EXPLORING THE ROLE OF GM-CSF IN THE HOST RESPONSE TO *C. DIFFICILE* INFECTION

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ABSTRACT

Clostridium difficile infection is increasingly dangerous and costly worldwide, yet the host response to the disease is poorly understood. Recent work has shed some light on the immune pathways involved in combating *C. diffiicle*-induced colitis, highlighting innate, pro-inflammatory signaling. In addition, it has been shown that the host response is characterized by leukocyte infiltration into the intestinal epithelium. Granuloctye-macrophage colony-stimulating factor (GM-CSF) is a cytokine that promotes leukocyte production and maturation, and has a role in inflammatory signaling. In this work, we sought to determine the effect that loss of GM-CSF signaling has on the intestinal innate immune response to *C. difficile* infection. Mice were made susceptible to infection via antibiotic treatment, treated with anti-GM-CSF antibody to deplete GM-CSF signaling, and infected with the 630g strain of *C. difficile*. We found that anti-GM-CSF treatment did not have an effect on the ability of *C. difficile* to colonize in the colon, but that it did lead to decreased inflammation and immune cell infiltration, as well as increased epithelial cell damage, coinciding with reduced inflammatory and epithelial-protective gene expression. These results provide evidence that GM-CSF plays a role in inflammatory signaling and epithelial protection during *C. difficile* colitis in mice, and indicate that more research into GM-CSF and related signaling pathways could further elucidate the host response to CDI.

INTRODUCTION

Intestinal Immune System Background

The gastrointestinal tract is the site of a tremendous amount of host-microbe interactions, and as such, it contains a variety of defense factors. In the large intestine, the first level of defense is a mucosal layer, generated by goblet cells, coating the intestinal epithelial cells (1). Epithelial cells produce antimicrobial peptides to prevent bacterial growth (1). In addition, they form a physical protective barrier, separating the contents of the intestinal lumen from the underlying tissue layers (2). They also play a part in recognizing microbes and stimulating host response. Leukocytes lie both within and underneath the epithelial barrier. Intraepithelial lymphocytes, most of which are T-cells, produce inflammatory and epithelial-protective signals (3). Lymphocytes under the epithelium, in the lamina propria of the large intestine, can initiate both inflammatory and anti-inflammatory responses (4). Lamina propria B-cells also secrete IgA, which plays a direct role in modulating the composition of the microbial population of the large intestine, known as the microbiota (4). The lamina propria also contains phagocytic cells, like dendritic cells (DCs), which help to determine whether host response will be anti-inflammatory or inflammatory, and macrophages to destroy pathogens (3). Together, the mucus layer, epithelial cell membrane, and underlying immune tissue make up the defense system needed to protect the gut against intestinal pathogens.

The resident gut microbiota plays its own vital role in the intestinal immune landscape. The microbiota has a bidirectional relationship with the intestinal immune

system; the microbiota influences both development and function of the immune system, while the immune system regulates the composition of the microbiota (5). A healthy gut microbiota is essential to the development and balance of the gut-associated lymphoid tissues (GALTs) discussed above, as well as effector T cells, T regulatory cells, and B cells (6). In addition to contributing to immune development, the microbiota also contributes to infection resistance (7). Commensal bacteria compete for nutrients such as amino acids, stunting pathogen growth (7). They promote the epithelial barrier by stimulating production of mucus and antimicrobial peptides (such as REGIIIγ), and by producing their own metabolites that block pathogens (7). Furthermore, the microbiota enhances the innate immune response by stimulating production of cytokines such as IL-1β and IL-17, and facilitates the adaptive immune response by promoting the development of the T cell subsets listed above (7). By directly competing with pathogens and stimulating the immune system, the microbiota protects the host from infection.

The innate immune response is a large component of intestinal immunity. The intestinal innate immune system is able to specifically target pathogenic bacteria while allowing the indigenous microbiota to flourish (8). It consists primarily of the epithelial barrier, including the mucus layer and antimicrobial peptides, and phagocytic cells in the lamina propria (9). Of these phagocytic cells, dendritic cells and macrophages are especially important for maintaining homeostasis in the gut (10). Their functions are dictated by signaling factors such as cytokines. When the homeostasis of the intestine is compromised due to infection or epithelial damage, inflammatory pathways are

activated, and neutrophils, macrophages, DCs and T cells are recruited in large numbers to the intestinal barrier (9). These response pathways, along with the mucus layer and antimicrobial defenses of the epithelium, allow the intestinal innate immune system to combat infection.

One of the factors involved in gut innate immune responses is the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF, also known as CSF2). GM-CSF has a host of functions, including promoting hematopoietic growth, promoting the survival and activation of macrophages, neutrophils and eosinophils, and mediating the maturation of DCs and the differentiation of invariant natural killer T cells (9, 11). GM-CSF usually requires stimulation (e.g. infection) to be found in detectable levels *in vivo,* and it is produced in especially high levels when the intestinal inflammatory response is activated (11, 12). A variety of cell types are responsible for GM-CSF production, such as fibroblasts, smooth muscle cells, endothelial cells, stromal cells, macrophages and osteoblasts—macrophages and endothelial cells being the most relevant in the gut, as well as myeloid cells along the GI tract. GM-CSF receptors are found mainly on macrophages, however, they are also found on a few types of non-hematopoietic cells. Specifically in terms of intestinal immunity, GM-CSF is important because of its function in the maturation of hematopoietic cells and maintenance of the intestinal barrier. During infection, GM-CSF produced in the intestinal mucosa regulates recruitment and survival of DCs, enhancing host protection (13). During DSS-induced colitis, GM-CSF increases systemic and mucosal type 1 IFN expression as part of the immune response, but causes reduced expression of some pro-imflammatory genes, ultimately

ameliorating disease (12). Overall, the role of GM-CSF during the innate immune response to intestinal disease includes granulocyte, monocyte, and dendritic cell survival and maturation, pro-inflammatory signaling, and some anti-inflammatory signaling as well.

