HHS Public Access

Author manuscript

Inflamm Bowel Dis. Author manuscript; available in PMC 2018 November 22.

Published in final edited form as:

Inflamm Bowel Dis. 2014 July ; 20(7): 1165–1176. doi:10.1097/MIB.0000000000000059.

CCL25/CCR9 Interactions Are Not Essential for Colitis Development but Are Required for Innate Immune Cell Protection from Chronic Experimental Murine Colitis

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Abstract

Background: The chemokine CCL25, and its receptor CCR9, constitute a unique chemokine/receptor pair, which regulates trafficking of T lymphocytes to the small intestine under physiological conditions and is an attractive target for small bowel Crohn's disease drug development. We have previously shown that CCL25/CCR9 interactions regulate the recovery from acute dextran sulfate sodium–induced colonic inflammation. In this study, we explored whether these interactions also regulate chronic colitis development in 2 independent murine models of experimental colitis.

Methods: Histological flow cytometry and qPCR analyses were performed to evaluate the role of CL25 and CCR9 in chronic colonic inflammation induced by serial exposures to dextran sulfate sodium salts or by adoptive transfer of $CD45RB^{hi}$ $CD4^{+}$ T cell into lymphopenic mice devoid of CCL25/CCR9 interactions.

Results: Chronic dextran sulfate sodium exposure results in exacerbated colitis in mice deficient for either CCR9 or CCL25 when compared with wild-type control mice. Although CCR9-deficient T cells traffic to the colon and induce severe colitis similar to wild-type T cells in the CD45RB transfer model, naive wild-type T cells induce more severe disease in recipient animals devoid of CCL25 expression.

Conclusions: CCL25/CCR9 interactions are required for modulating protection against large intestinal inflammation in 2 models of chronic colitis. These data may have implications for the potential effects of disrupting CCL25/CCR9 interactions in humans in the setting of intestinal disorders including inflammatory bowel disease.

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The authors have no conflicts of interest to disclose.

Keywords

CCL25; CCR9; colitis; T cell; innate immune cell

Human inflammatory bowel diseases (IBD) can be broadly subcategorized into Crohn's disease (CD) and ulcerative colitis, which affect the small and the large bowel, respectively.[1,2] These are complex chronic diseases associated with intestinal inflammation of unknown etiology, but several factors have been implicated including genetic predisposition, microbial dysbiosis, and other environmental triggers, and aberrant adaptive and/or innate mucosal immune function. Studies in various colitis models have highlighted a critical role for the balance between effector and regulatory T-cell populations and cross talk with innate immune cells in maintaining mucosal homeostasis and preventing intestinal inflammation.[3]

Chemokines and their receptors play a major role in recruiting T cells in health and disease to sites of injury.[4] In health, $CD4^+$ and $CD8^+$ effector/memory T-cell trafficking to the small intestine is mediated by CCR9- and α4β7-mediated cell surface interactions with CCL25[5–8] and MadCAM-1,[9] respectively, whereas trafficking to the large intestine is mediated at least in part by α4β7/MadCAM-1 interactions.[10–12] CCL25 is expressed constitutively by thymic and small intestinal epithelial cells in mice and in humans.[13–15] Upregulation of CCL25 expression, along with concomitant increase in $CCR9^+$ $CD4^+$ T cells, are found in the inflamed small bowel of wild-type (WT) mice during inflammatory states[16] or SAMP1/YitFC mice (which develop spontaneous enteritis) and in the small intestine of patients with CD,[17,18] suggesting that such interactions mediate recruitment of T cells to inflamed sites, further contributing to intestinal inflammation. Indeed, these interactions are the target of an anti-CCR9 agent that is being evaluated in patients with CD with active inflammation.[19]

Although augmented T-cell effector responses are associated with colonic inflammation in the setting of IBD,[20] the mechanisms underlying how T-cell migration to the large intestine is modulated during inflammation is largely unknown.[10–12] We previously reported increased colonic expression of CCL25 on recovery of acute dextran sodium sulfate (DSS)–mediated colitis in mice.[21] In addition, acute DSS administration in *Ccr9*$^{-/-}$ and *Ccl25*$^{-/-}$ mice led to exacerbated large intestinal inflammation.[21] These data suggested that CCL25/CCR9 interactions may be important for modulating T-cell responses in the *colon,* during induction and recovery phases of *acute* colitis. In this study, we aimed to determine the role of CCL25/CCR9 interactions in the setting of *chronic* inflammation using 2 independent models. Our results show that conventional and regulatory T cells (Tregs) do not require CCR9 expression to traffic into and function in the inflamed colonic lamina propria (cLP). However, colitic mice devoid of CCL25/CCR9 interactions display exacerbated colitis in association with altered innate immune cell distribution.

MATERIALS AND METHODS

Animals

The generation of *Ccr9*$^{-/-}$ and *Ccl25*$^{-/-}$ mice has been described previously.[8,22] *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). *Rag1*$^{-/-}$*Ccl25*$^{-/-}$

were generated by crossing *Ccl25*$^{-/-}$ onto *Rag1*$^{-/-}$ mice and kept at the homozygous state. All strains of mice including C57Bl/6 control mice were bred in our animal facility at Boston Children's Hospital, born and held in the same room under specific pathogen-free conditions. To avoid variations of commensal bacteria as a confounding factor in our experiments, littermate controls were used. All animal experiments were approved by Boston Children's Hospital Institutional Animal Care and Use Committee. Boston Children's Hospital's assurance number is A3303-01. Boston Children's Hospital is accredited by AAALAC International. All efforts were made to minimize suffering of animals.

DSS-mediated Chronic Colitis

Sex- and age-matched WT and *Ccr9*$^{-/-}$ mice received 1.5% to 2% DSS (36–50 kDa; MP Biomedicals, LCC, Solon, OH) in the drinking water for 4 cycles consisting of 7-day DSS administration followed by 7-day water administration. The animal weight was recorded daily. For flow cytometry, histology, and mRNA analysis, mice were euthanized at indicated time points, with day 0 corresponding to the initiation of DSS treatment.

T cell Transfer-mediated Chronic Colitis

For CD45RB^{hi} transfers and CD45RB^{hi} and CD45RB^{lo} cotransfers into *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice, spleens (SPLs) were harvested from WT donor mice. CD4^{+} T cells were enriched by negative selection using a depletion monoclonal antibody (mAb) cocktail (consisting of unconjugated anti-B220, anti-CD8α, anti-CD11b, anti-CD11c, anti-Gr-1, anti-Dx5, and anti-NK1.1 mAbs [BioXcell, West Lebanon, NH], followed by Rat anti-Mouse Igk chain conjugated magnetic beads [Miltenyi Biotec, Auburn, CA]). Enriched CD4^{+} T cells subsequently sorted by flow cytometry on a MoFlo Fluorescence Activated Cell Sorter (DakoCytomation, Carpinteria, CA) into 2 fractions: CD45RB^{hi} CD4^{+} to induce colitis in immunocompromised mice and CD45RB^{lo} CD4^{+} to prevent colitis induction when cotransferred with CD45RB^{hi} CD4^{+} T cells. Postsort purity was typically >98%. Age-matched lymphopenic recipient *Rag1*$^{-/-}$ mice (lacking or not CCL25 expression) were injected with 4×10^5 CD45RB^{hi} CD4^{+} T cells with or without 2×10^5 CD45RB^{lo} CD4^{+} T cells. Mice were typically analyzed between week 8 and 10 after transfer.

