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p40phox Expression Regulates Neutrophil Recruitment and Function during the Resolution Phase of Intestinal Inflammation

Kara L. Conway,*†‡§ Gautam Goel,†§,§,§,§ Harry Sokol,*†§,†§,‖ Monika Manocha,*‖ Emiko Mizoguchi,*† Cox Terhorst,*‖ Atul K. Bhan,*§# Agnès Gardet,*†‡§ and Ramnik J. Xavier*†‡§

NADPH oxidase is a multisubunit complex that assembles during phagocytosis to generate reactive oxygen species. Several components of this complex have been implicated in chronic granulomatous disease and Crohn’s disease, highlighting the importance of reactive oxygen species in regulating host immune response. In this study, we use genetically deficient mice to elucidate how p40phox, one subunit of the NADPH oxidase complex, functions during intestinal inflammation. We show that p40phox deficiency enhances inflammation in both dextran sulfate sodium-induced and innate immune-mediated murine colitis models. This inflammation is characterized by severe colonic tissue injury, increased proinflammatory cytokines, and increased neutrophil recruitment. We demonstrate that neutrophils are essential during the recovery phase of intestinal inflammation and that p40phox expression is necessary for this restitution. Lastly, using an integrative bioinformatic approach, we show that p40phox deficiency leads to upregulation of chemokine receptor 1 and downregulation of enzymes involved in glycan modifications, including fucosyltransferases and sialyltransferases, during inflammation. We propose that p40phox deficiency enhances intestinal inflammation through the dysregulation of these two pathways in neutrophils. The Journal of Immunology, 2012, 189: 3631–3640.

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1G.G and H.S. contributed equally to this work.

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Abbreviations used in this article: CD, Crohn’s disease; CGD, chronic granulomatous disease; Ce, threshold cycle; DAL, disease activity index; DKO, double knockout; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; iNOS, inducible NO synthase; FMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; WT, wild-type; XCGD, X-linked chronic granulomatous disease.
mation, particularly during the recovery phase. Additionally, p40<sup>phox</sup>-deficient mice are more susceptible to an innate immune model of colitis, which is dependent on anti-CD40 pathway engagement. We demonstrate that neutrophils are essential during the recovery phase of intestinal inflammation and that p40<sup>phox</sup> expression is necessary for the neutrophil-mediated restitution response. Based on these observations, we developed a bioinformatic approach that integrates analyses of clinical gene expression signatures in CGD patient neutrophils and temporal gene expression profiles during murine DSS colitis (18, 19). Using this method, we were able to identify novel mechanisms and regulators that profiled during murine DSS colitis (18, 19). Using this method, we natures in CGD patient neutrophils and temporal gene expression response. Based on these observations, we developed a bioinformatic expression is necessary for the neutrophil-mediated restitution re-

Materials and Methods

Animals

Mice were maintained in specific-pathogen-free facilities at Massachusetts General Hospital (Boston, MA). All animal studies were conducted under protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. p40<sup>phox</sup>-/- mice were provided by Phillip T. Hawkins (Babraham Institute, Cambridge, U.K.). Generation of this knockout line has been previously described, and these mice have been backcrossed to the C57BL/6 background (20). Rag1<sup>-/-</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained on food and water ad libitum. Mice were used between 7 and 9 wk and strains were age- and gender-matched for each experiment.

DSS colitis

Mice were fed 3% (w/v) DSS (MP Biomedicals; m.w., 36,000–50,000) dissolved in sterile, distilled drinking water ad libitum. Mice were treated with 5% DSS for 7 d, followed by 5 d regular drinking water. Animals were monitored daily for weight loss, survival, and disease activity. Disease activity index (DAI) was scored as previously described, based on the average of three parameters: stool consistency (0, 2, 4), fecal blood (0, 2, 4), and percentage weight loss (0–4) (21, 22). Animals that did not survive the full experimental course were not included in body weight and DAI analyses. Colon tissue was harvested at indicated time points for histology, RNA, and flow cytometry analyses.

Anti-CD40 colitis

Acute, innate-mediated colitis was induced as previously described (23). Briefly, Rag1<sup>-/-</sup> and p40<sup>phox</sup>-/- x Rag1<sup>-/-</sup> double knockout (DKO) mice were injected i.p. with 200 µg FGK4.5 anti-CD40 mAb (BioXCell, West Lebanon, NH). Age- and sex-matched control mice were treated with a rat IgG2a isotype control, 2A3 (BioXCell). Animals were monitored daily for weight loss and disease activity for 7 d. DAI was scored based on the sum of parameters previously detailed, including hunching (0, 1), wasting (0, 1), stool consistency (0–3), proximal colonic thickness (0–3), medial colonic thickness (0–3), and distal colonic thickness (0–3) (24). Colon was harvested for histology 7 d after induction of colitis.

Histology

Colon tissue was fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were cut and stained with H&E. Longitudinal sections were scored in a blinded fashion using methods previously established in each colitis model, with slight modification (23, 25). Colon tissues from DSS colitis experiments were scored by the following parameters: severity of inflammation (0–3), depth of injury/inflammation (0–3), and crypt damage (0–4). DSS histological scores were multiplied by a factor representing the percentage of tissue involvement: x1 (0–25%), x2 (26–50%), x3 (51–75%), and x4 (76–100%). Thus the maximum colitis score in the DSS model is 40 (25).

