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CCR7 is expressed in astrocytes and upregulated after an inflammatory injury

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ABSTRACT

Neurodegenerative or autoimmune diseases are frequently regulated by chemokines and their receptors, controlling both glial activation and immune cell infiltration. CCL19 and CCL21 have been described to mediate crucial functions during CNS pathological states, regulating both immune cell traffic to the CNS and communication between glia and neurons. Here, we describe the expression pattern and cellular sources of CCR7, receptor of CCL19 and CCL21, in the normal mouse brain. Moreover, we found that CCR7 is upregulated in reactive astrocytes upon intracerebral LPS, regulating early glial reactivity through its ligands CCL19 and CCL21. Our results indicate that CCR7 is playing an important role for the intercellular communication during the inflammatory activation in the CNS.

1. Introduction

Chemokines and their receptors are classified into four families: CXC, CC, C and CX3C chemokines (Rossi and Zlotnik, 2000). Signaling between a chemokine and its receptor is restricted to each family. For example, CCL21 (secondary lymphoid chemokine, exodus-2, 6Ckine) and CCL19 (Mip-3b, EBV-induced molecule 1 ligand (ELC)) are members of the CC family, activating CCR7 and having homeostatic activities in the periphery. However, CCL21 also interacts with CXCR3, being the only known exception of cross-family signaling (Soto et al., 1998). CXCR3 is also activated by its CXC ligands (CXCL9, CXCL10 and CXCL11)(Mantovani, 1999). CCL19 and CCL21 regulate traffic of B cells, naïve T cells and mature dendritic cell to secondary lymphoid organs (Nelson and Krensky, 2001). In the periphery, CCR7 controls the migration and functional activity of regulatory T cells and regulates the establishment of tolerance (Menning et al., 2007).

One of the main roles that has been assigned to chemokines and their receptors is the control of immune cells infiltration into the brain observed in several brain pathologies (Glabinski and Ransohoff, 1999; Mennicken et al., 1999). CCR7 is expressed in multiple sclerosis (MS) lesions and in T cells circulating in the CSF of MS patients (Kivisakk et al., 2004; Serafini et al., 2006). CCL19, ligand of CCR7, is expressed in the normal brain and is upregulated in both MS lesions and in the CSF from MS patients, its expression correlating with the severity of the disease (Krumbholz et al., 2007). Encephalitogenic T cells generated during EAE, an experimental model of MS, show surface expression of CCR7 and CXCR3, acting as receptors for the chemotactic production of CCL19 and CCL21 in venules of the blood brain barrier (Alt et al., 2002). A similar crosstalk between CCR7 and CCL19 has been described controlling leukaemic T cell infiltration into the CNS (Buonamici et al., 2009). Thus, the expression of chemokines CCL19 and CCL21 and their receptors CCR7 and CXCR3 are key features in the control of immune cells traffic into the damaged CNS, being their participation in the local glial inflammatory reaction being an important issue yet to be addressed.

Microglial and astroglial reactivity are a common feature of various nervous system pathologies of autoimmune or neurodegenerative origin (Ransohoff and Perry, 2009). The induction of CCL21 in endangered neurons links CNS early pathological events with glial activation (de Jong et al., 2005). Ischemic mouse neurons express CCL21 to attract CXCR3-expressing microglia, with no direct implication of CCR7 (Bruce-Keller, 1999; Biber et al., 2001; Rappert et al., 2002). CXCR3 is functionally expressed in both microglia and astrocytes (Biber et al., 2002), enabling them to respond to CCL21 in a CCR7-independent manner (Rappert et al., 2002). Stimulation of microglial cells with CCL21 results in increased intracellular calcium transients and chemotaxis through CXCR3 activation (Biber et al., 2001; Rappert et al., 2002; Dijkstra et al., 2004). Moreover, microglial activation in response to an enthorinal cortex lesion was prevented in CXCR3−/− mice, evidencing the impact of this system in the glial response (Rappert et al., 2004).

The expression of CCR7 in the CNS is a controversial issue that remains to be determined. CCR7 expression has been described in the spinal cord in different models of EAE, located in mononuclear cells

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(Bielecki et al., 2007) or microglia (Dijkstra et al., 2006). However, other studies reported no CCR7 expression in the CNS (de Haas et al., 2008). Considering the potential of this system to regulate inflammatory responses within the CNS, a careful description of the expression of CCR7 in the CNS appears necessary. Here, we describe that, in the normal brain, CCR7 was expressed in astrocytes, becoming upregulated after intraventricular LPS administration. CCR7-expressing astrocytes coexisted, in the lesion sites, with activated microglial cells, actively expressing the chemokines CCL19 and CCL21. Taken together, these results suggest that CCR7 expression in astrocytes is a key feature of the neuroimmune crosstalk in the CNS.

2. Materials and methods

2.1. Animals

C57BL/6 mice were originally purchased from Harlan Labs (Barcelona, Spain) and bred in-house (Cajal Institute, Madrid, Spain) for colony establishment and maintenance. Mice were kept on food and water *ad libitum* in a 12-h light/dark cycle. Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC) and the Cajal Institute animal welfare committee.