Preparation of Cell Suspensions

SPLs and mesenteric lymph nodes (mLNs) were harvested in HBSS with Ca^{2+} and Mg^{2+} supplemented with 2% fetal calf serum and 10 mM HEPES. For CD4^{+} T cell investigation, SPL and mLN cell suspensions were prepared with frosted slides and filtered through nylon mesh after red blood cell lysis. For analyses of innate immune cell, SPL and mLN suspensions were obtained by collagenase VIII digestion (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Colonic lamina propria lymphocyte suspensions were obtained as previously described[21]: colons were flushed in phosphate-buffered saline 1× and opened longitudinally. To remove intraepithelial lymphocytes and epithelial cells, intestinal pieces were incubated in HBSS without Ca^{2+}/Mg^{2+} supplemented with 10 mM EDTA, 10 mM HEPES, 0.5% fetal calf serum, and 1.5 mM DTET, for 2 × 20 minutes at 37°C. Intestinal pieces were digested in HBSS with Ca^{2+}/Mg^{2+}, 20% fetal calf serum, 100 U/mL collagenase VIII, and 5 μg/mL DNase (Sigma-Aldrich) for 60 to 90 minutes at 37°C. Lamina propria

lymphocytes were purified over a 40% to 100% Percoll gradient (GE Healthcare Bio-Sciences Corp, Piscataway, NJ).

Flow Cytometry Analysis

Cell suspensions from SPL, mLN, and cLP were analyzed by flow cytometry. Conventional $CD4^+$ T cells and Tregs were stained with anti-CD3ε and anti-CD4 mAbs (BD Biosciences, San Jose, CA) and intracytoplasmic anti-foxp3 mAbs (eBioscience, San Diego, CA). Neutrophils were stained with Gr-1-FITC, CD11b-PECy7, and Ly6G-AF647 mAbs (eBioscience). Plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) were stained with MHCII-FITC, CD11b-PECy7, PDCA-1-AF647, and CD11c-APCCy7 mAbs with a lineage-negative mAb cocktail containing B220-biotin, CD3ε-biotin, Ly6G-biotin (eBioscience) and PerCP-Cy5.5-conjugated streptavidin. The cDC subsets were stained with MHCII-FITC, CD11c-APCCy7, CD11b-PECy7, and CD103-PE mAbs with a lineage-negative mAb cocktail containing B220-biotin, CD3ε-biotin, Ly6G-biotin (eBioscience) and PerCP-Cy5.5-conjugated streptavidin (eBioscience). Monocytes and macrophages (MΦ) were stained with Gr-1-FITC, MHCII-PE, Ly6C-PerCP-Cy5.5, CD11b-PECy7, Ly6G-APC, and CD11c-APCCy7 mAbs (eBioscience). Blocking of FcγR binding was performed using mouse and rat serum (Jackson ImmunoResearch, West Grove, PA). Cells were analyzed on a FACS Canto II (BD Biosciences). Data were collected using FACS Diva software (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

Histology and IBD and Clinical Scoring

Intestinal samples of the distal colon were harvested, fixed in 10% formalin, and embedded in paraffin for hematoxylin and eosin (H&E) staining. Histological IBD scoring was performed blindly by a pathologist as follows: IBD scores corresponding to 0 = normal, 1 = mild, 2 = moderate, and 3 = severe were attributed to activity grade, changes of crypt architecture, basal lymphoplasmacytosis, expansion of lamina propria, and epithelial hyperplasia. Scores were graphed in a total range of 0–15.

mRNA Quantification

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Quantification of total RNA was performed with a NanoDrop spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE). For real-time RT-PCR, cDNA was synthesized with iScript Select cDNA Synthesis kit (Biorad, Hercules, CA). Amplification was performed with the CFX96 quantitative PCR System (Biorad) and iQ SYBR Green Supermix (Biorad) on 50 ng cDNA. Transcripts were quantified using HPRT mRNA for normalization. Primer sequences are available upon request.

Statistical Analysis

Weight loss, IBD scoring, and cellular distributions were compared using either Student's *t* test or ANOVA. Differences with $P < 0.05$ were considered significant. Statistical analysis was performed using Prism (Graph Pad Software, La Jolla, CA).

RESULTS

DSS-mediated Chronic Colitis Is Exacerbated in *Ccr9*$^{-/-}$ Mice

We previously reported that *Ccr9*$^{-/-}$ and *Ccl25*$^{-/-}$ mice are more susceptible to *acute* DSS colitis than WT controls.[21] As human ulcerative colitis is associated with signs of *chronic* colonic inflammation, we sought to assess whether the increased susceptibility to acute inflammation associated with defective CCL25/CCR9 interactions would also translate into increased susceptibility to chronic inflammation. WT and Ccr9$^{-/-}$ mice were exposed to DSS in drinking water for 4 cycles and monitored daily (Fig. 1). As previously reported, *Ccr9*$^{-/-}$ mice showed delayed weight loss recovery after the first DSS exposure (Fig. 1A, arrowhead, and Wurbel et al[21]), but these mice recovered without any evidence of increased chronic inflammation after each of 3 additional DSS cycles. However, 3 to 4 weeks after the last DSS exposures, *Ccr9*$^{-/-}$ mice and *Ccl25*$^{-/-}$ mice stopped thriving and failed to gain weight (Fig. 1A and data not shown). In addition, analysis of H&E-stained colonic sections revealed that *Ccr9*$^{-/-}$ mice exhibited severe colonic inflammation, whereas WT mice had fully recovered without evidence of histological signs of colitis (Fig. 1B, C). As evidenced by qPCR RNA analyses from colonic tissue, the exacerbation of chronic colonic inflammation in CCR9$^{-/-}$ animals was associated with a mixed Th1/Th17 immune response (Fig. 1D). In contrast, regulatory cytokines (IL-10 and TGFb2) and CCL25 transcripts were down-regulated in tissues from *Ccr9*$^{-/-}$ mice when compared with WT mice (Fig. 1D, E). Taken together, these data indicate that CCL25/CCR9 interactions are required to modulate the severity of DSS-mediated chronic colonic inflammation.

CD4^{+} T cells Home to the Large Bowel and Induce Colitis Independent of CCR9

We next used the CD45RB^{hi} transfer model to assess the role CCL25/CCR9 interactions in regulating a T cell–mediated chronic colitis model. In this model, colitis induction by naive CD45RB^{hi} CD4^{+} cells into lymphopenic mice can be prevented by the cotransfer of CD45RB^{lo} CD4^{+} T cells (which contain naturally occurring thymically derived FOXP3^{+} regulatory T cells, nTregs).[3] To determine the role of CCR9 on colonic homing and effector T-cell colitic activity, we adoptively transferred naive WT or *Ccr9*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells into *Rag1*$^{-/-}$ recipient mice (Fig. 2). *Rag1*$^{-/-}$ recipient animals receiving either WT (gray squares) or *Ccr9*$^{-/-}$ (black squares) CD45RB^{hi} CD4^{+} T cells developed comparable signs of inflammation (Fig. 2A–C). Both groups of mice gradually lost 10% to 20% of their initial body weight (Fig. 2A) and developed severe inflammation (Fig. 2B, C). The percentages of CD4^{+} T cells in SPL, mLN, and cLP after transfer of WT (gray bars) or *Ccr9*$^{-/-}$ (black bars) CD45RB^{hi} CD4^{+} colitogenic T cells into *Rag1*$^{-/-}$ recipient mice were comparable (Fig. 2D, E). Inflammatory cytokine elevations were also similar in colitic colons independent of CCR9 expression in recipient mice after CD45RB transfer (see Fig., Supplemental Digital Content 1, http://links.lww.com/IBD/A470). CCL25 transcripts were markedly reduced in colitic mice upon transfer of either WT or *Ccr9*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells (Fig. 2F). As this chemokine is expressed by both thymic and intestinal epithelial cells,[13] this finding may result from altered CCL25 epithelial expression in the setting of inflammation. To assess the role of CCR9 on nTreg activity, we adoptively cotransferred WT CD45RB^{hi} naive CD4^{+} T cells with either WT or *Ccr9*$^{-/-}$ CD45RB^{lo}, CD4^{+} T cells. In cotransfer experiments, both WT and *Ccr9*$^{-/-}$ CD45RB^{lo} prevented colitis development as

depicted by absence of both weight loss (Fig. 2A) and evidence of intestinal inflammation (Fig. 2B, C), indicating normal trafficking and in vivo suppressive function of Tregs in the absence of CCR9 expression. Cotransfer of WT CD45RB^{hi} CD4^{+} T cells with either WT CD45RB^{lo} (open gray bars) or Ccr9$^{-/-}$ CD45RB^{lo} (open black bars) CD4^{+} T cells resulted in similar distribution of CD4^{+} T cells in lymphoid tissues (Fig. 2E). The maintenance of Tregs was also not affected by CCR9 expression in cotransfer experiments (data not shown). In addition, T-cell suppression assays indicated that the suppressive function of CCR9-deficient Tregs in vitro was not impaired (data not shown). Taken together, these data suggest that in vivo trafficking and function of colitogenic T cells and Tregs to the large intestinal mucosa occur *independent* of CCR9 expression.

