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CCR6 Regulates the Migration of Inflammatory and Regulatory T Cells

Tomohide Yamazaki,* Xuexian O. Yang,* Yeonsoek Chung,* Atsushi Fukunaga,* Roza Nurieva,* Bhanu Pappu,* Natalia Martin-Orozco,* Hong Soon Kang,‡ Li Ma,§ Athanasia D. Panopoulos,* Suzanne Craig,‡ Stephanie S. Watowich,* Anton M. Jetten,‡ Qiang Tian,§ and Chen Dong2*

Th17 and regulatory T (Treg) cells play opposite roles in autoimmune diseases. However, the mechanisms underlying their proper migration to inflammatory tissues are unclear. In this study, we report that these two T cell subsets both express CCR6. CCR6 expression in Th17 cells is regulated by TGF-β and requires two nuclear receptors, RORγt and RORα. Th17 cells also express the CCR6 ligand CCL20, which is induced synergistically by TGF-β and IL-6, which requires STAT3, RORγt and IL-21. Th17 cells, by producing CCL20, promote migration of Th17 and Treg cells in vitro in a CCR6-dependent manner. Lack of CCR6 in Th17 cells reduces the severity of experimental autoimmune encephalomyelitis and Th17 and Treg recruitment into inflammatory tissues. Similarly, CCR6 on Treg cells is also important for their recruitment into inflammatory tissues. Our data indicate an important role of CCR6 in Treg and Th17 cell migration. The Journal of Immunology, 2008, 181: 8391–8401.

The CD4+ Th cells are central organizers in immune responses. Naive T cells, upon activation by APCs, differentiate into cytokine-expressing effector Th cells, which have been historically classified into Th1 and Th2 lineages based on their cytokine secretion and immune regulatory function (1, 2). Th1 cells regulate cellular immunity and Ag presentation through secreting IFN-γ, while Th2 cells mediate the humoral and allergic responses by producing IL-4, IL-5, and IL-13. Th1 and Th2 cells differ not only in their immune function but also in their migratory regulation; they express distinct chemokine receptors which mediate their selective recruitments into tissues depending on the types of pathogen infections or immune responses (3).

In addition to Th1 and Th2, a third subset of Th cells, namely, Th17, was recently identified (4–6). Th17 cells were first marked by IL-17 production and subsequently were found to also secrete IL-17F and IL-22 (5, 7–9). IL-17 has been previously found to be involved in host defenses against bacterial infection (10). Recently, Th17 cells have been strongly associated in autoimmune diseases, such as animal models for multiple sclerosis and rheumatoid arthritis (5, 6, 11, 12). Neutralizing IL-17 strongly inhibited experimental autoimmune encephalomyelitis (EAE) (5, 6).

Th17 differentiation is potently driven by TGF-β and IL-6 (13–15), which is reinforced by IL-23 (16, 17). Recently, IL-21 was reported as an autocrine factor induced by IL-6 to regulate Th17 differentiation (17–19). For cytokine-mediated Th17 differentiation, STAT3 was found to be a necessary transcription factor (16, 20). STAT3 may function by regulating the expression of lineage-specific master transcription factor(s), such as RORγt and RORα, two orphan nuclear receptors that were recently shown to regulate Th17 differentiation (21, 22).

In contrast to autoimmunity-promoting Th17 cells, thymus-derived natural regulatory T cells (nTreg) represent a unique subpopulation of CD4+ T cells that inhibits T cell proliferation and autoimmune responses (23). The hallmark of nTreg cells is the expression of Foxp3 transcription factor, which serves as a master regulator of Treg cell development and function (24). Under the influence of TGF-β, Foxp3 can be also induced in naive T cells’ periphery and the resulting inducible Treg (iTreg) cells exhibit the same suppressive phenotype as nTreg (24). Interestingly, both iTreg and Th17 cell development depends on TGF-β. Thus, there is not only functional antagonism between Th17 cells and Treg cells in autoimmunity, but also a reciprocal regulation in the generation of these cells. Although TGF-β induces Foxp3 expression, IL-6 and IL-21 inhibit this regulation and along with TGF-β drive Th17 differentiation.

Despite recent progress on understanding the regulation and function of Treg and Th17 cells, it is unclear how their recruitments into inflammatory sites are regulated. In this study, we report that both Treg and Th17 cells express CCR6. Th17 cells also express the CCR6 ligand CCL20, through which Th17 promotes the migration of Th17 and Treg cells. CCR6 deficiency in T cells

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3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; nTreg, natural regulatory T, Treg, regulatory T; iTreg, inducible Treg; KO, knockout; BM, bone marrow; MOG, myelin oligodendrocyte glycoprotein; LN, lymph node; WT, wild type.

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decreases the susceptibility to autoimmune diseases. Lack of CCR6 in Th17 cells inhibits their own as well as Treg recruitment into inflammatory tissues. Similarly, CCR6 on Treg cells is also important for their recruitment into inflammatory tissues. Our data thus indicate an essential role of CCR6 in Treg and Th17 migration in autoimmune disease and suggest that Th17 induces both amplification and inhibition mechanisms on inflammatory responses via CCR6.

Materials and Methods

Mice
C57BL/6 mice, CCR6 knockout (KO), Rag1 KO, B6.129-Ptprc<sup>Pepc</sup> /Boy1 (CD45.1) mice were purchased from The Jackson Laboratory. RORγ KO, RORα/δ double mutant mice, STAT3 conditional KO, and IL-21 KO mice have been previously described (16, 19, 22). All animal experiments have been conducted under the animal protocols approved by M. D. Anderson Cancer Center Institutional Animal Care and Usage Committee.

