

# The importance of CCR4 and CCR6 in experimental autoimmune encephalomyelitis

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## ABSTRACT

Chemokine receptors (CCRs) play important roles in the pathogenesis of immune-mediated diseases, as well as in normal immune response. We examined the role of CCR6 and CCR4 in experimental autoimmune encephalomyelitis (EAE) by using CCR6<sup>-/-</sup>CCR4<sup>-/-</sup> double knockout (DKO) and single knockout mice. DKO mice developed less severe EAE and presented repressed recall response in the induction phase, especially in the activity of T helper 17 (Th17) cells. CCR6 expression in central nervous system (CNS)-infiltrated cells was diminished in DKO. Our results suggest that CCR6 and CCR4 were involved in a more rapid progression of EAE and that their regulation might be a therapeutic target of human inflammatory demyelinating diseases.

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## 1. Introduction

C-C chemokines and chemokine receptors (CCRs) play important roles in the immune response by regulating leukocyte migration, infiltration to organs, lymphocyte differentiation, and cytokine production. Chemokine receptors have become clinical therapeutic targets. An anti-CCR4 antibody is now applied to patients with adult T cell lymphoma/leukemia and peripheral T cell lymphoma (Ishida and Ueda, 2006).

Experimental autoimmune encephalomyelitis (EAE) is a T helper 1/17 (Th1/Th17)-mediated autoimmune disease that can be induced in experimental animals as a disease model of human multiple sclerosis (Domingues et al., 2010). Many studies have been performed to evaluate the role of chemokine receptors in EAE, including CCR6 and CCR4 (Elhofy et al., 2009; Liston et al., 2009; Reboldi et al., 2009; Villares et al., 2009; Forde et al., 2011).

CCR4 is expressed in Th2/Th17 and considered to contribute to Th2 activation (Koelink et al., 2011). In atopic dermatitis patients, the number of CCR4<sup>+</sup> memory T cells is increased in the peripheral blood and correlated with clinical severity (Nakatani et al., 2001). On the other hand, Forde et al. reported that CCR4 knockout mice

developed less severe EAE (Forde et al., 2011). CCR6 is expressed in Th17 and dendritic cells and considered to regulate Th1/Th17 differentiation (Koelink et al., 2011). Indeed, Reboldi et al. and Liston et al. reported that CCR6 knockout mice developed less severe EAE and showed repressed recall response (Liston et al., 2009; Reboldi et al., 2009). However, Villares et al. reported that CCR6 knockout mice developed a severe course of EAE and Elhofy reported that CCR6 knockout mice failed to control the relapsing phase of EAE (Elhofy et al., 2009; Villares et al., 2009). The clinical relevance between inflammatory demyelinating disease and chemokine receptors is still controversial. Here, we examined the role of CCR6 and CCR4 in EAE, two major chemokine receptors expressed in pathogenetic Th17 in EAE (Aranami and Yamamura, 2008), using CCR6<sup>-/-</sup>CCR4<sup>-/-</sup> double knockout (DKO) mice, in addition to CCR6<sup>-/-</sup> and CCR4<sup>-/-</sup> single knockout mice.

## 2. Materials and methods

### 2.1. Mice

Mice used for this study were maintained in a specific pathogen-free (SPF) condition in the animal facility of the Kinki University School of Medicine. DKO mice were generated by mating C57BL/6-CCR6<sup>+/-</sup> mice and C57BL/6-CCR4<sup>+/-</sup> mice. CCR6<sup>-/-</sup> and CCR4<sup>-/-</sup> mice were originally obtained from Dr. Yoshie. Mice were backcrossed at least 8 generations onto C57BL/6 background. Genotyping of DKO mice was performed by polymerase chain reaction (PCR) as described elsewhere. All experiments were performed according to the guidelines of the institutional ethics committee. All mice used in the experiments were aged 8 to 16 weeks.

**Abbreviations:** 4KO, CCR4 knockout; 6KO, CCR6 knockout; DKO, CCR6<sup>-/-</sup>CCR4<sup>-/-</sup> double knockout; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; PCR, polymerase chain reaction; WT, wild type.

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## 2.2. Peptides

Peptides 35 to 55 of myelin-oligodendrocyte glycoprotein (MOG<sub>35–55</sub>; single-letter amino acid code: MEVGWYRSPFSRVVHLYRNGK) were purchased from Tore Research Institute (Tore Research, Tokyo, Japan). The peptides were >90% pure, as determined by high performance liquid chromatography (HPLC).

