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Elevated expression of granulocyte-macrophage colony-stimulating factor receptor in multiple sclerosis lesions

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease that disproportionately affects young adults, leading to disability and high costs to society. Infiltration of T cells and monocytes into the central nervous system (CNS) is critical for disease initiation and progression. However, despite a great deal of effort the molecular mechanisms by which immune cells initiate and perpetuate CNS damage in MS have not yet been elucidated. In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by pathogenic Th1 and Th17 cells is critical for the recruitment of monocytes into the CNS during the initial stage of disease. We and others have recently shown that, compared with healthy individuals, MS patients have greater numbers of $CD4^+$ and $CD8^+$ T cells that produce GM-CSF. Here, we describe the expression of GM-CSF and its receptor, GM-CSFR, in normal brain and MS lesions. Our data show that in acute and chronic MS lesions, microglia and astrocytes have upregulated expression of GM-CSFR; in addition, we show that GM-CSF-associated molecules are also upregulated in MS lesions. These findings further strengthen the argument that GM-CSF signaling contributes to MS pathogenesis.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS). MS is characterized by infiltration of immune cells into the CNS, where they cause damage to myelin, oligodendrocytes, and axons, as well as neuronal dysfunction (Imitola et al., 2006). Although the etiology of MS remains elusive, the risk of developing MS is associated with genetic and environmental factors, including allelic variants in over 100 genes (International Multiple Sclerosis Genetics et al., 2011). Environmental factors that increase risk of MS include vitamin D deficiency, Epstein-Barr virus infection early in life, and smoking (Ascherio, 2013). Current views of MS pathogenesis implicate trafficking of T cells into the brain, attracting other immune cells, primarily monocytes, and causing tissue damage. The migration of Leukocytes, including activated lymphocytes, monocytes and macrophages, into the brain is necessary to initiate pathology has been verified in humans by blocking antibody against the molecule VLA-4 (Natalizumab), which is critical for leukocytes migration. Natalizumab significantly decreases infiltration of immune cells into the CNS, leading to reduced disease activity, as shown by the decline in the number of gadolinium-enhancing lesions and improvement in disease activity (Nicholas et al., 2014). Conversely, if this medication is stopped, allowing migration of T cells into the CNS, disease activity invariably returns, with, in some instances, risk of severe exacerbation and progression. This indicates that migration of T cells into the CNS and their interaction with resident cells is critical to the pathological process in MS (Kivisakk et al., 2009). The mechanisms underlying the interaction of immune cells with resident CNS cells that leads to persistent immune activation are not known. It has been suggested that upon entry into the brain, T cells interact with microglia and astrocytes, and recruit monocytes and macrophages that play a critical role in initiating and maintaining an inflammatory environment that causes CNS damage (Frohman et al., 2006).

The mechanisms of T cell activation and generation of pathological T cells in MS have been elucidated in MS models such as experimental autoimmune encephalomyelitis (EAE). From this work, we have learned

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that granulocyte-macrophage colony-stimulating factor (GM-CSF) is critical for EAE to develop (El-Behi et al., 2011), in fact a recently described mouse model, where T cells were engineered to release GM-CSF led to invasion of inflammatory myeloid cells of the brain which was accompanied by the spontaneous development of severe neurological deficits (Spath et al., 2017).

GM-CSF is a pro-inflammatory cytokine essential for the development and progression of EAE. In EAE, monocytes infiltrate the CNS in large numbers and are critical in mediating pathogenesis by GM-CSF-producing T cells. We have recently shown than GM-CSF production by T cells is elevated in patients with MS, and that interferon-β, an approved MS medication, can reduce the production of GM-CSF in these cells (Rasouli et al., 2015).

In this study, we investigated the expression of GM-CSF receptor (GM-CSFR) in normal-appearing white matter (NAWM) and brain lesions of MS patients by gene expression analysis and immunohistochemistry. We found that both GM-CSFR subunits alpha (α) and beta (β) are highly expressed in the borders of active lesions compared with chronic lesions, NAWM and controls. Cell morphology and surface markers identified activated microglia/macrophages and astrocytes as GM-CSFR-expressing cells in MS lesions.