T cell differentiation
CD4<sup>+</sup>CD25<sup>-</sup>CD62<sup>hi</sup>CD44<sup>low</sup> cells were isolated by FACS sorting as described before (16, 19). Naive CD4<sup>+</sup> T cells were stimulated with anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/ml). For Th1 differentiation, IL-12 (10 ng/ml) and anti-IL-4 (5 μg/ml; 11B11) were used additionally. For Th2 differentiation, IL-4 (10 ng/ml) and anti-IFN-γ (5 μg/ml; XMG 1.2) were used additionally. For Th17 cell differentiation, IL-6 (50 ng/ml; Pepro-Tech), TGF-β (10 ng/ml; Pepro-Tech), IL-23 (50 ng/ml; R&D Systems), anti-IL-4 (5 μg/ml; 11B11), and anti-IFN-γ (5 μg/ml; XMG 1.2) were used additionally. For Treg differentiation, TGF-β (10 ng/ml), anti-IL-4 (5 μg/ml; 11B11), and anti-IFN-γ (5 μg/ml; XMG 1.2) were used additionally. To examine intracellular cytokine staining, at 4 days of the culture, the cells were washed and restimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiPlug (BD Biosciences) for 5 h. The cells were permeabilized with a Cytofix/Cytofix kit (BD Biosciences).

Quantitative real-time PCR
Total RNA was prepared from T cells with the use of TRizol reagent (Invitrogen). cDNAs were synthesized with Superscript reverse transcriptase and oligo(dT) primers (Invitrogen) and gene expression was examined with a Bio-Rad iCycler Optimal System using a iQ SYBER Green real-time PCR kit (Bio-Rad). The primers used were (5′–3′): CCR6: forward, CCTCACATCTTCTAGGCTGAGC and reverse, GGCAATCAGAGCTGACCTC, CCL20: forward, ATGGCCTGCGGTGGCAAGCGTCTG and reverse, ATGACGTGAGGAGGGTTCACAGCCCT. The data were normalized to β-actin gene expression as a reference.

In vitro migration assay
A migration assay was performed in 24-well plates (Costar) carrying Transwell-permeable supports with a 3-μm polycarbonate membrane for T cells. Recombinant CCL20 or the supernatants collected from Th17 cells stimulated with anti-CD3 for 3 days were placed on the lower chamber. Th1, Th2, Th17, Treg, or naive CD4<sup>+</sup> T cells from CCR6 KO mice or C57BL/6 mice were placed on the upper wells of Transwell membranes containing 100 μl of RPMI 1640 medium. A total of 5 × 10<sup>5</sup> T cells was incubated for 7 h at 37°C and 5% CO<sub>2</sub> atmosphere. T cells on the upper surface of membranes were completely removed by washing with distilled water. Migrated cells were fixed with methanol and stained with Giemsa (Sigma-Aldrich). Migrated cells were determined by counting the number of T cells stained with Giemsa in 10 randomly selected nonoverlapping fields of the wells under light microscope.

EAE induction
Bone marrow (BM) cells from female C57BL/6 or CCR6 KO mice were prepared by flushing the femurs and tibiae with RPMI 1640 medium (Life Technologies). After washing them with ice-cold PBS twice, the cells (5 × 10<sup>6</sup>) were resuspended with sterile PBS and injected i.v. into female Rag1 KO mice (6–7 wk old) that had been irradiated (750 rad) with a cesium irradiator. Around 5 wk later, reconstituted mice that had normal numbers of CD4 and CD8 in the blood were used for EAE induction. For induction of EAE, mice were immunized s.c. at the dorsal flanks with 150 μg of MOG<sub>35–55</sub> peptide (MEGVYGRSPFSHPHYLVRNGK) emulsified in CFA on days 0 and 7. Pertussis toxin was given i.p. on days 1 and 8 with a dose of 500 ng/mouse. Clinical EAE symptoms were scored according to a 1–5 scale as followed: 0, no symptoms; 1, limp tail or waddling tail; 2, wobbly gait; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; and 5, death.

Th17 transfer EAE
C57BL/6 or CCR6 KO mice were immunized s.c. at the dorsal flanks with 150 μg of MOG<sub>35–55</sub> peptide emulsified in CFA. The following day, pertussis toxin was injected i.p. at a dose of 500 ng/mouse. Seven days after immunization, a single-cell suspension of spleen cells was prepared from the mice and cultured with MOG<sub>35–55</sub> peptide and IL-23 for 7 days. MOG-reactive CD4<sup>+</sup> T cells were purified by AutoMACS (Miltenyi Biotec) with anti-CD4 microbeads (Miltenyi Biotec). Purified CD4<sup>+</sup> T cells (> 95% CD3<sup>+</sup>CD4<sup>+</sup>; 4 × 10<sup>5</sup>) were adoptively transferred into C57BL/6 mice followed by MOG immunization on the second day. Pertussis toxin was injected i.p. on the following day. Signs of EAE were assigned scores on a scale of 1–5 as mentioned above.

Treg transfer in EAE
CD4<sup>+</sup>CD25<sup>hi</sup>CD44<sup>low</sup> Treg cells from wild-type (WT) or CCR6<sup>−/−</sup> mice were isolated by FACS sorting. Isolated Treg cells were adoptively transferred into RAG<sup>−/−</sup> (0.5 × 10<sup>7</sup>/mouse along with 4.5 × 10<sup>5</sup> naïve T cells) or CD45.1<sup>−/−</sup> (1 × 10<sup>7</sup>/mouse) mice. The mice were immunized with MOG peptide with CFA on the second day. Pertussis toxin was injected on the following day.

Statistical analysis
Significant differences between two groups were analyzed by the Mann-Whitney U test. Values of p < 0.05 and p < 0.01 were considered significant.

Results
CCR6 is highly expressed on Th17 cells
In a gene expression profiling analysis of in vitro-differentiated Th1, Th2, and Th17 cells, we found that the expression of CCR6 was significantly elevated in Th17 cells compared with Th1 or Th2 cells (data not shown). We thus confirmed CCR6 expression in Th1, Th2, and Th17 cells. Assessed by real-time RT-PCR, Th17 cells, but not Th1 and Th2 cells, highly expressed CCR6 mRNA (Fig. 1A). IL-17 mRNA was also specifically expressed in Th17 cells, which indicated proper differentiation of Th17 cells in vitro.