Clostridium difficile Infection

Clostridium difficile is a gram-positive, spore-forming anaerobic bacterium, which causes toxin-mediated intestinal disease. *C. difficile* infection (CDI) can result in an array of clinical outcomes, ranging from asymptomatic colonization to mild diarrhea and abdominal pain to pseudomembranous colitis, toxic megacolon, sepsis, shock and death (14). CDI develops primarily after antibiotic treatment disrupts the intestinal microbiota, allowing for *C. difficile*, which is resistant to a variety of antibiotics, to colonize the gut (15). The bacteria is found mainly in infants, where it rarely causes clinical symptoms, and in elderly hospital patients on antibiotics, where it causes the most disease, though recently it has also emerged as a causative agent in adult antibiotic-associated diarrhea (14, 15). This is due in part to the spore-forming nature of *C. difficile*, which hinders sanitation efforts and increases the ease of transmission (16).

In the last 15 years, the incidence of severe CDI has increased dramatically. From 2000 to the present, the number of CDI diagnoses have increased from <150,000 to >500,000 cases per year in the US alone, with 15,000 to 20,000 of these cases resulting in death (14). This is costing the U.S. healthcare system over $2 billion per year (17). The most prominent *C. difficile* strain in North American infections is known as BI/NAP1/027

(14). CDI has historically been a hospital infection, but in recent years it is estimated that up to 50% of all initial CDI cases were onset outside a hospital or long-term care facility (17). Almost all infections are antibiotic/antimicrobial-associated, with the most commonly assicated medications being clindamycin, cephalosporins and fluoroquinolones (17).

C. difficile produces two large virulent toxins, TcdA and TcdB, which cause most of the disease's symptoms (14). TcdA seems to cause more severe disease (i.e. intestinal inflammation and cell death) in animal models, while TcdB is the main effector of disease in humans (18). Both toxins have the same mechanism: disruption of the actin cytoskeleton and tight junctions between epithelial cells, leading to a breakdown of epithelial membrane resistance (19). This is achieved by targeting host GTPases which regulate actin (20). The C-terminal and central regions of the toxin monoglucosylate threonine residues on GTPases, inactivating them and leading to cell death (21). The toxins also cause IECs, mast cells, and macrophages to release pro-inflammatory cytokines, leading to fluid accumulation and an influx of neutrophils into the epithelial lumen (19, 22). Eventually the combination of toxins bypassing the damaged barrier and the inflammatory response can lead to epithelial and immune cell apoptosis or necrosis and further mucosal damage (21).

Recent Research: Intestinal Innate Immune Response to *C. difficile*

C difficile infection in antibiotic pre-treated mice leads to the development of acute colitis, resulting in an innate immune response. This response is characterized by

neutrophil recruitment, tissue damage, increased mucosal permeability, and congestion and edema of the lamina propria (23). Numerous innate immune sensors and receptors, including the inflammasome, TLR4, TLR5, NOD1 and MyD88 signaling pathways are required to protect against severe disease (24). However, the blocking of neutrophil recruitment, as well as the down-regulation of various other inflammatory measures, has also resulted in increased protection from disease (25, 26). Importantly, intestinal damage seems to be caused by the inflammatory response itself, in addition to the direct cell damage from the toxin. Therefore, while the inflammatory response is important in host protection, overexuberant inflammation can have negative effects during *C. difficile*-induced colitis.

Previous research from our laboratory has shown that GM-CSF expression increases during *C. difficile* infection in mice (27). And as noted above, GM-CSF plays a key role in enteric innate immune response through its recruitment of DCs, macrophages, and neutrophils, and epithelial maintenance. It also plays a part in the inflammatory response in the gut. Cell recruitment, epithelial damage, and inflammation are characteristic of *C. difficile* infection. However, the role of GM-CSF during *C. difficile* infection is poorly understood. In the current study, we have used the mouse model previously outlined by Theriot et al. to test the role of GM-CSF signaling during *C. difficile*-induced colitis (28). We find that depleting GM-CSF signaling does not affect *C. difficile* colonization, but does lead to reduced epithelial inflammation and immune cell recruitment, while also leaving epithelial cells more vulnerable to damage and death.

MATERIALS AND METHODS

Animal Housing and Antibiotic Administration

C57BL/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were used to establish a breeding colony at the University of Michigan Medical School. They were housed under specific pathogen-free conditions and consumed clean food and water *ad libitum*. Male mice at 5–8 weeks of age were used for the current set of experiments. Beginning a week prior to infection, mice were treated with cefoperazone (0.5g/L) (Sigma) in distilled water (Gibco) for 5 days. The antibiotic water was replaced once during day 3. After antibiotic treatment, mice were given autoclaved drinking water without antibiotics for two days, after which, *C. difficile*-infected animals were infected with 630g *C. difficile* spores. Untreated mice received were neither infected nor antibiotic-treated.

During the antibiotic treatment and infection period, all animals were monitored for weight change once daily. *C. difficile* infected mice were monitored for symptoms of severe disease and humanely euthanized if moribund.

Histology and Necopsy

Mice were humanely euthanized via CO_2 asphyxiation four days post-infection. Samples were collected from excised colon tissue. Tissue for subsequent DNA analysis was immediately flash frozen and stored at -80^{o} C. Tissue for RNA analysis was stored in RNAlater solution (Ambion). The remaining excised colons were fixed in 10% formalin

solution overnight, then transferred to 70% ethanol solution. Cassettes were processed, paraffin embedded, sectioned, and used to prepare haematoxylin and eosin stained slides by McClinchey Histology Lab Inc.

Spore Preparation and Infection

Spores were taken from a stock of the 630g strain of *C. difficile* (ATCC BAA-1382). This strain was chosen due to its less virulent nature, which allowed for a more nuanced look at changes in immune response. The infection procedure was adapted from the model set forth by Theriot et al (28). The spores were serially diluted, then heated to 60^{O} C for 20 minutes to destroy any residual vegetative cells. Each animal was then infected with approximately $5*10^{4}$ spores. The inoculum was then serially diluted and plated on tarocholate-cefoxitin-cycloserine-fructose-agar (TCCFA) plates (Anaerobe Systems) overnight at 37^{O} C to confirm the actual infectious dose.

Antibody Administration

Mice in the anti-GM-CSF-treated group were given intraperitoneal injections of monoclonal anti-GM-CSF antibody MP1-22E9 mixed with sterile saline as previously described in the literature (29, 30). Each mouse received a total of 250 ug of antibody per injection. Injections were given every other day, beginning one day pre-infection and concluding three days post-infection. Mice in the untreated and CDI-treated control groups were not given injections.

