Interleukin-6 drives neutrophil-mediated pulmonary inflammation associated with bacteraemia in murine models of colitis.

Sean W. Mateer [1, 2, 3], Andrea Mathe [1, 2, 3], Jessica Bruce [1, 2, 3], Gang Liu [1, 2, 3], Steven Maltby [1, 2], Michael Fricker [1, 2, 4], Bridie J. Goggins [1, 2, 3], , Hock L. Tay [1, 2], Ellen Marks [1, 2, 3], Grace Burns [1, 2, 3], Richard Y. Kim [1, 2], Kyra Minahan [1, 2, 3], Marjorie M. Walker [2, 3, 4], Robert C. Callister[1,3], Paul S. Foster [1, 2], Jay C. Horvat [1, 2], Philip M. Hansbro[1, 2, *] and Simon Keely[1, 2, 3, *, †]

[1] School of Biomedical Sciences & Pharmacy, University of Newcastle, , NSW, Australia

[2] Hunter Medical Research Institute, New Lambton Heights, NSW, Australia

[3] Priority Research Centre for Digestive Health and Neurogastroenterology, University of Newcastle, Newcastle, NSW, Australia

[4] School of Medicine & Public Health, University of Newcastle, Newcastle, NSW, Australia

* Authors contributed equally

†Correspondence to: Simon Keely, Ph.D., School of Biomedical Sciences & Pharmacy, University of Newcastle, NSW, Australia. Office phone: (02) 40420229 Fax: (02) 4042 0024 E-mail: simon.keely@newcastle.edu.au

Financial Support: National Health and Medical Research Council (NHMRC) Project Grant APP1128487) and Cancer Institute NSW Career Development Fellowship (SK). Hunter Medical Research Institute Bowel of the Ball Grant (SK, AM, GL). Emlyn and Jennie Thomas Postgraduate Medical Research Scholarship (BJG). **Disclosures**: There are no financial disclosures or conflicts of interest for any author.

Abstract

Inflammatory bowel disease (IBD) is associated with a number of immune-mediated extra-intestinal manifestations (EIM). Over half of all IBD patients have some form of respiratory pathology, most commonly neutrophil-mediated diseases such as bronchiectasis and chronic bronchitis. Using murine models of colitis, we aimed to identify the immune mechanisms driving pulmonary manifestations of IBD. We found increased neutrophil numbers in lung tissue associated with the pulmonary vasculature in both trinitrobenzenesulfonic acid (TNBS) and dextran sulfate sodium (DSS)-induced models of colitis. Analysis of systemic inflammation identified that neutrophilia was associated with bacteraemia and pyrexia in animal models of colitis. We further identified IL-6 as a systemic mediator of neutrophil recruitment from the bone marrow of DSS animals. Functional inhibition of IL-6 led to reduced systemic and pulmonary neutrophilia, but did not attenuate established colitis pathology. These data suggest that systemic bacteraemia and pyrexia drive IL-6 secretion, which is a critical driver for pulmonary manifestation of IBD. Targeting IL-6 may reduce neutrophil associated EIMs in IBD patients.

Introduction

Inflammatory bowel disease (IBD) is the umbrella term for a number of chronic inflammatory conditions that predominately affect the gastrointestinal (GI) tract, including Crohn's disease (CD) and ulcerative colitis (UC). In addition to pathology within the gastrointestinal tract, IBD patients also exhibit secondary organ pathologies. These are termed "extra-intestinal manifestations" (EIM) and develop as inflammatory responses in skin, eyes, joints, liver and lungs [1]. EIM are diagnosed in approximately 45% of IBD patients and can contribute significantly to the morbidity associated with IBD [2]. In particular, there is a growing appreciation of the prevalence of pulmonary EIMs in IBD. A 2007 systematic review by Black *et al*. reported cases of respiratory disease in 35% of IBD patients assessed [3]. Other studies that have aimed to directly access the prevalence of respiratory pathologies in IBD patients report that up to 64% of IBD patients examined have some form of respiratory pathology, although many of these may be subclinical in nature and thus are missed in routine diagnosis [4-9]. This has led to a general consensus that respiratory pathologies in IBD patients are more common than currently appreciated, prompting considerable research interest [2-5, 7-19].

The causes and conditions that drive pulmonary EIMs are not known, with current hypotheses suggesting that inflammatory and microbial "spill-over" from the GI tract, due to increased intestinal permeability, drives systemic inflammation and immune cell mis-homing to extra-intestinal organs [10]. Bacteraemia and endotoxaemia are pathologies associated with IBD [20-22] while intestinal permeability, which would promote passage of bacteria to the serosa, has been associated with dermatological, muscloskeletal and hepatic manifestations of IBD [23-25]. Animal studies suggest that pyrexia associated with bacteraemia can promote immune cell mis-homing [26]. Inappropriate immune cell homing is a feature of many EIMs of IBD. In the lung, leukocyte-mediated diseases affecting the central airways, such as bronchiectasis and chronic bronchitis,

are the most common EIMs of IBD [27]. While these diseases are quite distinct they do share a similar immunophenotype, characterised by an initial local cytokine secretion and neutrophil recruitment and extravasation into the lungs [28, 29].

Much of our understanding of the pathology of IBD comes from animal models that mimic immune-pathologies observed in CD and UC. For instance, the trinitrobenzenesulfonic acid (TNBS) and dextran sulfate sodium (DSS) murine models of colitis develop systemic inflammation driven by bacteraemia and elevated systemic inflammatory cytokines [30, 31]. While the subclinical nature of pulmonary EIM limits our understanding of the pathology, there is also a lack of basic research modelling the mechanisms of gut-lung crosstalk. Given that intestinal permeability and systemic inflammation are characteristic pathologies of animal models of colitis, we hypothesized that subclinical pulmonary pathology would occur in murine models of colitis and thus provide a model to investigate the pathogenesis of colitis-induced respiratory disease. In this study, we aimed to characterise the physiological and immunological features of colitis-induced respiratory pathologies. We examined leukocyte populations and cytokine networks present in the pulmonary and circulatory systems of murine models of colitis and identified IL-6 as a factor necessary for neutrophilia associated with pulmonary pathology during intestinal inflammation. Further, we found evidence of increased bacterial load and induction of neutrophil chemokines in the lungs of mice with colitis. These data support the hypothesis that EIMs are driven by a combination of increased intestinal permeability and systemic inflammatory signalling and demonstrate that animal models of colitis offer opportunities to study EIMs associated with IBD.

Methods

Murine models of colitis

For the DSS model of colitis, six-week-old female C57BL/6 mice were given 2.5% DSS *ad libitum* in drinking water as previously described [32]. Age matched controls received drinking water. Mice received DSS for 5 days and were sacrificed at day 7. For the TNBS colitis model, six-week-old female BALB/C mice were sensitised by epicutaneous application of 1% TNBS in acetone/olive oil solution (4:1). After seven days, mice were anesthetised with isoflourane and intrarectally administered 5 µL/g body weight of a 2.5% TNBS in 50% ethanol solution as previously described [33]. Control animals received an equivalent volume of 50% ethanol alone. Core body temperature was measured by infrared thermometer as previously described [31]. Mice were sacrificed 7 days post-intrarectal TNBS administration. For both models, body weight, faecal occult blood and stool consistency were recorded daily for each mouse to determine the disease activity index (DAI) as described previously [31, 34]. At the experimental end-point the colon, lungs, bone marrow and blood were collected for analysis.

Anti-IL-6 treatment models

For anti-IL-6 treatments 200 µg of anti-IL-6 (clone MP5-20F3; BioXCell, USA) was injected intraperitoneal (ip) on day 3 and day 5 after initiation of DSS exposure. Rat IgG1 (clone HRPN; BioXCell, USA) was administered as an isotype control.

Histopathological scoring

For subsets of experiments, the colon was excised, formalin fixed, embedded in paraffin and cut longitudinally into 5 µm sections. For lung pathology, lungs were perfused with 0.9% saline by cardiac puncture with a 19-gauge needle and fixed by intratracheal inflation with 1.5mL of 10% buffered formalin. The lungs were then excised, formalin fixed, embedded in paraffin, cut

longitudinally into 5µm sections. Sections were stained with hematoxylin and eosin (H&E) for histopathological assessment and were scored by previously validated and published criteria outlined in **Table 1**, for colon sections [33] and **Table 2** for lung sections [35, 36]. Structural damage to alveoli and lung parenchyma was calculated using the mean linear intercept (MLI) method as previously described [37, 38].