We further analyzed which cytokine regulates CCR6 expression in Th17 cells. Time-dependent levels of CCR6 mRNA were analyzed in T cells after treatment with various Th17-regulating cytokines for 1, 2, or 5 days. TGF-β, but not any other tested cytokine, rapidly and strongly induced CCR6 mRNA expression in T cells at early time points of T cell activation such as days 1 and 2 (Fig. 1B). IL-6 or IL-21 did not further enhance this induction by TGF-β. These results indicate that TGF-β is a main factor for induction of CCR6 expression in Th cells. On day 5, the expression of CCR6 mRNA was reduced in T cells. Although T cells treated with TGF-β alone still exhibited elevated levels of CCR6 mRNA expression, cells treated with both TGF-β and IL-6 showed the highest CCR6 and IL-17 mRNA expression (Fig. 1B). These results indicate that although CCR6 is highly expressed by Th17, its induction kinetics and cytokine regulation appear to differ from those of IL-17.

RORγ and RORα are key transcription factors mediating Th17 cell differentiation (20–22). We thus assessed whether they regulate CCR6 expression in Th17 cells. RORγ-deficient, RORα/ RORγ doubly mutant, or their littermate control T cells were differentiated into Th17 cells, and CCR6 mRNA expression was examined as above. RORγ-deficient T cells exhibited enhanced CCR6 mRNA expression compared with their WT controls (Fig. 1C). Also, T cells carrying an RORα gene mutation did not have impaired CCR6 expression either (data not shown). However, T cells defective in both RORα and RORγ greatly reduced CCR6 mRNA expression, indicating that these two nuclear receptors are redundant in CCR6 regulation.
We also assessed the effect of IL-21, an autocrine Th17 differentiation inducer, on CCR6 regulation. IL-21-deficient T cells exhibited more enhanced CCR6 expression than WT cells when exposed to Th17 differentiation conditions (Fig. 1C). These results indicate that IL-21 would not be necessary in CCR6 expression.

In addition to mRNA expression, CCR6 protein expression was assessed in in vitro-differentiated Th1, Th2, and Th17 cells. CCR6 was only expressed in a small percentage of Th1 and Th2 cells, but was highly expressed in Th17 cells (Fig. 1D). In addition, CCR6 expression was assessed on IL-17- or IFN-γ-producing cells in the spleen, draining lymph node (LN), or CNS of mice in which experimental encephalomyelitis (EAE) diseases were induced. CCR6 expression was detected on IL-17+ cells in the spleen, LN, and CNS of mice with EAE diseases. C57BL/6 mice were immunized with MOG peptide to induce EAE. CCR6 expression was analyzed with allophycocyanin-labeled anti-IFN-γ and PE-labeled IL-17A in IL-17+ or IFN-γ+ CD4+ T cells. The data are expressed as the mean ± SD of three mice. The results shown are a representative of at least two independent experiments.

**FIGURE 1.** CCR6 is highly expressed by Th17 cells. A. In vitro-differentiated Th1, Th2, and Th17 cells were restimulated with anti-CD3 for 4 h for real-time RT-PCR analysis. The data are expressed as the mean ± SD of triplicate samples. Data shown were repeated twice with consistent results. B. FACS-sorted naïve T cells were cultured with the indicated cytokines for 1, 2, and 5 days. After restimulation with anti-CD3, CCR6 and IL-17 expression was analyzed by real-time PCR. The data are expressed as the mean ± SD of triplicate samples. Data shown were repeated twice with consistent results. C. Naïve CD4+ T cells from RORγ KO, RORαγ double mutant, IL-21 KO, or WT mice were differentiated under Th17 conditions for 4 days. CCR6 expression was analyzed by real-time PCR. Real-time PCR results in A–C were normalized to actb gene expression and relative gene expression levels are indicated. The data are expressed as the mean ± SD of triplicate samples. A–C. The expression level in Th0 of WT or KO cells was set at 1. Data shown were repeated twice with consistent results. D, CCR6 expression was analyzed with allophycocyanin-labeled anti-CCR6 or allophycocyanin-labeled rat IgG in Th1, Th2, or Th17 cells. E. CCR6 expression on Th17 cells in the spleen, LN, and CNS of mice with EAE diseases. C57BL/6 mice were immunized with MOG peptide to induce EAE. The cells from spleen, LN, and CNS of mice that developed EAE were restimulated with MOG peptide for 1 day. CCR6 expression was analyzed with allophycocyanin-labeled anti-IFN-γ and PE-labeled IL-17A in IL-17+ or IFN-γ+ CD4+ T cells. The data are expressed as the mean ± SD of three mice. The results shown are a representative of at least two independent experiments.

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**CCR6 expression on Treg cells**

Since TGF-β induces Foxp3 expression in T cells, we thus assessed CCR6 expression in Treg cells. CD4+CD25+CD62LhiCD44low naive T cells were isolated by FACS sorting and differentiated into iTreg cells with anti-CD3 and anti-CD28 with TGF-β, anti-IFN-γ, and anti-IL-4 for 5 days. Those activated with just anti-CD3/CD28 (Th0) served as controls. A high level of CCR6 mRNA expression was found in iTreg cells (Fig. 2A), when compared with naive CD4+ T cells and Th0 cells. CCR6 expression was also found on iTreg cells at the protein level (Fig. 2B). In addition, CCR6 mRNA was also highly expressed in sorted CD4+CD25+ nTreg cells (Fig. 2A). CD4+CD25+ cells in the spleen and peripheral LN, most of
which were Foxp3⁺, expressed CCR6 protein as well (Fig. 2C). Thus, in addition to Th17 cells, Treg cells generated in vitro and in vivo also express CCR6, consistent with a previous report by Kleinewietfeld et al. (25).

**CCL20 is expressed in Th17 but not Treg cells**

CCL20, also called MIP-3α, is the only ligand for CCR6 (26). In addition to CCR6, we also attempted to analyze the expression of CCL20 in Th17 and Treg cells. CCL20 mRNA was specifically expressed in Th17 cells, but not Treg and other effector Th subsets (Fig. 3A). Moreover, we used a Th17 reporter mouse we recently reported, in which a red fluorescent protein sequence was inserted into the IL-17F gene (27). By real-time PCR, CCL20 mRNA was strongly expressed in red fluorescent protein-positive cells generated in vitro (data not shown), confirming that CCL20 is indeed produced by Th17 cells. After anti-CD3 restimulation, Th17 cells produced CCL20 protein in a time-dependent manner (Fig. 3B). Thus, mouse Th17 cells, similar to their human counterparts (28, 29), express CCL20.