CCL25 Deficiency Leads to Exacerbated T cell–mediated Chronic Colitis

We next sought to assess the effect on chronic colitis development in animals that were devoid of the CCR9 ligand, CCL25. We adoptively transferred sorted WT CD45RB^{hi} CD4^{+} T cells into either *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice (Fig. 3). WT CD45RB^{hi} CD4^{+} T cell transfer into *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice, when compared with *Rag1*$^{-/-}$ recipient mice, developed increased weight loss (Fig. 3A), splenomegaly, and exacerbated colonic inflammation (Fig. 3B, C and see Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A471). Moreover, the overall cellularity in SPL, mLN, and cLP was significantly higher in *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice when compared with *Rag1*$^{-/-}$ mice (data not shown). In addition, the overall number of CD4^{+} T cells was increased in the SPL, mLN, and cLP of *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice (Fig. 3D). Transfer of WT CD45RB^{hi} CD4^{+} T cells into *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice was also associated with increased proinflammatory cytokine transcript levels when compared with *Rag1*$^{-/-}$ recipient mice (Fig. 3E). In contrast to studies by Mizuno et al[36] who reported small bowel inflammation in *Rag2*$^{-/-}$*Ccr9*$^{-/-}$ recipient mice that received WT CD45RB^{hi} CD4^{+} T cells, ileitis was not observed in *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice after T cell transfer (see Fig., Supplemental Digital Content 2, http://links.lww.com/IBD/A471). Together, incorporating the results from the last section, these data suggest that while CCR9 expression on T cells does not modulate T cell–mediated colitis in immunodeficient hosts, expression of CCL25 in recipient mice regulates the severity of T cell–mediated colitis development.

Treg Development and Function Are Independent of CCL25/CCR9 Interactions

To assess the role of CCL25 expression on the function of WT Tregs in suppressing colitogenic T cells, WT CD45RB^{hi} CD4^{+} T cells were cotransferred with WT CD45RB^{lo} CD4^{+} T cells (containing nTregs) in either *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice. Colitis development was similarly suppressed in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice indicating that WT nTreg function in vivo is independent of CCL25/CCR9 interactions (Fig. 3A–C). One potential explanation for the increased colitis observed in *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice after naive CD4^{+} T cell transfer might be the aberrant generation of inducible Tregs (iTregs) from naive T cells in the setting of CCL25 deficiency. Therefore, to test whether generation of iTreg is deficient in *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ host mice after naive CD45RB^{hi} CD4^{+} T cells, we analyzed the generation of CD4^{+} Foxp3^{+} T cells 8 weeks after transfer (Fig. 4). Generation of iTreg was similar between *Rag1*$^{-/-}$ (white bars) and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$

(gray bars) recipient mice (Fig. 4A, B). Collectively, these data indicate that the development and function of Tregs is independent of CCL25/CCR9 interactions.

Increased Neutrophils in Colitic Mice Devoid of CCL25/CCR9 Interactions

Neutrophil recruitment and activation are key steps in the intestinal innate immune response observed in IBD,[23] and studies with animal models of colitis highlight the relationship between neutrophil infiltration and disease severity.[24,25] We characterized neutrophil infiltration in chronic DSS-mediated colitis in *Ccr9*$^{-/-}$ mice and in *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice upon naive CD4^{+} T cell transfer (Fig. 5). Flow cytometry analyses revealed increased Gr-1^{hi} CD11b^{hi} neutrophils in the SPL, mLN, and cLP of *Ccr9*$^{-/-}$ DSS-treated mice (Fig. 5A, B) and in the SPL and mLN, but not cLP, of *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice upon naive CD4^{+} T cell transfer (Fig. 5C, D). Together, these data demonstrate that enhanced neutrophil levels correlate with colitis severity seen in 2 models of colitis associated with defective CCL25/CCR9 interactions.

Altered Conventional Dendritic Cell Subset Distribution in Colitic Mice Devoid of CCL25/CCR9 Interactions

We next hypothesized that CCL25 CCR9 interactions may play a role in innate immune cell distribution upon colonic inflammation because CCL25/CCR9 interactions were not necessary in effector and regulatory functions of CD4^{+} T cells. We analyzed the distribution of dendritic cell (DC) populations in SPL, mLN, and cLP of *Ccr9*$^{-/-}$ DSS-treated mice and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice upon naive CD4^{+} T cell transfer (Fig. 6). The percentage of pDC and cDC were comparable in all lymphoid organs isolated from DSS-treated *Ccr9*$^{-/-}$ mice and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice upon naive CD4^{+} T cell transfer (Fig. 6B, D). Recently, the characterization of conventional DC subsets in the intestinal LP has been intensely studied. Based on the differential expression of CD103 and CD11b markers, cDC subset display distinct functions (reviewed in Persson et al[26]). We analyzed cDC subsets and observed that CD103^{+}CD11b^{-} cDCs were decreased in cLP, whereas CD103^{-}CD11b^{+} cDCs were increased in the inflamed colons of DSS-treated *Ccr9*$^{-/-}$ intestinal mucosa and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice receiving WT naive T cells (Fig. 6C, E). In addition, the overall percentage of CD103^{+} cDCs (CD11b^{+} and CD11b^{-}), a DC subset that has been implicated in the generation and function of iTregs,[26] was decreased in the inflamed cLP of both DSS-treated *Ccr9*$^{-/-}$ mice (28.2 ± 3.2% *Ccr9*$^{-/-}$ cLP versus 42.3 ± 4.3% WT cLP, $P = 0.0120$) and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice after WT T cell transfer (28.2 ± 2.6% *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ cLP versus 62.3 ± 3.7% *Rag1*$^{-/-}$ cLP, $P = 0.0022$). These data suggest that chronic colonic inflammation alters cDC distribution in mice devoid of CCL25/CCR9 interactions.

Distribution of Proinflammatory and Anti-inflammatory Colonic Macrophages Is Independent of CCL25/CCR9 Interactions

We have previously reported that acute DSS exposure leads to increased frequencies of intestinal inflammatory monocytes in *Ccr9*$^{-/-}$ mice.[21] Here, we wanted to determine whether the frequencies of proinflammatory and anti-inflammatory MΦ were altered in mice lacking CCL25/CCR9 interactions in the setting of chronic colitis. We did not observe any statistically significant altered distribution in proinflammatory MΦ (Ly6C^{hi}MHCII^{hi}) and

anti-inflammatory MΦ (Ly6C^{lo}MHCII^{hi}) in the cLP of *Ccr9*$^{-/-}$ mice upon DSS treatment and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice after naive T cell transfer (Fig. 7). This suggests that the distribution of proinflammatory and anti-inflammatory MΦ in the LP of colitic mice is independent of CCL25/CCR9 interactions.

