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

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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.

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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 bioinformatics approach that integrates analyses of clinical gene expression signatures in CDG patient neutrophils and temporal gene expression profiles during murine DSS colitis (18, 19). Using this method, we approach that integrates analyses of clinical gene expression signature is necessary for the neutrophil-mediated restitution response. By applying this analysis to our functional animal model, we propose that p40<sup>phox</sup> is essential for the resolution of inflammation through downregulation of Ccr1 and upregulation of enzymes involved in glycan modifications in neutrophils.

**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 multiplex 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'-AAGACGGTCAAGG-ACTGGCA-3', reverse, 5'-CAGATGTAAGCCGGTCCAG-3'; Tnfα, forward, 5'-GACCTGGAACCTGGCAGAAG-3', reverse, 5'-TTGGTTGTTTGGATGTTGAG-3'; Ifng, forward, 5'-GATACAACCCTTCATGCC-3', reverse, 5'-GGACCCACAGTCTTGTCCT-3'; Il-1β, forward, 5'-GGCCATCTCTGTAAGTACT-3', reverse, 5'-AGGCAAGATTCTTGCAC-3'; IL-6, forward, 5'-CTGAGATGAGCTTTGAGAAGC-3', reverse, 5'-AGAGTATGAGCTTGGCAGAAA-3'; IL-17A, forward, 5'-TTTACCTCTTTGCGAAAACA-3', reverse, 5'-GTCCTCTCCCGCATGAC-3'; inducible NO synthase (iNOS), forward, 5'-CTGGTTATGTGTTGAGCAAGA-3', reverse, 5'-CATGAAATTTCTCCTCAGCC-3'; Ccl5, forward, 5'-ACGTCAGAGTATTTCTCAC-3', reverse, 5'-GATATGTCTTTGAGCAGA-3'; Ccl3, forward, 5'-AGATTCGCGCAACATCTCACTC-3'; reverse, 5'-CTCAAGGGCTCTGCTTAC-3'; T1gαl, forward, 5'-GCCCTTCCAAGAACAGGAC-3', reverse, 5'-CGCCTAAGAAGAGCAGAC-3'; T1gβ4, forward, 5'-GCCCTCAAAAGAAGCAGAC-3', reverse, 5'-GATGCGCAAGATGAAGGAC-3'; S100a1, forward, 5'-CTGGCCAAGGAGAATCAG-3', reverse, 5'-AGGCGTCTTCTGTTGACT-3'; Flt4, forward, 5'-GCCATTCTGCTGTAAGCT-3', reverse, 5'-TTGGTGACAGTAAAGGAAAG-3'; Furf, forward, 5'-CTTCTGTCGTTGAGCCAAA-3', reverse, 5'-GAAATGCTTGGCCATAGGTG-3'; Hexa, forward, 5'-GGGCGTACTGCTGGATAGGT-3'; reverse, 5'-ACCTCTTCATCCTTGTCG-3'. The threshold cycle (C<sub>t</sub>) for each sample was calculated for each gene and was normalized to the C<sub>t</sub> value of the endogenous housekeeping gene GAPDH. Data were calculated using the 2<sup>ΔΔCt</sup> method (26, 27).

**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 on polystyrene tubing (Becton Dickinson, Franklin Lakes, NJ). After being washed in calcium- and magnesium-free PBS, colon tissues were digested in calcium- and magnesium-free PBS, colon tissues were digested in calcium- and magnesium-free PBS. Colon tissues were digested 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 × g, 15 min) (28).

**Flow cytometry**

Colon lamina propria cells (2 × 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% FBS (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-PE, 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 Jose, CA) and analyzed using FlowJo analysis software (Tree Star, Ashland, OR).
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-week-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 C3bi-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 < 1283) 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 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 CCR1 antagonist treatment

