Inflammatory bowel disease (IBD) is associated with several immune-mediated extraintestinal manifestations. More than 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 and dextran sulfate sodium induced models of colitis. Analysis of systemic inflammation identified that neutrophilia was associated with bacteremia and pyrexia in animal models of colitis. We further identified IL-6 as a systemic mediator of neutrophil recruitment from the bone marrow of dextran sulfate sodium animals. Functional inhibition of IL-6 led to reduced systemic and pulmonary neutrophilia, but it did not attenuate established colitis pathology. These data suggest that systemic bacteremia and pyrexia drive IL-6 secretion, which is a critical driver for pulmonary manifestation of IBD. Targeting IL-6 may reduce neutrophil-associated extraintestinal manifestations in IBD patients.

Inflammatory bowel disease (IBD) is the umbrella term for several chronic inflammatory conditions that predominately affect the gastrointestinal (GI) tract, including Crohn disease and ulcerative colitis. In addition to pathology within the GI tract, IBD patients also exhibit secondary organ pathologies. These are termed extraintestinal manifestations (EIMs) and develop as inflammatory responses in skin, eyes, joints, liver, and lungs. EIMs are diagnosed in approximately 45% of IBD patients and can contribute significantly to the morbidity associated with IBD. 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. 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. This has led to a general consensus that respiratory pathologies in IBD patients are more common than currently appreciated, prompting considerable research interest.

The causes and conditions that drive pulmonary EIMs are not known, with current hypotheses suggesting that inflammatory and microbial spillover from the GI tract, attributable to increased intestinal permeability, drives systemic inflammation and immune cell mishoming to
extraintestinal organs.\textsuperscript{10} Bacteremia and endotoxemia are pathologies associated with IBD,\textsuperscript{20–22} whereas intestinal permeability, which would promote passage of bacteria to the serosa, has been associated with dermatologic, musculoskeletal, and hepatic manifestations of IBD.\textsuperscript{23–25} Animal studies suggest that pyrexia associated with bacteremia can promote immune cell mishoming.\textsuperscript{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.\textsuperscript{27} Although these diseases are distinct, they share a similar immunophenotype, characterized by an initial local cytokine secretion and neutrophil recruitment and extravasation into the lungs.\textsuperscript{28,29}

Much of our understanding of the pathology of IBD comes from animal models that mimic immune pathologies observed in Crohn disease and ulcerative colitis. For instance, the trinitrobenzenesulfonic acid (TNBS) and dextran sulfate sodium (DSS) murine models of colitis develop systemic inflammation driven by bacteremia and elevated systemic inflammatory cytokines.\textsuperscript{30,31} Although the subclinical nature of pulmonary EIM limits our understanding of the pathology, there is also a lack of basic research modeling the mechanisms of gut-lung cross talk. 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 characterize the physiological and immunologic features of colitis-induced respiratory pathologies. Leukocyte populations and cytokine networks present in the pulmonary and circulatory systems of murine models of colitis were examined, and IL-6 was identified as a factor necessary for neutrophilia associated with pulmonary pathology during intestinal inflammation. Furthermore, there was evidence of increased bacterial load and induction of neutrophil chemo- kines 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 signaling and demonstrate that animal models of colitis offer opportunities to study EIMs associated with IBD.

