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The role of Gr-1$^+$ cells and tumour necrosis factor-α signalling during *Clostridium difficile* colitis in mice

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Summary

The host response to *Clostridium difficile* infection in antibiotic-treated mice is characterized by robust recruitment of Gr-1$^+$ cells, increased expression of inflammatory cytokines including tumour necrosis factor-α (TNF-α), and the development of severe epithelial damage. To investigate the role of Gr-1$^+$ cells and TNF-α during *C. difficile* colitis, we treated infected mice with monoclonal antibodies against Gr-1 or TNF-α. Mice were challenged with vegetative cells of *C. difficile* strain VPI 10463 following treatment with the third-generation cephalosporin ceftriaxone. Ceftriaxone treatment alone was associated with significant changes in cytokine expression within the colonic mucosa but not overt inflammatory histopathological changes. In comparison, *C. difficile* infection following ceftriaxone treatment was associated with increased expression of inflammatory cytokines and chemokines including *Cxcl1*, *Cxcl2*, *Il1b*, *Il17f* and *Tnfa*, as well as robust recruitment of Ly6C^{Mid} Gr-1^{High} neutrophils and Ly6C^{High} Gr-1^{Mid} monocytes and the development of severe colonic histopathology. Anti-Gr-1 antibody treatment resulted in effective depletion of both Ly6C^{Mid} Gr-1^{High} neutrophils and Ly6C^{High} Gr-1^{Mid} monocytes: however, we observed no protection from the development of severe pathology or reduction in expression of the pro-inflammatory cytokines *Il1b*, *Il6*, *Il33* and *Tnfa* following anti-Gr-1 treatment. By contrast, anti-TNF-α treatment did not affect Gr-1$^+$ cell recruitment, but was associated with increased expression of *Il6* and *Il1b*. Additionally, *Ffar2*, *Ffar3*, *Tslp*, *Tff* and *Ang4* expression was significantly reduced in anti-TNF-α-treated animals, in association with marked intestinal histopathology. These studies raise the possibility that TNF-α may play a role in restraining inflammation and protecting the epithelium during *C. difficile* infection.

Keywords: colitis; epithelium; microbiome; mucosal inflammation; neutrophil

Introduction

The inflammatory signals that support leucocyte recruitment, intestinal histopathology and inflammatory cytokine production during *Clostridium difficile* infection are still not completely understood. Recent studies have identified key roles for Myeloid Differentiation Primary Response 88, Nucleotide Binding Oligomerization Domain 1, and Caspase Recruitment Domain Containing Protein in promoting neutrophil recruitment and the production of inflammatory cytokines in response to *C. difficile* infection.[1–3] Additionally, signalling of Toll-like

Abbreviations: GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor; TNF-α, Tumor Necrosis Factor Alpha; IL, Interleukin; IFNγ, Interferon Gamma; CCL, Chemokine (C-C Motif) Ligand; CXCL, Chemokine (C-X-C Motif) Ligand; CSF, Colony Stimulating Factor; MIF, Macrophage Migration Inhibitory Factor; iNOS, Inducible Nitric Oxide Synthase (NOS2); RegIIIγ, Regenerating Islet-Derived 3 Gamma; SLPI, Secretory leukocyte peptidase inhibitor; Tgfβ1, Transforming Growth Factor beta 1; Ang4, Angiogenin 4; TCCFA, Taurocholate Cycloserine Cefoxitin Fructose Agar; TNBS, 2,4,6-trinitrobenzenesulfonic acid; BHIS, Brain Heart Infusion Supplemented with 0.1% Cysteine; SSC, Side Scatter; FSC, Forward Scatter

receptors 4 and 5 has been shown to prevent the development of severe intestinal histopathology during *C. difficile* infection.[4,5] However, the role of tumour necrosis factor-α (TNF-α) in promoting epithelial damage, leucocyte recruitment and inflammatory cytokine expression during *C. difficile* infection has yet to be investigated.

Antibiotic pre-treatment is required to infect conventional mice with *C. difficile*.[1–4,6–11] However, antibiotic treatment is also capable of modulating inflammatory responses and immune cell function.[12–16] *In vitro* studies have demonstrated reduced expression of inflammatory mediators from monocytes stimulated with live fungal[15] or heat-killed bacterial[13] cells following moxifloxacin and co-trimoxazole treatment, respectively. Macrolide antibiotics can also affect inflammatory functions of pulmonary epithelial cells and modulate TNF-α, interleukin-8 (IL-8) and Granulocyte-macrophage colony stimulating factor production by these cells.[16–19] Ceftriaxone is a third-generation cephalosporin with bactericidal activity against both Gram-positive and Gram-negative bacteria.[20–22] Cefoperazone, another third-generation cephalosporin, can markedly alter the composition of the intestinal microbiota and render mice susceptible to *C. difficile* infection.[8,10] In the current study, we investigated the ability of ceftriaxone to permit *C. difficile* infection, and whether ceftriaxone treatment alone was sufficient to induce colonic inflammation.

Tumour necrosis factor-α promotes leucocyte recruitment and the expression of inflammatory cytokines during mucosal inflammation.[23–25] TNF-α expression is significantly increased during acute *C. difficile* colitis,[9] and macrophage TNF-α production is also enhanced by exposure to *C. difficile* toxins.[26] Gr-1 is an epitope found on both Ly6C and Ly6G, and is expressed on neutrophils, inflammatory monocytes, and plasmacytoid dendritic cells.[9,27–29] Gr-1$^+$ cells are recruited in large numbers to the large intestine in response to *C. difficile* infection,[1–3,9] and protect against bacterial dissemination and mortality.[2,3] However, much remains unknown about the contributions of Gr-1$^+$ cells and TNF-α in promoting intestinal histopathology, leucocyte recruitment, and the expression of inflammatory cytokines during *C. difficile* colitis.

Materials and methods

Bacterial culture and growth conditions

Clostridium difficile was prepared for infection as described previously.[8,10] Briefly, an overnight culture of *C. difficile* strain VPI 10463 (ATCC 43255) was back-diluted 1 : 10 in fresh brain–heart infusion broth supplemented with 0.1% cysteine and grown for 4–6 hr. The culture was then collected, washed three times in deoxygenated PBS, and diluted to the desired dose. Mice were challenged via oral gavage. The inoculum was serially diluted and plated on brain–heart infusion supplemented with 0.1% cysteine to confirm dosage. *Clostridium difficile* was grown and prepared for gavage in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI).

Animals and housing

C57BL/6 male mice aged 5–9 weeks at the time of antibiotic pre-treatment were used in the current study. All experiments were conducted under a protocol approved by the University Committee on Use and Care of Animals at the University of Michigan. All mice were purchased directly from Jackson Laboratories (Bar Harbor, ME) or obtained from an in-house colony founded by Jackson breeders. Mice were housed with autoclaved bedding, food and water. All animal manipulations were carried out in a laminar flow hood.

Antibiotic treatment and infection

For ceftriaxone and *C. difficile* infection studies, mice were treated with ceftriaxone (0·5 g/l) (Sigma, St Louis, MO) given *ad libitum* in their drinking water for 4 days. Antibiotic water was replaced every other day. Mice were then given a 2-day recovery period on drinking water without antibiotic before infection with *C. difficile* as described previously.[8,10] Ceftriaxone-treated mice were given the antibiotic regimen only, and untreated animals were not manipulated at all.

