IMMUNOLOGY ORIGINAL ARTICLE

Role of interferon-γ and inflammatory monocytes in driving colonic inflammation during acute *Clostridium 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-γ (IFN-γ) and the recruitment of myeloid cells including Ly6C^{High} monocytes. IL-23 knockout 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. *Clostridium difficile*-infected CCR2$^{-/-}$ (CCR2 KO) mice showed a significant defect in Ly6C^{High} Ly6G^{Mid} monocyte recruitment to the colon in response to *C. difficile*. Although 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 IFN-γ through the administration of anti-IFN-γ monoclonal antibody resulted in a significant reduction in the expression of the IFN-γ-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-IFN-γ treatment. Additionally, *Inos* and *Slpi* expression were unchanged following anti-IFN-γ treatment, suggesting that *Inos* and *Slpi* regulation is independent of IFN-γ during *C. difficile* colitis. Taken together, these data strongly suggest that IL-23 and CCR2 signalling are required for monocyte recruitment during *C. difficile* colitis. Additionally, these studies also suggest that monocytes, but not IFN-γ, are necessary for full expression of *Inos* and *Slpi* in the colon.

Keywords: bacterial; cytokines; inflammation; mucosa.

Introduction

Monocyte-derived cells are key mediators of inflammatory responses in the gastrointestinal 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 tumour necrosis factor-α (TNF-α) production during both pulmonary and colonic inflammation.[6,7] Monocyte recruitment is also required for full production of inflammatory cytokines including interleukin-1β (IL-1β) and IL-6, during dextran sulphate sodium (DSS) colitis.[9] Furthermore, the development of intestinal histopathology during DSS colitis is partially dependent on monocyte recruitment.[8,9]

Clostridium 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 signalling 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] Studies suggest that monocyte recruitment to the large intestine is not required for host survival during *C. difficile* colitis,[10] but 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, remain poorly understood.

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

Interleukin-23 is a known driver of innate inflammation at mucosal sites.[25,26] It 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 signalling.[25] Work by Buonomo *et al.* 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-23 KO 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 IFN-γ in mediating host protection during *C. difficile* infection,[24] but the role of IFN-γ in driving innate inflammatory responses to *C. difficile* colitis remains poorly understood. As such, we investigated the role of IFN-γ signalling 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 IFN-γ 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) mice 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. difficile* 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 culture (2 ml) was then used to inoculate 40 ml of Clospore[30] sporulation medium, and the culture was then allowed to grow for 7 days at 37° anaerobically. Spores were recovered by washing the resulting pellet at least four times to remove residual vegetative cells. Stocks were stored at 4° 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 to 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, St Louis, MO) at a concentration of 0·5 g/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 before infection with *C. difficile*. Untreated animals received neither *C. difficile* challenge nor antibiotic pretreatment.

For *C. difficile* infection studies, mice received approximately 10^6 colony-forming units 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 IFN-γ *in vivo*, mice were given 500 μg of anti-IFN-γ monoclonal antibody (clone XMG.1.2) via intraperitoneal injection 1 day before and 1 day after infection (Day –1 and Day 1, respectively).

Colonic leucocyte isolation

Leucocytes 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 20 ml of Hanks' balanced salt solution (HBSS) supplemented with 1 mM dithiothreitol, 5 mM EDTA, and 2·5% fetal calf serum for 20 min at 37°. Tissue was then washed, and subsequently incubated with 20 ml of HBSS supplemented with 0·5 mg/ml DNase (Roche, Basel, Switzerland), 400 U/ml collagenase type 3 (Worthington Biochemicals, Lakewood, NJ), and 2·5% fetal calf serum for 1 hr at 37°. After washing, samples were then resuspended in 20% Percol (Sigma) in PBS and spun at 900 *g* for 30 min 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 unlabelled FcRIII/II. After blocking, cells were stained with fluorescently labelled antibodies for 30 min at 4°. Cells were washed, and resuspended in stabilizing fixative (BD Biosciences, Franklin Lakes, NJ). All samples were acquired on a three-laser FACSCanto II using FACS-DIVA software (BD Biosciences, Franklin Lakes, NJ). All data analysis was performed in FLOWJO (Treestar, Ashland, OR). 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 (San Diego, CA).

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 leucocyte subsets is also highlighted in the Supplementary material (Fig. 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 hr and then transferred to 70% ethanol. Cassettes were processed, paraffin-embedded, sectioned and used to prepare haematoxylin and eosin stained slides by McClinchey Histology Lab. Inc. (Stockbridge, MI).

