Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Interleukin-23 (IL-23), independent of IL-17 and IL-22, drives neutrophil recruitment and innate inflammation during *Clostridium difficile* colitis in mice

Andrew J. McDermott,[1] Nicole R. Falkowski,[2] Roderick A. McDonald,[2] Chinmay R. Pandit,[2] Vincent B. Young[1,3] and Gary B. Huffnagle[1,2]

[1]*Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI,* [2]*Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, and* [3]*Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA*

doi:10.1111/imm.12545
Received 15 June 2015; revised 17 September 2015; accepted 5 October 2015.
Correspondence: Gary B. Huffnagle, 6220 MSRB III, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-5642, USA. Email: ghuff@umich.edu
Senior author: Dr. Gary B. Huffnagle

Summary

Our objective was to determine the role of the inflammatory cytokine interleukin-23 (IL-23) in promoting neutrophil recruitment, inflammatory cytokine expression and intestinal histopathology in response to *Clostridium difficile* infection. Wild-type (WT) and p19$^{-/-}$ (IL-23KO) mice were pre-treated with cefoperazone in their drinking water for 5 days, and after a 2-day recovery period were challenged with spores from *C. difficile* strain VPI 10463. Interleukin-23 deficiency was associated with significant defects in both the recruitment of CD11b^{High} Ly6G^{High} neutrophils to the colon and the expression of neutrophil chemoattractants and stabilization factors including *Cxcl1*, *Cxcl2*, *Ccl3* and *Csf3* within the colonic mucosa as compared with WT animals. Furthermore, the expression of inflammatory cytokines including *Il33*, *Tnf* and *Il6* was significantly reduced in IL-23-deficient animals. There was also a trend towards less severe colonic histopathology in the absence of IL-23. The induction of *Il17a* and *Il22* was also significantly abrogated in IL-23KO mice. Inflammatory cytokine expression and neutrophilic inflammation were not reduced in IL-17a-deficient mice or in mice treated with anti-IL-22 depleting monoclonal antibody. However, induction of *RegIIIg* was significantly reduced in animals treated with anti-IL-22 antibody. Taken together, these data indicate that IL-23, but not IL-17a or IL-22, promotes neutrophil recruitment and inflammatory cytokine and chemokine expression in the colon in response to *C. difficile* infection.

Keywords: *Clostridium difficile*; colitis; innate inflammation; mucosal inflammation; neutrophils.

Introduction

Marked neutrophil recruitment is one of the most prominent host responses to *Clostridium difficile* infection in antibiotic pre-treated mice.[1–6] This neutrophil recruitment appears to be protective to the host, as numerous studies have demonstrated reduced survival during *C. difficile* infection following interventions that reduced neutrophil recruitment.[1–3] Although recent studies have demonstrated roles for Myeloid Differentiation Primary Response 88,[3] Apoptosis-Associated Speck-Like Protein Containing a CARD,[1] Nucleotide Binding Oligomerization Domain 1[2] and interleukin-22 (IL-22) and CD160[6] in driving neutrophil recruitment to the large bowel during *C. difficile* infection, our understanding of the host signals promoting neutrophil recruitment remains incomplete.

Neutrophil mobilization and recruitment are intimately associated with *C. difficile* infection in humans as well. Neutrophilic infiltration is prominent in large colon tissue from patients with pseudomembranous colitis,[7] and increased neutrophilia in the bloodstream is common during *C. difficile* colitis.[8] Furthermore, the mobilization of neutrophils may play a role in determining the outcome of disease, as a recent study has reported a strong association between increased neutrophil numbers in the blood and increased risk of mortality.[9]

Neutrophil recruitment is a rapid and common host response to insult at mucosal sites.[2,4,5,10–13] Neutrophils are myeloid cells that are rapidly recruited to sites of inflamma-

tion,[1–6,10] and are characterized by high levels of Ly6G and CD11b expression.[1–3,10,14] The influx of neutrophils into peripheral tissues is often associated with expression or production of two potent neutrophil chemokines, CXCL1 and CXCL2, which activate neutrophils and promote their egress from the bone marrow.[1,3–5,11,14–16]

Interleukin-23 is a heterodimeric cytokine comprised of a p19 and a p40 subunit,[17] which promotes innate inflammatory responses during mucosal inflammation at numerous sites.[10,11,18–20] Neutrophil recruitment during both *Pseudomonas aeruginosa*[18,19] and bleomycin-mediated[20] pulmonary inflammation is partially dependent upon IL-23 signalling. Specific to intestinal inflammation, IL-23 also promotes neutrophil recruitment in response to *Salmonella typhimurium* typhlocolitis[11] and Dextran Sodium Sulphate-induced colitis.[10] Interleukin-23 can also promote the development of severe intestinal histopathology in response to infectious insult.[21] Recent studies have also reported IL-23 secretion by bone marrow-derived dendritic cells in response to stimulation with a combination of microbial pathogen-associated molecular patterns and *C. difficile* toxins,[22] and reduced mortality and morbidity following *C. difficile* infection of IL-23-deficient mice.[23] However, the role of IL-23 in driving neutrophil recruitment during *C. difficile* colitis has yet to be investigated.

Both IL-17 and IL-22 also promote neutrophil recruitment to mucosal sites, and are induced in response to mucosal inflammation. Interleukin-17 is known to promote neutrophil recruitment and epithelial damage during inflammatory responses in both the lung and the intestine.[12,13,24–26] Interleukin-22 signalling can promote neutrophil recruitment during pulmonary inflammation[24] as well as drive neutrophil-attractive chemokine expression from colonic cells;[27,28] IL-22 also protects against severe histopathology during infectious murine colitis.[29] Furthermore, IL-23 can drive the induction of both IL-22 and IL-17 in numerous models of mucosal inflammation.[10,11,19,21,29]

In the current study, our objective was to investigate the role of IL-23 in driving neutrophil recruitment, inflammatory cytokine expression and intestinal histopathology during *C. difficile* colitis. If so, our objective was to investigate the relative contributions of IL-17 and IL-22, two cytokines whose induction at sites of mucosal inflammation is controlled by IL-23, in driving neutrophil recruitment, colonic histopathology and inflammatory cytokine expression in the colon in response to *C. difficile* infection.

Materials and methods

Animals and housing

Male C57BL/6 mice aged 5–11 weeks, and male and female IL-17a$^{-/-}$ (IL-17KO) and p19$^{-/-}$ (IL-23KO) mice aged 5–14 weeks were used in the current study. C57BL/6 mice were obtained from an in-house colony founded by Jackson breeders, and IL-17a$^{-/-}$ (IL-17KO) and p19$^{-/-}$ (IL-23KO) on a C57BL/6 background were likewise obtained from a breeding colony maintained at the University of Michigan. The breeding pairs of IL-23$^{-/-}$ mice were a kind gift from Ben Segal at the University of Michigan. Mice were maintained under specific pathogen-free conditions, and autoclaved food, water and bedding were provided *ad libitum*. All animal manipulations were carried out in a laminar flow hood. All experiments were conducted in accordance with a protocol approved by the University Committee on Use and Care of Animals at the University of Michigan.