To understand CCL20 regulation in T cells, naïve T cells were cultured with various cytokines for up to 5 days and then analyzed for CCL20 mRNA expression. Although TGF-β or IL-6 alone weakly induced the expression of CCL20 mRNA on days 1 and 2, the combination of TGF-β and IL-6 or IL-21 exhibited strong synergy in inducing CCL20 expression (Fig. 3C). CCL20 expression was maintained in T cells in the presence of TGF-β/IL-6 or TGF-β/IL-21 combination on day 5 after activation (Fig. 3C).

To determine the mechanisms underlying CCL20 expression in Th17 cells, the effect of Th17 differentiation regulating transcriptional factors such as RORα, RORγt, and STAT3(16) was assessed. Naïve T cells from STAT3-deficient, RORγt-deficient, both RORα/γ-deficient mice, or their WT controls were differentiated under Th17 conditions and CCL20 expression was analyzed by real-time RT-PCR. CCL20 mRNA expression was abolished in STAT3-deficient T cells (Fig. 3D). RORγt-deficient as well as RORα/γ mutant T cells also showed greatly reduced CCL20 mRNA expression (Fig. 3E). Conversely, RORγ overexpression in T cells enhanced CCL20 mRNA expression (Fig. 3E).

Because CCL20 mRNA expression was regulated by IL-21 along with TGF-β, we also evaluated the effect of IL-21 deficiency on CCL20 expression in T cells. CCL20 mRNA expression was substantially decreased in IL-21-deficient T cells when they were activated under Th17-polarizing conditions (Fig. 3F).

Our above results indicated that in addition to CCR6, Th17 cells also highly expressed its ligand CCL20. CCR6 and CCL20 expression appears to be differentially regulated by cytokines and transcription factors.

**CCL20 does not regulate Th17 differentiation or their cytokine production**

A role has been reported for chemokines in costimulation and subsequent IL-2, IFN-γ, or IL-4 production as well as proliferation of T cells (30). We assessed whether CCL20 acts on Th17 differentiation and cytokine production of Th17 cells. Naïve CD4⁺ T cells were differentiated into Th1 or Th17 cells with or without recombinant CCL20. The frequency of IFN-γ- or IL-17-producing cells was not significantly different between T cells with or without recombinant CCL20 (Fig. 4A). To assess the CCL20 effect in cytokine production from Th17 cells, T cells cultured with Th17 differentiation condition for 4 days were restimulated with anti-CD3 or anti-CD25 and recombinant CCL20. No significant difference was found in IL-17 or IL-17F production between T cells with or without recombinant CCL20 (Fig. 4B). Also, WT or CCL6-deficient naïve CD4⁺ T cells were cultured with Th17 differentiation conditions. The frequency of Th17-producing cells was not different between WT T cells and CCR6-deficient T cells, which indicated that CCR6-deficient T cells did not have impairment in Th17 differentiation (Fig. 4C). These results indicated that CCL20 has no effect on Th17 differentiation and cytokine production of Th17 cells.

**Th17 cells regulate the migration of Th17 and Treg cells through CCR6**

We thus focused on the function of CCR6 in T cell directional chemotaxis (31). To determine the function of CCR6 in Th17 and Treg cells, their migratory responses to CCL20 were first examined by a Transwell assay. The chemotaxis was measured using the method described by Boyden (32). Migration of Th17, but not Th1...
**FIGURE 3.** CCL20 is specifically expressed by Th17 cells. A, Th1, Th2, Th17, nTreg, and iTreg cells were analyzed for CCL20 expression by real-time PCR. Real-time PCR results were normalized to Actb gene expression and relative gene expression levels are indicated. The expression level in iTreg was set at 1. The data are expressed as the mean ± SD of triplicate samples. Data shown were repeated twice with consistent results. B, In vitro-differentiated Th17 cells were restimulated with anti-CD3 for up to 72 h. The cell culture supernatants were analyzed for CCL20 production by ELISA at the indicated time points. The data are expressed as the mean ± SD of triplicate wells. Data shown were repeated twice with consistent results. C, Naive T cells were cultured with the indicated cytokines for 5 days. After restimulation with anti-CD3, CCL20 expression was analyzed by real-time PCR. The expression level in naive T cell was set at 1. The data are expressed as the mean ± SD of triplicate samples. Data shown were repeated twice with consistent results. D–F, Naive CD4⁺ T cells from STAT3 KO (D), RORγ KO (E), RORα/γ KO (E), or IL-21 KO mice (F) and their appropriate control mice were cultured for Th17 differentiation for 4 days. CCL20 expression was analyzed by real-time PCR after restimulation of T cells with anti-CD3. E, CCL20 expression was assessed in CD4⁺ T cells overexpressing RORγt with real-time PCR. Real-time PCR results in C–F were normalized to Actb gene expression and relative gene expression levels are indicated. The expression levels in Th0 of WT cells were set at 1. The data are expressed as the mean ± SD of triplicate samples. The results shown (D–F) are a representative of two independent experiments.

or Th2 cells, was detected in response to recombinant CCL20 (Fig. 5A). Likewise, nTreg cells, but not naive CD4⁺ T cells, also showed significant migration to CCL20 (Fig. 5B). CCR6-deficient Th17 or Treg cells completely lost the ability to migrate to CCL20 (Fig. 5C). This result indicated that the migration of Th17 and Treg cells in response to CCL20 is dependent on CCR6.

