Journal of Neuroimmunology 257 (2013) 53–58

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

The importance of CCR4 and CCR6 in experimental autoimmune encephalomyelitis

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ARTICLE INFO

Article history:
Received 4 October 2012
Received in revised form 19 January 2013
Accepted 11 February 2013

Keywords:
Experimental autoimmune encephalomyelitis
Chemokine receptor
Th17
CCR6
CCR4

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 $CCR6^{-/-}CCR4^{-/-}$ 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.

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 $CCR4^{+}$ 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 $CCR6^{-/-}CCR4^{-/-}$ double knockout (DKO) mice, in addition to $CCR6^{-/-}$ and $CCR4^{-/-}$ 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-$CCR6^{+/-}$ mice and C57BL/6-$CCR4^{+/-}$ mice. $CCR6^{-/-}$ and $CCR4^{-/-}$ 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, $CCR6^{-/-}CCR4^{-/-}$ double knockout; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; PCR, polymerase chain reaction; WT, wild type.

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http://dx.doi.org/10.1016/j.jneuroim.2013.02.002

2.2. Peptides

Peptides 35 to 55 of myelin-oligodendrocyte glycoprotein ($MOG_{35–55}$; 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 flan, bilaterally, with 0.2 mL of inoculums containing 100 mg of $MOG_{35–55}$ 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 (5×10^5 cells/well). The cells were restimulated with peptide for 48 h at 37 °C under a humidified air condition with 5% CO_2. To measure cellular proliferation, [^{3}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, 2×10^6 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. $CCR6^{-/-}CCR4^{-/-}$ DKO mice generation

$CCR6^{-/-}CCR4^{-/-}$ mice were generated by mating $CCR6^{+/-}$ and $CCR4^{+/-}$ mice. Intercrosses between the $CCR6^{+/-}CCR4^{+/-}$ 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 $CCR6^{-/-}$ mice, and a 1089-bp product from the WT allele and a 197-bp product from the CCR4 null allele in $CCR4^{-/-}$ 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), $CCR6^{-/-}$ (6KO, n=17), $CCR4^{-/-}$ (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

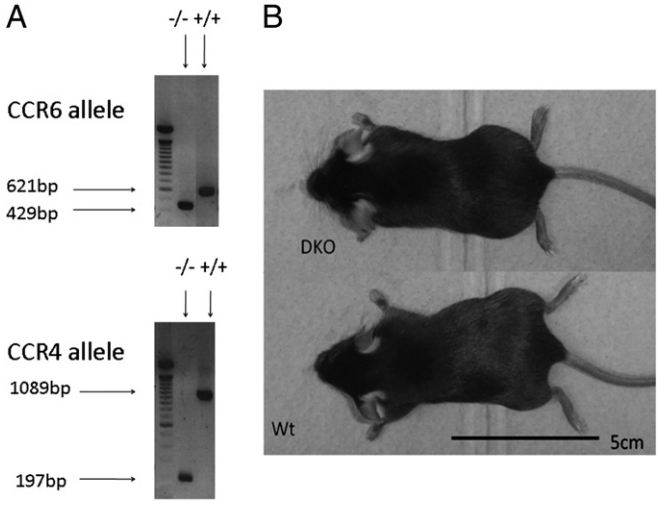

Fig. 1. Generation of double knockout (DKO) mice. A, Genotyping by polymerase chain reaction using genomic DNA. The upper picture shows a 621-bp product from the wild type (WT) allele and a 429-bp product from the CCR6 null allele, and the lower picture shows a 1089-bp product from the WT allele and 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.