2. Materials and methods

2.1. Brain tissue

Human brain tissues were obtained from the Rocky Mountain MS Center (RMMSC). Four specimens from patients with MS (two with active and two with chronic disease) and two specimens from individuals without neurological disease (controls) were investigated in this study. Clinical features are summarized in Table 1. Information about the nature of activity of the MS lesions, was obtained from the autopsy reports. Patients with active disease had MS plaques that were described as active or reactivated inflammation with predominant perivascular inflammation. Patients with chronic disease had lesions that were inactive without perivascular inflammation. In addition to the reports by the pathologist, we examined the MS plaques for inflammation and demyelination using haematoxylin & eosin staining (HE) and luxol-fast blue staining (LFB), respectively. Active lesions were characterized by areas of demyelination, perivascular inflammation with infiltration of lymphocytes and macrophages into the demyelinated areas and in their borders. Chronic inactive lesions were characterized by the lack of inflammatory infiltrates or microglial activation. We also studied normal appearing white matter (NAWM) adjacent to the MS lesions (Lassmann et al., 1998).

2.2. Immunohistochemistry and confocal microscopy

To identify GM-CSFR expression by different cell populations, macroscopic lesions from MS brains were identified; the lesions were demarcated by visual inspection and carefully dissected, catalogued and kept at − 80 °C until further use. The dissected tissue was cut in cryostat into 25 μm sections and stored on slides at − 80 °C until staining. Slides were fixed in acetone for 5 min; histological staining with HE and LFB was done according to routine protocols (Li et al., 2007). The sections were next immunostained with anti-GM-CSFR Abs after an initial blockade with horse serum to avoid nonspecific staining. To examine the expression of GM-CSFRα subunit, two specific Abs (clone C18, rabbit Polyclonal IgG at 1:100 from Santa Cruz Biotechnology for co-staining with GFAP and CD68. To confirm the staining and upregulation, we used clone K12B7.17A at 1:100 from Abcam). To stain microglia/macrophages we used an Ab against CD68 (clone Y1/82A, Mouse monoclonal IgG), for astrocytes we used anti-GFAP Ab (Clone 4A-11, Mouse monoclonal IgG) both from BD/Pharmingen. Isotype-matched Abs were used as control. After washing in PBS, sections were incubated with primary Abs for 1 h at room temperature or overnight at 4 °C. The sections were then incubated with blocking solution and with secondary Abs, Alexa Fluor 546-labelled donkey anti-rabbit and donkey anti-mouse Abs (both from Invitrogen, CA, USA). Negative controls omitting the first, then the second, primary Ab, as well as isotype control, were included in all the double-labeling experiments. The sections were then washed in phosphate-buffered saline (PBS), mounted in anti-fading mounting media and observed with a Zeiss Confocal LSM 510 microscope.

To study the expression of GM-CSFR, we used Quantitative Confocal Microscopy (QCM) methods with high resolution combined with stereology, as in our prior experiments (Imitola et al., 2011; Kivisakk et al., 2009; Pluchino et al., 2008; Rasmussen et al., 2011; Wang et al., 2008). Acquisition parameters for confocal microscopy were established for all experiments relative to negative control that was always included to control for non-specific staining. We used 2.5D pixel intensity analysis to determine the relative expression of GM-CSFR in a given microglia or astrocyte cell. We then used line pixel intensity to determine the expression of GM-CSFR on the surface and the processes of microglia and astrocytes (Starossom et al., 2012). Finally, we determined co-localization of GM-CSFR with orthogonal planes and a co-localization index that allows measurement of individual channels with high signal-to-noise ratio, without unspecific co-labeling.

2.3. RNA extraction and real-time PCR assay

Total RNA was extracted from cells or tissues with RNeasy Mini Kit (Qiagen, Valencia, California, USA), followed by reverse transcription with Superscript III (Invitrogen) according to the manufacturer's protocol. Taqman real-time PCR was performed with Taqman Fast Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA) on ABI 7500 system (Applied Biosystems). Primers and probes sets for human *STAT5B*, *CEPBA*, and *GMCSFR* were obtained from Applied Biosystems.

2.4. Human microglia isolation

The studies on human microglia were performed in accordance with the guidelines of the Thomas Jefferson University Hospital Institutional

Table 1
Patient demographics and disease activity in MS tissues.

Age at death	Sex	Type of lesion autopsy description	Disease duration	Clinical severity as per autopsy report
43	M	Extensive severe demyelination with chronic active smoldering lesions, moderate atrophy.	15 years	Severe disease
37	M	Active extensive severe demyelination involving much of the supratentorial white matter, severe perivascular lymphocytosis and macrophages in the edge of the plaque with reactivation, minimal atrophy.	6 years	Severe disease clinically active at time of dead
87	F	Chronic inactive plaques, no perivascular lymphocytes, mild cerebral atrophy	n/a	Chronic
61	F	Chronic inactive plaques, no perivascular lymphocytes, mild cerebral atrophy	n/a	Chronic
45	F	Control	–	–
89	M	Control	–	–