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Role of Gamma Interferon and Inflammatory Monocytes in Driving Colonic Inflammation During Acute *C. difficile* Infection in Mice

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Summary

The inflammatory response to the colonic pathogen *Clostridium difficile* is characterized by the induction of inflammatory cytokines including Interleukin-23 (IL-23) and interferon gamma (IFNγ) and the recruitment of myeloid cells including Ly6C^{High} monocytes. IL-23KO mice showed reduced expression of the monocyte chemokines *Ccl4* and *Ccl7*, but not *Ccl2*, as well as reduced Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the colon in response to *C. difficile* colitis. *C. difficile*-infected CCR2$^{-/-}$ (CCR2KO) mice showed a significant defect in Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the colon in response to *C. difficile*. While there was no decrease in expression of the inflammatory cytokines *Il1b*, *Il6*, or *Tnf* or reduction in the severity of colonic histopathology associated with ablation of monocyte recruitment, *Slpi* and *Inos* expression was significantly reduced in the colons of these animals. Additionally, neutralization of IFNγ through the administration of anti-IFNγ mAb resulted in a significant reduction in the expression of the IFNγ-inducible chemokines *Cxcl9* and *Cxcl10*, but not a reduction in the neutrophil chemokines *Cxcl1*, *Cxcl2*, and *Ccl3* or the monocyte chemokine *Ccl2*. Consistently, monocyte and neutrophil recruitment were unchanged following anti-IFNγ treatment. Additionally, *Inos* and *Slpi* expression were unchanged following anti-IFNγ treatment, suggesting that *Inos* and *Slpi* regulation is independent of IFNγ during *C. difficile* colitis. Taken together, these data strongly suggest that IL-23 and CCR2 signaling are required for monocyte recruitment during *C. difficile* colitis. Additionally, these studies also suggest that monocytes, but not IFNγ, are necessary for full expression of *Inos* and *Slpi* in the colon.

Introduction

Monocyte-derived cells are key mediators of inflammatory responses in the GI tract[1]. Monocyte recruitment is often associated with the development of inflammation and epithelial damage at mucosal sites[2-9]. Monocytes and macrophages contribute to TNFα production during both pulmonary and colonic inflammation[6, 7]. Monocyte recruitment is also required for full production of

inflammatory cytokines including IL-1β and IL-6, during DSS colitis[9]. Furthermore, the development of intestinal histopathology during DSS colitis is partially dependent on monocyte recruitment[8, 9].

Clostridium difficile (*C. difficile*) infection in mice results in innate large bowel inflammation, characterized by increased inflammatory cytokine expression, marked histopathology, and rapid, robust recruitment of innate immune cells including monocytes to the large bowel[2-5, 10-18]. MyD88 signaling is crucial for monocyte recruitment to the large intestine in response to *C. difficile* colitis, and CCR2-deficient mice show a significant defect in monocyte recruitment when challenged with *C. difficile*[10]. While recent studies suggest that monocyte recruitment to the large intestine is not required for host survival during *C. difficile* colitis[10], the role of recruited monocytes in promoting inflammatory cytokine expression and epithelial damage, as well as the host signals driving monocyte recruitment during *C. difficile* colitis remains poorly understood.

Interferon gamma (IFNγ) is a potent mediator of innate inflammation at mucosal sites, including in the gastrointestinal tract[19-21]. IFNγ signaling is required for full recruitment of neutrophils and production of CXCL1 in response to *Streptococcus pneumoniae* infection in the lung[20]. IFNγ is also required for CCL2 production and neutrophil recruitment to the colon during chemically-induced colitis[19]. Furthermore, recruited neutrophils produce IFNγ in response to both *C. difficile*[15] and *Salmonella typhimurium*[22] infection. Specific to the host response to *C. difficile*, Ishida and colleagues reported reduced TNFα and CXCL1 expression in IFNγKO mice following administration of *C. diffiicle* Toxin-A to ligated ileal loops[21]. Consistently, numerous studies from our own laboratory have reported increased IFNγ expression in the colonic mucosa in response to *C. difficile* infection[5, 11, 23]. Additionally, a recent study by Abt and colleagues has suggested a critical role for IFNγ-producing type I innate lymphoid cells (ILC1s) in mediating host survival during *C. difficile* colitis[24]. However, the role of IFNγ in modulating innate inflammatory responses, especially myeloid cell recruitment and inflammatory

cytokine and chemokine expression in response to infection with metabolically active *C. difficile*, is largely unknown.

IL-23 is a known driver of innate inflammation at mucosal sites[25, 26]. IL-23 drives inflammatory myeloid cell recruitment to the lung in response to chemical[27] and microbial[28] challenge. Additionally, IL-23 is required for the recruitment of inflammatory monocytes to the spleen in response to *Listeria monocytogenes* infection[29]. Furthermore, CD11b^{High} myeloid cell recruitment is markedly ablated during chemically-induced colitis in the absence of IL-23 signaling[25]. Work by Buonomo and colleagues has suggested a clear role for IL-23 in promoting severe outcomes during experimental *C. difficile* infection[18], and recent studies from our laboratory have demonstrated a role for IL-23 in driving neutrophil recruitment and contributing to colonic histopathology during *C. difficile* infection[26]. However, the role of IL-23 in driving the recruitment of other myeloid populations during *C. difficile* colitis, including monocytes, is poorly understood.

