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Colonic Eosinophilic Inflammation in Experimental Colitis Is Mediated by Ly6C<sup>high</sup> CCR2<sup>+</sup> Inflammatory Monocyte/Macrophage-Derived CCL11

Amanda Waddell,* Richard Ahrens,* Kris Steinbrecher, † Burke Donovan,* Marc E. Rothenberg,* Ariel Munitz,*‡ and Simon P. Hogan*

Recent genome-wide association studies of pediatric inflammatory bowel disease have implicated the 17q12 loci, which contains the eosinophil-specific chemokine gene CCL11, with early-onset inflammatory bowel disease susceptibility. In the current study, we employed a murine model of experimental colitis to define the molecular pathways that regulate CCL11 expression in the chronic intestinal inflammation and pathophysiology of experimental colitis. Bone marrow chimera experiments showed that hematopoietic cell-derived CCL11 is sufficient for CCL11-mediated colonic eosinophilic inflammation. We show that dextran sodium sulfate (DSS) treatment promotes the recruitment of F4/80<sup>+</sup>CD11b<sup>+</sup>CCR2<sup>+</sup>Ly6C<sup>high</sup> inflammatory monocytes into the colon. F4/80<sup>+</sup>CD11b<sup>+</sup>CCR2<sup>+</sup>Ly6C<sup>high</sup> monocytes express CCL11, and their recruitment positively correlated with colonic eosinophilic inflammation. Phenotypic analysis of purified Ly6C<sup>high</sup> intestinal inflammatory macrophages revealed that these cells express both M1- and M2-associated genes, including Il6, Ccl4, Cxcl2, Arg1, Chi3l3, Ccl11, and Il10, respectively. Attenuation of DSS-induced F4/80<sup>+</sup>CD11b<sup>+</sup>CCR2<sup>+</sup>Ly6C<sup>high</sup> monocyte recruitment to the colon in CCR2<sup>−/−</sup> mice was associated with decreased colonic CCL11 expression, eosinophilic inflammation, and DSS-induced histopathology. These studies identify a mechanism for DSS-induced colonic eosinophilia mediated by Ly6C<sup>high</sup>CCR2<sup>+</sup> inflammatory monocyte/macrophage-derived CCL11. The Journal of Immunology, 2011, 186: 5993–6003.

The inflammatory bowel diseases (IBD) Crohn’s disease (CD) and ulcerative colitis (UC) are chronic relapsing gastrointestinal (GI) inflammatory diseases that cause substantial morbidity and decreased quality of life. Over 30 years ago, Rutgeerts and colleagues (1) observed that aphthous ulcers containing an eosinophil infiltrate and blunted villi were the earliest endoscopic signs of recurrence in the neoterminal ileum and anastomosis following surgical resection for CD. Eosinophils usually represent only a small percentage of the infiltrating leukocytes (2, 3), but their level has been proposed to be a negative

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diagnostic marker of disease activity (4). They are often elevated in patients with CD (5–10). Increased numbers of tissue eosinophils associated with clinical relapse within 3 mo in CD (5). Since these initial studies, there have been a number of studies suggesting eosinophil involvement in IBD. Elevated levels of eosinophils have been observed in colon biopsy samples from UC patients, and increased numbers of this cell and eosinophil-derived granule proteins MBP, ECP, EPO, and EDN have been shown to correlate with ultrastructural evidence of activation has also been observed in patients with CD (11–13). Consistent with this clinical observation, dextran sodium sulfate (DSS)-induced histopathology is attenuated in mice deficient in eosinophils (14–16).

Genome-wide association studies of pediatric and adult IBD have revealed a number of IBD susceptibility genes associated with innate (CARD15, ATG16L1, and IRGM) and adaptive (IL23R, IL10, IL12B, and STAT3) immunity. Furthermore, a recent investigation identified a significant association between the C-C motif chemokine cluster on 17q12 loci, which contains the eosinophil-specific chemokine gene CCL11, and early-onset CD (17). CCL11 is a member of the CC chemokine family (18) and is a relatively potent and specific eosinophil chemoattractant (19–21). CCL11 is constitutively expressed in a variety of tissues that contain eosinophils such as the GI tract and thymus (22–25). Genetic deletion of CCL11 abrogates eosinophil recruitment during eosinophil-associated pulmonary and GI disease, suggesting an important role for CCL11 in eosinophil trafficking during disease (26–29). Consistent with this, clinical investigations by us and others demonstrate increased CCL11 mRNA levels in sputum and intestinal biopsy samples from asthmatic and eosinophilic GI disorder patients. Importantly, the levels of CCL11 positively correlated with tissue eosinophilia (14, 25, 26).

**Abbreviations used in this article:** BM, bone marrow; CD, Crohn’s disease; DAL, disease activity index; DSS, dextran sodium sulfate; eGFP, enhanced GFP; GI, gastrointestinal; hpf, high-power field; IBD, inflammatory bowel diseases; Mφ, macrophages; PDL1, programmed death ligand 1; PHIL, mice deficient in eosinophils; SC, stromal compartment; UC, ulcerative colitis; WT, wild-type.

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Although a link between CCL11 and eosinophils in IBD has been established, the cellular source of CCL11 and molecular regulation of CCL11 expression in experimental colitis is not yet delineated. In the current study, we employed a model of DSS-induced colitis, which features a pronounced CCL11-dependent eosinophilic inflammation, to decipher the molecular regulation of CCL11 in inflammation, to decipher the molecular regulation of CCL11 in experimental colitis.

