GASTROENTEROLOGY 2006;131:1518–1529

Antibody Blockade of CCL25/CCR9 Ameliorates Early but not Late Chronic Murine Ileitis

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Background & Aims: CCL25 mediates the homeostatic recruitment of CCR9-expressing lymphocytes to the small intestine, but the function of this chemokine/receptor pair during chronic small intestinal inflammation has yet to be determined. Furthermore, although clinical trials to evaluate the efficacy of targeting the CCL25/CCR9 axis for the treatment of Crohn's disease are being conducted, preclinical data in animal models of IBD are lacking. **Methods:** In the current studies, we investigated the expression of CCL25 and CCR9 as a function of disease progression in a spontaneous murine model of chronic ileitis (SAMP1/YitFc) using flow cytometry, real-time reverse-transcription polymerase chain reaction, enzyme-linked immunosorbent assay, and immunohistochemistry. In addition, we assessed the functional role of the axis in the overall disease process through therapeutic studies that target the chemokine or the receptor during early and late disease. **Results:** The percentage of CCR9-expressing lymphocytes increased during early disease, accompanied by the appearance of a population of $CCR9^{high}$ lymphocytes, predominantly within $CD8^+$ T cells. Yet different from patients with primary sclerosing cholangitis, the expression of CCL25 remained restricted to the small intestine, even in mice with inflammation of the biliary tree. Neutralization of the receptor or the chemokine attenuated early disease but showed no therapeutic efficacy during the later stages, when overall CCR9 expression decreased and the $CCR9^{high}$ population was absent. **Conclusions:** Our studies show that the role of this chemokine axis is not limited to homeostatic recruitment, as previously believed. However, these molecules appear to play their most crucial role during the early stages of chronic murine ileitis.

The inflammatory bowel diseases (ie, Crohn's disease [CD] and ulcerative colitis) affect distinct intestinal segments. Ulcerative colitis is strictly a colonic disease, whereas CD involves predominantly the small intestine.[1,2] Therefore, the recirculating effector/memory cell pool, which is responsible for perpetuating the chronic inflammatory process, must express a particular repertoire of adhesion molecules and chemokine receptors ("address code") that allows distinction between the small and large bowels.[3] Recirculation to the intestine in general is mediated by the gut-homing integrin $\alpha_4\beta_7$, which interacts with its ligand MAdCAM-1, expressed on the intestinal microvascular endothelium and associated lymphoid tissues.[4] However, the molecules that enable recirculation specifically to the small intestine and may explain the preferential small intestinal localization of CD have just begun to be identified.

The expression of the chemokine CCL25/TECK is restricted to the thymus and small intestine.[5] This expression pattern provides molecular evidence for the potential dichotomization of intestinal trafficking into distinct small and large intestinal compartments.[3,6] CCL25 is produced by small intestinal epithelial cells and serves as a homing beacon for the homeostatic recruitment of lymphocyte subpopulations (eg, immunoglobulin [Ig] A antibody-secreting cells, $CD8\alpha\alpha$ and T cells) to the small intestine.[7–12] However, it is not known whether CCL25 or its receptor CCR9 participate in effector T-cell recruitment to the chronically inflamed small intestine. To that effect, recent reports have shown that patients with small intestinal CD have an increased number of $CCR9^+$ T cells in the peripheral blood.[13] It has also been shown that CCL25 is induced aberrantly in the chronically inflamed hepatic microvasculature of patients with primary sclerosing cholangitis, a chronic immune-mediated disease of the biliary tree frequently associated with inflammatory bowel disease.[14] These findings imply that the role of CCL25/CCR9 may not be limited to homeostatic recruitment, but rather that this chemokine/receptor pair also participates in chronic inflammatory trafficking.

In the current studies, we used the SAMP1/YitFc mouse model, which develops spontaneous chronic inflammation in the terminal ileum (ie, chronic murine ileitis), to investigate the roles of CCL25/CCR9 during lymphocyte trafficking to the chronically inflamed small intestine. First, we determined whether the expression of CCR9 or of its ligand CCL25 is influenced by the development of chronic inflammation. We found that the number of CCR9-expressing cells increased with disease progression, and a $CCR9^{high}$ population was present only in inflamed mice at the peak of the disease (8–10 weeks). Total tissue expression of CCL25 was similarly increased compared with control mice and also as the disease progressed. However, the specific localization of CCL25 to the small intestine was preserved even in mice with inflammatory involvement of the biliary tree. Therapeutic studies probed the functional role of the chemokine/receptor pair in ileitis by targeting the chemokine or its receptor. These studies demonstrate therapeutic efficacy during early but not late disease, suggesting that in the late stages the recruitment process becomes less dependent on this predominantly homeostatic chemokine axis.

Abbreviations used in this paper: FSC, forward scatter; IL, interleukin; mAb, monoclonal antibody; MFI, mean fluorescence index; MLN, mesenteric lymph node; SSC, side scatter.

0016-5085/06/$32.00
doi:10.1053/j.gastro.2006.08.031

Materials and Methods

Mice

The SAMP1/YitFc substrain was generated after more than 30 generations of continuous inbreeding from 2 breeding pairs of SAMP1/Yit mice provided by Dr S. Matsumoto (Yakult Institute for Microbiological Research, Tokyo, Japan). Mice were kept under specific pathogen-free conditions at the University of Virginia.[15] Because most identifiable genes were AKR derived, age-matched AKR/J mice were used as controls.[16] Fecal samples from SAMP1/YitFc mice were consistently negative for *Helicobacter hepaticus*, *Helicobacter bilis*, and other murine *Helicobacter* species, as well as for protozoa and helminthes. All animals were handled according to procedures approved by the institutional committee for animal use. CCR9$^{-/-}$ mice were obtained from Dr Paul Love (National Institute of Child Health and Human Development, National Institutes of Health), integrin $\alpha_E^{-/-}$ mice from Dr Lynn Bry (Brigham & Women's Hospital), and integrin $\beta_7^{-/-}$ mice from Jackson Laboratories (Bar Harbor, ME).[17–20]

Tissue Collection and Histologic Analyses

Mice were anesthetized and killed at the times required by the experimental design. The distal ilea (10 cm) were resected, opened, rinsed of debris, and oriented from distal to proximal over a glass slide using HistoGel (Richard-Allan Scientific, Kalamazoo, MI) to prevent recoiling of intestinal tissue. Tissues were fixed in 10% buffered formalin or Bouin's fixative, embedded in paraffin, cut into 3- to 5-μm sections, and stained with H&E. Histologic assessment of ileal inflammation was performed by a single pathologist in a blinded fashion, using a standardized semi-quantitative scoring system, as described previously.[21]

Generation and Characterization of Rat Anti-CCR9 Monoclonal Antibody (9B-1)

A synthetic peptide comprising the 25 N-terminal amino acids (Met1-Phe25) of murine CCR9 was coupled to keyhole limpet hemocyanin using *N*-succinimidyl-4-(maleimidomethyl)-cyclohexanecarboxylate. Lewis rats were then immunized with the mCCR9-KLH conjugate, and hybridomas were generated using splenocytes and the mouse myeloma X63-AG8.653 cell line. Antibodies were screened for their ability to selectively bind to synthetic mCCR9 peptide and to L1.2-CCR9 cells, which overexpress CCR9 (kindly provided by Dr Michael J. Bevan, Seattle, WA). To determine whether monoclonal antibody (mAb) 9B-1 neutralizes mCCR9 function, 1×10^6 Fluo3-AM–loaded L1.2-CCR9 cells or RBL-C5aR cells[22] were incubated with 25 μg 9B-1 or an isotypic control mAb in 0.5 mL buffer and agonist-induced (50 nmol/L CCL25 or 20 nmol/L C5a, respectively) intracellular calcium mobilization was measured as described.[22,23]

Immunohistochemistry

Terminal ilea and colon from SAMP1/YitFc mice were harvested, snap frozen, and sectioned (5 μm) on a cryostat (Microm HM505N, Walldorf, Germany) and then incubated with goat polyclonal antibody against CCL25 (AF-481) or goat IgG in the presence or absence of recombinant mouse CCL25 (R&D Systems, Minneapolis, MN). Secondary staining was conducted with rabbit anti-goat horseradish peroxidase–labeled antibody (Vector Laboratories, Burlingame, CA). Normal rabbit serum was used to reduce nonspecific binding (Sigma Chemical Co, St Louis, MO).