RNA Isolation and Gene Expression Analysis

RNA isolation and gene expression analysis were conducted as previously described (26). Colon snips obtained during necropsy were homogenized into TRIzol RNA reagent (Life Technologies). An RNeasy Mini kit (Qiagen) was used according to manufacturer instructions to isolate RNA from the samples. An Agilent Bioanalyser (Aglilent Technologies) and a Nanodrop instrument (Thermo Fisher) were used to determine quality and concentration of isolated RNA, respectively. Complementary DNA (cDNA) was generated from isolated RNA using an RT^2 First Strand kit (Qiagen). Gene expression levels were measured using a custom mouse RT^2 Profiler PCR card (Qiagen) containing twelve replicated sets of 32 primer pairs. Each well was loaded with cDNA reaction product. The card was run on a LightCycler 480 RT-PCR system (Roche). Relative RNA expression levels were determined from C_t values.

DNA Isolation and Quantification of *C. difficile* Colonization

DNA was isolated from colon snips obtained during necropsy using a DNeasy kit (Qiagen) according to manufacturer instructions. *C. difficile* colonization was measured using species-specific qPCR of isolated colon DNA. Each 10 µL reaction included 2 µL of isolated DNA, 6.25 pmol F/R-TcdB primers, and 1 pmol TcdB probe. The following cycling conditions were used: Activation for 1 cycle, 95^{o} C for 15 minutes; Cycling for 45 cycles of 95^{o} C for 15 seconds, 60^{o} C for 20 seconds, and 72^{o} C for 10 seconds; Hold for 30 seconds at 72^{o} C. Raw C_t values were normalized to mouse TNFα gene content, used as a single-copy host internal control gene, to generate ΔC_t values. ΔC_t values were then

converted to “detectable genomes/g host tissue” using a standard curve. The standard curve was generated by calculating ΔC_t values for samples containing known amounts of vegetative *C. difficile* cells and mouse colonic tissue.

RESULTS

C. difficile Colitis in the Absence of GM-CSF Signaling

We first wanted to examine the effect that the loss of GM-CSF signaling has on *C. difficile* colonization of the large intestine. Mice were given cefaperazone drinking water (0.5 g/L) for five days (Figure 1). Two days after the end of antibiotic treatment, mice were challenged with approximately $5*10^4$ colony-forming units of *C. difficile.* The infection was allowed to progress for 4 days, and colonic tissue was harvested. Next, qPCR was used to determine the level of colonization of the experimental groups. qPCR results were originally calculated as ΔC_t values, corresponding to the difference in signal between the target *C. difficile* sequence and a single-copy host gene. We established a standard curve to convert ΔC_t values into units of CFU of *C. difficile* per gram of mouse tissue. Colonic tissue samples from untreated mice were weighed to determine the tissue mass. A series of dilutions containing known quantities of *C. difficile* were added to the tissue samples. Then, a species-specific quantitative polymerase chain reaction (qPCR) was used to determine the ΔC_t values that corresponded to the concentration of bacteria per gram of host tissue. The result was a standard curve that allowed for conversion of ΔC_t values from qPCR into a more useful metric for determining colonization, CFU per gram of colonic tissue.

Untreated animals were not detectably colonized with *C. difficile*. *C. difficile* infected wild-type and *C. difficile* infected anti-GM-CSF treated animals were colonized with approximately $1.5*10^5$ and $5*10^4$ CFU of *C. difficile* per gram of colonic tissue, respectively (Figure 2). Both infected groups had statistically significantly higher colonization than the untreated group, as determined by an ANOVA test. Notably, there was no significant difference in colonization levels between the two infected groups. These results provide evidence that depletion of GM-CSF signaling does not significantly affect the ability of *C. difficile* to colonize in the large intestine of cefaperazone-treated mice. They also confirm that this model is useful in studying disease caused by *C. difficile*, because the pathogen loads were equivalent between the infected groups.

The first measure of disease that we examined was weight loss. Mice were monitored for weight changes for seven days before inoculation, and four days after. All experimental groups showed consistent weight gain (5-7% of initial body weight) for the first seven days, regardless of antibiotic treatment (Figure 3). During the four days after infection, untreated animals continued to gain weight (~2% of initial body weight), while *C. difficile* infected animals lost ~8% of body weight during the peak of infection, around three days after exposure to the bacteria. Interestingly, anti-GM-CSF treated mice with CDI displayed intermediate weight loss, losing ~5% of initial body weight during the peak of infection. While both the CDI and anti-GM-CSF CDI groups displayed a sharp weight loss during the first three days of infection, mice treated with anti-GM-CSF antibody trended toward less weight loss than the wild-type group, indicating that anti-GM-CSF treatment may reduce the weight loss caused by CDI.

Histopathology Changes Due to Anti-GM-CSF Treatment during CDI

We next wanted to examine the effect of anti-GM-CSF treatment on intestinal damage during *C. difficile* colitis. Examination of intestinal histopathology revealed that uninfected animals showed signs of healthy colonic epithelial tissue, as characterized by a lack of tissue damage and inflammatory cell infiltration (Figures 4A and 4B, left). *C. difficile*-infected wild-type mice developed typical signs of *C. difficile*-induced disease, including inflammatory cell recruitment and submucosal edema (Figure 4A, middle, black arrows). Remarkably, anti-GM-CSF treated mice displayed much less inflammatory cell infiltration into epithelial tissue, indicating a decrease in inflammatory response in the absence of GM-CSF signaling (Figure 4A, right, black arrows). As expected, wild-type mice also some exhibited signs of colonic epithelial cell damage (Figure 4B, middle). Notably, treatment with anti-GM-CSF caused increased epithelial damage beyond normal levels seen in wild-type mice (Figure 4B, right, grey arrows). This data suggests that GM-CSF signaling not only promotes inflammatory signaling, but also has some epithelial protective function during *C. difficile* colitis.