Airway inflammation assessment

Airway inflammation was determined by enumerating leukocytes in the bronchoalveolar lavage fluid (BALF). BALF was collected by intratracheal lavage with PBS as previously described [37]. The collected fluid was centrifuged (300xg, 10min, 4^0C) and the cell pellet resuspended in 500 µL of red blood cell lysis buffer (Tris-buffer NH_4CI) and incubated on ice for 5 minutes. Following incubation the cell suspension was pelleted, resuspended in 250 µL of PBS and viable (trypan blue negative) leukocytes enumerated by haemocytometer count.

Cellular analysis of lungs, blood and bone marrow

Single cell suspensions were generated from lung tissue, blood and bone marrow and stained for flow cytometry analysis as previously described [39]. Briefly, lung tissue was digested in HEPES buffer containing collagenase D (Sigma-Aldrich; St Louis, MO, USA) and DNAse for 1 hour at 37 ^{o}C, then passed through a 70 µm strainer. Cardiac puncture blood was collected in EDTA-coated microvette collection tubes (Sarstedt; Numbrecht, Germany). Bone marrow cells were isolated by flushing femurs with PBS/2% FCS. Following isolation, red blood cell lysis was performed and cells were enumerated by haemocytometer. After generation of single cell suspensions, cells were treated with anti-FcϒRIII/II Fc block (BD Biosciences, San Jose, USA) and incubated with fixable viability dye eFlour® 506 (Ebiosciences, Thermo Fisher, USA) for 20 minutes. The cells were then stained with combinations of fluorochrome-conjugated

antibodies, for 45 minutes. Cells were stained to assess myeloid populations (FITC-conjugated CD45, APC-conjugated Gr-1, PerCP-Cy5.5-conjugated CD11b, APC-Cy7-conjugated Ly6C and PE-conjugated Siglec-F) or lymphoid populations (PE-Cy7-conjugated CD45, PE-conjugated CD3, APC-conjugated CD4, PercP-conjugated CD8a and FITC-conjugated B220) or progenitor populations (APC-Cy7-conjugated CD45, PE-Cy7-conjugated Sca-1, PerCP-Cy5.5-conjugated c-kit, PE-conjugated CD150, FITC-conjugated CD48 and APC-conjugated lineage mixture containing (CD3e, CD11b, B220, Ly-76, & Gr-1)). All antibodies were supplied by BD Biosciences. Following antibody incubations the cells were washed and fixed in PBS/2% FCS/0.1% paraformaldehyde. Cells were analysed on a BD FACSCanto II flow cytometer, using FACS Diva software (BD Biosciences, San Jose, USA) or FlowJo (BD Biosciences, San Jose, USA). Cell phenotype was resolved utilizing the gating strategy in **Figure 3A**.

Bone Marrow Colony Forming Unit (CFU) Assays

Murine bone marrow was obtained by flushing femurs with DMEM supplemented with 2% FCS. After red blood cell lysis, a single cell suspension of bone marrow cells were plated at 2 x 10^4 cells/plate in Methocult GF3534 (Stem Cell Technologies, Canada). Plates were cultured for 7 days and myeloid colonies (CFU-granulocyte [CFU-G]/CFU-monocyte [CFU-M]/CFU-granulocyte/macrophage [CFU-GM]) were distinguished and counted by light microscopy based on cell size and colony morphology in accordance with the manufacturer's instructions [39].

RNA and protein analysis

RNA analysis was performed by quantitative polymerase chain reaction (qPCR) on a ViiA7 real-time PCR machine (Life Technologies, Waltham, USA) using SYBR green reagents (Biorad). Primers used were: *tnf* Fw: CCC ACT CTG ACC CCT TTA CT. *tnf* Rv: TTT GAG

TCC TTG ATG GTG GT *ifng* Fw: TCT TGA AAG ACA ATC AGG CCA TCA *ifng* Rv: GAA TCA GCA GCG ACT CCT TTT CC *il6* Fw: CTA CCC CAA TTT CCA ATG CT *il6* Rv: ACC ACA GTG AGG AAT GTC CA. Primer sets were designed to cross exon boundaries to specifically amplify mRNA products and expression was normalized to the reference gene beta-actin *actb* Fw: GGA GAA AAT CTG GCA CCA CA *actb* Rv: AGA GGC GTA CAG GGA TAG CA. Tissue and blood protein levels were measured by BCA assay (Pierce Biotechnology, Waltham, USA) and normalised. Colon, lung and blood were assayed for protein cytokine levels of IL-6, IL-10, CCL2, IFN-γ, and TNF-α using the mouse inflammation cytometric bead array kit (BD Biosciences, San Jose, USA) according to manufacturer's specifications and analysed on a BD FACSCanto II flow cytometer and analysed using the FCAP array software (BD Biosciences, San Jose, USA). KC (R&D Systems, Minneapolis, USA) and endotoxin (Lonza, Basel, Switzerland) were measured by ELISA.

Adoptive transfer of mesenteric lymph node cell isolate

Adoptive transfer of leukocytes was peformed by methods adapted from Miyabe *et al.*[40]. Mesenteric lymph nodes (MLN) were collected from both control and DSS colitis mice on day 7 after induction of DSS colitis. MLN were mechanically homogenised and debris was removed by filtering tissue through a 70μm nylon filter. The total MLN cell suspension was then centrifuged at (187g, 10mins, 4^{o}C) and resuspended at a concentration of 1x10^{7} cells/mL in PBS with 5 μM of carboxyfluorescein succinimidyl ester (CFSE) (5 min at room temperature). Cells were collected by centrifugation, washed in HBSS and resuspended in 1 mL of PBS and counted. Cell suspensions were diluted and 100 μL (1x10^{6} cells) was injected intravenously (i.v.) into control and DSS colitis-treated mice (day 7 post induction). 24 hours post injection, mice were sacrificed and the lungs collected for analysis by flow cytometry.

Statistical analysis

Comparisons between two groups were made using unpaired *t*-Tests. Comparisons between multiple groups were made using a one-way ANOVA. For analysis of data with two independent variables (such as weight loss data) a two-way ANOVA was utilized. Correlations were analysed by Pearson coefficient. Analyses were performed using GraphPad Prism Software (San Diego, California).

Results

Pulmonary inflammation occurs in murine colitis.

Initially we sought to examine whether structural or biochemical changes in the lung were associated with murine models of colitis. To do this, we utilised an acute 7-day model of DSS colitis, exhibiting characteristic weight loss (mean ± SD (% original weight); Control 101.51±2.881, DSS 83.44±3.96, p<0.0001) **(Figure 1A)** and colon shortening (mean ± SD (cm); Control 7.28±0.84, DSS 5.54±0.87, p=0.012) **(Figure 1B)** and increased Disease Activity Index scores (mean ± SD (arbitrary units); Control 0.43±0.20, DSS 7.71±0.29, p<0.0001) **(Figure 1C)** compared to control mice. Structural pathology as measured by histopathology scoring of H&E sections **(Figure 1D)** was significantly higher in DSS mice compared to controls (mean ± SD (arbitrary units); Control 0.34±0.51, DSS 7.34±1.75, p<0.005) **(Figure 1E)**. Levels of TNF-α **(Figure 1F)**, IFN-γ **(Figure 1G)** and IL-6 **(Figure 1H)** inflammatory cytokines were quantified in colon tissue, as these cytokines are up-regulated in colonic biopsies from IBD patients and are acknowledged to contribute to disease pathology and progression [41]. The protein levels of TNF-α (mean ± SD (pg/mL); Control 0.77± 0.79, DSS 64.08±64.51, p=0.0371), IFN-γ (mean ± SD (pg/mL); Control 0.0001±0.0001, DSS 3.01±2.22, p=0.0436) and IL-6 (mean ± SD (pg/mL); Control 3.86±0.81, DSS 94.50±75.43, p=0.0147) were significantly increased in colon tissue from DSS colitis mice compared to healthy controls.