For *C. difficile* infection studies, mice received $5{\cdot}06 \pm 0{\cdot}31$ Log_{10} colony-forming units vegetative *C. difficile* via oral gavage on day 2 (Fig. 1). *Clostridium difficile*-infected animals were monitored for signs of severe disease (hunched posture, lethargy, weight loss exceeding 20% of baseline body weight) and were humanely killed if moribund. All surviving animals were killed on day 4 for subsequent analysis (Fig. 1).

Antibody administration

Mice were given intraperitoneal injections of 250 μg of anti-TNF-α monoclonal antibody (mAb; clone MP6-XT3) 1 day before infection with *C. difficile* or injections of 250 μg of anti-Gr-1 mAb (clone RB6-8C5) 1 day before and 1 day after infection. Mouse serum (Sigma) injections were administered to control mice.

Histology

Colonic tissue was collected from the midpoint of the colon and either flash-frozen in liquid nitrogen for subsequent DNA extraction or stored in RNAlater (Life Technologies, Grand Island, NY) for RNA analysis. Whole colons were excised from representative mice and prepared for histological analysis as described previously.[8,10] Cassettes were processed, paraffin embedded,

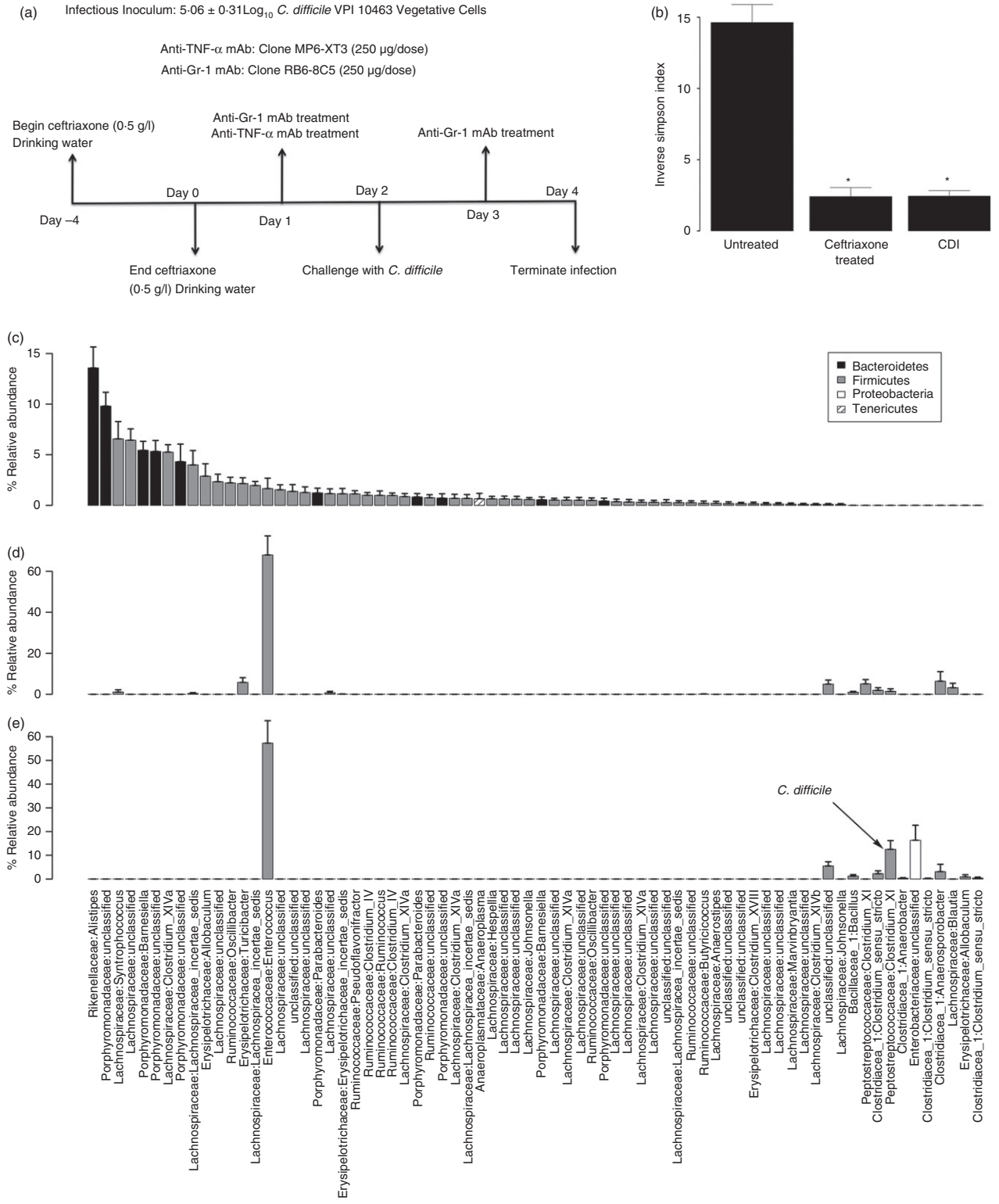

Figure 1. (a) Experimental approach and timeline. (b–e) Effect of ceftriaxone treatment and *Clostridium difficile* infection on the colonic microbiota (day 4). (b) Diversity (Inverse Simpson Index) of the mucosa-associated bacterial communities of untreated, ceftriaxone-treated, or ceftriaxone-treated and *C. difficile* infected mice. (c–e) Relative abundance of the mucosa-associated bacterial communities of the mice in panel b. The operational taxonomic units (OTUs) were ordered by decreasing abundance of genera in the untreated group. Data are shown as mean ± SEM relative abundance. The Family and Genus-level taxonomies are displayed along the *x*-axis and the Phylum-level taxonomy is identified in the shading of individual bars.

sectioned and used to prepare haematoxylin & eosin stained slides (McClinchey Histology Lab Inc., Stockbridge, MI).

Representative images were acquired on an Olympus BX40 light microscope (Olympus Corporation, Center Valley, PA) using a QIMAGING MICROPUBLISHER RTV 5·0 5 megapixel camera (QImaging Corporation, Surrey, BC, Canada) at a total magnification of × 400. Images were acquired using QCAPTURE SUITE PLUS (QImaging Corporation) version 3.1.3.10. Image and panel assembly were performed in ADOBE PHOTOSHOP CS5, version 12.0 (Adobe, San Jose CA). Image processing was restricted to global adjustments of brightness, contrast and image size.