Materials and Methods

Mice

Male and female, 6- to 8-week-old C57BL/6, C57BL/6 (The Jackson Laboratory, Bar Harbor, ME), BALB/c, C57BL/6 (BALB/c) (30), CXCR1+/GFP (The Jackson Laboratory), and Nzeg-enhanced GFP (eGFP) (31) mice were used. All mice were housed under specific pathogen-free conditions and treated according to institutional guidelines.

DSS-induced colonic injury and histopathologic examination

DSS (ICN Chemicals; 40–45 kDa) was administered in the drinking water as a 2.5–5% (w/v) solution for up to 8 d. Disease monitoring and histopathologic changes in the colon were scored as previously described (14).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (14, 32). In brief, frozen sections were fixed in 10% acetone for 10 min, rinsed in PBS, blocked with 3% goat serum/PBS for 2 h at room temperature, and incubated with primary Ab rat anti-mouse F4/80 (5 µg/ml; eBioscience, San Diego, CA) in 3% normal goat serum/PBS. Sections were incubated with isotype control alone in place of primary Ab as a negative control. After an overnight incubation at 4˚C, sections were washed with 0.1% BSA and 0.05% Tween/PBS and incubated with goat anti-rat Alexa Fluor 594 (Invitrogen, Carlsbad, CA) in 3% normal goat serum/PBS. Sections were incubated with primary Ab rat anti-mouse F4/80 (5 µg/ml; eBioscience, San Diego, CA) and immediately sorted using an FACSaria cell sorter (BD Biosciences, San Jose, CA) for CXCR1+eGFP and Ly6C. Purity of CXCR1+Ly6Chigh cells was >95% as assessed by flow cytometry. RNA was isolated using the Qiagen RNeasy micro kit for cDNA synthesis (Qiagen) and RT-PCR analysis was performed as described above.

Monocyte enrichment

Peripheral blood was collected in K2EDTA tubes, and RBCs were lysed by using RBC Lysing Buffer (Sigma-Aldrich, St. Louis, MO). Monocytes were enriched using the StemCell Technologies monocyte enrichment kit following the manufacturer’s protocol (StemCell Technologies). Purity was assessed by flow cytometry at >80% F4/80+CD11b+. RNA was isolated using the Qiagen RNeasy micro kit (Qiagen), and cDNA was generated for RT-PCR analysis as described above.

FACS analysis

Single-cell suspensions were washed with FACS buffer (PBS/1% FCS) and incubated with combinations of the following Abs: PE-anti-mouse F4/80 (clone CI.A3-1; AbD Serotec), PE-Cy7 anti-mouse CD11b (clone M1/70; BD Pharmingen), Alexa Fluor 647 anti-mouse Ly6C (clone ER-MP20; AbD Serotec), FITC anti-mouse CD206 (MRSD3; Biologend, San Diego, CA), allopolyocyanin anti-mouse CD11c (clone HL3; BD Pharmingen), FITC anti-mouse programmed death ligand 1 (PD1L) (clone MIH6; AbD Serotec), and goat anti-mouse CC1R (polyclonal; GeneTex, Irvine, CA) followed by donkey anti-goat FITC (Jackson ImmunoResearch Laboratories, West Grove, PA), and biotinylated anti-mouse TLR2 (clone nT2;7; eBioscience), followed by streptavidin-FITC (BD Pharmingen), allopolyocyanin anti-mouse CCR3 (clone 83101; R&D Systems), and PE anti-mouse Siglec F (clone E50-2440; BD Pharmingen). The following Abs were used as appropriate isotype controls: FITC rat IgG2a (clone B39-4; BD Pharmingen), PE rat IgG2a (clone 53-6;7; BD Pharmingen), PE-Cy7 rat IgG2b (clone DTA-1; BD Pharmingen), and Alexa Fluor 647 rat IgG2a (clone R35-95; BD Pharmingen). Cells were analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA), and analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Generation of BM chimeras

BM was isolated from Nzeg-eGFP (BALB/c) (31) and CCL11−/− mice (BALB/c). Lethally irradiated (two doses of 1100 cGy) at 475 and 475 rad, 3 h
apart) wild-type (WT) or CCL11−/− BALB/c recipients were injected i.v. with 5–10 × 10⁶ BM cells/mouse. Engraftment was checked by eGFP⁺ (donor)/eGFP⁺ (recipient) cells from the peripheral blood, mesenteric lymph node, and colon by flow cytometry. Seven to 8 wk postirradiation, the mice were administered 5% DSS for 7 d, and colonic eosinophil accumulation was assessed.

Statistical analysis

Data were analyzed by means of ANOVA, followed by the Tukey post hoc test, and correlative analysis was performed using a Spearman rank order correlation coefficient analysis with GraphPad Prism 5 (GraphPad, San Diego, CA). Data are presented as the mean ± SE. The p values < 0.05 were considered statistically significant.