DISCUSSION

T-cell homing to intestinal compartments requires expression of chemokine receptors on the surface of leukocytes and the attraction offered by chemokine receptor–specific ligands secreted predominantly by epithelial cells.[10–12] The gut homing receptor CCR9 and its ligand CCL25 have been shown to play an important role in regulating the homing of lymphocytes in health and in disease.[27] Moreover, because patients with CD have increased expression of CCL25 in the intestine and increased numbers of infiltrating CCR9^{+} T cells, blockade of this pathway has been postulated as a therapeutic target for CD.[14,28] Although a phase 2 study using a CCR9 antagonist (Vercirnon) demonstrated clinical efficacy in inducing remission in moderate to severe CD,[19] preliminary reports of a phase 3 study in patients with CD (including those with moderate to severe small bowel and/or colonic involvement) failed to show improvement in clinical response and clinical remission.[29] Moreover, CCR9 antagonist administration showed dose-dependent *increase* in adverse reactions among all treatment groups.[29] These data highlight that further investigation is required to determine whether blockade of CCL25/CCR9 interactions will be a safe and effective target for the treatment of CD.

CCR9 expression appears to be important for both effector and regulatory T-cell trafficking to the small bowel,[17,30] but not to the large bowel.[31] In our present study, we show that CCR9 expression on naive T effector cells is not required to mediate colitis when transferred into lymphopenic hosts. Moreover, such expression is also not required for generation and function of Tregs because CCR9-deficient Tregs are able to home to the cLP and suppress colitis. These data imply that CCR9 expression on T cells is not required for trafficking to the cLP or either effector/memory T-cell functions or regulatory T-cell functions. Recently, the G-coupled protein receptor 15 has been implicated in mediating T-cell trafficking (in particular Tregs) to the large bowel.[32] The unique trafficking functions of CCR9 and G-coupled protein receptor 15 in T-cell migration suggest that these molecules may be the restrictive elements targeting leukocytes to specific intestinal compartments.

Our data suggest that CCL25/CCR9 interactions regulate innate immune cell trafficking/function. Both DSS-treated *Ccr9*$^{-/-}$ mice and *Rag1*$^{-/-}$*CCL25*$^{-/-}$ recipient mice after naive WT T cell transfer are more susceptible to colitis than their respective controls. Since, as described above, CCL25/CCR9 interactions appear not to regulate T-cell colonic trafficking and function, exacerbation of colitis in these mice suggest that these interactions may influence the trafficking/function of other hematopoietic cells. Indeed, when compared with controls, *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice transferred with naive CD4^{+} T cells and DSS-treated *Ccr9*$^{-/-}$ mice display increased numbers of neutrophils. Although neutrophils can have an anti-inflammatory role in certain IBD models,[33] most studies have suggested that neutrophils play an important proinflammatory role in the pathogenesis of IBD and correlate with disease severity.[23,25]

The pDCs were not affected in DSS-treated *Ccr9*$^{-/-}$ mice and *Rag1*$^{-/-}$*CCL25*$^{-/-}$ recipient mice after naive T cell transfer. CCR9-expressing pDCs have been reported to home to small intestinal tissues in steady state and in the setting of intestinal inflammation and can modulate immune responses in extraintestinal sites.[34–36] Mizuno et al observed that *Rag2*$^{-/-}$*Ccr9*$^{-/-}$ recipient mice develop ileitis, as well as colitis, when transferred with WT CD45RB^{hi} CD4^{+} T cells.[36] The authors attributed the ileitis to altered localization and function of regulatory pDCs within the small intestine of *Rag2*$^{-/-}$*Ccr9*$^{-/-}$ recipient mice.[36] Although in our studies *Rag1*$^{-/-}$*CCL25*$^{-/-}$ recipient mice after T cell transfer developed colitis but not ileitis, we did not assess the effects of the absence of CCR9 expression directly on innate immune cells.

Although overall cDC numbers were unaffected, we demonstrated a decrease in CD103^{+}CD11b^{-} cDC and an increase in CD103^{-}CD11b^{+} cDC subsets in colonic tissues of mice devoid of CCL25/CCR9 interactions in 2 independent chronic colitis models. In addition, we observed a decrease in the overall percentage of CD103^{+} cDCs (CD11b^{+} and CD11b^{-}). An imbalance in cDC subsets has been reported in humans and mice with colonic inflammation[37] and suggests that inflammatory conditions may lead to and/or result from an altered balance in tolerogenic/inflammatory DC subpopulations. CD103^{+}CD11b^{-} cDCs have been reported to play a crucial role in promoting tolerance to commensal bacteria and food antigens with enhanced capacity to generate iTregs from naive CD4^{+} T cells.[26] The decrease in the CD103^{+} cDC subset seen in both chronic IBD models was accompanied by an increase in CD103^{-}CD11b^{+} cDCs, a fraction reported to increase in the sensitivity of CD4^{+} T-cell responses to bacterial antigens.[38] All in all, the exacerbated colitis observed in Rag1$^{-/-}$Ccl25$^{-/-}$ mice upon transfer of naive T cells and in *Ccr9*$^{-/-}$ mice after chronic DSS exposure may result from altered cDC subset distribution, which is associated with reduced Treg function and augmented effecter T cell function.

Monocytes migrate into the intestinal LP in a CCR2-dependent manner and undergo a sequential differentiation process into proinflammatory and anti-inflammatory intestinal MΦ.[39] Interestingly, Tamoutounour et al[39] show that in the setting of a T cell–mediated colitis, MΦ differentiation is impaired and is associated with an increase in proinflammatory MΦ. The signals that regulate proinflammatory versus anti-inflammatory MΦ differentiation remain unknown, but our results show that this transition does not require CCL25/CCR9 interactions. Nonetheless, activated MΦ are known to respond to CCL25 chemotactic gradients,[40] and CCL25 appears to drive CCR9^{+} MΦ differentiation in human inflammatory conditions.[41] CCR9^{+} MΦ can display both proinflammatory and anti-inflammatory functions: for example, CCR9^{+} MΦ can drive acute murine liver inflammation and fibrosis in some conditions,[42,43] whereas CCR9^{+} MΦ can also prevent peritoneal sepsis in association with reduction of inflammatory cytokines and accumulation of neutrophils.[44] Therefore, these studies suggest that further investigation is required to understand how CCR9/CCL25 interactions regulate MΦ differentiation and/or function.

Together, we have demonstrated that murine T cells lacking CCR9 expression can induce large intestinal inflammation and that Treg trafficking and function are not affected by CCR9 deficiency. However, mice lacking CCR9 have exaggerated colonic inflammation in response to DSS and those devoid of CCR9/CCL25 interactions have enhanced

colitogenicity after naive T cell transfer. In both the chronic colitis models resulting from aberrant CCR9/CCL25 interactions, enhanced neutrophil numbers correlate with the degree of intestinal inflammation. Although overall pDC and cDC numbers are not significantly different than in WT control mice, cDC proinflammatory and anti-inflammatory subsets are regulated by CCR9/CCL25 interactions. Finally, CCR9/CCL25-dependent innate immune cell lineage specificity and lineage-dependent functions will be aided significantly by targeted deletions of CCR9/CCL25 in innate immune cell and/or epithelial compartments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Drs. Dror Shouval, Jeremy Goettel, and Amlan Biwas for critical review of the data and the article.

Supported by a Pilot Project Grant from the Harvard Digestive Diseases Center (P30 DK034854) and a Crohn's and Colitis Foundation of America Career Development Award # 2821 (M.A.W.); by a NIH grant AI075037 (E.F.); S.B.S. is supported by NIH Grants HL59561, DK034854, and AI50950, the Helmsley Charitable Trust, and the Wolpow Family Chair in IBD Treatment and Research.