Histopathological examination

Histological sections were coded, randomized and scored in a blinded manner. The slides were first scored categorically on a 0–5 scale for epithelium damage and for inflammation, using defined criteria. Epithelium damage was scored as follows: 0, intact epithelium; 1, minimal, scattered goblet cell loss with no significant epithelium destruction and no histologically defined loss of surface integrity; 2, widespread moderate goblet cell loss with no significant epithelium destruction and no histologically defined loss of surface integrity; 3, moderate to extensive widespread goblet cell loss with scattered epithelium destruction and histologically defined loss of surface integrity; 4, extensive epithelium and goblet cell destruction with histologically defined loss of surface integrity; 5, severe epithelium destruction and goblet cell destruction with widespread histologically apparent loss of surface integrity. Inflammation was scored as follows: 0, no inflammation; 1, minimal multifocal leucocytic infiltrates; 2, moderate multifocal leucocytic infiltrates and low level oedema (greater submucosal involvement); 3, significant multifocal leucocytic infiltrates, oedema, submucosal involvement; 4, extensive multifocal leucocytic infiltrates, oedema, extensive submucosal involvement; 5, severe multifocal leucocytic infiltrates, extensive oedema and submucosal involvement with luminal involvement and/or abscess formation. The slides were then assigned an overall score using a rank-order scoring system. The total of both categorical scores (epithelium damage and inflammation) were used to rank all the slides in the study in order of increasing severity of histopathological changes (1 = least, 16 = most). This method offers significant advantages over straight categorical scoring systems for comparing histological changes between groups and has been previously reported for scoring *C. difficile*-induced intestinal pathology.[10,30,31]

RNA isolation and expression analysis

RNA was isolated and purified from colonic tissue as described previously.[9] RNA quality was assessed using an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA), and the concentration of RNA was assessed using a Nanodrop instrument (Thermo Fisher Scientific, Waltham, MA). Complementary DNA was generated using the RT^2 First Strand kit (Qiagen, Valencia, CA), and gene expression was then determined using RT^2 PCR cards (Qiagen). To account for any variation between RT^2 PCR cards, cross-card normalization was performed as described previously.[9,32] The mean Ct values of two internal control genes was subtracted from the Ct of the gene in question to generate ΔCt (dCt) values.[33] The 2^{-ddCt} method was used to calculate fold change gene expression for all comparisons.[34] All reactions were run on a LightCycler 480 (Roche, Indianapolis, IN).

DNA isolation, amplicon library preparation, 454 pyrosequencing and microbiome community analysis

All procedures and analyses were performed as previously described.[9] Briefly, DNA was isolated from rinsed colonic tissue and V3-V5 16S ribosomal RNA gene amplicon libraries were generated. They were then sequenced on a Roche 454 GS Junior Titanium platform according to the manufacturer's specifications. Bacterial 16S rRNA gene sequences were processed using the microbial ecology software suite *MOTHUR*[35] to generate operational taxonomic units (OTUs) at a 3% level of difference (approximating species-level differences). These data were then imported into the software package R and analysed using the R add on-package VEGAN.[36] Rank abundance plots were generated by selecting for the OTUs that contributed to > 0·5% of the population. The content of each treated tissue was ordered according to the average rank order of its untreated counterpart. Taxonomic classification of an OTU was assigned within *MOTHUR* by identifying the consensus sequence of the OTU and assigning taxonomy using a Bayesian classifier trained on an Ribosomal Database Project training set (classify.otu).

Quantification of C. difficile colonization

Mucosal *C. difficile* colonization was determined using a species-specific quantitative PCR of DNA isolated from colonic tissue. The *C. difficile*-specific qPCR was performed as described previously.[8,37] All reactions were carried out in a total volume of 10 μl. Each reaction contained 2 μl of template primers, 6·25 pmol forward and reverse tcdB primers, and 1 pmol tcdB probe. The cycling conditions and probe and primer sequences are identical to those used previously.[8] Raw Ct values were normalized to signal from a single-copy host internal control gene to generate dCt values.[8,34] The dCt values were then converted to '*C. difficile* genomes/g tissue' using a standard curve generated with known amounts of vegetative *C. difficile* and colonic tissue.

Colonic leucocyte isolation

Colonic leucocytes were isolated as described previously[2,9,38] with modification. Colonic tissue was minced with serrated scissors and incubated in 20 ml of Hanks' balanced salt solution supplemented with 2·5% fetal bovine serum, 5 mM EDTA and 1 mM dithiothreitol for 20 min at 37°. After washing, tissue was incubated in 20 ml Hanks' balanced salt solution supplemented with 2·5% fetal bovine serum, 400 U/ml collagenase type 3 (Worthington Biochemical, Freehold NJ), and 0·5 mg/ml DNase I (Roche) for 60 min at 37°. Samples were then resuspended in 20% Percoll (Sigma) in PBS, and centrifuged at 900 ***g*** for 30 min at room temperature. The resulting single-cell suspensions were stained for analysis of cell surface marker expression via flow cytometry.

Flow cytometry and staining

Flow cytometric staining was performed as described previously.[9] Briefly, cells were blocked with unlabelled FcRIII/II, and subsequently stained with fluorescently labelled antibodies for 30 min. Cells were then washed and finally suspended in stabilizing fixative (BD Biosciences, San Jose, CA). All data were collected on a three-laser Canto II using FACSDiva software (BD Biosciences). All subsequent analysis was performed using FlowJo (Treestar, Ashland, OR). The following antibodies were used for flow cytometric analysis of intestinal leucocytes. CD11c (clone HL3), CD45 (clone 30-F11), CD11b (clone M1/70), Gr-1 (clone RB6-8C5), and Ly6C (clone AL-21) as well as FcRIII/II (clone 2·4G2). All antibodies were purchased from BD Biosciences and Biolegend (San Diego, CA).

Statistical analysis

Unpaired two-tailed *t*-tests were used to identify statistically significant differences in gene expression between untreated and ceftriaxone-treated mice. For all other analyses, statistically significant changes were identified using a one-way analysis of variance with Tukey's post hoc test for multiple comparisons. For all quantitative PCR data (colonic gene expression and *C. difficile* colonization), statistical analysis was performed on normalized dCt values. Significance was set at $P \leq 0{\cdot}05$ in all analyses.

Results

Effect of ceftriaxone treatment on the colonic microbiota

To assess the effect of ceftriaxone treatment on the diversity and membership of the colonic microbiota, animals were given ceftriaxone (0·5 g/l) in their drinking water for 4 days and analysed 4 days later (Fig. 1a). 454-pyrosequencing analysis of 16S amplicon libraries generated from colonic tissue from untreated and ceftriaxone-treated mice allowed for characterization and comparisons of the colonic mucosa-associated microbiota between groups.[39] This analysis revealed that 4 days of ceftriaxone treatment was sufficient to significantly decrease the diversity of the mucosa-associated microbiota, compared with untreated mice, at 4 days post-antibiotic (Fig. 1b). While untreated animals possessed a complex microbiota comprised primarily of members of the phyla Bacteroidetes and Firmicutes (Fig. 1c), ceftriaxone treatment was associated with a marked shift in the membership of the community towards a composition dominated by a single Firmicute; Enterococcaceae (Fig. 1d). These experiments demonstrated that ceftriaxone treatment was sufficient to significantly decrease the diversity and markedly alter the membership of the colonic microbiota even 4 days after the cessation of antibiotic treatment.