**Materials and Methods**

**Murine Models of Colitis**

For the DSS model of colitis, 6-week—old female C57BL/6 mice were given 2.5% DSS ad libitum in drinking water, as previously described.\textsuperscript{32} Age-matched controls received drinking water. Mice received DSS for 5 days and were sacrificed at day 7. For the TNBS colitis model, 6-week—old female BALB/C mice were sensitized by epicutaneous application of 1% TNBS in acetone/olive oil solution (4:1). After 7 days, mice were anesthetized with isoflurane and intrarectally administered 5 \( \mu \)L/g body weight of a 2.5% TNBS in 50% ethanol solution, as previously described.\textsuperscript{33} Control animals received an equivalent volume of 50% ethanol alone. Core body temperature was measured by infrared thermometer, as previously described.\textsuperscript{31} Mice were sacrificed 7 days after intrarectal TNBS administration. For both models, body weight, fecal occult blood, and stool consistency were recorded daily for each mouse to determine the disease activity index, as described previously.\textsuperscript{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 \( \mu \)g of anti—IL-6 (clone MP5-20F3; BioXCell, West Lebanon, NH) was injected intraperitoneally on day 3 and day 5 after initiation of DSS exposure. Rat IgG1 (clone HRPN; BioXCell) 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 sections (5 \( \mu \)m thick). For lung pathology, lungs were perfused with 0.9% saline by cardiac puncture with a 19-gauge needle and fixed by intratracheal inflation with 1.5 mL of 10% buffered formalin. The lungs were then excised, formalin fixed, embedded in paraffin, cut longitudinally into sections (5 \( \mu \)m thick). Sections were stained with hematoxylin and eosin for histopathological assessment and were scored by previously validated and published criteria outlined in Table 1 for colon sections\textsuperscript{33} and in Table 2 for lung sections.\textsuperscript{35,36} Structural damage to alveoli and lung parenchyma was calculated using the mean linear intercept method, as previously described.\textsuperscript{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 phosphate-buffered saline (PBS), as previously described.\textsuperscript{37} The collected fluid was centrifuged (300 \( \times \) g, 10 minutes, 4\( ^\circ \)C), and the cell pellet was resuspended in 500 \( \mu \)L of red blood cell lysis buffer (Tris-buffer NH4Cl) and incubated on ice for 5 minutes. After incubation, the cell suspension was pelleted and resuspended in 250 \( \mu \)L of PBS, and viable (trypan blue—negative) leukocytes were enumerated by hemocytometer 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.\textsuperscript{39} Briefly, lung tissue was digested in HEPES buffer containing collagenase D (Sigma-Aldrich, St. Louis, MO) and DNase for 1 hour at 37\( ^\circ \)C, then passed through a 70-\( \mu \)m 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% fetal calf serum. After isolation, red blood cell lysis was performed and cells were enumerated by hemocytometer. After generation of single-cell suspensions, cells were treated with anti-Fc γRIII/II Fc block (BD Biosciences, San Jose, CA) and incubated with fixable viability dye eFlour 506 (Ebiosciences, Thermo Fisher Scientific, Waltham, MA) for 20 minutes. The cells were then stained with combinations of fluorochrome-conjugated antibodies, for 45 minutes. Cells were stained to assess myeloid populations (fluorescein isothiocyanate–conjugated CD45, allophycocyanin (APC)-conjugated Gr-1, PerCP-Cy5.5–conjugated CD11b, APC-Cy7–conjugated Ly6C, and phosphatidylethanolamine (PE)–conjugated Siglec-F), lymphoid populations (PE-Cy7–conjugated CD45, PE-conjugated CD3, APC-conjugated CD4, PerCP-conjugated CD8a, and fluorescein isothiocyanate–conjugated B220), or progenitor populations (APC-Cy7–conjugated CD45, PE-Cy7–conjugated Sca-1, PerCP-Cy5.5–conjugated c-kit, PE-conjugated CD150, fluorescein isothiocyanate–conjugated CD48, and APC-conjugated lineage mixture containing CD3e, CD11b, B220, Ly-76, and Gr-1). All antibodies were supplied by BD Biosciences (San Jose, CA). After antibody incubations, the cells were washed and fixed in PBS/2% fetal calf serum/0.1% paraformaldehyde. Cells were analyzed on a BD FACSCanto II flow cytometer, using FACS Diva software version 8.0 (BD Biosciences) or FlowJo version 10.4.1 (BD Biosciences).

### Bone Marrow CFU Assays

Murine bone marrow was obtained by flushing femurs with Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum. After red blood cell lysis, a single-cell suspension of bone marrow cells was plated at 2 × 10⁴ cells/plate in Methocult GF3534 (Stemcell Technologies, Vancouver, BC, Canada). Plates were cultured for 7 days, and myeloid colonies [colony-forming unit (CFU)–granulocyte/macrophage] were distinguished and counted by light microscopy on the basis of cell size and colony morphology, in accordance with the manufacturer’s instructions.

### RNA and Protein Analysis

RNA analysis was performed by real-time quantitative PCR on a ViiA7 real-time PCR machine (Life Technologies, Waltham, Massachusetts). Protein analysis was performed by western blotting using standardized procedures.