## 2.3. Induction and evaluation of EAE

Mice were injected subcutaneously in the flank, bilaterally, with 0.2 mL of inoculums containing 100 mg of MOG<sub>35–55</sub> and 0.5 mg of mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI) in incomplete Freund's adjuvant. Mice were given 300 ng of pertussis toxin (Sigma-Aldrich, St. Louis, MO) intraperitoneally on day 0 and day 2 postimmunization. Immunized mice were examined daily and scored as follows: 0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; and 5, moribund or dead. Mice were examined daily for signs of EAE in a blind fashion (Miyamoto et al., 2001).

## 2.4. T cell proliferation assay

For the proliferation assay, mice were immunized with peptides/CFA as described above, but they were not treated with pertussis toxin. A single-cell suspension was prepared from the draining lymph nodes (LNs) and spleen on day 11 after immunization. Cells were cultured in RPMI GlutaMAX (Gibco, Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS), and seeded onto 96-well flat-bottomed plates ( $5 \times 10^5$  cells/well). The cells were restimulated with peptide for 48 h at 37 °C under a humidified air condition with 5% CO<sub>2</sub>. To measure cellular proliferation, [<sup>3</sup>H]-thymidine was added (1 µCi/well) and uptake of the radioisotope during the final 18 h of culture was counted with a beta-1205 counter (Pharmacia, Uppsala, Sweden). To evaluate proliferative responses of LN and spleen cells to the peptide, we determined the Δcpm values for cells in each well by subtracting the background cpm and used the mean of these values to represent each mouse.

## 2.5. Cytokine capture beads assay

In parallel, the LN cells from immunized mice were cultured with peptide concentrations of 0, 1, 10, and 100 µg/mL. Supernatants from the cultures were harvested 48 h post activation and tested for the presence of various cytokines. The concentrations of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17a, interferon (IFN)-γ, and tumor necrosis factor (TNF) were measured by using a capture beads assay (BD Bioscience). The assay was performed according to the manufacturer's guidelines.

## 2.6. Pathological analysis

On day 35 after immunization, mice were sacrificed. Brains and spinal cords were harvested and fixed in 10% neutral buffered formalin. Paraffin sections were stained with hematoxylin and eosin (HE) to assess inflammation, and Klüver–Barrera stain to assess demyelination.

## 2.7. Analysis of infiltrating cells isolated from the central nervous system

Wild type (WT) and DKO mice were anesthetized with diethyl ether on day 14 after induction of EAE. After perfusion with PBS, brains and spinal cords were harvested and homogenized. After washing with PBS, mononuclear cells were isolated using Percoll gradient (GE Healthcare, Uppsala, Sweden) and counted (Miyamoto et al., 2006). As a control, the central nervous system (CNS)-infiltrated cells in naïve mice were also analyzed following the same method.

## 2.8. Flow cytometry

Single-cell suspensions were obtained from the spleen and CNS as described above. Briefly,  $2 \times 10^6$  cells were placed in each tube with 100 µL of 3% FBS in PBS, and flow cytometric analysis was performed. Three-color analysis was performed with a combination of fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, allophycocyanin (APC)-conjugated anti-mouse CD3, and phycoerythrin (PE)-conjugated anti-mouse CCR6, CCR4, or purified hamster IgG (BD Pharmingen, San Diego, CA). After Fc-block, samples were stained on ice with predetermined optimal concentrations of antibodies for 30 min, and analyzed on a flow cytometer (BD FACS Calibur). The assay for detecting regulatory T cell was performed using a mouse regulatory T cell staining kit according to the manufacture's guidelines (eBioscience).