Effect of ceftriaxone treatment on colonic gene expression

To investigate the effect of ceftriaxone treatment and the associated loss of microbial diversity on immune regulation the colonic mucosa, quantitative RT-PCR was used to examine colonic cytokine expression following ceftriaxone treatment. Ceftriaxone treatment was associated with significantly increased expression of the chemokines *Ccl5* and *Cxcl9*, and significantly decreased expression of *Ccl11*, *Ccl3* and *Cxcl2* (Fig. 2a,b). Additionally, *Il2*, *Il10*, *Il5* and *Il3* expression was all significantly increased following ceftriaxone treatment, while expression of *Il23a*, *Il17f*, *Il1b*, *Il17a*, *Tgfb* and *Slpi* was decreased (Fig. 2c–e). Examination of histopathological sections from the colons of ceftriaxone-treated mice revealed no evidence of cellular infiltration or inflammation in these animals (see Supporting information, Figure S1). Taken together, these data demonstrate that ceftriaxone treatment alone induces significant changes in the cytokine expression pattern in the colonic mucosa but does not result in overt inflammatory histopathological changes.

Clostridium difficile infection following ceftriaxone treatment

To determine if ceftriaxone treatment was sufficient to permit colonization and infection by *C. difficile*, ceftriaxone-treated mice were challenged with vegetative cells (10^5 colony-forming units) from *C. difficile* strain VPI 10463 2 days after the cessation of ceftriaxone treatment and were followed for an additional 2 days (Fig. 1a). Significant colonization by *C. difficile* was detected in the colonic mucosa of infected mice via quantitative PCR by

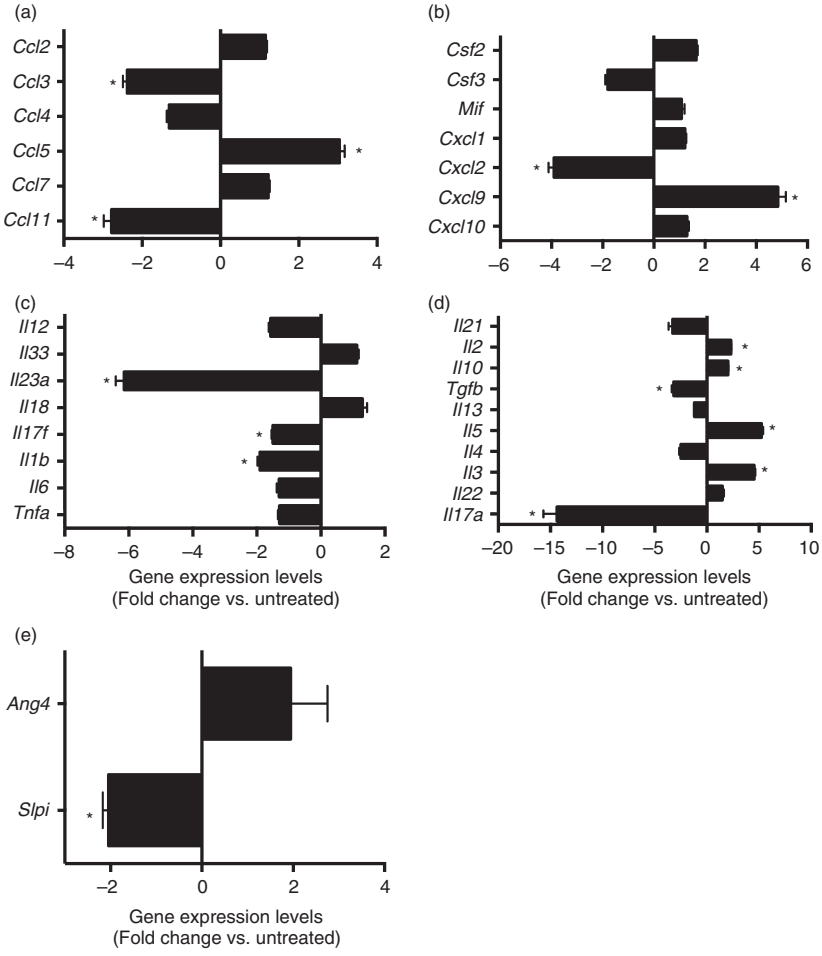

Figure 2. (a–e) Effect of ceftriaxone treatment on colonic inflammatory gene expression (day 4). Mice were treated as outlined in Figure 1. Host gene expression was measured by quantitative PCR as described in the methods; $n \geq 9$ per group. Data are shown as mean ± SEM fold change gene expression of ceftriaxone-treated animals compared with untreated mice. *$P < 0{\cdot}05$ as compared with untreated animals.

2 days post challenge (Fig. 3a). Colonization did not cause a shift in the membership (Fig. 1c–e) or diversity (Fig. 1b) of the colonic microbiota beyond that attributable to ceftriaxone treatment alone. *Clostridium difficile* colonization of ceftriaxone-treated mice resulted in an influx of inflammatory cells into the colon and marked epithelial damage, indicative of active infection (Fig. 4). Taken together, these data demonstrate that ceftriaxone treatment renders mice susceptible to *C. difficile* colonization with the concomitant development of colitis.

Host responses to *C. difficile* colitis

In order to characterize the mucosal response to *C. difficile* colitis following ceftriaxone treatment, colonic gene expression was examined. Infection with *C. difficile* was associated with increased expression of *Inos*, *Slpi*, *Il1b*, *Il6*, *Il17f*, *Ifng*, *Il17a*, *Il22*, *Il2*, *Il33* and *Tnfa* (Fig. 7 and 8c). Expression of *Il18*, *Il23*, *Il12*, *Il3*, *Il4*, *Il5*, *Il13*, *Il10*, *Tgfb* and *Ang4* was not induced in response to *C. difficile* infection (Fig. 7 and 8c). Consistent with the robust cellular recruitment observed in histological sections from *C. difficile*-infected mice (Fig. 4), there was increased expression of the CC chemokines *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5* and *Ccl7*, but not *Ccl11* or *Ccl24* (Fig. 6a,b) and the expression of the CXC chemokines *Cxcl1* and *Cxcl2*, as well as the neutrophil stabilization factors *Csf2* and *Csf3* were also increased (Fig. 6c,d). Hence, these data indicate that the mucosal response to *C. difficile* infection is characterized by increased expression of CC and CXC chemokines and inflammatory cytokines including *Il1b*, *Il6*, *Il17f* and *Tnfa*.

