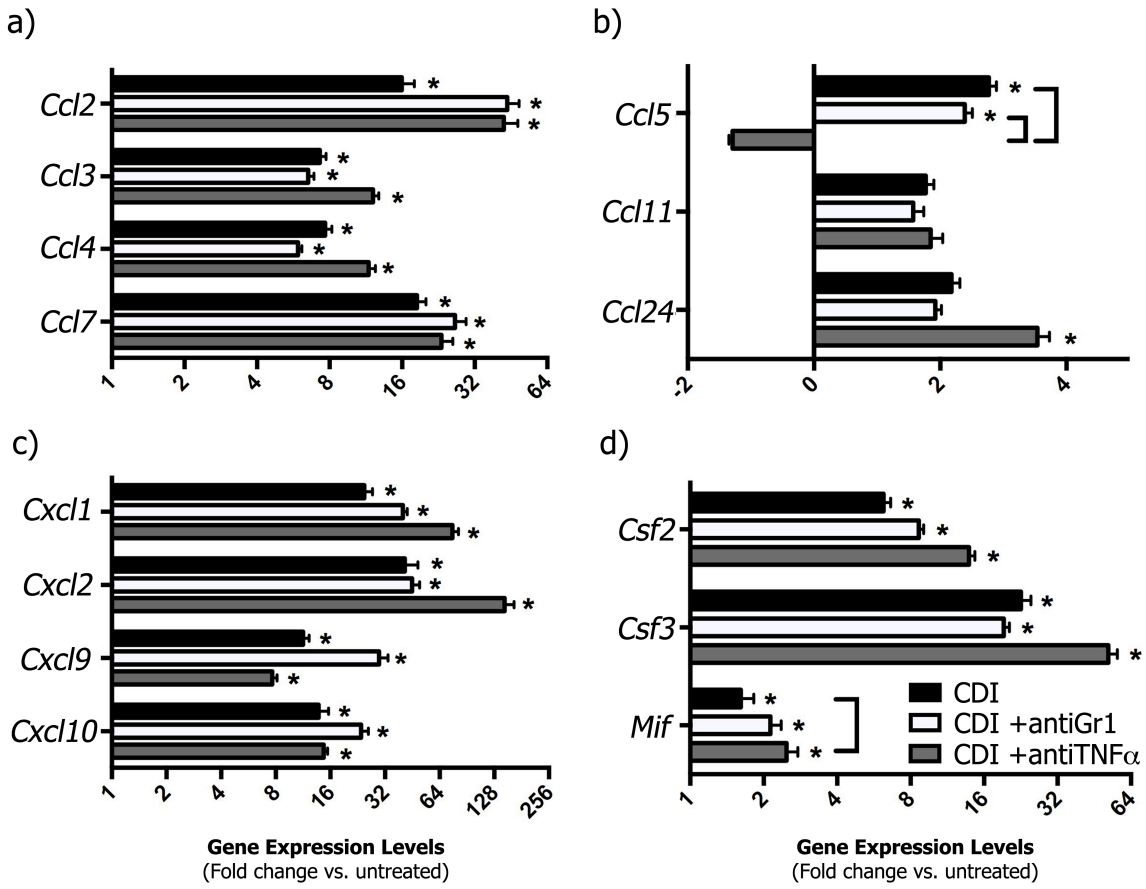

Figure 4-7: **Effect of anti-Gr-1 and anti-TNFα Treatment on Colonic Inflammatory Chemokine Expression During *C. difficile* Colitis.**
Colonic gene expression was measured via qPCR as outlined in the methods. n ≥ 8 per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile* infected (black bars), *C. difficile* infected and anti-Gr-1 treated (white bars), and *C. difficile* infected and anti-TNFα treated (gray bars) animals as compared to untreated mice. * $p<0.05$ as compared to untreated animals.] Brackets indicate $p<0.05$ for the differences between the indicated groups.

