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IL-9 signaling affects central nervous system resident cells during inflammatory stimuli

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ABSTRACT

Interleukin (IL) 9, a dominant cytokine in Th9 cells, has been proven to play a pathogenic role in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), by augmenting T cell activation and differentiation; however, whether IL-9 signaling affects central nervous system (CNS)-resident cells during CNS autoimmunity remains unknown. In the present study, we found that the IL-9 receptor (IL-9R) was highly expressed in astrocytes, oligodendrocyte progenitor cells (OPCs), oligodendrocytes and microglia cells, and that its expression was significantly upregulated in brain and spinal cord during EAE. In addition, IL-9 increased chemokine expression, including CXCL9, CCL20 and MMP3, in primary astrocytes. Although IL-9 had no effect on the proliferation of microglia cells, it decreased OPC proliferation and differentiation when in combination with other pro-inflammatory cytokines, but not with IFN-γ. IL-9 plus IFN-γ promoted OPC proliferation and differentiation. These findings indicate that CNS-restricted IL-9 signaling may be involved in the pathogenesis of MS/EAE, thus providing a potential therapeutic target for future MS/EAE treatment through disruption of CNS cell-specific IL-9 signaling.

1. Introduction

Interleukin (IL) 9 (IL-9) was originally described as a growth factor for T lymphocytes (Uyttenhove et al., 1988). $CD4^+$ T cells have been shown to be a major source of IL-9 (Monteyne et al., 1997). Although it has been reported that Th2, Th17, regulatory T cells (Tregs), mast cells, or even natural killer T cells can produce IL-9 (reviewed in Noelle and Nowak, 2010), the major source of IL-9 are Th9 cells. The T9 cell owes its name to its secretion of dominant cytokine IL-9, and it is recognized as a distinct helper T cell subset as it neither co-expresses cytokines IL-4, IL-5, IL-13 (Th2), IL-17a (Th17) or IFN-γ (Th1) with IL-9 upon activation (Chang et al., 2010; Dardalhon et al., 2008; Staudt et al., 2010; Veldhoen et al., 2008) nor subset-determining transcription factors including T-bet (Th1), GATA3 (Th2), RORγt (Th17), and FoxP3 Treg cells (Dardalhon et al., 2008; Veldhoen et al., 2008).

Despite a high amount of IL-10 secretion, Th9 cells are not known to have regulatory properties (Dardalhon et al., 2008). Th9 cells exert their biological role through the dominant cytokine IL-9, which has been reported to be involved in many diseases. Accumulating data indicate that IL-9 plays a role in the pathogenic process of allergy, in particular asthma (Knoops et al., 2005; Nicolaides et al., 1997; Wilhelm et al., 2011). IL-9 could drive T-cell mediated colitis by working with its receptor in the intestinal epithelial cells (Gerlach et al., 2014), and IL-9/IL-9 receptor signaling is involved in the pathogenesis of ulcerative colitis (Nalleweg et al., 2014). Functional interactions between IL-9 and mast cells that lead to VEGF release contribute to the initiation/propagation of the pathogenesis of atopic dermatitis (Sismanopoulos et al., 2012).

In addition, Th9/IL-9 involvement in several autoimmune diseases has been reported (Li and Rostami, 2010), e.g., increased IL-9 and Th9 cells have been found in systemic lupus erythematosus (Ouyang et al., 2013). Our previous work showed that IL-9 is important for T cell activation and differentiation in the central nervous system (CNS) autoimmune disease (Li et al., 2011); neutralization of IL-9 would thus ameliorate experimental autoimmune encephalomyelitis (EAE) (Li et al., 2010). It has been reported that CNS-restricted inflammatory signaling plays an important role in EAE (Ding et al., 2015; Yan et al., 2012); however, whether the IL-9 signal pathway in CNS cells is involved in disease development remains unknown. As the actions of IL-9 are mediated through its interaction with IL-9 receptor (IL-9R) (Renauld et al., 1992), in this study, we compared IL-9R expression in CNS tissues during EAE and characterized its expression on different

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CNS cells. In order to further investigate the pathophysiological mechanisms of MS/EAE, we then tested the effect of IL-9 stimulation of various CNS-resident cells in combination with other inflammatory cytokines.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice, 8–10 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Committee of Thomas Jefferson University.