Results

BM-derived CCL11 is sufficient to reconstitute eosinophil recruitment to the colon of CCL11−/− mice during DSS-induced colitis

We have previously reported a pathological role for eosinophils in DSS-induced colonic injury and that eosinophil recruitment into the colon following DSS treatment was dependent on CCL11 (14). With the emerging experimental and clinical data demonstrating DSS-induced colonic injury and that eosinophil recruitment into the colon during DSS-induced colitis was sufficient to reconstitute DSS-induced colonic eosinophilic inflammation, we were interested in defining the cellular source of CCL11 in experimental colitis. To assess if hematopoietic or stromal compartment expressed CCL11, we irradiated recipient CCL11−/− BALB/c recipients with WT BM. In these mice, referred to as SC−/−, only cells derived from transferred WT BM are CCL11 sufficient (+), whereas the radioresistant MΦs and SC native to the recipient CCL11−/− mice do not express functional CCL11 (−). Conversely, in SC⁺/⁺ mice, we restricted CCL11 signaling to only the SC compartment by irradiating WT mice and reconstituting with CCL11−/− BM. As controls, WT or CCL11−/− mice were irradiated and reconstituted with their own BM type (SC⁺/⁺ and SC−/−, respectively). Seven to 8 wk following BM reconstitution, chimeric mice were exposed to 5% DSS, and eosinophilic inflammation was evaluated. To facilitate analysis of chimerism in BALB/c mice, we transferred BM from BALB/c Nζg-eGFP mice to WT and CCL11−/− BALB/c mice. All cells from the Nζg-eGFP mice are constitutively GFP positive (31) and can be detected by flow cytometric analysis of autofluorescence. In chimeric mice, eGFP⁺ cells derived from the donor BM are distinguished from any remaining recipient eGFP⁺ cells. The degree of chimerism at 7 wk as determined by flow cytometry was 98.2 ± 0.2% and 95.4 ± 2.1% for peripheral blood monocytes and eosinophils, respectively, and 72.0 ± 0.6% for mesenteric lymph nodes (CD4⁺ T cells) (mean ± SEM; n = 3 to 4 mice per group) (Supplemental Fig. 1A–C). Percent reconstitution of colonic MΦs at baseline and following DSS was 66.9 ± 2.8% and 93.3 ± 1.2%, respectively, as determined by flow cytometry (Supplemental Fig. 1D, 1E). Chimerism was also determined for baseline colonic CD4⁺ T cells, B cells, and eosinophils (Supplemental Fig. 1D, 1E). Following the verification of reconstitution, mice were exposed to DSS for 7 d, and colonic eosinophil inflammation was quantitated. DSS treatment of SC⁺/⁺ and SC−/− BM mice induced a significant increase in colonic eosinophil levels compared with control-treated mice (Fig. 1A, 1B; SC⁺/⁺ baseline 8.0 ± 1.0 versus SC−/− BM DSS 17.2 ± 2.9 eosinophils/high-power field (hpf), p < 0.05; SC−/− baseline 3.4 ± 0.1 versus SC−/− BM 13.2 ± 1.2 eosinophils/hpf, p < 0.05; n = 3 to 5 mice baseline; n = 7 to 8 mice DSS). Similarly, DSS treatment of CCL11−/− mice reconstituted with WT BM (SC−/− BM) induced a 3-fold increase in eosinophil recruitment in the distal colon compared with SC−/− BM mice (Fig. 1A, 1B; SC−/− BM baseline 12.4 ± 1.6 eosinophils/hpf versus SC−/− BM 3.8 ± 1.3 eosinophils/hpf; n = 3 to 4 mice per group). Eosinophils were also significantly increased in SC−/− BM mice (Fig. 1B). Importantly, DSS-induced colonic eosinophilic inflammation was attenuated in CCL11−/− mice reconstituted with CCL11−/− BM (SC−/− BM; Fig. 1B). These data indicate that BM-derived CCL11 is sufficient to drive eosinophilic recruitment into the colon during DSS-induced colonic injury.

DSS-induced colonic injury promotes the specific recruitment of F4/80⁺CD11b⁺Ly6chigh monocytes

Following our demonstration that BM-derived CCL11 expression was sufficient to reconstitute DSS-induced colonic eosinophilic inflammation, we were next interested in identifying the hematopoietic source of CCL11 that drove DSS-induced colonic eosinophilic inflammation. We have previously demonstrated CCL11 expression in F4/80⁺ myeloid cells within the lamina propria of...
the colon of mice following 7 d of exposure to 2.5% DSS (14). We therefore assessed the relationship between myeloid cell and DSS-induced eosinophilic inflammation. First, we performed flow cytometry analysis on peripheral blood monocytes and colonic MΦs at baseline and following DSS exposure (Fig. 2A). Under homeostatic conditions, the peripheral blood was predominantly composed of F4/80+CD11b+Ly6C<sup>high</sup> myeloid cells, whereas the colon consisted of F4/80+CD11b+Ly6C<sup>low</sup> and F4/80+CD11b+Ly6C<sup>high</sup> myeloid cells (Fig. 2A). The predominant F4/80+CD11b+Ly6C<sup>low</sup> myeloid population (>80%) within the colon was CX3CR1<sup>high</sup>PDL1<sup>+</sup>TLR-2<sup>−/−</sup>CD206<sup>−/−</sup>CD11c<sup>−/−</sup>PDL1<sup>−/−</sup> (Fig. 2B), consistent with the resident intestinal MΦ phenotype (35). DSS exposure (5 d) induced a significant influx of F4/80+CD11b+Ly6C<sup>high</sup> monocytes (Fig. 2A). Notably, the increase in colonic F4/80+CD11b+Ly6C<sup>high</sup> monocyte/MΦ cell numbers (control 7,497±1,565 versus DSS 39,996±8,708, p<0.01; mean ± SEM; n=5 to 6 per group) occurred in the absence of any change in F4/80+CD11b+Ly6C<sup>low</sup> MΦ levels (control 55,979±12,490 versus 42,818±7,190; mean ± SEM; n=5 to 6 per group) (Fig. 2A). The infiltrating F4/80+CD11b+Ly6C<sup>high</sup> monocyte/MΦ population was predominantly CCR2<sup>−/−</sup>CX3CR1<sup>−/−</sup>TLR-2<sup>−/−</sup>CD206<sup>−/−</sup>CD11c<sup>−/−</sup>PDL1<sup>−/−</sup> (Fig. 2B, Supplemental Fig. 2). We next assessed the colonic eosinophil population following DSS exposure. We show that eosinophils were a distinct population characterized by forward light scatter<sup>low</sup> and side scatter<sup>high</sup>. Eosinophil lineage was confirmed as the cells were Siglec F<sup>+</sup>CCR3<sup>+</sup> double positive. Notably, the colonic eosinophil population was CD11b<sup>−/−</sup>, F4/80<sup>−/−</sup>, and CCR2<sup>−/−</sup> (Fig. 2C).

