

A Functional Role for CCR6 on Proallergic T Cells in the Gastrointestinal Tract

ANA BELÉN BLÁZQUEZ,* ADINA KAY KNIGHT,* HOHETEBERHAN GETACHEW,* JONATHAN S. BROMBERG,[‡] SERGIO A. LIRA,[‡] LLOYD MAYER,[‡] and M. CECILIA BERIN*[‡]

*Jaffe Food Allergy Institute and [‡]Immunology Institute, Mount Sinai School of Medicine, New York, New York

BACKGROUND & AIMS: CCL20 is a chemokine that regulates the homeostatic and inflammatory trafficking of leukocytes to the small intestine and regulates the development of the gastrointestinal lymphoid architecture. T cells expressing T helper cell (Th) 2 cytokines are critical for experimental food allergy, and we hypothesized that CCL20 is involved in the localization of these cells to the gut. **METHODS:** We evaluated the role of CCR6 in allergic diarrhea induced by sensitization and oral challenge with ovalbumin (OVA) using CCR6^{+/+} and CCR6^{-/-} mice. **RESULTS:** CCR6^{-/-} mice were protected from OVA-induced diarrhea but surprisingly were not impaired in mastocytosis or allergen-specific immunoglobulin E. CCR6^{-/-} mice were also protected from T cell-mediated diarrhea induced by anti-CD3 antibody. Allergic diarrhea was associated with an increased expression of Th2 cytokines within the intestinal mucosa that was significantly reduced in CCR6^{-/-} mice. Inhibition of lymphocyte homing by treatment with FTY720 did not impair allergic diarrhea, indicating that reactivation of T cells could occur locally within the small intestine. Finally, T-cell transfer studies demonstrated that CCR6 was required both on the transferred T cells and in the recipient mouse to manifest allergic disease in the gastrointestinal tract. **CONCLUSIONS:** These studies highlight a mast cell- and immunoglobulin E-independent role for CCR6-bearing T cells in the pathogenesis of gastrointestinal allergic disease.

Food allergic reactions are initiated by allergen cross-linking of immunoglobulin (Ig) E bound to intestinal mast cells, mast cell degranulation, and release of mast cell products that act directly on the intestinal epithelium, or indirectly through enteric nerves, to induce changes in intestinal ion secretion and barrier function.^{1,2} Mice systemically sensitized to ovalbumin (OVA) and repeatedly orally challenged with OVA develop a mast cell and IgE-dependent acute diarrhea associated with a T helper cell (Th) 2 inflammation in the small intestine.³ We have previously shown that mesenteric lymph node (MLN) CD4⁺ T cells from mice with allergic diarrhea can transfer allergic disease to naïve mice,⁴ highlighting the role of T lymphocytes in an IgE- and mast

cell-driven model system. Forbes et al recently showed that transgenic expression of the single T-cell cytokine interleukin (IL)-9 within the intestine could lead to a local mastocytosis and diarrhea replicating experimental models of allergen-driven experimental food allergy.⁵ Inhibition of IL-4 and IL-13 given early during repeated oral allergen challenge can also inhibit allergic symptoms.⁶ Allergen-specific T cells producing Th2 cytokines have been shown to be present in the intestinal mucosa of human subjects with food allergic diseases,^{7,8} including non-IgE-mediated food allergic disease. The factors responsible for recruitment of pathogenic T cells to the intestine in food allergic disorders are not known, and we hypothesized that mucosally expressed chemokines would be critical for the homing of T cells to the gut in experimental food allergy.

CCL20 (MIP-3α) is a chemokine that is expressed by gastrointestinal epithelium,⁹ is regulated by nuclear factor (NF)-κB,¹⁰ and is overexpressed in inflammatory bowel disease.^{10,11} We have recently shown that ligation of the low-affinity IgE receptor on intestinal epithelial cells leads to release of functional CCL20.¹² Expression of CCL20 is highest in the follicle-associated epithelium of the Peyer's patch,^{13,14} but it is also expressed by mouse and human enterocytes.⁹ The cognate receptor for CCL20 is CCR6 and is expressed on memory T cells, B lymphocytes, and dendritic cells (DCs). CCR6^{-/-} mice have impaired mucosal but not systemic humoral responses to immunization and rotavirus infection.¹⁵ In addition, CCR6^{-/-} mice have alterations in the architecture of organized lymphoid tissue in the gastrointestinal tract, including Peyer's patches, isolated lymphoid follicles, and cryptopatches.^{16–18} We hypothesized that this ubiquitous mucosal chemokine would play a role in the homing of T lymphocytes to the gastrointestinal tract in

Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; Ig, immunoglobulin; IL, interleukin; mRNA, messenger RNA; MLN, mesenteric lymph node; NF, nuclear factor; OVA, ovalbumin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse-transcription; Th, T helper cell; TNF, tumor necrosis factor.

experimental food allergy and tested this using CCR6^{+/+} and CCR6^{-/-} mice.

Materials and Methods

Allergic Diarrhea

CCR6^{-/-} mice were generated previously by S. Lira,¹⁵ backcrossed for 10 generations to the Balb/c background, and maintained in specific pathogen-free conditions. Balb/c mice were purchased from the National Cancer Institute (Frederick, MD). All experiments were performed with the approval of the Institutional Animal Care and Use Committee. Female age-matched CCR6^{+/+} and CCR6^{-/-} mice (5–8 weeks of age) were sensitized to OVA as previously described.⁴ Symptoms were monitored for 30 minutes after feeding, and diarrhea was marked as present or absent.

Cholera Toxin and CD3-Induced Diarrhea

Cholera toxin-induced diarrhea was generated by administering 50 μ g of cholera toxin (List Biological Laboratories, Campbell, CA) by intragastric gavage to CCR6^{+/+} or CCR6^{-/-} mice. After 3 hours, mice were killed, and 3 intestinal loops/mouse prepared for weight/length and wet/dry weight ratios. Intestinal segments were weighed, dried in an oven (80°C, 48 hours), and reweighed. Wet/dry weight ratios are reflective of fluid secretion and edema.

T cell-mediated diarrhea was induced as previously described.^{19,20} Briefly, mice were administered 0.2 mg of anti-CD3 (eBioscience, San Diego, CA; functional grade) by intraperitoneal injection or phosphate-buffered saline (PBS) as control. After 2 hours, mice were killed, and intestinal loops were prepared as above.

Cell Culture

MLN were isolated, and cells were cultured with media alone or OVA (100 μ g/mL) for 72 hours. Supernatants were collected for cytokine determination by enzyme-linked immunosorbent assay (ELISA) (eBioscience).

Adoptive Transfer

Donor mice were sensitized and fed with OVA to induce allergic diarrhea. MLN cells were isolated and restimulated with OVA as above prior to washing and intravenous transfer to naïve mice (4×10^6 cells/mouse). For CD4⁺ T-cell transfer, CD4⁺ T cells were negatively selected (StemCell, Vancouver, BC, Canada) prior to transfer of 2×10^6 cells/mouse. Recipient mice were orally challenged with OVA every second day starting 24 hours after transfer, and diarrhea symptoms were monitored as above.

Alternatively, 3×10^6 CD4⁺ T cells from DO11.10 mice were carboxyfluorescein succinimidyl ester (CFSE)-labeled and transferred to naïve CCR6^{+/+} or CCR6^{-/-} mice. After 24 hours, recipient mice were fed with 50 mg

of OVA. After 72 hours, cells in the MLN were isolated for analysis of proliferation.

FTY720 Administration

FTY720 was a gift from V. Brinkmann (Novartis Pharma AG, Basel, Switzerland) to J.S.B. To block lymphocyte egress from the lymph nodes, mice were treated orally with 0.3 mg/kg FTY720 daily beginning 1 day prior to initiation of oral OVA challenges and daily thereafter. Efficacy was verified by measurement of circulating CD4⁺ and CD8⁺ T cells.

Reverse-Transcription Polymerase Chain Reaction

RNA was isolated from jejunum, and real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed as previously described.⁴

Histology and Immunostaining

Jejunal segments were fixed in formalin and paraffin. Jejunal mast cells were detected by chloroacetate esterase staining according to published protocols of mast cell detection.²¹

IgE Measurement

OVA-specific IgE in serum and intestinal lavage fluid was measured by capture ELISA as previously described.⁴

Results

The CCL20-CCR6 Chemokine Axis Is Necessary for Gastrointestinal Allergic Symptoms

In a screen of local chemokine expression during the course of allergic diarrhea, CCL20 messenger RNA (mRNA) expression was significantly up-regulated compared with control mice that were sensitized but unfed with OVA (Figure 1A). Immunostaining for CCL20 showed a diffuse positive expression pattern in enterocytes and an intense immunoreactivity in M cells as has previously been reported¹⁴ (Figure 1B). CCL20 is known to be an important chemokine for mucosal lymphocyte homeostasis, and, to test the role of this chemokine in allergic diarrhea, we used mice that were genetically deficient for the receptor CCR6. Wild-type mice sensitized to OVA developed acute self-limiting symptoms beginning after the third oral challenge with OVA (>50% of mice had symptoms by the third feed and approximately 90% by the fifth feed). In contrast, CCR6^{-/-} mice were significantly protected from the onset of allergic diarrhea (Figure 1C).