Having confirmed intestinal pathology, we next sought to examine the airways for associated pulmonary manifestations of DSS colitis. Histopathology was quantified based on leukocyte congregation around the airway vasculature and parenchyma using a previously validated scoring [35] of H&E stained lung sections **(Figure 1I)**. A significant increase in histopathological scores were observed in the lungs of DSS colitis animals (mean ± SD (arbitrary units); Control

0.60 ±0.55, DSS 3.0±1.87, p=0.0249) **(Figure 1J)**, with the majority of inflammation localised around the pulmonary vasculature. To examine whether the vascular inflammation influenced lung morphology, alveoli diameter was calculated using the MLI method, however there was no change in alveoli diameter between control and DSS colitis animals **(Figure 1K)**. Given the histological evidence of immune infiltration, we assessed bronchoalveolar lavage fluid (BALF) for increases in leukocytes **(Figure 1L)** and found increased BALF cell numbers in DSS animals, compared to controls (mean±SD (cells/mL), Control 35.45 x 10^4±17.06 x10^4 DSS 51.49 x 10^4±14.26 x 10^4, p<0.035).

To examine whether colitis-associated pulmonary pathology was a feature specific to the DSS model of colitis, we extended our analysis into the TNBS model of colitis. TNBS colitis led to early, significant weight loss **(Figure 2A)**, and disease activity (mean ± SD (arbitrary units); Control 1.50±0.55, TNBS 5.17±1.47, p=0.0002) **(Figure 2B)** remained significantly increased in TNBS-treated animals on day 7, when compared to Ethanol (EtOH)-treated control groups. Colonic inflammation was also evident in TNBS-treated animals on day 7 **(Figure 2C)**, and histopathology scoring showed a significant increase in inflammatory pathology when compared to ethanol-treated controls (mean ± SD (arbitrary units); Control 0.67±0.52, TNBS 5.34±1.21, p<0.0001) **(Figure 2D)**. Upon histopathological examination of lung pathology in the TNBS colitis model **(Figure 2E)**, significant increases in pulmonary pathology were observed by pathology scoring (mean ± SD (arbitrary units); Control 0.84±0.75, TNBS 2.50±1.05, p=0.01) **(Figure 2F)**, consistant with findings in the DSS model. Also consistant with findings in the DSS model, we found increased leukocyte cell numbers in the BALF of TNBS animals, compared to controls **(Figure 2G)** (mean±SD (cells/mL), Control 15.43 x 10^4±7.58 x 10^4 TNBS 28.63 x 10^4±11.69 x 10^4, p<0.0179). Together these data demonstrate that leukocyte recruitment to the airway is a feature of animal models of colitis.

Myeloid, but not lymphoid cells, are recruited to the lung in acute colitis models

Our next aim was to determine the phenotype of leukocytes observed in the lungs in murine colitis models. Flow cytometry staining panels were designed to allow quantification **(Figure 3A)** of myeloid cell populations. A significant increase in the percentage of neutrophils (mean ± SD (%); Control 7.35±1.65, DSS 12.63±1.95, $p<0.001$) and inflammatory monocytes (mean ± SD (%); Control 3.68±0.63, DSS 7.72±1.14, $p<0.0028$) in the lung of DSS colitis mice was observed **(Figure 3B)**. These increases were also evident when comparing cell numbers (mean ± SD (cell numbers); Neutrophils: Control $1.57 \times 10^5 \pm 0.65 \times 10^5$, DSS $3.49 \times 10^5 \pm 1.19 \times 10^5$, $p< 0.0058$, Inflammatory Monocytes: Control $0.76 \times 10^5 \pm 0.24 \times 10^5$, DSS $2.18 \times 10^5 \pm 0.85 \times 10^5$, $p<0.0028$). Resident monocyte and eosinophil numbers were not altered between groups. Similarly, in TNBS animals neutrophil populations were increased (mean ± SD (%); Control 12.21±2.96, TNBS 20.27±2.11, $p=0.006$) but in contrast to DSS animals, there was no significant change in either inflammatory or tissue lung monocyte populations compared to healthy controls **(Figure 3C)**. No change was observed in lymphocyte populations in the lungs of DSS **(Figure 3D)** or TNBS **(Figure 3E)** mice, compared to controls. These data indicate increases in predominantly neutrophil leukocyte populations entering the lungs of animals during colitis.

Colitis-driven bacteraemia drives pulmonary inflammation

Having identified increases in the proportion of neutrophils in the lungs of DSS colitis animals, we next examined whether neutrophil recruitment to the lungs was an active or passive phenomenon. To this end, we performed an adoptive transfer of CFSE-stained MLN cells from healthy and DSS animals into healthy or DSS recipient animals and evaluated cell populations homing to the lungs of recipient animals **(Figure 4A)**. A total of $1x10^6$ cells unsorted MLN cells were transferred from control or DSS-treated animals. Within the transferred cell

population, this represents 40,080±18,730 neutrophils from control MLNs and 48,050±21,314 neutrophils transferred from MLNs isolated from DSS-treated mice. There was no significant increase in the percentages of CFSE-stained neutrophils in the lungs of either DSS donor to healthy recipient mice (mean ± SD (% transferred cells); 0.3505 ±0.1283) or healthy donor to DSS recipient mice (mean ± SD (cells); 0.3246±0.1748), when compared to healthy to healthy transfers (mean ± SD (cells); 0.1313±0.0725). However, there were significantly higher percentages of CFSE-stained neutrophils in the lungs of animals for the DSS donor to DSS recipient groups (mean ± SD (cells); 1.613±0.5479, $p<0.0001$) compared to all other groups. There were no differences in the numbers of other CFSE-stained myeloid or lymphoid cell populations. Together these data suggest that increased homing of neutrophils to the lung requires both local lung and systemic factors.

We next investigated local mediators of airway inflammation in DSS colitis. Gene expression analysis showed significant increases in *tnfa* (mean ± SD (fold change); Control 1.1±0.53, DSS 7.60±4.49, p=0.028), *ifng* (mean ± SD (fold change); Control 1.23±0.97, DSS 6.83±5.5, p=0.012), *il6* (mean ± SD (fold change); Control 1.06±0.35, DSS 7.62±6.38, p=0.011) and *il1β* (mean ± SD (fold change); Control 1.05±0.38, DSS 3.80±2.74, p=0.013) expression in the lungs of the DSS model compared to healthy controls **(Figure 4B)**. However, protein analysis of these cytokines showed increases in IL-1β alone (mean ± SD (pg/mL); Control 35.70±5.92, DSS 50.66±5.90, p=0.024), with no significant increases in TNF-α, IFN-γ or IL-6 protein levels with **(Figure 4C)**. Further assessment of inflammatory mediators identified a significant increase in both CCL2 (mean ± SD (pg/mL); Control 53.84±5.541, DSS 132.9±17.01, p=0.0013) and KC (mean ± SD (pg/mL); Control 15.67±2.66, DSS 41.50±17.69, p=0.0054) protein levels in the lungs of DSS mice **(Figure 4C)**, but no changes in protein levels of IL-12p40 or IL-17. As we have previously shown bacteraemia in animal models of colitis and

because CCL2 and KC are proinflammatory mediators during pulmonary inflammation induced by bacterial cell wall components[42], we also assessed endotoxin levels in the lung tissue of DSS mice, compared to controls **(Figure 4D)**. DSS mice exhibited a 37-fold increase in lung endotoxin (mean±SD (EU/mL); Control 0.016±0.03, DSS 0.606±0.036, $p<0.0001$). Given this increase, we next performed microbial culture on BALF from control and DSS animals and found an 18-fold increase in bacterial colony forming units in the BALF of DSS animals, when compared to controls **(Figure 4E)** (mean±SD (CFU/mL); Control 15.17±13.66, DSS 269.3±276.7, $p=0.0484$). Taken together, these data demonstrate an increased bacterial load in the lungs of DSS animals is associated with increased inflammatory and chemotactic signalling molecules, which may drive recruitment of inflammatory cells during colitis.