Lymphocyte Isolation

Mesenteric lymph node (MLN) and spleens were aseptically removed at the time of necropsy. Single-cell suspensions were obtained by gently pressing the MLN or spleen against a 100-μm cell strainer. Spleen red blood cells were lysed by 15-minute incubation in 1× ammonium chloride lysing reagent (BD PharM Lyse; BD Biosciences/PharMingen, San Jose, CA).

Flow Cytometry

Cells from indicated compartments were incubated with phycoerythrin-conjugated rat anti-mouse CCR9 (clone 242503) (R&D Systems) including anti-CD4 (clone GK1.5), CD8a (clone 53-6.7), CD19 (clone 1D3) for gating of lymphocyte populations, anti–L-selectin (clone MEL-14), and anti-integrin β_7 (clone M293; BD Biosciences/PharMingen). Cells were fixed with 2% paraformaldehyde, and 3–4 color analyses were performed using the FACS Calibur System (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Further analyses were performed using FLOWJo software (Tree Star Inc, Ashland, OR).

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from homogenized tissue or cell pellets using the RNeasy Mini Kit (Qiagen, Valencia, CA) and converted to complementary DNA with the GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA), along with random hexamers (0.75 μg of total RNA; final reaction volume, 20 μL). The levels of complementary DNA were quantified by real-time reverse-transcription polymerase chain reaction using an iCycler detection system (Bio-Rad, Hercules, CA). Primers for CCL25 were designed with Beacon Designer software (Premier Biosoft International; Palo Alto, CA) as follows: CCL25 forward 5′-CGTGCTGTGAGATTCTACTTCC-3′, reverse 5′- CTCCTCACGCTTGTACTGTTG-3′. For real-time polymerase chain reaction, 400 nmol/L of each primer and 5% of the volume of the first-strand complementary DNA synthesis were used in a total volume of 25 μL that included iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's directions. Each amplification reaction was performed in triplicate. Thermocycling conditions for the targets were as follows: 95°C for 3 minutes (iTaq DNA polymerase activation) and 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. The expression of 18S RNA was measured in each sample as an endogenous control. The ratio of messenger RNA expression was calculated for each group of mice by the $\Delta\Delta$Ct method (user bulletin no. 2, Applied Biosystems).

CCL25 Protein Extraction and Enzyme-Linked Immunosorbent Assay

Terminal ilea from SAMP/YitFc and AKR mice were collected, snap frozen in liquid nitrogen, and homogenized in T-PER Tissue Protein Extraction Buffer (Pierce Biotechnology, Inc, Rockford, IL) supplemented with a protease inhibitor cocktail (Complete Roche Diagnostics, Penzberg,

Germany) to prevent degradation of proteins during and after homogenization. Homogenates were centrifuged at 12,000 rpm, supernatants were collected, and total protein content was assayed using the Quick Start Bradford kit (Bio-Rad). Tissue homogenates were examined for CCL25 protein levels by a 3-step sandwich enzyme-linked immunosorbent assay as per the manufacturer's instructions (R&D Systems).

T-cell Culture and Cytokine Assay

Lymphocytes were cultured in 96-well round-bottom plates at 10^6 cells/mL in complete medium (RPMI 1640 with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin) with or without anti-CD3ε stimulation (clone 145-2C11, 5 μg/mL; PharMingen, San Diego, CA). Supernatants were collected after 48 hours and stored at −70°C. A bead-based multiplex immunoassay (Upstate, Charlottesville, VA) was used to determine cytokine concentrations from cell culture supernatants. Bound cytokines were detected using a Luminex 100 array reader (Bio-Rad), and results were analyzed using the BioPlex Manager bead array software (Bio-Rad).

Therapeutic Interventions

SAMP1/YitFc mice at 7 and 39 weeks of age were injected intraperitoneally every other day for 3 days with mAbs (200 μg each) against CCR9 (clone 9B-1, rat IgG1) and CCL25 (clone 89818, rat IgG2b; R&D Systems) or with irrelevant corresponding isotype control mAb. Mice were killed 1 week later, 16–18 hours after the last injection.

Statistics

Statistical analyses were performed using the 2-tailed Student *t* test or 2-way analysis of variance. Data are expressed as mean and SEM. Statistical significance was set at $P < .05$.

Results

CCR9 Expression Is Increased in SAMP1/YitFc Mice During Disease Induction

We examined the surface expression of CCR9 on $CD4^+$ and $CD8^+$ cells isolated from the spleen and MLN of SAMP1/YitFc mice and compared it with that of noninflamed AKR control mice at 4–6 weeks of age. Phycoerythrin-labeled isotype antibody (mean fluorescence index [MFI] $< 10^1$, not shown) and lymphocytes isolated from the respective organs of CCR9-deficient mice were used as controls (Figure 1, dashed lines). $CCR9^+$ T cells were identified in MLN and spleen of both control AKR and SAMP1YitFc mice (Figure 1). An increase in the percentage of $CCR9^+$ lymphocytes was observed in SAMP1/YitFc lymphocyte subsets (70% and 68% increase within the $CD8^+$ population for MLN and spleen, respectively) compared with age-matched AKR mice (Figure 1*A* and *B*). The percentage of CCR9-expressing cells within the $CD4^+$ population increased only within the MLN compartment, whereas within the spleen the differences were not significant. The predominant CCR9-expressing population was identified within $CD8^+$ T cells of the MLN, as shown in Figure 1*A*. Furthermore, a $CCR9^{high}$ subpopulation (MFI $> 10^2$) was present in both $CD4^+$ and $CD8^+$ T cells isolated from the MLN of SAMP1/YitFc mice, but not in similarly gated cells from control AKR mice without inflammation (Figure 1*A*).

CCL25 Levels Are Increased Within the Terminal Ileum of SAMP1/YitFc Mice Compared With Age-Matched Noninflamed AKR Mice

To determine whether the presence of inflammation influenced the expression of CCL25 within the terminal ileum, we assayed the tissue protein concentration of CCL25 using an enzyme-linked immunosorbent assay as described in Materials and Methods. CCL25 levels significantly increased in the inflamed ilea of SAMP1/YitFc mice compared with those of noninflamed AKR mice at 4 and 20 weeks of age (Figure 1*C*).