Representative photomicrographs were acquired using an Olympus BX40 light microscope (Olympus corporation, Tokyo, Japan) using a QImaging MicroPublisher RTV 5.0 5 megapixel camera. All photomicrographs were acquired at a total magnification of 400×. QCAPTURE SUITE PLUS version 3.1.3.10 (QImaging , Surrey, BC, Canada) was used for image acquisition. All panels were assembled in ADOBE PHOTOSHOP CS5, version 12.0 (Adobe Systems Incorporated, San Jose, CA 95110-2704). 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 was assessed as described previously.[4,5,11,23,26] Briefly, samples of colonic tissue approximately 1 cm^2 were excised from the midpoint of the colon and stored in

RNAlater (Ambion, Austin, TX) for further analysis. To isolate RNA, samples were homogenized in TRIzol reagent (Life Technologies, Grand Island, NY), and RNA was 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, Waltham, MA) and Agilent Bioanalyzer (Agilent, Santa Clara, CA), respectively. RNA was converted to cDNA using the RT² first strand kit (Qiagen, Hilden, Germany) and colonic gene expression was assessed using RT² Profiler PCR Assays (Qiagen). All PCR were run on a Roche LightCycler 480. For RT² 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 two 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 analysis of variance (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

To investigate the role of IL-23 in promoting monocyte recruitment during *C. difficile* colitis, wild-type (WT) and IL-23KO mice were infected with *C. difficile* as described previously.[26] Briefly, mice were given 0·5 g/l cefoperazone in their drinking water for 5 days and after a 2-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 2 days, at which point the infection was terminated and all samples were collected.

RT-PCR analysis was used 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 (Fig. 1a). The absence of IL-23 did not affect the *C. difficile* burden within the colon (see Supplementary material,

Fig. S2). Flow cytometric analysis of colonic CD45$^+$ leucocytes revealed a significant reduction in the frequency of CD11b^{High} CD11c^{Low} myeloid cells recruited to the colon in IL-23KO mice compared with WT animals (Fig. 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 (Fig. 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.
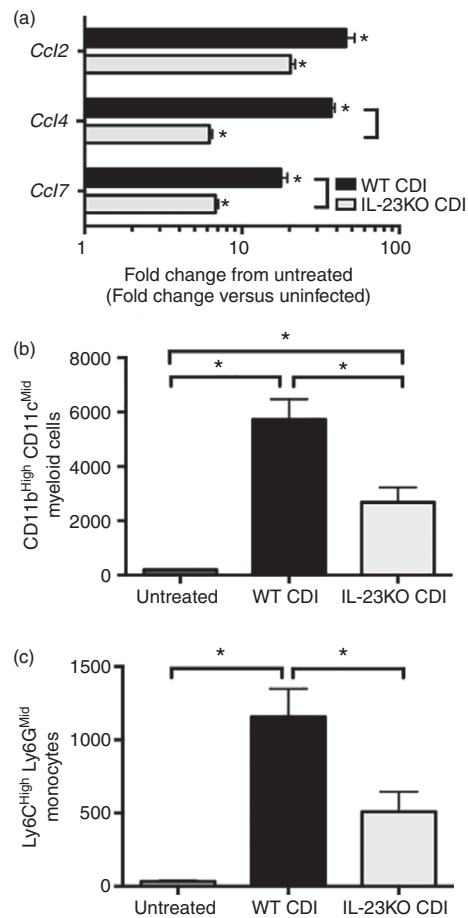

Figure 1. Colonic monocyte recruitment and colonic chemokine expression during *Clostridium difficile* infection in interleukin-23 (IL-23) -deficient mice. (a) Colonic chemokine expression as assessed by quantitative PCR. $n \geq 6$ per group. Data are shown as fold change in gene expression in the indicated group compared with untreated wild-type (WT) animals. Black bars: WT *C. difficile*-infected (CDI), white bars: IL-23 knockout (KO) CDI. *$P < 0.05$ for the difference between the indicated group and untreated WT animals. Square brackets indicate $P < 0.05$ for the differences in expression levels between the indicated groups. (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.