### Table 1 Gastrointestinal Histopathological Scoring System

<table>
<thead>
<tr>
<th>Score 1: inflammation</th>
<th>Score 2: injury</th>
<th>Score 3: colitis activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No evidence for inflammation</td>
<td>Colitis activity scored on a scale of 0–3, on the basis of the properties below, where 0 = no colitis and 3 = maximal colitis</td>
</tr>
<tr>
<td>1</td>
<td>Low level of inflammation with scattered infiltrating mononuclear cells (1 to 2 foci only)</td>
<td>Hypervascularization</td>
</tr>
<tr>
<td>2</td>
<td>Moderate inflammation with multiple foci</td>
<td>Presence of mononuclear cells</td>
</tr>
<tr>
<td>3</td>
<td>High level of inflammation with increased vascular density and marked wall thickening</td>
<td>Epithelial hyperplasia</td>
</tr>
<tr>
<td>4</td>
<td>Maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells</td>
<td>Epithelial injury</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Score 4: lymphoid aggregates</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoid aggregates; scored on a scale of 0–3</td>
<td>(score 1 + score 2 + score 3 + score 4)</td>
</tr>
</tbody>
</table>

### Table 2 Histopathological Scoring System for Mouse Lungs

<table>
<thead>
<tr>
<th>Score 1: airway inflammation</th>
<th>Score 2: vascular inflammation</th>
<th>Score 3: parenchymal inflammation (×10 magnification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Lack of inflammatory cells around airways: absent</td>
<td>0 Lack of inflammatory cells around vessels: absent</td>
<td>0 &lt;1% Affected</td>
</tr>
<tr>
<td>1 Some airways have small number of cells: mild</td>
<td>1 Some vessels have small number of cells: mild</td>
<td>1 1%–9% Affected</td>
</tr>
<tr>
<td>2 Some airways have significant inflammation: moderate</td>
<td>2 Some vessels have significant inflammation: moderate</td>
<td>2 10%–29% Affected</td>
</tr>
<tr>
<td>3 Most airways have some inflammation: marked</td>
<td>3 Most vessels have some inflammation: marked</td>
<td>3 30%–49% Affected</td>
</tr>
<tr>
<td>4 Most airways are significantly inflamed: severe</td>
<td>4 Most vessels are significantly inflamed: severe</td>
<td>4 &gt;50% Affected</td>
</tr>
</tbody>
</table>

Total score = (score 1 + score 2 + score 3).
MA) using SYBR Green reagents (Bio-Rad Laboratories, Hercules, CA). Primers used were as follow: tnf (forward) and 3'-TTTGAGTCCTTGATGGTGGT (reverse); ifng (forward) and 3'-GAATCAGCAGCGACTCCTTTTCC (reverse); and il6 (forward) and 3'-CCCACTCTGACCCCTTTACT (reverse). Primer sets were designed to cross exon boundaries to specifically amplify mRNA products, and expression was normalized to the reference gene β-actin: actb (forward) and 3'-GGAGAAAATCTGGCACCA (reverse). Tissue and blood protein levels were measured by bicinchoninic acid assay (Pierce Biotechnology, Waltham, MA) and normalized. Colon, lung, and blood were assayed for protein cytokine levels of IL-6, IL-10, chemokine (C-C motif) ligand (CCL) 2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α using the mouse inflammation cytometric bead array kit (BD Biosciences), according to the manufacturer’s specifications, analyzed on a BD FACSCanto II flow cytometer, and analyzed using the FCAP array software version 3.0 (BD Biosciences). Keratinocyte chemoattractant (KC; R&D Systems, Minneapolis, MN) and endotoxin (Lonza, Basel, Switzerland) were measured by enzyme-linked immunosorbent assay.