Neutrophilia underpins the systemic inflammation in DSS colitis

As leukocytes utilise the circulatory system to enter and exit tissues of interest, we next sought to investigate the effect of colitis on systemic inflammation and determine whether there is a relationship between the systemic and pulmonary inflammation induced by DSS colitis. Mice with DSS colitis exhibited significantly higher proportions of circulating neutrophils compared to control animals (mean±SD (%); Control 12.91±2.8, DSS 18.01±5.91, $p<0.05$, **Figure 5A**) but in contrast to the lung tissue of DSS animals there was no change in the proportion of monocytes. Furthermore, we identified a significant correlation between the percentage of neutrophils in the blood and the percentage of neutrophils in the lung (Pearson r^2 value =0.628, P=0.0021, **Figure 5B**), suggesting a possible relationship between systemic and pulmonary neutrophelia in DSS colitis mice. Given the correlation between lung and blood neutrophils, we investigated whether the same trends existed with serum cytokine levels. We found significant increases in systemic IL-6 (mean±SD (pg/mL); Control 3.89±0.34, DSS 20.16±16.57, $p<0.037$), IL1-β (mean±SD (pg/mL); Control 7.03±5.62, DSS 69.99±39.32,

p=0.023) and KC (mean±SD (pg/mL); Control 1.45±0.88, DSS 10.68±7.16, p=0.043) but not TNF-α, IFN-γ, CCL2, IL-12p70 or IL-17 **(Figure 5C)**, indicating that systemic inflammation is not a consequence of cytokine "spill over" into the circulation from either intestinal or pulmonary inflammation. As we have previously identified systemic IL-6 to be associated with pyrexia in animal models of colitis and because of the increased levels of bacteria and endotoxin observed in the lungs of DSS animals, we next investigated core body temperature as an indicator of a hyperthermic response and found that DSS animals had a significantly higher peak body temperature over the course of disease, when compared to controls (mean ± SD (Δ^{o}C); Control 0.01±0.57, DSS 1.28±0.45, p=0.0016) **(Figure 5D)**, suggesting the DSS model of colitis is associated with a pyrexic response. In order to verify the presence of circulating bacteria which drive pyrexia, we cultured CFU from the blood of control and DSS animals and found a 66-fold increase in bacterial colony forming units in the blood of DSS animals, when compared to controls (mean±SD (CFU/mL); Control 1.67±3.20, DSS 109.5±47.69, p=0.0003) **(Figure 5E)**. This supports the hypothesis that systemic bacteraemia (potentially due to loss of barrier function), leads to an increased burden in the lung and drives pulmonary inflammation.

Myeloid hematopoiesis is increased in DSS colitis.

Given the proportional increase in neutrophils in both the circulatory and pulmonary system with intestinal inflammation, we also characterised the influence of intestinal inflammation on myeloid cell progenitor populations in the bone marrow during DSS colitis. DSS mice had a small but significant increase in bone marrow lineage-negative cells, (mean±SD (%); Control 6.11±0.39, DSS 6.73±0.46, p=0.032) **(Figure 5F)**. This increase was attributed to an increase in multi-potent progenitor cells (MPP; $CD48^{+}CD150^{-}$) (mean±SD (%); Control 0.116±0.026, DSS 0.1995±0.034, p<0.001), witth no change in hematopoietic stem cells (HSC; $CD48^{-}$

CD150^{+}). We also performed bone marrow colony forming unit (CFU) assays to functionally quantify myeloid progenitor cell numbers. Bone marrow from DSS had significantly higher total CFUs (CD48^{+}CD150^{+}) than control animals (mean±SD; Control 83.25±9.39, DSS 108.13±10.54, p=0.0125) which was attributed to increases in granulocyte/monocyte CFU (CFU-GM; mean±SD; Control 32.38±4.15, DSS 42.88±5.94, p=0.027) and monocyte CFU (CFU-M; mean±SD; Control 28.63±4.31,DSS 39.25±4.37, p=0.013) populations but not granulocyte CFU (CFU-G) **(Figure 5G)**. Cellular analysis of the proportion of mature myeloid cells in the bone marrow revealed a significant decrease in the proportion of neutrophils (mean±SD (%); Control 35.28±1.80, DSS 28.71±4.34, p=0.0065) and an increase in the proportion of monocytes (mean±SD (%) Control 19.30±2.33 DSS 27.10±4.53, p=0.0038) **(Figure 5H)**. These results indicate that myeloid cell production is increased in bone marrow during DSS colitis and may indicate a rapid egress of neutrophils from the bone marrow niche to the circulatory system.

IL-6 is critical for pulmonary neutrophilia in DSS colitis.

Given the predominance of neutrophils in the lung and circulatory system of DSS animals and the increases in systemic IL-6, which can drive neutrophil mobilization, we next investigated whether IL-6 neutralization after the induction of colitis influenced the neutrophilia observed in DSS colitis. Serum levels of IL-6 were measured by CBA assay and were significantly reduced in anti-IL-6 treated animals compared to other groups (mean ± SD (pg/mL); Control 0.78±0.65, DSS (naïve) 19.33±8.24, DSS (isotype) 16.27±7.95 DSS (anti-IL6) 4.07±0.93) **(Figure 6A)**. DSS mice treated with anti-IL-6 lost significantly less weight compared to DSS mice treated with isotype controls or with untreated DSS mice (mean ± SD (% original weight); DSS (isotype) 79.34±1.59, DSS (anti-IL-6) 86.50±4.96, p<0.0001, DSS (untreated) 78.74±2.14, p<0.0001) **(Figure 6B)** however, no change in colon shortening **(Figure 6C),**

Disease Activity Index **(Figure 6D)** or histopathology score **(Figure 6E-F)** was observed between the naïve DSS, anti-IL-6 and isotype groups**,** suggesting that IL-6 is important in systemic responses during colitis but for maintaining local GI inflammation. In agreement with this, there was no change in the protein levels of inflammatory cytokines TNF-α, IFN-γ or CCL2 in the colon between the DSS isotype controls and DSS anti-IL-6 treated mice **(Figure 6G)**.

We next performed cellular analysis of the lungs of anti-IL-6 treated mice to assess whether neutralization of IL-6 reduced colitis-induced pulmonary immunopathology. Anti-IL-6 treated DSS mice exhibited significant decreases in both the proportion and total number of lung neutrophils compared to both the isotype-treated controls and untreated DSS mice (mean ± SD (%); DSS (anti-IL-6) 8.813±3.65, DSS (isotype) 15.70±1.79, p=0.003, DSS (naïve) 13.78±2.39, p=0.049) **(Figure 7A)**. However, there was no difference in the proportions of monocytes in the lungs of anti-IL-6-treated DSS mice, compared to isotype-treated controls **(Figure 7B)**. Given the increase in neutrophil recruiting mediators KC and CCL2 observed in the lungs of DSS colitis animals, we next examined whether IL-6 treatment had any effect on CCL2 **(Figure 7C)** and KC **(Figure 7D)** protein levels, but observed no significant differences with anti-IL-6 treatment compared to isotype controls. We also examined IL-1β in the lungs of anti-IL-6-treated DSS animals but found no differences compared to untreated or isotype control groups **(Figure 7E).** We next investigated whether the decrease in neutrophils entering the lung was a consequence of an overall decrease in circulating leukocytes due to neutralization of IL-6. The proportion and numbers of circulating neutrophils were significantly decreased following anti-IL-6 antibody administration, compared to isotype treated groups (mean ± SD (%); DSS (isotype) 23.83±9.77, DSS (anti-IL-6) 12.00±3.23, p=0.0297) **(Figure 7F).** In contrast, there was no significant difference in the proportion or

numbers of circulating monocytes in anti-IL-6- treated animals compared to isotype controls **(Figure 7G)**.

IL-6 mediates egress of neutrophils from the bone marrow during colitis

Because colitis induced the proliferation of neutrophil progenitor cells in the bone marrow, we hypothesized that the increase in neutrophils in the circulatory system was a result of IL-6-mediated stimulation of myeloid progenitor cells. To test this hypothesis, we examined whether IL-6 neutralization influenced neutrophil development in the bone marrow of DSS animals. Interestingly, anti-IL-6-treated animals had significantly higher bone marrow MPP cell proportions compared to isotype-treated groups (mean ± SD; DSS (isotype) 4.72±1.89, DSS (anti-IL-6) 11.34±1.31, $p<0.0001$) **(Figure 7H)**. Further, the proportion of mature neutrophils in the bone of anti-IL-6 treated animals were significantly greater than in isotype-treated groups (mean ± SD; DSS (isotype) 14.63±7.72, DSS (anti-IL-6) 39.30±6.28, $p=0.026$) **(Figure 7I)**. Together these data suggest that IL-6 signaling during DSS colitis mediates egress of neutrophils from the bone marrow, which contributes to systemic and extra-intestinal pathology during intestinal inflammation.