Flow cytometry was used to further delineate the leucocyte populations recruited to the colon following *C. difficile* infection. Analysis of the side scatter (SSC) and forward scatter (FSC) parameters of CD45$^+$ leucocytes revealed a drastic influx of FSC^{Mid} SSC^{High} leucocytes in response to *C. difficile* infection (Fig. 5a). Nearly all of the recruited FSC^{Mid} SSC^{High} cells were CD11b^{High} CD11c^{Low} inflammatory myeloid cells (Fig. 5b). Within the CD11b^{High} CD11c^{Low} population, there were two populations, the largest of which were Ly6C^{Mid} Gr-1^{High} neutrophils, and a smaller population of Ly6C^{High} Gr-1^{Mid} monocytes (Fig. 5c). Hence, these data demonstrate marked recruitment of two distinct Gr-1$^+$ leucocyte populations, Ly6C^{Mid} Gr-1^{High} neutrophils and Ly6C^{High}

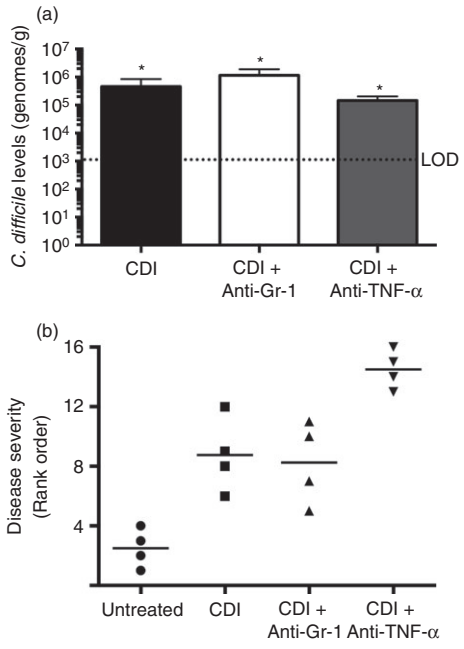

Figure 3. (a) Mucosal *Clostridium difficile* colonization as determined by species-specific quantitative PCR (Day 4). $n \geq 9$ mice per group. Data are shown as mean ± SEM. CDI = *C. difficile* infected. LOD = Limit of Detection. $^*P < 0{\cdot}05$ as compared with untreated animals. Mice were treated as outlined in Fig. 1 and the methods. (b) Rank order analysis of colonic histopathological sections from Untreated, *C. difficile*-infected, *C. difficile*-infected and anti-Gr-1-treated, *C. difficile* infected and anti-tumour necrosis factor-α (TNFα) treated animals (day 4). Slides were ordered on the basis of disease severity, with 16 having the most severe histopathology. $n = 4$ mice per group.

Gr-1^{Mid} monocytes, to the colon in response to *C. difficile* colitis.

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

To determine the role of Gr-1$^+$ cells in supporting the mucosal inflammatory response to *C. difficile* infection, animals were treated with an anti-Gr-1 mAb (clone RB6-8C5) 1 day before and 1 day after *C. difficile* infection (Fig. 1a). There was a marked reduction in the frequency of CD11b^{High} CD11c^{Low} inflammatory myeloid cells, as well as a reduction in both the Ly6C^{Mid} Gr-1^{High} neutrophil and Ly6C^{High} Gr-1^{Mid} monocyte populations following anti-Gr-1 treatment (Fig. 5b,c). However, anti-Gr-1 treatment had no effect on *C. difficile* levels in the colonic mucosa (Fig. 3a).

Compared with *C. difficile* infection alone, *Il5*, *Il12* and *Il22* expression was significantly reduced, and *Il3* expression was significantly increased, in *C. difficile*-infected anti-Gr-1-treated mice (Fig. 7c–e). However, expression of *Il1b*, *Il6*, *Csf2*, *Csf3*, *Tnfa* and *Il33* was unchanged following anti-Gr-1 treatment (Fig. 6 and 7). Expression of *Cxcl1*, *Cxcl2*, *Ccl2* and *Ccl3* was also not significantly affected by anti-Gr-1 treatment (Fig. 6a,c). Anti-Gr-1 treatment did not protect against the colonic histopathology that developed during the normal course of infection (Fig. 3b and 4). Taken together, these data indicate that the expression of CC and CXC chemokines, the expression of inflammatory cytokines including *Tnfa*, *Il1b* and *Il6*, and the development of severe colonic histopathology during *C. difficile* colitis are all independent of the presence of Gr-1$^+$ cells within the colon.

The role of TNF-α during *C. difficile* colitis

To examine the role of TNF-α in promoting inflammatory myeloid cell recruitment and epithelial damage in response to *C. difficile* infection, mice were treated with a TNF-α neutralizing mAb (clone MP6-XT3) 1 day before infection (Fig. 1a). *Clostridium difficile* colonization within the colonic mucosa was equivalent between anti-TNF-α-treated and *C. difficile*-infected animals (Fig. 3a). There was near identical recruitment of CD11b^{High} CD11c^{Low} inflammatory myeloid cells following anti-TNF-α treatment compared with that seen in response to *C. difficile* infection alone (Fig. 5b). Ly6C^{Mid} Gr-1^{High} neutrophil and Ly6C^{High} Gr-1^{Mid} monocyte frequencies were also unchanged following anti-TNF-α treatment (Fig. 5c). Consistent with this observation, *Cxcl1*, *Cxcl2*, *Ccl2* and *Ccl3* expression was not reduced in these mice (Fig. 6a,c). While the expression of *Il33*, *Ifng* and *Il17f* was not significantly reduced by anti-TNF-α treatment, expression of *Mif*, *Il1b* and *Il6* was all significantly increased following anti-TNF-α treatment (Fig. 6d and 7). Furthermore, expression of *Ccl5*, *Il5* and *Il22* was all significantly lower in anti-TNF-α-treated mice (Fig. 6b and 7d,e).

Epithelial damage was prominent in anti-TNF-α-treated mice (Fig. 4) and anti-TNF-α treatment was associated with more severe colonic histopathology (Fig. 3b). Anti-TNF-α treatment was also associated with a significant decrease in the expression levels of the colonic goblet cell gene *Ang4*, the free fatty acid receptors *Ffar2* and *Ffar3*, epithelium-derived effector and signal molecules encoded by *Tslp*, *Tff*, *Defb3* and *Lyz1*, in addition to a trend toward higher expression levels of *Slpi* compared with *C. difficile* infection alone (Fig. 8). Additionally, there was a trend towards decreased expression of the goblet cell-specific gene *Muc2* after anti-TNF-α treatment (Fig. 8a). Taken together, these data indicate that anti-TNF-α treatment was associated with enhanced colonic epithelial dysfunction/destruction and can augment the epithelial inflammatory milieu during *C. difficile* infection.

Discussion

Together, these data demonstrate that ceftriaxone-treated mice are a robust model for investigating the innate acute