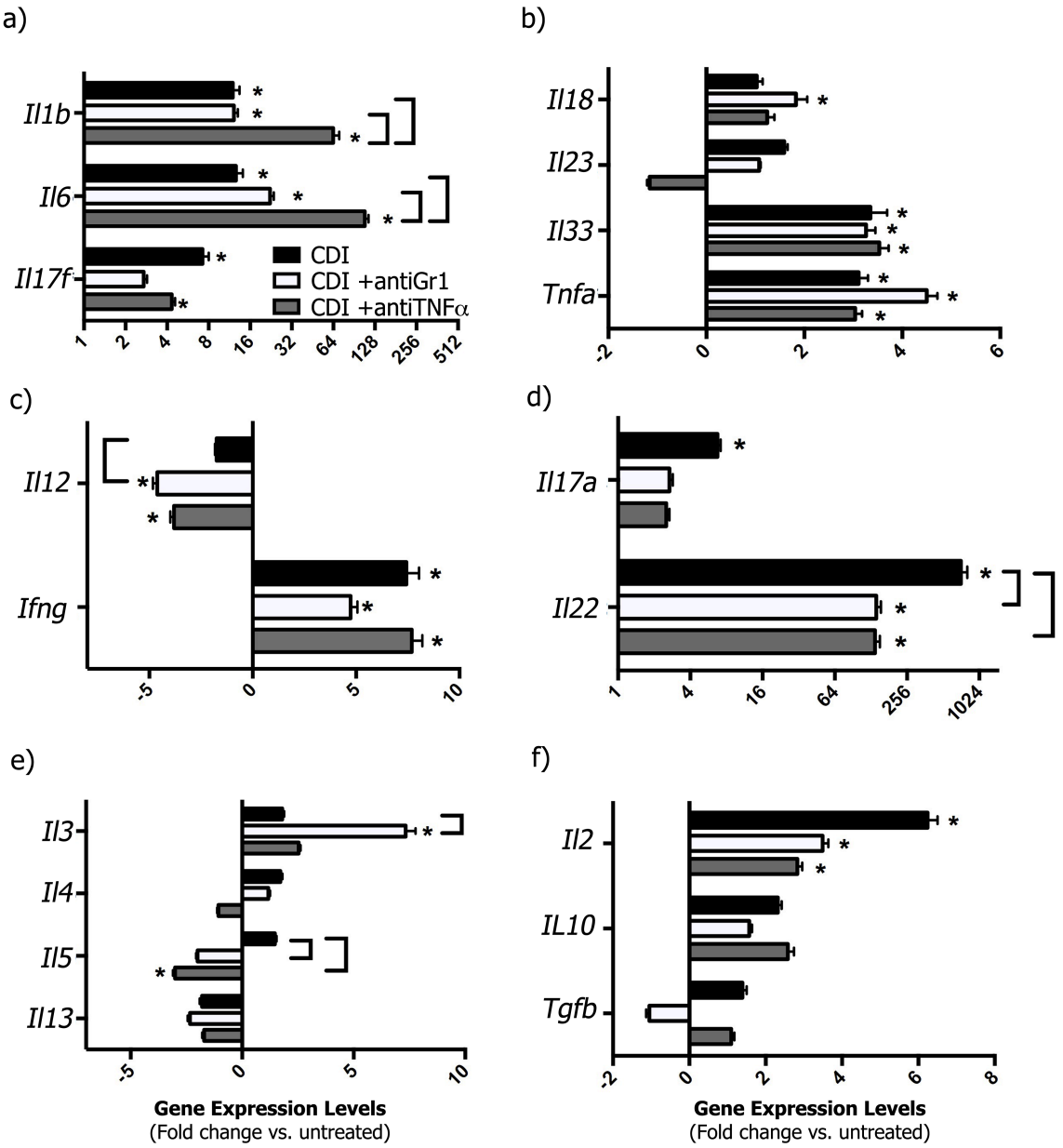

Figure 4-8: Effect of anti-Gr-1 and anti-TNFα Treatment on Colonic Inflammatory Cytokine Expression During *C. difficile* Colitis.
Host gene expression was measured via qPCR as described in the methods. n ≥ 7 per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile* infected (black bars), *C. difficile* infected and anti-Gr-1 treated (white bars), and *C. difficile* infected and anti-TNFα treated (gray bars) animals as compared to untreated mice. CDI = *C. difficile* infected * $p<0.05$ as compared to untreated animals.] Brackets indicate $p<0.05$ for the differences between the indicated groups.

