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CXCR7 suppression modulates microglial chemotaxis to ameliorate experimentally-induced autoimmune encephalomyelitis

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ABSTRACT

Multiple sclerosis (MS) is the prototypical inflammatory demyelinating disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE), widely used as an animal model of MS, classically manifests as an ascending paralysis that is characterized by extensive infiltration of the CNS by inflammatory cells. Although several studies uncover the significant role of microglia in the development of EAE, the cellular mechanisms of microglia that govern EAE pathogenesis remain unknown. In the current study, we report that CXCR7 expression is dynamic regulated in activated microglia during CNS autoimmunity and positively correlates with the clinical severity of EAE. In addition, microglial chemotaxis is mediated by CXCR7 during CNS autoimmunity, signaling through extracellular signal-regulated kinase (ERK)1/2 activation, whereas p38 mitogen-activated protein kinase (MAPK) and (c-Jun N-terminal kinase) JNK are not involved. Most importantly, CXCR7 neutralizing treatment ameliorates the clinical severity of EAE along with ERK1/2 phosphorylation reduction. Collectively, our data demonstrate that CXCR7 suppression modulates microglial chemotaxis to ameliorate EAE.

1. Introduction

Multiple sclerosis (MS) is the prototypical inflammatory demyelinating disease of the central nervous system (CNS). It is estimated to affect up to two million people worldwide [1]. In order to better understand the immunopathology of MS, various experimental models have been established with the most renowned being experimental autoimmune encephalomyelitis (EAE). EAE classically manifests as an ascending paralysis that is characterized by extensive infiltration of the CNS by inflammatory cells [2]. The inflammatory reaction in the central nervous system (CNS) is driven by induction of auto-reactive immune cells which survey and penetrate the brain [3].

Among kinds of immune cells, microglia are the brain-resident immune cells and represent about 10% of the total brain cell population [4]. Resting microglia have ramified morphology and can actively survey their surroundings sending out long processes to sense events occurring in their microenvironment in the CNS [5]. For several decades, substantial evidences indicate that microglial activation has been observed in both active demyelinating lesions and inflammatory non demyelinating areas of MS brain, persisting for the whole course of the disease [6,7]. Upon activation, microglia alter their morphology, change several surface markers, and secrete diverse cytokines which can modulate cells in a paracrine manner [8]. Although it was shown by others that microglia have the ability to migrate to sites during EAE [9], the actual mechanisms involved in attracting microglia to these sites remain ill defined.

It has been demonstrated that CXCR7 is expressed in many cell types including microglia in the mouse brain and upregulates in EAE, indicating that CXCR7 has a significant role in pathophysiology of MS [10]. Conflicting results raise the debate whether CXCR7 acts as a signaling or non-signaling “decoy” receptor [11]. On the one hand, several studies show that CXCR7 is a decoy receptor and functions as a scavenger to remove extracellular SDF-1 or via hetero-dimerization with CXCR4 indirectly regulating CXCR4 signaling [11–13]. On the other hand, CXCR7 is reported to be capable of triggering SDF-1-mediated macrophages, cancer cell and neuron migration [14–16]. Heretofore, these current studies only focus on the decoy role of CXCR7 as a scavenger to sequester

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extracellular SDF-1 in the autoimmune model EAE, however, CXCR7-mediated chemotaxis and signaling are still unknown, especially in microglia. Furthermore, SDF-1 and I-TAC, the ligands of CXCR7, have also been reported to play crucial roles in MS/EAE [17,18].

Intrigued by these findings, we were prompted to explore the crucial roles of CXCR7 and its implications in the pathogenesis of EAE.

2. Materials and methods

2.1. Animals, induction, treatment and clinical assessment of EAE

C57BL/6 mice were purchased from Vital River. All mice were housed in a specific pathogen-free facility at our campus. All experimental procedures and protocols were approved by the Institutional Authority for Laboratory Animal Care of Wenzhou Medical College.

EAE was induced by MOG_{35-55} in female mice used between 8 and 10 weeks of age. Briefly, each mouse was immunized subcutaneously with 300 μg of MOG_{35-55} emulsified with an equal volume of complete Freund's adjuvant (CFA, total 300 μg of Mycobacterium tuberculosis, strain H37RA, Difco) and then injected with 400 ng of pertussis toxin (Sigma) intraperitoneally at the time of immunization and 2 days later. Mice were weighed and examined for clinical scoring daily by the same investigator after immunization.