CCR6-Independent IgE and Mast Cell Responses

It has previously been demonstrated³ that diarrhea symptoms in this model are dependent on mast cells

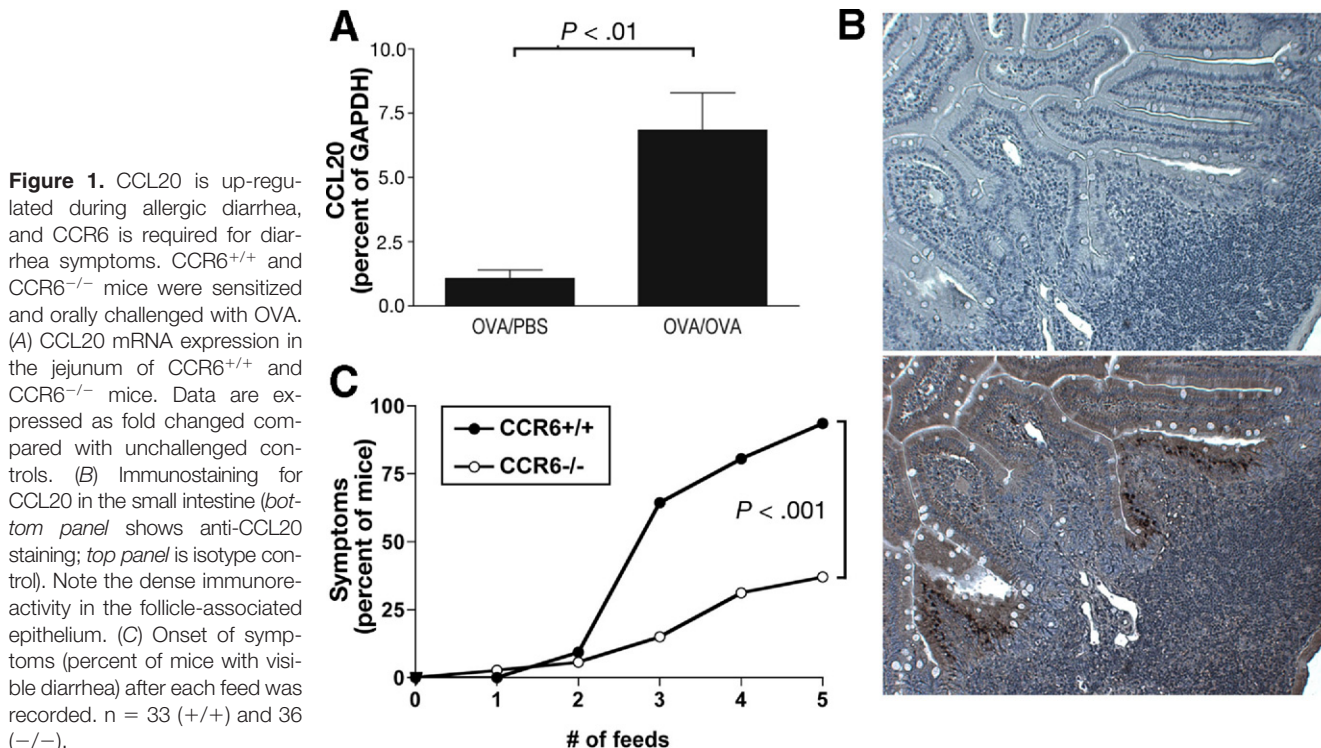


Figure 1. CCL20 is up-regulated during allergic diarrhea, and CCR6 is required for diarrhea symptoms. CCR6^{+/+} and CCR6^{-/-} mice were sensitized and orally challenged with OVA. (A) CCL20 mRNA expression in the jejunum of CCR6^{+/+} and CCR6^{-/-} mice. Data are expressed as fold changed compared with unchallenged controls. (B) Immunostaining for CCL20 in the small intestine (*bottom panel* shows anti-CCL20 staining; *top panel* is isotype control). Note the dense immunoreactivity in the follicle-associated epithelium. (C) Onset of symptoms (percent of mice with visible diarrhea) after each feed was recorded. $n = 33$ (+/+) and 36 (-/-).

and IgE. Surprisingly, we observed that neither gastrointestinal mast cells nor serum IgE were significantly impaired in CCR6^{-/-} mice (Figure 2). Serum OVA-specific IgE was measured by ELISA in mice killed after 0 to 4 oral challenges (every second day) with OVA. OVA-specific IgE was significantly boosted after the third oral challenge and was not significantly different between CCR6^{+/+} and CCR6^{-/-} mice. This was also true for OVA-specific IgE detected in intestinal lavage (data not shown). A significant increase in tissue mast cells was also observed, beginning after the third OVA challenge. CCR6^{-/-} mice were not impaired in tissue mast cells (Figure 2B). This was confirmed by mRNA expression of the mast cell protease MMCP-1, which was significantly up-regulated in both CCR6^{+/+} and CCR6^{-/-} mice (Figure 2C). Mast cell numbers were further confirmed by flow cytometry (Supplementary Figure 1). Therefore, we conclude that CCR6 deficiency does not prevent the onset of allergic diarrhea by interfering with either tissue mast cells or allergen-specific IgE.

CCR6 Is Required for T Cell-Mediated, but Not Toxin-Driven, Diarrhea

To determine whether CCR6^{-/-} mice have an impairment in their epithelial secretion mechanisms, we tested 2 other models of acute diarrhea. Mice were orally administered 50 μ g cholera toxin, and, after 3 hours, ligated loops were prepared to quantify fluid secretion by wet/dry weights. CCR6^{+/+} mice responded to cholera toxin with a significant fluid loss throughout the small intestine, and this was independent of CCR6 (Figure 3).

We next used a T cell-driven model of diarrhea induced by administration of anti-CD3 antibodies. Naïve CCR6^{+/+} and CCR6^{-/-} mice were injected with 0.2 mg of anti-CD3, and, 2 hours later, intestinal loops were prepared to assess fluid secretion in the small intestine (Figure 3). Administration of 0.2 mg of anti-CD3 antibody to CCR6^{+/+} mice led to fluid secretion in the proximal and mid-small intestine by 2 hours postinjection in CCR6^{+/+} mice. In contrast, CCR6^{-/-} mice were protected from CD3-induced diarrhea, similar to our findings with allergic diarrhea. We next isolated cells from the lamina propria of CCR6^{+/+} and CCR6^{-/-} mice. There was no difference in either the total number of lamina propria cells or the percentage of CD4⁺ T cells (Supplementary Figure 2). When isolated cells from the lamina propria were stimulated *in vitro* with anti-CD2/CD28 antibodies, cytokine secretion was also not significantly different in cells isolated from CCR6^{-/-} mice (Supplementary Figure 2). Therefore, CCR6 deficiency does not appear to be associated with a change in the constitutive homing of effector T cells into the small intestinal mucosa.

CCR6 Is Required for Local Th2 Cytokine Expression in the Jejunum

Because T cell-driven diarrhea was CCR6-dependent, we next assessed cytokine expression in the jejunum of mice with allergic diarrhea. CCR6^{+/+} and CCR6^{-/-} mice were sensitized to OVA and given 4 oral challenges of OVA or were unfed as controls. Real-time PCR was performed for the cytokines IL-4, IL-13, and IL-10 because we and others have previously shown that these