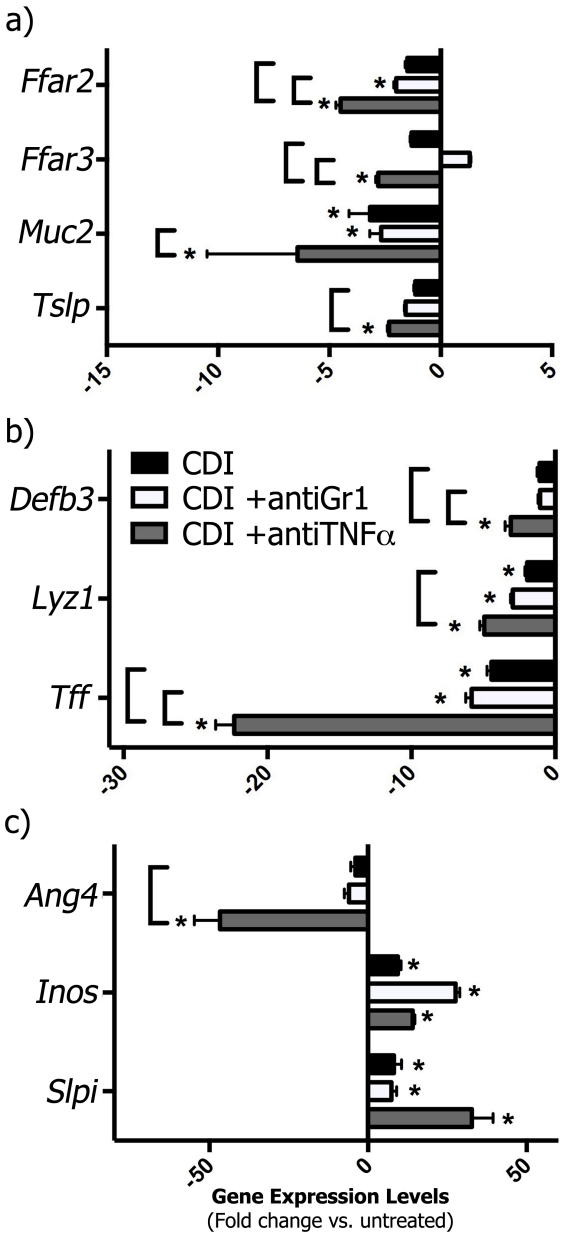

Figure 4-9: Effect of anti-Gr-1 and anti-TNFα Treatment on Colonic Gene Expression During *C. difficile* Colitis.

Host gene expression was measured via qPCR as described in the methods. n ≥ 7 per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile* infected (black bars), *C. difficile* infected and anti-Gr-1 treated (white bars), and *C. difficile* infected and anti-TNFα treated (gray bars) animals as compared to untreated mice. CDI = *C. difficile* infected * $p<0.05$ as compared to untreated animals.] Brackets indicate $p<0.05$ for the differences between the indicated groups.