In the current study, our initial goal was to determine the role of IL-23 in driving Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the large intestine during *C. difficile* colitis. Having demonstrated reduced monocyte recruitment in IL-23KO mice, we next sought to determine if ablation of CCR2-dependent monocyte recruitment alone was sufficient to ameliorate the severity of colonic inflammatory gene expression or colonic histopathology. Additionally, recent studies have suggested a role for IFNγ in mediating host protection during *C. difficile* infection[24], but the role of IFNγ in driving innate inflammatory responses to *C. difficile* colitis remains poorly understood. As such, we investigated the role of IFNγ signaling in driving neutrophil and monocyte recruitment, inflammatory cytokine expression, and colonic histopathology during acute *C. difficile* colitis in mice. Collectively, these studies reveal the role of IL-23 in driving monocyte recruitment during *C. difficile* infection, as well as identify the roles of CCR2-dependent monocyte recruitment and IFNγ in driving inflammatory cytokine expression, colonic histopathology, and inflammatory myeloid cell recruitment during acute *C. difficile* colitis.

Materials and Methods

Animals and Housing

C57BL/6 male mice aged 5-12 weeks from a colony maintained at the University of Michigan founded by Jackson breeders were used in the current study. Male and female CCR2$^{-/-}$ (CCR2KO) and p19$^{-/-}$ (IL-23KO) on a C57BL/6 background aged 5-14 weeks were used in the current study. Both CCR2KO and IL-23KO mice were obtained from in house breeding colonies at the University of Michigan. All mice were permitted autoclaved water and food *ad libitum*, and were maintained under specific pathogen-free conditions with autoclaved bedding. All animal manipulations were performed in a laminar flow hood, and all experiments were performed in accordance with a protocol approved by the University Commission on the Use and Care of Animals at the University of Michigan.

Bacterial Culture and Growth Conditions

Clostridium difficile spores were prepared for infection as previously described[11, 26]. Briefly, an existing spore stock of *C. diffiicle* strain VPI 10463 was plated on Taurocholate Cefoxitin Cycloserine Fructose Agar and cultured overnight to generate vegetative cells. An individual colony was used to inoculate an overnight culture in Columbia broth. This overnight (2ml) was then used to inoculate 40ml of Clospore[30] sporulation medium, and the culture was then allowed to grow for 7 days at 37^{0}C anaerobically. Spores were recovered by washing the resulting pellet at least 4 times to remove residual vegetative cells. Stocks were stored at 4^{0}C in water until use.

Quantification of *C. difficile* colonization

Mucosal *C. difficile* colonization was assessed as described previously[4, 11, 31, 32] using a *C. difficile* specific PCR of DNA isolated from host colonic tissue. Reaction volumes,

cycling conditions, and primer and probe sequences are identical as those used previously[4, 31, 32]. Raw Ct values were normalized to a single copy per genome host gene used as an internal control to generate dCt values[32, 33]. Normalized dCt values were then converted to "*C. difficile* genomes per gram of host tissue" using a standard curve developed using known quantities of host tissue and vegetative *C. difficile*.

Antibiotic Treatment and Infection

For all experiments, animals were given cefoperazone (Sigma) at a concentration of 0.5g/L in their drinking water for 5 days as described previously[11, 13, 23, 26]. After the antibiotic treatment, mice were permitted a 2 day recovery period on regular drinking water prior to infection with *C. difficile*. Untreated animals received neither *C. difficile* challenge nor antibiotic pretreatment.

For *C. difficile* infection studies, mice received approximately 10^6 CFU of VPI 10463 spores by oral gavage on Day 0. Infected animals were monitored for any sign of undue stress including lethargy, hunched posture, and weight loss exceeding 20% of baseline body weight, and any moribund animals were humanely killed. All experimental samples were collected on Day 2.

Neutralizing antibody

In order to neutralize IFNγ in vivo, mice were given 500μg of anti-IFNγ monoclonal antibody (mAb; clone XMG.1.2) via intraperitoneal injection one day prior and one day post infection (Day -1 and Day 1 respectively).

Colonic Leukocyte Isolation

Leukocytes were isolated from colonic tissue as described previously[4, 5, 23, 26, 34], with certain modifications. Briefly, colonic tissue was excised and physically disrupted

using serrated scissors. Minced tissue was then incubated in 20ml of Hanks' Balanced Salt Solution (HBSS) supplemented with 1mM DTT, 5mM EDTA, and 2.5% FCS for 20 minutes at 37^0C. Tissue was then washed, and subsequently incubated with 20ml of HBSS supplemented with 0.5mg/ml DNase (Roche), 400U/ml collagenase type 3 (Worthington Biochemicals), and 2.5% FCS for 1 hour at 37^0C. After washing, samples were then resuspended in 20% Percol (Sigma) in PBS and spun at 900g for 30 minutes without brake. The resulting single cell suspension was stained for surface marker expression by flow cytometry.

Flow Cytometry Staining and Analysis

Flow cytometry staining was performed as described previously[4, 5, 23, 26]. Briefly, cells were plated at a concentration of approximately 10^6 cells per well in a 96 well plate, and were blocked with unlabeled FcRIII/II. After blocking, cells were stained with fluorescently labeled antibodies for 30 minutes at 4^0C. Cells were washed, and resuspended in stabilizing fixative (BD Biosciences). All samples were acquired on a 3 laser FACSCanto II using FACS-Diva software. All data analysis was performed in FlowJo (Treestar) Cells were stained with the following antibody clones: CD45 (clone 30-F11), CD11c (clone HL3), CD11b (clone M1/70), Ly6C (clone AL-21), and Ly6G (clone 1A8). All antibodies were purchased from BD Biosciences or Biolegend.