Since Th17 cells express CCL20, we reasoned that activated Th17 cells may regulate the migration of Th17 or Treg cells. To address this hypothesis, Th17, Treg, or Th1 cells from C57BL/6 or CCR6-deficient mice were tested with the culture supernatants from anti-CD3-stimulated Th17 cells in Transwell plates. CCL20 (5 ng/ml) was detected in the culture supernatant of stimulated Th17 (data not shown). WT Th17 and Treg, but not Th1, cells migrated in response to the culture supernatants of activated Th17 cells (Fig. 5D). However, this migratory response was completely abolished in CCR6-deficient Th17 and Treg cells (Fig. 5D). This result indicates that Th17 cells produce CCL20 to attract other Th17 as well as Treg cells through CCR6.

**CCR6 regulates migration and function of Th17 cells in EAE**

Although the populations of Th17 and Treg cells in lymphoid tissues have been well characterized, the distribution of Th17 and Treg cells in nonlymphoid tissues and the underlying mechanisms were poorly understood. To determine whether CCR6 plays any role for the tissue localization of Th17 and Treg cells, CD4⁺ cells that express IL-17 or Foxp3 were analyzed in a variety of tissues in WT or CCR6-deficient mice on C57BL/6 background. The population of Treg cells was significantly reduced in the mesenteric LN (by 25%), Peyer’s patch (by 47%), and lung (by 56%). On the other hand, the population of Th17 cells was also significantly
KO mice exhibited significantly reduced EAE disease compared with C57BL/6 mice in the early phase of EAE. However, CCR6 KO mice caught up with C57BL/6 mice in the early phase of EAE. Since CCR6 is also expressed by non-T cells, to determine CCR6 function directly in T cells during inflammatory diseases, Rag1−/− mice were reconstituted with BM cells from WT or CCR6-deficient mice. Five weeks later, CD4+ or CD8+ T cell populations in the blood of BM-transferred mice were analyzed by flow cytometry. The frequencies of CD4+ or CD8+ cell populations were similar between mice transferred with WT and KO BM and also similar to those of C57BL/6 mice (C57BL/6 CD4: 38.9 ± 6.9%; p = 0.457 compared with WT CD4, p = 0.088 compared with KO CD4; WT CD4: 39.3 ± 4.4%, p = 0.125 compared with KO CD4; KO CD4: 45.1 ± 4.6%; C57BL/6 CD8: 18.9 ± 1.5% p = 0.309 compared with WT CD8, p = 0.370 compared with KO CD8; WT CD8: 17.3 ± 4.1%, p = 0.212 compared with KO CD8; KO CD8: 16.4 ± 3.8%). Mice with good constitution of lymphocytes were immunized with MOG peptide and CFA to induce EAE. CCR6-deficient chimeric mice showed significantly reduced EAE disease compared with WT mice (Fig. 6A). Because the disease score in CCR6-deficient mice caught up with the disease score in WT mice in the later time point, the other chemokines expressed by Th17 and the inflammatory site might help the migration of Th17 cells. To understand the underlying causes of reduced disease in CCR6-deficient chimeric mice, infiltrating cells in the CNS and the cytokine production by CD4+ cells in both CNS and spleen were analyzed. Although CD4+ or CD11b+ cell percentages in the CNS were not different (data not shown), the absolute cell numbers of

Th17 cells have been recently shown as a major pathogenic T cell subset in various autoimmune diseases, including EAE (12). Although there has been considerable progress on the regulation of Th17 differentiation programs, regulation on Th17 migration to the inflammatory tissues has been unclear. To assess the CCR6 effect in inflammatory tissue, CCR6 KO and control C57BL/6 mice were immunized with MOG peptide with CFA to induce EAE. CCR6

**FIGURE 4.** CCL20 does not regulate the differentiation or cytokine production of Th17 cells. A, FACS-sorted naive CD4+ T cells were stimulated with anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/mL) under Th1 conditions (20 ng/ml IL-12 and 5 μg/ml anti-IL-4) or Th17 conditions (10 ng/ml TGF-β, 50 ng/ml IL-6, 30 ng/ml IL-23, 5 μg/ml anti-IFN-γ, and 5 μg/ml anti-IL-4) in the presence or absence of CCL20 (10 ng/ml) for 4 days. Intracellular cytokine staining was performed with allophycocyanin-labeled anti-IFN-γ and PE-labeled IL-17 after permeabilization. The results are representative of three independent experiments. B, In vitro-differentiated Th17 cells were restimulated with anti-CD3 (3 μg/ml) with or without CCL20 (10 ng/ml) for 3 days. The production of IL-17 and IL-17F in the culture supernatants was measured by ELISA. The data has no statistical difference between T cells in the presence of CCL20 and absence of CCL20 (IL-17, p = 0.322; IL-17F, p = 0.391). Similar results were obtained from three independent experiments. C, Naive CD4+ T cells from WT or CCR6−/− mice were differentiated to Th17 cells with anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/ml) in the presence of TGF-β (10 ng/ml), IL-6 (50 ng/ml), IL-23 (30 ng/ml), anti-IFN-γ (5 μg/ml), and anti-IL-4 (5 μg/ml). Four days after culture, the cells were analyzed for Th17 differentiation in flow cytometry by staining with allophycocyanin-labeled IFN-γ and PE-labeled IL-17. The results are representative of two independent experiments.