For CXCR7 neutralizing treatment, mice were divided randomly into five groups (n = 8 in each group) (a) sham; EAE induction treated with (b) sheep IgG isotype control (R&D Systems) (10 μg); (c) anti-CXCR7 antibody (R&D Systems) (0.1 μg); (d) anti-CXCR7 antibody (1 μg); (e) anti-CXCR7 antibody (10 μg). Treatments began on the day of immunization, and were given by intraperitoneal injection every day until day 25. At the end of the study, mice were sacrificed. Microglia isolated in the equal amount of spinal cords were then counted by chemiluminescence using the luciferase-containing reagent Cell-Titer Glo (Promega).

Neurological assessments were reported using a five-point standardized rating scale to evaluate motor deficit: 0, no deficit; 1, tail paralysis; 2, incomplete hind limb paralysis; 3, complete hind limb paralysis; 4, complete hind limb paralysis and partial forelimb paralysis; 5, moribund state or death.

2.2. Cell preparation, culture and stimulation

The spinal cords of normal or EAE mice were isolated, homogenized, filtrated, centrifuged, and suspended in 70% Percoll (GE Healthcare) and overlaid with 37 and 30% gradient. After centrifugation, the majority of mononuclear cells, found in the interface of 37 and 70% Percoll, were collected, and then were washed twice and resuspended in PBS containing 0.5% bovine serum albumin (BSA) (Sigma). Untouched primary cells were purified by positive selection using Microglia Isolation Kit (Miltenyi Biotec). Subsequently, the purity of isolated monocytes was above 95%, as confirmed by fluorescence-activated cell sorter analysis.

Isolated microglia were cultured in microglial culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum), penicillin (50 U/mL), streptomycin (50 μg/mL), sodium pyruvate (1 mM) and L-glutamine (2 mM). To obtain activated microglia, cells were stimulated with LPS (100 ng/ml) for indicated time. Cultures were maintained in an incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air.

2.3. Real-time reverse transcription PCR (RT-PCR)

Total RNA was isolated using Trizol reagent according to the manufacturer's protocol, and 2 μg of total RNA was converted to cDNA by SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). Brilliant SYBR-Green was combined with cDNA and corresponding primers (Supplementary Table S1) specific for indicated genes and real-time PCR amplification was performed using the ABI Prism 7000 (Applied Biosystems). The expression levels of target genes were measured using comparative Ct method (ddCt) normalized against GAPDH.

2.4. Enzyme-linked immunosorbent assay (ELISA)

2.4.1. ELISA for total and phosphorylated ERK, p38 MAPK and JNK

Total and phosphorylated ERK, p38 MAPK and JNK were measured using ERK1/2 (Total/Phospho (Thr202/Tyr204, Thr185/Tyr187)) InstantOne™ ELISA, p38 MAPK (Total/Phospho (Thr180/Tyr182)) InstantOne™ ELISA and Phospho-JNK (Total/Phospho (Thr183/Tyr185)) InstantOne™ ELISA (eBioscience) separately following the manufacturer's instructions. Detailed procedures are given in the Supplementary material and methods.

2.4.2. Direct ELISA for specificity of anti-CXCR7 antibody

Details are given in the Supplementary material and methods.

2.5. Chemotaxis assay

Chemotaxis assays were performed using 6.5-mm Transwell tissue culture inserts with a 5 μm pore size (Corning). Microglia were suspended at 1×10^6 cells/ml in RPMI 1640 with 0.1% BSA, and 100 μl of cell suspension was added to an insert in a well. The lower compartment was placed with 600 μL of medium containing SDF-1 or I-TAC, the plates were then incubated for 180 min. Microglia found on the bottom of filters were counted as cells that had carried out chemotaxis. Cells were fixed, and stained with the Three Step Stain Set (Richard-Allan Scientific). The migrant cells were counted in five randomly selected high-power fields (400×) per well. The chemotaxis index was calculated as the number of cells that migrated to the sample medium divided by the number of cells that migrated to the control medium. For the chemotaxis inhibition assay, cells were pretreated with indicated inhibitors, a neutralizing anti-CXCR7 antibody or sheep IgG isotype control, and then loaded into the upper chamber.

2.6. Western blot

Details are given in the Supplementary material and methods.

2.7. Statistical analysis

Details are given in the Supplementary material and methods.

3. Results

3.1. CXCR7 expression is induced in microglia activation and is dynamic regulated during EAE

To investigate the role of CXCR7 in microglia activation, primary microglia were stimulated with LPS, a well-established microglia activation inducer. As shown in Fig. 1A, LPS treatment markedly induced CXCR7 mRNA expression upregulating. Specifically, CXCR7 mRNA in microglia was induced as early as 3 h in response to LPS, reached a maximal level at 24 h, and then decreased to a level still significantly higher than that of time-controlled unstimulated cells

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