Mice with a Selective Deletion of the CC Chemokine Receptors 5 or 2 Are Protected from Dextran Sodium Sulfate-Mediated Colitis: Lack of CC Chemokine Receptor 5 Expression Results in a NK1.1+ Lymphocyte-Associated Th2-Type Immune Response in the Intestine


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Mice with a Selective Deletion of the CC Chemokine Receptors 5 or 2 Are Protected from Dextran Sodium Sulfate-Mediated Colitis: Lack of CC Chemokine Receptor 5 Expression Results in a NK1.1+ Lymphocyte-Associated Th2-Type Immune Response in the Intestine


The chemokine receptors CCR2 and CCR5 and their respective ligands regulate leukocyte chemotaxis and activation. To determine the role of these chemokine receptors in the regulation of the intestinal immune response, we induced colitis in CCR2- and CCR5-deficient mice by continuous oral administration of dextran sodium sulfate (DSS). Both CCR2- and CCR5-deficient mice were susceptible to DSS-induced intestinal inflammation. The lack of CCR2 or CCR5 did not reduce the DSS-induced migration of macrophages into the colonic lamina propria. However, both CCR5-deficient mice and, to a lesser degree, CCR2-deficient mice were protected from DSS-induced intestinal adhesions and mucosal ulcerations. CCR5-deficient mice were characterized by a greater relative infiltration of CD4+ and NK1.1+ lymphocyte in the colonic lamina propria when compared to wild-type and CCR2-deficient mice. In CCR5-deficient mice, mucosal mRNA expression of IL-4, IL-5, and IL-10 was increased, whereas that of IFN-γ was decreased, corresponding to a Th2 pattern of T cell activation. In CCR2-deficient mice, the infiltration of Th2-type T cells in the lamina propria was absent, but increased levels of IL-10 and decreased levels of IFN-γ may have down regulated mucosal inflammation. Our data indicate that CCR5 may be critical for the promotion of intestinal Th1-type immune responses in mice. The Journal of Immunology, 2000, 164: 6303–6312.

Lymphocyte migration into the intestinal mucosa depends on adhesion molecules and is regulated by chemotactic cytokines (chemokines) and their receptors (1). Evidence is emerging that individual chemokines attract specific leukocyte populations through processes determined by ligand specificity and the expression patterns of the corresponding receptors (1). In addition to their ability to induce adhesion and migration of leukocytes, recent data have demonstrated that chemokines are involved in the activation of lymphocytes and mast cells (2, 3).

A number of chemokines have been assumed to contribute to the regulation of intestinal immune responses and mucosal inflammation, largely based on their increased expression during mucosal inflammation and their known properties as leukocyte chemoattractants and activators in vitro (4). However, determination of the role of individual chemokines and/or receptors in the regulation of mucosal immune cells in vitro has been hampered by the apparent redundancy in the chemokine/chemokine receptor network (5). Thus, the function of specific chemokine ligand/receptor systems in the attraction of leukocyte subpopulations in the healthy mucosa and during intestinal inflammation remains largely unclear.

Chemokines exert their effects through specific receptors that are differentially expressed on cell populations of hemopoietic origin (1). The CC chemokine receptors CCR2 and CCR5 are involved in both the regulation of monocyte- and macrophage-mediated immune responses and in the modulation of T cell migration and activation. In humans, two related receptors, CCR2A and CCR2B (6), mediate responses to monocyte chemoattractant protein-1 (MCP-1), MCP-2, and MCP-3. Interestingly, they also serve as necessary coreceptors for several HIV isolates (7, 8). Increased expression of MCP-1 and MCP-3 has been found in colonic tissue from patients with inflammatory bowel disease (IBD), and these chemokines possess functional activity that could contribute to the inflammatory process in the affected mucosa (9, 10). MCP-1 is a potent chemoattractant and activator of monocytes (11, 12), NK cells (13), and memory T cells (14) in vitro. MCP-3 is able to induce activation of dendritic cells, eosinophils, basophils, and neutrophils, whereas MCP-2 stimulates both eosinophils and...
basil Care at Massachusetts General Hospital and Harvard Medical School.

CCR5 binds the CC chemokines macrophage-inhibitory protein (MIP)-1α, MIP-1β, and RANTES (20), which are released from intestinal epithelial cells upon exposure to inflammatory mediators (21, 22). Expression of both MIP-1α and RANTES is increased in the intestinal mucosa in IBD (23–25). Experiments performed in vitro suggest that all three CCR5 ligands are involved in the generation of Th1 immune responses (26), a property which may be relevant to both murine colitis models and human Crohn’s disease in which increasing evidence suggests a predominant Th1 response. In addition, CCR5-binding chemokines preferentially attract memory and activated CD4+ and CD8+ T cells (27, 28), which is consistent with the presence of the CCR5 receptor on these lymphocytes (29). In this study, mutant mice lacking either CCR2 or CCR5 expression were used to determine the role of these chemokine receptors in intestinal inflammation.