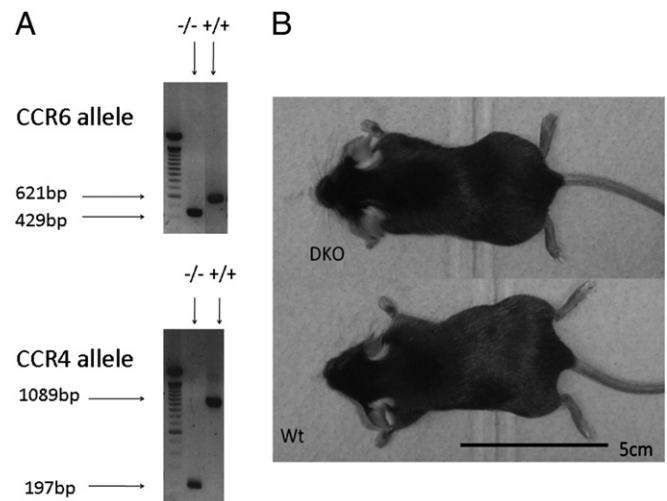
## 3. Results

### 3.1. CCR6<sup>-/-</sup>CCR4<sup>-/-</sup> DKO mice generation

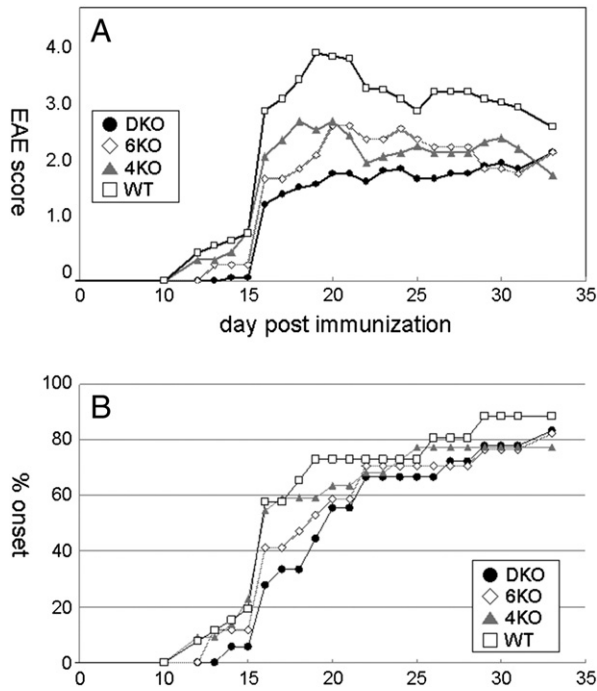
CCR6<sup>-/-</sup>CCR4<sup>-/-</sup> mice were generated by mating CCR6<sup>+/-</sup> and CCR4<sup>+/-</sup> mice. Intercrosses between the CCR6<sup>+/-</sup>CCR4<sup>+/-</sup> mice gave rise to 34 DKO homozygous, 96 CCR6 homozygous, 90 CCR4 homozygous, and 295 mice of other genotypes, nearly proportional to the Mendelian inheritance pattern of 1:3:3:9. The genotype of DKO mice was confirmed by employing a PCR method using tail genomic DNA to amplify a 621-bp product from the WT allele and a 429-bp product from the CCR6 null allele in CCR6<sup>-/-</sup> mice, and a 1089-bp product from the WT allele and a 197-bp product from the CCR4 null allele in CCR4<sup>-/-</sup> mice (Fig. 1A). DKO mice were healthy and no particular changes were found in their gross appearance (Fig. 1B).

### 3.2. Disease severity was suppressed in DKO mice

To examine the role of CCR6 and CCR4 in EAE, C57BL/6 WT control mice (WT, n = 26), CCR6<sup>-/-</sup> (6KO, n = 17), CCR4<sup>-/-</sup> (4KO, n = 22), and DKO (n = 18) were immunized to establish EAE. There were no significant differences in disease incidence (DKO, 77.8%; 6KO, 82.4%; 4KO, 77.3%; and WT, 84.5%). However, DKO exhibited delayed disease onset and less severe disease activity (Fig. 2A). Twenty days after immunization was required to develop neurological symptoms in 50% of DKO mice, whereas 50% of WT mice required 16 days



**Fig. 1.** Generation of double knockout (DKO) mice. A, Genotyping by polymerase chain reaction using genomic DNA. The upper picture shows a 621-bp product from the wild type (WT) allele and a 429-bp product from the CCR6 null allele, and the lower picture shows a 1089-bp product from the WT allele and a 197-bp product from the CCR4 null allele. B, Gross appearance of 8-week-old female DKO and WT mice. No particular change was found.



**Fig. 2.** EAE was induced in double knockout (DKO,  $n = 18$ ), CCR6 knockout (6KO,  $n = 17$ ), CCR4 knockout (4KO,  $n = 22$ ), and wild type (WT,  $n = 26$ ) mice by immunization with MOG<sub>35–55</sub> in complete Freund's adjuvant and monitored daily for symptoms of EAE. A, DKO mice developed less severe EAE. Summaries of disease features are shown in Table 1. B, Delayed disease onset in DKO mice is shown. In order to note neurological symptoms in 50% of DKO, 6KO, 4KO, and WT mice, 20, 18, 16, and 16 days were required, respectively.