Clostridium difficile spore preparation

VPI 10463 spore stocks were generated by plating an earlier spore preparation on taurocholate cefoxitin cycloserine fructose agar (TCCFA) plates anaerobically. Single colonies were isolated, and grown overnight in Columbia broth. Two millilitres of the overnight culture was inoculated into 40 ml of Clospore broth,[30] and the culture was allowed to grow for 7 days. Spores were collected by centrifugation, and washed to remove vegetative cell debris. All spore stocks were stored in water at 4° until used.

Clostridium difficile infection

Mice were given a 5-day course of cefoperazone (0·5 g/l) in their drinking water to permit *C. difficile* infection as described previously.[31] After a 2-day recovery period, mice were challenged via oral gavage with $5{\cdot}70 \pm 0{\cdot}25$ $\log_{10}$ *C. difficile* spores from strain VPI 10463. Animals were followed for an additional 2 days, and all samples were collected at 2 days post infection. Inoculum dosage was confirmed by serially diluting and plating an aliquot of the inoculum on TCCFA plates anaerobically. Animals were monitored following infection for signs of severe disease, including lethargy, hunched posture and $> 20\%$ weight loss. Any animals found moribund were humanely euthanized. Untreated animals did not receive antibiotics or *C. difficile* challenge.

Anti-IL-22 treatment

Animals were given two intraperitoneal injections of anti-IL-22 monoclonal antibody (mAb; clone 8E11). Each mouse received 150 μg antibody 1 day before and 1 day after infection.[29] The anti-IL-22 mAb was a kind gift from Dr Wenjun Ouyang.

Histology

Colonic tissue was fixed in 10% formalin for at least 24 hr, and then transferred to 70% ethanol. Tissue was

processed, paraffin embedded, sectioned and used to prepare haematoxylin & eosin stained slides by McClinchey Histology Labs Inc., Stockbridge, MI. Representative images were acquired using an Olympus BX40 light microscope (Olympus Corporation, Center Valley, PA) and a QIMAGING MICROPUBLISHER RTV 5.0 5 megapixel camera. All images were acquired at a total magnification of 400 ×. Panels were assembled in ADOBE PHOTOSHOP CS5, version 12.0. Image processing was restricted to global adjustments of brightness, contrast and image size.

Histological scoring

Light microscopic evaluation of haematoxylin & eosin stained colonic sections was performed by a board-certified veterinary pathologist. The pathologist was blinded to experimental groupings at the time of the evaluation, and sections were scored using a previously established system,[32,33] Oedema: 0 no oedema, 1 mild, focal or multifocal oedema with minimal submucosal expansion (< 2×), 2 moderate multifocal oedema with moderate submucosal expansion (2–3 ×), 3 severe multifocal to coalescing oedema with severe submucosal expansion (> 3 ×), 4 same as 3 with diffuse submucosal expansion. Inflammation: 0 no inflammation, 1 minimal, multifocal neutrophilic infiltration, 2 moderate, multifocal neutrophilic infiltration (greater submucosal involvement), 3 severe multifocal to coalescing neutrophilic infiltration (greater submucosal ± mural involvement), 4 same as 3 with abscesses or extensive transmural involvement. Epithelial damage: 0 no epithelial damage, 1 mild multifocal, superficial damage (vacuolation, increased apoptosis, villus tip attenuation/necrosis), 2 moderate, multifocal superficial damage (same qualitative changes as above), 3 severe multifocal to coalescing mucosal damage ± pseudomembrane formation (intraluminal aggregate of neutrophils and sloughed epithelium in a fibrinous matrix covering eroded or ulcerated mucosa), 4 same as 3 with extensive pseudomembrane or ulcer formation.

RNA isolation and expression analysis

Colonic tissue samples (~ 1 cm^2) were collected from the centre of the colon and stored in RNAlater (Ambion, Austin, TX). RNA isolation and purification from colonic tissue was performed as described previously.[4–6,31] Tissue was homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA) and the resulting RNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of the purified RNA was determined using a Nanodrop instrument (Thermo Fisher, Waltham, MA). Synthesis of cDNA, using the purified RNA as a template, was performed using the RT^2 First Strand kit (Qiagen), and colonic gene expression was assessed using RT^2 Profiler PCR arrays (Qiagen). All reactions were run on a Roche Lightcycler 480. To correct for variation between RT^2 Profiler PCR arrays, cross card normalization was performed as described previously.[5,34] ΔC_t (dC_t) values were calculated by subtracting the geometric mean of two internal control genes from the Ct value of the gene of interest.[35] The 2^{-ddCt} method was used to calculate fold change gene expression in treatment groups compared with untreated animals for all comparisons.[36]

Leucocyte isolation

Leucocytes were isolated from colonic tissue as described previously.[4] Isolated colonic tissue was minced with serrated scissors to physically disrupt the tissue, and was subsequently incubated in 20 ml Hanks' balanced salt solution (HBSS) supplemented with 2·5% fetal bovine serum, 5 mM EDTA and 1 mM dithiothreitol for 20 min at 37°. Tissue was then incubated in 20 ml of a digest solution consisting of HBSS supplemented with 2·5% fetal bovine serum, 400 U/ml collagenase type 3 (Worthington Biochemical, Lakewood, NJ) and 0·5 mg/ml DNAse I (Roche, Basel, Switzerland) for 60 min at 37°. Samples were then resuspended in 20% Percoll (Sigma, St Louis, MO) in PBS, and centrifuged at 900 **g** for 30 min at room temperature without brake. The resulting single cell suspensions were stained for flow cytometric analysis.

Flow staining and analysis

Single-cell suspensions were plated at a concentration of approximately 10^6 cells per well in a 96-well plate. Cells were blocked with unlabelled FC RIII/II, and then stained with fluorescently labelled antibodies for 30 min. Cells were washed to remove excess antibody, and resuspended in stabilizing fixative (BD Biosciences, Franklin Lakes, CA). Data were collected on a three-laser Canto II using FACSDIVA software (BD Biosciences). All data analysis was performed in FLOWJO (Treestar, Ashland, OR). Isolated colonic cells were stained with the following antibodies: CD45 (clone 30-F11), CD11b (clone M1/70) and Ly6G (clone IA8) as well as Fc RIII/II (clone 2·4G2). All antibodies were purchased from eBioscience (San Diego, CA), BD Pharmingen (Franklin Lakes, CA) and Biolegend (San Diego, CA).

Total number of neutrophils per colon was calculated by multiplying the frequency of $CD45^{High}$ $CD11b^{High}$ $Ly6G^{High}$ neutrophils as defined by flow cytometry by the total number of cells in the colon in question. For all animals, the entirety of the colon was taken and processed for leucocyte isolation and analysis by FACS.

Statistical analysis

Statistically significant differences in gene expression were determined using a one-way analysis of variance with Tukey's *post hoc* test for multiple comparisons. For all quantitative PCR data, statistical tests were performed on normalized dC_t values.[4,31] A one-way analysis of variance with Tukey's *post hoc* test was also used to identify significant differences in the number of neutrophils per colon. Significant differences in histopathological scoring were determined using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. For all analyses, significance was set at $P \leq 0{\cdot}05$.