Colon tissues from anti-CD40 colitis experiments were prepared in the same manner and scored by the following parameters: epithelial hyperplasia (0–3), goblet cell depletion (0–3), lamina propria infiltrate (0–3), and epithelial cell damage (0–3). Proximal, medial, and distal portions of the colon were individually scored in this model, and the colitis score is the sum of all three sections (maximum, 36).

Real-time quantitative PCR

Proximal, medial, and distal colon tissues were harvested at the end of each course of colitis and stored in RNalater (Ambion, Austin, TX) per the manufacturer’s protocol prior to RNA isolation. RNA was extracted from homogenized tissues using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. All RNA samples were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Using the iQ SYBR Green Supermix (Bio-Rad) for quantitative PCR, mRNA levels were determined using the iCycler with an iQ5 multicolor real-time PCR detection system (Bio-Rad). The reaction conditions consisted of 37 cycles of PCR with an annealing temperature of 59°C. The following primers were used: Ccr1, forward, 5′-AAGAGCTCTGAC-GACTGGGACA/-3; reverse, 5′-CAGATGTAAGGGCTGCAGA/-3; TNF-α, forward, 5′-GACCTGGAACGTGCAGAGA/-3, reverse, 5′-TTGGTGTG- TTTGAACTTGAG-3; IFN-γ, forward, 5′-ATGAAACGTACACTG-CATC/-3, reverse, 5′-CCATCTCTTGCCATGCCCTC/-3; IL-1β, forward, 5′-GCCCATCTCTCTGTAAGCTCAT/-3, reverse, 5′-AGCAGGAAGTATTTTGTCGC-3; IL-6, forward, 5′-GATGCTAGTGTAATGACTTCCAGA/-3, reverse, 5′-AGCAGAAGCACTGAGGA/-3; IL-10, forward, 5′-TTTAAAATCCTG- CTTTGGGAAA/-3, reverse, 5′-TTTCCCTTCCCGATTGAC/-3; inducible NO synthase (iNOS), forward, 5′-GCTTGTAACTGAGGCGACA/

Bacterial translocation

Spleen and mesenteric lymph nodes from DSS-treated mice were weighed and homogenized in 500 µl sterile PBS. Homogenates were plated on tryptic soy broth agar plates and incubated overnight at 37°C before quantification. Data are shown as CFUs per gram of tissue.

Lamina propria isolation

Colon lamina propria cells were isolated as previously described (28). Briefly, colons were harvested at indicated time points and inverted onto propylene tubing (Becton Dickinson, Franklin Lakes, NJ). After being washed in calcium- and magnesium-free PBS, colons were incubated with DTt (Sigma-Aldrich, St. Louis, MO), followed by 30 mM EDTA, all at room temperature. The remaining tissue was further digested using type IV collagenase (108 U/ml; Sigma-Aldrich) for 90 min. The filtered cells from the digested tissue were then layered on a 45/72% Percoll (GE Healthcare, Waukesha, WI) gradient and harvested at the interface after centrifugation (650 x g, 15 min) (28).

Flow cytometry

Colon lamina propria cells (2 x 10<sup>6</sup>) were washed in PBS supplemented with 3% FBS. Cells were first incubated with 2.4G2 mouse Fc block in PBS supplemented with 3% PBS (BD Pharmingen, San Diego, CA) for 20 min at 4°C. Cells were then washed and stained with fluorescent-conjugated Abs for 20 min at 4°C. The following Abs were purchased from BD Pharmingen and used for our analysis: Ly-6G-FITC, F4/80-PER, and CD11b-allophycocyanin. The anti-Ccr1 Ab and isotype control were purchased from R&D Systems and used per the manufacturer’s recommendations. Fluorescently labeled lamina propria cells were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA) and analyzed using FlowJo analysis software (Tree Star, Ashland, OR).

Neutrophil cytosis

Total lamina propria cells were isolated from mice treated with DSS as described above, and Ly-6G<sup>CD11b+</sup>F4/80<sup>-</sup> cells were FACS sorted. Cells (1 x 10<sup>5</sup>) were spun down onto a slide using a Cytospin 3 (Thermo Shandon, Waltham, MA) and subjected to Wright’s staining.

p40<sup>phox</sup> AND INTESTINAL INFLAMMATION
Neutrophil depletion
Where indicated, mice were treated with anti-Ly-6G mAb (clone 1A8, BioXCell) 24 h prior to DSS or anti-CD40 administration (4 mg/kg body weight i.p.). Age- and sex-matched littermates were injected with PBS. For neutrophil depletion in the DSS model, 24 h postdepletion, mice were administered 3% DSS for 7 d, followed by regular drinking water for 5 d. Mice received the same dose of anti-Ly-6G every 3 d during the course of the experiment. For neutrophil depletion in the anti-CD40 model, 24 h postdepletion, mice were administered anti-CD40 as described above. Mice received the same dose of anti-Ly-6G on day 3 of the 7-d course. Neutrophil depletion efficiency was determined by FACS staining in blood, spleen, bone marrow, and lamina propria.