Colonic Ly6C<sup>high</sup> MΦs express Ccl11

To directly assess if Ly6C<sup>high</sup> colonic MΦs were a source of CCL11, we purified F4/80+CD11b+Ly6C<sup>high</sup> MΦs from the colon of DSS-treated mice by using CX3CR1<sup>eGFP<sup>+</sup></sup> mice. Previous studies have demonstrated that inflammatory (Ly6C<sup>high</sup>CCR2<sup>−</sup>CX3CR1<sup>low</sup>) and noninflammatory (Ly6C<sup>low</sup>CCR2<sup>−</sup>CX3CR1<sup>hi</sup>) tissue MΦs can be distinguished based upon the level of CX3CR1 expression (35). Consistent with this, the resident noninflammatory F4/80+CD11b+Ly6C<sup>low</sup> MΦ population of CX3CR1<sup>eGFP<sup>+</sup></sup> mice was CX3CR1<sup>high</sup> whereas the DSS-induced colonic F4/80+CD11b+Ly6C<sup>high</sup> MΦ population was CX3CR1<sup>low</sup> (Fig. 2B). In conjunction, the CX3CR1<sup>low</sup> cells within the colon of DSS-treated mice were found to be F4/80+CD11b+Ly6C<sup>high</sup>, consistent with the infiltrating monocyte population (Fig. 3A).

Following our confirmation that CX3CR1 and Ly6C expression could distinguish between the two intestinal myeloid subpopulations, we purified CX3CR1<sup>low</sup>Ly6C<sup>high</sup> cells from the colons of CX3CR1<sup>eGFP<sup>+</sup></sup> mice following DSS exposure using flow sorting (Fig. 3B). Purity of CX3CR1<sup>low</sup>Ly6C<sup>high</sup> cells was >95% as assessed by flow cytometry (data not shown). For compara-

FIGURE 2. DSS exposure induces the recruitment of F4/80+CD11b+Ly6C<sup>high</sup> monocyte population. A, Representative flow cytometry plots of the F4/80+CD11b+Ly6C<sup>high</sup> monocyte/MΦ populations from the peripheral blood (top panel), colon at baseline (middle panel), and colon following DSS exposure (bottom panel). MΦ were initially gated by side scatter (SSC) versus forward light scatter (FSC) followed by F4/80+CD11b+. The double-positive cell populations from baseline and DSS-treated colons were assessed for Ly6C. B, Ly6C<sup>high</sup> and Ly6C<sup>low</sup> colonic MΦ expression of CCR2, CX3CR1, TLR2, PDL1, and CD206 by flow cytometry at baseline and following DSS. C, Characterization of F4/80, CD11b, Siglec F, CCR3, and CCR2 on colonic eosinophils from DSS-treated mice. Data represent the mean ± SEM of n=5 to 6 mice per group from duplicate experiments. Significant differences (*p<0.05) between groups.
tive analyses, we also purified blood CX3CR1\textsuperscript{low}F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocytes from CX3CR1\textsuperscript{eGFP/+} mice at baseline and on day 5 of DSS (Fig. 4A, 4B). PCR analyses revealed no detectable Ccl11 mRNA expression in CX3CR1\textsuperscript{low}Ly6Chigh peripheral blood monocytes at baseline or following DSS (Fig. 3C). Ccl11 mRNA expression was induced in the colonic CX3CR1\textsuperscript{low}Ly6Chigh cells following infiltration into the colon during DSS-induced colitis (Fig. 3C). Assessment of other genes expressed by the Ly6Chigh myeloid population revealed that recruited F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh M\textsubscript{F}s had a similar phenotype to the peripheral blood population, as they were positive for Chi3l3, Trem1, Cxcl10, Tnfa, and Pdgfb mRNA transcripts with no detectable Ccl17 expression (Fig. 3C and data not shown). Interestingly, DSS exposure did not significantly influence the Ly6Chigh peripheral blood population, as we observed no significant difference in the gene profile between control and DSS-treated Ly6Chigh peripheral blood cells (Fig. 3C). However, we detected increased mRNA transcripts for Arg1, Il10, Ccl4, Il6, Cxcl2, Retnla, and Ccl22 in the Ly6Chigh colonic population compared with the Ly6Chigh peripheral blood population (Fig. 3C).