Discussion

The aim of this study was to identify and characterize lung immunopathology associated with animal models of colitis. We have previously demonstrated that loss of intestinal permeability in murine models of colitis is associated with increased bacteremia, characterized by recovery of bacterial CFU from distal organs [31]. Here, guided by the prevalence of neutrophilic pulmonary pathology reported in IBD patients, we hypothesized that the bacteremia and systemic inflammation in animal models of colitis would predispose to lung pathologies identifiable by structural and biochemical assessment. We identified a vascular accumulation of neutrophils common to the DSS and TNBS models of colitis. Neutrophil recruitment to the lung was associated with increased bacteria in the lung and elevated levels of IL-1β, CCL2 and KC, and dependent on systemic IL-6 signaling. However, ablation of systemic IL-6 did not prevent increases in inflammatory mediators in the lung, suggesting that pulmonary manifestations of IBD are a consequence of overall systemic inflammation.

Pulmonary pathologies have previously been identified in rat models of colitis with elevated ICAM-1 identified in lung tissue sections of a rat TNBS model [43] and increases in TNF-α and VEGF in both rat DSS and TNBS models [44]. Importantly both of these models suggested vascular involvement in the disease, but did not identify a mechanism analogous to the pulmonary manifestations observed in IBD patients. In our current study, the predominance of neutrophils in the lung infiltrate of the colitis models is analogous to pulmonary pathologies associated with IBD. For instance, bronchiectasis and chronic bronchitis are two of the most common pulmonary manifestations of IBD, irrespective of smoking status [27] and both are associated with elevated CCL2, IL-8 and neutrophil extravasation into the respiratory system [45], with the subsequent activation of these cells inducing structural pathologies. The inflammation observed in our murine DSS and TNBS models was associated with increases in

CCL2 and KC along with infiltration of neutrophils in the lung. Both CCL2 and KC are key mediators of neutrophil recruitment during pulmonary infection [42, 46] and here, we hypothesize that increases in intestinal permeability and bacteremia drive pyrexia, which coupled with secondary local inflammation in the lung activates circulating, primed neutrophils. In support of this hypothesis, targeted depletion of the key pyrogen IL-6 was sufficient to inhibit egress of neutrophils from the bone marrow in DSS animals, but did not reduce local inflammatory signaling in the lung. In addition, while anti-IL-6 treatment did not affect gastrointestinal inflammation it did reduce colitis-associated wasting, an observation consistent with previous studies [47-49]. The ability of IL-6 blockade to reduce wasting in colitis may be attributed to the pyrogenic effect elicited by IL-6 [50]. Indeed, we have previously shown IL-6 to be associated with bacteremia and pyrexia in mouse models of colitis [31]. Importantly, LPS-induced toll-like receptor 4 signaling drives the synthesis of pyrogenic cytokines, including IL-6, which is a key mediator of the pyrexic response [51]. Previous studies have demonstrated that treatment with anti-IL-6 neutralizing antibodies prevents the development of LPS-induced fever in rodents [52] and this is mirrored in IL-6 deficient animals [53]. In both cases, and despite the lack of a fever response, TNF-α and IL-1β levels increased with LPS challenge, suggesting a central importance for IL-6 in mediating fever. Indeed, evidence from experiments in PBMC cultures suggest that IL-6 can supress both IL-1β and TNF-α at the transcriptional level [54] and Diao *et al.* [55] have shown that in septic shock models, IL-6 limits infection through suppression of TNF-α. This may explain the absence of a TNF-α response in our model, as IL-6 may be suppressing TNF-α responses.

Systemic IL-6 inhibition has previously been shown to be protective in DSS colitis with reduced severity of colitis reported in *Il-6* deficient mice [47, 56] and attenuated disease associated with antibody depletion of both IL-6 [48] and the IL-6 signal transducer; gp130 [49]. Although,

here, anti-IL-6 treatment had no influence on the severity of GI pathology, this may be attributed to the timing of the interventions, as anti-IL-6 antibody was administered after colitis was initiated. In studies by Sanders *et al.*, where gp130, the common signal transducer for IL-6, was deleted in a DSS model, loss of IL-6 signalling delayed and attenuated acute disease, but only marginally affected leukocyte infiltration by day 7. Despite this, loss of IL-6 reduced circulating leukocytes and DSS-induced wasting. The study concluded that IL-6 was important for intestinal leukocyte recruitment in the acute phase of disease [49], and this is largely attributed to early activation of colonic resident myeloid [49]. Colitis also alters hematopoietic processes in the bone marrow, which enhances production of granulocytes and monocytes that are released in the circulatory system [57]. However, the mechanism for this process is not well understood and the specific soluble mediators that promote the release of neutrophils into the circulation during colitis are not known. Systemic levels of IL-6, driving fever can increase circulating neutrophils [58], which in turn, may lead to accumulation of neutrophils within peripheral tissues, including the lung[26, 59]. Neutralization of IL-6 attenuated the systemic neutrophilia associated with colitis and was associated with an increase in mature neutrophils in the bone marrow. The role of IL-6 in controlling granulopoiesis and by extension accumulation of neutrophils at inflammatory sites has been shown by Liu *et al.* and Chou *et al.* [60, 61]. Under homeostatic conditions, neutrophil egress from the bone marrow is mediated by CXCR4/SDF-1 interactions but in the context of inflammation, a number of chemokines may enable chemotaxis of neutrophils across the bone marrow sinusoidal endothelium. Our data may indicate that while IL-6 is important for driving neutrophil egress during bacteremia/pyrexia, it is not key to the increase in granulocytes in the bone marrow. Alternatively, the timing of our treatment, after induction of colitis, may have facilitated an early IL-6 mediated accumulation of neutrophils in the bone marrow, but prevented the post-treatment release into the circulation.

Overall, our data suggest that pyrexia and systemic bacteremia due to loss of intestinal barrier integrity play a role in extra-intestinal manifestation of colitis. We propose a two-hit hypothesis, facilitated by both increases in the pyrogen IL-6 and local secretion of neutrophil chemokines, such as IL-1β, KC/IL-8 and CCL2. The importance of a local factor is supported by the observation that adoptive transfer of neutrophils from DSS animals, there was significantly increased homing to the lung in DSS recipient animals but not healthy controls. Because the population of neutrophils transferred from healthy to DSS animals did not accumulate in the lung in significant numbers, it would appear that neither systemic responses nor local responses in isolation, are sufficient to promote pulmonary manifestations of colitis. While IL-6 does not directly mediate tissue pathology, depletion of systemic IL-6 reduces the number of neutrophils in the circulation that can migrate to the lung. In considering the clinical relevance of these findings, it is worth noting that bacteraemia and fever are common pathologies in episodes of IBD relapse, and IBD patients with pulmonary pathology commonly present with bronchiectasis and chronic bronchitis, both of which are neutrophil driven diseases. Whether these pathways are specific to pulmonary manifestations of IBD is unclear, but there is evidence of neutrophil involvement in the pathology of hepatic morbidity in IBD[62]. We have recently shown that lung disease, caused by chronic cigarette smoke, leads to systemic ischemia that drives intestinal dysfunction, predisposing to colitis [63]. Whether pulmonary damage initiated by colitis may perpetuate disease in a similar manner is unclear, but would be worth investigating. The utility of targeting IL-6 as an intervention for pyrexic disease has already been established and the IL-6 receptor antagonist tocilizumab has been investigating as a therapy for pyrexia and cachexia associated with cancers [64, 65]. In the context of IBD, our studies indicate that targeting IL-6 for the early management of bacteremia in IBD could reduce the risk of developing extra-intestinal complications.

Acknowledgements

This work was supported by funding from the National Health and Medical Research Council (NHMRC Project Grant APP1128487) as well as a Cancer Institute NSW Career Development Fellowship (SK) the Hunter Medical Research Institute grant from Bowel of the Ball (SK, AM, GL) and an Emlyn and Jennie Thomas Postgraduate Medical Research Scholarship (BJG).