Inflammatory Gene Expression in the Absence of GM-CSF Signaling

In order to further examine the trends seen in the histology slides, we next investigated the effect of anti-GM-CSF treatment on the expression of inflammatory genes. Isolated RNA from colonic tissue of untreated, CDI treated, and anti-GM-CSF CDI treated mice was analyzed as describe in Materials and Methods. Along with the

inflammatory cell recruitment seen in the CDI groups, increased expression of various pro-inflammatory cytokine genes was detected in these animals (Figure 5A). Strikingly, and consistent with the reduced epithelial cell infiltration noted in the histopathological analysis, anti-GM-CSF treated mice showed reduced expression of pro-inflammatory genes. *Tnfα* expression in GM-CSF-depleted mice was just 1.2-fold higher than untreated animals, compared to a nearly 5 fold change in wild-type mice. *Il-1β* showed a less dramatic, but still significant drop in expression in GM-CSF-depleted mice versus wild-type mice. *Ifng* displayed an intermediate phenotype in GM-CSF-depleted mice; expression was not significantly higher than in untreated animals, but not significant lower than wild-type.

We observed a similar pattern in inflammatory chemokine expression as well. Infection of wild-type animals resulted in increased expression of *Cxcl1, Cxcl2, Cxcl10, Ccl2, and Ccl4* (Figure 5B). However, in mice treated with anti-GM-CSF, chemokine *Ccl4* displayed an intermediate phenotype, while *Cxcl2* expression was significantly reduced compared to the wild type. Taken together, these data strongly suggest that GM-CSF signaling drives inflammatory cytokine and chemokine gene expression during *C. difficile* colitis.

Epithelial Protective Gene Expression in the Absence of GM-CSF Signaling

In addition to inflammatory cytokines and chemokines genes, we also wished to investigate the effect of reduced GM-CSF signaling on the expression of genes involved in epithelial protection. Gene expression data confirmed that *Reg3γ* and *SLPI* were

significantly up-regulated during *C. difficile* infection in wild-type mice (Figure 6). Reg3γ is an antimicrobial peptide; SLPI is a protease inhibitor that protects the host from its own defensive enzymes. In addition, *NOS2*, which codes for nitric oxide synthase, and is also involved in epithelial protection, was significantly up-regulated. *Reg3γ* expression levels in GM-CSF-depleted mice were comparable to wild-type mice, but both *SLPI* and *NOS2* levels were significantly lower in the GM-CSF-depleted group, which is consistent with the increased epithelial damage seen in the histopathology analysis (Figure 4). These data suggest that GM-CSF plays a role in epithelial protection during *C. difficile* infection, and support the histological data showing that reduced GM-CSF signaling results in more epithelial damage during infection.

DISCUSSION

The current study was undertaken in order to examine the role of GM-CSF signaling during *C. difficile* colitis. Pathogen colonization was confirmed by quantifying detectable *C. difficile* genomes and murine colonic cells via qPCR and comparing the levels to a standard growth curve. Anti-GM-CSF depletion was shown to have no significant effect on levels of colonization, validating the utility of this experimental model in studying disease due caused by equivalent *C. difficile* burden. Weight loss data provided evidence that anti-GM-CSF treatment may reduce the severity of disease due to CDI. The effect of GM-CSF depletion on infection was investigated using histological preparations of colonic tissue to look at microscopic changes in the epithelial tissue, and an RT-qPCR assay to assess changes in gene expression. When GM-CSF signaling was

ablated through the use of a depleting antibody, we observed decreased cellular infiltrate. Consistently, we also observed reduced expression of pro-inflammatory myeloid cell chemokines in GM-CSF-depleted mice. Anti-GM-CSF treatment also caused an increase in epithelial cell damage, coinciding with a decrease in expression of genes involved in epithelial protection. This data supports the conclusion the GM-CSF serves both pro-inflammatory and protective functions during *C. difficile* infection in mice.

The histological data from this study provides evidence for decreased neutrophil and monocyte recruitment during CDI due to GM-CSF depletion, which is consistent with the role of GM-CSF as a granulocyte and macrophage stimulating cytokine (Figure 4). Neutrophil and monocyte (especially macrophage) recruitment are well-documented hallmarks of the intestinal immune response to *C. difficile* (27, 31, 32). Histopathology analysis also showed an increase in damage to epithelial cells in the absence of GM-CSF (Figure 4). Together, these results indicate that GM-CSF may play a role in the inflammatory response and epithelial protective response to CDI.

The gene expression data from this study indicated significant down-regulation of notable pro-inflammatory cytokines and chemokines in *C. difficile* infected tissue in the absence of GM-CSF signaling, as compared to infected wild-type mice. TNFα, which was reduced to almost baseline levels due to GM-CSF depletion, is involved in neutrophil recruitment during airway epithelial inflammation, and is connected, along with Il-22, to neutrophil recruitment in response to intestinal colitis (33, 34). *Il-22* expression was not significantly higher in infected GM-CSF-depleted mice than in untreated mice, while it was significantly higher in infected wild-type mice, indicating

that anti-GM-CSF treatment reduces both *TNFα* and *IL-22*. *CXCL2*, which produces a chemokine that also contributes to neutrophil infiltration during *C. difficile* colitis, was also significantly downregulated (35). It can be concluded from these data that GM-CSF plays a role in neutrophil recruitment during *C. difficile* colitis, likely due to decreased expression of *TNFα*, *IL-22*, and *CXCL2*.

Il-1β, which codes for a pro-inflammatory cytokine known to cause intestinal tight junction permeability, was also downregulated upon GM-CSF depletion (36). Disruption of tight junctions is a staple of *C. difficile* colitis, but it is possible that it is toxin-mediated and not due to *Il-1β* (21). *IFNγ* displayed an intermediate phenotype, showing a trend of lower expression when GM-CSF was depleted. Interferon-gamma has been shown to be a key mediator of TcdA-induced colitis due to its attenuation of TNFα and chemokine secretion, and so this result could warrant more investigation (37). These results all suggest that in addition to stimulating neutrophil recruitment, GM-CSF could be a part of the inflammatory response during CDI through a variety of other routes.