To determine the relationship between the intestinal inflammatory F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocytes/M\textsubscript{F}s and eosinophil recruitment in DSS colitis, we quantified colonic F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh M\textsubscript{F}s and eosinophil levels following DSS-induced colonic injury. We found a positive correlation between numbers of colonic F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh M\textsubscript{F}s and eosinophils (Fig. 4; \(p < 0.005\)). Notably, levels of colonic F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh M\textsubscript{F}s did not correlate with colonic neutrophil (F4/80\textsuperscript{−}CD11b\textsuperscript{+}Ly6G+) levels (Fig. 4), indicating a specific link between F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocyte recruitment and colonic eosinophilic inflammation. For F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocytes/M\textsubscript{F}s to drive eosinophil recruitment into the colon would require the influx of F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocyte/M\textsubscript{F} cells prior to eosinophil infiltration. We have previously reported that eosinophil infiltration of the colon occurs at day 5 of DSS exposure (14). Assessment of F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocyte/M\textsubscript{F} cells on days 3 and 4 of DSS exposure in the absence of increased eosinophil numbers (Supplemental Fig. 3). These data indicate that F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocyte/M\textsubscript{F} recruitment into the colon precedes eosinophil infiltration.

**FIGURE 3.** Colonic Ly6Chigh monocytes/M\textsubscript{F}s express CCL11 and are a mixed M1/M2 phenotype in DSS-induced colonic injury. A, CX3CR1\textsuperscript{eGFP/+} expression in F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh colonic monocytes/M\textsubscript{F}s. B, Representative FACS plot of CX3CR1\textsuperscript{low}Ly6Chigh colonic monocyte/M\textsubscript{F} flow sorted on day 5 of DSS. C, cDNA was made, and gene expression of Ccl11, Il1b, Tnf, Il6, Ccl4, Cxcl12, Arg1, Il10, Retnla, and Ccl22 was analyzed by RT-PCR. Three to four mice were pooled per sample. Peripheral blood baseline (6 samples), peripheral blood day 5 DSS (3 samples), and colon day 5 DSS (7–10 samples) monocyte/M\textsubscript{F} expression was analyzed. Data represent the mean ± SEM. Significant differences (*\(p < 0.05\), **\(p < 0.01\)) between groups. ND, below limit of detection.

**FIGURE 4.** DSS-induced F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocyte/M\textsubscript{F} levels positively correlated with colonic eosinophilic inflammation. Correlative analyses of eosinophils, neutrophils, and F4/80\textsuperscript{+}CD11b\textsuperscript{+}Ly6Chigh monocyte/M\textsubscript{F} levels in the colon following DSS exposure. Cells were gated as described in Fig. 1.
DSS-induced colonic injury recruitment of F4/80+CD11b+ Ly6C\textsuperscript{high} monocytes is CCR2-dependent

We next assessed the relative contribution of F4/80+CD11b+ Ly6C\textsuperscript{high} blood monocytes to DSS-induced colonic eosinophilic inflammation and colitis in CCR2\textsuperscript{-/-} mice, as CCR2 is important for Ly6C\textsuperscript{high} monocyte mobilization from the BM to the peripheral circulation and tissue inflammatory sites (36). Consistent with this, basal homeostatic levels of peripheral blood Ly6C\textsuperscript{high} monocytes were 6-fold lower in CCR2\textsuperscript{-/-} mice compared with WT mice (Fig. 5A, 5B). DSS-induced recruitment of F4/80+CD11b+Ly6C\textsuperscript{high} M\textsubscript{φ}s into the colon was attenuated in CCR2\textsuperscript{-/-} mice (Fig. 5C, 5D). Immunofluorescence analyses of F4/80\textsuperscript{+} cells in the colonic lamina propria of DSS-treated WT mice and CCR2\textsuperscript{-/-} mice revealed a loss of the large F4/80\textsuperscript{+} infiltrate in the CCR2\textsuperscript{-/-} mice (Fig. 5E). This reduction was specific for Ly6C\textsuperscript{high} monocytes, as recruitment of F4/80+CD11b\textsuperscript{+} cells (neutrophils) was not impaired (Fig. 5F, 5G). The reduction in intestinal M\textsubscript{φ} levels was not due to decreased levels of homeostatic intestinal M\textsubscript{φ}s, as the levels of resident F4/80+CD11b\textsuperscript{+}Ly6C\textsuperscript{low} colonic M\textsubscript{φ}s were comparable between WT and CCR2\textsuperscript{-/-} mice (Fig. 5H, 5I). Collectively, these data indicate that homeostatic resident F4/80+CD11b+Ly6C\textsuperscript{low} colonic M\textsubscript{φ} levels are independent of CCR2, whereas F4/80+CD11b+Ly6C\textsuperscript{high} monocyte recruitment to the colon during DSS-induced colitis is CCR2 dependent.