References

[1] Levine JS, Burakoff R: Extraintestinal manifestations of inflammatory bowel disease. Gastroenterol Hepatol (N Y) 2011, 7:235-41.
[2] Danese S, Semeraro S, Papa A, Roberto I, Scaldaferri F, Fedeli G, Gasbarrini G, Gasbarrini A: Extraintestinal manifestations in inflammatory bowel disease. World J Gastroenterol 2005, 11:7227-36.
[3] Black H, Mendoza M, Murin S: Thoracic manifestations of inflammatory bowel disease. Chest 2007, 131:524-32.
[4] Eade OE, Smith CL, Alexander JR, Whorwell PJ: Pulmonary function in patients with inflammatory bowel disease. Am J Gastroenterol 1980, 73:154-6.
[5] Herrlinger KR, Noftz MK, Dalhoff K, Ludwig D, Stange EF, Fellermann K: Alterations in pulmonary function in inflammatory bowel disease are frequent and persist during remission. Am J Gastroenterol 2002, 97:377-81.
[6] Marvisi M, Borrello PD, Brianti M, Fornarsari G, Marani G, Guariglia A: Changes in the carbon monoxide diffusing capacity of the lung in ulcerative colitis. Eur Respir J 2000, 16:965-8.
[7] Ates F, Karincaoglu M, Hacievliyagil SS, Yalniz M, Seckin Y: Alterations in the pulmonary function tests of inflammatory bowel diseases. Turk J Gastroenterol 2011, 22:293-9.
[8] Bonniere P, Wallaert B, Cortot A, Marchandise X, Riou Y, Tonnel AB, Colombel JF, Voisin C, Paris JC: Latent pulmonary involvement in Crohn's disease: biological, functional, bronchoalveolar lavage and scintigraphic studies. Gut 1986, 27:919-25.
[9] Tzanakis N, Bouros D, Samiou M, Panagou P, Mouzas J, Manousos O, Siafakas N: Lung function in patients with inflammatory bowel disease. Respir Med 1998, 92:516-22.
[10] Keely S, Talley NJ, Hansbro PM: Pulmonary-intestinal cross-talk in mucosal inflammatory disease. Mucosal Immunol 2012, 5:7-18.
[11] Zhao Y, Wang J, Liu Z, Lin H, Shi Y, Sun X: Pulmonary dysfunction in 114 patients with inflammatory bowel disease. Medicine (Baltimore) 2017, 96:e6808.
[12] Wang H, Liu JS, Peng SH, Deng XY, Zhu DM, Javidiparsijani S, Wang GR, Li DQ, Li LX, Wang YC, Luo JM: Gut-lung crosstalk in pulmonary involvement with inflammatory bowel diseases. World J Gastroenterol 2013, 19:6794-804.
[13] Romano C, Cardile S: Pulmonary implications in inflammatory bowel disease: not a rare event. Expert Opin Drug Saf 2016, 15:1001-2.
[14] Majewski S, Piotrowski W: Pulmonary manifestations of inflammatory bowel disease. Arch Med Sci 2015, 11:1179-88.
[15] Ji XQ, Wang LX, Lu DG: Pulmonary manifestations of inflammatory bowel disease. World J Gastroenterol 2014, 20:13501-11.
[16] Ji XQ, Ji YB, Wang SX, Zhang CQ, Lu DG: Alterations of pulmonary function in patients with inflammatory bowel diseases. Ann Thorac Med 2016, 11:249-53.
[17] Gut G, Ben-Tov A, Lahad A, Soferman R, Cohen S, Tauman R, Sivan Y: Pulmonary functions in children with inflammatory bowel disease. Eur J Gastroenterol Hepatol 2016, 28:708-13.
[18] Gelberg J, Stather DR: Pulmonary manifestations of inflammatory bowel disease. Can Respir J 2013, 20:84.
[19] Al-Mendalawi MD: Alterations of pulmonary function in patients with inflammatory bowel diseases. Ann Thorac Med 2017, 12:129.

[20] Lodes MJ, Cong Y, Elson CO, Mohamath R, Landers CJ, Targan SR, Fort M, Hershberg RM: Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest 2004, 113:1296-306.
[21] Pastor Rojo O, Lopez San Roman A, Albeniz Arbizu E, de la Hera Martinez A, Ripoll Sevillano E, Albillos Martinez A: Serum lipopolysaccharide-binding protein in endotoxemic patients with inflammatory bowel disease. Inflammatory bowel diseases 2007, 13:269-77.
[22] Gardiner KR, Halliday MI, Barclay GR, Milne L, Brown D, Stephens S, Maxwell RJ, Rowlands BJ: Significance of systemic endotoxaemia in inflammatory bowel disease. Gut 1995, 36:897-901.
[23] Sheth T, Pitchumoni CS, Das KM: Musculoskeletal manifestations in inflammatory bowel disease: a revisit in search of immunopathophysiological mechanisms. J Clin Gastroenterol 2014, 48:308-17.
[24] Tornai T, Palyu E, Vitalis Z, Tornai I, Tornai D, Antal-Szalmas P, Norman GL, Shums Z, Veres G, Dezsofi A, Par G, Par A, Orosz P, Szalay F, Lakatos PL, Papp M: Gut barrier failure biomarkers are associated with poor disease outcome in patients with primary sclerosing cholangitis. World J Gastroenterol 2017, 23:5412-21.
[25] Pietrzak D, Pietrzak A, Krasowska D, Borzecki A, Franciszkiewicz-Pietrzak K, Polkowska-Pruszynska B, Baranowska M, Reich K: Digestive system in psoriasis: an update. Arch Dermatol Res 2017, 309:679-93.
[26] Rice P, Martin E, He JR, Frank M, DeTolla L, Hester L, O'Neill T, Manka C, Benjamin I, Nagarsekar A, Singh I, Hasday JD: Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental gram-negative bacterial pneumonia. Journal of immunology (Baltimore, Md : 1950) 2005, 174:3676-85.
[27] Mateer SW, Maltby S, Marks E, Foster PS, Horvat JC, Hansbro PM, Keely S: Potential mechanisms regulating pulmonary pathology in inflammatory bowel disease. Journal of leukocyte biology 2015, 98:727-37.
[28] Richman-Eisenstat JB, Jorens PG, Hebert CA, Ueki I, Nadel JA: Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. Am J Physiol 1993, 264:L413-8.
[29] Eller J, Lapa e Silva JR, Poulter LW, Lode H, Cole PJ: Cells and cytokines in chronic bronchial infection. Ann N Y Acad Sci 1994, 725:331-45.
[30] Alex P, Zachos NC, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X: Distinct Cytokine Patterns Identified from Multiplex Profiles of Murine DSS and TNBS-Induced Colitis. Inflammatory bowel diseases 2009, 15:341-52.
[31] Keely S, Campbell EL, Baird AW, Hansbro PM, Shalwitz RA, Kotsakis A, McNamee EN, Eltzschig HK, Kominsky DJ, Colgan SP: Contribution of epithelial innate immunity to systemic protection afforded by prolyl hydroxylase inhibition in murine colitis. Mucosal Immunol 2014, 7:114-23.
[32] Marks E, Naudin C, Nolan G, Goggins BJ, Burns G, Mateer SW, Latimore JK, Minahan K, Plank M, Foster PS, Callister R, Veysey M, Walker MM, Talley NJ, Radford-Smith G, Keely S: Regulation of IL-12p40 by HIF controls Th1/Th17 responses to prevent mucosal inflammation. Mucosal Immunol 2017, 10:1224-36.
[33] Marks E, Goggins BJ, Cardona J, Cole S, Minahan K, Mateer S, Walker MM, Shalwitz R, Keely S: Oral delivery of prolyl hydroxylase inhibitor: AKB-4924 promotes localized mucosal healing in a mouse model of colitis. Inflammatory bowel diseases 2015, 21:267-75.
[34] Campbell EL, MacManus CF, Kominsky DJ, Keely S, Glover LE, Bowers BE, Scully M, Bruyninckx WJ, Colgan SP: Resolvin E1-induced intestinal alkaline phosphatase promotes resolution of inflammation through LPS detoxification. Proc Natl Acad Sci U S A 2010, 107:14298-303.