2.2. Antibodies and reagents

Antibodies for CNS-resident cell staining were from the following companies: anti-GFAP (2A5) from StemCell Technologies (BC, Canada); anti-NeuN from Millipore (Billerica, MA); anti-CD11b, antiA2B5 and anti-OSP (oligodendrocyte-specific protein) from Abcam (Cambridge, MA) and antibody for IL-9R from Biolegend (San Diego, CA). Fluorescent-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant IL-9, IL-17, IL-1β, IFN-γ, TNF-α, PDGF, bFGF, NT3 and M-CSF were from PeproTech (Rocky Hill, NJ). T3 and T4 were from Sigma-Aldrich (St. Louis, MO).

2.3. EAE induction

For EAE induction, mice were immunized subcutaneously (s.c.) on the back with 200 μg of MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA (Difco Lab, Detroit, MI) containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco). Two hundred nanograms of pertussis toxin (List Biological Lab, Epsom, England) was given i.p. on days 0 and 2 post-immunization (p.i.). Mice were scored daily for appearance of clinical signs of EAE on a scale from 0 to 5: 0, no clinical signs; 1, fully limp tail; 2, paralysis of one hind limb; 3, paralysis of both hind limbs; 4, paralysis of trunk; and 5, moribund or death.

2.4. Primary CNS cell purification, culture and cytokine treatment

For oligodendrocyte progenitor cell (OPC) culture, the whole brain of mouse embryos (E16) was harvested and dissociated with a Neural Tissue Dissociation Kit purchased from Miltenyi Biotec Inc. (Auburn, CA) following the manufacturer's instructions. OPCs were purified with anti-A2B5 microbeads (Miltenyi Biotec Inc.) following the manufacturer's MACS instructions. The purified OPCs were centrifuged at 300 *g* for 10 min, then resuspended with D-MEM/F12 (Mediatech, Inc., Manassas, VA), plus B27 (Invitrogen, Grand Island, NY), 20 ng/ml PDGF, 20 ng/ml bFGF, 2 mmol/L L-Glutamine, 100 I.U./ml penicillin and 100 μg/ml Streptomycin (Mediatech, Inc., Manassas, VA), and seeded on poly-lysine pre-coated 60-mm dishes at a density of 1×10^6 cells/dish. For differentiation, cells were cultured in differentiation media containing D-MEM/F12, B27, 30 ng/ml T3, 30 ng/ml T4, 10 ng/ml NT-3, 2 mmol/L L-Glutamine, 100 I.U./ml penicillin and 100 μg/ml Streptomycin.

For isolation, purification and culture of astrocytes and microglia primary cells, the whole brain of mice embryos (E16) was harvested and dissociated with the Neural Tissue Dissociation Kit (Miltenyi Biotec Inc., Auburn, CA) following the manufacturer's instructions. For astrocyte purification, the dissociated cells were centrifuged at 300 *g* for 10 min, then resuspended with astrocyte culture media, and seeded on poly-lysine-coated 60-mm dishes at a density of 1×10^6/dish. The astrocyte culture media contained D-MEM (Mediatech, Inc.), 10% fetal bovine serum (Invitrogen), 2 mmol/L L-Glutamine, 100 I.U./ml penicillin and 100 μg/ml Streptomycin. For astrocyte passage, after 7 days' culture, the remaining cultures were trypsinized and replanted in Petri dishes. Cultures that had been passaged twice were used as astrocytes. Microglia cells were purified with anti-CD11b microbeads (Miltenyi Biotec Inc., Auburn, CA) following the manufacturer's MACS instructions. Purified microglia cells were centrifuged at 300 *g* for 10 min, then resuspended with D-MEM/10% FBS plus 5 ng/ml M-CSF (PeproTech), and seeded on 60-mm dishes at a density of 1×10^6/dish. After 7 days, cultures were trypsinized and replated in Petri dishes. Cells from cultures that had been passaged once were used as microglia cells.