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 scores assessed on the basis of stool consistency, occult blood, colon shortening, and colon weight loss. D: Representative images of colon pathology, with arrows denoting epithelial injury, immune infiltration, and vascular thickening. Boxed areas are shown at higher magnification in the right column (700 × 450-μm area). E: Histopathology scoring of the extent of disease, as outlined in Table 1. F–H: Inflammatory cytokine levels quantified by cytometric bead array for tumor necrosis factor (TNF)-α (F), interferon (IFN)-γ (G), and IL-6 (H) in colon tissue. I: Representative images of pulmonary histopathology, with arrows denoting inflammation. Boxed areas are shown at higher magnification in the bottom row (900 × 600-μm area). J: Quantitative analysis of lung histopathology. K: The diameter of alveoli in the lungs measured by the mean linear intercept method. L: Airway inflammation determined by enumeration of leukocytes in bronchoalveolar lavage fluid (BALF). Statistical analysis was performed by two-way analysis of variance (A) or unpaired t-test (B, C, E–H, and J–L). Data are representative of two pooled experiments. Data are expressed as means ± SEM (B, C, E–H, and J–L), n = 6 to 14 (A–C, E–H, and J–L). *P < 0.05, **P < 0.01, and ***P < 0.005 versus control. Original magnification: ×20 (D, left column); ×10 (D, right column, and I, bottom row); ×4 (I, top row).
Adoptive Transfer of MLN Cell Isolate

Adoptive transfer of leukocytes was performed by methods adapted from Miyabe et al. Mesenteric lymph nodes (MLNs) were collected from both control and DSS colitis mice on day 7 after induction of DSS colitis. MLNs were mechanically homogenized, and debris was removed by filtering tissue through a 70-μm nylon filter. The total MLN cell suspension was then centrifuged at 187 × g for 10 minutes at 4°C and resuspended at a concentration of 1 × 10^7 cells/mL in PBS with 5 μmol/L of carboxyfluorescein succinimidyl ester (CFSE; 5 minutes at room temperature). Cells were collected by centrifugation, washed in Hanks’ balanced salt solution, resuspended in 1 mL of PBS, and counted. Cell suspensions were diluted, and 100 μL (1 × 10^6 cells) was injected intravenously into control and DSS colitis–treated mice (day 7 after induction). Twenty-four hours after injection, mice were sacrificed and the lungs were 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 analysis of variance. For analysis of data with two independent variables (such as weight loss data), a two-way analysis of variance was used. Correlations were analyzed by Pearson coefficient. Analyses were performed using GraphPad Prism Software version 7.0 (GraphPad, San Diego, CA).

Results

Pulmonary Inflammation Occurs in Murine Colitis

Initially, it was examined whether structural or biochemical changes in the lung were associated with murine models of colitis. An acute 7-day model of DSS colitis was used. This model exhibited characteristic weight loss [means ± SD (% original weight): control, 101.51 ± 2.881; DSS, 83.44 ± 3.96; *P* < 0.0001] (Figure 1A) and colon shortening [means ± SD (cm): control, 7.28 ± 0.84; DSS, 5.54 ± 0.87; *P* = 0.012] (Figure 1B) and increased disease activity index scores [means ± SD (arbitrary units): control, 0.43 ± 0.20; DSS, 7.71 ± 0.29; *P* < 0.0001] (Figure 1C) compared with control mice. Structural pathology, as measured by histopathology scoring of hematoxylin and eosin sections (Figure 1D), was significantly higher in DSS mice compared with controls [means ± 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, because these cytokines are up-regulated in colonic biopsy specimens from IBD patients and are acknowledged to contribute to disease pathology and progression. The protein levels of TNF-α [means ± SD (pg/mL): control, 0.77 ± 0.79; DSS, 64.08 ± 64.51; *P* = 0.0371], IFN-γ [means ± SD (pg/mL): control, 0.0001 ± 0.0001; DSS, 3.01 ± 2.22; *P* = 0.0436], and IL-6 [means ± 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 with healthy controls.

Having confirmed intestinal pathology, the airways were examined for associated pulmonary manifestations of DSS colitis. Histopathology was quantified on the basis of leukocyte congregation around the airway vasculature and parenchyma using a previously validated scoring of hematoxylin and eosin–stained lung sections (Figure 1I). A significant increase in histopathological scores was observed in the lungs of DSS colitis animals [means ± SD (arbitrary units): control, 0.60 ± 0.55; DSS, 3.0 ± 1.87; *P* = 0.0249] (Figure 1J), with most inflammation localized around the pulmonary vasculature. To examine whether the vascular inflammation influenced lung morphology, alveoli diameter was calculated using the mean linear intercept method; however, there was no change in alveoli diameter between control and DSS colitis animals (Figure 1K). Given the histologic evidence of immune infiltration, BALF was assessed for increases in leukocytes (Figure 1L), and increased BALF cell numbers were found in DSS animals, compared with controls [means ± SD (cells/mL): control, 35.45 × 10^4 ± 17.06 × 10^4; DSS, 51.49 × 10^4 ± 14.26 × 10^4; *P* < 0.035].