Materials and Methods

Animals

129/Ola mice with a targeted deletion of the CCR2 gene were created as previously described (30) and subsequently backcrossed onto a C57BL/6J background. CCR5-deficient mice were generated by the insertion of a neomycin resistance gene in the first intron of the CCR5 gene, causing a deletion of the entire second exon and a portion of the 3’ untranslated region. Correctly targeted embryonic stem cells were selected, and male chimeras were generated and mated to C57BL/6J females. F1 heterozygotes were mated to obtain homozygous CCR5-deficient mice (W. A. Kuziel, T. Dawson, and N. Maeda, manuscript in preparation). Control animals were generated by crossing F1 wild-type progeny of 129/Ola and C57BL/6J matings. The presence of a BglII restriction-length polymorphism was used to confirm that the CCR2 and CCR5 gene cluster in the control animals were derived from the 129/Ola strain (30).

The clinical course of the dextran sodium sulfate (DSS) colitis in the animal groups (Figs. 1-4) was determined in wild type (n = 10), CCR2 (n = 9), and CCR5 (n = 9) mice. Flow cytometry for the characterization of lymphocyte phenotypes was conducted in wild type (n = 16), CCR2 (n = 17), and CCR5 (n = 14). The determination of the expression of lymphocyte differentiation markers, chemokines, and cytokines in colonic tissues was conducted in at least five animals per group. All mice were female and 10–12 wk old at the beginning of the trial. All animal experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital and Harvard Medical School.

Induction of colitis

Colitis was induced in all groups by the addition of DSS (2.5% weight to volume ratio dissolved in distilled water; m.w. 40,000; ICN Biomedicals, Aurora, OH; lot no. 3073B) to their drinking water, as described previously (31). The mean DSS/water consumption and daily weights were recorded. Stool consistency was assessed daily using the following four-point scale: 0, normal; 1, soft; 2, very soft but formed; and 3, liquid. Fecal blood was assessed daily as follows: a single fecal pellet was suspended in 400 μl of H2O and centrifuged at maximum in a bench-top microcentrifuge (Eppendorf, model 5415C, Brinkman Instruments, Westbury, NY). A 40-μl aliquot was then added to a 0.5 × 0.5-cm piece of Hemocult SENSA paper, allowed to air dry, and developed with one drop of the provided SENSA developer solution (SmithKline Diagnostics, San Diego, CA). The SENSA color change was scored by observers blinded to the identity of the samples, were assessed in a blind fashion by two investigators (P.G.A. and H.-C.R.), who examined in a blind fashion by two investigators (P.G.A. and H.-C.R.), who counted the number of positive cells per five separate high-powered fields. Statistical analysis was performed as described below.

Lamina propria leukocyte isolation and flow cytometry

Leukocytes were extracted from the lamina propria of the large intestine as previously described (36). For flow cytometry, 2 × 107 colonic lamina propria cells were washed in PBS containing 0.2% BSA and 0.1% sodium azide. They were subsequently incubated first with blocking buffer (10% normal hamster, rat, and mouse serum; and 1 μg anti-CD16/32 mAb) at 4°C for 20 min, and then with FITC- and PE-tagged mAbs at 4°C for 30 min. After washing, cells were analyzed using Lysys II software on FACScan (Becton Dickinson, Mountain View, CA). The mAbs (PharMingen) used in this study were FITC-CD3 (145-2C11) and -B220 (RA3-6B2), and PE-CD4 (RM4-4), -CD8α (53-67), and -NK-1.1 (PK136).

RNA analysis

Whole colonic tissue RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the instructions of the manufacturer. RNase protection assays (RPAs) were performed using the Riboquant multiprobe RPA system (PharMingen) according to the instructions of the manufacturer. Mouse cluster of differentiation-1 or mouse cluster of chemokine-5 probes were synthesized and incubated with 10–15 μg of total RNA from intestinal tissue cut from these tissues were then fixed in acetone and incubated with primary and subsequently secondary Abs as previously described (35). Purified primary Abs against CD4 (RM4–5) were purchased from Pharmingen (San Diego, CA). Anti-F4/80 Ab was purchased from Serotec (Raleigh, NC). The secondary Ab was a biotinylated rabbit anti-rat Ig (Vector, Burlingame, CA). Each immunohistochemical section was subsequently examined in a blind fashion by two investigators (P.G.A. and H.-C.R.), who counted the number of positive cells per five separate high-powered fields. Statistical analysis was performed as described below.

Correlation coefficient was greater than 0.9 when the scores obtained by two independent investigators (P.L.B. and P.G.A.), blinded to the identity of the samples, were compared.