For calculating number of $CD11b^{High}$ $CD11c^{Low}$ cells per 100,000 events, the frequency of $CD45^+$ events was multiplied by the frequency of $CD11b^{High}$ $CD11c^{Low}$ events, and the resulting number was multiplied by 100,000.
For calculating the number of $Ly6C^{High}$ $Ly6G^{Mid}$ monocytes or $Ly6C^{Mid}$ $Ly6G^{High}$ neutrophils per 100,000 events, the frequency of $CD45^+$ events was multiplied by the frequency of $CD11b^{High}$ $CD11c^{Low}$ events, and the frequency of either $Ly6C^{High}$ $Ly6G^{Mid}$ monocytes or $Ly6C^{Mid}$ $Ly6G^{High}$ neutrophils. The resulting number was then multiplied by 100,000.

The gating strategy for identification and enumeration of colonic leukocyte subsets is also highlighted in Figure S1.

Preparation and Examination of Colonic Histological Sections

Excised colonic tissue was prepared for histological analysis as described previously[4, 5, 11, 23, 26]. Colonic tissue was fixed in 10% formalin for a minimum of 24 hours and then transferred to 70% ethanol. Cassettes were processed, paraffin embedded, sectioned, and used to prepare hematoxylin and eosin stained slides by McClinchey Histology Lab Inc. (Stockbridge, MI).

Representative photomicrographs were acquired using an Olympus BX40 light microscope (Olympus corporation) using a QImaging MicroPublisher RTV 5.0 5 megapixel camera. All photomicrographs were acquired at a total magnification of 400X. QCapture Suite PLUS version 3.1.3.10 was used for image acquisition. All panels were assembled in Adobe Photoshop CS5, version 12.0. Processing of images was restricted to global adjustments of brightness, contrast, and image size for each photomicrograph.

RNA isolation and expression analysis

RNA was purified from colonic tissue and gene expression assessed as described previously[4, 5, 11, 23, 26]. Briefly, samples of colonic tissue approximately $1cm^2$ were excised from the midpoint of the colon and stored in RNAlater (Ambion) for further analysis. To isolate RNA, samples were homogenized in TRIzol reagent (Life Technologies), and RNA purified using the RNeasy Mini Kit (Ambion) according to the manufacturers' instructions. Purified RNA was assessed for concentration and purity using a nanodrop instrument (Thermo Fisher) and Agilent Bioanalyzer (Agilent), respectively. RNA was converted to cDNA using the RT^2 first strand kit (Qiagen) and colonic gene expression was assessed using RT^2 Profiler PCR Assays (Qiagen). All PCR reactions were run on a Roche LightCycler 480. For RT^2 Profiler

PCR Assays, cross card normalization was performed to control for card-to-card variability[35]. ΔCt (dCt) values were calculated by subtracting the mean Ct value of 2 internal control genes from the gene in question[33, 36].

Statistical Analysis

Statistically significant differences in gene expression were determined by performing a one-way ANOVA with Tukey's post-hoc test on normalized dCt values[4, 11, 26]. Statistically significant difference in colonic *C. difficile* colonization were likewise identified by performing a one-way ANOVA with Tukey's post-hoc test on normalized dCt values[4] A one-way ANOVA with Tukey's post-hoc test was also used to identify statistically significant differences in the number of particular cellular subsets per 100,000 cells. For all analyses, statistical significance was set at $p \leq 0.05$.

Results

Effect of IL-23 deficiency on colonic monocyte recruitment

In order to investigate the role of IL-23 in promoting monocyte recruitment during *C. difficile* colitis, WT and IL-23$^{(-/-)}$ (IL-23KO) mice were infected with *C. difficile* as described previously[26]. Briefly, mice were given 0.5g/L cefoperazone in their drinking water for five days and after a two-day recovery period on regular water were infected with spores of the *C. difficile* strain VPI 10463 by oral gavage. The mice were followed for an additional two days, at which point the infection was terminated and all samples collected.

RT-PCR analysis was utilized to examine the effect of IL-23 deficiency on the expression of known monocyte-recruiting chemokines in the colon following *C. difficile* infection. The absence of IL-23 was associated with significantly reduced expression of *Ccl4* and *Ccl7*, but not *Ccl2*, in the colonic mucosa in response to *C. difficile* infection (Figure 1a). The absence of IL-23 did not affect the *C. difficile* burden within the colon (see supporting information, Figure S2). Flow cytometric analysis of colonic CD45^{+} leukocytes revealed a significant reduction in the frequency of CD11b^{High} CD11c^{Low} myeloid cells recruited to the colon in IL-23KO

mice as compared to WT animals (Figure 1b). Subsequent analysis of the CD11b^{High} CD11c^{Low} population demonstrated that IL-23 deficiency was also associated with a significant defect in the recruitment of Ly6C^{High} Ly6G^{Mid} monocytes (Figure 1c). Taken together, these data suggest that IL-23 promotes monocyte recruitment and the expression of monocyte-recruiting chemokines in the colon during *C. difficile* colitis.

Colonic monocyte recruitment during *C. difficile* colitis in CCR2 deficient mice

To further investigate the role of recruited monocytes during *C. difficile* colitis, CCR2$^{-/-}$ (CCR2KO) mice were infected as described in the Methods. *C. difficile* infection in WT animals was associated with significant recruitment of CD11b^{High} CD11c^{Low} myeloid cells (Figure 2a center panel and 3a). This CD11b^{High} CD11c^{Low} population contained both Ly6C^{High} Ly6G^{Mid} monocytes and Ly6C^{Mid} Ly6G^{High} neutrophils, and the frequency of both these populations was significantly increased above baseline following *C. difficile* infection (Figure 2b center panel and 3b,c). While neither CD11b^{High} CD11c^{Low} myeloid cell nor Ly6C^{Mid} Ly6G^{High} neutrophil recruitment were significantly altered in CCR2KO mice (Figure 2a,b right column and 3a,c), Ly6C^{High} Ly6G^{Mid} monocyte recruitment was significantly abrogated in CCR2KO mice infected with *C. difficile* (Figure 2b right panel, 3b). There was no difference in the frequency of total CD45^{+} leukocytes between groups (data not shown). These data indicate that Ly6C^{High} Ly6G^{Mid} monocyte recruitment, but not CD11b^{High} CD11c^{Low} myeloid cell or Ly6C^{Mid} Ly6G^{High} neutrophil recruitment, is significantly reduced during *C. difficile* colitis in the absence of CCR2 signaling.