GM-CSF depletion also has an effect on the expression of genes involved in epithelial protection. *SPLI*, which had significantly lower expression in GM-CSF-depleted mice, encodes a protease inhibitor that limits tissue damage and facilitates healing during colitis (38). *NOS2* expression was also reduced in the absence of GM-CSF. NOS2 produces nitric oxide, which is essential for the antimicrobial properties of macrophages (39). Taken together, this data indicates that knocking out GM-CSF signaling during CDI reduces the expression of epithelial protective genes.

In the current study, we have investigated the role of GM-CSF signaling in the innate immune response to *C. difficile* colitis. However, the nature of the cellular infiltrate and the sources and targets of GM-CSF signaling during CDI remain unknown. Thus, further work is required. Broadening the variety of genes examined could reveal other key signaling molecules involved in the innate response to *C. difficile*. The addition of an experimental group with overexpression of GM-CSF could strengthen the connections between GM-CSF and other signaling and protective molecules that have been indicated in this work. Histological scoring would allow for a more quantitative look at microscopic damage to the intestinal epithelium, and could confirm that anti-GM-CSF treatment reduces inflammation and increases epithelial cell damage. Flow cytometry of colonic tissue samples would be very advantageous in assessing the changes in inflammatory cell population in response to changes in GM-CSF signaling levels, with the expectation that fewer neutrophils and macrophages would be present when GM-CSF signaling is reduced. Microbiome sequencing could be useful in detecting changes in the commensal bacteria due to depletion of GM-CSF, both before and during infection, which could have an effect on the course of the disease. Based on the clear connections between GM-CSF and the inflammatory response to *C. difficile* laid out in this work, more investigation is warranted to further understand the intestinal innate immune response to CDI.

FIGURE 1: Experimental Timeline

A timeline for *C. difficile* infection of anti-GM-CSF treated mice. See Materials and Methods for a detailed description.

FIGURE 2: Effect of Infection of Body Weight

The effect of *C. difficile* infection following five day cefaperazone treatment on mouse body weight. Baseline body weight was determined prior to antibiotic treatment, one week before infection. Mice were weighed again two days pre-infection, the day of infection, and every day for four days post-infection. Plotted points with error bars represent mean body weight ± SEM from three independent experiments with n=8 per group.

FIGURE 4: *C. difficile* Colonization

C. difficile colonization of colonic tissue following cefaperazone treatement. Colonization of *C. difficile* was quantified by species-specific qPCR, the signal from which was converted to detectable *C. difficile* genomes per gram of mouse colonic tissue through the use of a pre-establish standard curve [see Materials and Methods]. Bars represent mean ± SEM from three independent experiments with n=8 per group. The limit of detection is approximately 10^4 CFU/g.

FIGURE 5: Histopathology of Mouse Colonic Tissue

Photomicrographs of haemotoxylin and eosin stained colon epithelial tissue. Colonic tissue was removed during necropsy and preserved in formalin, then ethanol. Black arrowheads indicate cellular infiltrate; gray arrowheads indicate epithelial damage due to *C. difficile* colitis. Magnification 100x.

FIGURE 6: Inflammatory Gene Expression

Changes in colonic inflammatory gene expression during *C. difficile* colitis due to GM-CSF depletion. Colonic gene expression was measured using reverse transcriptase quantitative PCR assays. A) Inflammatory cytokine expression B) Inflammatory chemokine expression. Asterisks indicate a statistically significant increase in gene expression compared to untreated expression levels. Brackets indicate a statistically significant difference in expression between CDI-treated and GM-CSF-depleted CDI-treated groups for the denoted gene. Bars represent mean ± SEM from three independent experiments with n=8 per group.

FIGURE 7: Epithelial Protective Gene Expression

Changes in colonic epithelial protection gene expression during *C. difficile* colitis due to GM-CSF depletion. Colonic gene expression was measured using reverse transcriptase quantitative PCR assays. Asterisks indicate a statistically significant increase in gene expression compared to untreated expression levels. Brackets indicate a statistically significant difference in expression between CDI-treated and GM-CSF-depleted CDI-treated groups for the denoted gene. Bars represent mean ± SEM from three independent experiments with n=8 per group.