General assessment of colitis

All animals were sacrificed by carbon dioxide narcosis. The entire colon was removed, and the length was recorded as previously described (32). The number of adhesions between the colon and other peritoneal structures was also recorded. The extent of adhesions was scored as follows: 0, nil; 1, adhesions to one organ; 2, adhesions to two organs and/or two distinct areas of adhesions; 3, adhesions to three organs and/or three distinct areas of adhesions. Colons were subsequently opened longitudinally, and the extent and severity of the colitis was assessed macroscopically as follows: 0, normal; 1, erythema of the mucosa; 2, presence of ulcers; and 3, diffuse ulceration. The extent of ulceration was determined by measuring the percentage of the colon and cecum that had macroscopic evidence of colitis.

The colons were divided into three sections: distal, mid-colon, and proximal colon. The distal colon was used for myeloperoxidase (MPO) assay, the mid-colon for histology, and the proximal colon for isolation of RNA. Tissue sections were examined in a blind fashion. Each section was scored for severity and extent of ulceration, and the tissue thickness from the muscularis propria to the luminal border was determined. Lesion severity was graded using a modification of a previously defined scoring system (33) with a scale of 0–3: 0, normal; 1, mild; 2, moderate; and 3, severe. Mild lesions contained small, focal, or widely dispersed areas of inflammation and/or fibrosis above the muscularis mucosa. Moderate lesions were multifocal or locally extensive and contained inflammation or fibrosis extending into the submucosa. Severe was defined as inflammatory cells extending into the muscularis propria. The reliability and reproducibility of this semiquantitative evaluation was assessed by comparing the scores of two independent investigators (P.L.B. and P.G.A.); the Pearson correlation coefficient was 0.787 (95% confidence interval (CI), 0.580–0.898).

MPO assay

This assay was performed as previously described (34). Absorbance at 460 nm was determined for three separate 30-s intervals. One unit of MPO activity was defined as 1 μmol H2O2 broken down to H2O and O2 by MPO (a change in absorbance of 1.13 × 10–5).

Immunohistochemistry

Tissue samples were frozen in OCT compound (Ames, Elkhart, IN). Sections cut from these tissues were then fixed in acetone and incubated with primary and secondary Abs as previously described (35). The expression of lymphocyte phenotypes was conducted in wild type (n = 16), CCR2 (n = 17), and CCR5 (n = 14). The expression of lymphocyte differentiation markers, chemokines, and cytokines in colonic tissues was conducted in at least five animals per group. All mice were female and 10–12 wk old at the beginning of the trial. All animal experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital and Harvard Medical School.
In contrast, the CCR5-deficient animals remained active through the duration of the study and experienced minimal weight loss (Fig. 1, A–C). In contrast, the CCR5-deficient animals remained active throughout the course of the study and experienced minimal weight loss (Fig. 1A). This difference became statistically significant after day 5 of the trial. Diarrhea and hemocult scores of the CCR5-deficient mice were also significantly lower than those of the wild-type animals (Fig. 1, B and C). In aggregate, these findings suggest that CCR5-deficient mice experience clinically less severe DSS-mediated colitis than wild-type mice. By day 7, the CCR2-deficient mice lost an amount of weight comparable to that of wild-type animals and developed quantitatively similar stool diarrhea and hemocult scores. However, the development of diarrhea and bloody stools was delayed (Fig. 1, A and B), with a significantly reduced hemocult score on day 4 of the trial and significantly diminished diarrhea on days 1, 3, and 4.

After 7 days of DSS ingestion, macroscopic examination showed significantly fewer intraabdominal adhesions in CCR5- and CCR2-deficient mice compared with those in wild-type mice (Fig. 2A). Their colons were also less erythematous and less ulcerated (Fig. 2A). This was especially notable in the CCR5-deficient mice, whose colons frequently looked normal. These observations paralleled the extent of measured affected colon. CCR5- and CCR2-deficient mice exhibited significantly less macroscopic disease in both the cecum and distal colon than wild-type mice (Fig. 2B).

Histological examination of the colon confirmed the reduced severity of inflammation and ulceration in CCR2- and CCR5-deficient mice when compared with wild-type mice (Fig. 3). As demonstrated in Fig. 4A, panels 1–3, wild-type animals often had extensive deep ulcerations characterized by near obliteration of the mucosa and rarely had areas of normal-appearing mucosa. In contrast, the colonic mucosa of CCR5-deficient mice was characterized by a diminished inflammatory infiltrate, and many areas appeared normal. The average percentage of ulcerated tissue in both the CCR5- and CCR2-deficient animals was less than that in the wild-type controls at 3 and 7 days of DSS ingestion (Fig. 3A). The difference between the CCR5 and wild-type mice was statistically significant at both day 3 and day 7, whereas the difference between CCR2 knockout mice and wild-type mice was significant only at day 3. Assessment of mucosal inflammation revealed a significantly diminished average depth of inflammatory cell penetration in CCR5- and CCR2-deficient animals at both 3- and 7-day time points (Fig. 3B).