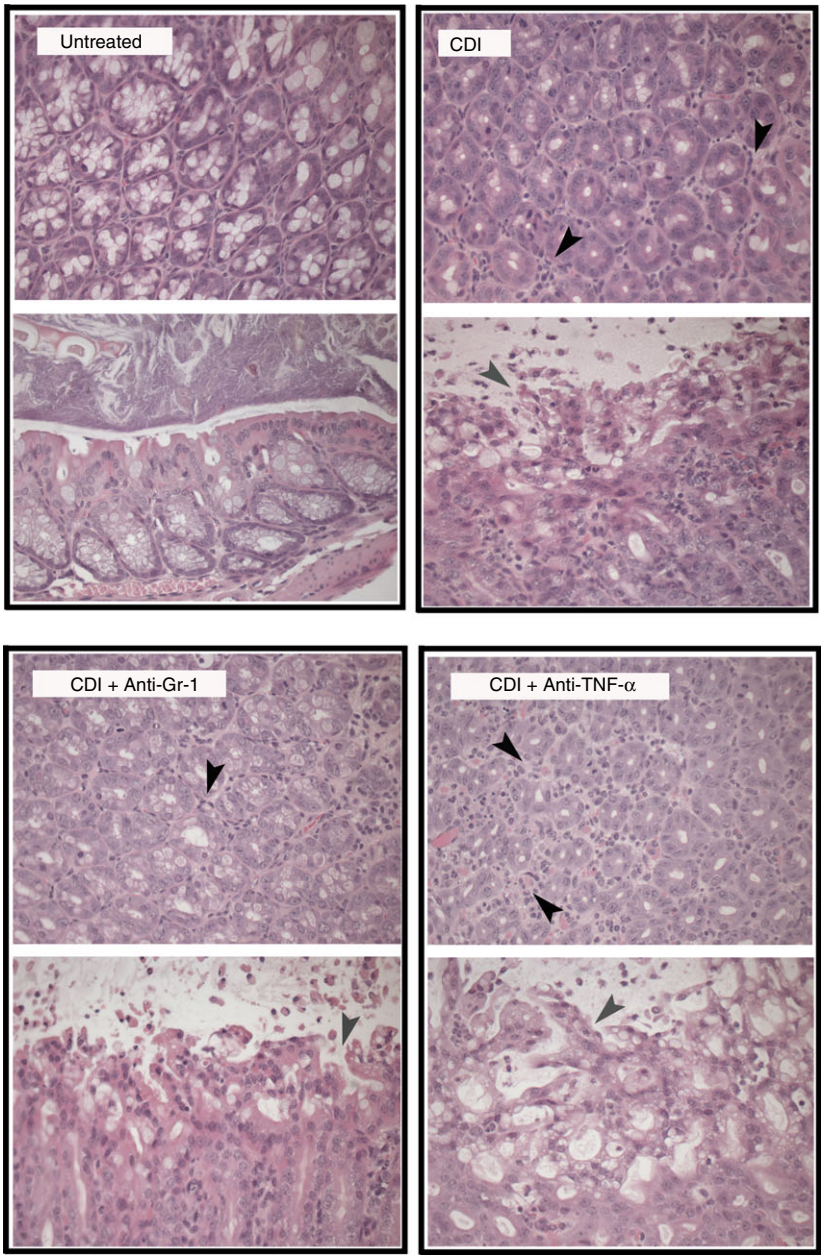

Figure 4. Photomicrographs of representative haematoxylin & eosin (H&E) -stained colonic sections from Untreated, *Clostridium difficile*-infected, *C. difficile*-infected and anti-Gr-1-treated, *C. difficile*-infected and anti- tumour necrosis factor-α (TNF-α)-treated animals (day 4). Cross-sections of colonic crypts (upper images) and longitudinal sections of the epithelial–luminal interface (lower images) are shown for each treatment. Black arrowheads indicate infiltrating inflammatory cells, while grey arrowheads indicate areas of epithelial damage. All animals were treated as outlined in Fig. 1. Total magnification for all images is 400 ×. CDI = *C. difficile* infected.

inflammatory response to *C. difficile* infection. In addition to altering the composition of the colonic microbiome, ceftriaxone treatment alone was also capable of modulating colonic cytokine and chemokine expression 4 days after the cessation of antibiotic treatment. While ceftriaxone treatment was associated with significantly altered expression of several genes induced during *C. difficile* colitis, the induction of the inflammatory cytokines and chemokines associated with *C. difficile* infection was significantly greater than that seen with ceftriaxone treatment alone (see Supporting information, Figure S2). Additionally, ceftriaxone treatment alone did not produce any histopathological changes in the colonic mucosa (see Supporting information, Figure S1). Hence, while ceftriaxone treatment is capable of modulating colonic gene expression, we propose it is not a confounding factor in evaluating the host inflammatory response in this model of infectious colitis.

Anti-TNF-α treatment was associated with an augmented epithelial inflammatory response to *C. difficile* infection. Previous studies have reported a role for TNF-α signalling in promoting myeloid cell recruitment during mucosal inflammation.[23,24] TNF-α can enhance the expression of CCL3 during chemically induced pulmonary inflammation,[24] and interference with TNF-α signalling reduces neutrophil recruitment in response to acute allergic airway inflammation.[23] TNF-α can contribute to inflammatory cytokine expression and tissue damage

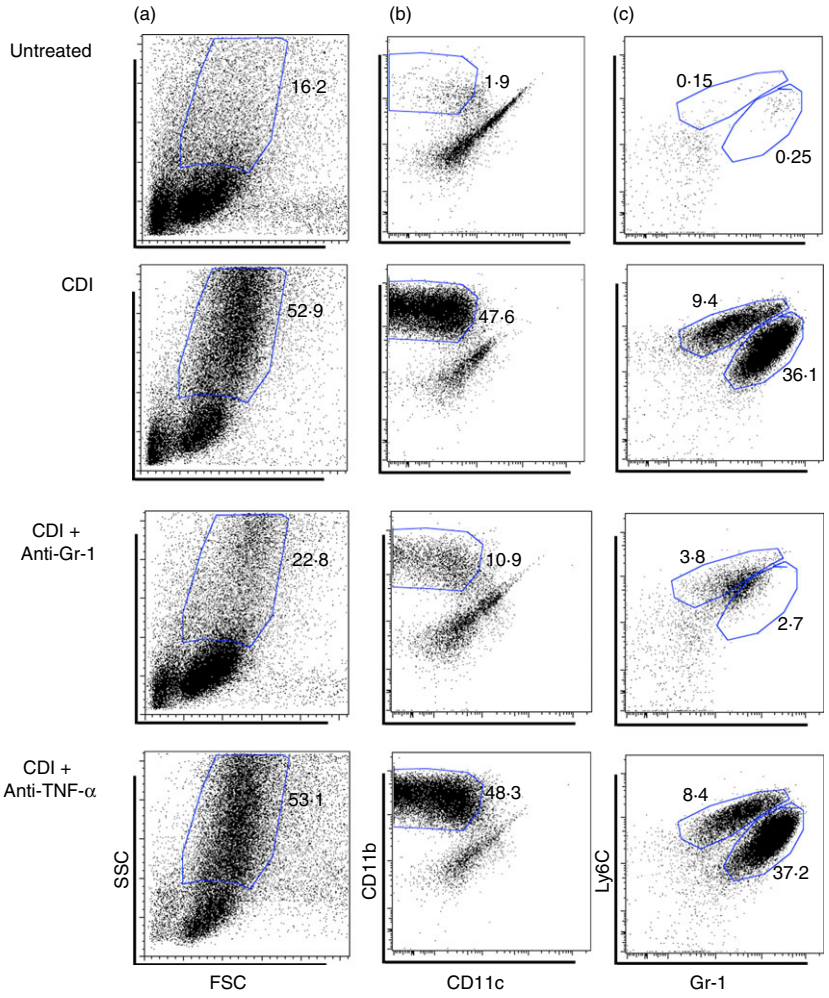

Figure 5. Flow cytometric analysis of colonic leucocytes from Untreated, *Clostridium difficile*-infected, *C. difficile*-infected and anti-Gr-1-treated, *C. difficile* infected and anti-tumour necrosis factor (TNF-α) -treated animals (day 4). (a) Analysis of forward-scatter (FSC) and side-scatter (SSC) properties of total colonic $CD45^+$ leucocytes. (b) Analysis of CD11b and CD11c expression profiles of SSC^{High} FSC^{Low} $CD45^+$ leucocytes as defined in panel (a). (c) Analysis of Ly6C and Gr-1 expression profiles of the $CD11b^{High}$ $CD11c^{Low}$ population defined in panel (b). CDI = *C. difficile* infected. The number in bold type indicates the percentage of total $CD45^+$ leucocytes contained within the indicated gate.