An Inflammation-Dependent $CCR9^{high}$ Population With MFI Similar to That of Thymocytes Was Present Only in Inflamed Mice and Coexpressed Integrin $\alpha_E\beta_7$ and Low Levels of L-selectin

We then compared the fluorescence intensity for CCR9 within the $CD8^+$ subpopulations of cells isolated from the MLN and spleen with that of thymocytes (MFI $>10^2$) and that of similarly gated CCR9-deficient cells (Figure 2*A*). A subpopulation of cells with an MFI similar to that of thymocytes ($CCR9^{high}$) was identified only within the MLN of SAMP1/YitFc mice (Figure 2*A*, SAMP MLN) but not in MLN cells from AKR mice (Figure 1*A*). Further analyses showed that these $CCR9^{high}$ cells expressed low levels of L-selectin (Figure 2*A*) and integrin β_7 and α_E (Figure 2*B*). Because β_7 integrin pairs only with α_E or α_4 integrins, we may conclude that most $CD8/CCR9^+$ T cells coexpress the $\alpha_E\beta_7$ integrin heterodimer.

To begin to understand the role of the $CD8^+$ T-cell subpopulation in the disease process, we magnetically enriched for this population (96% purity) and determined their contribution to the overall cytokine profile after anti-CD3 stimulation compared with that of unfractionated lymphocytes. $CD8^+$ T cells produced comparable levels of interferon gamma and significantly higher levels of tumor necrosis factor α (Figure 2*C*), in support of their important role in the pathogenesis of ileitis.

Restricted Expression of CCL25 to the Small Intestine Is Preserved in Early and Late Chronic Murine Ileitis

It was previously believed that CCL25 expression was restricted to the small intestine and thymus[3,6,8,13,24,25]; however, a recent report has confirmed that in the setting of chronic inflammation, CCL25 may be aberrantly induced within the hepatic microvasculature of patients with primary sclerosing cholangitis.[14] These data suggest that specific inflammatory mediators may induce aberrant CCL25 expression at sites where it is not physiologically expressed. SAMP1/YitFc mice develop inflammatory infiltrates within their intrahepatic and extrahepatic biliary tree (Figure 3*A* and *B*), sharing some features with human primary sclerosing cholangitis (unpublished results). Late in the course of the disease, mice also develop colonic infiltrates (caecitis,[15] not depicted) as well as hypertrophy of the MLN,[26] where most of the $CCR9^+$ T cells are found. We thus inquired whether

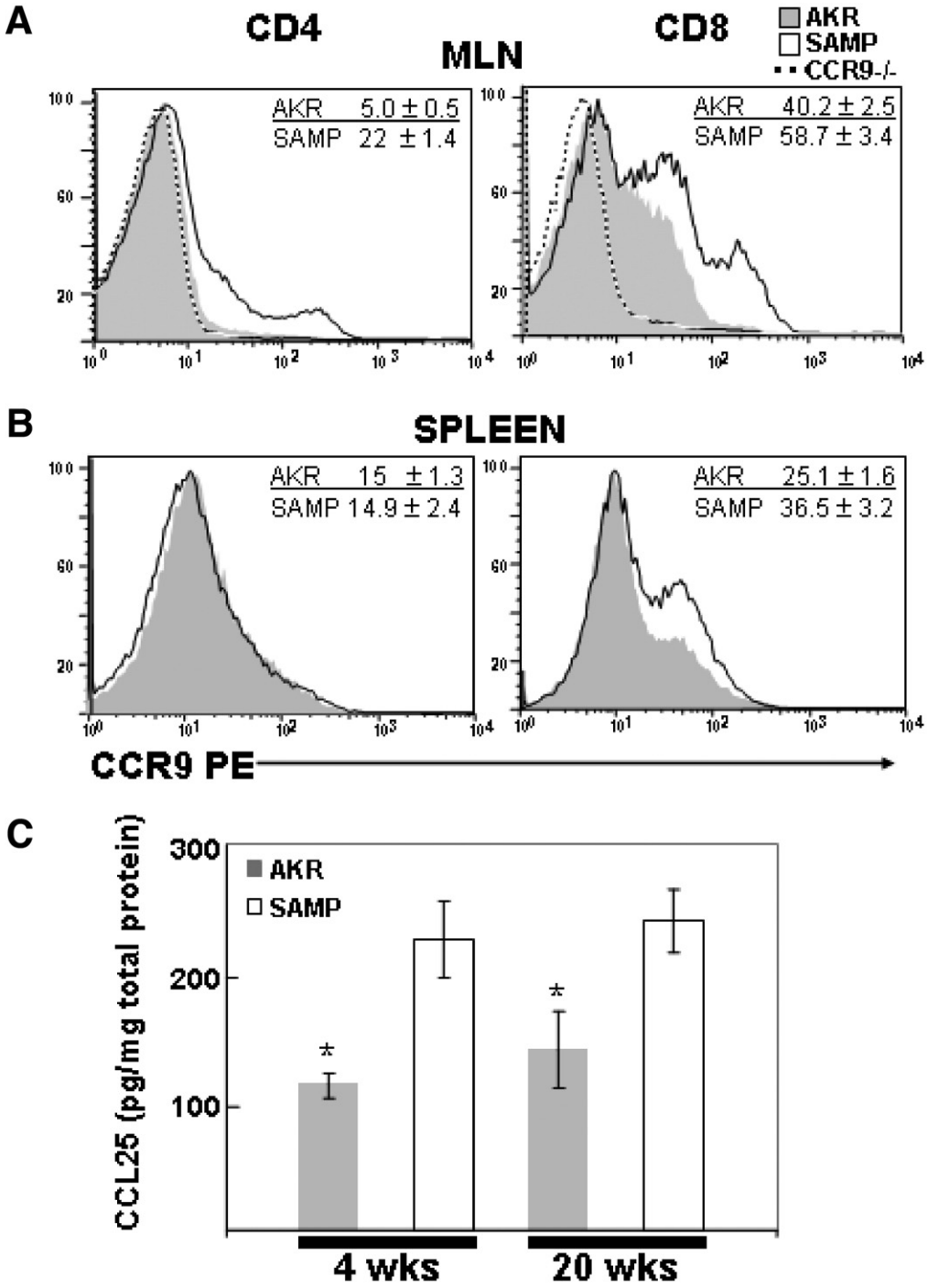

Figure 1. CCR9 expression and CCL25 levels increased in SAMP1/YitFc (SAMP) mice compared with noninflamed age-matched AKR mice (AKR). (*A* and *B*) Lymphocytes isolated from the indicated lymphoid compartments were incubated with CD4, CD8, and phycoerythrin-labeled anti-CCR9 mAb and analyzed by flow cytometry using CCR9-deficient lymphocytes (*dashed line*) from the respective organs and isotype antibody (MFI $< 10^1$, not shown) as controls. Cells were gated on forward scatter (FSC), side scatter (SSC), and indicated populations. Representative data obtained from 3–4 mice at 4–6 weeks of age from each mouse strain run in duplicate. (*C*) CCL25 protein concentration within the terminal ilea was determined by enzyme-linked immunosorbent assay as per methods (n = 6 per strain and time point, $^*P < .05$).

CCL25 might be aberrantly expressed at these sites, enabling recruitment of CCR9-expressing lymphocytes. Tissues from SAMP1/YitFc mice with established ileitis (20 weeks of age) and from age-matched AKR mice were assayed for CCL25 messenger RNA using real-time reverse-transcription polymerase chain reaction. CCL25 expression was detected in the duodenum and ileum but not in the MLN, liver, or colon in mice with or without inflammation (Figure 3*C* and *D*).