(Fig. 2B). With regard to the severity of EAE, DKO showed least severe EAE among all groups and a significantly lower score on day 19 (DKO,  $1.29 \pm 0.31$ ; 6KO,  $1.38 \pm 0.36$ ; 4KO,  $1.66 \pm 0.34$ ; WT,  $2.95 \pm 0.37$ , [mean  $\pm$  SEM]). The cumulative EAE score was as follows: DKO,  $23.0 \pm 3.3$ ; 6KO,  $29.9 \pm 5.1$ ; 4KO,  $34.1 \pm 4.0$ ; and WT,  $43.5 \pm 4.5$ . The maximum clinical score was as follows: DKO,  $2.82 \pm 0.25$ ; 6KO,  $3.10 \pm 0.28$ ; 4KO,  $3.18 \pm 0.23$ ; and WT,  $3.52 \pm 0.18$ . Pathological changes in spinal cord sections were also evaluated. DKO mice exhibited less mononuclear infiltration than WT mice. Summaries of disease features are shown in Table 1 and Fig. 2.

### 3.3. Lack of CCR6 and CCR4 reduces uptake of [ $^3$ H]-thymidine in MOG-specific T cells

The proliferative response of draining LNs and spleens of in vitro DKO, or WT immunized with MOG<sub>35–55</sub> was evaluated. On day 11 after immunization, bilateral inguinal LNs and spleens were harvested and the cells of prepared single-cell suspensions were counted. No particular change was found in spleens collected from DKO mice compared with naïve mice, whereas WT mice developed splenomegaly. The cell count from spleens of DKO mice was lower than that of WT mice

(DKO,  $n = 6$ ,  $83.0 \pm 8.5 \times 10^6$ ; WT,  $n = 7$ ,  $101.6 \pm 9.9 \times 10^6$ , Fig. 3A). Uptake of [ $^3$ H]-thymidine was also significantly downregulated in spleen cells of DKO mice ( $9100 \pm 868$  versus  $15923 \pm 3799$ ,  $P < 0.05$ , Fig. 3B). In cells from LNs, there was no significant difference.

### 3.4. IL-6 and IL-17 production was downregulated in DKO mice

The cytokine concentrations in the culture supernatants were estimated by using a cytometric bead assay (CBA). IL-6 and IL-17a production was significantly lower in DKO mice than in WT mice (IL-6,  $16.1 \pm 4.9$  versus  $49.4 \pm 9.2$ ; IL-17a,  $17.4 \pm 2.5$  versus  $38.3 \pm 7.4$ ; DKO,  $n = 8$ ; WT,  $n = 7$ ;  $P < 0.01$ ). IFN- $\gamma$  and TNF levels were also slightly lower, although not significant (IFN- $\gamma$ ,  $295 \pm 131$  versus  $384 \pm 94$ ; TNF,  $72.9 \pm 17.5$  versus  $100.4 \pm 14.2$ ; DKO,  $n = 8$ ; WT,  $n = 7$ ). IL-2, IL-4, and IL-10 were not detected. This finding might suggest that Th17 activity, rather the Th1 activity, was suppressed in DKO mice. A summary of the data is shown in Fig. 4.

### 3.5. Cellular infiltration into the CNS was repressed in DKO mice

We examined the isolated cells from the immunized murine CNS to analyze the role of CCRs on migration of inflammatory cells into the CNS. The number of mononuclear cells in the CNS isolated from DKO mice was less than that from WT mice (Fig. 5; DKO,  $n = 6$ ,  $12.5 \pm 3.1 \times 10^5$ ; WT,  $n = 6$ ,  $17.7 \pm 1.4 \times 10^5$ ; naïve,  $10.2 \pm 2.2 \times 10^5$ ,  $n = 6$ ). In parallel, lymphocytes isolated from the CNS and spleens were analyzed by flow cytometry. Compared with peripheral T cells, CCR6 expression of infiltrated T cells in WT was increased, whereas no CCR6 expression was detected in both CNS and peripheral T cells of DKO mice (Fig. 6). Next, we evaluated the number of CD4 $^+$ CD25 $^+$ Foxp3 $^+$  regulatory T cells in spleens; however, there was no significant difference between the groups (data not shown).