Effect of CCR2 deficiency on inflammatory cytokine and chemokine expression

RT-PCR analysis was utilized to examine the effect of CCR2 deficiency on colonic inflammatory cytokine and chemokine expression in response to *C. difficile* colitis. Consistent with the significant influx of neutrophils observed in these animals (Figure 2b and 3c), *C. difficile* infection in WT mice was associated with increased expression of the neutrophil chemokines *Ccl3*, *Cxcl1*, and *Cxcl2* (Figure 4a). Expression of the inflammatory cytokines *Il1b*, *Il6*, *Il17f*, and *Tnf* were all

significantly increased in WT *C. difficile* infected animals (Figure 4b). *Arg1*, *Slpi,* and *Inos* were also significantly induced during *C. difficile* colitis (Figure 4c).

CCR2 deficiency did not result in any defects in the induction of the myeloid cell chemokines *Ccl3*, *Cxcl1*, or *Cxcl2*, (Figure 4a). Furthermore, CCR2 deficient animals displayed no reduction in *Il1b, Il6*, *Tnf,* or *Arg1* expression (Figure 4b,c). The expression levels of *Slpi* and *Inos*, however, were significantly reduced in CCR2KO mice as compared to WT animals infected with *C. difficile* (Figure 4c). Consistent with the gene expression, we observed no reduction in the severity of colonic histopathology (see Supporting information, Figure S3, left and middle columns) or levels of *C. difficile* colonization in CCR2KO animals (see Supporting information, Figures S2). Additionally, the absence of CCR2 was associated with a significant decrease in *ifng* expression within the colonic mucosa in response to *C. difficile* infection (Figure 4d). Collectively, these data suggest that while CCR2 signaling is not required for the induction of inflammatory mediators including *Cxcl1, Cxcl2*, *Il1b*, and *Il6*, the full expression of *Inos* and *Arg1* is dependent on CCR2 signaling during *C. difficile* colitis.

Effect of anti-IFNγ treatment on myeloid cell recruitment and inflammatory cytokine expression within the colon

In order to investigate the role of IFNγ in promoting monocyte and neutrophil recruitment, as well as inflammatory cytokine and chemokine expression during *C. difficile* colitis, animals were treated with anti-IFNγ mAb one day prior and one day post infection. As in previous experiments, all samples were collected 2 days post infection.

Analysis of $CD45^{+}$ colonic leukocytes revealed no defect in $CD11b^{High}$ $CD11c^{Low}$ myeloid cell recruitment in anti-IFNγ treated animals (Figure 5a). Further analysis of Ly6C and Ly6G expression within the $CD11b^{High}$ $CD11c^{Low}$ myeloid cell population revealed equivalent levels of $Ly6C^{High}$ $Ly6G^{Mid}$ monocyte and $Ly6C^{Mid}$ $Ly6G^{High}$ neutrophil recruitment following anti-IFNγ treatment (Figure 5b). Taken together, these data suggest that the recruitment of inflammatory myeloid cells,

including Ly6C^{High} Ly6G^{Mid} monocytes and Ly6C^{Mid} Ly6G^{High} neutrophils is independent of IFNγ signaling during acute *C. difficile* colitis.

Colonic inflammatory cytokine and chemokine expression following anti-IFNγ treatment was also examined using RT-PCR. Anti-IFNγ treatment was associated with significantly reduced expression of the IFNγ-inducible chemokines *Cxcl9* and *Cxcl10* in response to *C. difficile* infection (Figure 6a). However, in agreement with the high level of monocyte and neutrophil recruitment seen in these animals, anti-IFNγ treatment did not ablate expression of *Ccl3*, *Cxcl1*, *Cxcl2*, *Ccl2*, or *Ccl4* (Figure 6b,c). Consistently, the expression of inflammatory cytokines including *Il6* was not reduced in anti-IFNγ treated animals (Figure 6d). Additionally, we observed no change in *C. difficile* burden or reduction in the severity of colonic histopathology following ablation of IFNγ (see Supporting information, Figures S2 and S3).

Furthermore, induction of *Arg1* and *Slpi* was also unchanged following anti-IFNγ treatment (Figure 6e). Surprisingly, *Inos* expression was independent of anti-IFNγ treatment as well (Figure 6e). These data indicate that IFNγ is not a major driver of inflammatory cytokine and chemokine expression within the colon during acute *C. difficile* infection.

Discussion

In the current study, we reported a significant reduction in both CD11b^{High} CD11c^{Low} myeloid cell and Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the colon, as well as significant defects in the induction of the monocyte chemokines *Ccl4* and *Ccl7*, within the colonic mucosa of IL-23KO mice. Additionally, we observed significantly reduced monocyte recruitment in CCR2 deficient mice infected with *C. difficile*. Despite the drastic reduction in monocyte recruitment, the induction of inflammatory cytokines and chemokines including *Il1b*, *Il6*, *Cxcl1*, and *Cxcl2* were not significantly altered in CCR2 deficient animals. Furthermore, CCR2KO mice were not protected from the development of severe intestinal histopathology during *C.*

difficile colitis. Collectively, these data strongly suggest that IL-23 signaling promotes Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the colon in response to *C. difficile* colitis, but that the monocytes themselves are not major drivers of inflammatory cytokine expression or intestinal histopathology during *C. difficile* colitis.