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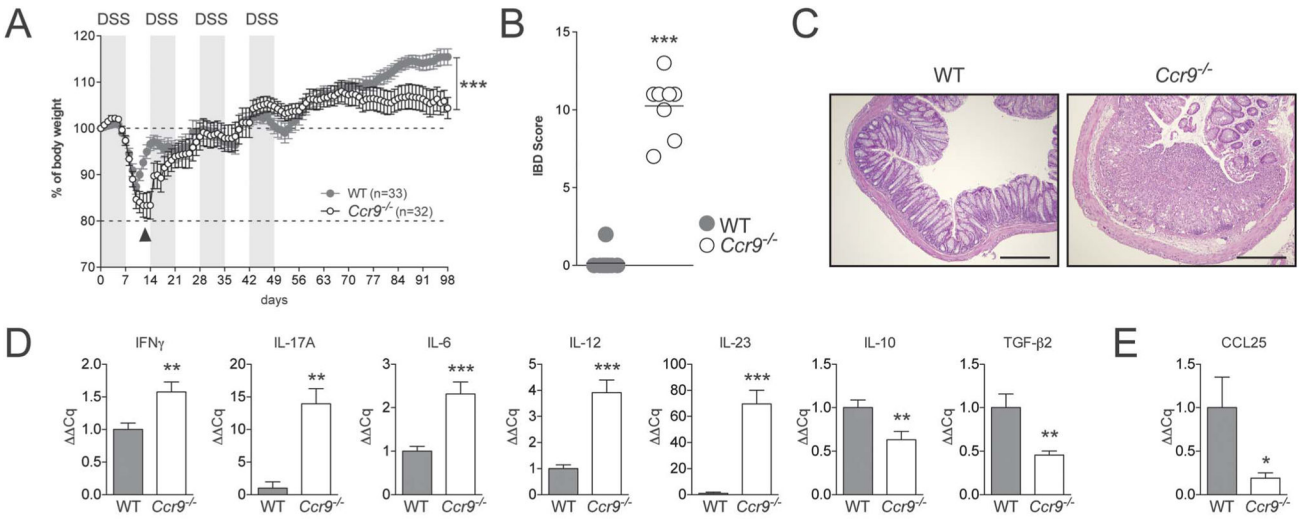

FIGURE 1.

Ccr9$^{-/-}$ mice show signs of exacerbated DSS-mediated chronic colitis. A, WT and *Ccr9*$^{-/-}$ mice were subjected to 4 DSS cycles (consisting of 2% DSS administration for 7 d in drinking water followed by water for 7 d). Weight was monitored daily and weight loss was calculated as percent of initial body weight. Data represent the mean of 4 independent experiments. Arrowhead highlights differences between WT and *Ccr9*$^{-/-}$ mice after first DSS cycle. B, IBD scores of chronic DSS-treated WT and *Ccr9*$^{-/-}$ mice were determined blindly by a pathologist after H&E staining of distal colonic tissue sections. C, Representative H&E staining of colonic tissue sections harvested in WT (left) and *Ccr9*$^{-/-}$ mice (right) >100 days after initiation of DSS exposure (10× magnification). D, mRNA profiling of proinflammatory and anti-inflammatory cytokines in colonic tissues from DSS-treated WT and *Ccr9*$^{-/-}$ mice. The mRNA transcripts were quantified by qPCR and normalized to the housekeeping gene HPRT. Data represent 1 out of 4 experiments. E, CCL25 transcript qPCR analysis in colons harvested from WT and *Ccr9*$^{-/-}$ mice treated with DSS. Data represent an individual experiment out of 4. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

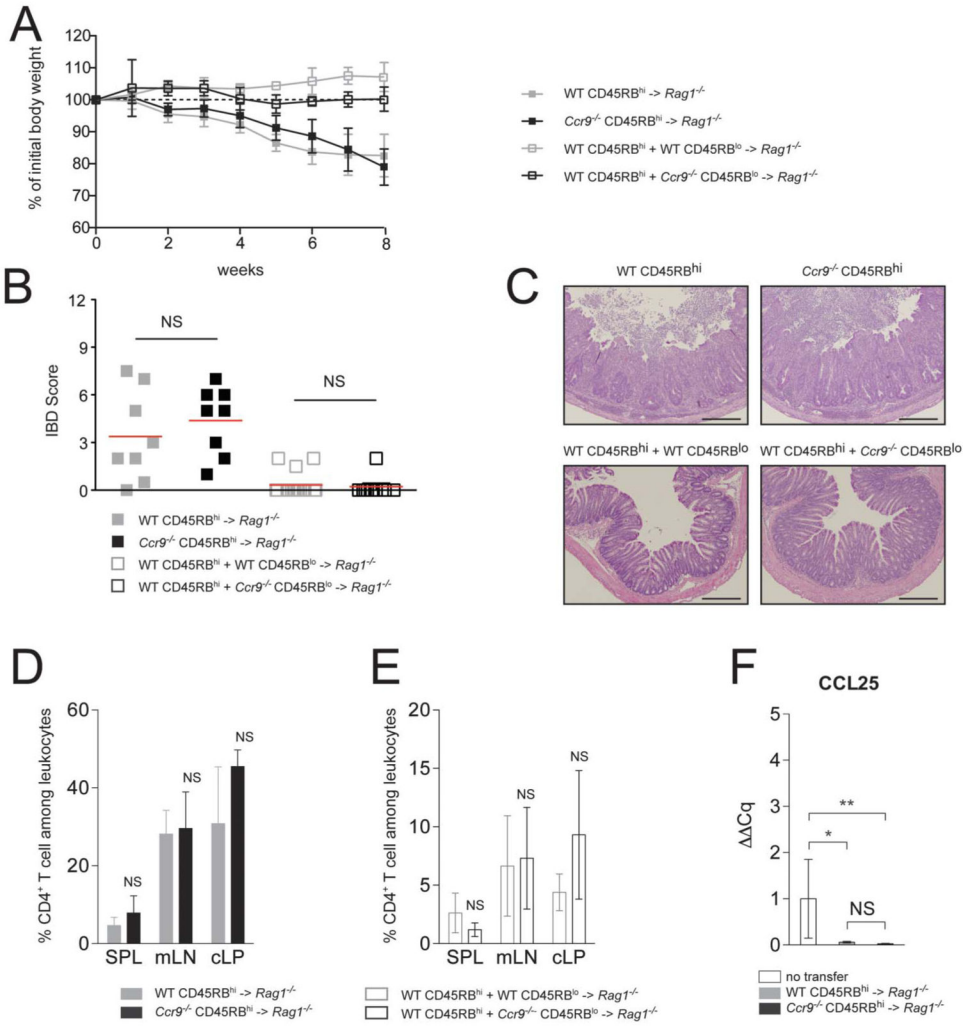

FIGURE 2.

CD45RB^{hi} CD4$^+$ T cells and CD45RB^{lo} CD4$^+$ T cell home to the large bowel independent of CCR9 expression. A, Weight loss monitoring of *Rag1*$^{-/-}$ recipient mice adoptively transferred with either WT CD45RB^{hi} (filled gray squares) or *Ccr9*$^{-/-}$ CD45RB^{hi} (filled black squares) CD4$^+$ T cells and in *Rag1*$^{-/-}$ recipient mice cotransferred with WT CD45RB^{hi} CD4$^+$ T cells and either WT CD45RB^{lo} (open gray squares) or *Ccr9*$^{-/-}$ CD45RB^{lo} (open black squares) CD4$^+$ T cells. Mice were monitored weekly and weight loss was reported and expressed as percentage of initial body weight (mean ± SD). Data represent 3 pooled experiments (n = 15). B, IBD scores of distal colons of paraffin-embedded sections of *Rag1*$^{-/-}$ mice are after transfer of either WT CD45RB^{hi} CD4$^+$ T cell transfer alone (filled gray squares) or with WT CD45RB^{lo} CD4$^+$ T cells (open gray squares), with *Ccr9*$^{-/-}$ CD45RB^{lo} CD4$^+$ T cells (open black squares), or *Ccr9*$^{-/-}$ CD45RB^{hi} CD4$^+$ T cell transfer alone (filled black squares) (mean ± SD). Data represent 3 pooled experiments. NS, not significant (*P* values). C, Representative H&E staining of colonic sections harvested in *Rag1*$^{-/-}$ mice transferred with either WT CD45RB^{hi} or *Ccr9*$^{-/-}$ CD45RB^{hi} CD4$^+$T cells alone, or WT CD45RB^{hi} T cells cotransferred with either WT CD45RB^{lo} or *Ccr9*$^{-/-}$ CD45RB^{lo} CD4$^+$ T cells (10× magnification). D, Percentage of CD4$^+$ T cells among