## 4. Discussion

Chemokines and CCRs play important roles in mediating various autoimmune diseases, including multiple sclerosis (MS). Many studies were conducted to identify an association between EAE and CCRs, expecting they become therapeutic targets in MS. Here, we demonstrated that DKO, CCR6 $^{-/-}$ , and CCR4 $^{-/-}$  mice developed less severe EAE. This finding suggests that CCR6 and CCR4 play an important role in deteriorating the course of EAE, especially in activating MOG-specific T cells in the induction phase. The recall response showed an inhibited T cell proliferation response and decreased Th17-associated expression of cytokines in DKO mice. The number of CNS-infiltrated cells was significantly lower in DKO mice than in WT mice. In WT mice, the CCR6 expression was increased in CNS-infiltrated T cells compared with that of splenic T cells. These results might reflect either repressed recall response or relevance of CCR6 and CCR4 to blood-brain barrier migration, which would be confirmed by adoptive transfer EAE.

Our results and the report of Forde et al. showed that CCR4 knockout reduced the severity of EAE, although no obvious CCR4 expression was observed in our study. CCR4 has been conventionally considered as a marker of Th2 activation (Koelink et al., 2011). Indeed, administration of an anti-CCR4 antibody to human healthy subjects reduced Th2-mediated cytokine production; however, it had no effect on the production of Th1-mediated cytokines (Yamane-Ohnuki and Satoh, 2009). On the other hand, Shimizu et al. reported that the numbers of CD4 $^+$ CCR4 $^+$  cells and CD8 $^+$ CCR4 $^+$  cells in peripheral blood of MS patients were higher in the relapsing phase than in the remission phase or in healthy controls (Shimizu et al., 2011). Recent studies showed that Th17 cells co-express CCR4 and CCR6 and that the proportions of CCR4 $^+$ CCR6 $^+$ CD4 $^+$  correlate with the disease activity of MS (Mehling et al., 2010). These findings might indicate that CCR4 was necessary in activating pathogenic Th1/Th17 cells, and inhibition of Th2 cells did not necessarily mean Th1 stimulation. Recently

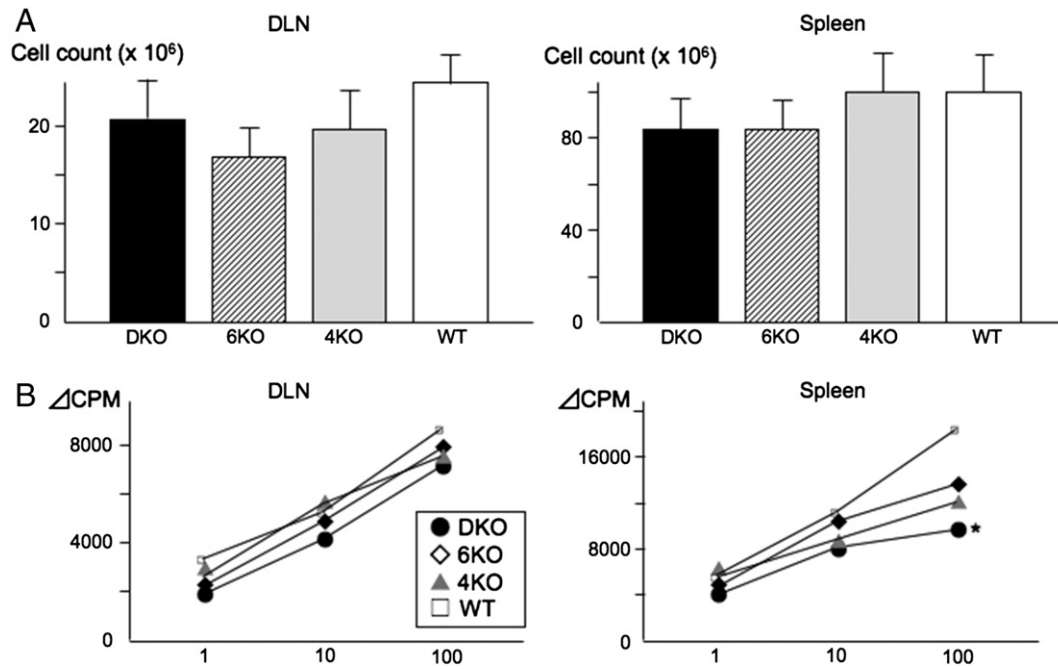
**Table 1**  
Summary of clinical experimental autoimmune encephalomyelitis (EAE).