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 Materials and methods. *Clostridium difficile* infection in WT animals was associated with significant recruitment of CD11b^{High} CD11c^{Low} myeloid cells (Fig. 2a centre panel and Fig. 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 (Fig. 2b centre panel and Fig. 3b,c). Whereas neither CD11b^{High} CD11c^{Low} myeloid cell nor Ly6C^{Mid} Ly6G^{High} neutrophil recruitment were significantly altered in CCR2KO mice (Fig. 2a,b right column and Fig. 3a,c), Ly6C^{High} Ly6G^{Mid} monocyte recruitment was significantly abrogated in CCR2KO mice infected with *C. difficile* (Fig. 2b right panel, Fig. 3b). There was no difference in the frequency of total CD45$^+$ leucocytes 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 signalling.

Effect of CCR2 deficiency on inflammatory cytokine and chemokine expression

RT-PCR analysis was used 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 (Figs 2b and 3c), *C. difficile* infection in WT mice was associated with increased expression of the neutrophil chemokines *Ccl3*, *Cxcl1* and *Cxcl2* (Fig. 4a). Expression of the inflammatory cytokines *Il1b*, *Il6*, *Il17f* and *Tnf* were all significantly increased in WT *C. difficile* infected animals (Fig. 4b). *Arg1*, *Slpi* and *Inos* were also significantly induced during *C. difficile* colitis (Fig. 4c).

CCR2 deficiency did not result in any defects in the induction of the myeloid cell chemokines *Ccl3*, *Cxcl1* or *Cxcl2*, (Fig. 4a). Furthermore, CCR2-deficient animals displayed no reduction in *Il1b, Il6, Tnf* or *Arg1* expression (Fig. 4b,c). The expression levels of *Slpi* and *Inos*, however, were significantly reduced in CCR2KO mice compared with WT animals infected with *C. difficile* (Fig. 4c). Consistent with the gene expression, we observed no reduction in the severity of colonic histopathology (see Supplementary material, Fig. S3, left and middle columns) or levels of *C. difficile* colonization in CCR2KO animals (see Supplementary material, Fig. 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 (Fig. 4d). Collectively, these data suggest that while CCR2 signalling 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 signalling during *C. difficile* colitis.

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

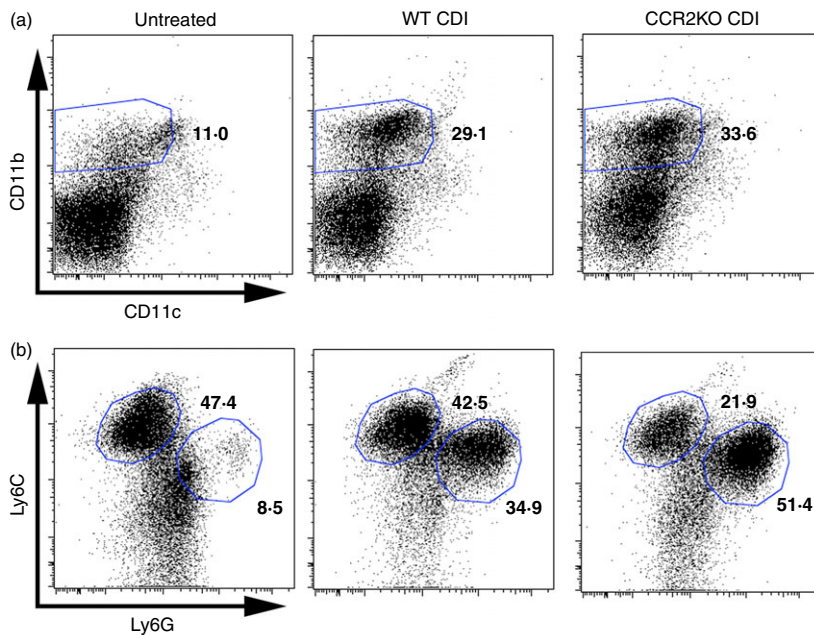To investigate the role of IFN-γ in promoting monocyte and neutrophil recruitment, as well as inflammatory