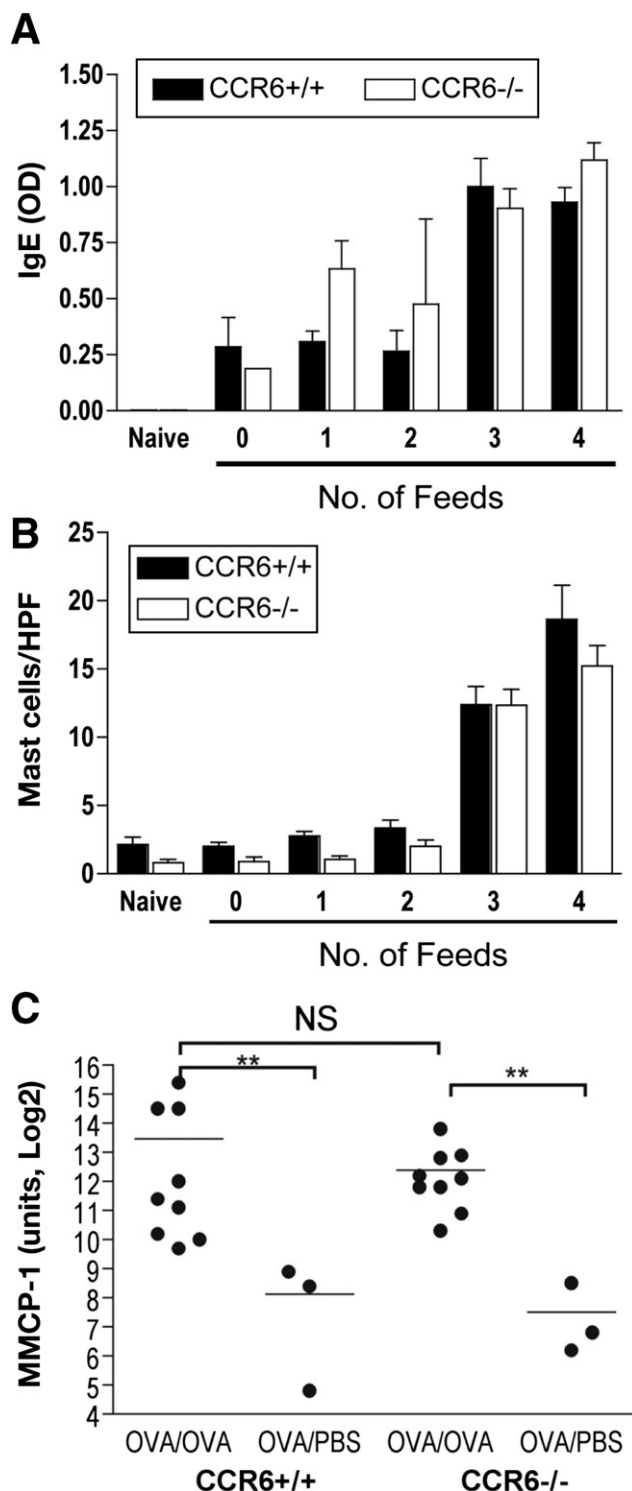


Figure 2. Allergen-induced jejunal mast cells and serum IgE are CCR6 independent. CCR6^{+/+} and CCR6^{-/-} mice were sensitized and with OVA. Four mice were killed after each feed of OVA (from 0 to 4). *Top panel:* Serum OVA-specific IgE. *Middle panel:* Jejunum mast cell counts per high-power field (HPF). *Bottom panel:* MMCP-1 mRNA expression in jejunum from mice killed after 4 (OVA/OVA) or 0 (OVA/PBS) feeds of OVA. ***P* < .01; ns = not significant.

cytokines are locally up-regulated during allergic diarrhea.^{3,4} CCR6^{+/+} mice had a significant up-regulation of IL-4, IL-13, and IL-10 that was significantly reduced in CCR6^{-/-} mice (Figure 4). Interferon (IFN)- γ , IL-17, tumor necrosis factor (TNF)- α , and IL-18 were neither significantly up-regulated during allergic diarrhea nor significantly different between CCR6^{+/+} and CCR6^{-/-} mice (Supplementary Figure 3). This was observed throughout the OVA challenge period as shown by time course of cytokine expression. Thus, the characteristic Th2-skewed inflammation in the jejunum of mice with allergic diarrhea was impaired by CCR6 deficiency.

CCR6 Does Not Influence Th2 Reactivation in the MLN

We have previously shown that activation of Th2 lymphocytes in the allergic diarrhea model occurs in the

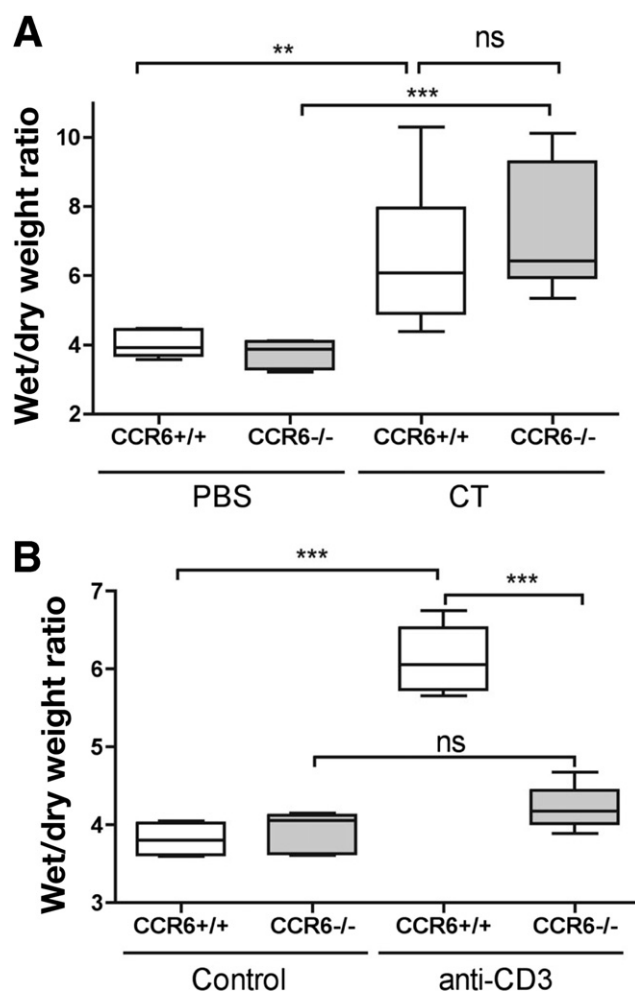


Figure 3. CCR6 is required for T cell-mediated but not toxin-induced diarrhea. (A) CCR6^{+/+} and CCR6^{-/-} mice were fed 50 μ g of cholera toxin or PBS as control. After 3 hours, mice were killed, and ligated loops were prepared from small intestine. Wet/dry weights were calculated as in Materials and Methods section. *n* = 3/group. (B) CCR6^{+/+} and CCR6^{-/-} mice were injected with 0.2 mg of anti-CD3 antibody or left uninjected as control. After 2 hours, ligated loops were prepared as above. *n* = 7/group. ***P* < .01; ****P* < .001; ns = not significant.

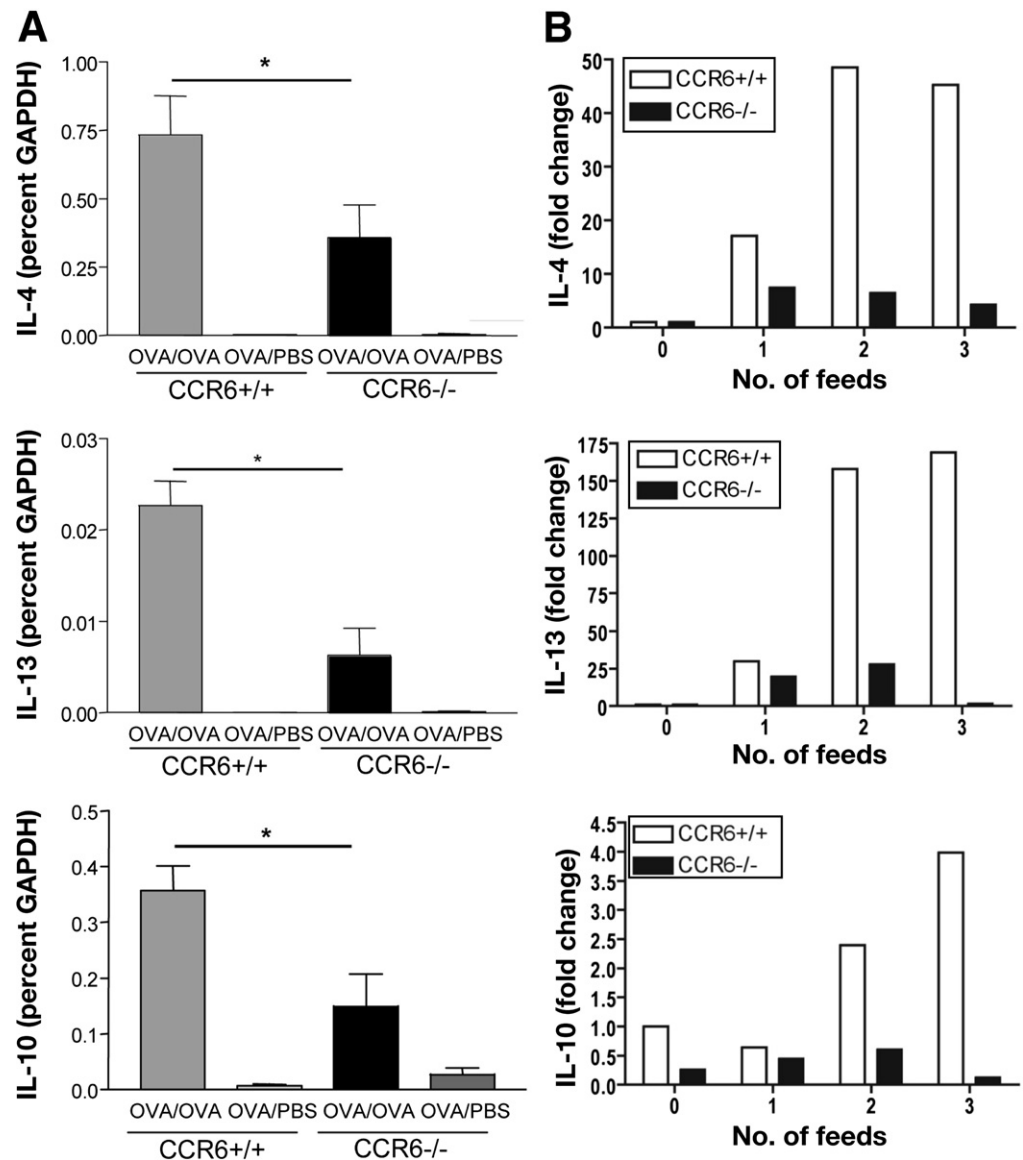


Figure 4. Allergen-induced Th2 cytokine expression in the small intestine is impaired in CCR6^{-/-} mice. *Left panels:* CCR6^{+/+} and CCR6^{-/-} mice were sensitized to OVA and challenged with OVA (OVA/OVA) or PBS (OVA/PBS) as control. Mice were killed within 1–2 hours after symptom onset. (n = 10 samples/group). *Right panels:* Mice were killed after each OVA feeding (4 mice/group). RNA was isolated from jejunum, and RT-PCR for IL-4 (top panel), IL-13 (middle panel), and IL-10 (bottom panel) was performed on individual samples (left panels) or pooled samples from the time course (right panels).