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FIGURE 1

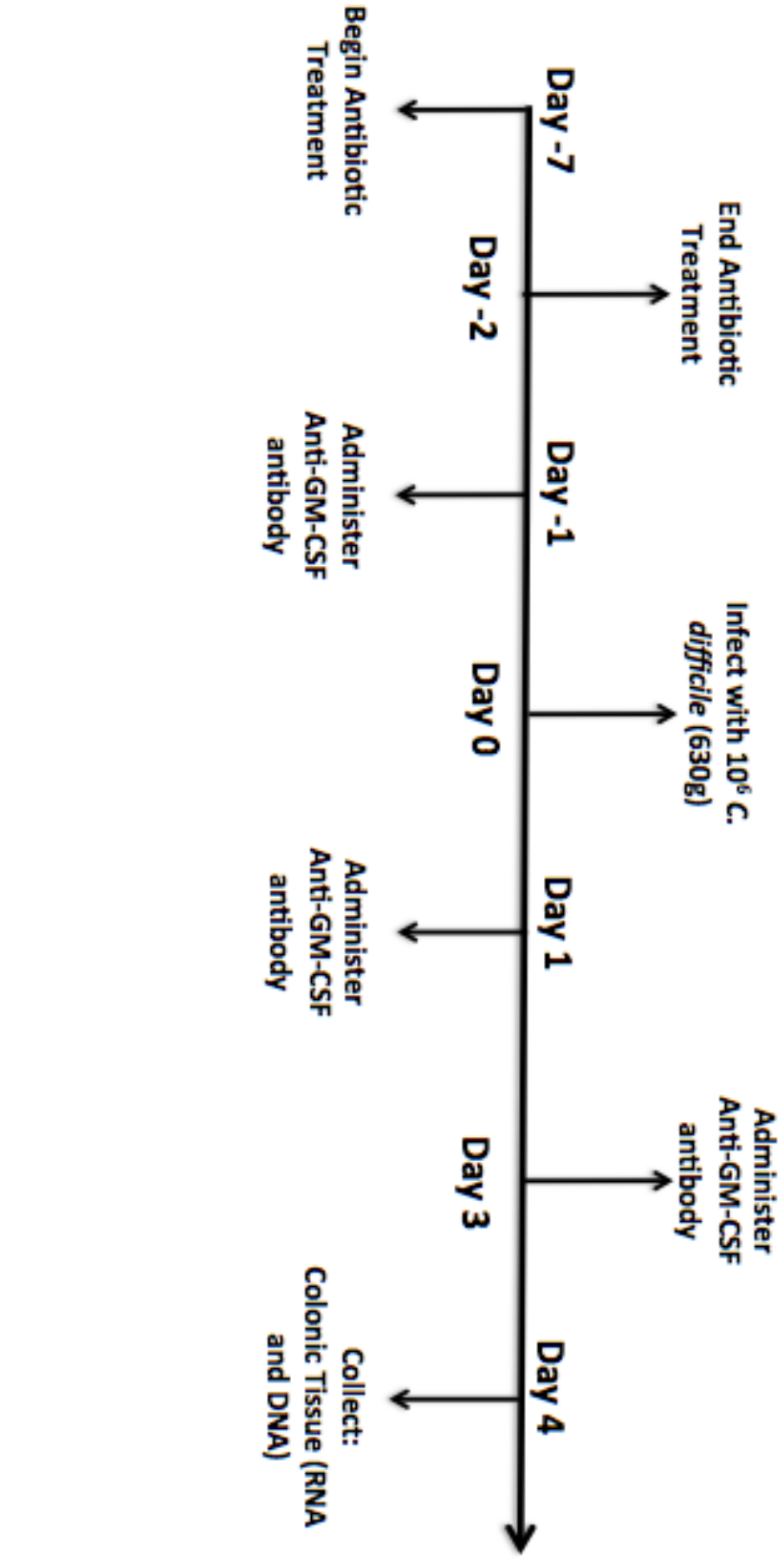

Day -7
Begin Antibiotic Treatment
Day -2
Administer Anti-GM-CSF antibody
Day -1
End Antibiotic Treatment
Day 0
Infect with 10^6 *C. difficile* (630g)
Day 1
Administer Anti-GM-CSF antibody
Day 3
Administer Anti-GM-CSF antibody
Day 4
Collect: Colonic Tissue (RNA and DNA)