To examine whether colitis-associated pulmonary pathology was a feature specific to the DSS model of colitis, the analysis was extended into the TNBS model of colitis. TNBS colitis led to early significant weight loss (Figure 2A), and disease activity [means ± 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 with ethanol-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 with ethanol-treated controls [means ± SD (arbitrary units): control, 0.67 ± 0.52; TNBS, 5.34 ± 1.21; *P* < 0.0001] (Figure 2D). On histopathological examination of lung pathology in the TNBS colitis model (Figure 2E), significant increases in pulmonary pathology were observed by pathology scoring [means ± SD (arbitrary units): control, 0.84 ± 0.75; TNBS, 2.50 ± 1.05; *P* = 0.01] (Figure 2F), consistent with findings in the DSS model. Also consistent with findings in the DSS model, increased leukocyte cell numbers were found in the BALF of TNBS animals, compared with controls (Figure 2G) [means ± SD (cells/mL): control, 15.43 × 10^4 ± 7.58 × 10^4; TNBS, 28.63 × 10^4 ± 11.69 × 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

The phenotype of leukocytes was next 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 [means ± SD (%): control, 7.35 ± 1.65; DSS, 12.63 ± 1.95; \( P < 0.001 \)] and inflammatory monocytes [means ± 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 [means ± SD (cell numbers): neutrophils: control, \( 1.57 \times 10^5 \) ± \( 0.65 \times 10^5 \); DSS, \( 3.49 \times 10^5 \) ± \( 1.19 \times 10^5 \); \( P < 0.0058 \); inflammatory monocytes: control, \( 0.76 \times 10^5 \) ± \( 0.24 \times 10^5 \); DSS, \( 2.18 \times 10^5 \) ± \( 0.85 \times 10^5 \); \( P < 0.0028 \)]. Resident monocyte and eosinophil numbers were not altered between groups. Similarly, in TNBS animals, neutrophil populations were increased [means ± 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 with healthy controls (Figure 3C). No change was observed in lymphocyte populations in the lungs of DSS (Figure 3D) or TNBS (Figure 3E) mice, compared with controls. These data indicate increases in predominantly neutrophil leukocyte populations entering the lungs of animals during colitis.
Colitis-Driven Bacteremia Drives Pulmonary Inflammation

Having identified increases in the proportion of neutrophils in the lungs of DSS colitis animals, it was next examined whether neutrophil recruitment to the lungs was an active or passive phenomenon. An adoptive transfer of CFSE-stained MLN cells was performed from healthy and DSS animals into healthy or DSS recipient animals, and cell populations homing to the lungs of recipient animals were evaluated (Figure 4A). A total of \(1 \times 10^6\) 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 [means ± SD (% transferred cells): 0.350 ± 0.1283] or healthy donor to DSS recipient mice [means ± SD (cells): 0.3246 ± 0.1748], when compared with healthy to healthy transfers [means ± 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 [means ± SD (cells): 1.613 ± 0.5479; \(P < 0.0001\)] compared with 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.