Bioinformatic analysis
To identify NADPH oxidase-regulated genes underlying pathogenesis in the DSS colitis model, two publicly available datasets were analyzed. The first dataset provides temporal, whole-genome expression profiling during DSS colitis treatment in mice (18). Colon tissue from 12- to 14-wk-old DSS-treated C57BL/6J mice was collected at 0, 2, 4, and 6 d. The second dataset provides global gene expression patterns in polymorphonuclear neutrophils (PMNs) from X-linked chronic granulomatous disease (XCGD) patients and healthy control individuals (19, 29). PMNs were profiled at 0, 90, 180, and 360 min after stimulation with IgG and C3b-coated latex beads to activate phagocytosis. Both datasets were normalized separately using a GC robust multi-array average routine in MATLAB. Probes were filtered with low absolute expression values, low entropy, and variance less than the 10th percentile. DSS colitis data were analyzed for differential expression using a two-tailed, two-sample \( t \) test between pairs of data collected from consecutive time points (Supplemental Fig. 1A). Significant genes \((q < 0.05)\) were identified with expression change \(>2\)-fold and an adjusted \( p \) value of \( <0.05\). We followed a similar strategy for the PMN data and performed a differential-of-differential analysis to identify 124 genes that were significantly differentially expressed in healthy PMNs relative to XCGD PMNs (Supplemental Fig. 1B).

Differentially expressed genes identified in the DSS colitis dataset were clustered into three groups: early responders, middle-to-late responders, and late responders (Supplemental Fig. 2). Each of these groups was then separately analyzed for gene set overlap using the canonical pathway gene sets from MSigDB (30). The differentially expressed genes in the PMN dataset were similarly clustered into two groups: genes that were induced or genes that were suppressed in the absence of ROS (Supplemental Fig. 3). This was followed by a gene set overlap analysis (see Fig. 6C).

To identify NADPH oxidase-dependent genes that were important during DSS-induced colitis, the intersection of the two lists of differentially expressed genes from both DSS and PMN datasets was determined and yielded a set of 10 genes (see Fig. 6C).

Enrichment score
Enrichment scores were calculated as previously described (31).

Isolation of lamina propria neutrophils
Lamina propria cells were isolated from wild-type (WT) and p40phox−/− mice as described above. Ly-6G+ cells were isolated via MACS positive enrichment scores were calculated as previously described (31).

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Lamina propria cells were isolated from wild-type (WT) and p40phox−/− mice as described above. Ly-6G+ cells were isolated via MACS positive selection cell separation kits (Miltenyi Biotec, Auburn, CA). Isolated neutrophils were washed twice with ice-cold PBS and cell pellets were stored at \(-80°C\) until RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. Quantitative RT-PCR was performed as described above.

In vivo CCRI antagonist treatment
One hour prior to DSS treatment, WT C57BL/6 mice were injected i.p. with 10 mg/kg body weight of the CCRI antagonist J113863 (Tocris Bioscience, Bristol, U.K.) dissolved in PBS, as described with other CCRI antagonists (32). Mice were injected once daily thereafter. Assays from treated mice were conducted as described above.

Statistical analysis
Unless otherwise stated, a Student \( t \) test was used to assess the significance of observed differences. A \( p \) value \(<0.05\) was considered significant. A log-rank test was used to assess significance in survival curve comparisons. A two-sided Wilcoxon rank sum test was used to assess significance for DAI scores. The test rejected the null hypothesis at the 5% significance level \((p = 0.0095)\). All error bars represent SD.

Results
p40phox−/− mice develop severe colitis during the recovery phase of DSS-induced colitis
We first assessed the role of p40phox in intestinal inflammation through the use of an epithelial injury model, consisting of 3% DSS treatment for 7 d followed by 5 d recovery. p40phox−/−-deficient mice lost significantly more weight than did their WT littermates by day 6 during DSS colitis and were unable to recover from this weight loss (Fig. 1A). Additionally, p40phox−/−-deficient mice exhibited a higher mortality rate throughout the course of DSS treatment (Fig. 1B). p40phox−/−-deficient mice had significantly more severe disease activity based on a composite index (Fig. 1C) and more severe colonic inflammation on day 12 as shown by a significant decrease in colon length (Fig. 1D) and increase in histology score (Fig. 1E). H&E staining of colon sections revealed inflammation in p40phox−/− mice characterized by epithelial ulceration and leukocyte infiltration (Fig. 1F).

To determine whether NADPH oxidase deficiency affects epithelial permeability during intestinal inflammation, we assessed bacterial translocation to the spleen and mesenteric lymph nodes during DSS colitis. p40phox−/− mice had significantly greater numbers of CFU per gram of tissue in both spleen and mesenteric lymph node, suggesting an inability to effectively clear microbes (Fig. 11). p40phox−/− mice show no spontaneous defect in intestinal permeability, as no bacteria were detected in the peripheral organs without DSS treatment, similar to WT mice (data not shown). Collectively, the data demonstrate that p40phox is necessary for the resolution of DSS-induced inflammation and that p40phox deficiency enhances proinflammatory cytokine production during intestinal inflammation.