The anti-CCR9 mAb 9B1 inhibited CCL25-induced calcium mobilization in L1.2-CCR9 cells. To determine whether

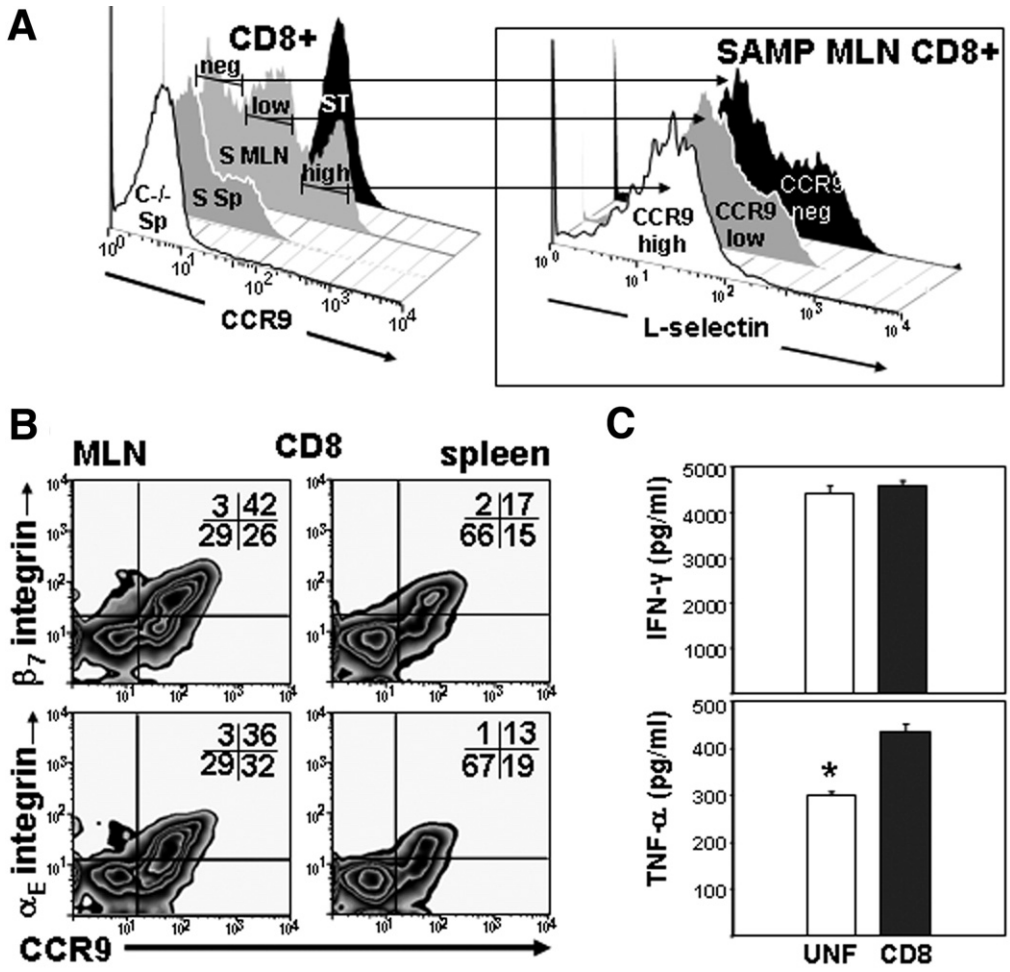

Figure 2. A CD8$^+$/CCR9^{high} population from SAMP1/YitFc MLN coexpressed integrins α_E, β_7, and low L-selectin. (*A* and *B*) Lymphocytes isolated from SAMP thymus (ST), SAMP MLN (S MLN), SAMP spleen (S sp) were incubated with indicated mAb and analyzed by flow cytometry using CCR9$^{-/-}$ splenocytes (C$^{-/-}$ sp), integrins $\beta_7^{-/-}$, or α_E-deficient lymphocytes from the respective organs and isotype antibodies (MFI $< 10^1$) as controls. Cells were gated on FSC, SSC, and CD8. L-selectin expression was determined within indicated subpopulations (*left*) of MLN CD8$^+$ T cells based on their intensity of expression of CCR9 (neg, low, high). Representative data obtained from 3–4 mice run in duplicate. (*C*) Cytokine production by indicated populations of 10-week-old SAMP1/YitFc lymphocyte mice was determined as described in Materials and Methods (cells harvested from 4 mice were cultured individually under anti-CD3 stimulation before [unfractionated, unf] or after magnetic enrichment for CD8$^+$ and assayed independently; *$P < .001$).

mAb 9B-1 specifically neutralized CCR9 function, L1.2-CCR9 cells, which overexpress CCR9, were incubated with 25 μg 9B-1 or an isotypic control mAb (rat IgG2a/κ) and agonist (50 nmol/L CCL25) and the CCL25-induced intracellular calcium mobilization was determined by spectrofluorometry as described.[22,23] RBL-C5aR cells[22] similarly stimulated with 20 nmol/L C5a served as controls (Figure 4*A* and *B*). Only the anti-mCCR9 mAb 9B-1 inhibited CCL25-induced calcium mobilization in L1.2-CCR9 cells but failed to inhibit C5a-induced (20 nmol/L) calcium mobilization in RBL-C5aR cells (Figure 4*A* and *B*).

Immunoblockade of CCL25 and CCR9 Attenuated Induction but Not Maintenance of Chronic Murine Ileitis in SAMP1/YitFc Mice

To evaluate whether CCL25/CCR9 played a role in lymphocyte recruitment during the early or late stages of chronic murine ileitis, we administered 3 doses of function-blocking antibodies against CCR9, CCL25, or their respective isotype control mAbs to 7-week-old and 39-week-old SAMP1/YitFc mice. This treatment regimen effectively attenuated ileitis during prior studies, when the adhesion molecules PSGL-1 (mAb 4RA10) or the α_4 integrins (mAb PS-2) were targeted.[27,28] Terminal ilea were harvested 1 week later, 16 hours after the final injection. The severity of ileitis was determined by a pathologist in a blinded fashion, as previously described.[21] Compared with the isotype-treated controls, 8-week-old SAMP1/YitFc mice treated with anti-CCR9 showed significantly attenuated villous distortion (4.2 ± 0.6 vs 6) and active (4.8 ± 0.5 vs 6), chronic (2.6 ± 0.8 vs 5.1 ± 0.9), and total (11.6 ± 1.5 vs 17 ± 0.9) inflammatory indices (Figure 5*A*). Likewise, anti-CCL25 mAb attenuated villous distortion (2.8 ± 0.4 vs 4.8 ± 0.6) and active (2.8 ± 0.4 vs 4.9 ± 0.6), chronic (2.8 ± 0.5 vs 4.9 ± 0.6), and total (8.4 ± 1.3 vs 13.5 ± 1.8) inflammatory indices compared with control mice that were administered isotype antibodies (Figure 5*B*). Both treatments partially restored intestinal architecture (Figure 5*C*). By contrast, inflammation was unaffected by the