Figure 2. Flow cytometric analysis of colonic CD45$^+$ leucocytes from untreated, wild-type *Clostridium difficile*-infected (WT CDI), and CCR2 knockout (KO) CDI mice. (a) Analysis of CD11b and CD11c expression profiles on isolated colonic CD45$^+$ leucocytes. (b) Analysis of Ly6C and Ly6G expression profiles on the CD11b^{High} CD11c^{Low} population as defined in (a). The number in bold indicates the percentage of the parent population contained within the indicated gate. [Colour figure can be viewed at wileyonlinelibrary.com]
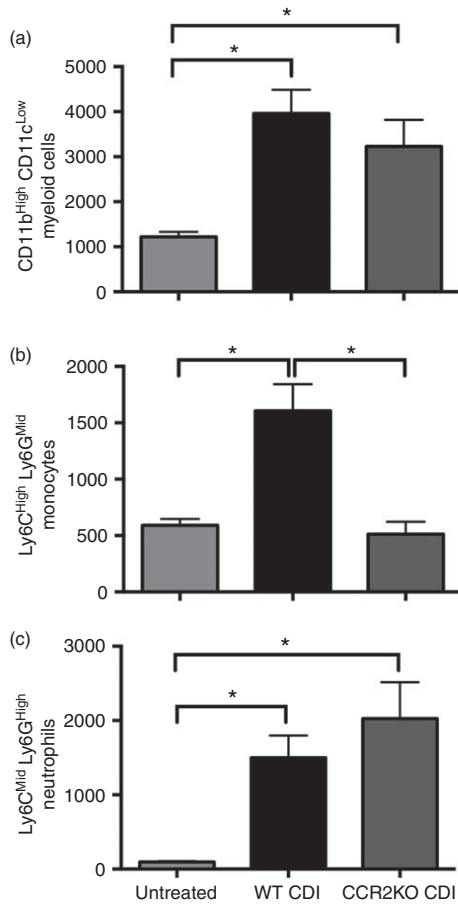
Figure 3. Quantification of colonic myeloid cell populations from Untreated, wild-type *Clostridium difficile*-infected (WT CDI), and CCR2 knockout (KO) CDI mice as defined in Fig. 2. (a) Number of CD11b^{High} CD11c^{Low}, (b) Ly6C^{High} Ly6G^{Mid} and (c) Ly6C^{Mid} Ly6G^{High} cells per 100 000 cells. All populations were defined as shown in Fig. 2. Bars represent mean ± SEM number of the indicated cell type per 100 000 cells. $n \geq 6$ per group. CDI = *C. difficile*-infected. *$P < 0.05$ for the differences between the indicated groups.

cytokine and chemokine expression during *C. difficile* colitis, animals were treated with anti-IFN-γ monoclonal antibody 1 day before and 1 day after infection. As in previous experiments, all samples were collected 2 days after infection.

Analysis of CD45$^+$ colonic leucocytes revealed no defect in CD11b^{High} CD11c^{Low} myeloid cell recruitment in anti-IFN-γ-treated animals (Fig. 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-IFN-γ treatment (Fig. 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 IFN-γ signalling during acute *C. difficile* colitis.
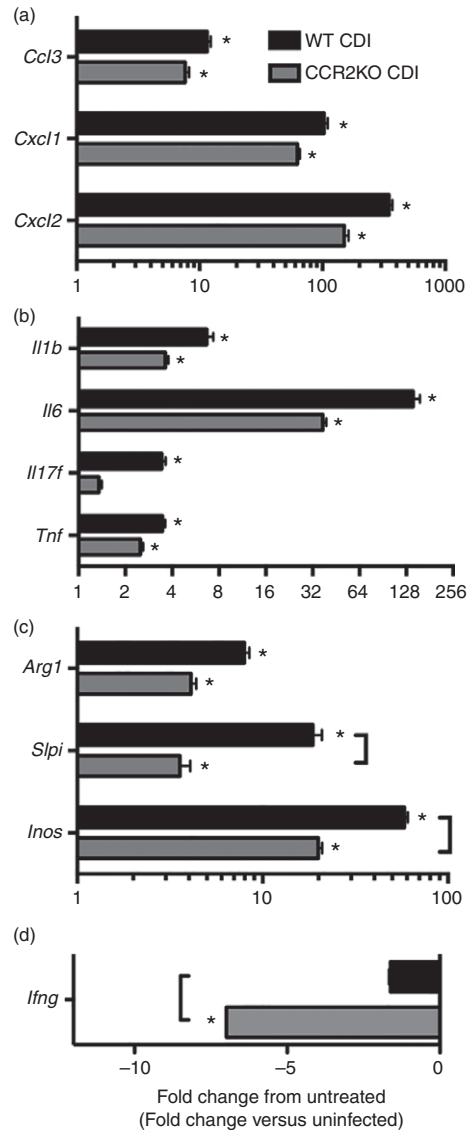

Figure 4. Inflammatory cytokine expression in wild-type (WT) and CCR2 knockout (KO) mice infected with *Clostridium difficile*. Expression of inflammatory cytokine genes in the colon was assessed via quantitative PCR as outlined in the Materials and methods. Data are shown as mean ± SEM fold change gene expression in the indicated group compared with untreated WT animals. $n \geq 7$ per group. Black bars: wild-type *C. difficile*-infected (WT CDI, grey 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.