MLN in response to oral OVA challenge.⁴ To test the role of CCR6 in T-cell reactivation in mucosal tissues, we restimulated MLN cells from CCR6^{+/+} and CCR6^{-/-} mice with OVA and determined cytokine output (Figure 5). CCR6^{+/+} mice with allergic diarrhea had a significant increase in OVA-specific IL-4, IL-5, IL-13, and IL-10 secretion that was unimpaired in CCR6^{-/-} mice. Naïve T-cell priming was also assessed by transferring CFSE-labeled DO11.10 CD4⁺ T cells to CCR6^{+/+} and CCR6^{-/-} mice. Feeding of mice resulted in a robust proliferation of DO11.10 cells in the MLN of both CCR6^{+/+} and CCR6^{-/-} mice (Figure 5). Up-regulation of the gut homing molecule $\alpha 4\beta 7$ on T cells activated within the MLN was also unimpaired in CCR6^{-/-} mice (data not shown). Thus, antigen delivery to the lymph node and presentation to T cells are not impaired in CCR6^{-/-} mice. In addition, we performed in vitro antigen presentation assays with purified lamina propria DCs and observed

that DCs from CCR6^{+/+} and CCR6^{-/-} mice were equal in their ability to induce cytokine secretion from responder CD4⁺ T cells (Supplementary Figure 4).

T-Cell Reactivation During Oral Challenge Does Not Require Homing From the Lymph Node

To investigate further the role of T-cell homing in induction of disease, we used the sphingosine 1-phosphate 1 receptor agonist FTY720 that has been shown to inhibit the egress of lymphocytes from lymph nodes, including MLN and Peyer's patches.^{22–24} FTY720 was administered orally to OVA-sensitized mice prior to oral OVA challenge. Efficacy of FTY720 was verified by measuring circulating CD4⁺ and CD8⁺ T cells (Figure 6). FTY720 resulted in an almost complete elimination of circulating CD4⁺ and CD8⁺ T cells, although there was no major effect of FTY720 on the resident CD4⁺ T-cell

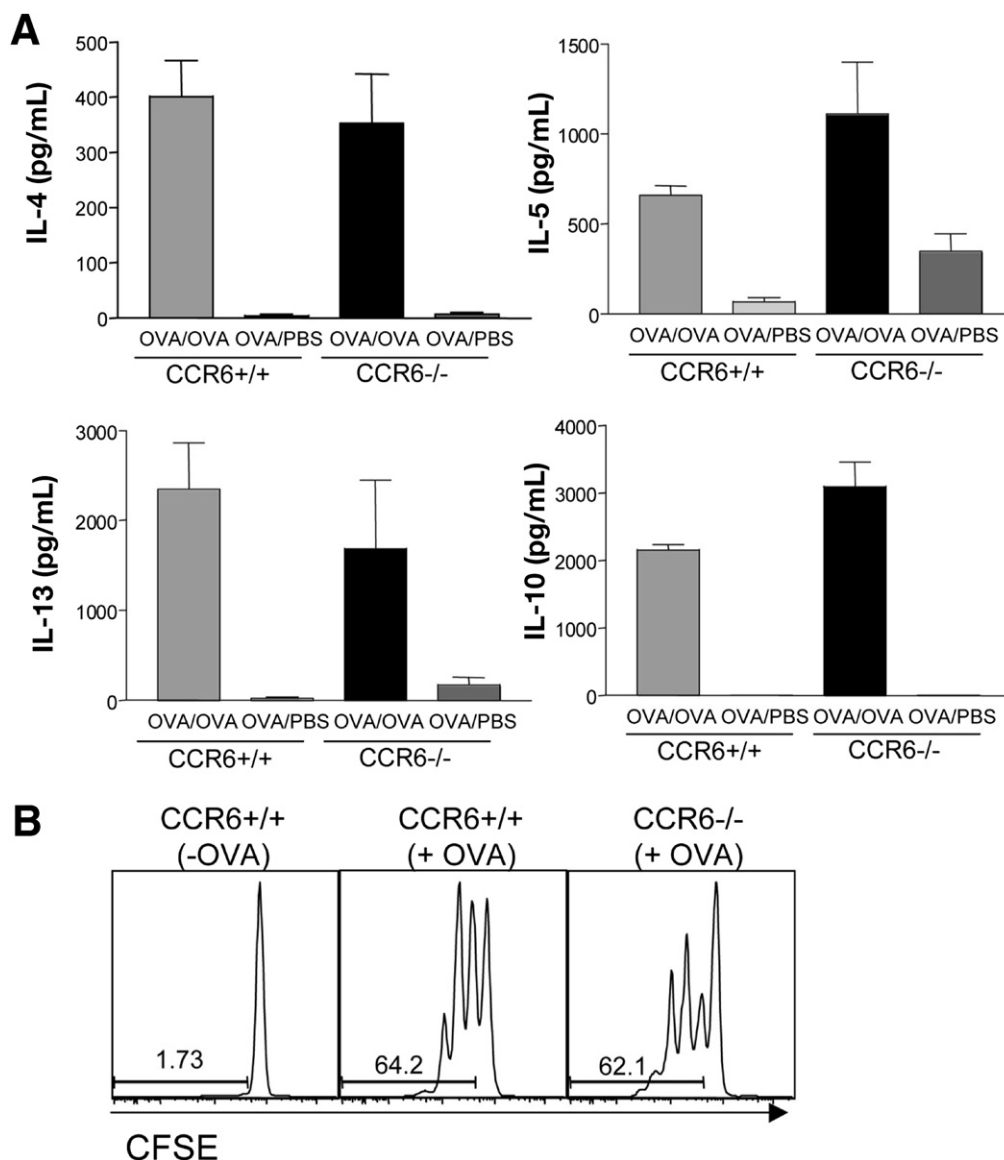


Figure 5. Th2 responses in the mesenteric lymph node are normal in CCR6^{-/-} mice. CCR6^{+/+} and CCR6^{-/-} mice were sensitized to OVA and challenged with OVA (OVA/OVA) or PBS (OVA/PBS) as control. MLN cells were restimulated with OVA; and IL-4, IL-5, IL-13, and IL-10 were measured in culture supernatants (panel A). CFSE-labeled OVA-specific DO11.10 cells were transferred to naïve CCR6^{+/+} and CCR6^{-/-} mice. Mice were fed 50 mg OVA (+OVA) or remained unfed as control (-OVA). After 96 hours, T-cell proliferation in the MLN was assessed by CFSE dilution (panel B).

population in the lamina propria (Supplementary Figure 5). Blockade of lymphocyte egress from the lymph nodes with FTY720 had no effect on onset of allergic diarrhea, tissue mast cells, or local Th2 cytokine production. Therefore, once sensitization has already occurred and memory has been established, reactivation of T cells can readily occur in the lamina propria without trafficking to and from lymph nodes.

T-Cell Transfers Demonstrate Requirement for CCR6 on Both Effector T Cells and in the Recipient for Disease Onset

Administration of MLN cells (total or CD4⁺ T cells) from mice with allergic diarrhea can transfer disease and bypass the need for systemic sensitization in naïve mice.⁴ MLN cells from CCR6^{-/-} mice have normal cytokine responses as compared with CCR6^{+/+} mice, so we tested the ability of these cells to transfer disease

(Figure 7). MLN cells from CCR6^{+/+} and CCR6^{-/-} mice (after sensitization and oral OVA challenge) were transferred to naïve Balb/c mice. Recipient mice were then repeatedly challenged with OVA. Mice receiving MLN cells from CCR6^{+/+} mice developed diarrhea. In contrast, mice receiving MLN cells from CCR6^{-/-} mice had significantly reduced onset of allergic diarrhea. Pretransfer IL-4 and IL-13 levels were confirmed to be similar in cells from CCR6^{+/+} and CCR6^{-/-} mice (Figure 7), suggesting that, whereas the effector function of these cells is not reduced, either homing of these cells to the small intestine or reactivation of these cells in vivo is defective. We next performed cell transfers to determine whether wild-type CCR6^{+/+} CD4⁺ T cells could restore the disease phenotype to CCR6^{-/-} mice (Figure 7). Wild-type mice receiving purified CD4⁺ T cells from CCR6^{+/+} MLN developed diarrhea in response to oral