### Results

**CCR2- and CCR5-deficient mice are protected from DSS-mediated colitis**

Mean consumption of 2.5% DSS in water was equal in all animals throughout the duration of the study: wild-type mice, 5.53 ± 1.6 ml/day; CCR2-deficient, 5.25 ± 1.3 ml/day (p > 0.1); and CCR5-deficient, 4.62 ± 1.7 ml/day (p > 0.1). Over the 7-day course of DSS, wild-type mice developed diarrhea and hemocult-positive stools, which were also often bloody. They became progressively more lethargic and lost almost 15% of their body weight (Fig. 1, A–C). In contrast, the CCR5-deficient animals remained active throughout the course of the study and experienced minimal weight loss (Fig. 1A). This difference became statistically significant after day 5 of the trial. Diarrhea and hemocult scores of the CCR5-deficient mice were also significantly lower than those of the wild-type animals (Fig. 1, B and C). In aggregate, these findings suggest that CCR5-deficient mice experience clinically less severe DSS-mediated colitis than wild-type mice. By day 7, the

### Table 1. PCR primers and conditions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequencesa</th>
<th>MgCl₂ (mM)</th>
<th>Cycle Parameters</th>
<th>Cycles</th>
<th>Size (bp)</th>
</tr>
</thead>
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<tr>
<td>IL-1β</td>
<td>F 5'-CAG GAT GAG GAC ATG AGC ACC-3' R 5'-CCT TGC AGA CTC AAA CTC CAC-3'</td>
<td>1.0</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>33</td>
<td>983</td>
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<td>IL-6</td>
<td>F 5'-CAC AAA GGC AGA GTC CTT CAG AGA-3' R 5'-CTA GGT TGG CCG AGT AGA TCT-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>40</td>
<td>226</td>
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<td>TGF-β</td>
<td>F 5'-CGG GGC GAC CTC GGC ACC ATC CAT GAC-3' R 5'-CTC CTC CAC GTC CCT GGC ACC CAC-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>30</td>
<td>406</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F 5'-ATG AGC ACA GAA AGC ATG ATC-3' R 5'-TAC AGG CTG TGC ACT CTA ATT-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>33</td>
<td>306</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F 5'-TAC TGC CAC GGC ACA GTG ATT GA-3' R 5'-GCA GGC ACT TTT CCG CTT CCT-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>33</td>
<td>406</td>
</tr>
<tr>
<td>IL-4</td>
<td>F 5'-AGC GAT GAT GTG CCA AAC GTC-3' R 5'-CGA GTA ATC CAT TGG CAT GAT GC-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>40</td>
<td>279</td>
</tr>
<tr>
<td>IL-5</td>
<td>F 5'-GCC ATG GAT ATT CCC ATG AGC ACA-3' R 5'-GCC TTC TCC CTA CAA CTC TGT AC-3'</td>
<td>2.0</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>40</td>
<td>338</td>
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<tr>
<td>IL-10</td>
<td>F 5'-GTG AAG ACT TTC TTT CAA ACA AAG-3' R 5'-CTG CTC CTC CAG TCT GCT ATT-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 56°C, 60 s; 72°C, 90 s; 72°C, 10 min</td>
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<td>274</td>
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<tr>
<td>GAPDH</td>
<td>F 5'-AGT CAA CCG ATT TGG TCG TAT-3' R 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'</td>
<td>1.5</td>
<td>94°C, 5 min 96°C, 36 s; 60°C, 45 s; 72°C, 90 s; 72°C, 10 min</td>
<td>30</td>
<td>307</td>
</tr>
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</table>

a F, Forward; R, reverse.
DSS colitis in CCR2- and CCR5-deficient mice is characterized by macrophage infiltration and neutrophil activation

Induction of DSS colitis in mice has been associated with the influx of macrophages into the intestinal mucosa. Therefore, we determined whether the diminished severity of DSS-mediated colitis in the CCR5-deficient and CCR2-deficient mice was associated with alterations in the composition of immune cell populations in the intestinal mucosa. Macrophage infiltration into the lamina propria was assessed by immunohistochemistry. Mucosal frozen tissue sections were stained with anti-F4/80 Abs to detect mucosal macrophages and with Abs to detect CD4+ T cells.

As demonstrated in Fig. 4, DSS-induced mucosal inflammation in all animal groups was characterized by a significant increase in F4/80+ macrophages in the lamina propria. The number of macrophages in the CCR2- and CCR5-deficient animals was not statistically different when compared with the wild-type controls at either day 3 or day 7 (Fig. 4, A panels 4–6 and B). In contrast, the number of mucosal CD4+ T cells during DSS-induced colitis in CCR2- and CCR5-deficient mice differed significantly from that in wild-type mice. The intestinal mucosa of CCR5-deficient mice was characterized by an enhanced infiltration of CD4+ lymphocytes after 7 days of DSS administration compared with either wild-type or CCR2-deficient mice (Fig. 4, A panels 7–9 and B). The intestinal mucosa of CCR2-deficient mice did not contain a significantly increased number of CD4+ T cells (Fig. 4B).