p40phox−/− × Rag1−/− mice develop severe colitis during the recovery phase of DSS-induced colitis
To determine the contribution of the innate immune compartment in this phenotype, we next assessed whether p40phox deficiency also promotes intestinal inflammation in a Rag1-deficient mouse model, which lacks an adaptive immune system. For these experiments, p40phox−/− × Rag1−/− DKO mice were treated with DSS as described above, and the resulting phenotypes were compared with Rag1−/− lymphocyte-deficient mice. As shown in Fig. 2A, DKO mice lost more weight during DSS treatment than did their Rag1−/− counterparts, most notably during the recovery phase (Fig. 2A). Additionally, when compared with the weight loss observed in p40phox−/− mice treated with DSS (Fig. 1A), DKO mice lost a comparable percentage at day 12 (p40phox−/−, 20 ± 2.1%; DKO, 22.1 ± 1.2%). Histological and clinical examination demonstrated greater inflammation during DSS in DKO mice than in their Rag1−/− littermates (Fig. 2B–D). The histological score for the DKO group, although significantly greater than the Rag1−/− control cohort, was lower than that seen previously in the lymphocyte-sufficient mice (Fig. 1E), suggesting a potential, albeit secondary, role for lymphocytes in colitis progression. These data indicate that p40phox deficiency renders mice more susceptible to intestinal inflammation, even in the absence of B and T lymphocytes.

p40phox−/− mice are more susceptible to anti-CD40–induced colitis
To define the contribution of p40phox in innate immunity, we next used an anti-CD40 model of colitis. Injection of CD40 mAb into T...
Bacterial translocation to MLNs and spleen was determined on day 12 after DSS administration. The number of CFUs per gram of tissue is shown (DSS. IL-17A was not detected in the absence of DSS treatment; therefore, IL-17A expression fold is relative to the WT DSS condition (termined by a two-sided Wilcoxon rank sum test; the test rejected the null hypothesis at the 5% significance level (enhanced inflammation of p40

hallmarks of this colitis model include leukocyte infiltration into

largely driven by local IL-23 production (23). The histological

intestinal inflammation and wasting disease within 2 d and is

recover from anti-CD40–induced colitis, DKO mice had signifi-

control littermates. However, DKO mice induced acute gastro-

Neutrophil recruitment in the colon is augmented in p40

expression is a key component in preserving intestinal

homeostasis in an innate immunity model of colitis.

Neutrophil recruitment in the colon is augmented in p40

Given the exacerbated response of p40

phox

phox

DKO mice were unable to recover similarly to their anti-CD40–

mice following DSS treatment, Ly-6G+CD11b+F4/80

cells possessed multilobulated nuclei, the hallmark characteristic

defining neutrophil morphology (Fig. 4B). Total neutrophil num-

the context of p40

phox

phox

the anti-CD40–treated Rag1

control littermates. However, DKO mice were unable to recover similarly to their anti-CD40–

mice injected with isotype control

Ab did not show weight loss (23). In addition to the inability to recover from anti-CD40–induced colitis, DKO mice had significantly greater DAI values as assessed by wasting, stool consistency, and colonic thickness (p < 0.001, Fig. 3B). Similar to the enhanced inflammation of p40

phox

phox

mice had a significantly higher histology score in comparison with Rag1

mice (Fig. 3C). Colonic tissues displayed greater inflamma-

in DKO mice, characterized by polymorphonuclear cell infiltration (Fig. 3D). Taken together, these data demonstrate that p40

expression is a key component in preserving intestinal homeostasis in an innate immunity model of colitis.

Neutrophil recruitment in the colon is augmented in p40

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DKO mice following DSS treatment.

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sufficient neutrophils play a protective role in intestinal inflammation

Given the increased susceptibility to DSS during the recovery phase and the enhanced neutrophil infiltration in p40

phox

phox

mice, we next addressed the role of neutrophils in intestinal inflammation in the context of p40

phox

deficiency. Neutrophils are responsible for initiating early responses in inflammation by aiding in pathogen-
Neutrophils are critical for resolution of intestinal inflammation, and that p40<sup>phox</sup>-deficient neutrophils are also necessary for recovery in this process. We next developed an integrative bioinformatic approach to identify novel NADPH oxidase-dependent genes and pathways important during intestinal inflammation, particularly in the neutrophil compartment (Fig. 6, Supplemental Fig. 1). We first assessed time course gene expression patterns from whole murine colon tissue to identify novel NADPH oxidase-dependent genes and pathways important during intestinal inflammation, particularly in the neutrophil compartment (Fig. 6, Supplemental Fig. 1). We first assessed time course gene expression patterns from whole murine colon tissue to identify novel NADPH oxidase-dependent genes and pathways important during intestinal inflammation, particularly in the neutrophil compartment (Fig. 6, Supplemental Fig. 1).

**FIGURE 2.** p40<sup>phox</sup><sup>−/−</sup> X Rag1<sup>−/−</sup> mice show increased susceptibility to DSS colitis. (A) Age- and weight-matched mice were given 3% DSS in drinking water ad libitum for 7 d, followed by 5 d regular drinking water. Changes in body weight were monitored daily (n = 10/group; *p < 0.01). (B) Animal weight, stool consistency, and stool blood content were assessed daily to determine DAI. The median DAI scores for days 6 and 12 are shown. Significance was determined by a two-sided Wilcoxon rank sum test; the test rejected the null hypothesis at the 5% significance level (n = 10/group; *p = 0.0095). (C) Colonic tissues were blindly scored for inflammation on day 12. Grading parameters include severity of inflammation, depth of injury, and crypt damage (n = 10/group; Rag1<sup>−/−</sup> average, 6.5 ± 2.4; DKO average, 12.3 ± 2.6; p = 0.005). (D) Representative colon sections stained by H&E (top panel, original magnification ×10; bottom panel, original magnification ×20). Unless otherwise noted, significance was determined using a Student t test. For all panels, data were generated from two independent experiments.