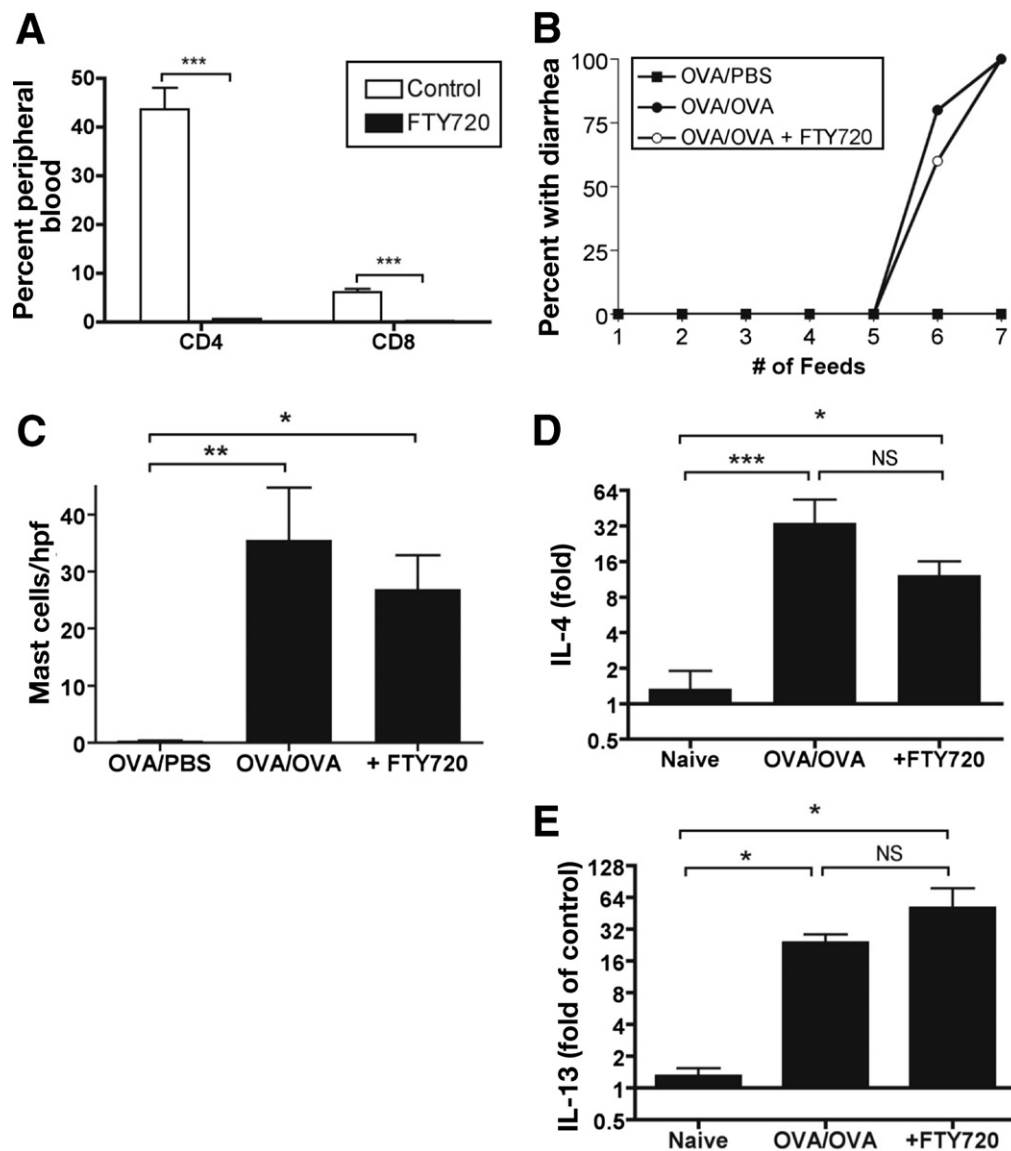


Figure 6. T-cell reactivation occurs locally in the absence of homing from lymph nodes. Mice were treated with FTY720 daily beginning 1 day prior to beginning the OVA feeds. Efficacy of the FTY720 treatment was checked by flow cytometry of peripheral blood (A), which showed a near abolishment of circulating CD4 and CD8 T cells. FTY720 treatment had no effect on onset of OVA-induced diarrhea (B), jejunal mastocytosis (C), or jejunal IL-4 (D) or IL-13 (E) expression. Data are representative of 2 independent experiments, with a total of 10 mice per group.

OVA challenge. In contrast, $CCR6^{-/-}$ mice were completely resistant to the transfer of allergic diarrhea using $CCR6^{+/+}$ T cells from the MLN.

Discussion

T lymphocytes play an important role in IgE-mediated food allergic disorders and have also been presumed to be central to the pathogenesis of non-IgE-mediated gastrointestinal allergic disorders such as eosinophilic gastrointestinal disorders. We hypothesized that recruitment of T cells to the intestinal mucosa through chemokine-driven pathways would be instrumental to the development of gastrointestinal allergic disease. We examined the role of the mucosal chemokine CCL20 and its receptor CCR6 in the development of experimental food allergy using $CCR6^{-/-}$ mice. CCR6 was necessary for allergen-induced diarrhea symptoms,

and our results point to a critical role for CCR6 in both T-cell homing and activation in the gastrointestinal tract.

$CCR6^{-/-}$ mice were significantly protected from the onset of allergic diarrhea, despite mounting a normal IgE and mast cell response. This suggests an alternative mechanism of disease pathogenesis in this model of allergic diarrhea. Brandt et al have clearly shown that mast cells and IgE are required for allergen-induced diarrhea.³ Our results show that T cells are also required and not solely upstream of the generation of IgE and mast cells. IL-4 and IL-13 have been shown to directly increase epithelial permeability and reduce glucose-coupled sodium absorption in mouse intestine.²⁵ This would be of significant clinical interest because there are several food-allergic disorders that have been loosely categorized as “cell-mediated” without having a clear mechanism explaining the pathway from allergen exposure to gastro-

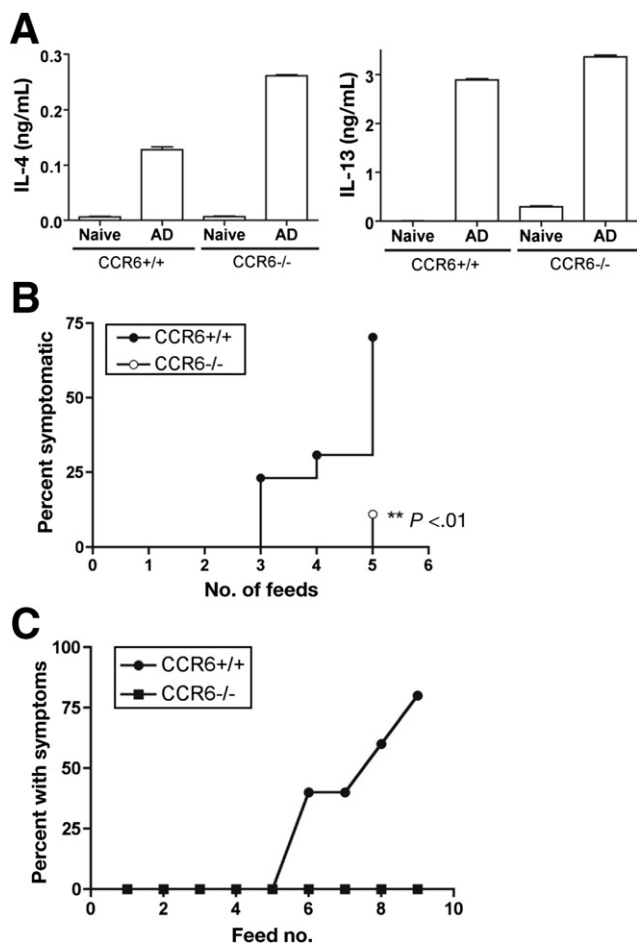


Figure 7. CCR6 on T cells is necessary, but not sufficient, for allergic diarrhea. MLN cells from OVA-sensitized and fed (AD) CCR6^{+/+} or CCR6^{-/-} mice were restimulated with OVA in vitro before transferring to naïve Balb/c mice. Mice were then fed with OVA every second day and diarrhea symptoms recorded. (A) The cytokine output of the transferred cells prior to transfer. (B) Symptoms of wild-type mice receiving primed CCR6^{+/+} or CCR6^{-/-} T cells as above. (C) Donor cells were CCR6^{+/+}, and recipients were CCR6^{+/+} or CCR6^{-/-} as indicated.

intestinal dysfunction. Recently, Brandt et al demonstrated that targeting IL-4 during oral challenge of mice could prevent allergic diarrhea, but, once diarrhea had been established, IL-4 was no longer required for acute symptom onset.⁶ Together, these results suggest that mast cells and Th2 cytokine production are both independently necessary for establishing allergic symptoms and inflammation in the gut.