One hour prior to DSS treatment, WT C57BL/6J mice were injected i.p. with 10 mg/kg body weight of the CCR1 antagonist JNJ113865 (Tocris Bioscience, Bristol, U.K.) dissolved in PBS, as described with other CCR1 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−/− mice exhibited a higher mortality rate throughout the course of DSS treatment (Fig. 1B). p40phox−/− 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). HE staining of colon sections revealed inflammation in p40phox−/− mice characterized by epithelial ulceration and leukocyte infiltration (Fig. 1F). Consistent with the inflammation observed in p40phox−/− mice, local TNF-α, IFN-γ, IL-1β, IL-6, iNOS, and IL-17A levels were significantly higher in p40phox−/− mice than in WT mice on day 12 (Fig. 1G, 1H). Notably, similar levels of the anti-inflammatory cytokine IL-10 were observed in DSS-treated WT and p40phox−/− colon after 12 d (data not shown). 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 CFUs per gram of tissue in both spleen and mesenteric lymph node, suggesting an inability to effectively clear microbes (Fig. II). p40phox−/− mice show 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 (p<0.05), 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
and B lymphocyte-deficient Rag1\(^{-/-}\) mice induces acute gastrointestinal inflammation and wasting disease within 2 d and is largely driven by local IL-23 production (23). The histological hallmarks of this colitis model include leukocyte infiltration into the lamina propria, goblet cell depletion, and marked epithelial hyperplasia (23). During the first 4 d following CD40 mAb treatment, p40\(^{phox/-}\) \(\times\) Rag1\(^{-/-}\) DKO mice lost weight with similar kinetics as did their Rag1\(^{-/-}\) control littermates. However, DKO mice were unable to recover similarly to their anti-CD40- treated Rag1\(^{-/-}\) littermates on days 5–7 (\(p < 0.01\), Fig. 3A). As previously reported, Rag1\(^{-/-}\) 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\(^{phox/-}\) mice in DSS, DKO mice had a significantly higher histology score in comparison with Rag1\(^{-/-}\) mice (Fig. 3C). Colonic tissues displayed greater inflammation in DKO mice, characterized by polymorphonuclear cell infiltration (Fig. 3D). Taken together, these data demonstrate that p40\(^{phox}\) 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\(^{phox/-}\) deficient mice during DSS colitis**

Given the exacerbated response of p40\(^{phox/-}\) deficient animals to murine models of colitis, we next assessed the cellular composition of the colonic lamina propria in WT and p40\(^{phox/-}\) mice after DSS colitis. Flow cytometric analysis revealed a population of Ly-6G\(^{+}\)CD11b\(^{+}\)F4/80\(^{-}\) cells in p40\(^{phox/-}\) deficient colon whose frequency was significantly greater than their WT counterparts (representative histograms shown in Fig. 4A; WT, 5.4 \(\pm\) 3%; p40\(^{phox/-}\), 39.9 \(\pm\) 5%). Based on cell surface molecule expression, Ly-6G\(^{+}\)CD11b\(^{+}\)F4/80\(^{-}\) cells would be defined as neutrophils; however, to definitively categorize this population, the morphology of FACS-sorted Ly-6G\(^{+}\)CD11b\(^{+}\)F4/80\(^{-}\) cells was examined on day 12 after DSS colitis in p40\(^{phox/-}\) mice. Wright’s staining revealed that the sorted Ly-6G\(^{+}\)CD11b\(^{+}\)F4/80\(^{-}\) cells possessed multilobulated nuclei, the hallmark characteristic defining neutrophil morphology (Fig. 4B). Total neutrophil numbers were increased in p40\(^{phox/-}\) lamina propria at day 6 (Fig. 4C; WT, 0.3 \(\times\) 10\(^5\) \(\pm\) 0.1 \(\times\) 10\(^5\); p40\(^{phox/-}\), 3.0 \(\times\) 10\(^5\) \(\pm\) 1 \(\times\) 10\(^5\)) and day 12 (Fig. 4D; WT, 1.2 \(\times\) 10\(^5\) \(\pm\) 0.5 \(\times\) 10\(^5\); p40\(^{phox/-}\), 3.7 \(\times\) 10\(^5\) \(\pm\) 2 \(\times\) 10\(^5\)). Thus, our data demonstrate enhanced neutrophil recruitment to the colon in p40\(^{phox/-}\) mice following DSS treatment.

**p40\(^{phox/-}\) 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\(^{phox/-}\) 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-


FIGURE 2. p40<sup>lox/lox</sup> × 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.