NADPH oxidase activity regulates expression of chemokine receptor 1 and enzymes involved in glycan modifications

significant difference in body weight loss with or without neutrophils (Fig. 5B, 5D), demonstrating that the protective role of neutrophils is dependent on p40<sup>phox</sup> expression. We next assessed the role of neutrophils in the anti-CD40 model and found that 100% of the neutrophil-depleted mice did not survive past day 4, indicating that neutrophils are also necessary for recovery in this colitis model (Fig. 5E–H). Taken together, these observations indicate that neutrophils are critical for resolution of intestinal inflammation and that p40<sup>phox</sup> expression is necessary for this process.
is largely neutrophil-specific, as we detected higher levels of Ly-6G+CD11b+F4/80 in murine intestinal inflammation (32, 40). Quantitative PCR confirmed that neutrophils and p40phox play key roles in intestinal homeostasis, we next analyzed data from a study chronicling global gene expression patterns in stimulated PMNs isolated from healthy controls and XCGD patients (19, 29). One hundred twenty-four differentially expressed genes were identified in intestinal homeostasis, we next analyzed data from a study chronicling global gene expression patterns in stimulated PMNs isolated from healthy controls and XCGD patients (19, 29). One hundred twenty-four differentially expressed genes were identified in XCGD neutrophils were enriched for members of the NF-kB, PI3K/Akt, and MAPK signaling pathways (Fig. 6B, Supplemental Fig. 3). Similarly, the expression of several NF-kB–dependent proinflammatory cytokines, including IL-6, was enhanced in p40phox mice during DSS colitis (Fig. 1G). Notably, HexA and HexB, two genes highly upregulated in healthy patients but not induced during phagocytosis in XCGD PMNs, belong to the glycosphingolipid biosynthesis pathway, a network highlighted in our DSS microarray analysis (Supplemental Figs. 2, 3).

Cross-comparison of these two functional analyses highlighted 10 genes regulated both by NADPH oxidase activity in human PMNs and by intestinal inflammation in the DSS colitis model: CCR1, ST3GAL6, SLC20A1, SOCS3, CDKN1A, IL1R1, TIM25, HEXB, TGM3, and NR1D2 (Fig. 6C). Most of these genes have enriched expression in neutrophils and myeloid cells, suggesting their relevance in the context of p40phox, which is also highly expressed in these cell types (Fig. 6D). Among them, CCR1, a neutrophil chemokine receptor, has been previously implicated in murine intestinal inflammation (32, 40). Quantitative PCR confirmed that Ccr1 expression in colonic tissue from DSS-challenged mice is more highly induced in p40phox−/− mice than in WT controls (Fig. 7A). This induction of Ccr1 expression is largely neutrophil-specific, as we detected higher levels of Ccr1 expression in isolated p40phox−/− lamina propria neutrophils compared with WT neutrophils during the acute phase of DSS colitis (Fig. 7B). Moreover, p40phox neutrophils isolated from the lamina propria expressed significantly higher levels of cell surface Ccr1 protein as detected by flow cytometry (Fig. 7C). Notably, no difference in expression was observed in the Ccr1 ligands Ccl3 and Ccl5 in WT and p40phox−/− colon during DSS colitis, suggesting an inherent increased capacity for p40phox−/− neutrophils to migrate (Fig. 7D). Thus, enhanced Ccr1 expression upon decreased NADPH oxidase activity may contribute to increased neutrophilic recruitment during DSS colitis.

Taken together, the bioinformatic and DSS data suggest that Ccr1 expression is regulated by ROS and that neutrophils are important for resolving inflammation. To directly examine the role of Ccr1 in DSS colitis, we next blocked Ccr1 using a molecular antagonist prior to DSS treatment in WT mice. As predicted, neutrophils were not recruited efficiently to the colon (Fig. 7E; FIGURE 5. Ly-6G+ neutrophils are necessary for recovery from DSS and anti-CD40 colitis. For DSS colitis, age- and weight-matched mice were pretreated with either PBS or Ly-6G neutrophil depletion Abs prior to DSS administration. Changes in body weight (A, B) and animal survival (C, D) were monitored daily (n = S/group). Significance was determined using a Student t test for weight (*p < 0.01) and a log-rank test for survival [p = 0.0002 for (G); p = 0.0043 for (H)]. For all panels, data were generated from two independent experiments.

FIGURE 4. Enhanced neutrophil recruitment during DSS colitis in p40phox−/− mice. (A) Colonic lamina propria cells were isolated after recovery from DSS (day 12) and stained for flow cytometry analysis. The percentages of Ly-6G+ cells in WT and p40phox−/− lamina propria are shown in the top histograms; CD11b and F4/80 expression of the Ly-6G+ populations are shown in the lower dot plots (n = 10/group; representative data shown). (B) Cell morphology was assessed in FACS-sorted Ly-6G+CD11b+F4/80 cells from p40phox−/− mice (day 12) using Wright’s staining after cytospin. Original magnification ×40. (C) The total numbers of Ly-6G+CD11b+F4/80 neutrophils were calculated during acute and recovery phases of DSS colitis. The average of 10 mice per group is displayed (*p < 0.001). Significance was determined using a Student t test. For all panels, data were generated from three independent experiments.
The glycosphingolipid biosynthesis pathway was highlighted in our analysis of both DSS and XCGD datasets, suggesting that this pathway is important in neutrophils during inflammation. A temporal microarray data from colonic tissues obtained from DSS-treated mice were normalized and analyzed to identify 124 differentially expressed genes. Genes enriched in this analysis are involved in the cytokine/chemokine receptor-ligand interactome, glycosphingolipid biosynthesis, and Wnt and sonic hedgehog (Shh) signaling pathways. Temporal profiles of gene expression in neutrophils stimulated for phagocytosis in healthy mice were enriched in this analysis.