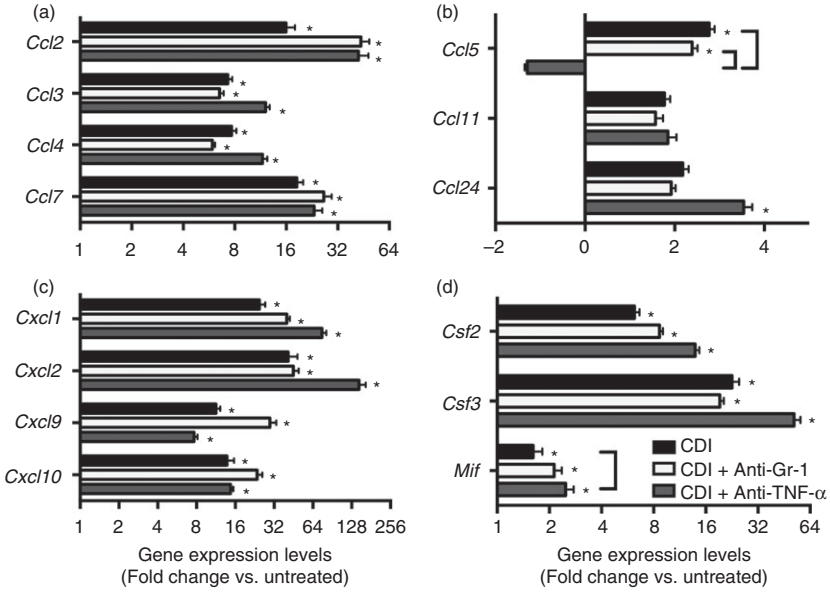

Figure 6. (a–d) Effect of anti-Gr-1 and anti-tumour necrosis factor-α (TNF-α) treatment on colonic inflammatory chemokine expression during *C. difficile* colitis (day 4). Colonic gene expression was measured via quantitative PCR as outlined in the Materials and methods. $n \geq 8$ per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile* infected (black bars), *C. difficile*-infected and anti-Gr-1-treated (white bars), and *C. difficile* infected and anti-TNFα treated (gray bars) animals as compared with untreated mice. $^*P < 0{\cdot}05$ compared with untreated animals. Brackets indicate $P < 0{\cdot}05$ for the differences between the indicated groups.

during mucosal inflammation.[25,40] Directly applicable to gastrointestinal inflammation, during 2,4,6-trinitrobenzenesulphonic acid colitis, TNF-α signalling promotes both IL-18 and TNF-α expression as well as the development of intestinal histopathology.[25] Anti-TNF-α treatment had no effect on *Cxcl1*, *Cxcl2*, *Ccl2* and *Ccl3*

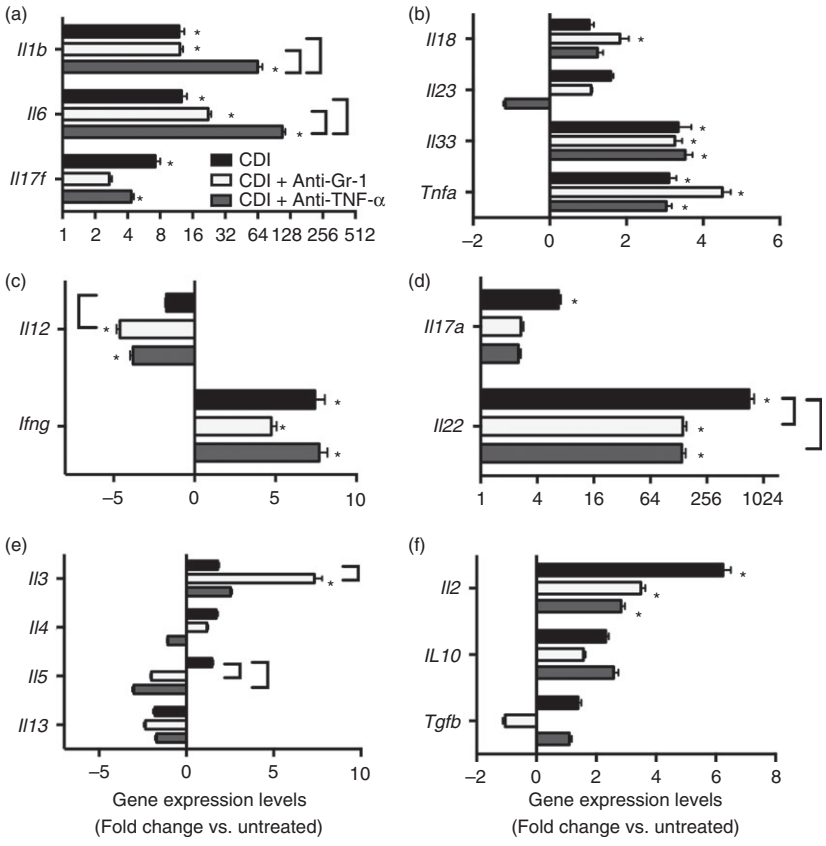

Figure 7. (a–g) Effect of anti-Gr-1 and anti-tumour necrosis factor-α (TNF-α) treatment on colonic inflammatory cytokine expression during *C. difficile* colitis. Host gene expression was measured via quantitative PCR as described in the methods; $n \geq 7$ per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile* infected (black bars), *C. difficile* infected and anti-Gr-1 treated (white bars), and *C. difficile* infected and anti-TNF-α treated (grey bars) animals as compared with untreated mice. CDI = *C. difficile* infected *$P < 0{\cdot}05$ compared with untreated animals. Brackets indicate $P < 0{\cdot}05$ for the differences between the indicated groups.

expression during *C. difficile* colitis, and we observed no reduction in neutrophil and monocyte recruitment under these conditions. However, colonic expression of *Il1b* and *Il6* was increased following anti-TNF-α treatment. Hence, despite its relatively low level of expression, TNF-α is involved in the inflammatory response during *C. difficile* colitis but the induction of CC and CXC chemokines and subsequent myeloid cell recruitment during infection are largely TNF-α-independent.

While TNF-α is generally held to be a pro-inflammatory mediator that can participate in indirect epithelial damage, there is precedent for an epithelium cytoprotective role for this cytokine, as our data suggest may occur in this infection model.[41–43] TNF-α signalling can protect against severe intestinal histopathology during *Citrobacter rodentium* infection.[41] Furthermore, cellular infiltration and intestinal epithelial damage during DSS colitis are enhanced in the absence of TNF-α.[42,43] Following anti-TNF-α treatment we observed decreased expression of the free fatty acid receptors *Ffar2* and *Ffar3*, as well as *Defb3*, *Lyz1* and *Tslp*, consistent with the severe colonic epithelial damage present in these animals. Anti-TNF-α treatment was also associated with significantly decreased expression of Ang4 and a trend towards decreased expression of Muc2, two genes whose expression is specific to goblet cells within the gastrointestinal tract,[44–46] suggesting reduced goblet cell function. Additionally, colonic histopathology was most severe following anti-TNF-α treatment. Hence, our data are consistent with these other models of colitis where TNF-α plays a role in protecting the colonic epithelium from damage during inflammation, and suggests an epithelial-protective role for TNF-α during acute, severe *C. difficile* infection.