CCR6 deficiency could potentially be acting at the level of the epithelial cell because it has been reported that CCL20 can influence epithelial ion secretion in human intestinal epithelial cell lines.²⁶ This may be particularly relevant for the alternative CCR6 ligand HBD-2, which is secreted lumenally and has been shown to promote epithelial restitution.²⁷ Murine β -defensin 14 has also been shown to have activity on CCR6,²⁸ but the expression of defensins in gastrointestinal allergic disease is not yet known. However, we think that epithelial CCR6 is un-

likely to be a major factor because 2 independent T cell-mediated models of diarrhea were CCR6-dependent, whereas toxin-induced diarrhea was not influenced by CCR6 deficiency. CD3 treatment mediates diarrhea by a TNF- α -dependent mechanism leading to alteration of epithelial transporters and manipulation of epithelial barrier function.^{20,29} Our data point to memory T-cell recruitment and/or activation of those memory T cells being defective in CCR6^{-/-} mice. When total cells were isolated from the lamina propria of naïve CCR6^{+/+} or CCR6^{-/-} mice, there was no defect in stimulation-induced cytokine secretion. These data suggest that activation of cells in vivo is deficient in CCR6^{-/-} mice, and we speculate that this is due to changes in mucosal architecture (loss of lymphoid structures) associated with CCR6 deficiency. We examined T-cell cytokine expression more closely in our allergic diarrhea model.

Our data show that allergen-induced Th2 cytokine expression in the small intestine is dependent on CCR6, despite normal Th2 cytokine expression in the draining lymph node. This suggests that either activation within the lamina propria or homing from the lymph node to the lamina propria is defective in CCR6^{-/-} mice. CCR6 is present on memory T cells and on a subset of CD4⁺ T cells in the lamina propria.¹⁷ CCR6 is up-regulated on proliferating OT-II transgenic T cells after activation in the mesenteric lymph node,³⁰ supporting a role for CCR6 in homing to the lamina propria after activation in the lymph node. There are clearly other mechanisms responsible for T-cell recruitment to the lamina propria, such as CCR9 and the homing molecule $\alpha 4\beta 7$, evidenced by the fact that CCR6^{-/-} mice do not have a major impairment in mucosal T cells and in fact had previously been reported to have increased intraepithelial and lamina propria CD4⁺ populations.¹⁵ We directly addressed the role of T-cell homing during allergen challenge by using FTY720, a sphingosine 1-phosphate receptor 1 agonist that blocks lymphocyte egress from the lymph nodes. FTY720 had no effect when it was administered after sensitization and during oral OVA challenge. These data support the conclusion that T-cell reactivation in response to oral OVA challenge is occurring locally within the intestinal mucosa. FTY720 has been shown to be effective on blocking egress from both MLN and Peyer's patch,²²⁻²⁴ and efficacy of FTY720 treatment was verified in our experiments by showing a near abolishment of circulating CD4 and CD8 T cells. FTY720 ameliorates experimental colitis,^{31,32} demonstrating efficacy in the gastrointestinal tract. FTY720 partially impairs allergic symptoms when given prior to allergen challenge in a model of allergic diarrhea localized to the large intestine,³³ and more complete inhibition was observed when FTY720 was administered prior to sensitization. Allergen-driven inflammation in the large intestine may be more likely to be dependent on reactivation within the lymph nodes because it is unlikely that significant

amounts of intact antigen would remain to be absorbed into the large intestinal lamina propria.

Transfer of primed Th2-secreting T cells to naïve mice eliminates the need for systemic sensitization with OVA and alum. Transfer of wild-type T cells to wild-type recipients resulted in allergic diarrhea when the mice were repeatedly fed with OVA. Transfer of CCR6^{-/-} cells that secreted similar levels of Th2 cytokines could not transfer disease. Thus, CCR6 on CD4⁺ T cells is necessary for transfer of allergic diarrhea. However, CCR6 on CD4⁺ T cells is not sufficient to transfer disease because we observed that adoptive transfer of wild-type CD4⁺ T cells (or whole MLN populations) to CCR6^{-/-} mice did not lead to OVA-induced diarrhea. These data are very similar to those previously shown by Lundy et al,³⁴ whereby splenic T cells from wild-type mice could transfer allergic airway hyperresponsiveness to wild-type mice but not CCR6^{-/-} mice. Those authors demonstrated that there was a deficiency in DC numbers in the CCR6^{-/-} lung and that the DCs from CCR6^{-/-} mice were less able to trigger IL-5 secretion from pulmonary T cells. In the gastrointestinal tract, we observed the previously reported result³⁵ that CCR6 is not expressed on lamina propria DCs. In addition, we did not observe any functional defect in the ability of DCs from CCR6^{-/-} mice to prime T cells in vivo as shown in Figure 5B or in vitro as shown in Supplementary Figure 6. Therefore our data do not support a role for CCR6 on DCs in OVA-specific T-cell activation. This is in contrast to the T-cell response to microbial pathogens, such as *Salmonella*, which is dependent on DC expression of CCR6.³ *Salmonella*, as particulate antigens, are more likely to be taken up via the Peyer's patch in which CCR6 is abundantly expressed on DCs. CCR6 deficiency has been shown to lead to changes in mucosal lymphoid tissue architecture. Number and size of Peyer's patches are reduced,¹⁸ isolated lymphoid follicles are reduced,¹⁶ and M-cell numbers are also decreased in CCR6^{-/-} mice.³⁶ Takayama et al directly addressed the role of Peyer's patches in allergic diarrhea and found in fact that they played a suppressive role through facilitating the expansion of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells.³⁷ Therefore, the diminished Peyer's patches in CCR6^{-/-} mice would be expected to enhance rather than reduce allergic diarrhea and inflammation. However, the role of isolated lymphoid follicles in allergic diarrhea or as an inductive or reactivation site for T lymphocytes has not been addressed. Mouse small intestine normally contains 100–200 of these structures that bring together B cells, T cells, and DCs.³⁸ CCR6 deficiency results in a decrease in the total number of these isolated lymphoid follicles.¹⁶ We hypothesize that these organized structures may be required for optimal reactivation of T lymphocytes in the gastrointestinal tract and explain why wild-type CD4⁺ cells transferred to CCR6^{-/-} mice do not fully achieve their pathogenic potential in the gastrointestinal tract and why CCR6^{-/-} mice are also

resistant to CD3-induced diarrhea, despite the normal function of these T cells in vitro. We speculate that an altered gut-associated lymphoid tissue anatomy could potentially inhibit the ability of memory T cells to be reactivated by either antigen or anti-CD3 antibodies.

In summary, we show that CCR6 plays a role in the development of gastrointestinal allergic disease, in part through expression on CD4⁺ T lymphocytes. Our data utilizing adoptive transfers indicate that, in a normal recipient, the absence of CCR6 on a single cell type (CD4⁺ T cells) prevents disease development. In addition, provision of wild-type pathogenic T cells cannot induce disease in CCR6^{-/-} mice. These findings suggest a role for CCR6 by 1 of 2 mechanisms: either in homing of T cells to appropriate mucosal locations for reactivation or, secondly, for optimal activation of T cells in the small intestine, which we speculate is due to the absence of organized solitary isolated lymphoid follicles in CCR6^{-/-} mice. In addition, our data point to intriguing mast cell and IgE-independent effects of T lymphocytes in gastrointestinal allergic disease that may be of particular relevance in the pathogenesis of "cell-mediated" allergic disorders of the gastrointestinal tract.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: [10.1053/j.gastro.2009.09.016](https://doi.org/10.1053/j.gastro.2009.09.016).

References

- Berin MC, Kiliaan AJ, Yang PC, et al. The influence of mast cells on pathways of transepithelial antigen transport in rat intestine. *J Immunol* 1998;161:2561–2566.
- Perdue MH, Masson S, Wershil BK, et al. Role of mast cells in ion transport abnormalities associated with intestinal anaphylaxis. Correction of the diminished secretory response in genetically mast cell-deficient W/W^v mice by bone marrow transplantation. *J Clin Invest* 1991;87:687–693.
- Brandt EB, Strait RT, Hershko D, et al. Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;112:1666–1677.
- Knight AK, Blazquez AB, Zhang S, et al. CD4 T cells activated in the mesenteric lymph node mediate gastrointestinal food allergy in mice. *Am J Physiol Gastrointest Liver Physiol* 2007;293:G1234–G1243.
- Forbes EE, Groschwitz K, Abonia JP, et al. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J Exp Med* 2008;205:897–913.
- Brandt EB, Munitz A, Orekov T, et al. Targeting IL-4/IL-13 signaling to alleviate oral allergen-induced diarrhea. *J Allergy Clin Immunol* 2009;123:53–58.
- Lin XP, Magnusson J, Ahlstedt S, et al. Local allergic reaction in food-hypersensitive adults despite a lack of systemic food-specific IgE. *J Allergy Clin Immunol* 2002;109:879–887.
- Beyer K, Castro R, Birnbaum A, et al. Human milk-specific mucosal lymphocytes of the gastrointestinal tract display a TH2 cytokine profile. *J Allergy Clin Immunol* 2002;109:707–713.
- Tanaka Y, Imai T, Baba M, et al. Selective expression of liver and activation-regulated chemokine (LARC) in intestinal epithelium in mice and humans. *Eur J Immunol* 1999;29:633–642.