To quantitate mucosal neutrophil infiltration, colonic MPO activity was determined after 7 days of DSS (Fig. 4C). No difference in colonic MPO activity between CCR2- and wild-type mice was observed: wild-type, 32.4 ± 9.2 U; CCR2-deficient, 32.8 ± 6.4 U. MPO activity in the colon of CCR5-deficient mice (28.7 ± 6.3 U) appeared to be lower when compared with wild-type and CCR2-deficient mice, but this difference did not reach statistical significance (Fig. 4C).
CCR5-deficient mice are characterized by an increase of CD4⁺ and NK1.1⁺ lymphocytes in the intestinal mucosa

To determine the DSS-induced regulation of lamina propria lymphocytes in wild-type, CCR2-deficient, and CCR5-deficient mice, lymphocytes were isolated from the colons of all three groups of mice before and at days 3 and 7 of DSS. The phenotype and the composition of mucosal lymphocyte before and during the development of DSS-induced colitis were assessed by FACS analysis (Fig. 5 and Table II). The expected number of isolated cells was proportional to the degree of inflammation in the colon. The CD4⁺ and CD8⁺ T cell populations were differentially regulated in the wild-type, CCR2-deficient, and CCR5-deficient animals. As demonstrated in Table II, CCR5-deficient animals were characterized by an increased number of CD4⁺ lymphocytes and a reduced number of CD8⁺ lymphocytes before and during DSS-induced colitis. The resulting CD4/CD8 ratio increased from 1.8 ± 0.3 before DSS treatment to 5.8 ± 2.3 at day 7 in CCR5-deficient animals. In contrast, the lamina propria of CCR2 deficient mice was characterized by an increase in the CD4/CD8 ratio from 0.9 ± 0.1 to 1.5 ± 0.4, whereas in wild-type mice the CD4/CD8 ratio changed from 1.0 ± 0.3 to 1.4 ± 0.1 over the 7-day DSS treatment. These data indicate that CCR5-deficient mice have an increased percentage of CD4⁺ cells in the lamina propria lymphocyte population, and this population may be further expanded during the course of the DSS-induced colitis. In all animal groups, we observed a decrease in CD4⁺ T cells in the intestinal mucosa at day 3 of the DSS treatment. This decrease was accompanied by an increased number of CD8⁺ cells in the wild-type and the CCR2-deficient animals.
additional analysis was performed by RPA using specific RNA probes deficient mice, the B cell population initially decreased from 34.0 to 43.5 percent of lamina propria B cells increased from 27.4 to 38.6 percent of the CCR5-deficient animals. Although the number of B cells in the wild-type mice decreased from 52.0 to 57.0 percent down to 34.5 to 54.0 percent at day 3 and rebounded back to 43.0 to 47.0 percent after DSS treatment. In contrast, the percentage of lamina propria B cells increased from 27.4 to 40.0% to 43.5 to 3.8% in the CCR2-deficient group from 47.4 to 43.3% to 8.2 to 4.5% and 5.4 to 0.0% before DSS treatment, at day 3, and at day 7, respectively. In all animal groups, the strongest influx or expansion of NK1.1+ lymphocyte in the intestinal mucosa occurred on day 3 of the DSS treatment.

The percentage of B cells in the lamina propria was also differentially regulated during DSS colitis in wild-type and chemokine receptor-deficient animals. Although the number of B cells in the wild-type mice decreased from 45.2 to 5.7% down to 34.5 to 5.4% at day 3 and rebounded back to 38.9 to 4.7% at day 7, the percentage of lamina propria B cells increased from 27.4 to 40.0% to 43.5 to 3.8% in the CCR2-deficient group (Table II). In the CCR5-deficient mice, the B cell population initially decreased from 34.0 to 14.2% down to 18.8 to 9.3% at day 3 and then increased back to 34.8 to 2.6% after 7 days of DSS treatment (Table II).

To further characterize lymphocytes within the intestinal mucosa, additional analysis was performed by RPA using specific RNA probes for TCR6, TCRc, CD3e, CD4, CD8e, CD8b, CD19, F4/80, and CD45 as well as for the two housekeeping genes L32 and GAPDH.

This increase in CD8+ T cells was absent in the CCR5-deficient animals (Table II). The total number of isolated CD3+ T cells was not different in the animal groups.

The increased percentage of CD4+ T cells may be due in part to an increased percentage of NK1.1+ T cells in the lamina propria of the CCR5-deficient animals. The mucosal cellular infiltrates in CCR5-deficient animals were characterized by a 2-fold higher number of NK1.1+ cells compared with the other animal groups. As demonstrated in Table II, the percentage of NK1.1+ lymphocyte increased from a mean ± SD of 4.7 ± 5.8% to 14.5 ± 6.1% at day 3 to reach 13.8 ± 1.3% percent after 7 days of DSS treatment. In contrast, the percentage of NK1.1+ lymphocyte increased in the wild-type mice from 2.7 ± 1.0% to 7.7 ± 3.2% and 6.1 ± 0.8% and in the CCR2-deficient group from 4.7 ± 4.3% to 8.2 ± 4.5% and 5.4 ± 3.0% before DSS treatment, at day 3, and at day 7, respectively. In all animal groups, the strongest influx or expansion of NK1.1+ lymphocyte in the intestinal mucosa occurred on day 3 of the DSS treatment.