Local mediators of airway inflammation in DSS colitis were next investigated. Gene expression analysis showed significant increases in *tnfa* [means ± SD (fold change): control, 1.1 ± 0.53; DSS, 7.60 ± 4.49; \(P = 0.028\)], *ifng* [means ± SD (fold change): control, 1.5 ± 0.25; DSS, 6.40 ± 2.52; \(P = 0.002\)], and *il1b* [means ± SD (fold change): control, 2.0 ± 0.32; DSS, 7.60 ± 2.80; \(P = 0.005\)]. These results suggest that local production of inflammatory cytokines contributes to the development of neutrophilic lung inflammation in DSS colitis.
(fold change): control, 1.23 ± 0.97; DSS, 6.83 ± 5.5; P = 0.012), iil6 [means ± SD (fold change): control, 1.06 ± 0.35; DSS, 7.62 ± 6.38; P = 0.011], and il1β [means ± 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 with healthy controls (Figure 4B). However, protein analysis of these cytokines showed increases in IL-1β alone [means ± 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 (Figure 4C). Further assessment of inflammatory mediators identified a significant increase in both CCL2 [means ± SD (pg/mL): control, 53.84 ± 5.54; DSS, 132.9 ± 17.01; P = 0.0013] and KC [means ± 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 bacteremia in animal models of colitis and because CCL2 and KC are proinflammatory mediators during pulmonary inflammation induced by bacterial cell wall components,42 endotoxin levels were assessed in the lung tissue of DSS mice, compared with controls (Figure 4D). DSS mice exhibited a 37-fold increase in lung endotoxin [means ± SD (EU/mL): control, 0.016 ± 0.03; DSS, 0.606 ± 0.036; P < 0.0001]. Given this increase, microbial culture was next performed on BALF from control and DSS animals and an 18-fold increase was found in bacterial colony-forming units in the BALF of DSS animals, when compared with controls (Figure 4E) [means ± SD (CFU/mL): control, 15.17 ± 13.66; DSS, 269.3 ± 276.7; P = 0.0484]. Taken together, these data demonstrate that an increased bacterial load

Figure 4  Airway inflammation in DSS colitis mice. A: Carboxyfluorescein succinimidyl ester (CFSE)—stained cells harvested from the mesenteric lymph nodes of control (CTRL) and DSS mice were injected into either naïve control or DSS colitis recipient mice. The percentage of total transferred neutrophils (CFSE+Gr-1+Cd11b+Ly6CintScint) in the lung was calculated 24 hours after transfer. B–E: Gene expression analysis of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-6, and IL-1β (B) and proteins levels of TNF-α, IFN-γ, IL-6, IL-1β, chemokine (C-C motif) ligand (CCL) 2, keratinocyte chemoattractant, IL-12p70, and IL-17 (C) measured in the lung. Endotoxin levels (D) and cultured bacterial colony-forming units (E) from the lungs of control and DSS animals. Statistical analysis was performed by one-way analysis of variance (A) or unpaired t-test (B–E). Data are expressed as means ± SEM. n = 6. *P < 0.05, **P < 0.01, and ***P < 0.005 versus control. BALF, bronchoalveolar lavage fluid.
in the lungs of DSS animals is associated with increased inflammatory and chemotactic signaling molecules, which may drive recruitment of inflammatory cells during colitis.