The reduced Ly6C^{High} Ly6G^{Mid} monocyte influx seen in CCR2KO mice infected with *C. difficile* was associated with significantly reduced expression of *Inos* and *Slpi* in the colon. iNOS production by CD11b^{High} monocyte/macrophages has been previously reported in the lungs following LPS-mediated pulmonary inflammation[37]. Ly6C^{High} monocytes recruited to the spleen following *L. monocytogenes* infection also produce iNOS[29]. Furthermore, several *in vitro* studies have reported Slpi induction in macrophages following stimulation with microbial products including LPS[38] and heat-killed *Mycobacterium tuberculosis*[39]. Consistent with these *in vitro* studies we observed decreased *Inos* and *Slpi* expression in CRR2-deficient mice infected with *C. difficile*. However, *Inos* and *Slpi* expression were not affected by anti-IFNγ treatment. These data suggest that Ly6C^{High} Ly6G^{Mid} monocytes are a major source of IFNγ-independent *Inos* and *Slpi* expression in the colon in response to *C. difficile* colitis.

We observed no reduction in inflammatory cytokine or chemokine expression in CCR2KO mice during *C. difficile* colitis, although Ly6C^{High} Ly6G^{Mid} monocyte recruitment was significantly reduced in these animals. Reduction of macrophage/monocyte recruitment during bleomycin-mediated pulmonary inflammation is associated with reduced TNFα production in the lungs[6]. Likewise, TNFα^{+} monocyte/macrophages are recruited to the colon in a CCR2-dependent manner during DSS colitis[7]. Furthermore, a recent study has suggested that Ly6C^{High} monocytes are largely responsible for colonic IL-6, IL-1β, and IFNγ production during DSS colitis[9]. Specific to *C. difficile*, stimulation of monocytes with *C. difficile* toxins *in vitro* results in rapid production of inflammatory cytokines including IL-8, IL-1β, and TNFα[40, 41]. However, in the current study there was no reduction in inflammatory cytokine expression in CCR2-deficient mice infected with *C. difficile*,

suggesting that Ly6C^{High} Ly6G^{Mid} monocytes are not a major source of inflammatory cytokines expression in the colon during acute *C. difficile* colitis.

Despite significantly reduced levels of Ly6C^{High} Ly6G^{Mid} monocyte influx, CCR2KO mice were not protected against the development of severe colonic inflammation and histopathology in response to *C. difficile* colitis. Numerous studies have reported reduced intestinal histopathology during DSS colitis following interference with CCR2-dependent monocyte influx[8, 9]. Furthermore, robust monocyte/macrophage recruitment has been previously reported during *C. difficile* infection in association with marked intestinal histopathology[2-5, 10]. A previous study from our group also reported no decrease in the severity of intestinal histopathology following the depletion of both monocyte and neutrophil populations by anti-Gr-1 mAb treatment[4]. In the current study we report no protection from robust intestinal histopathology in CCR2KO mice. Thus, the data presented here suggest that Ly6C^{High} Ly6G^{Mid} monocytes are not major drivers of intestinal histopathology during acute *C. difficile* colitis.

We report no reduction in monocyte and neutrophil recruitment or decreased inflammatory cytokine expression following anti-IFNγ treatment. IFNγ drives neutrophil recruitment in response to insult at mucosal sites[19-21], and IFNγ^{+} neutrophil influx has recently been reported in response to *Salmonella typhimurium* typhlocolitis[22] as well as *C. difficile* colitis[15]. Specific to the colon, neutrophil recruitment and CCL2 production during DSS colitis are both dependent upon IFNγ[19]. Additionally, CXCL1 expression and neutrophil recruitment to the ileum were blunted in response to *C. difficile* toxin A in IFNγKO mice[21]. Furthermore, a recent study has highlighted the crucial role of type-1 ILCs for host survival during experimental *C. difficile* infection[24]. However, in the current study, we observed no defect in myeloid cell recruitment or expression of *Il6* and *Il17f* following anti-IFNγ treatment, suggesting that innate IFNγ protects the host from mortality through other mechanisms.

One potential interpretation of the data presented in the current study is that IFNγ is largely redundant in the host response to *C. difficile* colitis. However, a

recent study by Abt et al has reported a marked defect in the induction of IFNγ in association with decreased survival in mice lacking Tbet^{+} ILC1 cells following challenge with *C. difficile*[24]. Additionally, Rag$^{-/-}$ IFNγ$^{-/-}$ mice also demonstrated a large increase in mortality, strongly suggesting that innate IFNγ signaling is critical for host survival in response to *C. difficile* infection[24]. Collectively, these data suggest that IFNγ is protective during *C. difficile* colitis via mechanisms independent of inflammatory cytokine expression and granulocyte recruitment.

We observed significantly reduced CD11b^{High} CD11c^{Low} myeloid cell and Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the colon during *C. difficile* colitis in IL-23 deficient mice. IL-23 has been previously demonstrated to drive TNFα^{+} inflammatory monocyte recruitment to the spleen in response to *Listeria monocytogenes* infection[29]. Specific to colonic inflammation, IL-23 signaling promotes CD11b^{High} myeloid cell recruitment during chemically induced colitis[25]. We report significantly reduced Ly6C^{High} Ly6G^{Mid} monocyte recruitment and expression of the monocyte chemotactic factors *Ccl4* and *Ccl7* in IL-23-deficient mice during *C. difficile* colitis. Thus, our data strongly suggest that IL-23 signaling drives monocyte recruitment to the colon in response to *C. difficile* infection.