**DSS-induced colonic inflammation and colitis is attenuated in CCR2\textsuperscript{-/-} mice**

To assess the contribution of F4/80+CD11b+Ly6C\textsuperscript{high} monocytes to DSS-induced colonic CCL11 expression and eosinophilia, we quantitated CCL11 and eosinophil levels in the colon of CCR2\textsuperscript{-/-} mice following DSS exposure. Importantly, colonic eosinophil levels and distribution at baseline were comparable between WT and CCR2\textsuperscript{-/-} mice, indicating no role for CCR2 in basal colonic eosinophil recruitment (Fig. 6A, 6B and data not shown). DSS treatment of WT mice induced a significant increase in colonic eosinophil levels (Fig. 6A, 6B). In contrast, there was no significant increase in eosinophil levels in DSS-treated CCR2\textsuperscript{-/-} mice (Fig. 6A, 6B). Notably, the reduction in colonic eosinophil levels associated with no significant increase in colonic CCL11 levels in colonic punch biopsies from DSS-treated CCR2\textsuperscript{-/-} mice (Fig. 6C: WT baseline 8.7 ± 1.2 pg/ml versus WT DSS 42.4 ± 9.4 pg/ml, p < 0.05; CCR2\textsuperscript{-/-} baseline: 12.6 ± 3.1 pg/ml; CCR2\textsuperscript{-/-} DSS: 26.4 ± 8.3 pg/ml vs. p < 0.001). DSS treatment was associated with a loss of CCL11 expression in the colon of WT mice (Fig. 6D).

**FIGURE 5.** DSS-induced recruitment of F4/80+CD11b+Ly6C\textsuperscript{high} monocytes in WT and CCR2\textsuperscript{-/-} mice. A. Flow cytometric analysis of F4/80+CD11b+ Ly6C\textsuperscript{high} peripheral blood monocytes in WT and CCR2\textsuperscript{-/-} mice. B. Quantification of percent Ly6C\textsuperscript{high} peripheral blood monocytes in WT and CCR2\textsuperscript{-/-} mice. C. Flow cytometric analysis of F4/80+CD11b+Ly6C\textsuperscript{high} colonic monocytes/M\textsubscript{φ}s at baseline and following DSS in WT and CCR2\textsuperscript{-/-} mice. D. Quantification of Ly6C\textsuperscript{high} colonic M\textsubscript{φ} numbers based on flow cytometry analysis. E. Colonic sections from WT and CCR2\textsuperscript{-/-} mice treated with vehicle and 2.5% DSS for 5 d were stained with either anti-F4/80 or isotype Ig and goat anti-rat Alexa Fluor 594. Slides were counterstained with nuclei stain DAPI. Images represent overlay DAPI/F480. Original magnification ×100. F. Representative flow cytometry plots of F4/80+CD11b+ cells (neutrophils) in colon at baseline and following DSS in WT and CCR2\textsuperscript{-/-} mice. G. Percent F4/80+CD11b+ in colon at baseline and following DSS in WT and CCR2\textsuperscript{-/-}. H. Colonic lamina propria cells were stained for flow cytometry with F4/80, CD11b, and Ly6C, and the number of triple-positive cells was examined. I. Quantification of percent of F4/80+CD11b+ and F4/80+CD11b+Ly6C\textsuperscript{high} WT and CCR2\textsuperscript{-/-} colonic M\textsubscript{φ}s at baseline. Data represent the mean ± SEM of n = 3 to 4 mice per group from duplicate experiments. Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) between groups.
CCL2 is a CC chemokine that binds to the CCR2 receptor, and experimental data indicate that this chemokine is important in the recruitment of monocytes and MΦs into inflamed tissues (37–39). Notably, DSS exposure induce a significant increase in colonic CCL2 protein levels (Supplemental Fig. 4A). To evaluate the relative contribution of CCL2 to DSS-induced Ly6C<sup>high</sup> monocyte recruitment into the colon and disease pathology, we examined CCL2<sup>−/−</sup> mice. Surprisingly, the levels of colonic F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes in the colon of CCL2-treated CCL2<sup>−/−</sup> mice were comparable to that of strain- and weight-matched DSS-treated WT mice (Supplemental Fig. 4B, 4C). Consistent with our data, recruitment of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes into the colon of CCL2<sup>−/−</sup> mice was associated with DSS-induced weight loss and disease activity (Supplemental Fig. 4D), disease pathology (Supplemental Fig. 4E, 4F), and colonic eosinophil infiltration (Supplemental Fig. 4G, 4H). Assessment of Ly6C<sup>high</sup> peripheral blood monocytes and colonic MΦs at baseline revealed comparable levels between WT and CCL2<sup>−/−</sup> mice, indicating that CCL2 does not contribute to either homeostatic resident intestinal F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> MΦ levels or DSS-induced recruitment of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes into the colon (Supplemental Fig. 5).

**Discussion**

In the current study, we have investigated the molecular regulation of CCL11 and colonic eosinophilic inflammation in an experimental mouse model of DSS-induced colitis. We demonstrate that reconstitution of CCL11<sup>−/−</sup> mice with BM-derived CCL11 is sufficient for DSS-induced colonic eosinophil inflammation. We show that DSS exposure promotes the influx of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes into the colon and that recruitment of this cell population positively correlated with colonic eosinophilic inflammation. Purification of the F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocyte population revealed that these cells express a mixture of M1- and M2-associated genes, including Chi3l3, Relmα, Il10, Il6, Il1b, Trem1, Cxcl2, and Ccl11. Abrogation of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocyte recruitment by genetic deletion of CCR2 was associated with decreased DSS-induced histopathology, CCL11 expression, and eosinophil recruitment. These studies indicate that colonic eosinophilic inflammation in experimental colitis is mediated by Ly6C<sup>high</sup>CCR2<sup>+</sup> inflammatory monocyte/MΦ-derived CCL11.