Results

Effect of IL-23 deficiency on colonic neutrophil recruitment

For these studies, WT and $p19^{-/-}$ (IL-23KO) mice were given cefoperazone (0·5 g/l) in their drinking water for 5 days as described previously.[6,31] Following a 2-day recovery period on regular water, mice were challenged with $5{\cdot}70 \pm 0{\cdot}25\log_{10}$ *C. difficile* spores (strain VPI 10463). Animals were followed for an additional 2 days, and all samples were collected at 2 days post-infection. All infected groups had a mean *C. difficile* colonization level of $\geq 10^5$ CFU/g host tissue (data now shown).

To determine the role of IL-23 in driving neutrophil recruitment in response to *C. difficile* colitis, flow cytometry was used to identify recruited leucocytes. Analysis of colonic leucocytes isolated from WT animals revealed a drastic influx of $CD11b^{High}$ $Ly6G^{High}$ neutrophils following *C. difficile* infection (Fig. 1a). In contrast, the frequency of the $CD11b^{High}$ $Ly6G^{High}$ neutrophil population was markedly reduced in IL-23KO animals (Fig. 1a). Further quantification of the total number of $CD11b^{High}$ $Ly6G^{High}$ neutrophils per colon revealed a statistically significant reduction in the total number of neutrophils recruited to the colons of IL-23KO animals compared with WT (Fig. 1b). These data demonstrate a significant reduction in neutrophil recruitment to the colon in response to *C. difficile* colitis in the absence of IL-23.

To investigate the effect of IL-23 in driving chemokine and granulocyte colony-stimulating factor (CSF3) expression during *C. difficile* colitis, quantitative RT-PCR was used to examine colonic cytokine expression at 2 days post infection. *Clostridium difficile* infection was associated with increased expression of the neutrophil chemokines *Cxcl1*, *Cxcl2* and *Ccl3*, as well as the neutrophil stabilization factor *Csf3* within the colonic mucosa (Fig. 1c). Consistent with the reduced neutrophilic influx observed in response to *C. difficile* infection in IL-23KO mice (Fig. 1a,b), *Cxcl1*, *Cxcl2*, *Ccl3* and *Csf3* expression

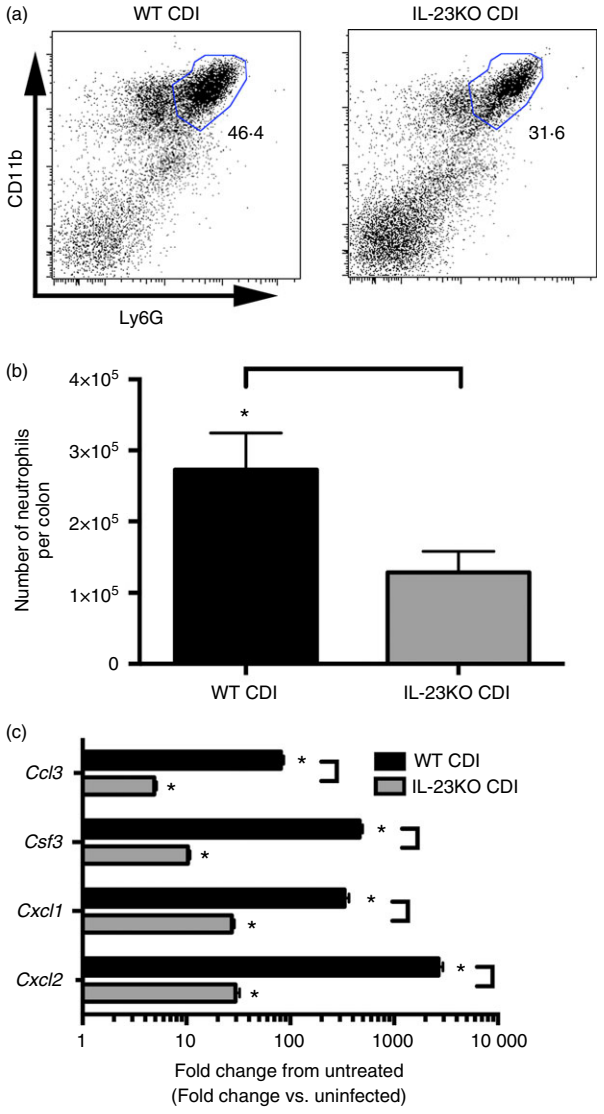

Figure 1. Colonic neutrophil recruitment and neutrophil chemokine expression in response to *Clostridium difficile* infection in the absence of interleukin-23 (IL-23) signalling. (a) Analysis of CD11b and Ly6G expression profiles of $CD45^+$ colonic leucocytes. The number in bold type represents the percentage of total $CD45^+$ leucocytes contained within the indicated gate. (b) Total number of recruited $CD11b^{High}$ $Ly6G^{High}$ neutrophils as defined in (a). Bars represent mean ± SEM number of recruited neutrophils for the indicated group. $n = 8$ per group. For all animals, the entire colon was taken for FACS analysis. (c) Colonic gene expression was assessed using quantitative PCR as outlined in the Materials and methods. $n \geq 6$ per group. Data are shown as mean ± SEM fold change gene expression of wild-type (WT) *C. difficile*-infected (black bars) and IL-23KO *C. difficile*-infected (grey bars) animals compared with untreated WT mice. CDI = *C. difficile* infected. For all analyses, $^*P < 0{\cdot}05$ compared with untreated WT animals and brackets indicate $P < 0{\cdot}05$ for the differences between indicated groups.

levels were significantly reduced in IL-23KO animals compared with WT (Fig. 1c). There was no defect in the expression of the eosinophil chemokine *Ccl11*, or the

T-cell chemokines *Cxcl9* and *Cxcl10* in IL-23-deficient animals (Fig. 2a). Furthermore, *Ccl24* expression was significantly increased in the absence of IL-23 (Fig. 2a). Taken together, these data demonstrate that IL-23 deficiency is associated with significant defects in both the recruitment of neutrophils to the colon and the expression of neutrophil chemoattractants within the colonic mucosa during *C. difficile* colitis.