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The amount of TCRc, CD3e, CD19, CD45, and F4/80 mRNA expression after DSS exposure was comparable to that of wild-type mice. The equivalent F4/80 mRNA expression suggests the presence of similar numbers of macrophages in the lamina propria of CCR5-deficient and wild-type mice.

Untreated CCR2-deficient mice had a 5-fold increase in the expression of TCRδ and CD8e compared with that in wild-type controls. After DSS exposure, CD8e mRNA expression was further increased and was comparable to that of wild-type mice (Fig. 6). Moreover, CCR2-deficient mice had a 10-fold increase in CD19 mRNA expression after 3 days of DSS that returned to baseline by day 7. The expression of mRNA of all other cell markers studied, including F4/80 and CD45, was not altered after DSS administration in either CCR2-deficient or wild-type animals, suggesting that the absence of CCR2 does not affect macrophage and overall leukocyte recruitment in this model (Fig. 6). All animal groups developed a DSS-dependent increase in TCRδ mRNA expression, although this pattern was less pronounced in the CCR2-deficient mice (Fig. 6).

CCR5-deficient mice develop a Th2 pattern of cytokine expression in DSS-induced colitis

To determine the pattern of cytokine expression in the knockout and wild-type animals, we performed RT-PCR with RNA derived

Table II. Analysis of lamina propria lymphocyte populations in CCR2- and CCR5-deficient mice during DSS colitis

<table>
<thead>
<tr>
<th>Animal</th>
<th>n</th>
<th>% CD3e</th>
<th>% CD4e</th>
<th>% CD8e</th>
<th>% CD4/CD8e</th>
<th>% B Cells</th>
<th>% NK Cells</th>
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</thead>
<tbody>
<tr>
<td>Wild-type: no DSS</td>
<td>6</td>
<td>50.9 ± 9.4</td>
<td>25.5 ± 6.9</td>
<td>20.7 ± 10</td>
<td>1.0 ± 0.3</td>
<td>45.2 ± 5.7</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td>Wild-type: 3 days DSS</td>
<td>5</td>
<td>49.4 ± 6.1</td>
<td>19.7 ± 0.9</td>
<td>22.8 ± 1.8</td>
<td>0.9 ± 0.1</td>
<td>34.5 ± 5.4</td>
<td>7.7 ± 3.2</td>
</tr>
<tr>
<td>Wild-type: 7 days DSS</td>
<td>5</td>
<td>48.8 ± 3.8</td>
<td>26.2 ± 8.4</td>
<td>15.7 ± 0.8</td>
<td>1.4 ± 0.1</td>
<td>38.9 ± 4.7</td>
<td>6.1 ± 0.8</td>
</tr>
<tr>
<td>CCR2+/−: no DSS</td>
<td>5</td>
<td>53.4 ± 4.3</td>
<td>25.4 ± 6.8</td>
<td>19.2 ± 5.1</td>
<td>0.9 ± 0.1</td>
<td>27.4 ± 4.0</td>
<td>4.7 ± 4.3</td>
</tr>
<tr>
<td>CCR2+/−: 3 days DSS</td>
<td>6</td>
<td>58.0 ± 8.0</td>
<td>16.9 ± 2.2</td>
<td>23.7 ± 7.2</td>
<td>0.8 ± 0.2</td>
<td>31.9 ± 7.0</td>
<td>8.2 ± 4.5</td>
</tr>
<tr>
<td>CCR2+/−: 7 days DSS</td>
<td>5</td>
<td>45.6 ± 2.6</td>
<td>23.5 ± 4.8</td>
<td>15.1 ± 2.3</td>
<td>1.5 ± 0.4</td>
<td>43.5 ± 3.8</td>
<td>5.4 ± 3.0</td>
</tr>
<tr>
<td>CCR5+/−: no DSS</td>
<td>5</td>
<td>58.7 ± 14.4</td>
<td>37.0 ± 12.5</td>
<td>17.8 ± 2.4</td>
<td>1.8 ± 0.3</td>
<td>34.0 ± 14.2</td>
<td>4.7 ± 5.8</td>
</tr>
<tr>
<td>CCR5+/−: 3 days DSS</td>
<td>5</td>
<td>47.0 ± 1.4</td>
<td>21.9 ± 10.5</td>
<td>13.0 ± 1.7</td>
<td>1.2 ± 0.1</td>
<td>18.8 ± 9.3</td>
<td>14.5 ± 6.1</td>
</tr>
<tr>
<td>CCR5+/−: 7 days DSS</td>
<td>5</td>
<td>41.6 ± 4.5</td>
<td>35.2 ± 18.9</td>
<td>5.1 ± 1.5</td>
<td>5.8 ± 2.3</td>
<td>34.8 ± 2.6</td>
<td>13.8 ± 1.3</td>
</tr>
</tbody>
</table>