A cross-comparison of the two functional analyses described in (A) and (B) identified 10 genes that are differentially regulated by NADPH oxidase activity and during DSS colitis. Heatmap displaying human gene expression enrichment scores of each gene was expressed at significantly lower levels in p40<sup>−/−</sup> mice during the recovery period, and are also involved in glycan modifications. We investigated the expression of these genes during DSS treatment and observed that each gene was expressed at significantly lower levels in p40<sup>−/−</sup> mice during colitis and, unlike WT mice, expression of these transferases remained low after the recovery phase at day 12.

FIGURE 7. p40<sup>−/−</sup> regulates neutrophil Ccr1 expression and this pathway is important for intestinal inflammation restitution. (A) Ccr1 transcript levels were assessed via quantitative PCR in WT and p40<sup>−/−</sup> mice on days 0, 6, and 12 during the DSS course (7 d 3% DSS followed by 5 d water). Fold expression changes are relative to day 0. Data were generated from four independent experiments (n = 12/group). (B) Ccr1 transcript levels were assessed via quantitative PCR in lamina propria-isolated Ly-6G<sup>+</sup> neutrophils from WT and p40<sup>−/−</sup> mice on days 6 and 12 during DSS colitis. Fold expression change is relative to Ccr1 expression in isolated WT neutrophils. Data were generated from four independent experiments (n = 5/group). (C) Lamina propria cells isolated 6 d after 3% DSS treatment were stained with both Ly-6G and Ccr1 Abs and analyzed by flow cytometry. The histogram shows an overlay of rat IgG2B isotype control (gray) and Ccr1 staining on Ly-6G<sup>+</sup> cells from WT (solid line) and p40<sup>−/−</sup> (dotted line) mice. Data were generated from two independent experiments (n = 5/group; representative data shown). (D) Quantitative PCR was run for Ccl3 and Ccl5, two Ccr1 ligands, in colon tissue from WT and p40<sup>−/−</sup> mice during DSS colitis. Fold expression changes are relative to day 0. Data were generated from four independent experiments (n = 12/group). (E-H) WT mice were treated with the CCR1 agonist J113863 (10 mg/kg body weight) 24 h prior to DSS administration and every day thereafter. Lamina propria Ly-6G<sup>+</sup> cell frequency was assessed in CCR1 agonist-treated mice (a representative histogram is shown) (E), body weights were monitored daily (F), colon histology was scored on day 12 (G), and bacterial translocation was quantified on day 12 (H). For (E)–(H), data were generated from two independent experiments (n = 4/group). Significance was determined using a Student t test (p < 0.001).

FIGURE 6. An integrative bioinformatic approach identifies neutrophil-specific ROS-dependent genes important during murine colitis. (A) Temporal microarray data from colonic tissues obtained from DSS-treated mice were normalized and analyzed to identify 1283 differentially expressed genes. Genes involved in PI3K/Akt and MAPK signaling, NF-κB regulation, and glycosphingolipid biosynthesis were normalized and analyzed to identify 1283 differentially expressed genes. Genes enriched in this analysis are involved in the cytokine/chemokine receptor-ligand interactome, glycosphingolipid biosynthesis, and Wnt and sonic hedgehog (Shh) signaling pathways. (B) Temporal profiles of gene expression in neutrophils stimulated for phagocytosis in healthy mice were enriched in this analysis. (C) A cross-comparison of the two functional analyses described in (A) and (B) identified 10 genes that are differentially regulated by NADPH oxidase activity and during DSS colitis. (D) Heatmap displaying human gene expression enrichment scores of the genes identified in (C).
that neutrophils (data not shown). These studies demonstrate modifications during DSS colitis.

Therefore, we next investigated the expression levels of the target enzymes involved in glycan modifications. (A) Expression of enzyme members of the glycosphingolipid biosynthesis pathway were assessed via quantitative PCR in WT and p40\textsuperscript{phox}\^{−/−} colon on days 0, 6, 8, and 12 during the DSS course described above. Fold expression changes at each time point are relative to day 0. Data were generated from four independent experiments (n = 12/group). (B) Expression of enzyme members of the glycosphingolipid biosynthesis pathway were assessed via quantitative PCR in WT and WT Ly-6G\textsuperscript{+} neutrophil-depleted colon on days 0, 6, 8, and 10 during the DSS course described above. Fold expression changes at each time point are relative to day 0. Data were generated from two independent experiments (n = 8/group).