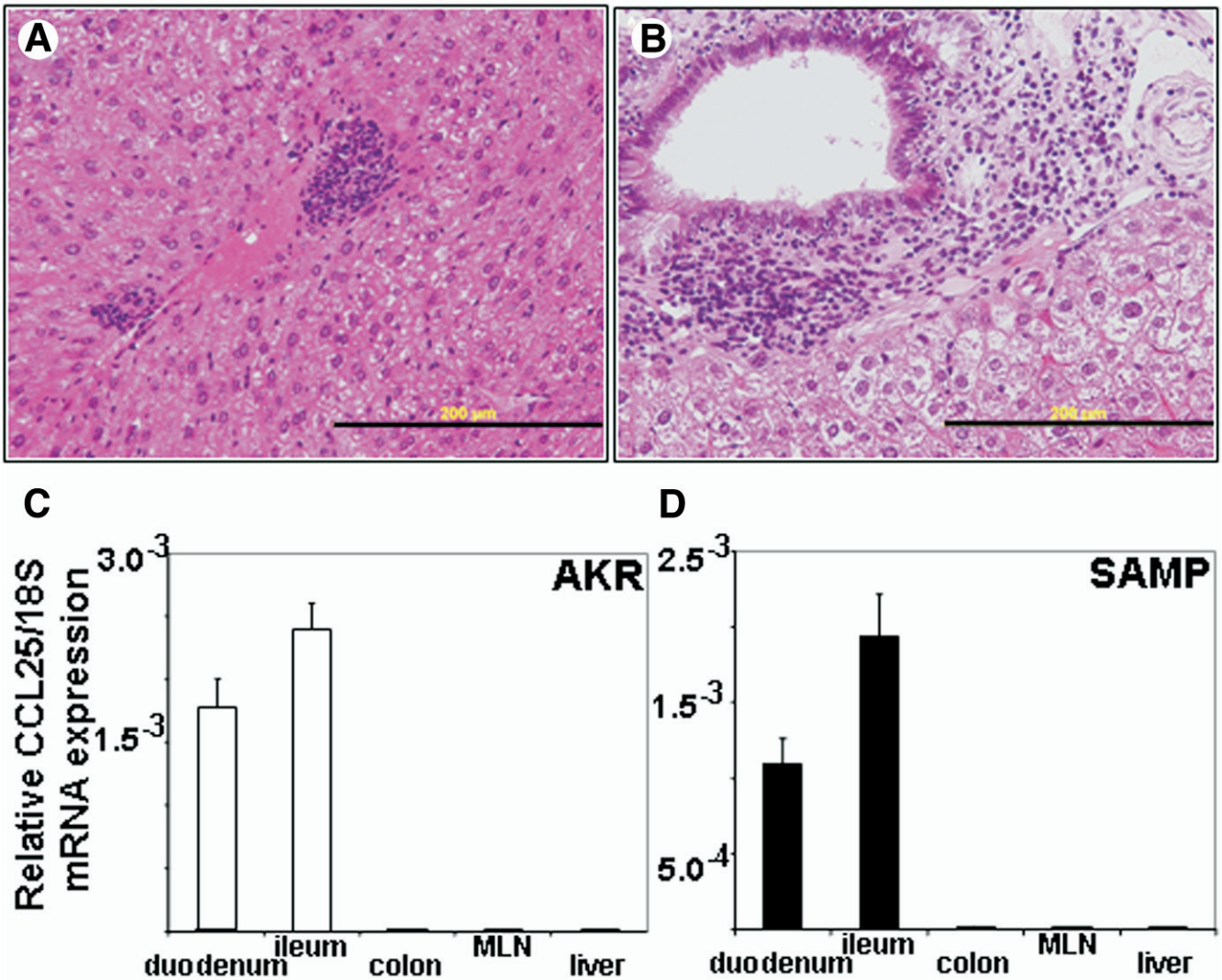

Figure 3. Lymphocytic infiltration of the biliary tree was not mediated by aberrant hepatic CCL25 expression in SAMP1/YitFc mice. (*A* and *B*) Liver tissues were stained with H&E. Representative (*A*) intrahepatic and (*B*) extrahepatic inflammatory infiltrates surrounding the bile ducts are shown (*bar* = 200 μm). (*C* and *D*) Total RNA was extracted from indicated tissues of 20- to 30-week-old AKR or SAMP1/YitFc (SAMP) mice (n = 7/strain), and CCL25 messenger RNA levels were determined using real-time reverse-transcription polymerase chain reaction, normalized to the 18s ribosomal RNA internal control within each sample. Data are expressed as mean ± SEM.

anti-CCR9 or anti-CCL25 mAb during late chronic murine ileitis (40-week-old mice; Figure 5*D*), suggesting that the disease becomes less dependent on this chemokine axis during its maintenance stages.

Expression of CCL25 Increased With Progression of Ileitis

The overall cytokine milieu in SAMP1/Yit mice is different during the early and late stages of the disease. Th1 polarization predominates early, followed by combined Th1/Th2 responses subsequently.[29] We therefore compared the expression of CCL25 in 4-week-old mice with that of 40-week-old mice, using immunohistochemistry and enzyme-linked immunosorbent assay, to determine whether differences in overall cytokine expression may alter the expression of CCL25 and result in the decreased therapeutic efficacy observed during late disease (Figure 6*A–E*). However, this was not the case, and even a further increase in CCL25 protein levels was observed in the ileum but not in the colon during late disease.

Decreased Levels of Lymphocytes Expressing CCR9 in SAMP1/YitFc Mice During Late Disease

We then compared the expression of CCR9 in lymphocytes isolated from the MLN and spleen during early disease (when immunoblockade was efficacious) with that seen in lymphocytes during late disease, when no therapeutic benefit was appreciated (Figure 5). CCR9 expression, which was increased in SAMP1/YitFc $CD4^+$ and $CD8^+$ populations during early disease compared with control mice (Figure 1*A* and *B*), decreased by 40 weeks of age (Figure 7*A* and *B*). In addition, the $CD4^+$ and $CD8^+$ $CCR9^{high}$ subpopulations, present at the peak of the disease, were virtually absent (Figure 7*A*).

