Journal of Neuroimmunology 236 (2011) 17–26

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

CCR4 contributes to the pathogenesis of experimental autoimmune encephalomyelitis by regulating inflammatory macrophage function

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ARTICLE INFO

Article history:
Received 25 March 2011
Received in revised form 13 April 2011
Accepted 19 April 2011

Keywords:
Experimental autoimmune encephalomyelitis
Multiple sclerosis
Central nervous system
Inflammatory macrophage
Chemokine receptor
CCR4

ABSTRACT

Chemokines and their receptors play a critical role in orchestrating the immune response during experimental autoimmune encephalomyelitis (EAE). Expression of CCR4 and its ligand CCL22 has been observed in ongoing disease. Here we describe a role for CCR4 in EAE, illustrating delayed and decreased disease incidence in CCR4$^{-/-}$ mice corresponding with diminished CNS infiltrate. Peripheral T cell responses were unaltered in CCR4$^{-/-}$ mice; rather, disease reduction was related to reduced CD11b^{+}Ly6C^{hi} inflammatory macrophage (iMϕ) numbers and function. These results provide evidence that CCR4 regulates EAE development and further supports the involvement of CCR4 in iMϕ effector function.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a T helper cell mediated autoimmune disease of the central nervous system (CNS) that shares many disease characteristics with multiple sclerosis (MS) (Hickey, 1999). The disease manifests itself clinically as ascending hind limb paralysis, induced by immunization with myelin antigen emulsified in complete Freund's adjuvant (CFA) or encephalitogenic CD4^{+} T cell transfer (Whitham et al., 1991; Baron et al., 1993; Bettelli et al., 2003). This results in infiltration of mononuclear cells, including but not limited to; CD4^{+} T cells, CD8^{+} T cells, macrophages (Mϕ) and dendritic cells (DC) into the spinal cord resulting in inflammation and demyelination (Brosnan et al., 1981; Hickey et al., 1983; Jensen et al., 1992; Abdul-Majid et al., 2003; Bailey et al., 2007).

Chemokines are small molecular weight chemoattractants that are produced under steady state and pathological conditions to maintain or influence cell recruitment to specific tissues (Karpus et al., 1995; Huang et al., 2001; Forster et al., 2008). Chemokines can function in differentiation and maturation of leukocytes, in addition to their well described role in cell trafficking and accumulation (Karpus et al., 1997; Marsland et al., 2005). Various chemokines and their cognate receptors are expressed during EAE, and have been shown to be important for disease progression (Karpus and Kennedy, 1997; Fife et al., 2000; Rottman et al., 2000; Fife et al., 2001; Huang et al., 2001; Carlson et al., 2008; Elhofy et al., 2009; Dogan et al., 2011). CCR4 and CCL22 transcripts are known to be expressed in the CNS of mice during EAE (Columba-Cabezas et al., 2002). Recently, we demonstrated inhibition of CCL22 ameliorated EAE in SJL mice by reducing the CNS accumulation of the inflammatory macrophage (iMϕ) population and/or reducing the inflammatory function of that cellular population (Dogan et al., 2011).

In the present study, we sought to distinguish between these two possibilities by utilizing CCR4$^{-/-}$ mice to determine whether CCR4 regulates iMϕ tissue accumulation or inflammatory function. Macrophages are critical for the development of EAE, as evidenced by depletion studies which resulted in greatly diminished disease (Huitinga et al., 1993). Additionally, our lab and others have demonstrated ameliorated disease in CCR2$^{-/-}$ and CCL2$^{-/-}$ mice due to defective macrophage migration (Fife et al., 2000; Huang et al., 2001). The Ly6C^{hi} subset of macrophages are considered inflammatory and are pathogenic during EAE (King et al., 2009), thus inhibition of this specific population of macrophages may provide a more strategic targeting for disease inhibition. Here we report that CCR4-deficient mice display a delay in clinical onset, while maintaining normal peripheral T cell responses. These data support a role for CCR4 in regulating iMϕ effector function during EAE.

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doi:10.1016/j.jneuroim.2011.04.008

2. Materials and methods

2.1. Mice

Female C57BL/6 (H-2^b) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). CCR4$^{-/-}$ (H-2^b) homozygous knockout mice were previously described (Chvatchko et al., 2000) and were a gift from Dr. Amanda Proudfoot. All mice were maintained in the Center for Comparative Medicine at Northwestern University. Mice were 6–8 weeks old at the initiation of experiments and were maintained on standard laboratory chow and water. Animal care was in accordance with Northwestern University Animal Care and Use committee and Public Health Service policies.

2.2. Antigen and antibodies

MOG 35–55 (MEVGWYRSPFSRVVHLYRNGK) (Slavin et al., 1998) was purchased from Peptides International (Louisville, KY). Amino acid composition was verified by mass spectrometry, and purity was determined to be >98%. Fluorochrome-conjugated monoclonal antibodies to murine CD45 (Ly-5), CD4 (RM4-5), CD11b (M1/70), Ly6C (AL-21), IL-17A (TC11-18H10), TNF (MP6-XT22), and IL-4 (BVD4-1D11) were purchased from BD PharMingen (San Diego, CA). Fluorochrome-conjugated monoclonal antibody to murine CCR4 (2G12) was purchased from BioLegend (San Diego, CA), and the isotype control antibody fluorochrome-conjugated antibody to hamster IgG was purchased from Caltag Laboratories (Burlingame, CA). Fluorochrome-conjugated monoclonal antibodies to murine IFNγ (XMG1.2) and Foxp3 (FJK-16s) were purchased from eBioscience (San Diego, CA).