FIGURE 8. p40\textsuperscript{phox} regulates expression of enzymes involved in glycan modifications. (A) Expression of enzyme members of the glycosphingolipid biosynthesis pathway were assessed via quantitative PCR in WT and p40\textsuperscript{phox}\^{−/−} colon on days 0, 6, 8, and 12 during the DSS course described above. Fold expression changes at each time point are relative to day 0. Data were generated from four independent experiments (n = 12/group).

These data confirm our bioinformatic predictions, in which we propose a set of ROS-dependent genes important during inflammation. However, based on the human dataset, we can only surmise that neutrophils are the relevant cell type driving this phenotype. Therefore, we next investigated the expression levels of the target genes in colon harvested from neutrophil-depleted WT mice after DSS. Expression of all six glycan-modifying proteins was significantly diminished in neutrophil-depleted WT mice compared with the nondepleted WT cohort (Fig. 8B). Additionally, no differences in expression were detected in p40\textsuperscript{phox}\^{−/−} mice with or without neutrophils (data not shown). These studies demonstrate that p40\textsuperscript{phox}-intact neutrophils are required for appropriate expression of genes important for glycan posttranslational modifications during DSS colitis.

Discussion

Mutations that impair NADPH oxidase function have been linked to CGD (2, 3), and recent genetic studies have identified associations between ileal CD and genomic regions containing NADPH oxidase genes (9–11). We investigated the role of the p40\textsuperscript{phox} subunit in intestinal homeostasis using gene-targeted mice and demonstrated that mice lacking the p40\textsuperscript{phox} subunit of NADPH oxidase are more susceptible to DSS-induced colitis. Additionally, p40\textsuperscript{phox}-deficient mice showed enhanced neutrophil infiltration, a hallmark of both inflammatory bowel disease (IBD) and CGD (34). To our knowledge, we report the first evidence that NADPH oxidase deficiency is crucial for the resolution phase, rather than the acute phase, of inflammation. Previous studies of DSS colitis that used genetically deficient mice for other NADPH oxidase subunits have reported different outcomes in their models (15–17). Kriegstein et al. (16) demonstrated that the absence of p47\textsuperscript{phox} had no effect on colitis severity and colonic myeloperoxidase production after 7 d of 3% DSS treatment. Furthermore, through the use of an iNOS inhibitor, the investigators showed that in the absence of p47\textsuperscript{phox} and iNOS, animals were protected from DSS colitis. In contrast, Bao et al. (15) reported less colonic tissue damage and reduced myeloperoxidase levels after 7 d 2.5% DSS in p91\textsuperscript{phox}-deficient mice compared with WT mice. Although these studies have reported that susceptibility to DSS colitis is not affected by p47\textsuperscript{phox} deficiency and is lessened in the absence of p91\textsuperscript{phox}, we hasten to note that the recovery phase of inflammation was not investigated in these studies (15–17).

In this study, we show that the absence of p40\textsuperscript{phox} in Rag1\textsuperscript{−/−} mice enhances susceptibility to anti-CD40–induced colitis, suggesting that ROS in the innate compartment is essential during intestinal inflammation. However, in p40\textsuperscript{phox}\^{−/−}×Rag1\textsuperscript{−/−}DKO mice, DSS-induced inflammation was less severe than in p40\textsuperscript{phox}\^{−/−} mice, implying a contribution from lymphocytes. Given that NADPH oxidase activity has recently been shown to play a role in T cell differentiation and activation, p40\textsuperscript{phox}-deficient mice may have altered T cell responses in addition to the neutrophil phenotype. p47\textsuperscript{phox} deficiency has been associated with impaired T regulatory cell induction (44, 45) and increased IL-23 and Th17 response (44, 45). Indeed, we observed 50% fewer peripheral T regulatory cells in p40\textsuperscript{phox}\^{−/−} mice than in WT mice (data not shown). Although our data highlight an important role for p40\textsuperscript{phox} in neutrophils, we recognize that NADPH oxidase activity may affect other components of the inflammatory response.

We demonstrate that neutrophil infiltration in p40\textsuperscript{phox}\^{−/−} mice is coincident with greater inflammation during DSS challenge. However, despite greater numbers of recruited lamina propria neutrophils in p40\textsuperscript{phox}\^{−/−} mice during DSS colitis, the ROS-deficient phagocytes are unable to control infiltrating bacteria, as demonstrated by greater bacterial translocation to peripheral lymph nodes. This inability to efficiently kill microbes during DSS colitis may contribute to the exacerbated inflammation described in this NADPH oxidase-deficient model. During inflammation, ROS produced by neutrophils are thought to not only assist in antibacterial clearance but also to damage surrounding tissue (33, 34). However, mice deficient in the neutrophil chemokine Cxcl1 fail to recruit neutrophils during DSS colitis and show more severe symptoms, highlighting a role for neutrophils in restoring mucosal barrier integrity (46). To address whether neutrophils are necessary for recovery from DSS- and/or anti-CD40–induced colitis, as well as to establish whether p40\textsuperscript{phox}\^{−/−} neutrophils are pathogenic, we performed in vivo neutrophil depletion experiments. Depletion of WT neutrophils increased disease severity and induced 100% mortality by day 10 during DSS colitis and by day 4.
during anti-CD40 colitis, suggesting that neutrophils are important in the resolution of intestinal inflammation. Neutrophil depletion in p40\(^{-/-}\) mice treated with DSS, however, induced no significant differences in weight loss and inflammation compared with their p40\(^{-/-}\) neutrophil-replete counterparts, suggesting that an inflammatory threshold had already been met in the absence of ROS. Previous reports using the RP-3 mAb suggested that neutrophil depletion in WT rats suppresses DSS colitis; however, this Ab targeted the Gr1 Ab, which consists of both Ly-6G and Ly-6C molecules and therefore depleted not only neutrophils but also a subset of macrophages. Thus, we propose that the observed differences may be due to the depletion of several immune populations in previous studies and/or the kinetics of Ab administration (47). Our experimental design, in which a Ly-6G neutrophil-specific depletion Ab was repeatedly injected throughout the DSS course, differs from one-time depletion studies in which new neutrophils may be generated and recruited to assist in the resolution of inflammation at later stages of DSS. Using another anti-Gr1 Ab (RB6-8C5), Qualls et al. (48) demonstrated protection from DSS at early time points, which was lost by day 7 of treatment. Thus we extend these reports by describing a role for neutrophils during the resolution phase of intestinal inflammation.