**Neutrophilia Underpins the Systemic Inflammation in DSS Colitis**

Because leukocytes use the circulatory system to enter and exit tissues of interest, the effect of colitis on systemic inflammation was next examined, and it was determined 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 with control animals [means ± 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, a significant correlation was identified 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 neutrophilia in DSS colitis mice. Given the correlation between lung and blood neutrophils, it was investigated whether the same trends existed with serum cytokine levels. Significant increases were found in systemic IL-6 [means ± SD (pg/mL): control, 3.89 ± 0.34; DSS, 20.16 ± 16.57; *P < 0.037], IL-1β [means ± SD (pg/mL): control, 7.03 ± 5.62; DSS, 69.99 ± 39.32; $P = 0.023$], and KC [means ± SD (pg/mL): control, 1.45 ± 0.88; DSS, 10.68 ± 7.16; *P = 0.0423], but not TNF-α, IFN-γ, CCL2, IL-12p70, or IL-17 (Figure 5C).
indicating that systemic inflammation is not a consequence of cytokine spillover into the circulation from either intestinal or pulmonary inflammation. Because systemic IL-6 is 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, core body temperature (as an indicator of a hyperthermic response) was next investigated. DSS animals had a significantly higher peak body temperature during the course of disease, when compared with controls [means ± SD (Δ°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. To verify the presence of circulating bacteria that drive pyrexia, CFU was cultured from the blood of control and DSS animals and a 66-fold increase in bacterial colony-forming units was found in the blood of DSS animals, when compared with controls [means ± SD (CFU/mL): control, 1.67 ± 3.20; DSS, 109.5 ± 47.69; \(P = 0.0003\)] (Figure 5E). This supports the hypothesis that systemic bacteremia (potentially attributable 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 systems with intestinal inflammation, the influence of intestinal inflammation was also characterized 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 [means ± SD (%): control, 6.11 ± 0.39; DSS, 6.73 ± 0.46; \(P = 0.032\)] (Figure 5F). This increase was attributed to an increase in multipotent progenitor cells (CD48\(^+\)CD150\(^+\)) [means ± SD (%): control, 0.116 ± 0.026; DSS, 0.1995 ± 0.034; \(P < 0.001\)], with no change in hematopoietic stem cells (CD48\(^-\)CD150\(^+\)). Bone marrow CFU assays were also performed to functionally quantify myeloid progenitor cell numbers. Bone marrow from DSS had significantly higher total CFUs (CD48\(^+\)CD150\(^+\)) than control animals [means ± SD: control, 83.25 ± 9.39; DSS, 108.13 ± 10.54; \(P = 0.0125\)], which was attributed to increases in granulocyte/macrophage CFUs [means ± SD: control, 32.38 ± 4.15; DSS, 42.88 ± 5.94; \(P = 0.027\)] and monocyte CFUs [means ± SD: control, 26.83 ± 4.31; DSS, 39.25 ± 4.37; \(P = 0.013\)] populations but not granulocyte CFUs (Figure 5G). Cellular analysis of the proportion of mature myeloid cells in the bone marrow revealed a significant decrease in the proportion of neutrophils [means ± SD (%): control, 35.28 ± 1.80; DSS, 28.71 ± 4.34; \(P = 0.0065\)] and an increase in the proportion of monocytes [means ± 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, but there is also 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 increased in systemic IL-6, which can drive neutrophil mobilization, it was 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 cytometric bead array assay and were significantly reduced in anti-IL-6–treated animals compared with other groups [means ± SD (pg/mL): control, 0.78 ± 0.65; DSS (naïve), 19.33 ± 8.24; DSS (isotype), 16.27 ± 7.95; DSS (anti-IL-6), 4.07 ± 0.93] (Figure 6A). DSS mice treated with anti-IL-6 lost significantly less weight compared with DSS mice treated with isotype controls or with untreated DSS mice [means ± 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 6, E and 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).

Cellular analysis of the lungs of anti-IL-6–treated mice was next performed 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 with both the isotype-treated controls and untreated DSS mice [means ± SD (%): DSS (anti-IL-6), 8.301 ± 3.65; DSS (isotype), 15.70 ± 1.79; \(P = 0.003\); DSS (naïve), 13.18 ± 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 with isotype-treated controls (Figure 7B). Given the increase in neutrophil recruiting mediators KC and CCL2 observed in the lungs of DSS colitis animals, it was next examined whether IL-6 treatment had any effect on CCL2 (Figure 7C) and KC (Figure 7D) protein levels. No significant differences were observed with anti-IL-6 treatment compared with isotype controls. IL-1β was also examined in the lungs of anti-IL-6–treated DSS animals, but no differences were found compared with untreated or isotype control groups (Figure 7E). It was next investigated whether the decrease in neutrophils entering the lung was a consequence of an overall decrease in circulating leukocytes because of neutralization of IL-6. The proportion and numbers of circulating neutrophils were significantly decreased after anti-IL-6 antibody administration, compared with isotype-treated groups [means ± SD (%): DSS (isotype), 23.83 ± 9.77; DSS (anti-IL-6), 12.00 ± 3.23; \(P = 0.0297\)] (Figure 7F).
contrast, there was no significant difference in the proportion or numbers of circulating monocytes in anti—IL-6—treated animals compared with 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, it was 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 multipotent progenitor cell proportions compared with isotype-treated groups [means ± SD: DSS (isotype), 4.72 ± 1.89; DSS (anti—IL-6), 11.34 ± 1.31; P < 0.0001] (Figure 7H). Furthermore, the proportion of mature neutrophils in the bone of anti—IL-6—treated animals was significantly greater than in isotype-treated groups [means ± 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 extraintestinal pathology during intestinal inflammation.