leukocytes determined in SPL, mLN, and cLP from *Rag1*$^{-/-}$ mice transferred with either WT or *Ccr9*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells. E, Percentage of CD4^{+} T cells among leukocytes was determined in SPL, mLN, and cLP determined in *Rag1*$^{-/-}$ mice transferred with either WT CD45RB^{hi} CD4^{+} T cells and WT CD45RB^{lo} CD4^{+} T cells or WT CD45RB^{hi} CD4^{+} T cells and *Ccr9*$^{-/-}$ CD45RB^{lo} CD4^{+} T cells. Mean ± SD values of 3 pooled experiments are shown. F, A qPCR analysis of CCL25 transcript levels in *Rag1*$^{-/-}$ colitic host mice transferred with either WT or *Ccr9*$^{-/-}$ CD45RB^{hi} CD4^{+} T cells after normalization to HPRT and to untransferred *Rag1*$^{-/-}$ mice (mean ± SD). Data represent of 1 out of 3 experiments. *$P < 0.05$; **$P < 0.005$.

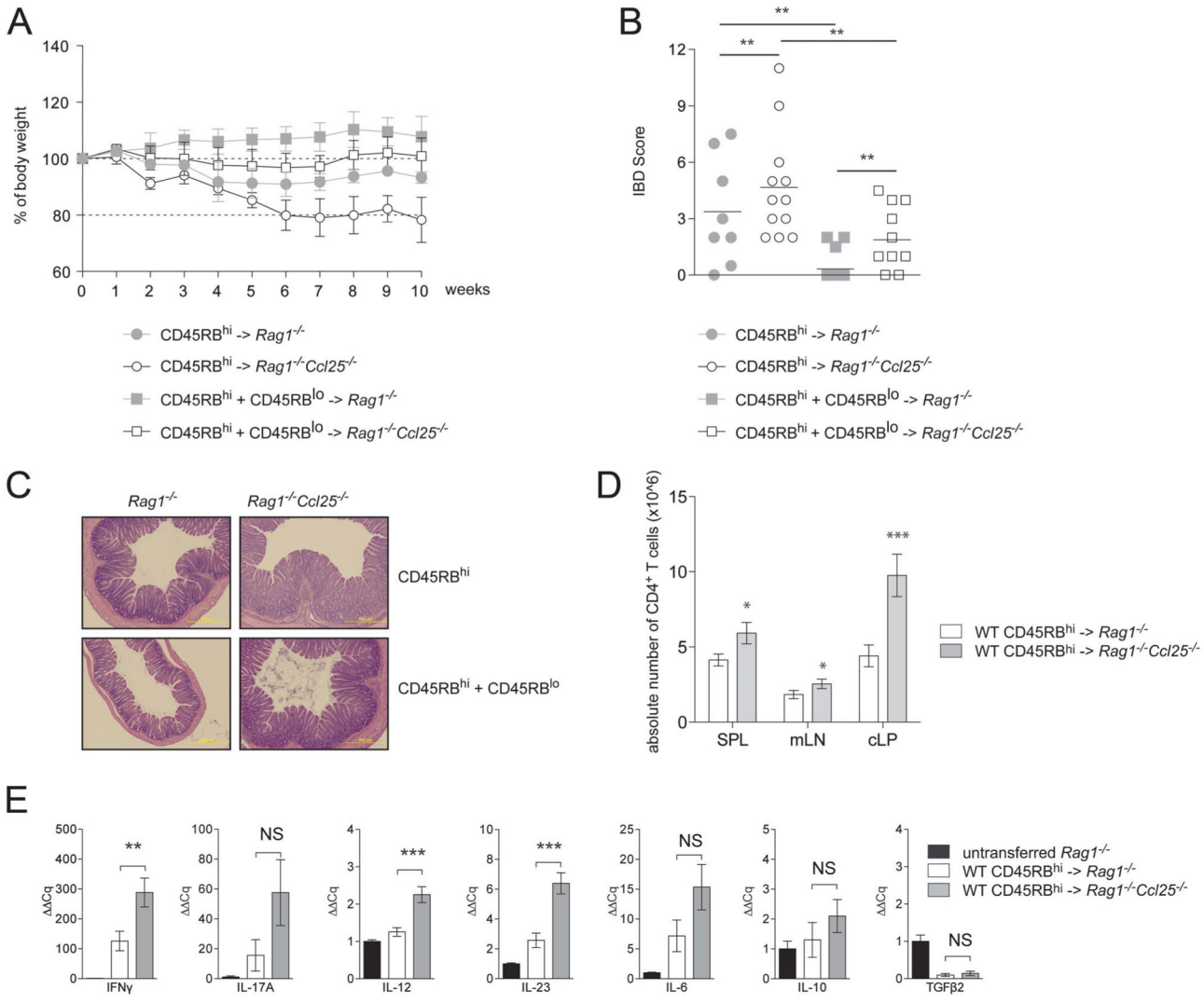

FIGURE 3.

Rag1$^{-/-}$*Ccl25*$^{-/-}$ mice display exacerbated T cell-mediated chronic colitis. A, Sex- and aged-matched *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice were adoptively transferred with WT CD45RB^{hi} or CD45RB^{hi} and CD45RB^{lo} CD4^{+} T cells to respectively induce or protect from experimental T cell-mediated colitis. Mice were monitored weekly and weight loss was reported and expressed as the percentage of initial body weight (mean ± SD). Data representative of 5 pooled experiments. B, Comparison of IBD scores obtained by H&E histological examination of paraffin-embedded sections from distal colons. *Rag1*$^{-/-}$ recipient mice are depicted in filled gray circles (after CD45RB^{hi} transfer) or filled gray squares (after CD45RB^{hi} and CD45RB^{lo} cotransfers), and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice are depicted in open circles (after CD45RB^{hi} transfer) or open squares (after CD45RB^{hi} and CD45RB^{lo} cotransfers). C, Representative H&E staining of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ recipient mice after transfer of CD45RB^{hi} CD4^{+} T cells or after cotransfer of CD45RB^{hi} and CD45RB^{lo} CD4^{+} T cells (10× magnification). D, Absolute CD4^{+} T-cell numbers among leukocytes quantified by flow cytometry in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ SPL, mLN, and

cLP after CD45RB^{hi} CD4^{+} T cell transfer (mean ± SD). Data are representative of 5 pooled experiments. E, The qPCR mRNA profiling in *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ colons after transfer of WT CD45RB^{hi} CD4^{+} T cells. Normalization was performed to HPRT and untransferred *Rag1*$^{-/-}$ control mice. Data represent of 1 of 5 experiments. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; NS, not significant.