One model which explains our observations is that IL-23 promotes the expression of the monocyte chemokines *Ccl4* and *Ccl7*, but not *Ccl2*, contributing to the recruitment of Ly6C^{High} Ly6G^{Mid} monocytes to the colon. These recruited monocytes are not required for the induction of inflammatory cytokines and chemokines including *Cxcl1*, *Cxcl2*, and *Il1b* or the development of severe intestinal histopathology, but are required for full expression of *Slpi* and *Inos* within the colon. A previous study from our laboratory reported reduced neutrophil recruitment and inflammatory cytokine and chemokine expression in the absence of IL-23 signaling[26], while an additional study noted no decrease in the severity of intestinal histopathology or inflammatory cytokine expression following the concomitant depletion of monocytes and neutrophils[4]. Taken together, these studies strongly suggest that 1) IL-23 is required for the recruitment of numerous myeloid populations including monocytes and neutrophils during *C. difficile* colitis 2)

monocytes and neutrophils are not major drivers of intestinal histopathology or inflammatory cytokine expression during *C. difficile* infection and 3) the reduced intestinal histopathology and inflammatory cytokine production seen in IL-23KO mice is independent of the reduced neutrophil and monocyte recruitment in these animals. Thus, IL-23-responsive host cells other than recruited myeloid populations are the main sources of inflammatory cytokine expression in the colon during *C. difficile* colitis.

Recent studies have reported a protective role of innate IFNγ[24] and a therapeutic benefit for enhanced eosinophil recruitment[17] in response to *C. difficile* infection, strongly suggesting a protective role for Type 1 and Type 2 responses, respectively. The role for Type 17 responses, and specifically the role of the Type 17-promoting cytokine IL-23, remains more difficult to define. IL-23 has been implicated in driving severe intestinal histopathology and mortality during *C. difficile* infection[18, 26], suggesting that interference with IL-23 signaling would be of therapeutic benefit to patients with clinical *C. difficile* infection. However, neutrophil recruitment, a host response repeatedly demonstrated to be critical for survival during *C. difficile* infection[3, 10], is also significantly reduced in the absence of IL-23 signaling[26], As such, interference with IL-23 signaling may ultimately worsen disease by impairing protective neutrophil recruitment, in addition to increasing the susceptibility of the patient to other infections. Therefore, any immunomodulatory treatment for *C. difficile* infection, either a biologic or microbiota-based, should seek to diminish the pathological aspects of IL-23 signaling while still retaining robust neutrophil recruitment. Future studies will focus on better understanding the inflammatory cascades driven by IL-23, with the ultimate goals of identifying IL-23 dependent host signals which promote histopathology and disease development but not neutrophil recruitment.

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Author Contributions

AJM and GBH conceived, designed, and interpreted the experiments. VBY, NRF, and RAM contributed to their design and interpretation. AJM, NRF, RAM, CRF, and CRP performed the experiments. AJM, NRF, CRF, and GBH analyzed the data. AJM and GBH prepared the manuscript, and all other authors provided comments and advice on the manuscript.

Disclosures

The authors declare no financial conflicts of interest

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Figure Legends

Figure 1:

Colonic monocyte recruitment and colonic chemokine expression during *C. difficile* infection in IL-23 deficient mice. (a) Colonic chemokine expression as assessed by qPCR. $n \geq 6$ per group. Data are shown as fold change in gene expression in the indicated group as compared to untreated WT animals. Black bars: WT CDI, white bars: IL-23KO CDI. * $p<0.05$ for the difference between the indicated group and untreated WT animals.] Brackets $p<0.05$ for the differences in expression levels between the indicated groups. CDI = *C. difficile* infected. (b) Number of $CD11b^{High}$ $CD11c^{Low}$ and (c) Number of $Ly6C^{High}$ $Ly6G^{Mid}$ cells per 100,000 cells. Bars represent mean ± SEM number of the indicated cell type per 100,000 cells. n = 8 for all groups. * $p<0.05$ for the differences between the indicated groups.

Figure 2:

Flow cytometric analysis of colonic $CD45^{+}$ leukocytes from Untreated, WT CDI, and CCR2KO CDI mice. (a) Analysis of CD11b and CD11c expression profiles on isolated colonic $CD45^{+}$ leukocytes. (b) Analysis of Ly6C and Ly6G expression profiles on the $CD11b^{High}$ $CD11c^{Low}$ population as defined in panel (a). CDI = *C. difficile* infected. The

bolded number indicates the percentage of the parent population contained within the indicated gate.

Figure 3:
Quantification of colonic myeloid cell populations in from Untreated, WT CDI, and CCR2KO CDI mice as defined in Figure 2. (a) Number of $CD11b^{High}$ $CD11c^{Low}$ (b) $Ly6C^{High}$ $Ly6G^{Mid}$ and (c) $Ly6C^{Mid}$ $Ly6G^{High}$ cells per 100,000 cells. All populations were defined as shown in Figure 2. Bars represent mean ± SEM number of the indicated cell type per 100,000 cells. n ≥ 6 per group. CDI = *C. difficile* infected. * $p<0.05$ for the differences between the indicated groups.

Figure 4:
Inflammatory cytokine expression in WT and CCR2KO mice infected with *C. difficile*. Expression of inflammatory cytokine genes in the colon was assessed via qPCR as outlined in the methods. Data are shown as mean ± SEM fold change gene expression in the indicated group as compared to untreated WT animals. n ≥ 7 per group. CDI = *C. difficile* infected. Black bars: WT CDI, gray bars: CCR2KO CDI. Brackets $p<0.05$ for the differences in expression levels between the indicated groups. * $p<0.05$ for the difference between the indicated group and untreated WT animals.