[35] Horvat JC, Beagley KW, Wade MA, Preston JA, Hansbro NG, Hickey DK, Kaiko GE, Gibson PG, Foster PS, Hansbro PM: Neonatal chlamydial infection induces mixed T-cell responses that drive allergic airway disease. Am J Respir Crit Care Med 2007, 176:556-64.
[36] Liu G, Cooley MA, Nair PM, Donovan C, Hsu AC, Jarnicki AG, Haw TJ, Hansbro NG, Ge Q, Brown AC, Tay H, Foster PS, Wark PA, Horvat JC, Bourke JE, Grainge CL, Argraves WS, Oliver BG, Knight DA, Burgess JK, Hansbro PM: Airway remodelling and inflammation in asthma are dependent on the extracellular matrix protein fibulin-1c. J Pathol 2017, 243:510-23.
[37] Beckett EL, Stevens RL, Jarnicki AG, Kim RY, Hanish I, Hansbro NG, Deane A, Keely S, Horvat JC, Yang M, Oliver BG, van Rooijen N, Inman MD, Adachi R, Soberman RJ, Hamadi S, Wark PA, Foster PS, Hansbro PM: A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. J Allergy Clin Immunol 2013, 131:752-62.
[38] Liu G, Cooley MA, Jarnicki AG, Hsu AC, Nair PM, Haw TJ, Fricker M, Gellatly SL, Kim RY, Inman MD, Tjin G, Wark PA, Walker MM, Horvat JC, Oliver BG, Argraves WS, Knight DA, Burgess JK, Hansbro PM: Fibulin-1 regulates the pathogenesis of tissue remodeling in respiratory diseases. JCI Insight 2016, 1.
[39] Maltby S, Hansbro NG, Tay HL, Stewart J, Plank M, Donges B, Rosenberg HF, Foster PS: Production and differentiation of myeloid cells driven by proinflammatory cytokines in response to acute pneumovirus infection in mice. Journal of immunology (Baltimore, Md : 1950) 2014, 193:4072-82.
[40] Miyabe Y, Kim ND, Miyabe C, Luster AD: Studying Neutrophil Migration In Vivo Using Adoptive Cell Transfer. Methods Mol Biol 2016, 1407:179-94.
[41] Neurath MF: Cytokines in inflammatory bowel disease. Nat Rev Immunol 2014, 14:329-42.
[42] van Zoelen MA, Verstege MI, Draing C, de Beer R, van't Veer C, Florquin S, Bresser P, van der Zee JS, te Velde AA, von Aulock S, van der Poll T: Endogenous MCP-1 promotes lung inflammation induced by LPS and LTA. Mol Immunol 2011, 48:1468-76.
[43] Liu Y, Wang XY, Yang X, Jing S, Zhu L, Gao SH: Lung and intestine: a specific link in an ulcerative colitis rat model. Gastroenterol Res Pract 2013, 2013:124530.
[44] Aydin B, Songur Y, Songur N, Aksu O, Senol A, Ciris IM, Sutcu R: Investigation of pulmonary involvement in inflammatory bowel disease in an experimental model of colitis. Korean J Intern Med 2016, 31:853-9.
[45] Rose CE, Jr., Sung SS, Fu SM: Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. Microcirculation 2003, 10:273-88.
[46] Singh IS, Gupta A, Nagarsekar A, Cooper Z, Manka C, Hester L, Benjamin IJ, He JR, Hasday JD: Heat shock co-activates interleukin-8 transcription. Am J Respir Cell Mol Biol 2008, 39:235-42.
[47] Naito Y, Takagi T, Uchiyama K, Kuroda M, Kokura S, Ichikawa H, Yanagisawa R, Inoue K, Takano H, Satoh M, Yoshida N, Okanoue T, Yoshikawa T: Reduced intestinal inflammation induced by dextran sodium sulfate in interleukin-6-deficient mice. International journal of molecular medicine 2004, 14:191-6.
[48] Sommer J, Engelowski E, Baran P, Garbers C, Floss DM, Scheller J: Interleukin-6, but not the interleukin-6 receptor plays a role in recovery from dextran sodium sulfate-induced colitis. International journal of molecular medicine 2014, 34:651-60.
[49] Sander LE, Obermeier F, Dierssen U, Kroy DC, Singh AK, Seidler U, Streetz KL, Lutz HH, Muller W, Tacke F, Trautwein C: Gp130 signaling promotes development of acute experimental colitis by facilitating early neutrophil/macrophage recruitment and activation. Journal of immunology (Baltimore, Md : 1950) 2008, 181:3586-94.

[50] Netea MG, Kullberg BJ, Van der Meer JWM: Circulating Cytokines as Mediators of Fever. Clinical Infectious Diseases 2000, 31:S178-S84.
[51] Evans SS, Repasky EA, Fisher DT: Fever and the thermal regulation of immunity: the immune system feels the heat. Nat Rev Immunol 2015, 15:335-49.
[52] Cartmell T, Poole S, Turnbull AV, Rothwell NJ, Luheshi GN: Circulating interleukin-6 mediates the febrile response to localised inflammation in rats. J Physiol-London 2000, 526:653-61.
[53] Kozak W, Kluger MJ, Soszynski D, Conn CA, Rudolph K, Leon LR, Zheng H: IL-6 and IL-1 beta in fever. Studies using cytokine-deficient (knockout) mice. Ann N Y Acad Sci 1998, 856:33-47.
[54] Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA: Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood 1990, 75:40-7.
[55] Diao H, Kohanawa M: Endogenous interleukin-6 plays a crucial protective role in streptococcal toxic shock syndrome via suppression of tumor necrosis factor alpha production. Infect Immun 2005, 73:3745-8.
[56] Grivennikov S, Karin E, Terzic J, Mucida D, Yu G-Y, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L, Karin M: IL-6 and Stat3 Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer. Cancer Cell 2009, 15:103-13.
[57] Trottier MD, Irwin R, Li Y, McCabe LR, Fraker PJ: Enhanced production of early lineages of monocytic and granulocytic cells in mice with colitis. Proc Natl Acad Sci U S A 2012, 109:16594-9.
[58] Capitano ML, Nemeth MJ, Mace TA, Salisbury-Ruf C, Segal BH, McCarthy PL, Repasky EA: Elevating body temperature enhances hematopoiesis and neutrophil recovery after total body irradiation in an IL-1-, IL-17-, and G-CSF-dependent manner. Blood 2012, 120:2600-9.
[59] Tulapurkar ME, Almutairy EA, Shah NG, He JR, Puche AC, Shapiro P, Singh IS, Hasday JD: Febrile-range hyperthermia modifies endothelial and neutrophilic functions to promote extravasation. Am J Respir Cell Mol Biol 2012, 46:807-14.
[60] Liu F, Poursine-Laurent J, Wu HY, Link DC: Interleukin-6 and the granulocyte colony-stimulating factor receptor are major independent regulators of granulopoiesis in vivo but are not required for lineage commitment or terminal differentiation. Blood 1997, 90:2583-90.
[61] Chou DB, Sworder B, Bouladoux N, Roy CN, Uchida AM, Grigg M, Robey PG, Belkaid Y: Stromal-derived IL-6 alters the balance of myeloerythroid progenitors during Toxoplasma gondii infection. Journal of leukocyte biology 2012, 92:123-31.
[62] Seibold F, Weber P, Schoning A, Mork H, Goppel S, Scheurlen M: Neutrophil antibodies (pANCA) in chronic liver disease and inflammatory bowel disease: do they react with different antigens? Eur J Gastroenterol Hepatol 1996, 8:1095-100.
[63] Fricker M, Goggins BJ, Mateer S, Jones B, Kim RY, Gellatly SL, Jarnicki AG, Powell N, Oliver BG, Radford-Smith G, Talley NJ, Walker MM, Keely S, Hansbro PM: Chronic cigarette smoke exposure induces systemic hypoxia that drives intestinal dysfunction. JCI Insight 2018, 3.
[64] Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH: Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 2013, 368:1509-18.
[65] Ando K, Takahashi F, Kato M, Kaneko N, Doi T, Ohe Y, Koizumi F, Nishio K, Takahashi K: Tocilizumab, a proposed therapy for the cachexia of Interleukin6-expressing lung cancer. PLoS One 2014, 9:e102436.