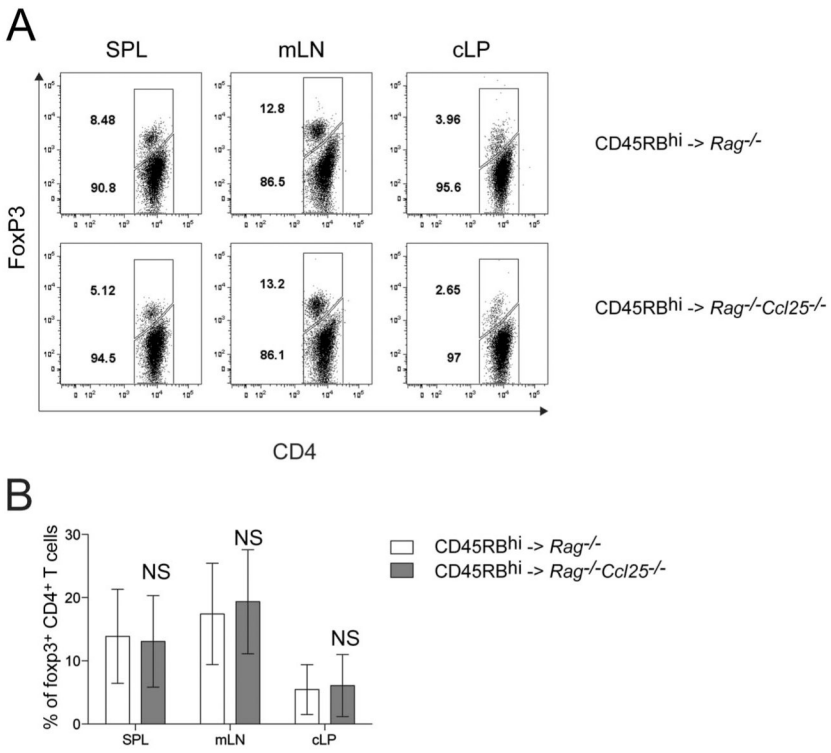

FIGURE 4.

Generation of inducible Tregs (iTregs) is not impaired in the absence of CCL25. A, Data represent the percentage of $CD4^+$ Foxp3-positive iTregs in SPL, mLN, and cLP of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice 8 weeks after transfer of naive $CD4^+$ T cells. Data show 1 representative experiment out of 5. B, Mean ± SD percentages of $CD4^+$ T cells that are $Foxp3^+$ pooled from 3 independent experiments. NS, not significant.

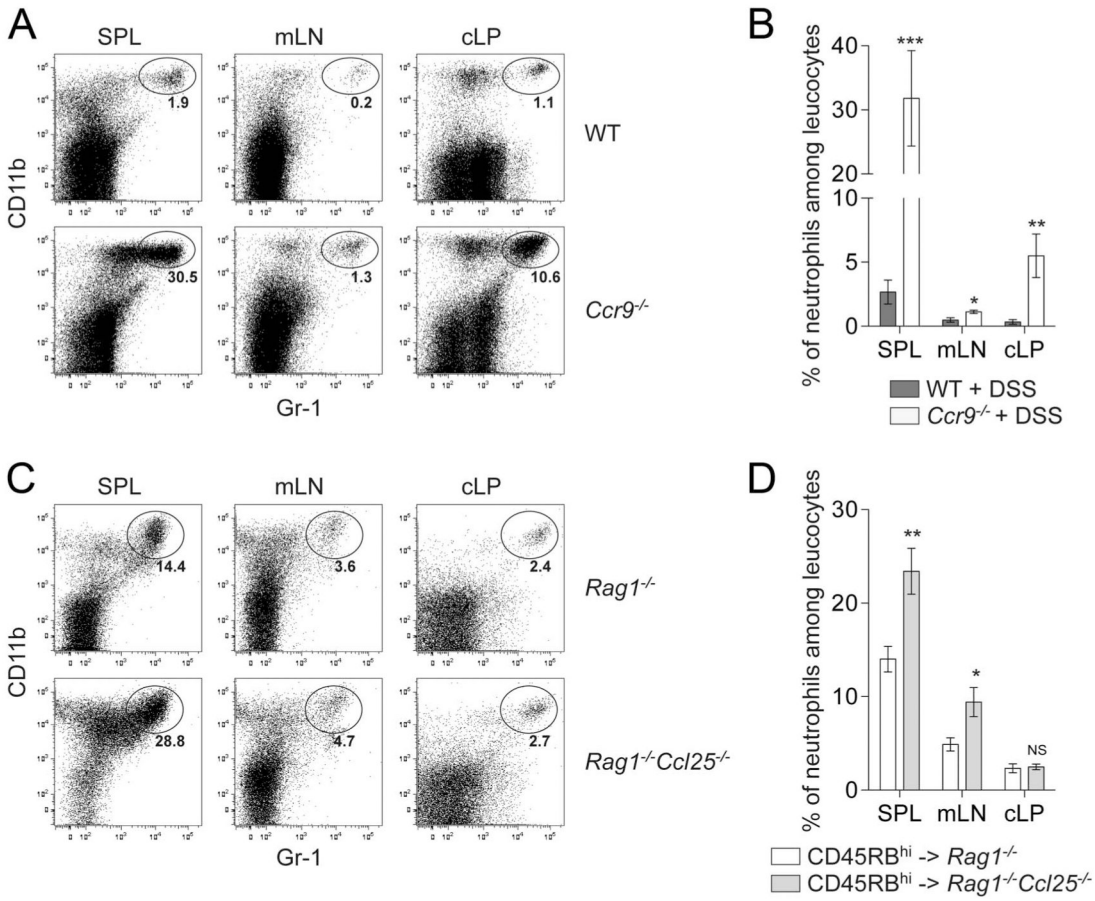

FIGURE 5.

Increased frequencies of circulating and intestinal granulocytes. Granulocytes defined by Gr-1^{hi} (Ly6G^{hi} Ly6C^{lo}) CD11b^{hi} CD11^{-} MHCII^{-} expression were analyzed by flow cytometry in SPL, mLN, and cLP of WT versus *Ccr9*$^{-/-}$ mice upon chronic DSS-mediated colitis (A and B) and of *Rag1*$^{-/-}$ versus *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice adoptively transferred with WT CD45RB^{hi} CD4^{+} T cells (C and D). A and C, Display representative flow cytometry dot plots of Gr-1^{hi} CD11b^{hi} granulocytes. B and D, Display granulocyte quantification in SPL, mLN, and cLP of mice developing chronic colitis (mean ± SD). Data representative of 5 pooled experiments. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; NS, not significant.