Figure 5:
Flow cytometric analysis of colonic $CD45^+$ leukocytes from WT CDI, and CDI + anti-IFNγ treated mice. (a) Analysis of CD11b and CD11c expression profiles on isolated colonic $CD45^+$ leukocytes. (b) Analysis of Ly6C and Ly6G expression profiles on the $CD11b^{High}$ $CD11c^{Low}$ population as defined in panel (a). CDI = *C. difficile* infected. The bolded number indicates the percentage of the parent population contained within the indicated gate.

Figure 6:

Effect of anti-IFNγ treatment on colonic inflammatory cytokine and chemokine expression. Data are shown as mean ± SEM fold change gene expression in the indicated group as compared to untreated WT animals. n ≥ 4 per group. CDI = *C. difficile* infected. Black bars: WT CDI, light gray bars: CDI+ anti-IFNγ. Brackets $p<0.05$ for the differences in expression levels between the indicated groups. * $p<0.05$ for the difference between the indicated group and untreated WT animals.

Figure 1S:

Gating strategy for flow cytometric analysis of colonic leukocytes. Results from a representative WT mouse infected with *C. difficile* are shown. (a) Total colonic leukocytes were identified as $CD45^+$ events. (b) Inflammatory myeloid cells were defined as $CD11b^{High}$ $CD11c^{Low}$ cells within the $CD45^+$ gate as defined in panel a. (c) Monocytes were defined as $Ly6C^{High}$ $Ly6G^{Low}$ cells and neutrophils were defined as $Ly6C^{Mid}$ $Ly6G^{High}$ cells within the $CD11b^{High}$ $CD11c^{Low}$ gate defined in panel b. The bolded numbers indicate percent of parent population within the indicated gate.

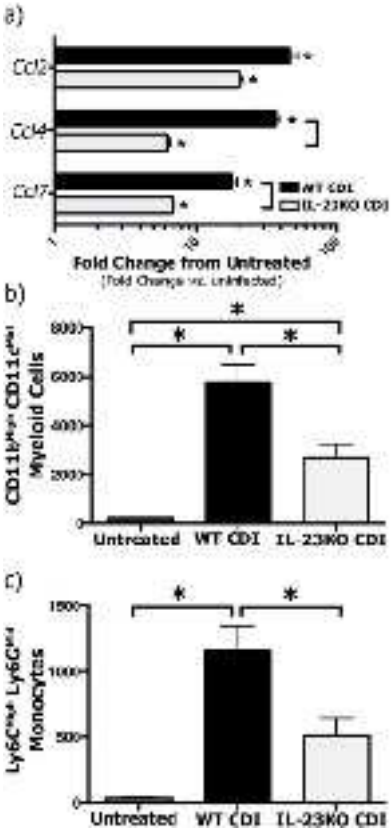
Figure 2S:

Colonic mucosal *C. difficile* colonization as determined by *C. difficile*-specific qPCR (2 days post-infection). n ≥ 6 for all groups. Data are shown as mean ± SEM number of *C. difficile* genomes per gram of host tissue. * $p<0.05$ for the difference between the indicated group and untreated WT animals. There were no statistically significant differences in *C. difficile* colonization levels between infected groups.

Figure 3S:

Photomicrographs of representative Hematoxylin and eosin-stained colonic sections from *C. difficile* infected WT (left column), *C. difficile* infected CCR2KO (center column), and *C. difficile* infected mice treated with anti-IFNγ. Upper images are cross-section of colonic crypts, while lower images are of the epithelial-luminal interface. Black arrowheads highlight inflammatory infiltrate, which grey arrowheads highlight epithelial damage. Total magnification for all images: 400X.

McDermott et al Figure 1



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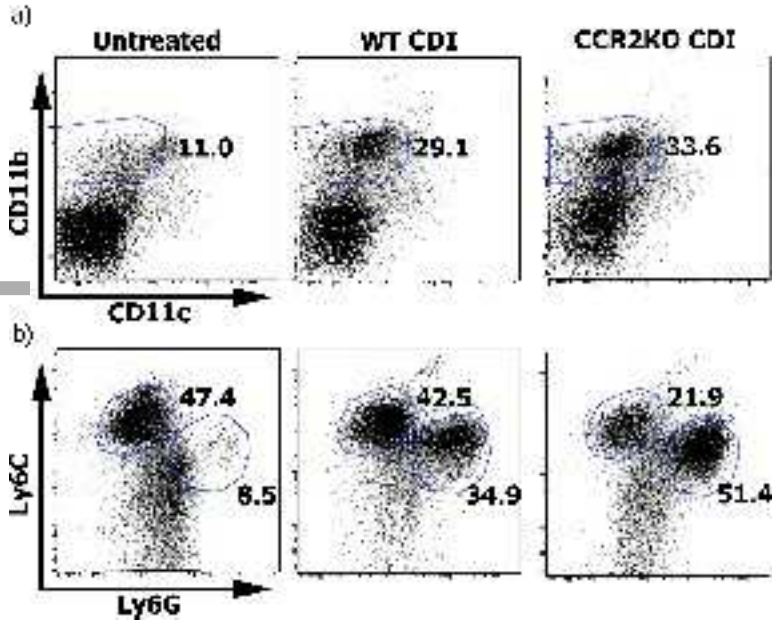
McDermott et al -igure 2