**FIGURE 5.** The migration of Th17 and Treg cells is mediated by CCR6 and CCL20 interaction. A, Transwell chemotaxis assay on Th1, Th2, and Th17 cells in the presence of CCL20. The migrated cells were analyzed at 7 h of culture. Data shown were repeated twice with consistent results. B, The migration of naive CD4+ T cells and nTreg cells was evaluated in response to CCL20. Data shown were repeated twice with consistent results. C, WT or CCR6-deficient Th1, Th17, and Treg cells were examined in chemotaxis assays in response to CCL20. Data shown were repeated twice with consistent results. D, The migration of naive CD4+ T cells and nTreg cells was evaluated in response to CCL20. Data shown were repeated twice with consistent results.
FIGURE 6. CCR6 deficiency in lymphocytes leads to inhibited EAE diseases. A, CCR6−/− or C57BL/6 mice (five mice in each group) were immunized with MOG peptide to induce EAE. Disease scores in each group were evaluated. The data are shown as the mean ± SD. The data represent one of two experiments with similar results. B–K, Rag1−/− chimeric mice reconstituted with BM cells from C57BL/6 or CCR6−/− mice were immunized with MOG peptide to induce EAE. B, Disease scores in five mice in each group were evaluated. The data are expressed as the mean ± SD of five mice. The data represent one of two experiments with similar results. C and D, The CNS-infiltrating cells were analyzed by staining with PerCP-labeled anti-CD4 and FITC-labeled anti-CD11b. The analysis was done on five mice in each group. E, The population of CD4+CD25+Foxp3+ cells was analyzed in the cells from the CNS. The analysis was done on five mice in each group. F–H, IL-17+ or IFN-γ+ cells in mononuclear cells in the CNS were analyzed in a CD4+ gate. The analysis was done on five mice in each group. Representative data from flow cytometry are shown in F. The data of cell percentage are expressed as the mean ± SD of five mice. The absolute cell numbers of IL-17+ cell and IFN-γ+ cells were shown in G and H. I–K, IL-17+ or IFN-γ+ cells in spleen cells were analyzed in a CD4+ gate. The data from flow cytometry was shown in I. The analysis was done on five mice in each group. The data of cell percentages are expressed as the mean ± SD of five mice. The absolute cell numbers of IL-17+ cell and IFN-γ+ cells were shown in J and K. The analysis was done on five mice in each group. B–J, Circles represent individual mice. The horizontal lines represent the mean. The results shown are representative of two independent experiments. *, p < 0.01 and **, p < 0.05.

CD4+ or CD11b+ infiltrating cells were significantly reduced in the CCR6−/− chimeric mice (Fig. 6, C and D), supporting reduced CNS inflammation in these mice. Absolute cell numbers of Treg cells, but not the percentages of Foxp3+ Treg cells in total cells in CNS (data not shown), were also significantly decreased in the KO chimeric mice (Fig. 6E). The percentages of IL-17+ but not IFN-γ+, cells were significantly reduced in the CNS of the KO chimeric mice (Fig. 6F), indicating that CCR6 may be more important for Th17 than Th1 cells. However, the absolute cell numbers of IL-17+ or IFN-γ+ cells in the CNS were both reduced in the KO chimeric mice (Fig. 6, G and H). Next, we examined T cells in the spleen by restimulation with MOG peptide and intracellular staining. The percentages of MOG-specific IFN-γ+ T cells in the spleen were significantly increased in the KO chimeric mice while IL-17+ cells were similar between WT and KO chimeric mice (Fig. 6I). Moreover, there appeared to be more total IL-17+ and IFN-γ+ cell numbers in the spleens of KO chimeric mice (Fig. 6, J and K). In addition, spleen cells were cultured with MOG peptide and MOG-specific T cell responses were measured. No significant difference was found in MOG-specific T cell proliferation or IL-2, IL-17, and IFN-γ production between WT and KO cells (data not shown). Thus, immune responses against MOG peptide were similar between WT and KO chimeric mice. These results indicated that although there was no defective expression of IL-17 in the spleen in the KO chimeric mice, Th17 cells appeared to be reduced in both numbers and percentages in the CNS.

The above results suggest that CCR6-mediated Th17 migration to inflamed tissues may be important for driving CNS inflammation. To directly confirm the role of CCR6 on Th17 cell migration and function in EAE, C57BL/6 and CCR6 KO were immunized with MOG peptide and Ag-specific Th17 cells were expanded in vitro with MOG and IL-23 and adoptively transferred into C57/BL6 recipient mice. After expansion by MOG and IL-23, C57BL/6 or CCR6-deficient T cells contained ~40% of IL-17+ cells, 5% of IFN-γ+ cells, and 10% of Foxp3+ cells, respectively. The recipient mice were subsequently immunized with MOG and...
CCR6<sup>−/−</sup> Th17 cells fail in induction of EAE. A, IL-23-cultured MOG-reactive CD4<sup>+</sup> T cells from C57BL/6 or CCR6 KO mice were adoptively transferred into five C57BL/6 mice in each group. The mice were immunized with MOG to induce EAE. A, Disease scores in five mice in each group were evaluated. The data are shown as the mean ± SD of five mice. B and C, CD4<sup>+</sup> (B) or CD11b<sup>+</sup> cells (C) in the mononuclear cells from CNS were analyzed in five mice in each group by staining with PerCP-labeled anti-CD4 and FITC-labeled anti-CD11b. D, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells were analyzed in the CNS of five mice in each group. E–G, IL-17<sup>+</sup> or IFN-γ<sup>+</sup> cells in the CNS of five mice in each group were analyzed in mononuclear cells in a CD4<sup>+</sup> gate. The data from flow cytometry are shown in E. The data of cell percentages are expressed as the mean ± SD of five mice. The absolute cell numbers of IL-17<sup>+</sup> cells and IFN-γ<sup>+</sup> cells in the CNS of five mice in each group were shown in F and G. H–J, IL-17<sup>+</sup> or IFN-γ<sup>+</sup> cells in spleen cells of five mice in each group were analyzed in a CD4<sup>+</sup> gate. The data from flow cytometry are shown in H. The data of cell percentages are expressed as the mean ± SD of five mice. The absolute cell numbers of IL-17<sup>+</sup> cells and IFN-γ<sup>+</sup> cells in spleen cells of five mice in each group are shown in I and J. B–J, Circles represent individual mice. The horizontal lines represent the mean. All results are representative of two independent experiments. *, p < 0.01 and **, p < 0.005.