FIGURE 2

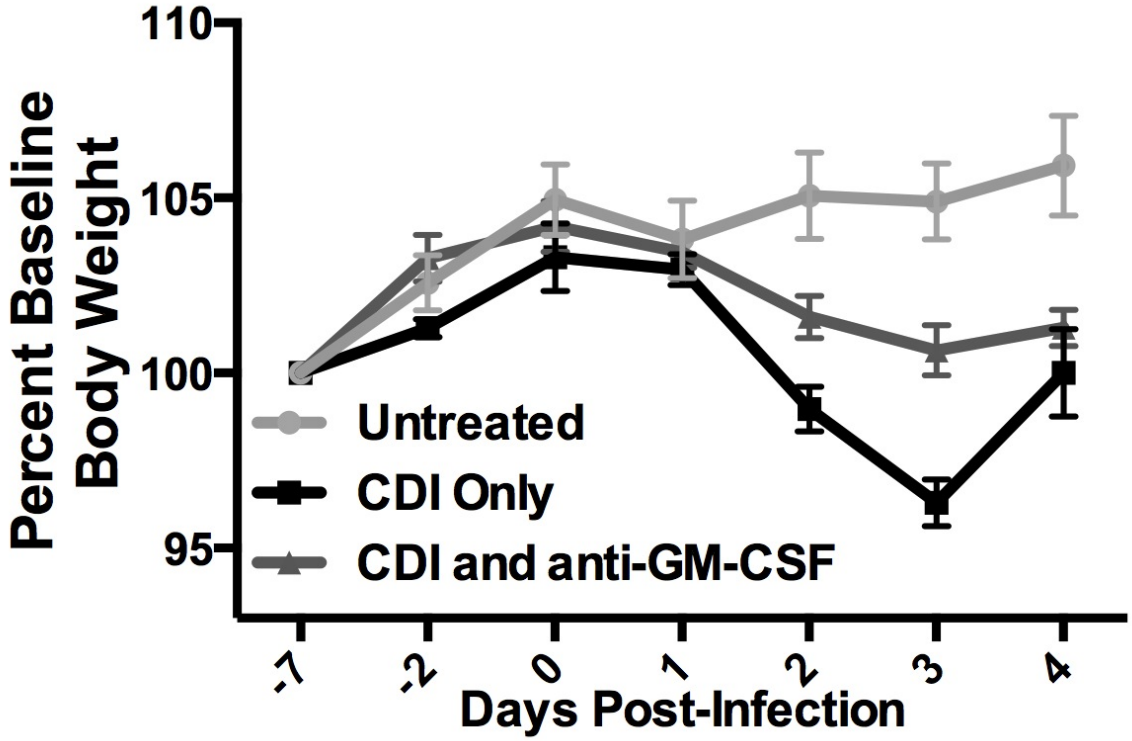


FIGURE 3

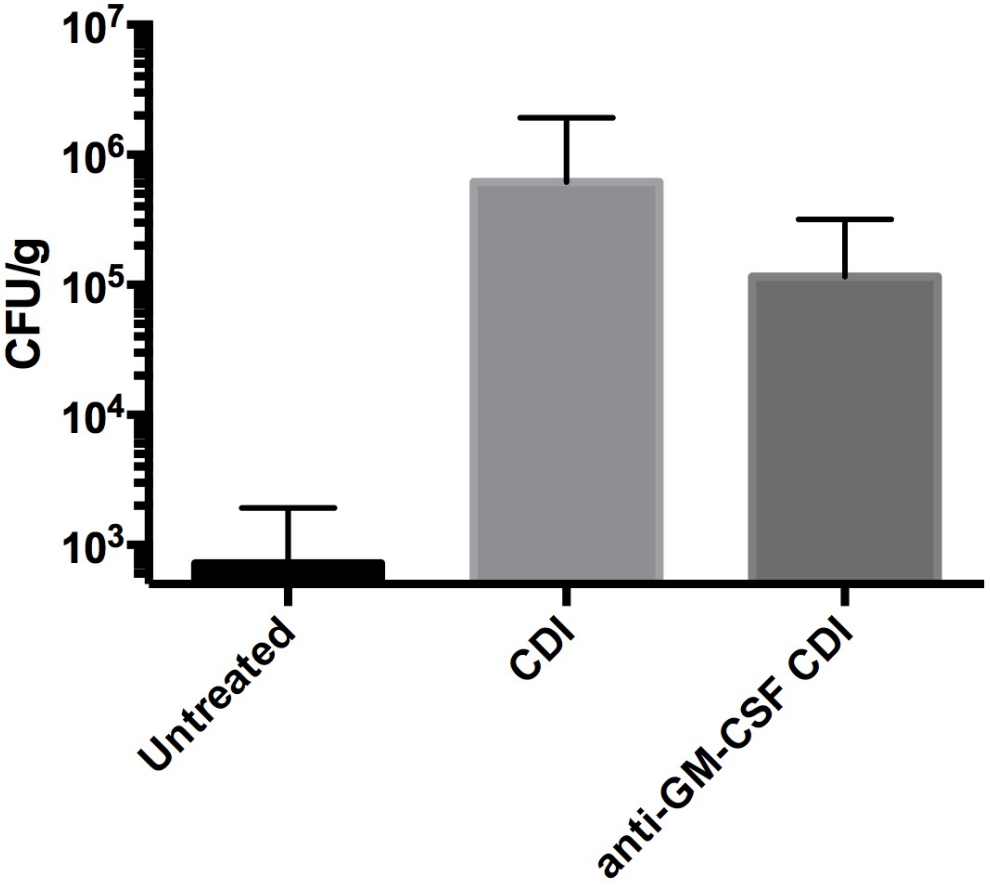


FIGURE 4

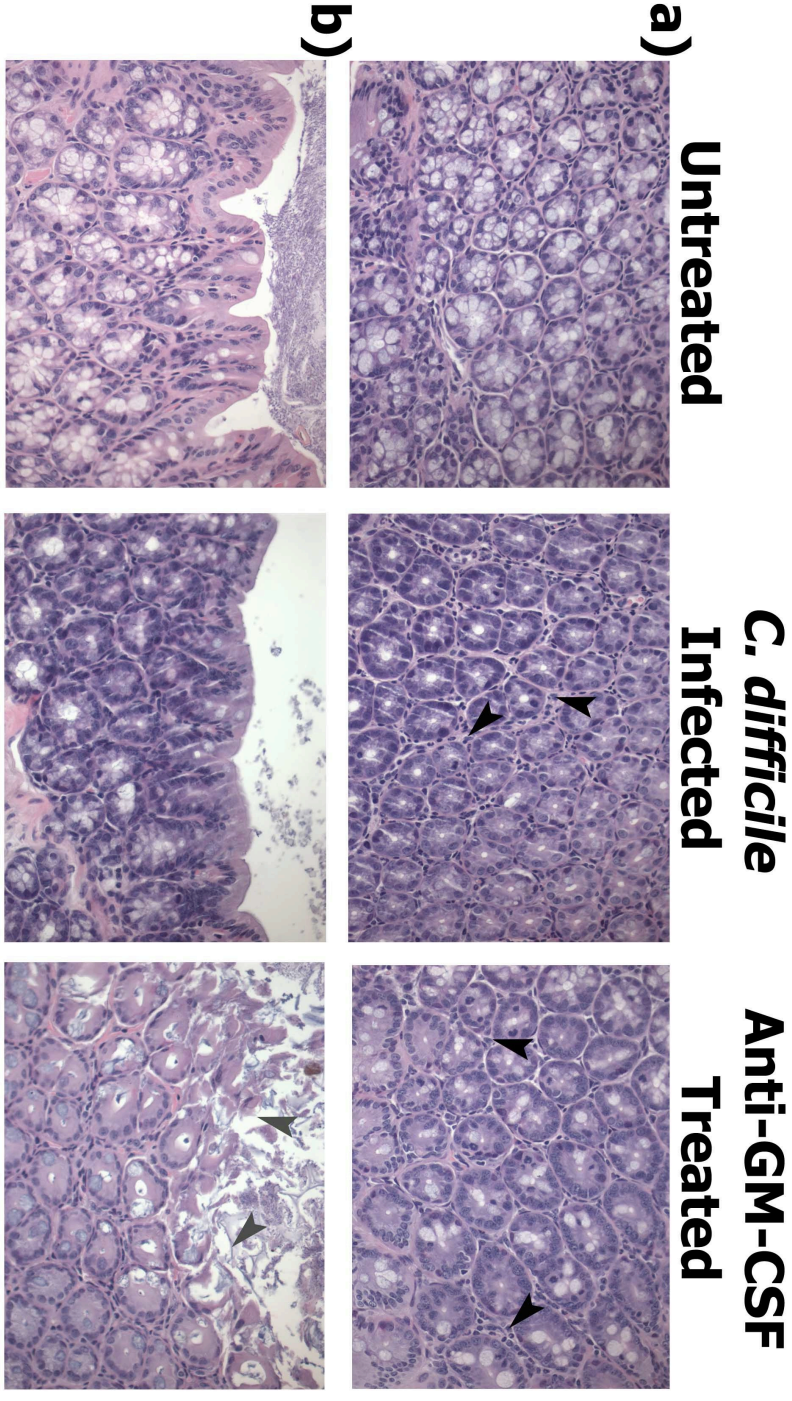
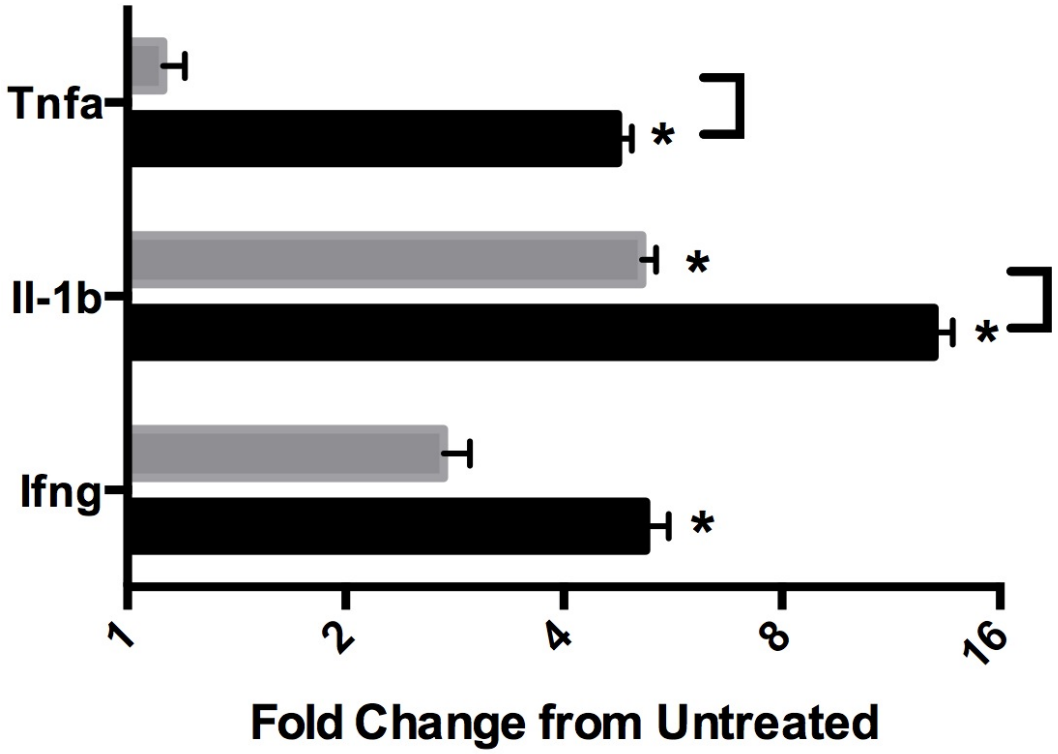


