The Chemokine Receptor CCR9 Is Required for the T-Cell–Mediated Regulation of Chronic Ileitis in Mice

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BACKGROUND & AIMS: A balance between effector and regulatory T-cell (Treg) responses is required to maintain intestinal homeostasis. To regulate immunity, T cells migrate to the intestine using a combination of adhesion molecules and chemokine receptors. However, it is not known whether the migration pathways of effector cells and Tregs are distinct or shared. We sought to determine whether interaction between the chemokine receptor 9 (CCR9) and its ligand, chemokine ligand 25 (CCL25), allows effectors or Tregs to localize to chronically inflamed small intestine. **METHODS:** By using a mouse model that develops Crohn's-like ileitis (tumor necrosis factor Δadenosine uracyl-rich element [TNFΔARE] mice) we examined the role of CCL25–CCR9 interactions for effector and Treg traffic using flow cytometry, quantitative reverse-transcription polymerase chain reaction, immunohistochemistry, immunoneutralization, and proliferation analyses. **RESULTS:** In TNFΔARE mice, expression of CCL25 and the frequency of CCR9-expressing lymphocytes increased during late-stage disease. In the absence of CCR9, TNFΔARE mice developed exacerbated disease, compared with their CCR9-sufficient counterparts, which coincided with a deficiency of $CD4^+$/$CD25^+$/forkhead box $P3^+$ and $CD8^+$/$CD103^+$ Tregs within the intestinal lamina propria and mesenteric lymph nodes. Furthermore, the $CD8^+$/$CCR9^+$ subset decreased the proliferation of $CD4^+$ T cells in vitro. Administration of a monoclonal antibody against CCR9 to TNFΔARE mice exacerbated ileitis in vivo, confirming the regulatory role of $CD8^+$/$CCR9^+$ cells. **CONCLUSIONS: Signaling of the chemokine CCL25 through its receptor CCR9 induces Tregs to migrate to the intestine. These findings raise concerns about the development of reagents to disrupt this pathway for the treatment of patients with Crohn's disease.**

Keywords: CD; Regulatory T Cells; Inflammation; Immune System.

Crohn's disease (CD) and ulcerative colitis result from an imbalance between effector and regulatory T-cell (Treg) responses.[1] Although agents that target lymphocyte recirculation have proven therapeutic efficacy in CD (eg, natalizumab),[2] none specifically target the traffic of effector T cells while sparing that of Tregs. Further understanding of the mechanisms that mediate effector and Treg recirculation may result in the development of novel therapeutics with better specificity than those in clinical use.

Lymphocyte recirculation to the intestine is mediated by integrin $\alpha_4\beta_7$, which interacts with mucosal addressin cell adhesion molecule-1.[3] However, it is not known how T cells specifically recognize the small intestinal microenvironment to maintain the preferential small-intestinal localization observed in CD. The expression of chemokine ligand 25 (CCL25) (thymus-expressed chemokine) is restricted to the small intestine and thymus, providing a potential molecular mechanism for a trafficking dichotomy into distinct small and large intestinal compartments.[4]

CCL25 attracts cells that express its cognate receptor, chemokine receptor 9 (CCR9). This might be of clinical relevance because patients with small-intestinal CD have increased numbers of $CCR9^+$ T cells in blood.[5] CCL25 is additionally induced in the liver of patients with primary sclerosing cholangitis.[6] Thus, it is conceivable that CCL25 serves as a chemoattractant for effector $CCR9^+$ T cells or, alternatively, CCL25 may recruit Tregs to help dampen dysregulated inflammation. Consistent with this hypothesis, Papadakis et al[7] recently identified a $CCR9^+$ T-cell population that produces interleukin-10.

Preliminary trial data (Prospective Randomized Oral Therapy Evaluation in Crohn's Disease Trial [PROTECT1]) suggest that a small molecule inhibitor of CCR9 (CCX282, Traficet-EN; ChemoCentryx, Mountain View, CA) might have a therapeutic effect in CD.[8] However, CCR9 deficiency had no effect on the severity of ileitis in tumor necrosis factor Δadenosine uracyl-rich element (TNFΔARE) mice,[9] whereas Traficet EN prevented disease onset. Thus, there is disagreement between the existing preclinical and clinical evidence.

The TNFΔARE model develops terminal ileitis, reminiscent of human CD in its histologic features and the pivotal role played by TNF in its pathogenesis.[10] Thus, we

Abbreviations used in this paper: CCR9, chemokine receptor 9; CCL25, chemokine ligand 25; FoxP3, forkhead box P3; LP, lamina propria; mAb, monoclonal antibody; MLN, mesenteric lymph node; TNFΔARE, tumor necrosis factor Δadenosine uracyl-rich element; Treg, regulatory T cell; WT, wild type.