10. Izadpanah A, Dwinell MB, Eckmann L, et al. Regulated MIP-3 α /CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G710–G719.
11. Puleston J, Cooper M, Murch S, et al. A distinct subset of chemokines dominates the mucosal chemokine response in inflammatory bowel disease. *Aliment Pharmacol Ther* 2005;21:109–120.
12. Li H, Chehade M, Liu W, et al. Allergen-IgE complexes trigger CD23-dependent CCL20 release from human intestinal epithelial cells. *Gastroenterology* 2007;133:1905–1915.
13. Anderle P, Rumbo M, Sierro F, et al. Novel markers of the human follicle-associated epithelium identified by genomic profiling and microdissection. *Gastroenterology* 2005;129:321–327.
14. Iwasaki A, Kelsall BL. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3 α , MIP-3 β , and secondary lymphoid organ chemokine. *J Exp Med* 2000;191:1381–1394.
15. Cook DN, Prosser DM, Forster R, et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity* 2000;12:495–503.
16. McDonald KG, McDonough JS, Wang C, et al. CC chemokine receptor 6 expression by B lymphocytes is essential for the development of isolated lymphoid follicles. *Am J Pathol* 2007;170:1229–1240.
17. Luger A, Kucharzik T, Soler D, et al. Lymphoid precursors in intestinal cryptopatches express CCR6 and undergo dysregulated development in the absence of CCR6. *J Immunol* 2003;171:2208–2215.
18. Varona R, Villares R, Carramolino L, et al. CCR6-deficient mice have impaired leukocyte homeostasis and altered contact hypersensitivity and delayed-type hypersensitivity responses. *J Clin Invest* 2001;107:R37–R45.
19. Radojevic N, McKay DM, Merger M, et al. Characterization of enteric functional changes evoked by in vivo anti-CD3 T-cell activation. *Am J Physiol* 1999;276:R715–R723.
20. Musch MW, Clarke LL, Mamah D, et al. T-cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na⁺/K⁺-ATPase. *J Clin Invest* 2002;110:1739–1747.
21. Friend DS, Ghildyal N, Gurish MF, et al. Reversible expression of tryptases and chymases in the jejunal mast cells of mice infected with *Trichinella spiralis*. *J Immunol* 1998;160:5537–5545.
22. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J Immunol* 1998;160:5037–5044.
23. Yanagawa Y, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. III. Increase in frequency of CD62L-positive T cells in Peyer's patches by FTY720-induced lymphocyte homing. *Immunology* 1998;95:591–594.
24. Henning G, Ohl L, Junt T, et al. CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. *J Exp Med* 2001;194:1875–1881.
25. Madden KB, Whitman L, Sullivan C, et al. Role of STAT6 and mast cells in IL-4- and IL-13-induced alterations in murine intestinal epithelial cell function. *J Immunol* 2002;169:4417–4422.
26. Yang CC, Ogawa H, Dwinell MB, et al. Chemokine receptor CCR6 transduces signals that activate p130Cas and alter cAMP-stimulated ion transport in human intestinal epithelial cells. *Am J Physiol Cell Physiol* 2005;288:C321–C328.
27. Vongsang RA, Zimmerman NP, Dwinell MB. CCR6 regulation of the actin cytoskeleton orchestrates human β defensin-2- and CCL20-mediated restitution of colonic epithelial cells. *J Biol Chem* 2009;284:10034–10045.
28. Rohrl J, Yang D, Oppenheim JJ, et al. Identification and biological characterization of mouse β -defensin 14, the orthologue of human β -defensin 3. *J Biol Chem* 2008;283:5414–5419.
29. Clayburgh DR, Barrett TA, Tang Y, et al. Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T-cell activation-induced diarrhea in vivo. *J Clin Invest* 2005;115:2702–2715.
30. Stenstad H, Ericsson A, Johansson-Lindbom B, et al. Gut-associated lymphoid tissue-primed CD4⁺ T cells display CCR9-dependent and -independent homing to the small intestine. *Blood* 2006;107:3447–3454.
31. Fujii R, Kanai T, Nemoto Y, et al. FTY720 suppresses CD4⁺CD44⁺ high CD62L-effector memory T cell-mediated colitis. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G267–G274.
32. Mizushima T, Ito T, Kishi D, et al. Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. *Inflamm Bowel Dis* 2004;10:182–192.
33. Kurashima Y, Kunisawa J, Higuchi M, et al. Sphingosine 1-phosphate-mediated trafficking of pathogenic Th2 and mast cells for the control of food allergy. *J Immunol* 2007;179:1577–1585.
34. Lundy SK, Lira SA, Smit JJ, et al. Attenuation of allergen-induced responses in CCR6^{-/-} mice is dependent upon altered pulmonary T-lymphocyte activation. *J Immunol* 2005;174:2054–2060.
35. Salazar-Gonzalez RM, Niess JH, Zammit DJ, et al. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. *Immunity* 2006;24:623–632.
36. Luger A, Floer M, Westphal S, et al. Absence of CCR6 inhibits CD4⁺ regulatory T-cell development and M-cell formation inside Peyer's patches. *Am J Pathol* 2005;166:1647–1654.
37. Takayama N, Igarashi O, Kweon MN, et al. Regulatory role of Peyer's patches for the inhibition of OVA-induced allergic diarrhea. *Clin Immunol* 2007;123:199–208.
38. Hamada H, Hiroi T, Nishiyama Y, et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol* 2002;168:57–64.

Received April 14, 2009. Accepted September 11, 2009.

Reprint requests

Address requests for reprints to: M. Cecilia Berin, PhD, Pediatric Allergy and Immunology, Box 1198, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029. e-mail: cecilia.berin@mssm.edu.

Acknowledgments

A.B.B. and A.K.K. contributed equally to the work.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by NIH funds DK071576 and AI044236, EPA grant R834064, support from the Food Allergy Initiative (to M.C.B.), and by a fellowship from the Crohn's and Colitis Foundation of America (to A.B.B.).

Supplementary Materials and Methods

Immunostaining

For CCL20 immunostaining, paraffin sections were hydrated, and antigen retrieval performed with citrate buffer. Sections were stained with anti-CCL20 (R&D Systems, Minneapolis, MN) or isotype control (rat IgG1; eBioscience, San Diego, CA), followed by biotinylated goat anti-rat antibody (Jackson ImmunoResearch, West Grove, PA), ABC reagent (Vector Laboratories, Burlingame, CA), and DAB chromogen (Vector Laboratories).

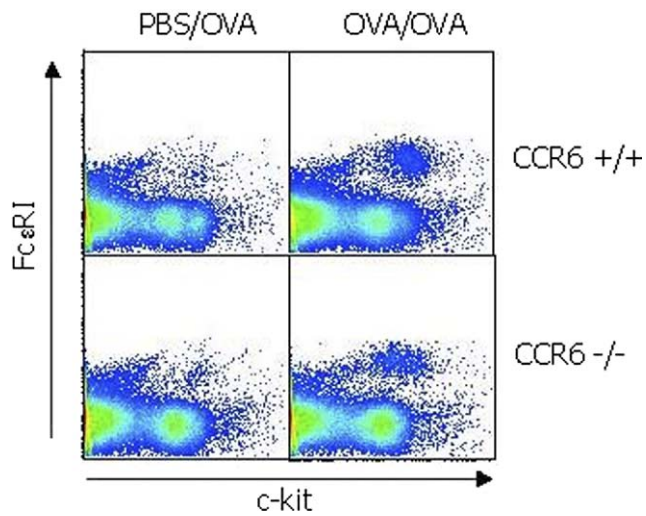
Flow Cytometry

Cells were isolated from small intestinal lamina propria. Peyer's patches were excised, and the epithelium removed by EDTA treatment. Remaining segments were treated with collagenase D and DNase (Roche, Indianapolis, IN), followed by separation on a discontinuous Percoll gradient (GE Life Sciences, Piscataway, NJ). Cells were stained with anti-CD11c, CD11b, CD8 α , CD4,

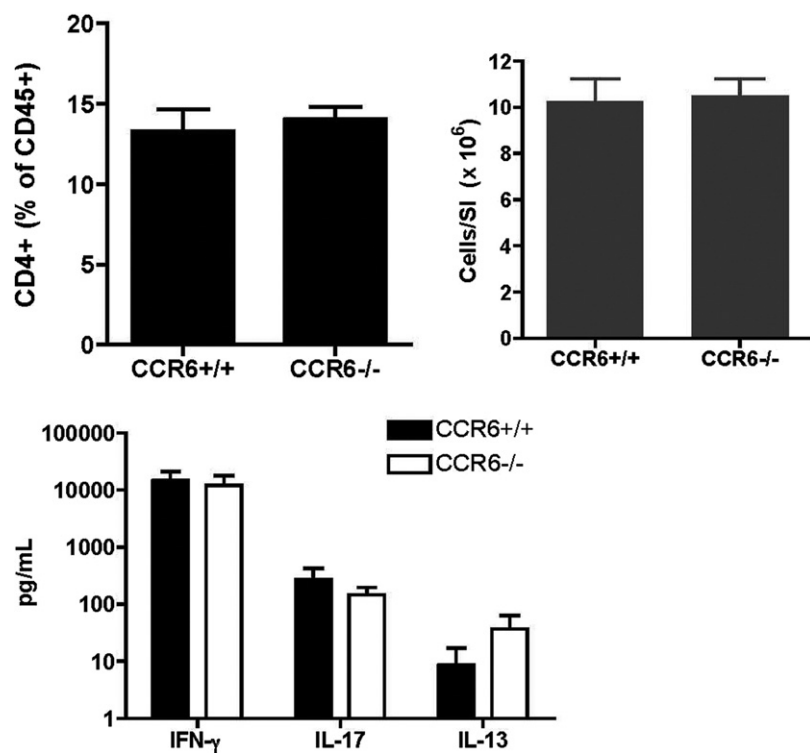
B220, CD62L, Fc ϵ RI, and c-kit (all from eBioscience) and CCR6 (BD Biosciences, San Jose, CA).