FIGURE 5

A


Tnfa
Il-1b
Ifng
*
1
2
4
8
16
Fold Change from Untreated

B

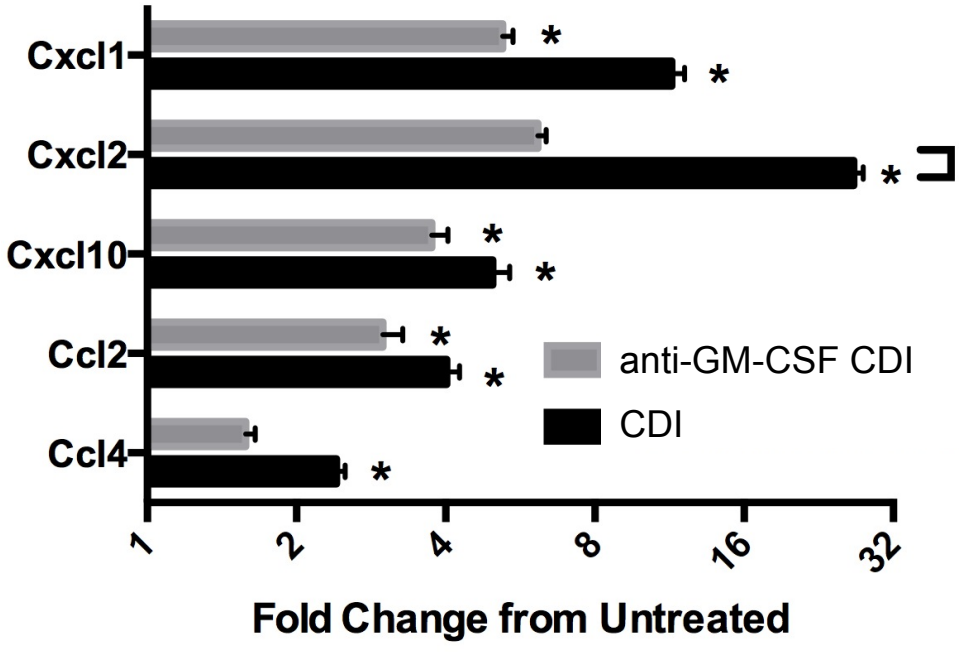
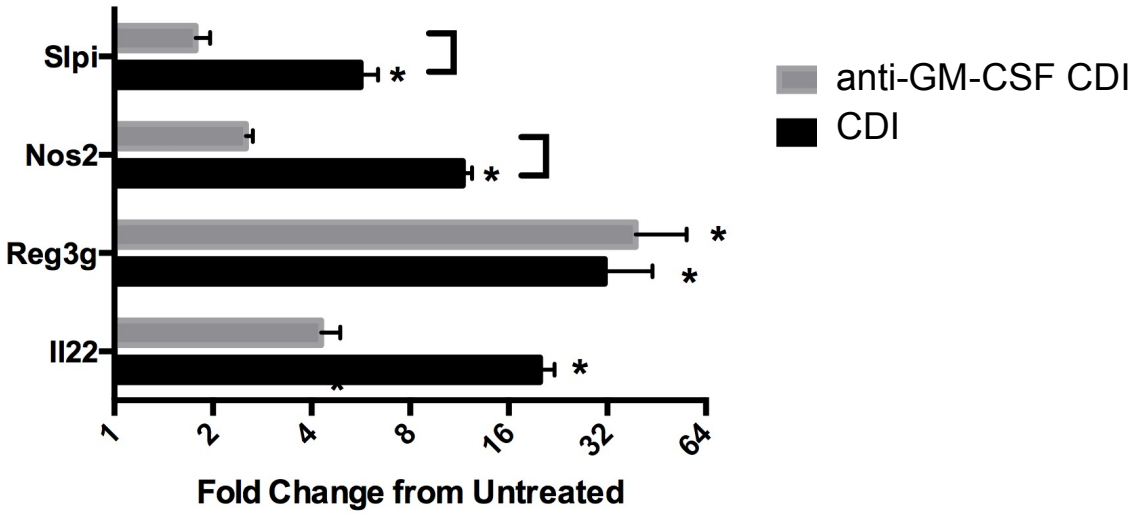

Cxcl1
Cxcl2
Cxcl10
Ccl2
Ccl4
*
anti-GM-CSF CDI
CDI
1
2
4
8
16
32
Fold Change from Untreated

FIGURE 6


Slpi
Nos2
Reg3g
Il22
*
1
2
4
8
16
32
64
Fold Change from Untreated
anti-GM-CSF CDI
CDI