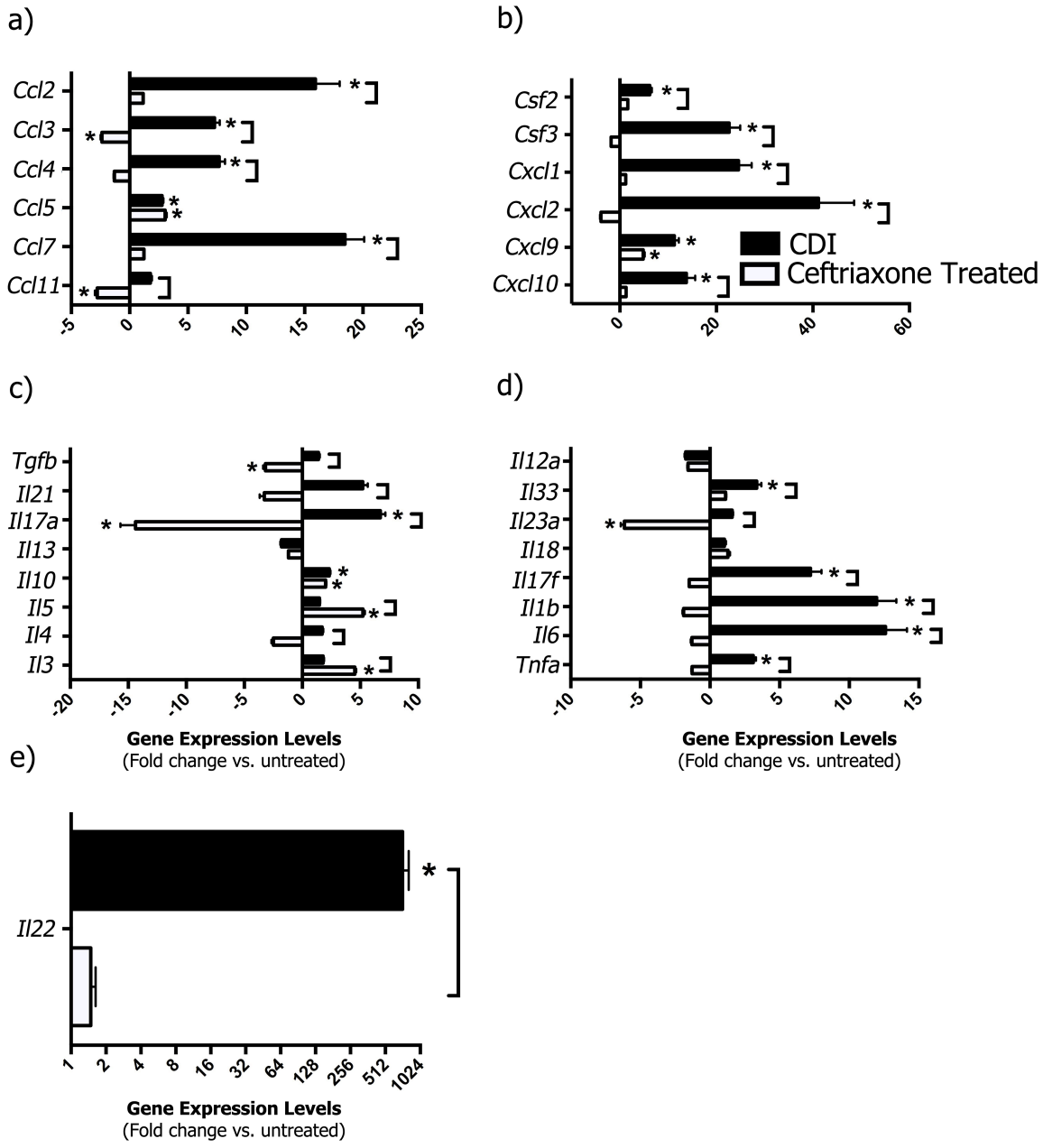

Figure 4-10: Comparison of Ceftriaxone Treatment Alone and *C. difficile* infection Following Ceftriaxone Treatment on Colonic Inflammatory Gene Expression.

Host gene expression was measured by qPCR as described in the methods. n ≥ 8 per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile infected* (black bars) and ceftriaxone treated (white bars) animals as compared to untreated mice. * $p<0.05$ as compared to untreated animals.] Brackets indicate $p<0.05$ for the differences between the indicated groups.

Discussion

Altogether, these data demonstrate that ceftriaxone treated mice are a robust model for investigating the innate acute inflammatory response to *C. difficile* infection. In addition to altering the composition of the colonic microbiome, ceftriaxone treatment alone was also capable of modulating colonic cytokine and chemokine expression four days after the cessation of antibiotic treatment. While ceftriaxone treatment was associated with significantly altered expression of several genes induced during *C. difficile* colitis, the induction of the inflammatory cytokines and chemokines associated with *C. difficile* infection was significantly greater than that seen with ceftriaxone treatment alone (Supplemental Figure 2). Additionally, ceftriaxone treatment alone did not produce any histopathological changes in the colonic mucosa (Supplemental Figure 1). Thus, while ceftriaxone treatment is capable of modulating colonic gene expression, we propose it is not a confounding factor in evaluating the host inflammatory response in this model of infectious colitis.

Anti-TNFα treatment was associated with an augmented epithelial inflammatory response to *C. difficile* infection. Previous studies have reported a role for TNFα signaling in promoting myeloid cell recruitment during mucosal inflammation (5, 6). TNFα can enhance the expression of CCL3 during chemically-induced pulmonary inflammation (6), and interference with TNFα signaling reduces neutrophil recruitment in response to acute allergic airway inflammation (5). TNFα can contribute to inflammatory cytokine expression and tissue damage during mucosal inflammation (7, 10). 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 (7). Anti-TNFα treatment had no effect on *Cxcl1*, *Cxcl2*, *Ccl2*, and *Ccl3* expression during *C. difficile* colitis, and we observed no reduction in neutrophil and monocyte recruitment under these conditions. However, colonic expression of *Il1b* and *Il6* were increased following anti-TNFα treatment. Thus, despite its relatively low level of expression, TNFα is involved in the inflammatory response during *C. difficile* colitis but the induction of CC and CXC chemokines and subsequent myeloid cell recruitment during infection are largely TNFα-independent