Flow cytomentry analyses identified the presence of two distinct monocyte populations in the peripheral blood of mice characterized by F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> and F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup> phenotype. We show that the F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> population was CCR2<sup>+</sup> and CX3CR1<sup>low</sup>, whereas the F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup> monocytes were CCR2<sup>−</sup> and CX3CR1<sup>high</sup>. The F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup> blood monocytes are a precursor to resident homeostatic tissue MΦs (35). Consistent with this, under homeostatic conditions, the colon predominantly consisted of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup> dendritic cells. Intestinal dendritic cells under homeostatic conditions also express CX3CR1 and F4/80. Analysis of CD11c expression revealed that F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup> cells consisted of 80% CD11c<sup>+</sup> and 20% CD11c<sup>−</sup> cells, indicating the presence of homeostatic tissue MΦs and dendritic cells within the F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup> population (Supplemental Fig. 2). The presence of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>low</sup>CD11c<sup>+</sup> dendritic cell population may have contributed to the gene expression heterogeneity seen within this cell population. The F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes are an inflammatory monocyte population and rapidly recruited into tissues following inflammatory insult. Large infiltrates of Ly6C<sup>high</sup>CX3CR1<sup>low</sup>CCR2<sup>+</sup> blood monocytes have been observed in the peritoneum and injured myocardium following immune stimulation (35, 40). Similarly, we observed the selective increase in the numbers of F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> cells within the colon following DSS exposure. Importantly, the F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> cells within the colon were CD11c<sup>−</sup>, indicating that this population did not contain a contaminating dendritic cell population (Supplemental Fig. 2). Consistent with previous investigations, we show that the recruitment of the inflammatory F4/80<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> monocytes/MΦs into inflamed tissues...
was dependent on CCR2 (35). Previous investigations have indicated that CCR2-dependent recruitment of this monocytic population is mediated by CCL2 (37, 41); however, the CCR2-mediated recruitment of F4/80+CD11b+Ly6Chigh monocytes/MFs into the colon was not dependent on CCL2. CCR2 is a promiscuous chemokine receptor binding multiple ligands, including CCL2, CCL8, CCL7, and CCL13 (42). Additionally, CCL2, CCL8, and CCL7 have been shown to be increased in the colon in inflamed tissue from IBD patients as well as in the DSS model of colitis (43, 44).

The demonstration that ablation of F4/80+CD11b+Ly6Chigh monocytes/MFs into the colon in CCR2−/− mice was associated with a reduction in proinflammatory cytokines (IL-6 and TNF-α) indicates that this cell population is an important source of cytokines that drive DSS-induced intestinal inflammation and colitic disease. Consistent with this, PCR analyses of purified Ly6Chigh monocytes/MFs revealed that these cells expressed high levels of proinflammatory markers Trem1 and TLR2, but not the anti-inflammatory markers CD206 or PDL1. Additionally, CCL2, CCL8, and CCL7 have been shown to be increased in the colon in inflamed tissue from IBD patients as well as in the DSS model of colitis (43, 44).

The demonstration that ablation of F4/80+CD11b+Ly6Chigh monocytes/MFs into the colon in CCR2−/− mice was associated with a reduction in proinflammatory cytokines (IL-6 and TNF-α) indicates that this cell population is an important source of cytokines that drive DSS-induced intestinal inflammation and colitic disease. Consistent with this, PCR analyses of purified Ly6Chigh monocytes/MFs revealed that these cells expressed high levels of proinflammatory markers Trem1 and TLR2, but not the anti-inflammatory markers CD206 or PDL1. Similar to our observations, Platt and colleagues (45) identified a TLR2+CCR2+ CX3CR1+Ly6ChighTNF-α+ monocyte/MΦ population in the colon following DSS exposure and that this cell type drives DSS-induced pathology. We have extended these observations, identifying a new cellular function for the F4/80+CD11b+Ly6Chigh TLR2+ population in driving colonic eosinophilic inflammation and histopathology associated with DSS-induced colitis. Furthermore, we have developed a protocol that permits purification of this cell population and demonstrated that the Ly6Chigh colonic monocyte/MΦs express Arg1, Retnla, Chi3l3, Ccl22 (MDC), and Il10. The Arg1 and Chi3l3 (YM1) mRNA expression of the infiltrating Ly6Chigh cell population is consistent with the previously reported DSS-induced total increase in tissue ARG1 and YM1 protein (46). The expression of Arg1 and Chi3l3 (YM1) suggests that the monocytes/MΦs are alternatively activated; however, we also observed evidence of classical activation as the cells expressed the proinflammatory cytokines/chemokines Il1b, Il6, Ccl4, and Cxcl2. Surprisingly, Tnfa mRNA expression was not increased in Ly6Chigh colonic monocytes compared with peripheral blood monocytes, although protein expression correlated with Ly6Chigh monocyte recruitment. However, it has previously been shown that Tnf mRNA stability and translation regulate its bioreactivity (47–50). Our results suggest that Ly6Chigh colonic monocytes/MΦs have a mixed classically/alternatively activated phenotype in the acute phase of DSS-induced colitis, further indicating that classical/alternatively activated phenotypes are part of a spectrum of activation as opposed to the strongly polarized phenotypes that are often seen in vitro (51, 52). Alternatively, the mixed phenotype could suggest the presence of subpopulations of Ly6Chigh colonic monocytes/MΦs within the colon. DSS-induced colitis has been linked with increased expression of the classical/alternative activation cytokines IL-4/IFN-γ (53, 54). In a mouse model of kidney injury, Chi3l3 was expressed in Ly6C+ MΦs in the kidney; however, other M2 genes, including Ccl17 and Ccl22, were only expressed at very low levels, whereas high levels of...
Ccl11 and Chi3l3 were expressed, indicating an M1-biased pattern (55). Further investigations are required to determine whether the Ly6C<sup>high</sup> monocyte/MΦ population consists of multiple polarized monocyte/MΦ subsets of different phenotypes or whether the Ly6C<sup>high</sup> monocyte/MΦ population is simply plastic and adapts to exogenous and endogenous stimuli within the microenvironment.