* Mean ± SD.
from whole colonic tissue (Fig. 7). The pattern of Th1- (IFN-γ) and Th2- (IL-4, IL-5, and IL-10) type cytokine mRNA expression differed significantly among the groups of mice. After correction for GAPDH, CCR5-deficient animals expressed one-fifth as much IFN-γ mRNA in the intestinal mucosa as wild-type animals after 3 and 7 days of DSS (Fig. 7). The expressed reduction of this Th1 cytokine was accompanied by a 75-fold increase in IL-4 mRNA, and a two-fold increase in IL-5 and IL-10 mRNA (Fig. 7). CCR2-deficient mice also had one-fifth as much IFN-γ mRNA expression as wild-type animals, a level comparable to that observed in CCR5-deficient mice. The level of IL-4 mRNA was much less than that of CCR5-deficient mice, but it was still 3-fold greater than that of wild-type animals. IL-10 mRNA expression in CCR2-deficient mice was 9-fold greater than in wild-type mice and over 4-fold more than in CCR5-deficient mice (Fig. 7). IL-5 mRNA expression in CCR2-deficient mice was equivalent to that of wild-type mice. All mouse types developed increased IL-1β, TNF-α, and IL-6 mRNA expression upon DSS exposure, although the level of IL-6 was 3-fold less in the CCR2-deficient mice. By contrast, the level of IL-15 mRNA expression remained relatively stable throughout the course of DSS treatment (Fig. 7). Together these data indicate that CCR2 and CCR5 regulate the composition of lamina propria mononuclear cells and determine the development of Th1- vs Th2-type immune responses in the intestinal mucosa.

**CCR2 and CCR5 regulate the expression of chemokine mRNA within the intestinal mucosa**

Because the migration of leukocytes into the intestinal mucosa may be in part regulated by chemokines, RPAs were used to assess intestinal chemokine mRNA expression in CCR2- and CCR5-deficient mice during DSS-mediated colitis (Fig. 8). RPAs were conducted to detect expression of lymphotactin (LTN), RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, L32, and GAPDH. Densitometry analysis revealed that baseline IP-10 mRNA expression was 2-fold less in both CCR2- and CCR5-deficient animals compared with that in controls. After DSS treatment, CCR5-deficient mice had a 3- to 4-fold increase in RANTES mRNA expression compared with that of wild-type animals. By densitometry, the average expression levels of the other chemokines before and during DSS treatment were comparable to those of the controls (Fig. 8). The strong induction of RANTES may have contributed to the enhanced migration of T cells into the lamina propria of CCR5-deficient mice after challenge with DSS (37).

In CCR2-deficient mice, baseline RANTES expression was 6-fold greater than in wild-type controls. However, after 3 days of DSS, CCR2-deficient mice had no increase in mucosal RANTES expression above baseline or above that of wild-type mice. By day 7, RANTES expression had increased considerably and was now 3-fold higher than that of wild-type mice (Fig. 8). When corrected for GAPDH and L32 mRNA expression, the mRNA expression of LTN, eotaxin, MIP-1β, MIP-1α, MIP-2, and TCA-3 was not different in CCR2-deficient mice and wild-type mice before or during DSS treatment (Fig. 8).

**Discussion**

The analysis of mutant mice lacking a single chemokine ligand or chemokine receptor gene has been useful for determining specific functions of chemokines and their receptors. Nonredundant roles in neutrophil and eosinophil recruitment (38–40), hematopoiesis (38–41), inflammatory response to viral infection (42), neutrophil-mediated host defense, granuloma formation, and cytokine balance (39) have been demonstrated in mice lacking the chemokine receptors CXC chemokine receptor 2 and CCR1 and the chemokine ligands MIP-1, stromal cell-derived factor-1, and eotaxin, respectively. However, alteration of intestinal immune responses in the absence of chemokine or chemokine receptor genes have not been characterized.
TGF-β has been shown to mediate immune suppression through expression of an enhanced influx or propagation of NK1.1^+ CD4^+ T cells of a predominantly Th2 phenotype. The increased percentage of NK1.1^+ CD4^+ T cells in the peripheral blood, the data suggest that they represent cells from the peripheral blood, the data suggest that they represent cells that extravasate into the lamina propria of these mice. NK1.1^+ CD4^+ T cells have been shown to produce IL-4 in vivo promptly upon injection of anti-CD3 and therefore have been speculated to play a role in initiating Th2 cell-mediated immunity. Increased percentage of mouse colitis (46). In these experiments, NK cells inhibited effector CD4^+ CD45RB^hi T cells in an effect that was dependent on perforin (46).