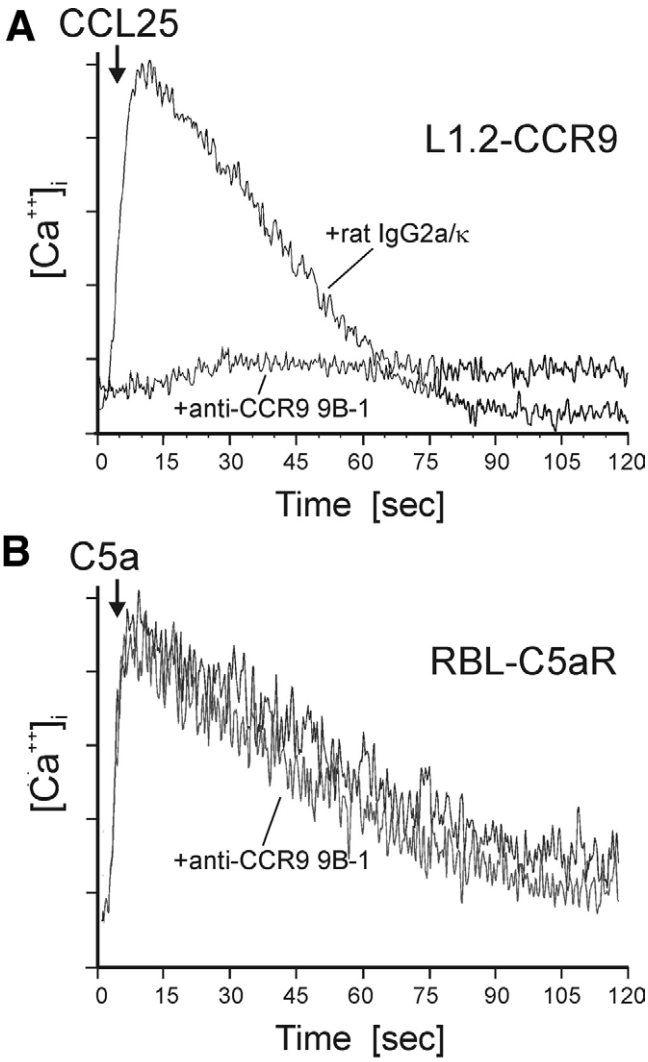

Figure 4. The mAb 9B-1 inhibits CCL25-induced calcium mobilization in L1.2-CCR9 cells. (*A*) Fluo3-AM–loaded L1.2-CCR9 cells were pretreated with 9B-1 (50 μg/mL) or an isotype control mAb (rat IgG2a/κ). Intracellular calcium mobilization within the first 2 minutes after stimulation of cells with 50 nmol/L CCL25 was determined by spectrofluorometry. (*B*) The anti-mCCR9 mAb 9B-1 did not inhibit C5a-induced (20 nmol/L) calcium mobilization from RBL-C5aR cells.

Decreased CCR9 Expression Occurs Despite Increased Production of Endogenous Interleukin-4 During Late Ileitis

The specific signals that trigger the expression of CCR9 and CCL25 above physiologic levels are not known. Interleukin (IL)-4 has been shown to increase the total percentage of cells expressing CCR9 and the surface density of receptor expression when added to cells in vitro.[30] To determine whether CCR9 expression may be regulated by autocrine mechanisms, we harvested lymphocytes from 8- and 40-week-old SAMP1/YitFc mice, cultured them under anti-CD3 stimulation for 48 hours, and assayed their IL-4 production from the culture supernatants. IL-4 production by cells isolated from mice during late disease was significantly higher compared with that of cells isolated from mice during early disease (Figure 7*C*). This pattern is different from that of CCR9 expression, which was higher during early disease. These data suggest that the regulation of CCR9 expression in vivo is more complex than that previously observed in vitro.

Discussion

We investigated whether CCL25/CCR9 play a role in chronic inflammatory trafficking to the small intestine using the SAMP1/YitFc model of chronic ileitis.[15,26] This strain differs from other animal models of inflammatory bowel disease, because the site of inflammation coincides with the restricted location of CCL25 expression.[31] We showed that the levels of CCL25 increased in inflamed small intestine compared with noninflamed mice and that further increases occur as the inflammation progresses. However, in this mouse model, different from prior reports in patients with primary sclerosing cholangitis,[14] we did not observe aberrant extraintestinal expression of CCL25. Lymphocytes expressing the receptor CCR9 underwent early inflammation-driven expansion, predominantly of the $CD8^+$ population, and a population of lymphocytes with an MFI for CCR9 similar to that of thymocytes was detected only in inflamed mice. Conversely, during late disease, the percentage of $CCR9^+$ cells decreased and the $CCR9^{high}$ subpopulation was absent, supporting potential extinction of pathway use.

We showed that the CCR9/CCL25 receptor/ligand pair predominantly plays its role during induction of spontaneous chronic murine ileitis, because neutralizing antibodies attenuate early disease when receptor expression is at its maximum. By contrast, blockade of CCR9 or CCL25 was not sufficient to attenuate inflammation during the late stages of the disease. At this stage, CCR9 is down-regulated and it is likely that other inflammatory/inducible chemokines and chemokine receptors offset the effect of CCL25/CCR9 immunoblockade.

The expression of MAdCAM-1, which also plays a pivotal role in intestinal trafficking, is increased in the chronically inflamed small and large intestine.[32] It is also aberrantly expressed in the chronically inflamed pancreas and liver, where it recruits lymphocytes that express integrin $\alpha_4\beta_7$ to extraintestinal sites.[33,34] Thus, chronic inflammatory signals increase the expression of adhesion molecules/chemokines at effector sites and trigger aberrant expression at sites where they are not physiologically expressed.[35] Although CCL25 has been considered a strictly homeostatic chemokine,[36,37] more recent data have led to the suggestion that its expression may be induced under the appropriate conditions.[14,38]

SAMP1/YitFc mice develop extraintestinal manifestations reminiscent of those seen in human inflammatory bowel disease (ie, pyoderma-like skin involvement, ocular lesions, joint inflammation, and intrahepatic and extrahepatic periportal infiltrates). We hypothesized that some of these extraintestinal manifestations could be mediated by recruitment of small intestinal–specific $CCR9^+$ T cells to these sites, where CCL25 may be aberrantly expressed. However, in this mouse model, CCL25 was not detected outside the small intestine, indicating that its restricted pattern of expression is conserved. Whether this is due to interspecies variation or whether sustained chronic inflammation is required for extraintestinal induction remains to be elucidated.