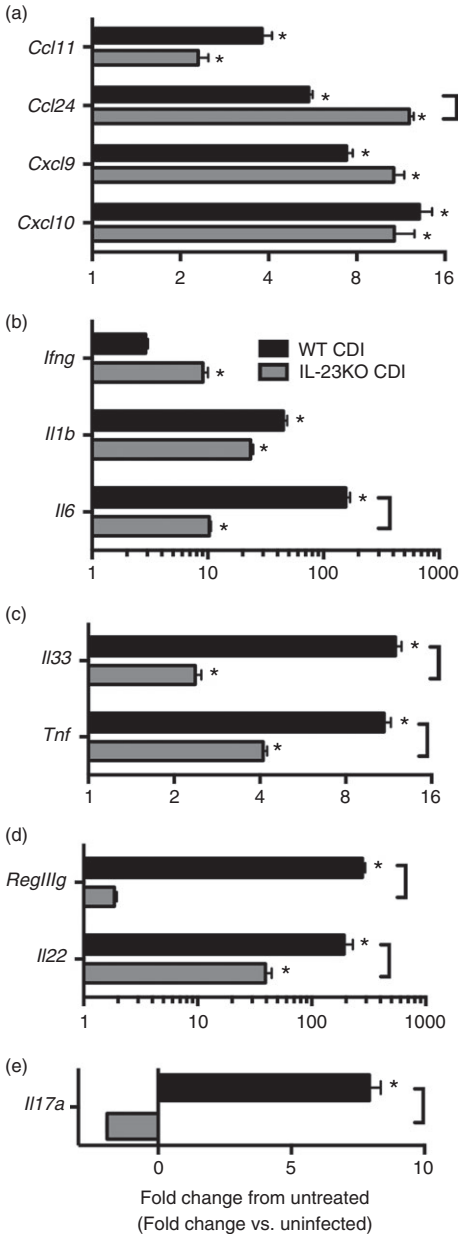

Figure 2. Effect of interleukin-23 (IL-23) deficiency on colonic (a) chemokine and (b–e) inflammatory cytokine expression during *Clostridium difficile* colitis. Host gene expression was measured as outlined in the methods. $n \geq 6$ per group. Data are shown as mean ± SEM fold change gene expression of wild-type (WT) *C. difficile*-infected (black bars) and IL-23KO *C. difficile*-infected (grey bars) animals compared with untreated WT mice. CDI = *C. difficile* infected. *$P < 0{\cdot}05$ compared with untreated WT animals. Brackets indicate $P < 0{\cdot}05$ for the differences between indicated groups.

Effect of IL-23 deficiency on colonic inflammatory cytokine expression

Analysis by RT-PCR was also used to determine the role of IL-23 in promoting inflammatory cytokine expression in response to *C. difficile* infection. Expression of the antimicrobial C-type lectin *RegIIIg* was significantly increased in response to *C. difficile* infection, and this induction was significantly reduced in IL-23KO mice (Fig. 2d). Interleukin-23-deficient animals displayed no reduction in *Il1b* or *Ifng* expression levels in response to *C. difficile* infection (Fig. 2b). However, expression levels of the inflammatory cytokines *Il6*, *Il33* and *Tnf* were all significantly reduced in the absence of IL-23 (Fig. 2b,c). Additionally, the increased expression of *Il17a* and *Il22* seen in WT animals was completely abrogated in IL-23KO mice (Fig. 2d,e). Hence, these data indicate that IL-23 promotes the induction of numerous inflammatory cytokines including *Il6*, *Il17a* and *Tnf*, as well as the pleiotropic cytokine *Il22*, in response to *C. difficile* colitis.

To assess the contribution of IL-23 signalling towards the development of intestinal inflammation and epithelial destruction during *C. difficile* colitis, sections of the colonic mucosa were examined for histopathological evidence of severe inflammation. In addition to significant neutrophilic influx (Fig. 1a,b), *C. difficile* infection was associated with significant epithelial damage and oedema, indicative of severe intestinal inflammation (Fig. 3). Interestingly, although the absence of IL-23 had no impact on the development of colonic epithelial damage (Fig. 3c), there was a trend towards reduced oedema in IL-23KO mice (Fig. 3b). Neutrophilic inflammation was also reduced in IL-23KO mice (data not shown). These data suggest that IL-23 also promotes the development of colonic oedema, but not epithelial damage, during *C. difficile* colitis.

The role of IL-17 during *C. difficile* colitis

To investigate the contribution of IL-17 in supporting neutrophil recruitment and mucosal inflammatory responses during *C. difficile* colitis, IL-17a$^{-/-}$ (IL-17KO) mice were infected with *C. difficile*. As in previous experiments, all samples were collected at 2 days post infection. Compared with *C. difficile* infection in WT animals, there was no reduction in expression of the neutrophil chemokines *Cxcl1*, *Cxcl2* and *Ccl3* within the colonic mucosa of IL-17KO mice (Fig. 4c). Consistently, cellular infiltrates were apparent in colonic sections from IL-17KO mice (Fig. 5a) and the levels of colonic neutrophilic inflammation were equivalent between IL-17KO and WT animals infected with *C. difficile* (Fig. 5b). These

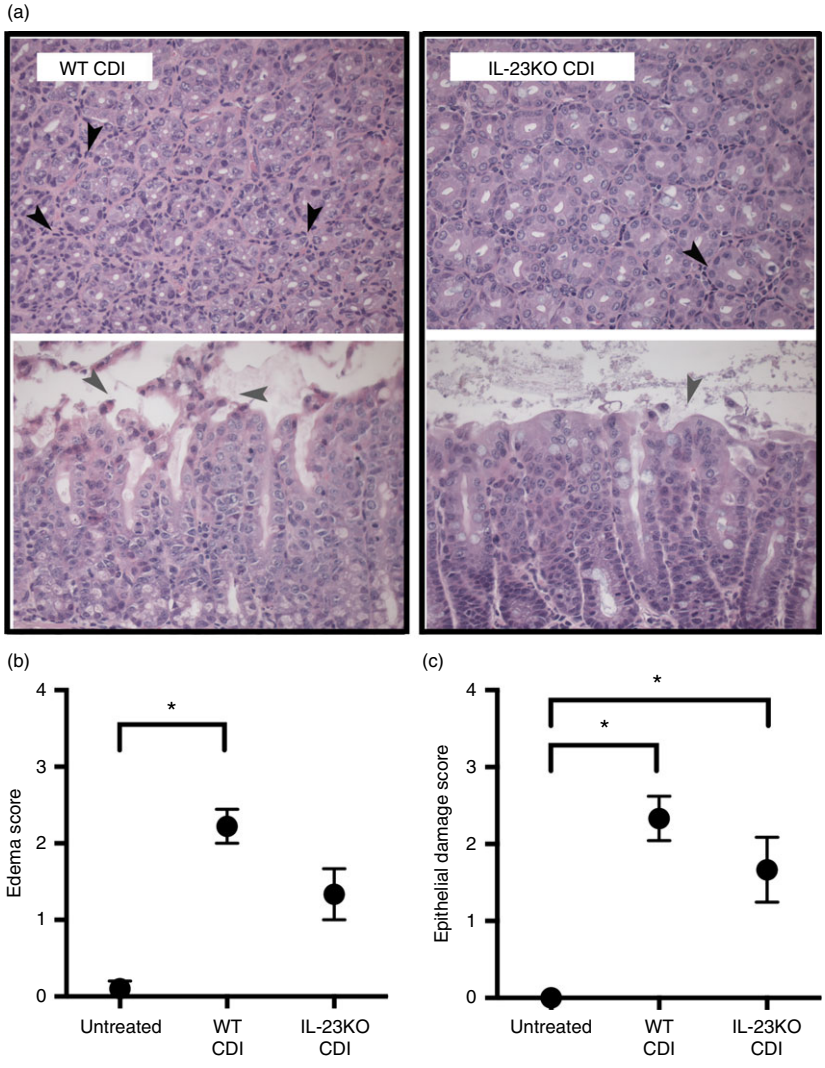

Figure 3. Colonic histopathology during *Clostridium difficile* infection in the absence of interleukin-23 (IL-23). (a) Representative photomicrographs of haematoxylin & eosin-stained colonic sections from wild-type (WT) *C. difficile*-infected and IL-23KO *C. difficile*-infected animals. Cross-sections of colonic crypts (upper images) and longitudinal sections of the epithelial–luminal interface (lower images) are shown for each genotype. Black arrowheads highlight cellular infiltrate, whereas grey arrowheads highlight epithelial damage. Total magnification for all images is 400×. (b, c) Histopathological scoring of colonic sections from Untreated, WT CDI, and IL-23KO CDI mice. Slides were scored for oedema (b) and epithelial damage (c) as described in the Materials and methods. Data are shown as mean ± SEM. $n \geq 6$ per group. CDI = *C. difficile* infected. Brackets indicate $P < 0{\cdot}05$ for the differences between indicated groups.