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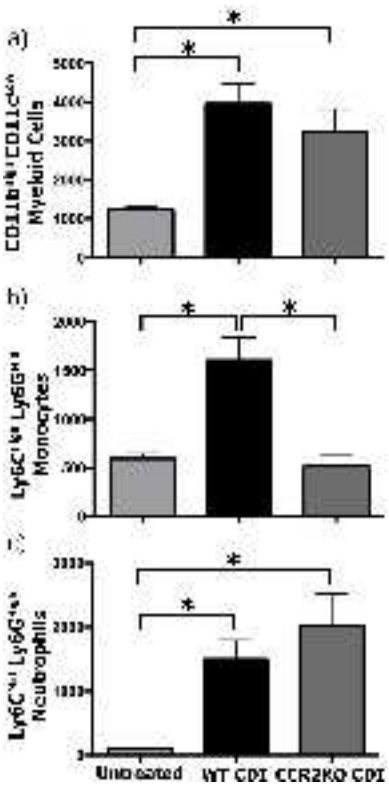
McDermott et al Figure 3



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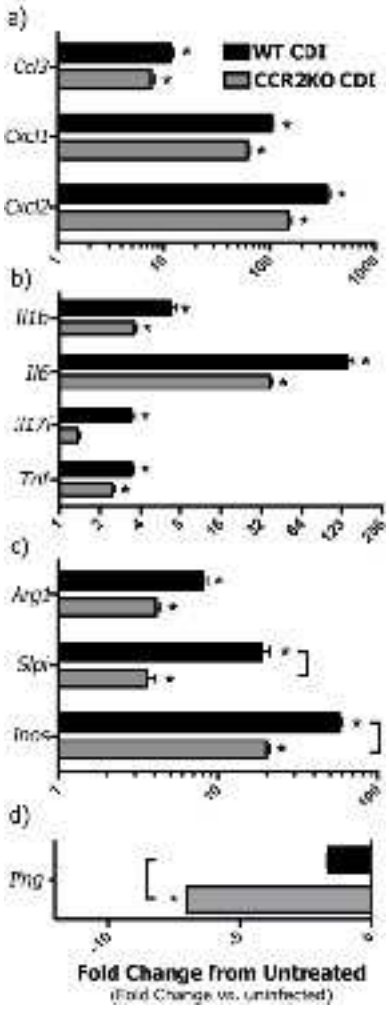
McDermott et al Figure 4



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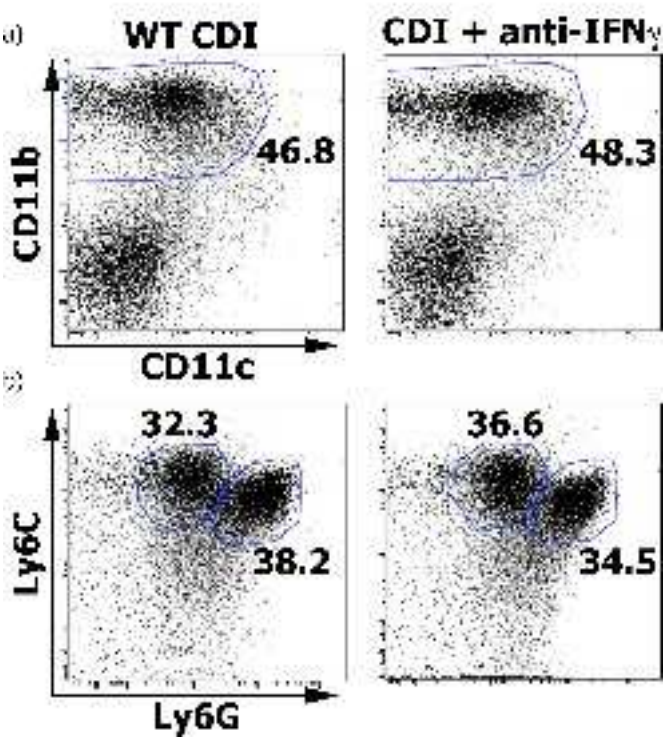
McDermott et al Figure 5



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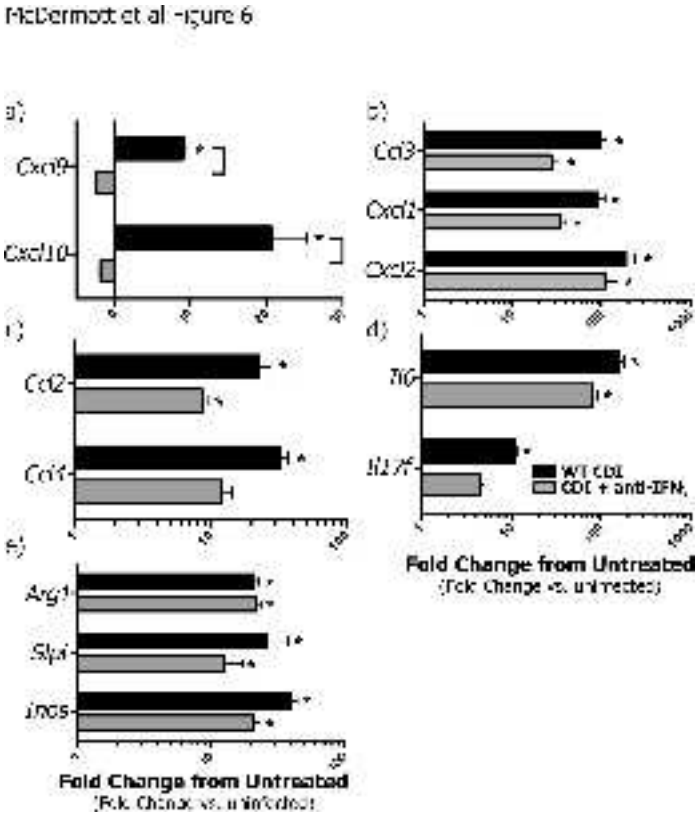

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