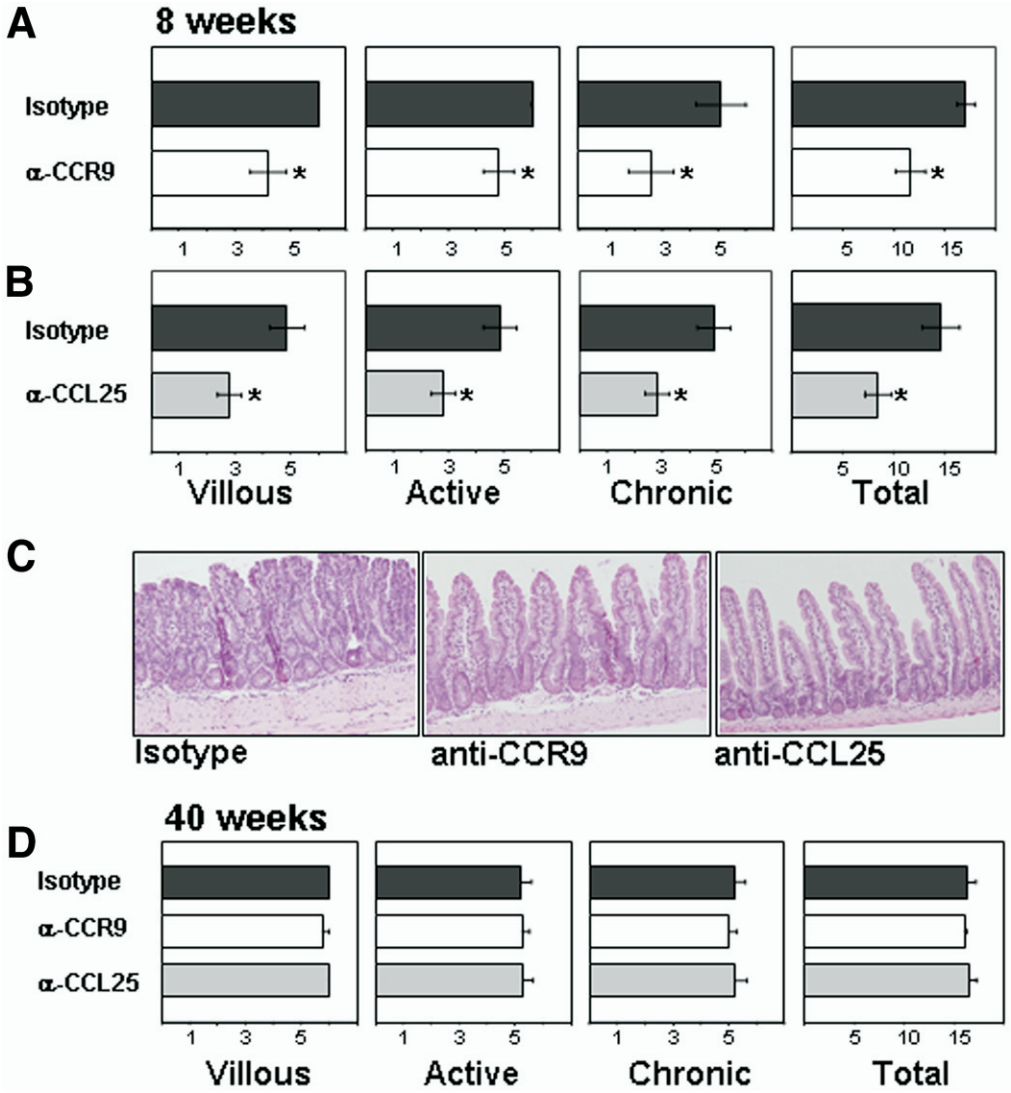

Figure 5. CCR9 and CCL25 immunoblockade attenuated inflammation during induction but not during maintenance of ileitis in SAMP1/YitFc mice. Mice at indicated ages received (*A*) IgG1 isotype mAb (n = 6), anti-CCR9 (α-CCR9, n = 6) or (*B*) IgG2b isotype mAb (n = 8), anti-CCL25 (α-CCL25, n = 8) or (*D*) IgG1 or IgG2b isotypes (pooled data, n = 12) anti-CCR9 (n = 8) or anti-CCL25 (n = 8). Terminal ilea were harvested and the severity of ileitis was determined as described in Materials and Methods (mean ± SEM from 4 independent experiments; $^*P < .05$) (*C*) Effect of indicated treatment on terminal ileal architecture of 8-week-old mice (representative micrographs; H&E; original magnification 20×).

The total percentage of $CCR9^+$ cells and the density of surface expression increased in ileitis, as shown by the presence of a $CCR9^{high}$ subpopulation that was unique to inflamed mice. However, the specific signals that mediate the up-regulation of CCR9 have yet to be identified. Data derived from in vitro studies suggest that IL-4 may be an important factor.[30] Nonetheless, these studies did not predict the in vivo scenario, because CCR9 expression decreased during late disease despite increased local levels[29] and endogenous IL-4 production by the assayed cells.

Our data show that throughout early disease, this predominantly homeostatic chemokine axis (already operational in the ileum) continues to be used as an important recruitment pathway by pathogenic lymphocytes. However, as the disease progresses, CCL25/CCR9 appear to be insufficient to sustain the demand for proinflammatory cells to the effector site, resulting in up-regulation of inducible molecules that respond to inflammatory signals (eg, MAdCAM-1, ICAM-1, and inflammatory chemokines).[39,40] Yet, there does not appear to be a switch from constitutive to inflammatory pathways but rather continuous overlap, without clear demarcation of the replacement of one axis by another. During the later phase, redundant inflammatory pathways are acquired that sustain continuous dysregulated recruitment to the effector site.[27] The recruitment process then becomes autonomous and refractory to regulation, resulting in chronic inflammation, tissue destruction, and loss of function.

A significant number of patients with CD become refractory to standard therapies, and even the most widely used biologic therapeutic agent (ie, infliximab, an antibody against tumor necrosis factor α) is effective in only 70% of patients.[41] Patients with refractory CD often require repeated use of corticosteroids, with potentially devastating side effects, or undergo palliative surgeries to remove strictures,

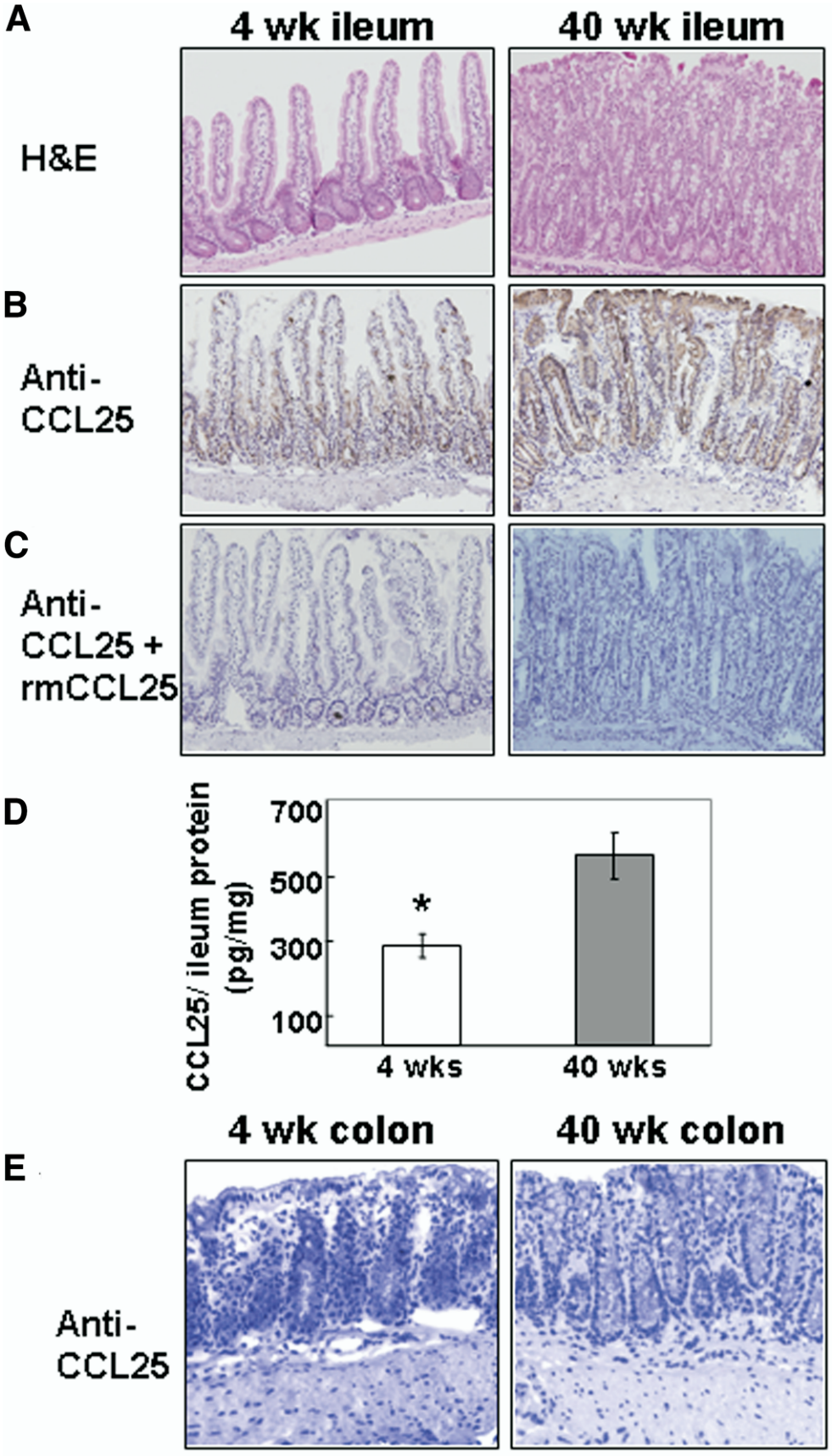

Figure 6. CCL25 expression increased from 4 to 40 weeks of age in SAMP1/YitFc mice ileum. (*A*, *B*, *C*, and *E*) Frozen sections from terminal ilea and colon were harvested from 3–4 mice at the indicated ages and (*A*) stained with H&E or incubated with anti-CCL25 antibody (*C*) with or (*B* and *E*) without recombinant mouse CCL25 (rmCCL25) as described in Materials and Methods. (*D*) CCL25 protein concentration within the terminal ilea of mice at the indicated ages was determined by enzyme-linked immunosorbent assay as described in Materials and Methods (n = 6/time point; $^*P < .05$).