Cytokine concentrations used for all treatments were as follows: IL-9, 20 ng/ml; IL-17, 50 ng/ml; IL-1β, 20 ng/ml; IFN-γ, 10 ng/ml; and TNF-α, 10 ng/ml. For chemokine expression in astrocytes, 1×10^6 cells/well, cells were treated with different combinations of cytokines for 12 h; cells were then harvested for RNA purification, cDNA synthesis and real-time PCR analysis. For proliferation assay, cells were treated with different cytokines for 3 days. For OPC differentiation experiments, cells were treated for 5 days.

2.5. Immunohistochemical staining

Immunohistochemical staining was performed as previously described (Yan et al., 2012), with some modifications. Briefly, spinal cords were carefully excised from the brain stem to the lumbar region and cryoprotected with 30% sucrose in PBS. The lumbar enlargement was identified and then transected at the exact midpoint of the lumbar enlargement to standardize a site along the longitudinal axis of the cord, ensuring that the same lumbar spinal cord regions were analyzed for all conditions. Transverse sections of the brain and spinal cord were cut, and immunohistochemistry was performed using different antibodies. Immunofluorescence controls were routinely generated with irrelevant IgGs as the first antibody. Finally, slides were covered with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA), containing 1 μM DAPI. Results were visualized by fluorescent microscopy (Eclipse 800, Nikon). The staining procedure for cells was similar to that for tissue slides; cells were incubated on coverslips pretreated with poly-lysine and were then stained following the same procedures mentioned above.

2.6. Real-time quantitative RT-PCR

Total RNA was isolated from tissues or cultured cells with TRIzol (Life Technologies, Gaithersburg, MD). For RT-PCR, 2 μg of total RNA was transcribed into cDNA. Then, quantitative real-time PCR was performed using the Applied Biosystems 7000 Real-time PCR system and SYBR Green detection. PCR reactions were performed (50 °C 2 min, 95 °C 10 min, followed by 40 cycles on 95 °C, 15 s; 60 °C, 1 min), after which melting curve reaction was performed to verify the specificity of amplification using the Power SYBR Green PCR master mix (Applied Biosystems). Transcript quantization was relative to HPRT standard. Error bars indicate SEM values calculated from $-\Delta\Delta$Ct values from triplicate PCR reactions, according to the Applied Biosystems protocols. PCR primer pairs were listed as follows: HPRT: 5′-GTAATGATCAGTCAACGGGGGAC-3′ and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′; CXCL1: 5′-CTTGCCTTGACCCTGAAGCTC-3′ and 5′-AGCAGTCTGTCTTCTTTCTCCGT-3′; CXCL2: 5′-CCCCCTGGTTCAGAAAATCA-3′ and 5′-GCTCCTCCTTTCCAGGTCAGT-3′; CCL20: 5′-GTGGCAAGCGTCTGCTCT-3′ and 5′-TGTACGAGAGGCAACAGTCG-3′; CXCL9: 5′-TGCACGATGCTCCTGCA-3′ and 5′-AGGTCTTTGAGGGATTTGTAGTGG-3′; CXCL10: 5′-CTCATCCTGCTGGGTCTGAG-3′ and 5′-CCTATGGCCCTCATTCTCAC-3′; CXCL12: 5′-AACCAGTCAGCCTGAGCTACC-3′ and 5′-CTGAAGGGCACAGTTTGGAG-3′; MMP3: 5′-GTTGGAGATGACAGGGAAGC-3′ and 5′-CGAACCTGGGAAGGTACTGA-3′; MMP9: 5′-CCAGATGATGGGAGAGAAGC-3′ and 5′-GGCCTTTAGTGTCTGGCTGT-3′.

2.7. Microglia and OPC proliferation assay

Microglia and OPC proliferation were assayed by ^{3}H-thymidine incorporation. Briefly, triplicate aliquots of 1×10^4 of cells in 96-well plates were stimulated with different combinations of cytokines in