data suggest that IL-17 signalling is not required for the expression of neutrophil chemokines or the development of neutrophilic inflammation in response to *C. difficile* colitis.

Analysis by RT-PCR was also used to investigate the role of IL-17 in promoting inflammatory cytokine expression in response to *C. difficile* infection. Interestingly, the absence of IL-17 was not associated with any reduction in inflammatory cytokines, including *Ifng*, *Il1b*, *Il6*, *Il33* and *Tnf* (Fig. 4b,d). Additionally, *Ccl24*, *Cxcl9* and *Cxcl10* expression levels were unchanged in IL-17KO mice (Fig. 4a). Consistent with the unaltered induction of inflammatory cytokines seen in these animals, IL-17KO mice were not protected against the development of significant colonic epithelial damage and oedema during *C. difficile* infection (Fig. 5c,d). Taken together, these data support the hypothesis that neutrophil recruitment, inflammatory cytokine expression, and the development of colonic histopathology during *C. difficile* colitis are independent of IL-17 signalling.

The role of IL-22 during *C. difficile* colitis

To determine the role of IL-22 in supporting mucosal inflammatory responses to *C. difficile* infection, mice were treated with an anti-IL-22 mAb (clone 8E11) 1 day before and 1 day after *C. difficile* infection. Animals were followed for 2 days post infection at which point all samples were collected. Colonic sections from anti-IL-22-treated mice were examined for signs of marked histopathology. Anti-IL-22 treatment was associated with no reduction in epithelial damage or oedema compared with WT *C. difficile*-infected animals (Fig. 5c,d). In agreement with these findings, colonic expression of numerous pro-inflammatory cytokines including *Il1b*, *Il6* and *Il33* were unchanged in anti-IL-22-treated mice (Fig. 4b,d). Interestingly, the expression levels of other inflammatory cytokines, most notably *Tnf* and *Ifng* as well as *Cxcl10*, were significantly increased following anti-IL-22 treatment (Fig. 4a,b,d,). *RegIIIg* expression was significantly reduced in anti-IL-22-treated mice (Fig. 4e), indicating that the anti-IL-22 mAb

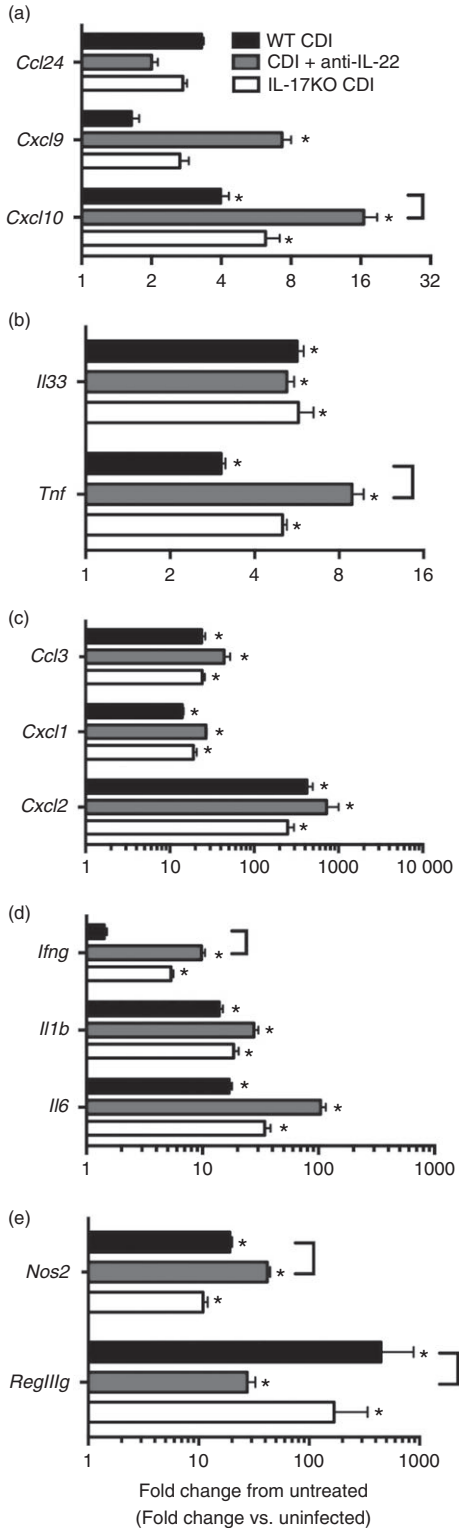

Figure 4. Effect of anti-interleukin-22 (IL-22) treatment or IL-17 deficiency on colonic (a, c) chemokine and (b, d, e) inflammatory cytokine expression during *Clostridium difficile* colitis. Colonic gene expression was assessed via quantitative PCR as outlined in the Materials and methods. $n \geq 6$ per group. Data are shown as mean ± SEM fold change gene expression of wild-type (WT) *C. difficile*-infected (black bars), *C. difficile*-infected and anti-IL-22-treated (grey bars), and IL-17KO *C. difficile*-infected (white bars) animals compared with untreated WT mice. CDI = *C. difficile* infected. *$P < 0{\cdot}05$ compared with untreated WT animals. Brackets indicate $P < 0{\cdot}05$ for the differences in expression levels between indicated groups.

treatment was sufficient to ablate IL-22 signalling *in vivo*. Colonic sections from anti-IL-22-treated mice were scored for neutrophilic inflammation. Anti-IL-22 treatment was not associated with any reduction in neutrophilic inflammation (Fig. 5a,b), and consistently, the expression levels of *Cxcl1*, *Cxcl2* and *Ccl3* were unchanged following anti-IL-22 treatment (Fig. 4c). Taken together, these data suggest that IL-22 does promote the induction of *RegIIIg*, but does not promote neutrophil recruitment or affect histopathology during the response to *C. difficile* colitis.