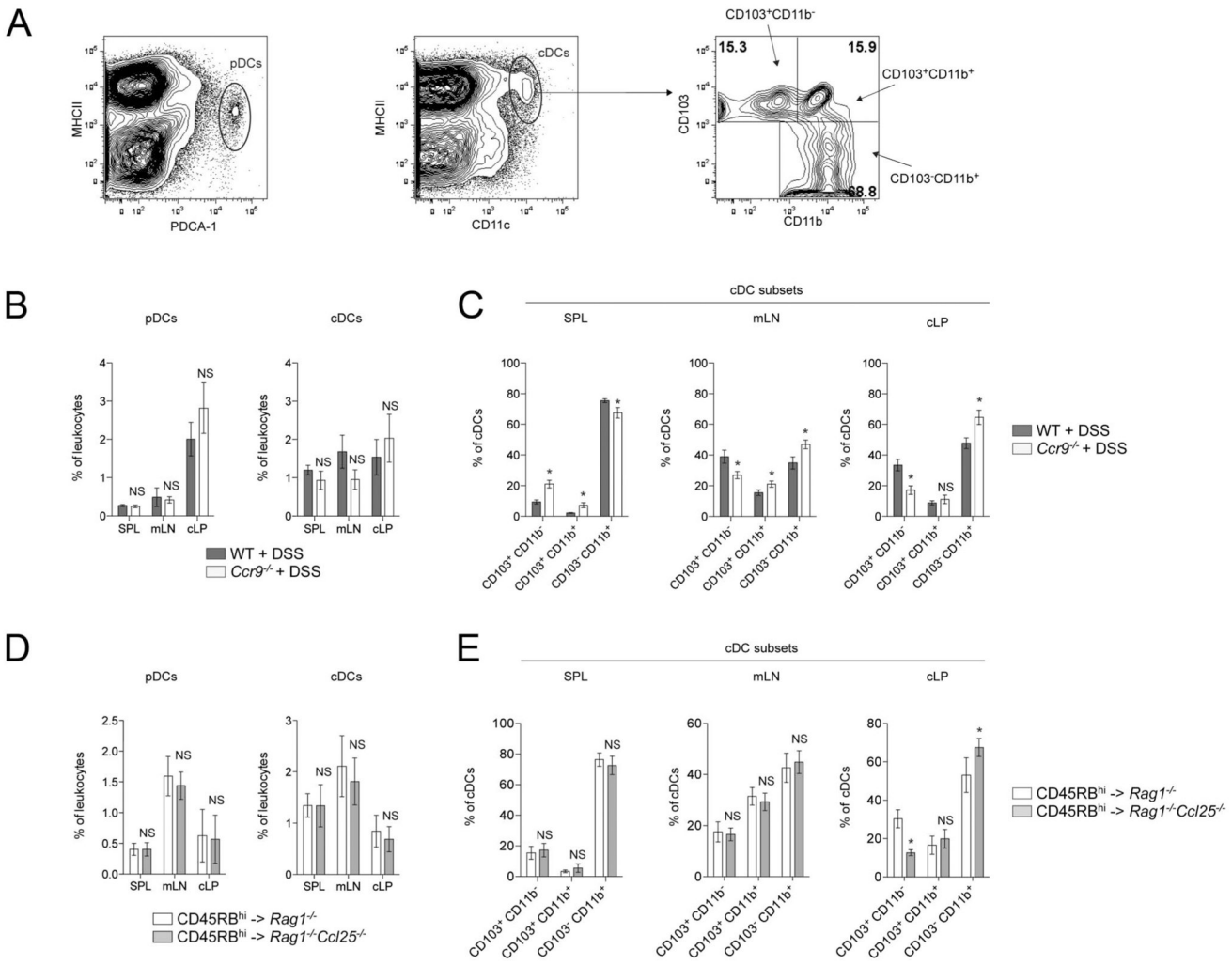

FIGURE 6.

Altered cDC subset distribution in colitic mice lacking CCL25/CCR9 interactions. A, Flow cytometry gating strategy to identify PDCA-1^{+} MHCII^{lo} pDCs (left), CD11c^{hi} MHCII^{hi} cDCs (middle), and cDC subsets (right). B, The pDC (left) and cDC (right) distribution analyses in SPL, mLN, and cLP of DSS-treated WT and *Ccr9*$^{-/-}$ mice. C, The cDC subsets distribution analyses in SPL, mLN, and cLP of DSS-treated WT and *Ccr9*$^{-/-}$ mice. D, The pDC (left) and cDC (right) distribution analyses in SPL, mLN, and cLP of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice transferred with WT CD45RB^{hi} CD4^{+} T cells. E, The cDC subsets distribution analyses in SPL, mLN, and cLP of *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Ccl25*$^{-/-}$ mice transferred with WT CD45RB^{hi} CD4^{+} T cells (mean ± SD). Data represent 5 pooled experiments. *$P < 0.05$; NS, not significant.

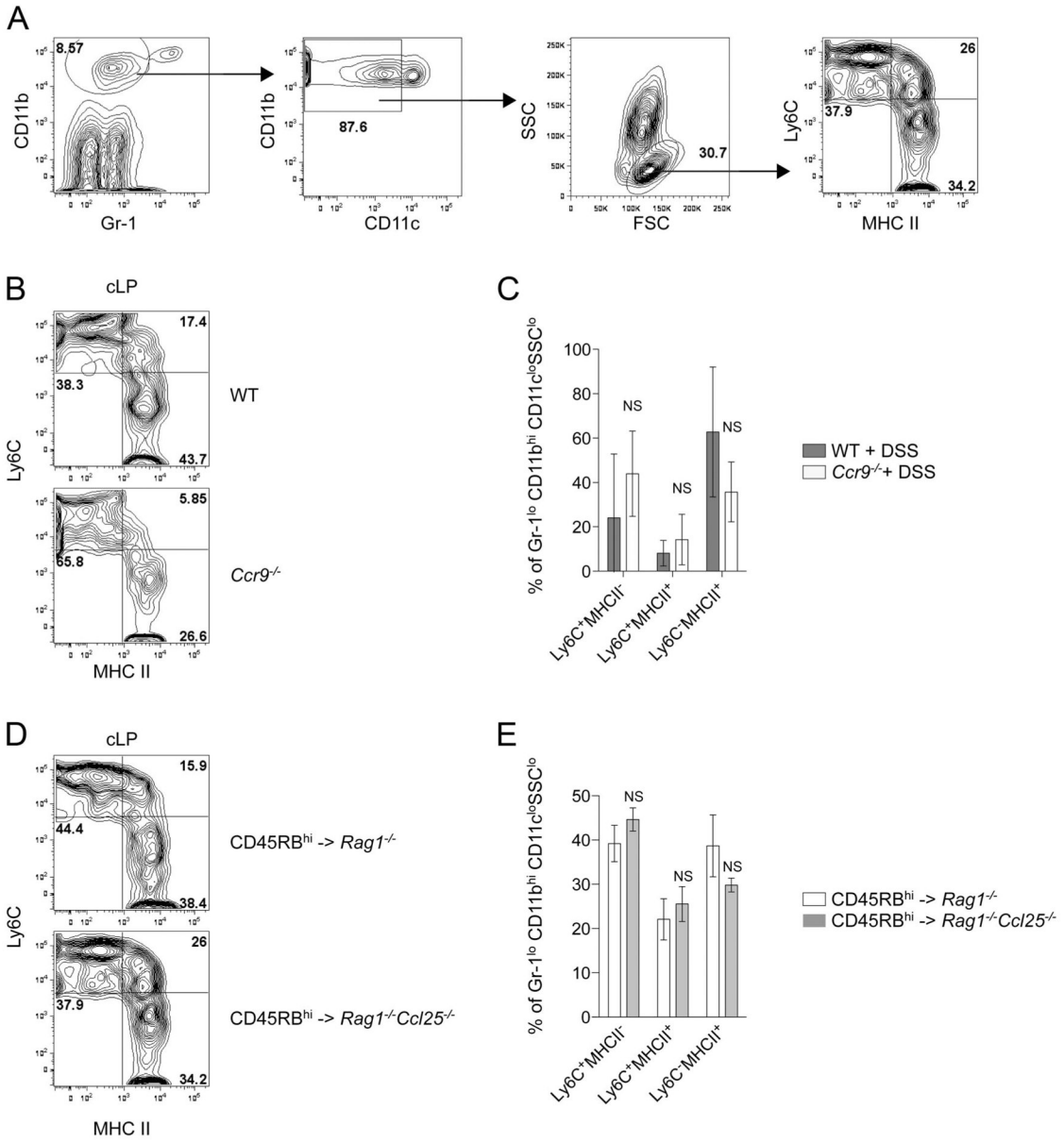

FIGURE 7.

Distribution of proinflammatory and anti-inflammatory colonic macrophages is independent of CCL25/CCR9 interactions. A, Flow cytometry gating strategy used to investigate proinflammatory and anti-inflammatory macrophages. B and D, Representative dot plot of proinflammatory and anti-inflammatory macrophages in cLP of DSS-treated WT and *Ccr9*$^{-/-}$ mice (B) and *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*xCcl25*$^{-/-}$ recipient mice transferred with WT CD45RB^{hi} CD4^{+} T cells (D). Indicated percentages are relative to gated Gr-1^{lo}CD11b^{hi}CD11c^{lo}SSC^{lo} leukocytes. C and E, Frequencies of proinflammatory and anti-inflammatory intestinal macrophages in DSS-treated WT and *Ccr9*$^{-/-}$ mice (C) and *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*xCcl25*$^{-/-}$ mice transferred with WT CD45RB^{hi} CD4^{+} T cells (E).

Pooled data from 5 (B) and 3 experiments (D) (mean ± SD). Data represent 5 pooled experiments. NS, not significant.