2.2. Cell culture of mouse astrocytes

Perinatal mouse astrocytes were cultured as previously described (Gomez-Nicola et al., 2008a). Briefly, the cerebral cortices were first mechanically dissociated, centrifuged and further cultured in DMEM/F12 medium (Sigma-Aldrich, St Louis, MO), supplemented with 10% (v/v) FBS, and penicillin/streptomycin (Sigma-Aldrich, St Louis, MO) at 37 °C and 5% CO2. Culture flasks were shaken at 280 rpm overnight at 37 °C, followed by washing and trypsinization (0.25% trypsin, 0.1% EDTA; Sigma-Aldrich, St Louis, MO). Dissociated cells were seeded on tissue culture flasks and treated with DMEM/F12 (24 h; control) or with LPS (lipopolysaccharide from *Escherichia coli*, Sigma L-3755, Serotype 026:B6; 500 ng/ml in DMEM/F12) for 12 or 24 h.

2.3. Intracerebral acute inflammation

CNS acute inflammatory injury was elicited by unilateral intraventricular injection of LPS (lipopolysaccharide from *Escherichia coli*, Sigma L-3755, Serotype 026:B6) as previously described (Gomez-Nicola et al., 2008a). Briefly, LPS solution in PBS (1 μg in 5 μl) was injected in the lateral ventricle of C57BL/6 adult male mice ($n=6$), using a Hamilton syringe. Another group of animals received PBS as a vehicle control ($n=6$). The mice were sacrificed after 4 days, as described for immunohistochemical analysis, examining their brains for correct lateral ventricle injection and increase in ventricular volume. Alternatively, mice were sacrificed to dissect its hippocampus, for western blotting techniques.

2.4. Immunohistochemistry

C57BL/6 mice perfussion, tissue processing and immunohistochemical analysis was performed as previously described (Gomez-Nicola et al., 2008a). The sections were treated successively with 1% methanol/30% H_2O_2 and 5% normal donkey serum/0.1% BSA, to block endogenous peroxidase and non-specific binding, respectively. After repeated rinses with PBS + Tween 20 (PBST; 0.1% v/v), they were incubated overnight at 4 °C with goat anti-mouse CCR7 (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), goat anti-mouse CCL19 (MIP3b; 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), rabbit anti-mouse CCL21 (6Ckine; 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), mouse anti-mouse GFAP (1:2000; Chemicon, Temecula, CA), mouse anti-mouse NeuN (1:2000; Chemicon, Temecula, CA) or biotinylated tomato lectin (1:100; Sigma-Aldrich, St Louis, MO). Following primary antibody incubation, the sections were washed with PBST and incubated with biotinylated donkey anti-goat antibody (1:200, Jackson ImmunoResearch, West Grove, PA), or with the appropriate Alexa 405, 488 or 594 conjugated secondary (1:1000; 1 h 4 °C; Molecular Probes, Leiden, The Nederlands), or Streptavidin-Alexa 488 (for lectin visualization) for cell type-specific triple immunofluorescence. For light microscopy, the sections were washed and incubated with Vectastain ABC complex (Vector Labs, Burlingame, CA) and visualized using diaminobenzidine precipitation. Sections for light microscopy were mounted with DePeX (BDH, Poole, UK) and visualized in an Olympus Provis AX70 microscope coupled to an Olympus DP71 image acquisition system. After immunofluorescence labelling, the sections were mounted with glycerol/DABCO mixture. The sections were visualized on a Leica TCS-SP5 confocal system, coupled to a Leica DMI6000CS microscope. As negative controls, to test goat anti-mouse CCR7 antibody specificity, sections without primary antibody or sections incubated with a non-immune goat antibody were used as negative controls (data not shown), finding absence of tissue labelling. Additionally, western blotting analysis was used to test the CCR7 antibody specificity.

The expression of CCR7 was quantified with the help of an image analysis system (AIS, Imaging Research Inc., Linton, England), using a 4× lens, as previously described (Gomez-Nicola et al., 2008a). The data are presented as percent proportional area (epitope-positive area/scan area). The proportional stained area for CCR7 was determined in each visible hippocampus, subventricular zone (SVZ) or corpus callosum in control animals (PBS) and in animals treated with LPS. Measurements were carried out in 6 animals per group and 5–6 sections/animal.

2.5. Western blotting

Tissue and cell protein extractions were performed as previously described (Gomez-Nicola et al., 2008a,b, 2010a). Protein samples (20 μg protein/lane) were electrophoresed and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). After blocking non-specific binding (5% non-fat milk; 30 min), the blots were incubated with goat anti-mouse CCR7 (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), using mouse anti-glyceraldehyde phosphate dehydrogenase (1:2000; GAPDH; Chemicon, Temecula, USA) as loading control. Blots were incubated with HRP-conjugated donkey anti-goat or goat anti-mouse antibodies (1:5000; Jackson Immunoresearch, Cambridgeshire, UK) and the protein bands were detected using Supersignal west pico or west femto chemiluminescent substrate (Pierce, Rockford, USA).

2.6. Statistical analysis

The analysis of CCR7 expression level was performed using 6 animals per group ($n=6$). Data were expressed as mean ± SEM and analyzed with the STATISTICA 6.0 software package from Statsoft Inc. (Tulsa, OK). For all data sets, normality and homocedasticity assumptions were reached, validating the application of the one-way ANOVA, followed by the Tukey post-hoc test for multiple comparisons. Differences were considered significant for $p<0.05$.

3. Results

3.1. CCR7 is expressed in astrocytes in the central nervous system

CCR7 protein expression in the normal brain, evidenced by immunohistochemical labeling, was found in cells with glial morphology (Fig. 1). The specificity of the antibody, tested as described in the methods section, did not evidence any unspecific labeling (data not shown). CCR7-expressing cells were located in neurogenic niches like the subventricular zone (SVZ; Fig. 1A, B), the rostral migratory stream (RMS; Fig. 1C) and the subgranular layer of the dentate gyrus