FIGURE 3. p40<sup>lox/lox</sup> × Rag1<sup>−/−</sup> mice show increased susceptibility to anti-CD40-induced colitis. (A) Age- and weight-matched mice were injected i.p. with either anti-CD40 monoclonal depletion Ab (Rag1<sup>−/−</sup> and DKO mice) or isotype control (Rag1<sup>−/−</sup> Iso) on day 0. Changes in body weight were monitored daily for 7 d (n = 24/group; *p < 0.01 between DKO and Rag1<sup>−/−</sup> mice injected with anti-CD40). (B) DAI was determined on day 7 by scoring hunching, wasting, stool consistency, and colon thickness. Significance was determined by a two-sided Wilcoxon rank sum test; the test rejected the null hypothesis at the 5% significance level (n = 24/group; *p = 0.0095). (C) Colonic tissue sections were blindly scored for inflammation on day 7. Grading parameters include extent of epithelial hyperplasia, goblet cell depletion, lamina propria infiltration, and epithelial cell damage (n = 14/group; Rag1<sup>−/−</sup> average, 12.8 ± 0.5; DKO average, 17.5 ± 0.5; *p < 0.001). (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 four independent experiments.

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

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 of C57BL/6 WT mice treated with DSS (18). After normalization and analysis of the microarray data, we identified 1283 differentially expressed genes (Supplemental Fig. 2). Most of the early responding genes (days 0–2) were suppressed by day 2 and were largely enriched for sonic hedgehog and Wnt signaling pathways (Fig. 6A, Supplemental Fig. 2). The group of middle- to late-responding genes (days 2–6) were enriched for the glycosphingolipid biosynthesis pathway (Fig. 6A, Supplemental Fig. 2). Eight of 26 genes in the glycosphingolipid biosynthesis pathway, including fucosyltransferases and sialyltransferases Fuc2, Fut9, Fut4, St3gal4, St3gal6, Abo, B3gal5, and B3galn5, were significantly differentially downregulated during this acute DSS phase (Supplemental Fig. 2). Finally, the late-responding genes showed significant enrichment for the cytokine–cytokine receptor interactome (Fig. 6A, Supplemental Fig. 2). Notably, these genes included chemotactic receptors and ligands such as Ccr1, Ccl2, and Ccr2. Thus, the analysis of temporal gene expression profiles


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- Killing ROS generation, epithelial imprinting, and immune cell recruitment (33, 34). During the resolution phase of inflammation, however, neutrophils promote wound healing via heterotypic cell interactions with the epithelium, inducing the expression of enzymes that facilitate the production of anti-inflammatory lipid mediators and mucins (35–38).

To complement our genetic model involving NADPH oxidase-deficient neutrophils and to determine whether neutrophils contribute to intestinal homeostasis, we next depleted neutrophils during DSS inflammation using a Ly-6G–specific depletion Ab (39). We monitored body weight and survival during DSS administration (7 d), followed by a water recovery period (5 d) (Fig. 5). Interestingly, WT mice that had been depleted of neutrophils prior to DSS treatment lost significantly more weight than did their neutrophil-intact counterparts and had a 100% mortality rate by day 10 (Fig. 5A, 5C), indicating a protective role for neutrophils during DSS colitis. In contrast, p40<sup>lox/lox</sup> mice exhibited no 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>lox/lox</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>lox/lox</sup> expression is necessary for this process.
is largely neutrophil-specific, as we detected higher levels of phox expression in isolated p40 of Ly-6G+CD11b+F4/80 confirmed that in murine intestinal inflammation (32, 40). Quantitative PCR of a neutrophil chemokine receptor, has been previously implicated expressed in these cell types (Fig. 6D). Among them, CCR1, phox challenged mice is more highly induced in p40 displayed (* recovery phases of DSS colitis. The average of 10 mice per group is shown 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.

in murine colon during DSS highlights important roles for hedgehog and Wnt signaling pathways, glycosphingolipid biosynthesis, and the activation of the chemokine reactome.

Having established 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 our analysis. We found that genes induced during phagocytosis in XCGD neutrophils were enriched for members of the NF-κB, PI3K/Akt, and MAPK signaling pathways (Fig. 6B, Supplemental Fig. 3). Similarly, the expression of several NF-κB–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, TRIM25, 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 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 FACSSorted 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.

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 = 8/group). Significance was determined using a Student t test for body weight (*p < 0.01) and a log-rank test for survival (p < 0.001 for (C); p = 0.399 for (D)). For anti-CD40 colitis, age-and weight-matched mice were pretreated with either PBS or Ly-6G neutrophil depletion Abs prior to anti-CD40 administration. Changes in body weight (E, F) and animal survival (G, H) were monitored daily (n = 5/group). Significance was determined using a Student t test for body 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.
compare with Fig. 4A). However, WT mice that received the CCR1 antagonist experienced significantly greater weight loss, more severe inflammation, and increased bacterial translocation compared with nontreated animals (Fig. 7F–H). Taken together, these data suggest that Ccr1 expression and subsequent neutrophil recruitment are required for DSS recovery, a process that is dysregulated in the absence of NADPH oxidase.