Discussion

In the current study, we reported decreased neutrophil recruitment in IL-23-deficient animals in response to *C. difficile* colitis. This decrease in neutrophil recruitment was associated with decreases in *Cxcl1* and *Cxcl2* expression, as well as with reduced expression of *Il-17a* and *Il-22*. However, neither *Cxcl1* and *Cxcl2* expression nor neutrophilic inflammation was reduced in either IL-17-deficient mice or mice treated with a depleting anti-IL-22 mAb. Hence, our data strongly suggest that IL-23, independent of IL-17 or IL-22, drives neutrophil recruitment and innate inflammatory responses during *C. difficile* colitis.

In the absence of IL-23, neutrophil recruitment was significantly reduced in response to *C. difficile* colitis. Recent studies have demonstrated increased levels of IL-23 in colonic biopsies from *C. difficile*-infected patients,[23] as well as increased levels of IL-23 production from myeloid cells stimulated with *C. difficile* toxins *in vitro*.[22] However, the role of IL-23 in supporting innate inflammatory responses, including neutrophil recruitment, remains poorly understood. In the current study, we observed reduced neutrophil recruitment in association with decreased expression of neutrophil chemotactic factors in the absence of IL-23. Previous studies have reported a role for IL-23 in supporting neutrophil recruitment and neutrophil chemokine production in other models of mucosal inflammation.[10,11,18–20,37,38] Interleukin-23 is required for the full recruitment of neutrophils to the large intestine in response to both *S. typhimurium* typhlocolitis,[11] as well as dextran sodium sulphate-induced colitis.[10] Additionally, neutrophil recruitment during pulmonary inflammation in response to both chemical[20] and microbial[18,19,38] chal-

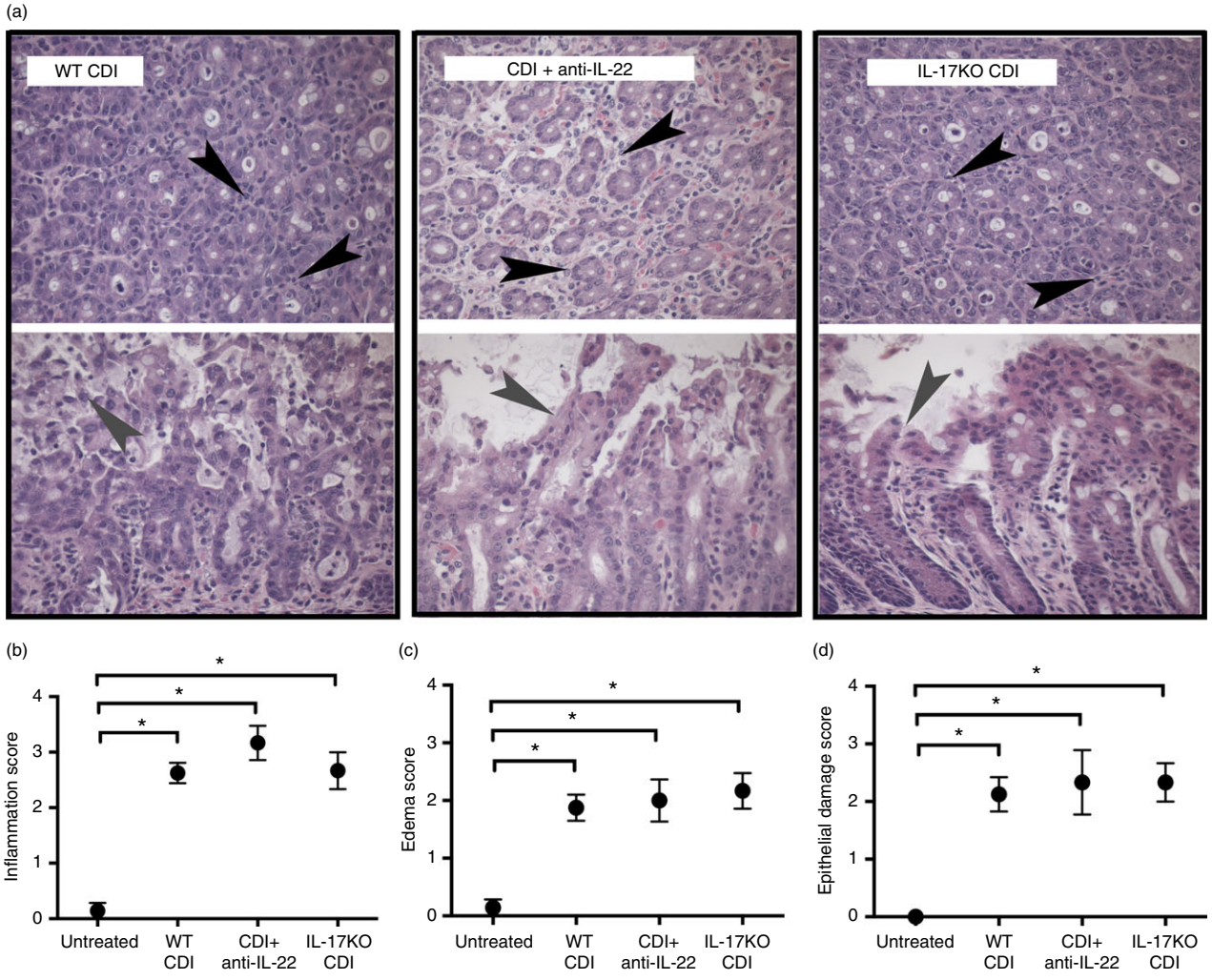

Figure 5. Colonic histopathology during *Clostridium difficile* infection in the absence of interleukin-17 (IL-17) or following anti-IL-22 treatment. Representative photomicrographs of haematoxylin & eosin-stained colonic sections from wild-type (WT) *C. difficile*-infected, *C. difficile*-infected and anti-IL-22-treated, and IL-17KO *C. difficile*-infected animals. Cross-sections of colonic crypts (upper images) and longitudinal sections of the epithelial–luminal interface (lower images) are shown for each genotype. Black arrowheads highlight leucocytic infiltrate, and grey arrowheads highlight areas of epithelial damage. Total magnification for all images is 400×. (b–d) Histopathological scoring of colonic sections from Untreated, WT CDI, CDI + anti-IL-22, and IL-17KO CDI mice. Slides were scored for neutrophilic inflammation (b), oedema (c), and epithelial damage (d) as described in the Materials and methods. Data are shown as mean ± SEM. $n \geq 6$ per group. CDI = *C. difficile* infected. Brackets indicate $P < 0{\cdot}05$ for the differences between indicated groups.

lenges is supported by IL-23. We observed a significant defect in the recruitment of $CD11b^{High}$ $Ly6G^{High}$ neutrophils, as well as the induction of the neutrophil chemokines *Cxcl1* and *Cxcl2*, in IL-23-deficient mice infected with *C. difficile*. Taken together, these data strongly suggest that IL-23 promotes neutrophil chemokine expression and neutrophil recruitment to the colon during *C. difficile* colitis.