CFA to boost the MOG-specific transferred cells in vivo. The development of EAE was greatly reduced in mice receiving KO Th17 cells than those injected with WT cells (Fig. 7A). The numbers of infiltrating CD4<sup>+</sup> cells in the CNS were greatly reduced in mice receiving CCR6-deficient T cells, but there was no significant difference in CD11b<sup>+</sup> cells (Fig. 7, B and C). The percentages of CD4<sup>+</sup> cells in the total CNS cells were also significantly decreased in the KO Th17 cell-transferred mice (data not shown). In contrast, the absolute numbers of Treg cells (Fig. 7D) as well as their percentages in total cells of the CNS (data not shown) also showed significant reduction. In addition, IL-17<sup>+</sup> or IFN-γ<sup>+</sup> cells in the CNS were reduced in percentages (Fig. 7E) and in total numbers in KO T cell-transferred mice (Fig. 7, F and G). In spleen, although the percentages of IL-17<sup>+</sup> cells were less in KO T cell-transferred mice (Fig. 7H), the total numbers of IL-17<sup>+</sup> cells were not significantly different between two experimental groups (Fig. 7I). In addition, there was no difference in the percentages of IFN-γ<sup>+</sup> cells in the spleen (Fig. 7H). Although the absolute cell numbers of IFN-γ<sup>+</sup> cells were not significantly different in KO T cell-transferred mice, IFN-γ<sup>+</sup> cells appear to be more in the spleen of KO T cell-transferred mice (Fig. 7J). These results indicated that CCR6-deficient autoreactive Th17 cells failed in migration to the CNS, which resulted in blockade of EAE development.

**CCR6 regulates the migration of Treg cells to inflamed site**

To understand the function of CCR6 in Treg cell migration in autoimmune disease, Rag<sup>1−/−</sup> mice were transferred with the mixture of WT naive CD4<sup>+</sup> T cells from CD45.1-congenic mice and WT Treg or CCR6<sup>−/−</sup> Treg cells. The mice were immunized with MOG to induce EAE. No significant difference was observed in the clinical scores between mice receiving WT and CCR6<sup>−/−</sup> Treg cells (data not shown). However, the frequency of Treg cells in the CNS was significantly lower in mice transferred with CCR6<sup>−/−</sup> Treg cells (data not shown). Also, the absolute cell numbers of CCR6<sup>−/−</sup> Treg cells in the CNS was significantly less than WT Treg cells (Fig. 8A). In contrast, the frequencies (data not shown) and absolute cell numbers of Treg cells in the spleen were not significantly different (Fig. 8B), although Treg cells appeared to be increased in the spleen of CCR6<sup>−/−</sup> Treg cell-transferred mice (Fig. 8B).

CCR6 function in the migration of Treg cells to the inflamed sites was also investigated in a different way. We transferred Treg from...
The absolute cell number of CD45.2 D cells and transferred into five RAG1 CCR6 /H11001 mice. After the mice were an-

The distribution of Treg cells in the CNS. Approximately 10% of Treg cells in the CNS produced IL-17 (44), the underlying mech-

The absolute cell number of CNS-infiltrating CCR6 /H11001 (1 cells and transferred into five RAG1 CCR6 /H11002 mice. The mice were immunized with MOG peptide to induce EAE. A. The absolute cell number of CNS-infiltrating Treg cells was analyzed in CD45 Foxp33 gate. The data of absolute cell number are expressed as the mean ± SD of five mice. B. The absolute cell number of Treg cells in the spleen were analyzed in the CD45 Foxp33 gate. The data of absolute cell number are expressed as the mean ± SD of five mice. C. The data of absolute cell number are expressed as the mean ± SD of two mice. D. The absolute cell number of CD45.2 Treg cells derived from WT or CCR6−/− mice were analyzed by staining with anti-CD45.2 and anti-Foxp3 in CD45 gate. The data of absolute cell number are expressed as the mean ± SD of two mice. E. Foxp3-GFP mice (three mice in each group) were immunized with MOG/CFA to induce EAE. After the mice reached EAE disease score 3, the cells in the CNS were harvested and analyzed for CCR6 and IL-17 expression. All data shown are representa-

either WT or CCR6−/− mice, that are CD45.2, into C57BL/6/mice congenic for CD45.1. These recipient mice were further immunized with MOG and CFA to induce EAE. No significant difference in the clinical scores was found in the mice transfected with WT or CCR6−/− Treg cells (data not shown). Similar to the result in Fig. 8, A and B, the CCR6−/− donor’s T Cells showed significantly lower percentages (data not shown) and the absolute cell numbers than their WT controls in the CNS (Fig. 8C). In contrast, no significant differ-
ence was observed in the frequencies (data not shown) and absolute cell numbers of Treg cells in the spleen (Fig. 8D).

It has been reported that human Treg cells produce IL-17 (33). To assess IL-17 production in CNS-infiltrating CCR6+ Treg cells of EAE mice, Foxp3-GFP mice (34) were induced EAE and when they reached EAE disease score 3 analyzed for their IL-17 produc-
tion in CCR6+ Treg cells in the CNS. Approximately 10% of CNS-infiltrating CCR6+ Treg cells produced IL-17 (10.84 ± 4.57%) and 9% of CCR6− Treg cells in the CNS produced IL-17 (9.14 ± 2.36%; Fig. 8E). Therefore, small populations of Treg cells express IL-17, regardless of CCR6 expression. These results indicated that CCR6 plays a critical role in Treg cells by mediating the migration of Treg cells to the inflamed sites.

**Discussion**

Our study presented here has demonstrated that CCR6 expressed on Th17 and Treg cells plays a critical role in Th17 and Treg migration to inflammatory sites. In the absence of CCR6, Th17 and Treg cells were impaired in infiltration into the CNS during EAE disease.

Differentiation of naïve CD4+ T cells to Th17 cells is mediated by TGF-β and IL-6 signaling via STAT3, RORγt, and RORα transcription factors, which is further enhanced and maintained by IL-23 and IL-21. Among Th1, Th2, and Th17 subsets, Th17 cells expressed both CCR6 and CCL20, which were also recently reported in human Th17 cells (28, 29). However, these two mole-

molecules appear to have different regulatory mechanisms. The expression of CCL20 on Th17 cells is regulated similarly as IL-17. It is induced by TGF-β along with IL-6 or IL-21, in which both RORγ and STAT3 were required. However, CCR6 expression in Th17 cells does not require RORγ, due to a redundant function of RORα. In addition, CCR6 was induced by TGF-β alone. Since TGF-β also induces Foxp3-expressing iTreg cells, we went on and found expression of CCR6 in both iTreg and nTreg cells, consistent with a previous report (25). CCR6 expression remains or even is increased in IL-21−/− cells, possibly due to the expression by iTreg cells. Expression of CCR6 in Th17 and Treg cells not only supports their intimate relationship, but also has a biological sig-

nificance in regulation of coordinated localization of pro- and anti-
inflammatory T cells.