The glycosphingolipid biosynthesis pathway was highlighted in our analysis of both DSS and XCGD datasets, suggesting that this pathway may be important in neutrophils during inflammation. Within this pathway, the overlap specifically highlighted HexB and St3gal6, enzymes that are involved in the synthesis and termination of carbohydrate chains and that modify glycolipids and glycoproteins. We confirmed in our DSS model that St3gal6 is downregulated during acute colitis; however, as inflammation resolved during the recovery period, St3gal6 transcript levels increased and were significantly higher in WT mice compared with p40phox−/− mice (Figs. 6C, 8A) (18). St3gal6 is a transferase involved in the biosynthesis of carbohydrates, particularly through sialylation, and has been implicated in diseases characterized by aberrant mucus production (41–43). In addition to St3gal6, analysis of the DSS time course dataset highlighted two additional sialyltransferases (St3gal4 and St6gal1) and two fucosyltransferases (Fut4 and Fut9) that are downregulated during acute colitis 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 p40phox−/− mice during colitis and, unlike WT mice, expression of these transferases remained low after the recovery phase at day 12.
that p40 subunit in intestinal homeostasis using gene-targeted mice and demonstrated that mice lacking the p40 subunit of NADPH oxidase are more susceptible to DSS-induced colitis. Additionally, p40-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 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 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 gp91-deficient mice compared with WT mice. Although these studies have reported that susceptibility to DSS colitis is not affected by p47 deficiency and is lessened in the absence of gp91, 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 in Rag mice enhances susceptibility to anti-CD40–induced colitis, suggesting that ROS in the innate compartment is essential during intestinal inflammation. However, in p40/×× Rag DKO mice, DSS-induced inflammation was less severe than in p40/ 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-deficient mice may have altered T cell responses in addition to the neutrophil phenotype. p47 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/ mice than in WT mice (data not shown). Although our data highlight an important role for p40 in neutrophils, we recognize that NADPH oxidase activity may affect other components of the inflammatory response.

We demonstrate that neutrophil infiltration in p40/ mice is coincident with greater inflammation during DSS challenge. However, despite greater numbers of recruited lamina propria neutrophils in p40/ 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/ 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

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 subunit in intestinal homeostasis using gene-targeted mice and demonstrated that mice lacking the p40 subunit of NADPH oxidase are more susceptible to DSS-induced colitis. Additionally, p40-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 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 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 gp91-deficient mice compared with WT mice. Although these studies have reported that susceptibility to DSS colitis is not affected by p47 deficiency and is lessened in the absence of gp91, we hasten to note that the recovery phase of inflammation was not investigated in these studies (15–17).

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**Figure 8.** p40 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/– 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 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).
during anti-CD40 colitis, suggesting that neutrophils are important in the resolution of intestinal inflammation. Neutrophil depletion in p40$^{lox/-}$ mice treated with DSS, however, induced no significant differences in weight loss and inflammation compared with their p40$^{lox/-}$ 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 Ag, 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$^{lox/-}$, 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-$k$$b$ 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$^{lox/-}$ 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$^{lox}$ 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$^{lox/-}$ 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$^{lox/-}$ 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 Ccl3 and Ccl5 chemotacticants present in the colon. As Ccr1 plays a key role in neutrophil infiltration (32, 40), its upregulation in p40$^{lox/-}$ neutrophils likely accounts for the observed enhanced neutrophil recruitment in p40$^{lox/-}$ colon during DSS. However, despite their greater recruitment, p40$^{lox/-}$ 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$^{lox}$ 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, Futt4, Futt9, and HexA) were significantly impaired in p40$^{lox/-}$ 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$^{lox}$-intact neutrophils are required for their expression during DSS inflammation. Sialyltransferases and fucosyltransferases such as St3gal4, St6gal1, Futt4, and Futt9 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$^{lox}$ 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, Futt4, and Futt9 have been implicated in intestinal mucin modification, and their impaired expression in p40$^{lox/-}$ mice may compromise the protective mucosal barrier integrity, resulting in enhanced inflammation (37, 58). Moreover, Futt4 contributes to epithelial wound repair during airway inflammation (59, 60), suggesting that its decreased expression in p40$^{lox}$ deficiency may contribute to impaired resolution of inflammation. Taken together, our observations indicate that p40$^{lox}$ 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$^{lox}$ 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