The role of monocytes/MΦs in eosinophil recruitment during chronic inflammatory responses and the relative contribution of CCL11 to this response are not yet fully delineated. Previous studies have demonstrated Ccl11 mRNA expression in MΦs in allergen-induced cutaneous biopsies in atopic patients (56) and in bronchial biopsies from asthmatic patients that possess eosinophils (57). Furthermore, a recent study also demonstrated CCL11 expression in lung MΦs from rhinovirus-infected allergic mice with pulmonary eosinophilic inflammation (58). Experimental analyses employing a Nippostrongylus brasiliensis infestation model have demonstrated a role for monocyte/MΦ populations in eosinophil recruitment into the peritoneum. Similar to our observations, eosinophil recruitment was associated with Retinla and Chi3l3 monocyte/MΦ expression but was not associated with Ccl11 expression (59). We show that during colonic inflammation, CX6CR1<sup>Ly6C<sub>high</sub></sup> colonic monocytes/MΦs are the primary cellular source of CCL11 and that this cell population is sufficient to mediate colonic eosinophilic inflammation. These observations suggest that DSS exposure stimulates CX6CR1<sup>Ly6C<sub>high</sub></sup> colonic monocyte/MΦ recruitment in the colon and that the recruited inflammatory monocytes/MΦs subsequently drive eosinophil infiltration via a CCL11-dependent pathway. We have previously characterized eosinophil infiltration of the colon following DSS exposure and reported that eosinophil levels begin to increase on day 5 of DSS exposure (14). Assessment of F4/80<sup>CD11b<sup>Ly6C<sub>high</sub></sup></sup> and eosinophil numbers in the colon of mice 3 and 4 d following DSS exposure revealed a significant influx of F4/80<sup>CD11b<sup>Ly6C<sub>high</sub></sup></sup> monocytes/MΦs prior to commencement of eosinophil recruitment (Supplemental Fig. 3). These kinetic data indicate that F4/80<sup>CD11b<sup>Ly6C<sub>high</sub></sup></sup> monocytes/MΦs are strategically positioned in the colon to regulate DSS-induced eosinophil recruitment. These studies strongly suggest that CCL11 is predominantly monocyte/MΦ-derived and is important in colonic eosinophilic recruitment in experimental colitis. We have previously reported a critical role for CCL11 and not the other eotaxin family members CCL24 and CCL26 in the regulation of chronic inflammatory responses and the relative contribution of other BM-derived cell populations; however, we have previously demonstrated that DSS-induced CCL11 expression in the colon was restricted to F4/80<sup>myeloid cells and not F4/80<sup>−</sup></sup>cells (14). Surprisingly, we observed a significant eosinophilic infiltrate in the colon following DSS exposure in WT mice reconstituted with BM from CCL11<sup>−/−</sup> mice, suggesting a role for structural cell-derived CCL11 in colonic eosinophilic inflammation. We postulate that this paradoxical observation is due to the radioresistance of monocytes/MΦs and the effects of radiation on monocyte/MΦ function. Many studies have demonstrated that tissue MΦs are radioresistant (62–64). For example, in the lung, it takes >90 d for alveolar MΦs to reach ∼80% reconstitution (64). Similarly, we found that Ly6C<sup>low</sup> colonic MΦs only reached ∼60% reconstitution at ∼50 d postirradiation and that ∼5% of monocytes/MΦs in the colon of DSS-treated mice were recipient-derived cells. Furthermore, irradiation has been shown to induce MΦ oxidative injury (65) as well as alter MΦ activation, which may lead to homeostatic resident MΦ involvement in eosinophil recruitment (66). We currently cannot rule out a contribution for non-BM cell-derived CCL11 to eosinophil recruitment during DSS-induced colitis; however, our previous data demonstrating CCL11 expression restricted to F4/80<sup>+</sup> cells in the lamina propria of the colon on day 7 of DSS exposure (14) and our observations in CCR2<sup>−/−</sup> mice indicate that the CCR2-dependent Ly6C<sup>high</sup> monocytes are sufficient to drive eosinophil recruitment in DSS-induced colonic injury.

Recently, a number of unique loci were identified to be associated with early-onset IBD susceptibility including the C-C motif chemokine cluster on 17q12 loci, which contains the eosinophil-specific chemokine gene CCL11 (17). Clinical and experimental data indicate: 1) a strong relationship between eosinophils and the exacerbation and severity to IBD; and 2) a pivotal role for MΦs in the augmentation of the intestinal inflammatory response associated with IBD (67). We provide evidence of a direct pathway involving Ly6C<sup>high</sup> colonic monocyte/MΦ-derived CCL11 in colonic eosinophilic inflammation and histopathology in experimental colitis. These studies provide significant rationale for the assessment of monocyte/MΦ-derived CCL11 in human IBD and the targeting of the monocyte/MΦ/CCL11 pathway as a therapeutic modality for the treatment and prevention of IBD.

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