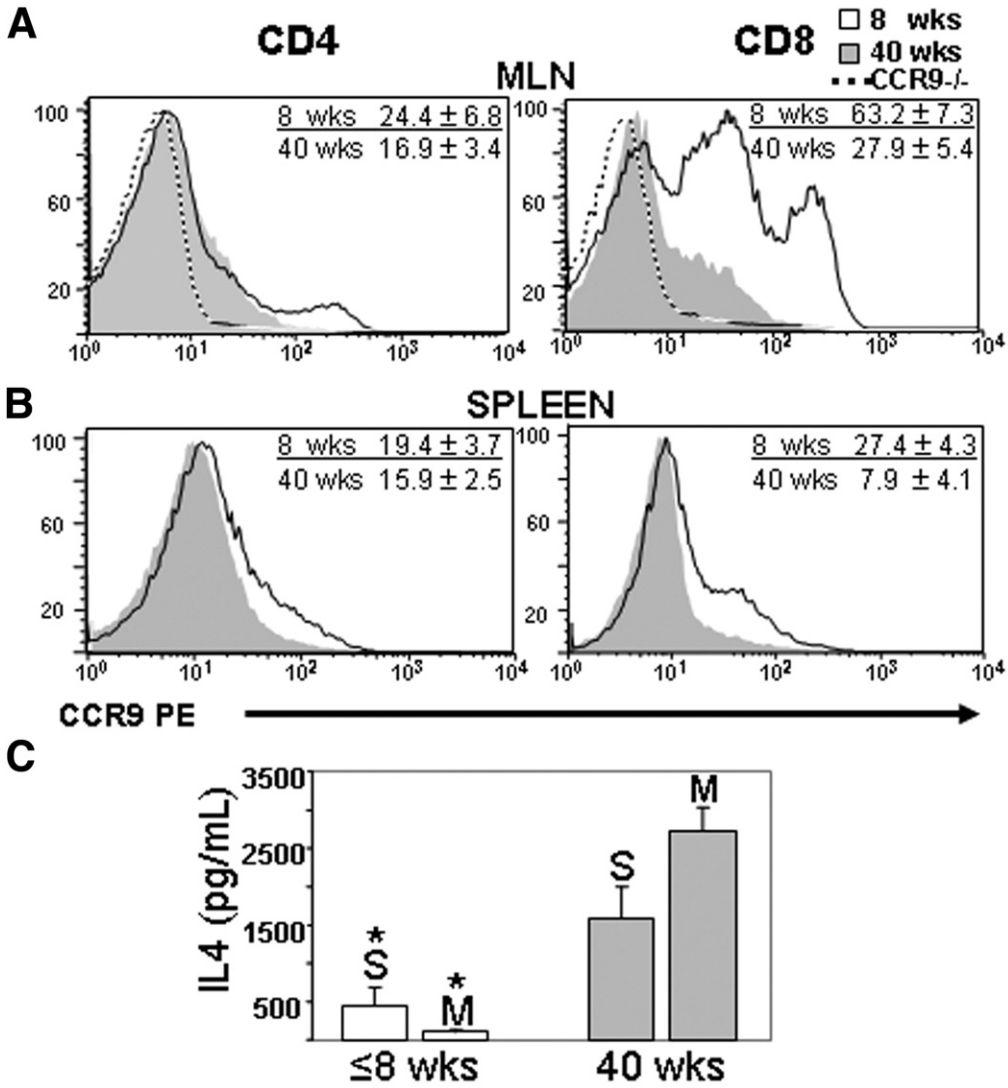

Figure 7. CCR9 expression decreased in 40-week-old SAMP1/YitFc mice lymphocytes compared with 8-week-old mice, despite increased production of IL-4. (*A* and *B*) Lymphocytes isolated from indicated lymphoid organs were incubated with indicated mAb and analyzed by flow cytometry using cells isolated from the respective organs of CCR9$^{-/-}$ mice (*dashed line*) and isotype control antibody (MFI $< 10^1$) as controls, after gating on FSC, SSC, and indicated populations. Representative data were obtained from 3–4 mice at each time point run in duplicate. (*C*) IL-4 production by cells isolated from spleen (S) or MLN (M) of mice at the indicated ages were determined as described in Materials and Methods (cells harvested from 3 or 4 mice were cultured individually under anti-CD3 stimulation and assayed independently [$^*P < .05$] compared with respective organ and time point).

treat abscesses, or correct fistulas. Unfortunately, other cytokine-based therapies, such as the anti-inflammatory cytokine IL-10 and recombinant IL-11, have had limited success.[42,43] Therefore, alternative therapeutic modalities that target other pathways of chronic intestinal inflammation (eg, trafficking) must be evaluated in CD.[44]

To that effect, clinical trials to test the safety and efficacy of the small molecule antagonist Traficet EN, CCX282, which targets the CCL25/CCR9 axis, have been completed (http://www.clinicaltrial.gov/ct/show/NCT00102921) in patients with CD. Yet, to our knowledge, little preclinical information to describe the efficacy of this therapeutic strategy has been made available and none has been published. It is worth noting that, because the chemokine is not known to be expressed in normal or inflamed colon, any data generated from colitic models may be of limited interest; yet models that develop inflammation in the small intestine may provide valuable information. Although caution is needed when extrapolating conclusions drawn from animal models, our data suggest that inclusion of patients with small intestinal CD and with increased circulating CCR9-expressing cells (as previously shown by Papadakis et al[45]) may enhance the response rate of subsequent clinical studies that target this chemokine/receptor axis.

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Received April 28, 2006. Accepted July 26, 2006.

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Supported by US Public Health Service/National Institutes of Health grants DK067254 and DK057880, by a Harold Amos MMFDP grant from the Robert Wood Johnson Foundation (to J.R-N.), by Deutsche Forschungsgemeinschaft grants SFB402/B7 (to M.O.) and GRK335 to (N.I.), and by the Administrative, Morphology, and Immunology Cores of the University of Virginia Digestive Health Research Center (DK 56703).

The authors thank Sharon Hoang, Elizabeth Wiznerowicz, and Oscar Castañón-Cervantes for valuable technical assistance; Dr Rusty Mize for the histologic assessment of intestinal inflammation; Margarita Mishina for the design of real-time reverse-transcription polymerase chain reaction primers; Dr Paul E. Love for providing CCR9$^{-/-}$ mice; Dr Lynn Bry for providing integrin $\alpha_E^{-/-}$ mice; Zheng Wei (Chemocentryx) for providing the anti-CCL25 antibody; Michael Solga and Joanne Lannigan for expert advice with flow cytometry; and Dr Sarah de la Rue for critical review of the manuscript.