Despite the robust increase in *Il22* expression within the colonic mucosa during *C. difficile* colitis, we observed no decrease in inflammatory cytokine and chemokine expression following anti-IL-22 treatment. Interleukin-22 promotes CXCL1 production from mouse tracheal epithelial cells,[39] and also supports neutrophil recruitment in response to chemical pulmonary challenge.[24] Additionally, IL-22 is capable of stimulating neutrophil chemokine expression from both colonic epithelial cells[28] and subepithelial myofibroblasts[27] *in vitro*. However, in agreement with a recent study by Hasegawa *et al.*,[14] we observed no decrease in expression of the neutrophil chemokines *Cxcl1* or *Cxcl2*, or reduced expression of the inflammatory cytokines *Il1b*, *Il6* or *Il33* following anti-IL-22 treatment. Neutrophilic inflammation, oedema, and epithelial damage levels were also unchanged following the administration of anti-IL-22. Furthermore, anti-IL-22 treatment was associated with increased expression of *Cxcl10*, *Ifng*

and *Tnf*. Hence, these data strongly suggest that IL-22 is not a major driver of neutrophil chemokine or inflammatory cytokine expression in response to *C. difficile* colitis.

Likewise, we observed no significant change in the severity of intestinal histopathology following anti-IL-22 treatment. The role of IL-22 during mucosal inflammation is pleiotropic: although IL-22 is protective against severe colonic histopathology and mortality during *Citrobacter rodentium* infection,[29] IL-22 drives severe intestinal histopathology and necrosis during *Toxoplasma gondii* infection.[21] Recent studies have reported no change in the severity of intestinal histopathology in IL-22KO animals[14] or following anti-IL-22 treatment[6] during *C. difficile* infection. Consistently, we found that anti-IL-22 treatment was not associated with a significant increase or amelioration of neutrophilic inflammation, colonic epithelial damage or oedema during *C. difficile* colitis. Hence, the data presented here indicate that the development of colonic histopathology is independent of IL-22.

The expression of the antimicrobial-peptide RegIIIγ was significantly decreased in both IL-23KO and anti-IL-22-treated IL-23-sufficient mice. *RegIIIg* induction during *Citrobacter rodentium* colitis is induced by IL-22 signalling, which is in turn induced by IL-23.[29] Marked induction of *RegIIIg* transcript has previously been reported in response to *C. difficile* infection of the large bowel.[5,6,31] Additionally, a recent study from our laboratory has demonstrated a significant reduction in *RegIIIg* expression during *C. difficile* colitis following neutralization of IL-22.[6] In addition to reduced *RegIIIg* expression following anti-IL-22 treatment, in the current study, we also observed a concomitant reduction in both *Il22* and *RegIIIg* expression in IL-23-deficient mice infected with *C. difficile*. Hence, these data strongly suggest that IL-23-dependent IL-22 signalling is required for full induction of *RegIIIg* expression during *C. difficile* colitis.

Interleukin-17 deficiency was not associated with any reduction in expression of inflammatory cytokines or neutrophil-attracting chemokines, including *Cxcl1* and *Cxcl2* within the colonic mucosal following *C. difficile* infection. Interleukin-17 has a well-documented role supporting neutrophil recruitment during inflammatory responses at mucosal sites.[12,13,24–26,40] Specific to the gut, IL-17 promotes CXCL1 expression in response to *S. typhimurium* typhlocolitis,[25] as well as supporting neutrophil recruitment during both dextran sodium sulphate-induced[12] and 2,4,6-trinitrobenzenesulphonic acid-induced[13] colitis. However, we observed no reduction in expression of the neutrophil chemoattractants *Cxcl1*, *Cxcl2* or *Ccl3* in the absence of IL-17. In agreement with the unaltered levels of chemokine expression, as well as the unaltered expression of the inflammatory cytokines *Il1b*, *Il6*, *Il33* and *Tnf*, IL-17 deficiency was not associated with a reduction in the severity of colonic histopathology. Taken together, our data support the hypothesis that IL-17 is dispensable for the recruitment of neutrophils, the induction of inflammatory cytokines, and the development of intestinal histopathology during *C. difficile* colitis.

In addition to decreased neutrophil recruitment, IL-23 deficiency was associated with decreased expression of the inflammatory cytokines *Il6*, *Il33* and *Tnf* as well as a trend towards decreased colonic oedema. Interleukin-23 contributes to IL-6 production in response to *Pseudomonas aeruginosa* pulmonary infection,[18] and IL-6 and IL-1β expression in response to *Toxoplasma gondii* ileitis is partially dependent upon IL-23.[21] Furthermore, interference with IL-23 signalling has been shown to reduce the severity of intestinal histopathology in both infectious[21] and chemical[10] models of gastrointestinal inflammation. In the current study, we report decreased colonic oedema in association with reduced inflammatory cytokine expression in IL-23-deficient animals. These data suggest that IL-23 contributes to the development of severe intestinal histopathology and drives the induction of inflammatory cytokines including *Il6* and *Il33* during *C. difficile* colitis.

The data presented in the current study suggest a clear role for IL-23 in supporting neutrophil recruitment, the induction of inflammatory cytokines, and the development of severe colonic histopathology during *C. difficile* infection. One possible model that could explain these phenomena is that neutrophils, recruited in part by IL-23 signalling, contribute to colonic histopathology and severe disease outcomes. Indeed, a recent study has demonstrated reduced morbidity and mortality in IL-23-deficient mice infected with *C. difficile*.[23] However, numerous previous studies have reported increased mortality during *C. difficile* infection following interventions that reduced neutrophilic influx,[1–3] suggesting a protective role for neutrophil recruitment. Furthermore, a recent study from our laboratory found no reduction in the severity of colonic histopathology during *C. difficile* colitis following anti-Gr-1 treatment.[4] Taken together these studies suggest that IL-23 signalling may ultimately play a dual role during *C. difficile* colitis by both promoting neutrophil recruitment as well as other innate responses that contribute to morbidity and intestinal histopathology during infection.

Acknowledgements

The authors would like to thank the staff of the University of Michigan Flow Cytometry Core for their assistance in performing the flow cytometry experiments. We thank the University of Michigan Unit for Laboratory Animal Medicine In-Vivo Animal Core, and specifically Dr Ingrid Bergin, for scoring of histological sections. We thank Dr Wenjun Ouyang for his kind gift of anti-IL-22 mAb. We thank Dr Paul Carlson Jr for supplying *C. difficile* spores

for this study. We thank Dr John Erb-Downward for his discussions and contributions to the project. We thank Charles Frank for technical contributions to the project. We also thank Dr Merritt Gillilland III for his critical reading of this manuscript. This work was supported by National Institutes of Health grants U19 AI090871 (GBH and VBY) and P30 DK034933 (GBH and VBY). Support was also provided by the Host-Microbiome Initiative (HMI) of the University of Michigan Medical School (GBH and VBY).

Author contributions

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

Disclosures

The authors declare no conflicts of interest.

References

1 Hasegawa M, Kamada N, Jiao Y, Liu MZ, Nunez G, Inohara N. Protective role of commensals against *Clostridium difficile* infection via an IL-1β-mediated positive-feedback loop. *J Immunol* 2012; **189**:3085–91.

2 Hasegawa M, Yamazaki T, Kamada N, Tawaratsumida K, Kim YG, Nunez G *et al.* Nucleotide-binding oligomerization domain 1 mediates recognition of *Clostridium difficile* and induces neutrophil recruitment and protection against the pathogen. *J Immunol* 2011; **186**:4872–80.