Prior studies have shown that CCR5 is involved in the regulation of Th1 lymphocyte function. CCR5 is expressed almost exclusively on Th1 cells (47). The CCR5 binding chemokine MIP-1α, and to a lesser extent MIP-1β and RANTES, induces selective Th1 lymphocyte migration in transwell chemotaxis assays (48). In addition, CCR5 is highly expressed in the T cell areas of the synovium of patients with rheumatoid arthritis, a predominantly Th1-mediated disease (49). Therefore, absence of CCR5 may result in diminished Th1 responses in the intestinal mucosa, resulting in a Th2-type cytokine profile. Consistent with this hypothesis, CCR5-deficient mice generate high amounts of RANTES, which may signal through CCR3 in the absence of CCR5 (37, 50), preferentially attracting Th2 cells (51–53). Decreased IFN-γ and increased IL-4 mRNA expression in turn may have acted to suppress macrophage activation (54).

In the DSS colitis model, a Th2-type mucosal immune response may result in less severe colonic damage. Th2-inducing agents decrease the severity of colitis in other murine models including trinitrophenyl-kehole limpet hemocyanin-induced colitis in IL-2-deficient mice and trinitrobenzene sulfonic acid colitis (55). Moreover, in mice given DSS, anti-IFN-γ and/or anti-TNF-α Abs significantly attenuate the severity of the colitis (56). In addition, BALB/c mice are characterized by a preferential Th2 immune response and a susceptibility to Leishmania infections (57) and develop less severe colonic damage in response to DSS administration than other mouse strains do (32).

Although the pathogenesis of DSS-mediated colitis is incompletely understood, the recruitment and activation of macrophages seems to have a critical role (43). Prior studies have found that CCR5 plays an important role in macrophage activation. CCR5-deficient mice display a reduced ability to clear Listeria infection and a resistance to LPS-induced endotoxemia (58). CCR5 seems to have less effect on the regulation of macrophage chemotaxis, in that CCR5-deficient mice develop normal glucan-induced granulomas in the lung and liver (58). Consistent with these results, DSS-induced migration of macrophages into the colonic lamina propria of CCR5-deficient mice was similar to that observed in wild-type controls. Therefore, a defect in macrophage activation caused by the absence of CCR5 may have contributed to the reduced colonic damage observed in CCR5-deficient mice.

Clinical signs of colonic injury in response to DSS administration were delayed for the first 3 days of the study in CCR2-deficient mice. By day 7, the clinical severity of the disease was similar to that observed in wild-type mice. This transient period of reduced disease severity coincided with increased CD19 mRNA expression and lamina propria B cells. Because prior studies have suggested a role for B cells in the regulation of experimental colitis (59), it is possible that the increased number of B cells acts to reduce the severity of the colonic inflammation. Further studies of the role of B cells in the development of DSS-mediated colitis will be necessary to verify this hypothesis.

Several studies have shown CCR2 to be critical for the induction of macrophage migration. CCR2-deficient mice show impaired macrophage migration in response to Mycobacterium bovis Ag (60), Schistosoma mansoni Ag (61) in the lung, and in the thio-glycollate model of peritoneal inflammation (62). In contrast to these studies, CCR2-deficient mice administered DSS were observed to develop colonic lamina propria macrophage infiltration.
similar to that seen in wild-type controls. Thus, in this model CCR2 does not play a critical role in macrophage chemotaxis. It is possible that in DSS colitis, the absence of CCR2 is compensated for by other receptor/ligand pairs, or that DSS itself may provide such a strong chemical signal that it may directly overcome the effects of CCR2 deficiency on macrophage chemotaxis. Although infiltration of T cells and macrophages into the colonic lamina propria of CCR2-deficient mice did not differ from that of wild-type mice, CCR2-deficient mice exhibited a shift in cytokine mRNA expression during DSS-induced colitis that was characterized by a reduction in IFN-γ and an increase in IL-10. A defect in IFN-γ production by CCR2-deficient mice has been demonstrated in the Schistosoma Ag-induced model of pulmonary granuloma formation (61). In addition, CCR2-deficient mice show a reduced Th1-type cytokine response after Mycobacterium bovis Ag challenge in the lung (60). This reduced Th1 response is associated with a decrease in IFN-γ production by splenocytes activated by Con A, suggesting that CCR2 may be directly involved in the induction of IFN-γ expression by T cells (60).

The lack of a complete shift to a Th2-type mucosal immune response in CCR2-deficient mice may explain the different clinical and morphological responses to DSS administration in CCR2- and CCR5-deficient mice. However, the diminished IFN-γ and increased IL-10 expression observed in the CCR2-deficient mice may have been sufficient to decrease attenuate the severity of the colonic damage by reducing macrophage inflammatory function (54). The proinflammatory role of IFN-γ in the activation of macrophages in animal models of IBD and in Crohn’s disease has been well established (55, 63). IL-10 is able to reduce macrophage se-

References