While TNFα is generally held to be a pro-inflammatory mediator that can participate in indirect epithelial damage, there is precedent for an epithelium cytoprotective role for this cytokine, as our data suggest may occur in this infection model (11-13). TNFα signaling can protect against severe intestinal histopathology during *Citrobacter rodentium* infection (11). Furthermore, cellular infiltration and intestinal epithelial damage during DSS colitis are enhanced in the absence of TNFα (12, 13). Following anti-TNFα treatment we observed decreased expression of the free fatty acid receptors *Ffar2* and *Ffar3*, as well as *Defb3*, *Lyz1*, and *Tslp*, consistent with the severe colonic epithelial damage present in these animals. Anti-TNFα treatment was also associated with significantly decreased expression of Ang4 and a trend towards decreased expression of Muc2, two genes whose expression is specific to goblet cells within the gastrointestinal tract(14-16), suggesting reduced goblet cell function. Additionally, colonic histopathology was most severe following anti-TNFα treatment. Thus, our data are consistent with these other models of

colitis where TNFα plays a role in protecting the colonic epithelium from damage during inflammation, and suggests an epithelial-protective role for TNFα during acute, severe *C. difficile* infection.

One model that could explain the associated between anti-TNFα treatment and increased inflammatory responses and epithelial damage is that TNFα signaling may restrain host inflammatory responses by promoting the clearance of translocated commensal bacteria. Previous studies have demonstrated the dissemination of commensal bacterial to distal sites during *C. difficile* infection(1, 2), and have also suggested a role for these translocated bacteria in promoting innate inflammatory responses, including the production of IL-1β (1). TNFα signaling can activate macrophages and enhance their bactericidal capacity(17), and mucosal macrophages represent a large resident population within the colonic lamina propria with the capacity to eliminate invading bacteria(18).Thus, in the absence of TNFα signaling, translocated commensal bacteria may be less efficiently cleared by macrophages within the colonic lamina propria, leading to increased activation of inflammatory pathways and enhanced colonic tissue damage.

Despite the robust recruitment of Ly6C^{Mid} Gr-1^{High} neutrophils and Ly6C^{High} Gr-1^{Mid} monocytes in response to *C. difficile* infection, we observed no reduction in colonic expression of CC and CXC chemokines or proinflammatory cytokines including *Il1b*, *Il6*, *Il33*, and *TNFα* following anti-Gr-1 treatment. Neutrophil recruitment is commonly associated with the development of inflammation at mucosal sites (5, 19, 20) and neutrophils are a well-documented source of inflammatory cytokines, including IL-6, as well as the neutrophil chemokine IL-8

(21-23). While several studies have investigated the role of neutrophils in reducing host mortality and preventing the translocation of bacteria during *C. difficile* infection(2, 3), to our knowledge the role of neutrophils as a cellular source of chemokine and cytokine expression has not been extensively investigated. Despite effective depletion of both neutrophils and monocytes following our anti-Gr-1 treatment, colonic CC and CXC chemokine expression as well as *Il1b*, *Il6*, *Il33*, and *TNFα* expression was unchanged. Consistent with the significant reduction in *Il22* expression, a cytokine with a vital role in promoting wound healing and preventing epithelial damage at mucosal sites (24-26), we observed no protection from intestinal epithelial damage in anti-Gr-1 treated mice. Taken together, our data strongly suggest that the recruited Gr-1$^+$ populations, including neutrophils, are not responsible for the robust inflammatory cytokine expression or epithelial damage observed during *C. difficile* infection.

One potential explanation for our observation that depletion of Gr-1$^+$ cell populations had minimal effect on colonic cytokine and chemokine expression is that Gr-1$^+$ cells, including neutrophils, are not a major cellular source of these inflammatory mediators during the host response to *C. difficile* colitis. A recent study has reported decreased intestinal IL-1β and CXCL1 production following neutrophil depletion (1). However, that study utilized a less severe model of disease with 100% survival of infected animals at five days-post infection(1), while the model used in the current study, similar to other models based on the third generation cephalosporin cefoperazone(27, 28), resulted in high morbidity by two days post infection. Thus, in the context of such an overwhelming infection, alternative

pathways and sources of inflammatory cytokine expression may respond and effectively supersede the cytokine production by neutrophils observed in less severe models. Additionally, Hasegawa et al utilized a combination of seven antibiotics to permit *C. difficile* infection(1), as compared to the single antibiotic, ceftriaxone, used in the current study. These two antibiotic treatments will likely result in different colonic microbial communities, whose members may differentially stimulate host cell subsets and result in the activation of distinct inflammatory pathways in response to the same challenge.

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