Table 1: Gastrointestinal histopathological scoring system

Score 1 - Inflammation	
0	No evidence for inflammation
1	Low level of inflammation with scattered infiltrating mononuclear cells (1–2 foci only)
2	Moderate inflammation with multiple foci
3	High level of inflammation with increased vascular density and marked wall thickening
4	Maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells.
Score 2 - Injury	
0	No epithelial injury
1	Occasional epithelial lesion
2	1–2 foci of ulcerations
3	Extensive ulcerations.
Score 3 – Colitis activity	
Colitis activity scored on a scale of 0 – 3, based on the properties below, where 0 = no colitis and 3 = maximal colitis	
	Hypervascularzation
	Presence of mononuclear cells
	Epithelial hyperplasia
	Epithelial injury
	Presence of neutrophils
Score 4 – Lymphoid aggregates	
	Lymphoid aggregates; scored on a scale of 0 - 3
Total score = (score 1 + score 2 + score 3 +score 4)	

Table 2: Histopathological scoring system for mouse lungs

Score	Scoring Criteria
Score 1 - Airways inflammation	
0	Lack of inflammatory cells around airways – Absent
1	Some airways have small number of cells – Mild
2	Some airways have significant inflammation – Moderate
3	Majority of airways have some inflammation – Marked
4	Majority of airways are significantly inflamed – Severe
Score 2 -Vascular inflammation	
0	Lack of inflammatory cells around vessels – Absent
1	Some vessels have small number of cells – Mild
2	Some vessels have significant inflammation – Moderate
3	Majority of vessels have some inflammation – Marked
4	Majority of vessels are significantly inflamed – Severe
Score 3 - Parenchymal inflammation (10x magnification)	
0	<1% affected
1	1-9% affected
2	10-29% affected
3	30-49% affected
4	>50% affected
Total score = (score 1 + score 2 + score 3)	

Figure Legends:

Figure 1: Intestinal and pulmonary pathology in a DSS murine model of colitis .

(A) Percentage weight loss in DSS animals normalized to controls over the time course of colitis. (B) Colon length at experimental end-point. (C) Disease Activity Index (DAI) scores assessed based on stool consistency, occult blood, colon shortening and weight loss. (D) Representative images of colon pathology with arrows denoting epithelial injury, immune infiltration and vascular thickening. (E) Histopathology scoring of the extent of disease as outlined in Table 1. Inflammatory cytokine levels quantified by CBA assay for (F) TNF-α, (G) IFN-γ and (H) IL-6 in colon tissue. (I) Representative images of pulmonary histopathology with arrows denoting inflammation. (J) Quantitative analysis of lung histopathology. (K) The diameter of alveoli in the lungs measured by the mean linear intercept (MLI) method. (L) Airway inflammation determined by enumeration of leukocytes in bronchoalveolar lavage (BAL) fluid. All data represented as mean ± S.E.M *=$p<0.05$ ***=$p<0.005$, (A) two-way ANOVA, (B, C, E-H and J-L) unpaired t-test. Data is representative of two pooled experiments, n=6-14.

Figure 2: Intestinal and pulmonary pathology in a TNBS murine model of colitis .

(A) Percentage weight loss in TNBS animals normalized to controls over the time course of colitis. (B) Disease Activity Index (DAI) scores assessed from stool consistency, occult blood, colon shortening and weight loss. (C) Representative images of colon pathology with arrows denoting epithelial injury and immune infiltration. (D) Histopathology scoring of the extent of disease. (E) Representative images of the pulmonary histopathology, arrows denote inflammation. (F) Quantitative analysis of lung histopathology. (G) Airway inflammation determined by enumeration of leukocytes in bronchoalveolar lavage fluid (BALF). All data

represented as mean ± S.E.M *=p<0.05 ***=p<0.005, (A) two-way ANOVA, (B, D, F and G) unpaired t-test. Data is representative of two pooled experiments (e) n=6, n=8-14 for all others.

Figure 3: Immune cell populations in the lungs of control and DSS colitis mice. (A) Representative gating strategy for the identification of leukocytes in lungs. Hematopoietic cells were identified as $CD45^+$ cells and Eosinophils ($SiglecF^+$), Neutrophils ($Gr\text{-}1^+CD11b^+Ly6C^{int}SSC^{int}$), inflammatory (inflam) monocytes ($Gr\text{-}1^+CD11b^+Ly6C^{hi}$ SSC^{lo}) and tissue resident (Res) monocytes ($Gr\text{-}1^{lo}CD11b+Ly6C^{lo}SSC^{lo}$) were quantified by flow cytometry (B) The proportion (%$CD45^+$ cells) and total number of myeloid cells in the lung. (C) The proportion of myeloid cells in the lung of TNBS colitis mice. (D) The proportion of lymphoid cells in the lungs of DSS colitis mice and (E) TNBS colitis mice, represented as %$CD45^+$ cells. All data represented as mean ± S.E.M. from two separate experiments (n = 6). *=p<0.05, unpaired t-test.

Figure 4: Airway inflammation in DSS colitis mice. (A) CFSE-stained cells harvested from the MLN of control and DSS mice were injected into either naïve control or DSS colitis recipient mice. The percent of total transferred neutrophils ($CFSE^+$ $Gr\text{-}1^+CD11b^+$ $Ly6C^{int}SSC^{int}$) in the lung were calculated 24 hours post-transfer. (B) Gene expression analysis of TNF-α, IFN-γ, IL-6 and IL-1β and (C) proteins levels TNF-α, IFN-γ, IL-6, IL-1β, CCL2, KC, IL-12p70 and IL-17 measured in the lung. (D) Endotoxin levels and (E) cultured bacterial colony forming units (CFU) from the lungs of control and DSS animals. All data represented as mean ± S.E.M. *=p<0.05 ***=p<0.005, ****=p<0.0001, (A) One-way ANOVA, (B-E) unpaired t-test. (n=6)

Figure 5: Systemic inflammation in DSS colitis. (A) The proportion of neutrophils and monocytes in the blood of DSS colitis mice, compared to controls, represented as %$CD45^+$

cells. (B) A correlation between the number of neutrophils in the blood and the lung neutrophil numbers quantified by flow cytometry. (C) Systemic cytokines TNF-α, IFN-γ, IL-6, IL-1β, CCL2, KC, IL-12p70 and IL-17 in serum measured by ELISA (IL-1β, KC) and cytometric bead array. (D) Mean peak body temperature, measured by infra-red thermometer over 7-day course of DSS model. (E) Bacterial colony forming units (CFU) cultured from whole blood of control and DSS animals. (F) Proportion of linage negative cells (Lin-), hematopoietic stem cells (HSC; Lin-CD48-CD150+) and multipotent progenitor stem (MPP; Lin-CD48+CD150-) cells in the bone marrow, measured by flow cytometry. (G) The number of granulocyte (CFU-G), macrophage (CFU-M) and granulocyte/macrophage (CFU-GM) colony forming units in bone marrow. (H) The proportion of mature neutrophils and monocytes in the bone marrow, measured by flow cytometry. All data represented as mean ± S.E.M. N=6 from two separate experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ ((A, C-H) unpaired t-test, (B) Pearson correlation

Figure 6: Influence of IL-6 depletion on lung and colon pathology in DSS colitis.

DSS-treated animals and DSS groups were treated with anti-IL-6 antibody or isotype control antibody. (A) Serum IL-6 levels measured by cytometric bead array. Animals were monitored for (B) weight loss normalized to naïve controls and (C) colon length, (D) Disease Activity Index (DAI) assessed by stool consistency, occult blood, colon shortening and weight loss. (E) Quantitative analysis of colon histopathology. (F) Representative images of histopathology observed in the colon, arrows denote epithelial damage and immune infiltration. (G) TNF-α, IFN-γ and CCL2 protein levels in the colon, measured by cytometric bead array and expressed as cytokine/total protein. All data represented as mean ± S.E.M, (A, C, D E and G) one-way ANOVA. (B) two-way ANOVA. All data represented as mean ± S.E.M. from two separate experiments (n = 8) *=$p<0.05$, **=$p<0.01$, ***=$p<0.005$, ****=$p<0.001$

Figure 7: Influence of IL-6 depletion on lung, blood and bone marrow cellularity in DSS colitis.

DSS animals were treated with anti-IL-6 or isotype control and compared to healthy control and naïve DSS animals. $CD45^{+ve}$ cells were isolated from the lungs, blood and bone marrow and assessed by flow cytometry. The proportion and total cell number of (A) neutrophils and (B) monocytes in the lungs. Protein levels of (C) CCL2, (D) KC and (E) IL-1β in the lungs, as measured by cytometric bead array. The proportion and total cell number of blood (F) neutrophils and (G) monocytes in the blood. (H) The number of multi-potent progenitor (MPP; Lin-CD48+CD150-) cells in the bone marrow. (I) The proportion and total cell number of mature neutrophil levels in the bone marrow s. All data represented as mean ± S.E.M. from two separate experiments (N=8) *=$p<0.05$, **=$p<0.01$, ***=$p<0.005$ One-way ANOVA.