One model that could explain the association between anti-TNF-α treatment and increased inflammatory responses and epithelial damage is that TNF-α signalling may restrain host inflammatory responses by promoting the clearance of translocated commensal bacteria. Previous studies have demonstrated the dissemination of commensal bacterial to distal sites during *C. difficile* infection,[1,2] and have also suggested a role for these translocated bacteria in promoting innate inflammatory responses, including the production of IL-1β.[1] TNF-α signalling can activate macrophages and enhance their bactericidal capacity,[47] and mucosal macrophages represent a large resident population within the colonic lamina propria with the capacity to eliminate invading bacteria.[48] Hence, in the absence of TNF-α signalling, translocated commensal bacteria may be less efficiently cleared by macrophages within the colonic lamina propria, leading to increased activation of inflammatory pathways and enhanced colonic tissue damage.

Despite the robust recruitment of Ly6C^{Mid} Gr-1^{High} neutrophils and Ly6C^{High} Gr-1^{Mid} monocytes in response

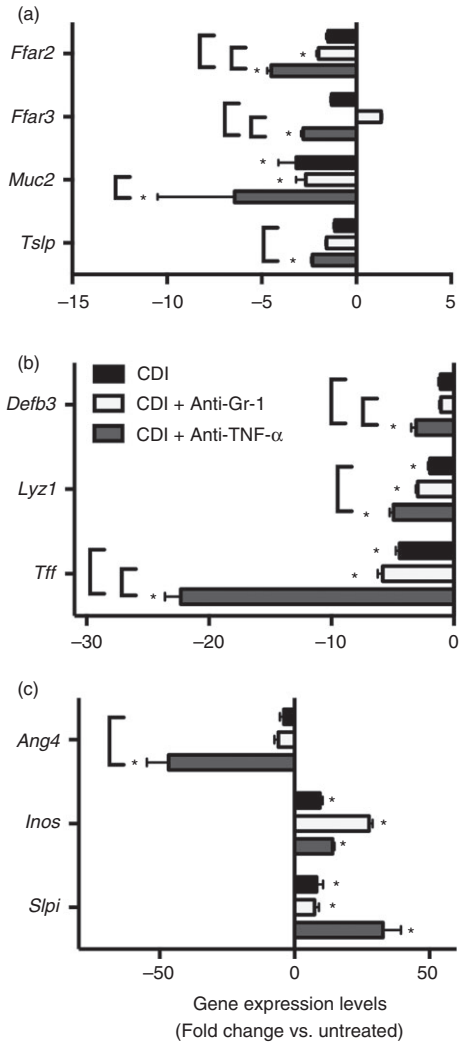

Figure 8. (a–c) Effect of anti-Gr-1 and anti-tumour necrosis factor-α (TNF-α) treatment on colonic gene expression during *Clostridium difficile* colitis. Host gene expression was measured via quantitative PCR as described in the Materials and methods; $n \geq 7$ per group. Data are shown as mean ± SEM fold change gene expression of *C. difficile*-infected (black bars), *C. difficile*-infected and anti-Gr-1-treated (white bars), and *C. difficile*-infected and anti-TNF-α-treated (grey bars) animals as compared to untreated mice. CDI = *C. difficile* infected $*P < 0{\cdot}05$ as compared to untreated animals. Brackets indicate $P < 0{\cdot}05$ for the differences between the indicated groups.

to *C. difficile* infection, we observed no reduction in colonic expression of CC and CXC chemokines or proinflammatory cytokines including *Il1b*, *Il6*, *Il33* and *Tnfa* following anti-Gr-1 treatment. Neutrophil recruitment is commonly associated with the development of inflammation at mucosal sites[23,49,50] and neutrophils are a well-documented source of inflammatory cytokines, including IL-6, as well as the neutrophil chemokine IL-8.[51–53] While several studies have investigated the role of neutrophils in reducing host mortality and preventing the translocation of bacteria during *C. difficile* infection,[2,3] to our knowledge the role of neutrophils as a cellular source of chemokine and cytokine expression has not been extensively investigated. Despite effective depletion of both neutrophils and monocytes following our anti-Gr-1 treatment, colonic CC and CXC chemokine expression as well as *Il1b*, *Il6*, *Il33* and *Tnfa* expression was unchanged. Consistent with the significant reduction in *Il22* expression, a cytokine with a vital role in promoting wound healing and preventing epithelial damage at mucosal sites,[54–56] we observed no protection from intestinal epithelial damage in anti-Gr-1-treated mice. Taken together, our data strongly suggest that the recruited Gr-1$^+$ populations, including neutrophils, are not responsible for the robust inflammatory cytokine expression or epithelial damage observed during *C. difficile* infection.

One potential explanation for our observation that depletion of Gr-1$^+$ cell populations had minimal effect on colonic cytokine and chemokine expression is that Gr-1$^+$ cells, including neutrophils, are not a major cellular source of these inflammatory mediators during the host response to *C. difficile* colitis. A recent study has reported decreased intestinal IL-1β and CXCL1 production following neutrophil depletion.[1] However, that study used a less severe model of disease with 100% survival of infected animals at 5 days-post infection,[1] while the model used in the current study, similar to other models based on the third-generation cephalosporin cefoperazone,[8,10] resulted in high morbidity by 2 days post infection. Hence, in the context of such an overwhelming infection, alternative pathways and sources of inflammatory cytokine expression may respond and effectively supersede the cytokine production by neutrophils observed in less severe models. Additionally, Hasegawa *et al.*[1] used a combination of seven antibiotics to permit *C. difficile* infection, compared with the single antibiotic, ceftriaxone, used in the current study. These two antibiotic treatments will likely result in different colonic microbial communities, whose members may differentially stimulate host cell subsets and result in the activation of distinct inflammatory pathways in response to the same challenge.

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Author contributions

AJM and GBH conceived, designed and interpreted the experiments; VBY, NRF and RAM contributed to their design and interpretation. AJM, JRE, NRM, KEH and RAM performed the experiments. AJM, RM, JRE, KEH and GBH analysed the data. AJM and GBH wrote the manuscript, and all other authors provided comments and advice on the manuscript.

Disclosures

Vincent B. Young is on the advisory board of ViroPharma in relation to the development of a non-toxigenic *C. difficile* strain for the management of *C. difficile* infection. All other authors declare no conflict of interest.

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Supporting Information

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

Figure S1. Photomicrographs of representative Hematoxylin and Eosin (H&E) stained colonic sections from both uninfected (untreated) and ceftriaxone treated mice (day 4).

Figure S2. (a–e) Comparison of ceftriaxone treatment alone and *Clostridium difficile* infection following ceftriaxone treatment on colonic inflammatory gene expression (day 4).