	Incidence	Day of onset	Max score	Cumulative score
DKO ( $n = 18$ )	77.8% (14/18)	$20.4 \pm 1.36$	$2.82 \pm 0.25^*$	$23.0 \pm 3.3^{**}$
6KO ( $n = 17$ )	82.4% (14/17)	$19.4 \pm 1.43$	$3.10 \pm 0.28$	$29.9 \pm 5.1^*$
4KO ( $n = 22$ )	77.3% (17/22)	$17.1 \pm 0.92$	$3.18 \pm 0.23$	$34.1 \pm 4.0$
WT ( $n = 26$ )	84.5% (22/26)	$18.2 \pm 1.01$	$3.52 \pm 0.18$	$43.5 \pm 4.5$

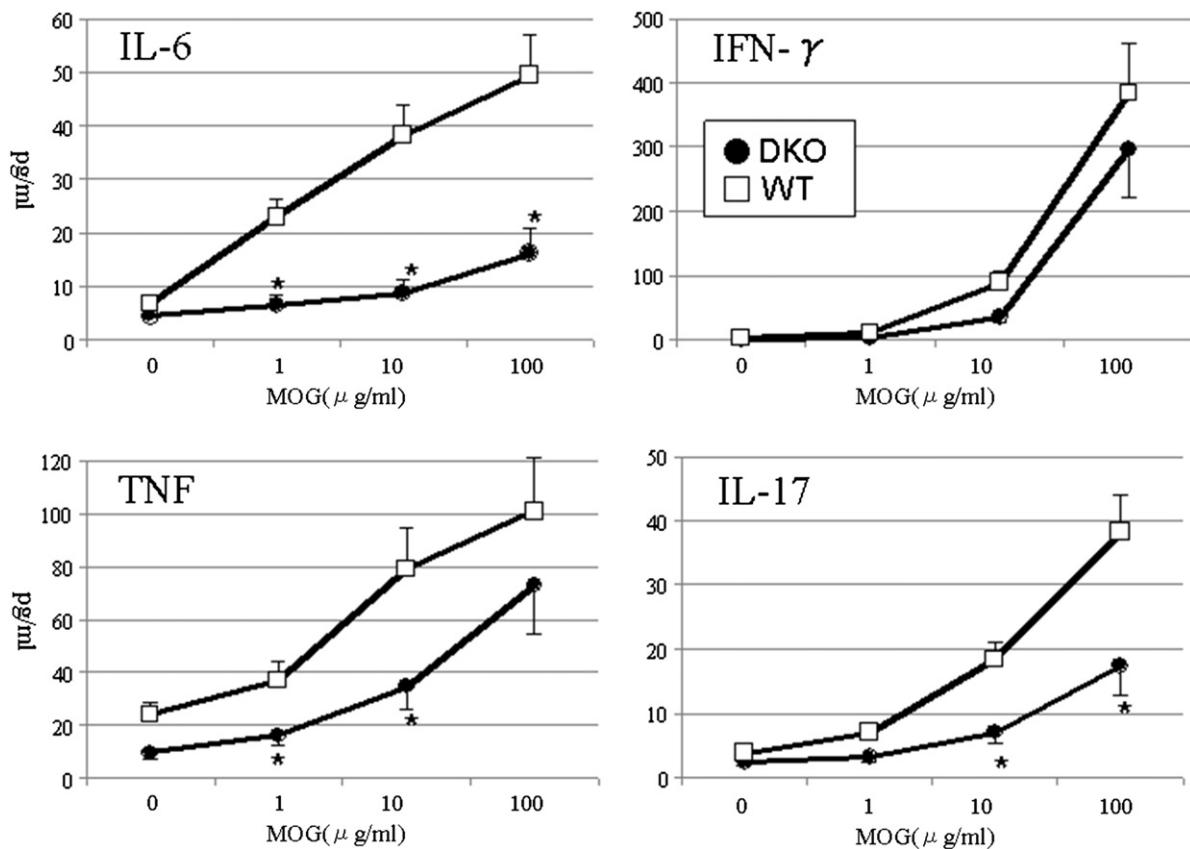
Mean  $\pm$  standard error.