Discussion

The aim of this study was to identify and characterize lung immunopathology associated with animal models of colitis.
The loss of intestinal permeability in murine models of colitis is associated with increased bacteremia, characterized by recovery of bacterial CFUs from distal organs. Herein, 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. A vascular accumulation of neutrophils common to the DSS and TNBS models of colitis was identified. Neutrophil recruitment to the lung was associated with increased bacteria in the lung and elevated levels of IL-1β, CCL2, and KC, and it was 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 intercellular adhesion molecule 1 identified in lung tissue sections of a rat TNBS model and increases in TNF-α and vascular endothelial growth factor in both rat DSS and TNBS models. More important, both of these models suggested vascular involvement in the disease, but they did not identify a mechanism analogous to the pulmonary manifestations observed in IBD patients. In the 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. Both are associated with elevated CCL2 and IL-8 and neutrophil extravasation into the respiratory system, with the subsequent activation of these cells inducing structural pathologies. The inflammation observed in the 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. Herein, 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

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 with healthy control and naive DSS animals. CD45<sup>+</sup> cells were isolated from the lungs, blood, and bone marrow and assessed by flow cytometry. A and B: The proportion and total cell number of neutrophils (A) and monocytes (B) in the lungs. C–E: Protein levels of chemokine (C-C motif) ligand (CCL) 2 (C), keratinocyte chemoattractant (D), and IL-1β (E) in the lungs, as measured by cytometric bead array. F and G: The proportion and total cell number of neutrophils (F) and monocytes (G) in the blood. H: The number of multipotent progenitor (MPP; Lin<sup>−</sup>CD48<sup>−</sup>CD150<sup>−</sup>) cells in the bone marrow. I: The proportion and total cell number of mature neutrophil levels in the bone marrow. All data represented as means ± SEM from two separate experiments (A–I), n = 8 (A–I). *P < 0.05, **P < 0.01, and ***P < 0.005 (one-way analysis of variance).
signaling in the lung. In addition, although anti–IL-6 treatment did not affect GI inflammation, it reduced colitis-associated wasting, an observation consistent with previous studies. The ability of IL-6 blockade to reduce wasting in colitis may be attributed to the pyrogenic effect elicited by IL-6. Indeed, IL-6 is associated with bacteremia and pyrexia in mouse models of colitis. More important, lipopolysaccharide-induced toll-like receptor 4 signaling drives the synthesis of pyrogenic cytokines, including IL-6, which is a key mediator of the pyrexic response. Previous studies have demonstrated that treatment with anti–IL-6 neutralizing antibodies prevents the development of lipopolysaccharide-induced fever in rodents, and this is mirrored in IL-6–deficient animals. In both cases, and despite the lack of a fever response, TNF-α and IL-1β levels increased with lipopolysaccharide challenge, suggesting a central importance for IL-6 in mediating fever. Indeed, evidence from experiments in peripheral blood mononuclear cell cultures suggests that IL-6 can suppress both IL-1β and TNF-α at the transcriptional level, and Diao and Kohanawa 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 this model, because 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 and attenuated disease associated with antibody depletion of both IL-6 and the IL-6 signal transducer, gp130. Although, herein, anti–IL-6 treatment had no influence on the severity of GI pathology, this may be attributed to the timing of the interventions, because anti–IL-6 antibody was administered after colitis was initiated. In studies by Sander et al, where gp130, the common signal transducer for IL-6, was deleted in a DSS model, loss of IL-6 signaling 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, and this is largely attributed to early activation of colonic resident myeloid. Colitis also alters hematopoietic processes in the bone marrow, which enhances production of granulocytes and monocytes that are released in the circulatory system. 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, which, in turn, may lead to accumulation of neutrophils within peripheral tissues, including the lung. 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. Under homeostatic conditions, neutrophil egress from the bone marrow is mediated by C-X-C chemokine receptor 4/stromal cell-derived factor 1 (SDF-1) interactions, but in the context of inflammation, several chemokines may enable chemotaxis of neutrophils across the bone marrow sinusoidal endothelium. These data may indicate that, although 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 the 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, these data suggest that pyrexia and systemic bacteremia attributable to loss of intestinal barrier integrity play a role in extraintestinal 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 in 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. Although 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 bacteremia 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. We have recently shown that lung disease, caused by chronic cigarette smoke, leads to systemic ischemia that drives intestinal dysfunction, predisposing to colitis. 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 investigated as a therapy for pyrexia and cachexia associated with cancers. 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 extraintestinal complications.

Supplemental Data

Supplemental material for this article can be found at https://doi.org/10.1016/j.ajpath.2018.03.016.
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