Colonic inflammatory cytokine and chemokine expression following anti-IFN-γ treatment was also examined using RT-PCR. Anti-IFN-γ treatment was associated with significantly reduced expression of the IFN-γ-inducible chemokines *Cxcl9* and *Cxcl10* in response to *C. difficile* infection (Fig. 6a). However, in agreement with the high level of monocyte and neutrophil recruitment seen in these animals, anti-IFN-γ treatment did not ablate
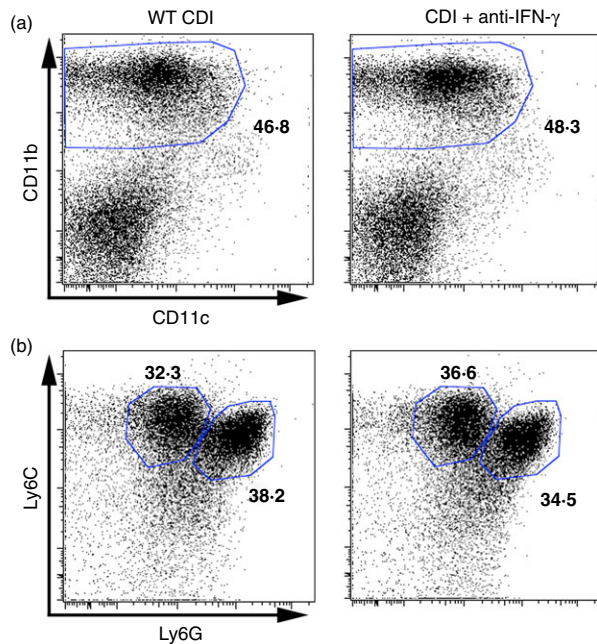
Figure 5. Flow cytometric analysis of colonic CD45$^+$ leucocytes from wild-type *Clostridium difficile*-infected (WT CDI), and CDI+ anti-interferon-γ (IFN-γ) -treated mice. (a) Analysis of CD11b and CD11c expression profiles on isolated colonic CD45$^+$ leucocytes. (b) Analysis of Ly6C and Ly6G expression profiles on the CD11b^{High} CD11c^{Low} population as defined in (a). The number in bold indicates the percentage of the parent population contained within the indicated gate. [Colour figure can be viewed at wileyonlinelibrary.com]

expression of *Ccl3*, *Cxcl1*, *Cxcl2*, *Ccl2* or *Ccl4* (Fig. 6b,c). Consistently, the expression of inflammatory cytokines including *Il6* was not reduced in anti-IFN-γ-treated animals (Fig. 6d). Additionally, we observed no change in *C. difficile* burden or reduction in the severity of colonic histopathology following ablation of IFN-γ (see Supplementary material, Figs S2 and S3).