Th17 cells have been implicated in various inflammatory dis-

eases that affect different target organs, including EAE, rheumatoid arthritis, allergic pulmonary inflammation, and psoriasis (35, 36). Once primed in lymphoid organs, how effector Th17 cells are re-
cruited to large numbers to these inflammatory tissues has been unclear. Our results indicate an essential function of CCR6 in the organization of inflammatory reactions. Chemotaxis assays demon-

strated that Th17 cells and Treg cells migrated in response to CCL20 in a CCR6-dependent manner, indicating that CCR6 on both cells are functional. Moreover, Th17 cells, by producing CCL20, could also attract other Th17 cells via CCR6. In addition to a Th17 source, CCL20 could be also induced in nonhemopoietic cells by IL-17 (6), dependent on Act1 adaptor protein (37). Therefore, we propose that the first wave of inflammatory Th17 cells, once entered into the target tissues and become reactivated by local APCs, will express CCL20 and IL-17. The latter will induce CCL20 expression by other residential cell types. Production of CCL20 leads to further recruitment of other CCR6-expressing Th17 cells and sustained chronic inflammation. Memory Th17 cells with different antigenic specificity may be recruited into the inflammatory site, possibly resulting in an epitope spreading. Our hypothesis is supported by our EAE experiments, where CCR6-deficient MOG-specific Th17 cells did not initiate CNS inflamma-

tion. Th17 cells were greatly reduced in CNS but still were found in large numbers in spleen. Thus, CCR6 is important for Th17 migration to inflammatory tissues and may mediate an amplifying regulation to sustain inflammatory responses.

In contrast, it has been reported that Treg cells inhibit various autoimmune diseases, such as EAE (38, 39), diabetes (40), colitis (41), gastritis (42), and collagen-induced arthritis (43). In EAE, Ag-specific Treg cells have been recently reported to accumulate in the CNS along with effector T cells (44), the underlying mech-

anism of which is still unknown. In this study, we showed that in

![FIGURE 8. CCR6 regulates the distribution of Treg cells in EAE. A and B, WT naive CD4+ T cells were mixed with either WT or CCR6−/− Treg cells and transferred into five RAG1−/− mice in each group. The mice were immunized with MOG peptide to induce EAE. A. The absolute cell number of CNS-infiltrating Treg cells was analyzed in CD45 Foxp33 gate. The data of absolute cell number are expressed as the mean ± SD of five mice. B. The absolute cell number of Treg cells in the spleen were analyzed in the CD45 Foxp33 gate. The data of absolute cell number are expressed as the mean ± SD of five mice. C. The data of absolute cell number are expressed as the mean ± SD of two mice. D. The absolute cell number of CD45.2 Treg cells derived from WT or CCR6−/− mice were analyzed by staining with anti-CD45.2 and anti-Foxp3 in CD45 gate. The data of absolute cell number are expressed as the mean ± SD of two mice. E. Foxp3-GFP mice (three mice in each group) were immunized with MOG/CFA to induce EAE. After the mice reached EAE disease score 3, the cells in the CNS were harvested and analyzed for CCR6 and IL-17 expression. All data shown are representa-

tive of two independent experiments. *, p < 0.01 and **, p < 0.05.](http://www.jimmunol.org/DownloadedFromHttp://www.Jimmunol.org)
addition to Th17 cells, Treg cells also expressed CCR6, although they did not express CCL20. Treg cells migrated toward to CCL20-producing Th17 in vitro, which was completely dependent on CCR6. In vivo during EAE disease, not only Th17 but also Treg cell numbers were reduced in the CNS, indicating that Treg migration into inflammatory tissues is also mediated by CCR6. Since in mice receiving CCR6−/− Th17 cells, the host Treg cells could still express CCR6, lack of Treg cells in the CNS supports a role of Th17 cells in initiation of inflammatory responses and in the recruitment of Treg cells. In this case, the absence of CCL20 rather than CCR6 expression is important in inhibition of Treg cell migration. In the result of CCR6−/− Treg cell transfer in EAE, CCR6−/− Treg cells were greatly reduced in their migration into the CNS during EAE disease. The clinical disease score of EAE was not different between the mice transferred with WT Treg and CCR6−/− Treg at this stage. It has been reported that migrated Treg cells to the CNS cannot regulate encephalitogenic effector T cells in EAE-developing mice. TNF-α and IL-6 produced by encephalitogenic effector T cells in the CNS suppress the function of Treg cells in the CNS (44). It is possible that transferred Treg cells were also suppressed Treg function by encephalitogenic effector T cells in the CNS of EAE-developing mice. Our study does not formally rule out the importance of other chemokine receptors in trafficking of Treg cells, considering CCR4 has also been reported to be expressed on Treg cells (45). Th1 cells were also reduced in numbers in mice receiving CCR6−/− Th17 cells, suggesting that Th17 may regulate Th1 cell migration in vivo through an unidentified mechanism. Our data not only demonstrate expression of CCR6 on Treg cells and its possible function in Treg cell localization and migration, but also suggest that CCL20-CCR6 interaction in EAE, by recruiting Treg cells, initiates a feedback anti-inflammatory mechanism in the CNS. This adds a complexity in the regulation of inflammatory responses in autoimmunity. One would be cautious about targeting CCR6 in autoimmune diseases as this may also interfere with the natural healing process of the hosts mediated by Treg cells.

In summary, our current work reveals that CCR6 is expressed on not only Th17 cells but also Treg cells. Th17 cells express CCL20 to promote migration of Th17 and Treg cells via CCR6. Our results indicate an important, previously unknown role of CCL20-CCR6 interaction in coordinating the migration of both proinflammatory and Treg cells in autoimmune diseases, which will benefit our understanding of tissue inflammation and autoimmunity.

Disclosures
The authors have no financial conflict of interest.

References