DKO, double knockout; 6KO, chemokine receptor 6 knockout; 4KO, chemokine receptor 4 knockout; WT, wild type.

\*\* $p < 0.001$ , \* $p < 0.05$  versus WT by Mann–Whitney  $U$  test.

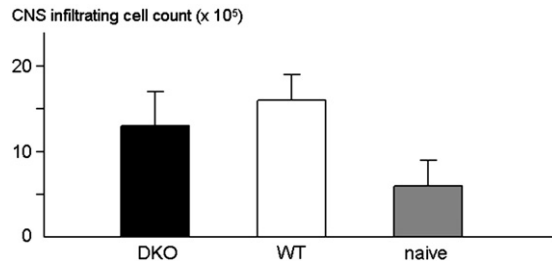


**Fig. 3.** Comparison of MOG<sub>35–55</sub> T cell responses in DKO and WT mice. **A**, Cell count of lymphocytes isolated from draining lymph nodes (LN) and spleens. The cell count of cells isolated from spleens of DKO mice was lower than that of WT mice (DKO,  $n=6$ ,  $83.0 \pm 8.5 \times 10^6$ ; WT,  $n=7$ ,  $101.6 \pm 9.9 \times 10^6$ ). The cell count of cells harvested from LNs was also slightly decreased in DKO mice (DKO,  $21.0 \pm 3.9 \times 10^6$ ; WT,  $24.4 \pm 3.5 \times 10^6$ ). **B**, The proliferative response was determined by the uptake of [<sup>3</sup>H] thymidine. The value of  $\Delta$ cpm was significantly decreased in DKO splenic cells ( $9100 \pm 868$ ,  $n=6$  versus  $15923 \pm 3799$ ,  $n=7$ ;  $P<0.05$  by Mann–Whitney *U* test). \* $p<0.05$  versus WT by Mann–Whitney *U* test.



**Fig. 4.** Cytokine concentrations of culture supernatants were measured by using a cytometric bead assay (CBA). The production of IL-6 and IL-17a was significantly suppressed in double knock out (DKO) mice (IL-6,  $16.1 \pm 4.9$  versus  $49.4 \pm 9.2$ ; IL-17a,  $17.4 \pm 2.5$  versus  $38.3 \pm 7.4$ ; DKO,  $n=8$ ; WT,  $n=7$ ;  $P<0.01$  by Mann–Whitney *U* test). The production of interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF) was also decreased, although not significantly (IFN- $\gamma$ ,  $295 \pm 131$  versus  $384 \pm 94$ ; TNF,  $72.9 \pm 17.5$  versus  $100.4 \pm 14.2$ ; DKO,  $n=8$ ; WT,  $n=7$ ). \* $p<0.01$  versus WT by Mann–Whitney *U* test.





**Fig. 5.** Infiltrated cell count in central nervous system. Cell count of mononuclear cells isolated from the CNS of immunized mice and naïve mice. Mononuclear cell count of the CNS isolated from double knockout (DKO) mice was less than that of WT mice (DKO,  $n = 6$ ,  $12.5 \pm 3.1 \times 10^5$ ; WT,  $n = 6$ ,  $17.7 \pm 1.4 \times 10^5$ ; naïve,  $10.2 \pm 2.2 \times 10^5$ ,  $n = 6$ ).

anti-CCR4 antibody has been applied in clinical trial of adult T cell leukemia. This antibody may become a novel therapeutic drug for MS.