3 Jarchum I, Liu M, Shi C, Equinda M, Pamer EG. Critical role for MyD88-mediated neutrophil recruitment during *Clostridium difficile* colitis. *Infect Immun* 2012; **80**:2989–96.

4 McDermott AJ, Higdon KE, Muraglia R, Erb-Downward JR, Falkowski NR, McDonald RA *et al.* The role of Gr-1^{+} cells and tumour necrosis factor-α signalling during *Clostridium difficile* colitis in mice. *Immunology* 2015; **144**:704–16.

5 Sadighi Akha AA, Theriot CM, Erb-Downward JR, McDermott AJ, Falkowski NR, Tyra HM *et al.* Acute infection of mice with *Clostridium difficile* leads to eIF2α phosphorylation and pro-survival signalling as part of the mucosal inflammatory response. *Immunology* 2013; **140**:111–22.

6 Sadighi Akha AA, McDermott AJ, Theriot CM, Carlson PE Jr, Frank CR, McDonald RA *et al.* Interleukin-22 and CD160 play additive roles in the host mucosal response to *Clostridium difficile* infection in mice. *Immunology* 2015; **144**:587–97.

7 Price AB, Davies DR. Pseudomembranous colitis. *J Clin Pathol* 1977; **30**:1–12.

8 Kelly CP, Pothoulakis C, LaMont JT. *Clostridium difficile* colitis. *N Engl J Med* 1994; **330**:257–62.

9 Walker AS, Eyre DW, Wyllie DH, Dingle KE, Griffiths D, Shine B *et al.* Relationship between bacterial strain type, host biomarkers, and mortality in *Clostridium difficile* infection. *Clin Infect Dis* 2013; **56**:1589–600.

10 Cox JH, Kljavin NM, Ota N, Leonard J, Roose-Girma M, Diehl L *et al.* Opposing consequences of IL-23 signaling mediated by innate and adaptive cells in chemically induced colitis in mice. *Mucosal Immunol* 2012; **5**:99–109.

11 Godinez I, Raffatellu M, Chu H, Paixao TA, Haneda T, Santos RL *et al.* Interleukin-23 orchestrates mucosal responses to *Salmonella enterica* serotype Typhimurium in the intestine. *Infect Immun* 2009; **77**:387–98.

12 Ito R, Kita M, Shin-Ya M, Kishida T, Urano A, Takada R *et al.* Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. *Biochem Biophys Res Commun* 2008; **377**:12–6.

13 Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis* 2006; **12**:382–8.

14 Hasegawa M, Yada S, Liu MZ, Kamada N, Munoz-Planillo R, Do N *et al.* Interleukin-22 regulates the complement system to promote resistance against pathobionts after pathogen-induced intestinal damage. *Immunity* 2014; **41**:620–32.

15 Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest* 2010; **120**:2423–31.

16 Burdon PC, Martin C, Rankin SM. The CXC chemokine MIP-2 stimulates neutrophil mobilization from the rat bone marrow in a CD49d-dependent manner. *Blood* 2005; **105**:2543–8.

17 Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol* 2005; **5**:521–31.

18 Dubin PJ, Kolls JK. IL-23 mediates inflammatory responses to mucoid *Pseudomonas aeruginosa* lung infection in mice. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**:L519–28.

19 Dubin PJ, Martz A, Eisenstatt JR, Fox MD, Logar A, Kolls JK. Interleukin-23-mediated inflammation in *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* 2012; **80**:398–409.

20 Gasse P, Riteau N, Vacher R, Michel ML, Fautrel A, di Padova F *et al.* IL-1 and IL-23 mediate early IL-17A production in pulmonary inflammation leading to late fibrosis. *PLoS ONE* 2011; **6**:e23185.

21 Munoz M, Heimesaat MM, Danker K, Struck D, Lohmann U, Plickert R *et al.* Interleukin (IL)-23 mediates *Toxoplasma gondii*-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. *J Exp Med* 2009; **206**:3047–59.

22 Cowardin CA, Kuehne SA, Buonomo EL, Marie CS, Minton NP, Petri WA Jr. Inflammasome activation contributes to interleukin-23 production in response to *Clostridium difficile*. *MBio* 2015; **6**:e02386–14.

23 Buonomo EL, Madan R, Pramoonjago P, Li L, Okusa MD, Petri WA Jr. Role of interleukin 23 signaling in *Clostridium difficile* colitis. *J Infect Dis* 2013; **208**:917–20.

24 Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *J Exp Med* 2010; **207**:1293–305.

25 Keestra AM, Godinez I, Xavier MN, Winter MG, Winter SE, Tsolis RM *et al.* Early MyD88-dependent induction of interleukin-17A expression during *Salmonella* colitis. *Infect Immun* 2011; **79**:3131–40.

26 Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P *et al.* Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 2001; **194**:519–27.

27 Andoh A, Zhang Z, Inatomi O, Fujino S, Deguchi Y, Araki Y *et al.* Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology* 2005; **129**:969–84.

28 Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM *et al.* IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**:G827–38.

29 Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q *et al.* Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* 2008; **14**:282–9.

30 Perez J, Springthorpe VS, Sattar SA. Clospore: a liquid medium for producing high titers of semi-purified spores of *Clostridium difficile*. *J AOAC Int* 2011; **94**:618–26.

31 McDermott AJ, Frank CR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB. Role of GM-CSF in the inflammatory cytokine network that regulates neutrophil influx into the colonic mucosa during *Clostridium difficile* infection in mice. *Gut Microbes* 2014; **5**:476–84.

32 Theriot CM, Koumpouras CC, Carlson PE, Bergin II, Aronoff DM, Young VB. Cefoperazone-treated mice as an experimental platform to assess differential virulence of *Clostridium difficile* strains. *Gut Microbes* 2011; **2**:326–34.

33 Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB. The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* infection. *Gut Microbes* 2011; **2**:145–58.

34 Sadighi Akha AA, Harper JM, Salmon AB, Schroeder BA, Tyra HM, Rutkowski DT *et al.* Heightened induction of proapoptotic signals in response to endoplasmic reticulum stress in primary fibroblasts from a mouse model of longevity. *J Biol Chem* 2011; **286**:30344–51.

35 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3**:RESEARCH0034.

36 Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc* 2008; **3**:1101–8.

37 Hue S, Ahern P, Buonocore S, Kullberg MC, Cua DJ, McKenzie BS *et al.* Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 2006; **203**:2473–83.

38 Wu Q, Martin RJ, Rino JG, Breed R, Torres RM, Chu HW. IL-23-dependent IL-17 production is essential in neutrophil recruitment and activity in mouse lung defense against respiratory *Mycoplasma pneumoniae* infection. *Microbes Infect* 2007; **9**:78–86.

39 Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* 2008; **14**:275–81.

40 Liu J, Feng Y, Yang K, Li Q, Ye L, Han L *et al.* Early production of IL-17 protects against acute pulmonary *Pseudomonas aeruginosa* infection in mice. *FEMS Immunol Med Microbiol* 2011; **61**:179–88.