To identify disease-relevant pathways mediated by p40\(^{box}\), we developed an integrative approach by combining functional studies of genetically deficient mice and murine models of IBD with cell-specific data extracted from clinical microarray studies of CGD. Bioinformatic analysis of the XCGD human PMN dataset revealed a signature in NF-κB signaling, suggesting that NADPH oxidase deficiency induces hyperactivity of this pathway. Data obtained from our murine model confirmed that proinflammatory cytokine expression was indeed higher in p40\(^{box}\) mice than in WT mice during DSS colitis, correlating with the degree of inflammation in the colon. Thus, our model mimics human disease and demonstrates a role for p40\(^{box}\) in the regulation of proinflammatory mediators.

We identified a subset of genes whose expression is affected both by impaired NADPH oxidase activity in XCGD neutrophils and by inflammation in mouse colon. Given our in vivo data implicating aberrant neutrophil recruitment, the neutrophil cell-specific analysis was key to unraveling the underlying mechanisms of increased colitis severity in p40\(^{box}\)-deficient mice. Validating the findings from our computational analysis, we confirmed that during early DSS colitis, induction of the neutrophil chemokine receptor Ccr1 was significantly higher in p40\(^{box}\)-/− colon compared with WT. Analysis of isolated lamina propria cells confirmed that in the context of NADPH oxidase deficiency, Ccr1 expression on neutrophils is significantly higher, despite equal levels of Cci3 and Cc15 chemoattractants present in the colon. As Ccr1 plays a key role in neutrophil infiltration (32, 40), its upregulation in p40\(^{box}\)-/− neutrophils likely accounts for the observed enhanced neutrophil recruitment in p40\(^{box}\)-/− colon during DSS. However, despite their greater recruitment, p40\(^{box}\)-/− neutrophils are unable to resolve intestinal inflammation. By using a Ccr1 antagonist in WT mice, we were able to block neutrophil recruitment to the colon during DSS colitis. Interestingly, we observed greater disease susceptibility in this setting, in agreement with a key role for neutrophils during healing. These data, as predicted by bioinformatic analyses, show not only that Ccr1 is regulated by p40\(^{box}\) but, moreover, that neutrophils are necessary during the restitution phase of DSS.

Our integrated bioinformatic analysis further identified NADPH oxidase activity as a novel regulator of the enzymes involved in the modification of glycan structures during intestinal inflammation. Six genes identified through our integrative genomics approach (St3gal6, St3gal4, St6gal1, Fut4, Fut9, and HexA) were significantly impaired in p40\(^{box}\)-/− mice during acute and recovery phases of DSS colitis. Moreover, by assessing these genes in neutrophil-depleted WT mice, we were able to demonstrate that p40\(^{box}\)-/− neutrophils are required for their expression during DSS inflammation. Sialyltransferases and fucosyltransferases such as St3gal4, St6gal1, Fut4, and Fut9 are key regulators of leukocyte trafficking through selectin ligand formation. For example, St3gal4 promotes neutrophil adhesion (49, 50), whereas St6gal1 deficiency has been reported to induce neutrophilic inflammation (51). Thus, decreased expression of sialyltransferases and fucosyltransferases in the absence of p40\(^{box}\) may contribute to aberrant neutrophil trafficking. Furthermore, alterations in mucosal thickness and mucin structure have been reported in IBD and other mucosal disorders due to aberrant glycosylation (41–43, 52–57). St3gal6, St3gal4, Fut4, and Fut9 have been implicated in intestinal mucin modification, and their impaired expression in p40\(^{box}\)-/− mice may compromise the protective mucosal barrier integrity, resulting in enhanced inflammation (37, 58). Moreover, Fut4 contributes to epithelial wound repair during airway inflammation (59, 60), suggesting that its decreased expression in p40\(^{box}\) deficiency may contribute to impaired resolution of inflammation. Taken together, our observations indicate that p40\(^{box}\) deficiency leads to alterations in the expression of enzymes modifying glycan structures, which may induce aberrant intestinal neutrophil recruitment and impaired wound healing.

By combining functional studies in genetically engineered murine models with computational analysis, we have demonstrated that p40\(^{box}\) deficiency induces an aberrant neutrophilic inflammation in response to intestinal injury, likely due to alterations in the mechanisms controlling leukocyte recruitment and wound healing.

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References