Antigen Presentation Assay

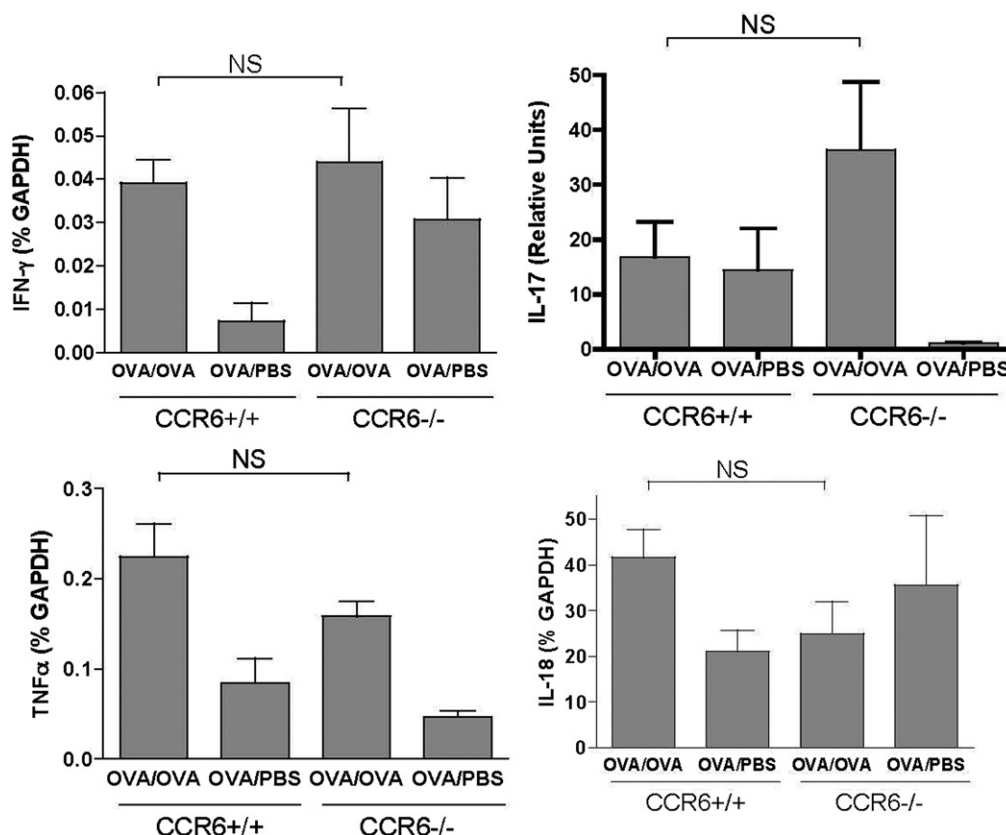
CD4⁺ T cells from the mesenteric lymph node (MLN), and spleen of DO11.10 ovalbumin (OVA) T-cell receptor (TCR) transgenic mice were purified by negative selection (StemCell, Vancouver, BC Canada) according to the manufacturer's instructions. CD4⁺ T cells were cocultured at a ratio of 1:10 with dendritic cells (DCs) purified from the lamina propria of CCR6^{+/+} and CCR6^{-/-} mice. DCs were purified by positive selection (Miltenyi Biotec, Auburn, CA). Cells were cultured with OVA₃₂₃₋₃₃₉ peptide (1000 ng/mL). After 72 hours, CD4⁺ T cells were restimulated with 3 μ g/mL plate-bound anti-CD3 monoclonal antibody (mAb) (eBioscience) plus 3 μ g/mL of soluble anti-CD28 (eBioscience). Cells were cultured in complete medium for 3 days, and supernatants were collected for detection of cytokines by enzyme-linked immunosorbent assay (ELISA) (all from eBioscience).



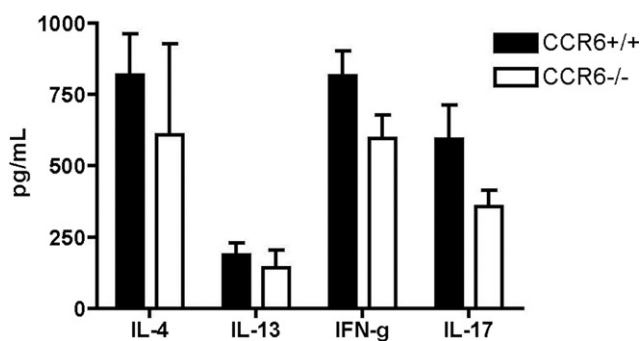
Supplementary Figure 1. Flow cytometric detection of lamina propria mast cells. Lamina propria cells were isolated from CCR6^{+/+} and CCR6^{-/-} mice with allergic diarrhea (OVA/OVA) or control (PBS/OVA) mice and stained with anti-FcεRI and c-kit antibodies. Mice with allergic diarrhea had an expansion of the FcεRI⁺c-kit⁺ population. This was also observed in CCR6^{-/-} mice that did not develop allergic diarrhea symptoms.



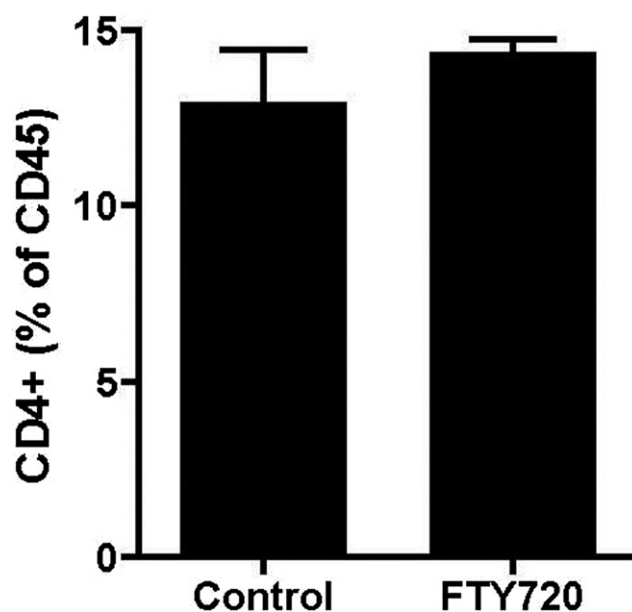
Supplementary Figure 2. Lamina propria T cells in CCR6^{+/+} and CCR6^{-/-} mice. Cells were isolated from the lamina propria of CCR6^{+/+} and CCR6^{-/-} mice (4/group). Cells were stained with the pan-leukocyte marker CD45 and CD4. The proportion of CD4⁺ T cells was unchanged in CCR6^{-/-} mice (*top left*). In addition, the total yield of cells per small intestine (*right*) was also unchanged. Cells were stimulated with anti-CD2 and anti-CD28 for 72 hours in complete media, and cytokine release measured by ELISA (*bottom panel*).



Supplementary Figure 3. Impact of CCR6 on lamina propria expression of Th1 and proinflammatory cytokines. CCR6^{+/+} and CCR6^{-/-} mice were sensitized and fed with OVA 4 times (OVA/OVA) or were sensitized and left unfed as controls (OVA/PBS). Jejunum was removed, RNA was isolated, and RT-PCR was performed for IFN- γ , IL-17, TNF- α , and IL-18. Expression was normalized to the housekeeping gene GAPDH.



Supplementary Figure 4. Induction of T-cell cytokine production by CCR6^{+/+} or CCR6^{-/-} DCs from the lamina propria. Lamina propria cells were isolated from CCR6^{+/+} and CCR6^{-/-} mice, followed by positive selection for CD11c⁺ DCs. CD4⁺ T cells were isolated from DO11.10 mice and cocultured with DCs plus OVA peptide. Cytokine secretion from CD3/CD28 restimulated T cells was measured by ELISA. Data are the mean + SEM from 3 individual isolations.



Supplementary Figure 5. Impact of FTY720 treatment on resident lamina propria CD4⁺ T cells. CCR6^{+/+} and CCR6^{-/-} mice were fed with FTY720 on 2 consecutive days prior to isolation of lamina propria cells from the small intestine. Cells were stained with the pan-leukocyte marker CD45 and CD4, and cells were acquired on a flow cytometer. Percent of CD4⁺ T cells among the total CD45⁺ population was calculated.