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further explored the role of the CCR9/CCL25 axis for the trafficking of T cells in this model because it is the first chemokine and receptor pair that may account for the lifelong terminal ileal localization shared with the human disease. First, we assessed the expression of CCR9 and CCL25 along the time course of the disease. We then generated CCR9-deficient TNFΔARE mice and assessed the severity of ileitis, noticing increased severity, when compared with CCR9-sufficient TNFΔARE mice. Thus, we hypothesized that Tregs might be more dependent on CCL25/CCR9 than effectors for homing into small bowel. To further explore this possibility we assessed the frequency of Tregs in CCR9-sufficient and CCR9-deficient mice and investigated a potential role for CCR9-expressing $CD8^+$ T cells in the regulation of Crohn's-like ileitis. Finally, the effect of CCR9 immunoneutralization on disease severity was evaluated.

Materials and Methods

Mice

The B6.129S-Tnf^{tm2Gkl}/Jarn strain was generated as previously described,[11] back-crossed to C57BL6/J for more than 30 generations, and kept under specific pathogerm-free conditions. Experimental animals were either heterozygous for the ΔARE mutation or homozygous wild-type (WT), which served as noninflamed controls. CCR9-deficient TNFΔARE mice were generated from CCR9-deficient mice[12] by mating TNFΔARE males to CCR9-/- females on a C57BL6/J background for 2 or more generations. CCR9 deficiency was confirmed by genotyping the progeny and by flow cytometry on isolated T cells. Fecal samples were negative for Helicobacter, protozoa, and helminthes. All animals were handled according to procedures approved by the Institutional Committee for Animal Use.

Tissue Collection and Histologic Analyses

The distal ilea (10 cm) were harvested and histologic assessment of ileal inflammation was performed by a single pathologist (P.J.) in a blinded fashion, using a semiquantitative scoring system, as described.[13]

Real-Time Reverse-Transcription Polymerase Chain Reaction

CCL25 transcript quantification was performed using TaqMan assays (Mm00436443_m1; Applied Biosystems, Foster City, CA) using an ABI PRISM 7300 Sequence Detection System (ABI, Carlsbad, CA). Each reaction was performed in duplicate. Thermocycling conditions were as per the manufacturer's instructions (TaqMan Reagents, product insert). The expression of 18s (Hs99999901-s1; Applied Biosystems) was measured as an endogenous control. The ratio of messenger RNA (mRNA) expression was calculated by the ΔΔCt method (user bulletin no. 2; Applied Biosystems).

Immunohistochemistry

Terminal ilea were snap-frozen, sectioned (5 μm), fixed with acetone, and incubated with goat polyclonal anti-CCL25 (AF-481; R&D Systems, Minneapolis, MN) or goat immunoglobulin (Ig)G, with or without recombinant mouse CCL25 (R&D Systems). Rabbit anti-goat horseradish-peroxidase–labeled antibody was used as secondary antibody (Vector Laboratories, Burlingame, CA). Normal rabbit sera was used to reduce nonspecific binding (Sigma Chemical Co, St Louis, MO).

Lymphocyte Isolation

Splenocytes, mesenteric lymph node (MLN), and lamina propria mononuclear cells were isolated as previously described.[14]

Flow Cytometry and Intracellular Staining

Cells were incubated with fluorescent rat or hamster anti-mouse antibodies with the following specificities: CD4 (GK1.5, RM4-5), CD8 (53-6.7), CD19 (6D5), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD103 (2E7) (BD Biosciences, San Jose, CA), forkhead box P3 (FoxP3) (FJK-16s) (e-Biosciences, San Diego CA), and CCR9 (242503) (R&D Systems), or CW-1.2 (e-Biosciences). FoxP3 staining was performed as per the manufacturer's instructions (e-Biosciences). Additional controls included cells isolated from CCR9-deficient mice whereas positive controls were collected from WT thymi. Cells were washed and fixed with 2% paraformaldehyde and 7 or 8 color analyses were performed using the BD FACSCanto II (BD Biosciences). Cell sorting was performed using the Moflo XDP (Beckman Coulter, Inc, Fullerton, CA). Further analyses were performed using FLOWJo software (Tree Star, Inc, Ashland, OR).

In Vitro T-Cell Proliferation Assay

Splenic and MLN $CD4^+$ and $CD8^+$/$CCR9^+$T cells were isolated from TNFΔARE mice, $CD19^+$ cells were depleted using anti-mouse CD19 microbeads (Miltenyi Biotec, Auburn, CA). The flow-through was stained with anti-mouse CD4, anti-mouse CD8, and anti-mouse CCR9. $CD4^+$ and $CD8^+$/$CCR9^+$subsets were separated using a Moflo XDP fluorescence-activated cell sorter (Beckman Coulter). $CD4^+$T cells were stained with carboxyfluorescein succinimidyl ester (0.5 μmol/L) and 1×10^6 cells/well were stimulated with anti-CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA) and cultured for 5 days alone or with $CD8^+$/$CCR9^+$ T cells (≥97% pure). Cells were stained with anti-CD4 and analyzed by flow cytometry. Cell proliferation was quantified using the cell proliferation analysis function of FlowJo software (Tree Star, Inc).

Treatment Study

TNFΔARE mice received 3 intraperitoneal doses (200 μg) of anti-CCR9 monoclonal antibody (mAb)

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