Furthermore, induction of *Arg1* and *Slpi* was also unchanged following anti-IFN-γ treatment (Fig. 6e). Surprisingly, *Inos* expression was independent of anti-IFN-γ treatment as well (Fig. 6e). These data indicate that IFN-γ 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 signalling 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. Inducible nitric oxide synthase production by CD11b^{High} monocyte/macrophages has been previously reported in the lungs following lipopolysaccharide-mediated pulmonary inflammation.[37] Ly6C^{High} monocytes recruited to the spleen following *L. monocytogenes* infection also produce inducible nitric oxide synthase.[29] Furthermore, several *in vitro* studies have reported Slpi induction in macrophages following stimulation with microbial products including lipopolysaccharide[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-IFN-γ treatment. These data suggest that Ly6C^{High} Ly6G^{Mid} monocytes are a major source of IFN-γ-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 TNF-α production in the lungs.[6] Likewise, TNF-α^+ 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 IFN-γ 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 TNF-α.[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 cytokine 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
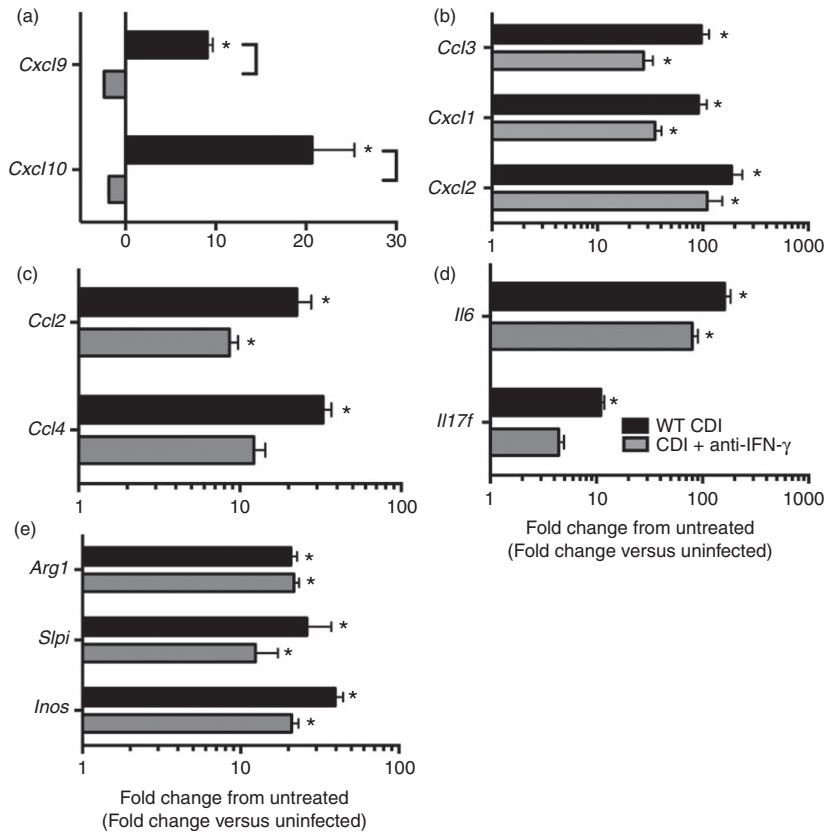
Figure 6. Effect of anti-interferon-γ (IFN-γ) treatment on colonic inflammatory cytokine and chemokine expression. Data are shown as mean ± SEM fold change gene expression in the indicated group compared with untreated wild-type (WT) animals. $n \geq 4$ per group. CDI = *Clostridium difficile*-infected. Black bars: WT CDI, light grey bars: CDI+ anti-IFN-γ. 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.

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 monoclonal antibody treatment.[4] In the current study we report no protection from robust intestinal histopathology in CCR2KO mice. Hence, 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-IFN-γ treatment. IFN-γ drives neutrophil recruitment in response to insult at mucosal sites,[19–21] and IFN-γ^+ 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 IFN-γ.[19] Additionally, CXCL1 expression and neutrophil recruitment to the ileum were blunted in response to *C. difficile* toxin A

in IFN-γKO mice.[21] Furthermore, a recent study has highlighted the crucial role of type 1 innate lymphoid cells 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-IFN-γ treatment, suggesting that innate IFN-γ protects the host from mortality through other mechanisms.

One potential interpretation of the data presented in the current study is that IFN-γ 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 IFN-γ in association with decreased survival in mice lacking Tbet^+ type 1 innate lymphoid cells cells following challenge with *C. difficile*.[24] Additionally, Rag^−/− IFN-γ^−/− mice also demonstrated a large increase in mortality, strongly suggesting that innate IFN-γ signalling is critical for host survival in response to *C. difficile* infection.[24] Collectively, these data suggest that IFN-γ 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. Interleukin-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 signalling 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. Hence, our data strongly suggest that IL-23 signalling 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 signalling,[26] whereas 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 (i) IL-23 is required for the recruitment of numerous myeloid populations including monocytes and neutrophils during *C. difficile* colitis, (ii) monocytes and neutrophils are not major drivers of intestinal histopathology or inflammatory cytokine expression during *C. difficile* infection, and (iii) 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. Hence, 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 IFN-γ[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. Interleukin-23 has been implicated in driving severe intestinal histopathology and mortality during *C. difficile* infection,[18,26] suggesting that interference with IL-23 signalling 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 signalling.[26] As such, interference with IL-23 signalling 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 biologically or microbiota-based method, should seek to diminish the pathological aspects of IL-23 signalling 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 that promote histopathology and disease development but not neutrophil recruitment.

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 analysed 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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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gating strategy for flow cytometric analysis of colonic leucocytes.

Figure S2. Colonic mucosal *Clostridium difficile* colonization as determined by *C. difficile*-specific quantitative PCR (2 days post-infection).

Figure S3. Photomicrographs of representative haematoxylin and eosin-stained colonic sections from *Clostridium difficile*-infected wild-type (WT) (left column), *C. difficile* infected CCR2 knockout (KO; centre column), and *C. difficile*-infected mice treated with anti-interferon-γ.