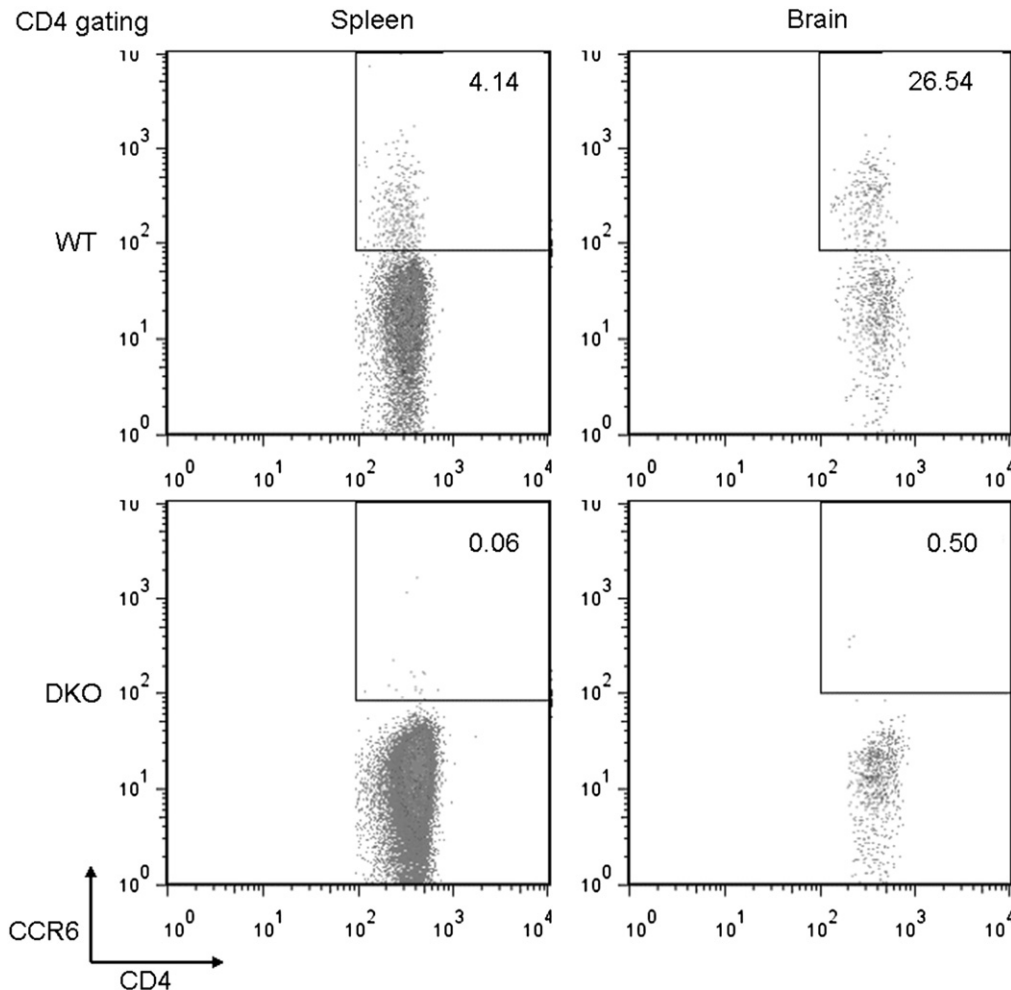
The results of previous reports of EAE in CCR6 single knockout mice are conflicting. This discrepancy might be explained by the complexity of immune mechanisms, i.e., a single cytokine or chemokine has contrary functions *in vitro*, and many different immunological pathways have a similar outcome. Because disruption of IFN- $\gamma$  signaling deteriorates EAE (Ferber et al., 1996), blockage of a single pathogenic pathway in the immune response might accelerate another pathway.

Our results, which focus mainly on analyzing pathogenic T cells in the priming phase, suggest that CCR6 and CCR4 are not essential for generating EAE; however, they play important roles in the more severe progression of EAE. Karpus and Kennedy showed that disease development of adoptive-transfer EAE correlated with CCL3, a ligand for CCR4 and that anti-CCL3 antibodies suppress the disease severity of EAE (Karpus and Kennedy, 1997). Buschmann et al. demonstrated that CCL3 expression correlated with demyelination and microglia/astrocyte activation in cuprizone-induced demyelination model (Buschmann et al., 2012). CCL20, a ligand for CCR6, is also known to be secreted by astrocyte and contribute to the disease course during EAE (Ambrosini et al., 2003). Our result might reflect abolishment of CCL3-CCR4 or CCL20-CCR6 signaling and subsequent inhibition of microglia/astrocyte activation.

Although further researches are required to assess the role of CCR in the effector phase of EAE and the involvement of other immune-mediating cell populations expressing CCRs or their ligands, their regulation might be a therapeutic target of human inflammatory demyelinating diseases.

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**Fig. 6.** Analysis of central nervous system (CNS)-infiltrated cells. In parallel, lymphocytes isolated from CNS and spleen were analyzed by flow cytometry. Compared with peripheral T cells (4.14%), CCR6 expression of infiltrated T cells in WT was increased (26.54%), whereas no CCR6 expression was detected in both peripheral (0.06%) and CNS (0.50%) T cells of DKO mice.

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