2.3. Histology

Mice were anesthetized, perfused intracardially with ice-cold PBS and spinal cords harvested. Spinal cords were embedded in optimal cutting temperature compound (OCT) and 8–10 μm sections were cut on a cryostat. Frozen sections were stained using standard H&E methodology as previously described (Karpus et al., 1995).

2.4. Disease induction and clinical evaluation

For active EAE, mice were immunized with 100 μg of $MOG_{35–55}$ emulsified in CFA containing 4 mg/ml *Mycobacterium tuberculosis* (Difco, Kansas City, MO) subcutaneously. Mice were given 200 ng of pertussis toxin (List Biological Laboratories) intraperitoneally on days 0 and 2 post immunization. Animals were graded daily according to clinical severity using the following scale: grade 0, no abnormality; grade 1, limp tail; grade 2, limp tail and hind limb weakness; grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; grade 5, death (Miller et al., 2010).

2.5. ELISA

CCL22 expression was quantified from spleen, draining lymph nodes (DLN), and spinal cord samples homogenized in PBS containing 0.05% Tween-20 (Sigma-Aldrich) and clarified by centrifugation at 400×*g* for 10 min. Levels of CCL22 from homogenates were quantified by ELISA. Briefly, flat bottom 96 well plates (Nunc, Rochester, NY) were coated with 200 ng/well monoclonal rat anti-mouse CCL22 (R&D Systems, Minneapolis, MN) in PBS overnight at 4 °C. Nonspecific binding sites were blocked with 2% BSA in PBS for 1 h at 37 °C. Dilutions of tissue homogenate were added in triplicate for 4 h at 37 °C, plates were washed and 5 ng/well biotinylated goat-anti mouse CCL22 (R&D Systems) was added for 1 h at 37 °C. Plates were developed using streptavidin–peroxidase at a 1:5000 dilution (Zymed, San Francisco, CA) for 30 min at 37 °C, followed by TMB substrate–chromogen (Dako, Denmark) and the reaction was stopped using sulfuric acid. The absorbance was read on a plate reader at 450 nm and CCL22 levels were quantified based on a standard curve expressed as pg/ml.

2.6. Flow cytometry

Spinal cords were isolated from the CNS of mice anesthetized and perfused intracardially with ice cold PBS. Mononuclear cells were isolated as previously described (Pope et al., 1996). Briefly, tissues were dissociated in Hank's Balanced Salt Solution (HBSS) by passage through a metal screen (100 mesh) and centrifuged at 1200 rpm for 10 min at 4 °C. The pellet was resuspended in a 30% isotonic Percoll gradient (Pharmacia Biotech, NJ) and centrifuged at 1200 rpm for 10 min at room temperature. Spleens and DLN were also subjected to mechanical disruption through metal screens, centrifuged and resuspended in Dulbecco's Modified Eagle Medium (DMEM). Red blood cells (RBC) from spleen samples were lysed with Tris–NH_4Cl (pH 7.3) and resuspended in DMEM. Cells were collected and washed followed by incubation with specific antibodies to extracellular antigens. For intracellular flow cytometry cells were treated in culture medium with 50 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h, and 1 μl of 1000× monensin (Ebioscience) was added 2 h into stimulation. Cells were stained for extracellular antigens and then fixed with 2% paraformaldehyde for 10 min at room temperature. Cells were permeabilized in 0.5% saponin solution and then stained intracellularly for cytokines and transcription factors. Data was collected on a FACSCanto flow cytometer (BD Biosciences) in the Interdepartmental Immunobiology Center Flow Cytometry facility at Northwestern University and was analyzed offline using FCS Express software (DeNovo Software, Thornhill, Ontario, Canada).

2.7. Statistical analysis

Clinical disease differences, sample mean, standard deviation and statistical significance were calculated using Prism 5.0 (Graphpad). Statistical significance between sample means was analyzed using the Student's t test and differences in disease incidence were analyzed using χ^2. P values <0.05 were considered significant.

3. Results

3.1. CCR4 and its ligand, CCL22, are elevated in the periphery and CNS during EAE

Previous reports have described an increase in CCR4 and CCL22 transcripts during EAE in the CNS of C57BL/6 mice (Columba-Cabezas et al., 2002). Although there are two known CCR4 ligands, CCL17 (TARC) (Imai et al., 1996) and CCL22 (MDC) (Andrew et al., 1998), only CCL22 has been shown to be highly expressed and play a functional role during disease (Columba-Cabezas et al., 2002; Dogan et al., 2011). We set out to evaluate expression of this receptor/ligand pair before and after disease induction in both the periphery and CNS of wild type (WT) mice. Tissue homogenates were harvested at key points in disease and analyzed for CCL22 by ELISA. Protein levels were found to be significantly increased over unimmunized controls at onset of clinical disease in each tissue tested, and at peak of disease in the spleen and CNS (Fig. 1A). We also evaluated CCR4 expression on leukocytes from DLN, spleens, and spinal cords after disease induction. Flow cytometric analysis comparing unimmunized and immunized animals illustrates a marked increase in CCR4 expression on leukocytes in both the periphery and CNS post immunization (Fig. 1B). These data confirm that CCR4 and its corresponding ligand CCL22 are expressed in the target organ during EAE in C57BL/6 mice, suggesting a role in disease development.

3.2. Delayed disease onset in the absence of CCR4

We confirmed leukocyte expression of CCR4 in key tissues following disease induction (Fig. 1B), next we investigated whether